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Identification and Structural Analysis of the Androgen Receptor from Normal and Androgen Insensitive Human Fibroblasts.

by

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Common Names and Abbreviations Used.

A.R., Analytical reagent
Arg., Arginine
Cys., Cysteine
DCC, Dextran coated charcoal
DHA, Dehydroepiandrosterone
DHT, 5α-dihydrotestosterone (17β-hydroxy-5α-androstane-3-one)
2-DGE, Two-dimensional gel electrophoresis
EDTA, Ethylenediaminetetraacetic acid
EC1, MEM + 1% Newborn calf serum
EC10, MEM + 10% Newborn calf serum
FPLC, Fast protein liquid chromatography
GSF, Genital skin fibroblasts
His., Histidine
HPLC, High performance liquid chromatography
IEF, Isoelectric focusing
Leu., Leucine
Lys., Lysine
MEM, Minimum essential medium (Glasgow modified Eagles medium)
Mibolerone, (17β-hydroxy-7α,17α-dimethly-4-estrene-3-one)
Mr., Relative molecular mass
NEPHGE, Nonequilibrium pH gradient gel electrophoresis
nt^-, Nuclear transfer deficient
nt^i, Increased nuclear transfer
NGSF, Nongenital skin fibroblasts
PEM, 10mM-KH_2PO_4, 1mM-EDTA, 12mM-monothioglycerol buffer
PEG, 10mM-KH_2PO_4, 1mM-EDTA, 10% glycerol buffer
Phe., Phenylalanine
pI, Isoelectric point
PMSF, Phenylmethylsulphonyl fluoride
r^-, Receptorless
Rs, Stokes radius
R1881, Methyltrienolone (17β-hydroxy-17α-methyl-4,9,11,-estrien-3-one)
SDS-PAGE, Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis.
Ser., Serine
TEMED, N,N,N',N'-tetramethylethylenediamine
Thr., Threonine
Tyr., Tyrosine

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SUMMARY.

Resistance to androgens *in utero* and at puberty causes a dysfunction of male sexual differentiation, and results in a form of male pseudohermaphroditism. From whole cell binding studies, using cultured human genital skin fibroblasts (GSF) a number of defects in the androgen receptor have been discovered, these have been classified as: absent, deficient, thermolabile, defective activation to the DNA/nuclear binding form, and finally, failure to "up-regulate" the basal binding level in response to prolonged (i.e. 24h) incubation of cells with hormone. There is, however, a need to study the receptor protein directly, without relying on the reversible binding of ^{3}H ligand. Therefore, in the present study the androgen receptor from human GSF was extracted using 0.5M-KCl, and partially purified by 35% ammonium sulphate preciptitation prior to further studies.

Normal and variant forms of the androgen receptor were then searched for by comparing the proteins in receptor enriched fractions from control and androgen insensitive cells, by a dual-labelling technique and high resolution two-dimensional gel electrophoresis (2-DGE). As a complement to the electrophoresis studies, normal and variant forms of the receptor were analysed on 5-20% linear sucrose density gradients and by ADP-Sepharose chromatography. The activated form of the androgen receptor from normal cells was also characterised by FPLC-anion exchange chromatography and HPLC-size exclusion chromatography. Finally, attempts were

made to covalently label the fibroblast receptor using the photoactive ligand [^{3}H]methyltrienolone (R1881): a synthetic steroid known to bind specifically to the androgen receptor.

After incubating cells in culture with [^{3}H] DHT or mibolerone, the partially purified receptor complex from normal cell lines was found to sediment at 4S on sucrose gradients and from gel filtration studies to have a relative molecular mass of around 60000, a Stokes radius of 3.16nm and a frictional ratio of between 1.21-1.43. After HPLC-gel filtration a second peak of bound steroid was observed (at about Mr.15000), believed to represent a fragment of the receptor containing the steroid binding domain.

The receptor complexes from androgen insensitive cell lines also sedimented at 4S on linear sucrose gradients. However, the receptor profiles from cells shown to have absent, deficient, or unstable binding in whole cell assays were quantitatively altered from controls. Furthermore, this procedure may be a useful means of distinguishing quantitative and qualitative defects of the androgen receptor, since for one cell line (TCF) found to have normal levels of receptor (Receptor positive, unstable binding) in the whole cell binding assay, the profile on sucrose gradients resembled that of receptor negative (Absent) cells. The complexes from one cell line (T4) diagnosed as receptor positive (whole cell studies) also sedimented at 4S. The receptor from these cells interacted with ADP-sepharaose in a manner indistinguisable from the receptor complexes from control cells (SW): both were eluted with

0.5-1.0M-KCl. From whole cell binding and *in vitro* studies it appears that the androgen receptor from this cell line is normal, implying that the androgen resistance mutation lies at some subsequent step in androgen action and that other factors could play important roles in steroid hormone action.

The comparison of proteins from receptor enriched fractions from control and androgen resistant cells labelled with [^{35}S]methionine and [^{75}Se]selenomethionine respectively, failed to show differences that could be related to the androgen receptor protein or the androgen insensitive phenotype. Similar results were seen for the comparison of two-dimensional patterns of whole cell protein, labelled with [^{35}S]methionine only.

Finally, after partial purification and U.V. irradiation, the receptor complexes from rat ventral prostate cytosol and calf uterus cytosol were succesfully photolinked with [^{3}H] R1881: peaks of specifically bound radioactivity were recovered after SDS-PAGE, at Mr. of 50000 and 100000 respectively. However subsequent attempts to covalently label the human GSF androgen receptor, either after partial purification or *in situ* were unsuccessful. This was thought to be due to instability of the receptor complexes during the partial purification protocol, and also a reflection of the low efficiency of the photoactivation reaction.

INTRODUCTION

1.1 Androgen Action

Androgens are C-19 steroids, which are synthesised and secreted mainly by the Leydig cells of the testes and to a lesser degree, by the adrenals and ovaries (Gower 1979). In common with other classes of steroid hormone, androgens act on the genome of target cells to effect a change in the pattern of gene transcription: this action being mediated through a specific intracellular receptor mechanism (Chan & O'Mally 1976; Higgins & Gehring 1978; Katzenellenbogen 1980; Mainwaring 1977;Yamamoto 1985; Yamamoto & Alberts 1976).

Jensen and associates (Jensen et al 1968) were the first to descibe the action of a steroid by the "two-step mechanism" (Fig.1.1). Although recently, the location of the receptor in the absence of hormone has been the source of growing controversy, the model in principal remains valid (Jensen 1984; Schrader 1984). Once inside the target cell the free steroid must first bind to the high-affinity, low capacity receptor sites, and secondly, the steroid-receptor complex must interact with sites in the nucleus to effect specific changes.

The two-step model was proposed originally to describe the action of oestrogen in the rat uterus (Jensen et al 1968; Jensen & de Sombre 1973), and the key features of this scheme were subsequently described for all classes of steroid hormone (Chan & O'Mally 1976; Higgins & Gehring 1978; Lan et al 1984). The conversion of testosterone, the main circulating andogen, to the more potent 5α-reduced metabolite dihydrotestosterone (DHT; Fig.1.1) in certain target tissues is a unique feature of

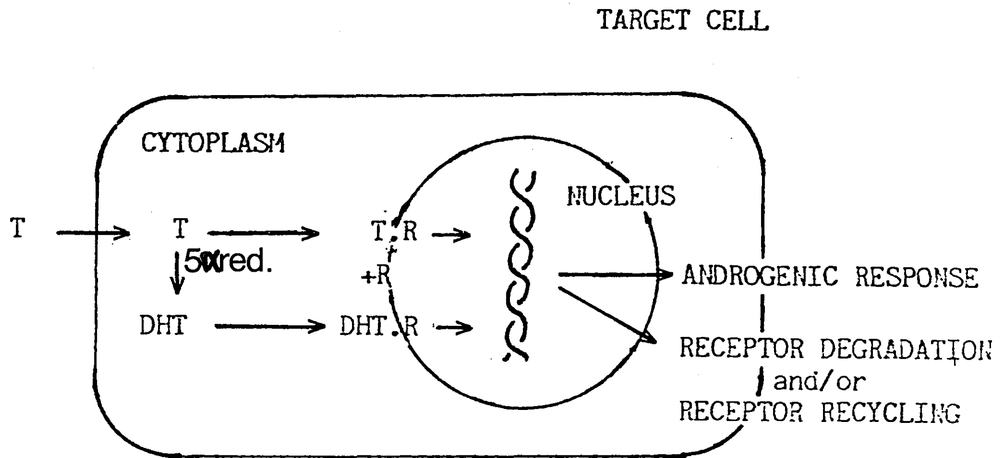


Figure 1.1 Scheme for androgen action.
T, testosterone; DHT, 5α-dihydrotestosterone; 5α-red., 5α-reductase; R, androgen receptor.

androgen action (Wilson and Glonyna 1970).

The relevance of the above model (Fig.1.1) to the mechanism of androgen action will now be discussed in more detail, with reference to other classes of steroid where appropriate.

A. Uptake of Steroid Hormones.

In Man and other higher vertebrates there are a number of circulating serum proteins that bind steroid hormones in a specific or non-specfic manner: sex hormone binding globulin (SHBG), corticosteroid binding globulin (CBG) and albumin (Anderson 1974). As a result, only a small percentage of the total circulating hormone will be free (i.e. 1 to 3% of total testosterone and oestradiol); it is this fraction that is biologically active and which determines the intracellular concentration of steroid (Anderson 1974).

It is generally thought that because of their lipophilic nature, steroid hormones enter cells by passive or simple diffusion (Gorski & Gannon 1976; Higgins & Gehring 1978; Katzenellenbogen 1980). Despite the technical difficulties inherent in studies of this phenomenon, passive uptake of progesterone, glucocorticoids, oestrogens and androgens by cells (hamster fibroblasts and rat hepatoma cells) grown in culture was demonstrated by Giorgi (1980; Giorgi & Stein 1981). The contamination of assays by serum binding proteins and intracellular binding sites has complicated the search for facilated or active transport mechanisms. Therefore, the physiological significance of studies apparently showing such uptake mechanisms remains, at best, unclear (Gorski & Gannon 1976; Higgins & Gehring 1978; Katzenellenbogen 1980).

B. Receptor Localisation.

Steroid receptors are intracellular proteins, characterised by their ability to reversibly bind steroids in a high affinity, low capacity manner. Evidence for their existence came initially from the use of radiolabelled steroids, which were preferentially retained by target tisses (Anderson & Liao 1968; Baulieu & Jung 1970 Bruchovsky & Wilson 1968; Fang, Anderson & Liao 1969; Higgins and Gehring 1978; Katzenellenbogen 1980; King & Gordon 1966; Mainwaring 1969a,b; Stumpf & Madhabananda 1975; Toft & Gorski 1966; Tveter & Attramadal 1968; Unhjem, Tveter & Aakvaag 1969). Further progress was made with the introduction of synthetic analogues of various hormones, which bound to receptors but not to contaminating serum proteins (Higgins & Gehring 1978; Katzenellenbogen 1980).

Since steroid receptors, in the absence of hormone, could be isolated in the soluble fraction of cell extracts, it was generally assumed that they were located in the cytoplasm in the absence of ligand ("cytoplasmic exclusion hypothesis"), and that translocation of receptor complexes to the nucleus occured after hormone binding (Jensen et al 1968; Katzenellenbogen 1980). However recent experimental evidence, from two independent groups, suggested that receptor molecules may always be associated with the nuclear compartment, irrespective of hormone binding status (Greene et al 1984; King & Greene 1984; Welshons, Lieberman & Gorski 1984).

Greene and co-workers (Greene et al 1984; King and Greene 1984) using a panel of five monoclonal antibodies (specific for

the oestrogen receptor) and an indirect immunoperoxidase technique, demonstrated that in frozen sections of human breast carcinoma, human and rabbit uterus, and MCF-7 cells, specfic staining was localised in the nucleus, in the presence or absence of oestrogen. Gorski and co-workers (Welshons et al 1984) using a different experimental approach, isolated "cytoplast" and "nucleoplast" fractions by cytochalasin B-induced ennucleation of GH_3 cells (derived from a rat pituitary tumour), and showed that the unoccupied oestrogen receptor was associated with the nuclear fraction. The presence of receptors in the soluble extracts of earlier studies could have been due to the isolation procedures used and/or the possible weak association of unoccupied receptors with nuclear components (Green et al 1984; Jensen 1984; Yamamoto 1985).

If the above results are shown to be relevant to steroid receptors in general the question that arises, is where in the nucleus are the unoccupied receptor molecules located; on the nuclear membrane, chromatin or nuclear matrix (scaffolding) structures ?

However, the findings of an other immunocytochemical study, using antisera raised against the glucocorticoid receptor, supported the more classical view of the intracellular distribution of receptor molecules. Antakly and Eisen (1984) observed specfic staining in both cytoplasm and nuclei of rat liver hepatocytes and cells of the anterior pituitary. The staining in hepatocyte nuclei was reduced in adrenalectomised animals, but could be recovered after cortisol treatment. These

authors suggested that the differences between their findings and those of the above groups may reflect: 1. differences in the distribution of oestrogen and glucocorticoid receptors in their respective target tissues; or 2. the antibodies raised against the oestrogen receptor only recognise antigenic determinants on the nuclear form of the receptor.

C. Receptor Structure.

Androgen binding has been extensively studied in the classical androgen target tissue, the rat ventral prostate (Baulieu & Jung 1968; Brinkmann et al 1985a,b; Davies 1983; Davies & Griffiths 1974; Davies et al 1980; Davies, Thomas & Griffiths 1976; Fang & Liao 1971; Fang et al 1969; Feit & Muldoon 1983; Goueli, Holtzman & Ahmed 1984; Katsumata & Goldman 1974; Liao et al 1973; Mainwaring 1969a,b; Mainwaring & Irving 1973; Mulder et al 1984; Shain & Boesel 1975; Unhjem et al 1979). Subsequently, androgen receptors have been found and characterised in a wide variety of tissues and species: the mechanism of androgen action was assumed to be similar in all tissues containing the androgen receptor (Mainwaring 1977). Table 1.1 gives a brief summary of the physico-chemical properties of the receptor protein from a number of different sources.

The androgen receptor (complex II; Fang & Liao 1971) has been shown to be essentially acidic in nature (Table 1.1; Chang and Tindal 1983; Chang et al 1982; Mainwaring and Irving 1973; Razel et al 1985; Valladares and Minguell 1975), and has been partially purified by ammonium sulphate precipitation (25 to 40% saturation; Chang and Tindal 1983; de Boer et al 1986; Kyakumoto

Table 1.1 Physico-chemical properties of the androgen receptor from different sources and laboratories.

Tissue	Kd[a] (nM)	S[b]	Mr.[c] x10-3	pI[d]	Ref.[e]
Rat Ventral Prostate	2.4-4.0	8.0	280	5.8	1,2,3
		4.2	100	6.5	
" " "	6.00	4.5	87	-	4
" " "	6.50	4.5	85	6.3	5
" " "	-	4.0	50	-	6,7
Prostatic Tumour	0.30	8.5-9.3	265	-	8
	-	4.4	120	-	
Rat Bone Marrow	5.90	3.0	-	4.9	9
Rat Uterus	2.10	6.0	167	5.9	10
Calf Uterus	0.26	4.5	80-100	-	7,11
Steer Seminal Vesicles	1.40	3.8	60	6.6	12
Mouse Kidney	1.70	3.6	-	-	13
Human Liver	0.95	-	75	-	14
Human Prostate	2.38	3.0	34	4.7	15
Human Foreskin	0.51	4.0	-	5.7	16
Human GSF	0.2-1.6	4.0	114.3	-	17,18

a. Equilibrium dissociation constant (ligand: DHT, Testosterone, R1881 or Mibolerone).
b. Sedimentation coefficient from 5-20% sucrose density gradient centrifugation; activated (3.0-4.5S) and unactivated (6.0-10.0S) complxes.
c. Relative molecular mass, determined from gel filtration or SDS-PAGE.
d. Isoelectric point.
e. References:

1. Mainwaring 1969a
2. Mainwaring 1969b
3. Mainwaring & Irving 1973
4. Goueli et al 1984
5. Chang et al 1983
6. Mulder et al 1983
7. Brinkmann et al 1985b
8. Rowley, Chang & Tindall 1984
9. Valladares & Minguell 1985
10. Chang & Tindall 1983
11. deBoer et al 1986
12. Chang et al 1982
13. Bullock & Bardin 1974
14. Bannister, Sheridan & Losowsky 1985
15. Lehoux, Benard & Elhilali 1985
16. Pazel et al 1975
17. Keenan et al 1975
18. Keenan, Greger & Hedge 1986

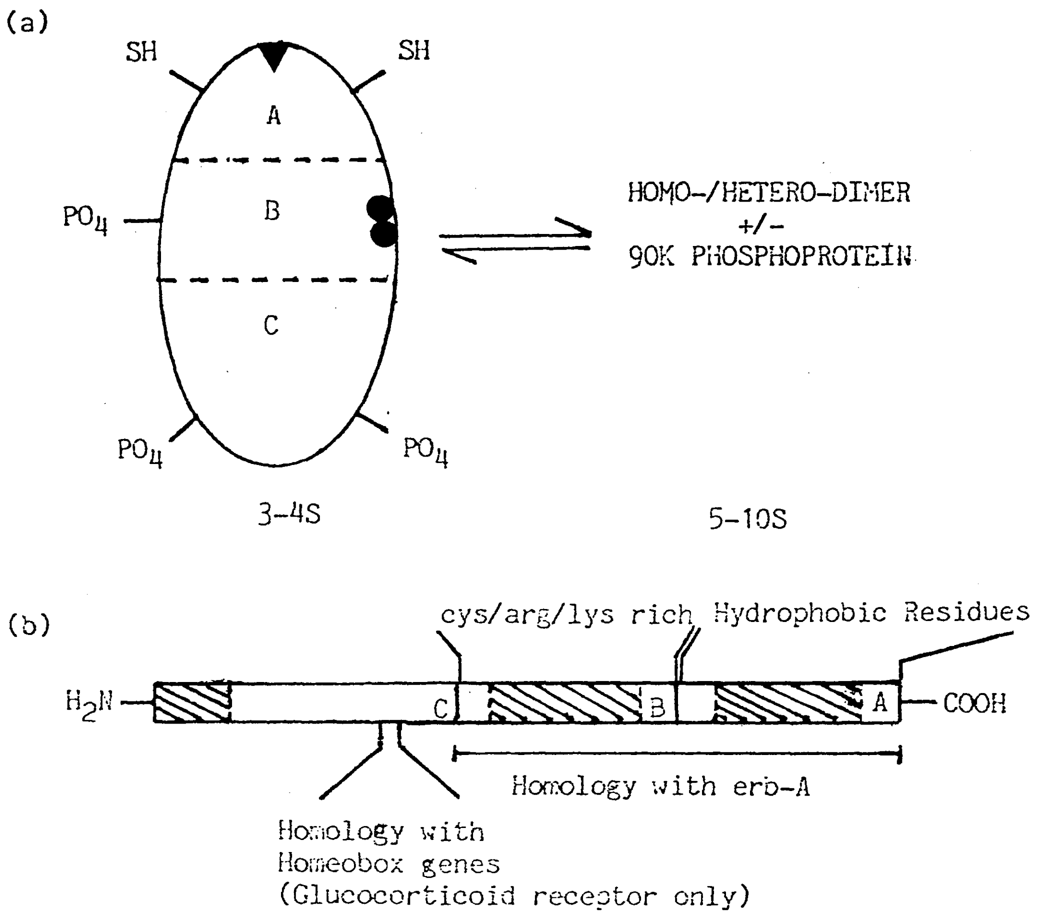
et al 1986), DNA-cellulose chromatography (Brinkmann et al 1985b, 1986; de Boer et al 1986a), 2'5' ADP-sepharose chromatography (Mulder et al 1984), FPLC-anion exchange chromatography (Brinkmann et al 1985a; Brinkmann et al 1986) and finally, by affinity chromatography (Chang et al 1982; de Larminat et al 1984).

It remains unclear whether the reported differences in receptor properties (Table 1.1) are a true reflection of tissue and/or species receptor heterogeneity or are simply the result of differences in experimental procedures.

Subunit nature of steroid receptors.

The observation that receptors can aggregate with themselves or with "cytosolic" proteins (Fig.1.2a), has led to the suggestion that they are oligomeric proteins (Higgins & Gehring 1978). O'Mally and co-workers (Schrader et al 1981) were the first to describe the detailed structure of a steroid receptor. They proposed that the avian progesterone receptor contained two dissimilar subunits, A and B, both of which bound steroid but differed in their affinities for nuclear structures. The A subunit (approximately 70000-daltons) bound to DNA, while the B subunit (approximately 110000-daltons) bound to chromatin (Schrader et al 1981). Further, two proteins having similar molecular masses were recovered after in situ photoaffinity labelling of the progesterone receptor from human breast cancer cells (Horwitz & Alexander 1983), and two peaks of specifically bound steroid were observed after non-denaturing gel electrophoresis (Smith et al 1986).

However, more recent evidence has led to a modification of



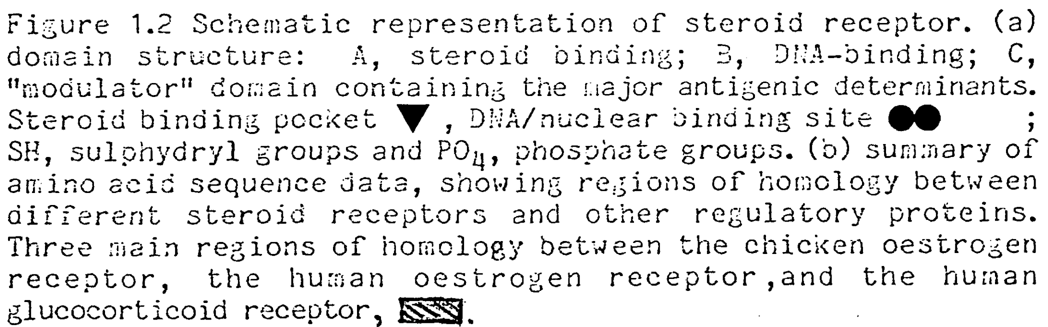


Figure 1.2 Schematic representation of steroid receptor. (a) domain structure: A, steroid binding; B, DNA-binding; C, "modulator" domain containing the major antigenic determinants. Steroid binding pocket ▼, DNA/nuclear binding site ●● ; SH, sulphydryl groups and PO_4, phosphate groups. (b) summary of amino acid sequence data, showing regions of homology between different steroid receptors and other regulatory proteins. Three main regions of homology between the chicken oestrogen receptor, the human oestrogen receptor, and the human glucocorticoid receptor, ▨.

the above model of the chicken oviduct receptor. Immunological studies have shown that the "activated" (4S form) receptor was a mixture of two steroid binding polypeptides (the A and B subunits above), while the "non-activated" (8S form) receptor was made up of a hormone binding polypeptide (A or B) associated with a non-steroid binding protein (two molecules per complex) (Renoir & Mester 1984). This non-steroid binding protein was found to be phosphorylated on serine residues and to have a molecular weight of 90000-daltons. It has also been described associated with the 8S forms of the androgen, oestrogen, and glucocorticoid receptors (Joab et al 1984; Puri, Dougherty & Toft 1984; Renoir & Mester 1984; Schuh et al 1985). This 90K protein was indistinguishable from a protein associated with the Rous Sarcoma Virus transforming protein ($pp60^{v-src}$) by peptide mapping and immunological studies, and it may also represent one of the major heat shock proteins, which are induced under a variety of stress conditions (Schuh et al 1985). The implications of these different associations to hormone action remain to be determined; however, it is tempting to speculate that cellular reponses to stress, mediated through steroid receptors, could be controlled via interactions involving this 90K phosphoprotein.

Milgrom and co-workers (Logeat et al 1985; Loosfelt et al 1984) have demonstrated that the rabbit uterine progesterone receptor contained only one steroid binding subunit (110K), if precautions were taken during homogenization to prevent proteolytic degradation of the receptor; if not, smaller fragments of 72000, 70000, and 64000-daltons were observed.

Finally, after large scale purification of the chicken oviduct progesterone receptor, both the A and B forms of the receptor were found to be immunologically similar (Gronemyer, Govindan & Chambon 1985).

The transformation of the 4S oestrogen receptor to the 5S form has been associated with dimerization of the 4S subunits (Miller et al 1985; Muller, Traish & Wotiz 1983; Scholl & Lippman 1984), or alternatively, with the interaction of the 4S receptor with an unidentified protein "X" (Bailly et al 1980). Further studies by Miller et al (1985), using chemical cross-linking and dense amino acid labelling, concluded that the 5S nuclear receptor form was a homodimer of 4S (65K) monomers.

Steroid receptor domains.

Gustafsson and co-workers (Carslstedt-Duke et al 1982; Wrange and Gustafsson 1978) showed that the glucocorticoid receptor contained three distinct domains: A, steroid-binding (19A); B, DNA-binding (36A); and C, "modulation" (Fig.1.2a). The wild-type receptor has a Stokes radius of about 6.0 nm (87-90K Mr; A+B+C) which could be converted, by partial proteolysis with trypsin or α-chymotrypsin, to a form of about 3.0 nm (39-50K Mr; A+B), while more extensive enzymic digestion resulted in a 2.0 nm fragment (20-30K Mr;A)(Carlstedt-Duke et al 1982; Wrange et al 1984). Subsequently, limited proteolysis has been used to separate the steroid and nuclear binding domains of the oestrogen (Greene et al 1984), progesterone (Protein B: Edwards et al 1984), and 1,25-dihydroxyvitamin D_3 (Mellon 1985) receptors.

Steroid-receptor binding is a reversible, second-order reaction (Higgins & Gehring 1978). Early work on the nature of

the androgen binding site, suggested that the receptor bound steroid from the α-face, β-face and peripheral sides, and that steric not electrostatic properties of the ligand were important in this interaction. The hormone therefore seemed to be "enveloped" by a hydrophobic pocket (Liao et al 1973; Tymoczko, Liang & Liao 1978). Studies with the rat ventral prostate androgen receptor showed that the conformation around the A:B ring junction had a marked effect on androgenic activity; steroids with a cis-conformation were not bound by the prostate receptor (Tymoczko et al 1978). Cunningham et al (1983) correlated plannar A and B rings and the presence of the 3-keto group with steroid binding. The presence of the 17β-hydroxy group was also important, and the addition of a 17β-hydroxy group enhanced binding. This latter group, together with a 7α-methyl group may explain the obseved tighter binding of the synthetic androgen dimethyl-nortestosterone (Mibolerone) over DHT (Fang et al, 1969; Hodgins and co-workers unpublished observations; Liao et al 1973; Traish, Muller & Wortiz 1986; Tymoczko et al 1978). Testosterone and DHT have both been shown to be bound by the same receptor, although DHT was found to have a higher relative binding affinity (Tymoczko et al 1978; Griffin, Leshin & Wilson 1982). Furthermore when the dissociation rate of steroid complexes in cultured genital skin fibroblasts (GSF) was measured, testosterone-receptor complexes were found to dissociate four times faster than DHT-complexes (Hodgins 1982; Kaufman and Pinsky 1983; Wilson & French 1976).

Recent advances exploiting immunological (hybridoma) and

genetic engineering techniques have enabled the isolation of mRNA and the cloning of cDNAs for the rat (Miesfeld et al 1984) and human (Hollenberg et al 1985) glucocorticoid receptors, chicken (Krust et al 1986) and human (Green et al 1986; Walter et al 1985) oestrogen receptors, and more recently the rabbit progesterone receptor (Loosfelt et al 1986). Analysis of the deduced amino acid sequences has, in turn, allowed the identification of putative functional domains, and homology with other known or suspected regulatory proteins (Fig.1.2b).

The hydrophobic nature of the C-terminus, suggested that this was the location of the steroid-binding domain. The predicted secondary structure included α-helices and β-strands, which were compatable with the formation of a hydrophobic pocket (Green et al 1986; Krust et al 1986; Weinberger et al 1985). The assignment of steroid-binding activity to this region was based on two pieces of evidence. The cDNAs for the human glucocorticoid receptor predicted two proteins (of 777 amino acids and 742 amino acids) which differed at their carboxy termini, and were found to differ in their ability to bind hormone (Hollenberg et al 1985). Secondly, the introduction of mutations in the relevant region of the oestrogen receptor cDNA was also found to impair hormone binding of the receptor protein after _in vitro_ translation (Kumar et al 1986).

The DNA-binding domain was defined as a cysteine, lysine, arginine-rich region near the middle of the receptor molecule (Fig.1.2b)(Green et al 1986; Hollenberg et al 1985; Krust et al 1986; Kumar et al 1986; Weinberger et al 1985). This domain was linked to the steroid binding domain by a "hinge-region", which

allowed direct contact between these two domains; this could be an important feature for the regulation of receptor function (Krust et al 1986). Analysis of the amino acid sequence of nucleic acid binding proteins has led to the discovery of repeated sequences that can form, so called, "metal-binding fingers", which it was suggested were capable of binding nucleic acids (Berg 1986; Hartsharne et al 1985). The basis of this repeated motif were two cysteine residues which could form a tetrahedral complex with another two cysteine or histidine residues and a Zn^{2+} ion; twelve to thirteen residues separated the pairs of cysteineand histidine residues, forming the finger structure (Berg 1986; Hartshorne et al 1985). The consensus sequences from a number of different nuclear binding proteins can be summarized as follows, Tyr-Phe-X-Cys-X_4-Cys-X_3-Phe-X_5-Leu-X_2-His-X_3-His-X_{2-6}. Although this motif was not found repeated within the suspected DNA-binding domain of the oestrogen or glucocorticoid receptors, a single consensus sequence did occur, and the high proportion of cysteine residues in the remainder of this region may form a finger-like domain by a different mechanism (Krust et al 1986).

Perhaps the most unexpected and intriguing finding to emerge from the primary structure of steroid receptors was the homology with other classes of protein. The glucocorticoid, the oestrogen and the progesterone receptors all have homology with the v-erb A gene product ($p75^{gag-erbA}$) from the oncogenic Avian Erythroblastosis Virus (Krust et al 1986; Loosfelt et al 1986; Weinberger et al 1985); and the human glucocorticoid receptor was

found to have limited homology with the products of the homeo-box genes, Antennepedia and fuhsi tarazu, from the fruit fly Drosophila (Weinberger et al 1985). Chambon and co-workers (Krust et al 1986) showed that there was 80% homology between the chicken and human oestrogen sequences, in three "highly conserved" regions; two of these regions (containing the steroid and DNA-binding sites) shared homology with the human glucocorticoid receptor and the v-erb A fusion product, and the third region (at the N-terminus) was absent from the truncated $p75^{gag-erbA}$ protein, but had homology with the glucocorticoid receptor. Although the function of the cellular erb A protein or how the product of the v-erb A gene enhances transformation in erythroblasts remain unknown, it would appear that the protooncogene and steroid receptors share a common ancestor (Krust et al 1986; Weinberger et al 1985).

Phosphorylation of steroid receptors.

Evidence supporting the role of phosphorylation in the regulation of receptor function has been reviewed recently by Dougherty, Puri & Toft (1985); both indirect and direct experimental evidence for the phosphorylation of receptor proteins was reported.

Indirect evidence comes from four lines of investigation: steroid binding activity of receptors has been correlated with ATP and cyclic nucleotide (cAMP, cGMP) levels (Dougherty et al 1985; Fleming, Blumenthal & Gurpide 1983; Holbrook, Bodwell & Munck 1983b; Munck & Brink-Johnsen 1968; Sando et al 1979); treatment of cytosolic glucocorticoid and progesterone receptors with alkaline phosphatase was found to reduce the ability of

these receptors to bind steroid (Dougherty et al 1985; Nielsen, Sando & Pratt 1977; Puri et al 1984); molybdate, a known inhibitor of phosphatase activity, prevented the loss of steroid binding activity of the unactivated (8S) form of the androgen, oestrogen, and glucocorticoid receptors (Noma et al 1980; Sando et al 1979); and lastly, signal sequences for cAMP-dependent phosphorylatilon (lys/arg-lys/arg-X-ser/thr) and also phosphorylation on tyrosine residues (tyr-acidic/basic residue) were found in the amino acid sequence of the human oestrogen receptor (Green et al 1986).

Auricchio and co-workers (Auricchio et al 1981; Auricchio et al 1984; Auricchio et al 1985; Migliaccio et al 1982) have purified a Ca^{2+}-dependent protein kinase from calf uterus and a nuclear phosphatase activity, which they claimed, regulated the binding of oestradiol by means of phosphorylation-dephosphorylation of the oestrogen receptor. Phospho-amino acid analysis showed phosphorylation on tyrosine residues (Auricchio et al 1985). The nonactivated, molybdate stabilised, progesterone receptor was resolved by DEAE-sephadex chromatography into two components (I and II): receptor A (80K) plus the 90K protein and receptor B (110K) plus the 90K protein respectively. Incorporation of [^{32}P]orthophosphate showed that receptor B and the 90K protein were phosporylated on serine residues, receptor A was also thought to be phosphorylated as both forms A and B were substrates in vitro for a cAMP-dependent protein kinase (Dougherty 1985; Puri et al 1984; Wiegel et al 1981). Similarly Goueli et al (1984), found that the purified androgen receptor

from rat ventral prostate was specifically phosphorylated by a nuclear cAMP-independent protein kinase. Finally the glucocorticoid receptor from L-cells (Housley & Pratt 1983) and rat liver (Grandics et al 1984; Kurl & Jacob 1984) has been shown to be phosphorylated by an endogenous protein kinase.

Phosphorylation - dephosphorylation cycles have long been recognised as important elements in metabolic regulation, controlling the activities of proteins and enzymes, therefore, it has been suggested that phosphorylation could regulate the binding of ligand by the unactivated and activated forms of the receptor (Dougherty et al 1985). Another intriguing possibility, is that phosphorylation could integrate steroid hormone action with other signal transducing mechanisms (Dougherty et al 1985). It is of interest, therefore that Ghosh-Dastidar et al (1984) observed that both the A and B forms of the hen oviduct progesterone receptor were phosphorylated by epidermal growth factor (EGF) via the EGF-receptor.

Receptor heterogeneity.

The rat prostate cytosol was found to contain at least two proteins that specifically bound DHT, which could be separated by ammonium sulphate precipitation (0-40% complex II; 55-70% complex I) and by gel filtration; only complex II was retained by the nucleus (Fang and Liao 1971). Two populations of high affinity DHT-binding protein have also been identified by Fiet and Muldoon (1983). They could be distinguished by their rates of association with steroid and the differential susceptability of the complexes to protamine sulphate precipitation. It was suggested that the interconversion of receptor forms was controlled by a "cytosolic

factor", which was ribonuclease resistant and precipitated by percholoacetate and (55-70%) ammonium sulphate (Feit & Muldoon 1983).

A second class of cytosolic and nuclear oestrogen binding sites (Type II) have also been described (Katzenellenbogen 1980). The cytoplasmic Type II sites had a 40-fold lower affinity for oestrogen than the classical receptor (Type I sites), with dissociation constants of 30nM and 0.8nM respectively: these sites were found in target tissues and at lower levels in other tissues. However, they were not translocated to the nucleus, and were thought to be a means of concentrating steroid in target cells. The Type II nuclear sites were not related to either Type I or II cytoplasmic proteins, and had a lower affinity for steroid compared to Type I sites (Kd=20nM and 0.6nM respectively) but were present at a much higher concentration: the phyisological role remained unclear (Katzenellenbogen 1980). More recently McNaught and Smith (1986) characterised a second oestrogen receptor species in the chicken oviduct; this receptor form (Y) had a slower rate of association than the higher affinity (X) form, and was apparently involved with increases in ovalbumin gene transcription (McNaught & Smith 1986; Raymoure, McNaught & Smith 1985).

Finally, Smith and Hermon (1985) using affinity labelling, immunoprecipitation and high resolution two-dimensional gel electrophoresis, were able to show at least two isoforms of the glucocorticoid receptor (binding protein) with isoelectric points of 5.7 and 6.0-6.5; analysis of crude cytosol revealed an add-

itional isoform with an isoelectric point of about 5.2. These species could be competed out when excess cold triamcinolone acetonide was included during the affinity labelling step: therefore charge heterogeneity was a feature of the structure, and possibly function, of the glucocorticoid receptor from the IM-9 lymphoid cell line.

D. Activation and Transformation of Receptor Complexes.

For the purposes of this discussion the term "activation" will be used to describe the conversion of the steroid-receptor complex into a form that bound tightly to nuclei in vivo, and was capable of binding to DNA-cellulose and translocating [^{3}H]steroid into target cell chromatin in vitro. The process could be mimicked in vitro by heating, increasing ionic strength, ammonium sulphate precipitation, gel filtration, ultracentrifugation, alkaline pH, dilution, ATP, and dialysis (Goidl et al 1977; Katzebnellenbogen 1980; Mainwaring and Irving 1973; Moudgil et al 1985; Munck and Foley 1979; Munck & Holbrook 1984).*

Posssible mechanisms for receptor activation that have been suggested include: dissociation of subunits and/or conformational changes (Bailly et al 1980; deBoer et al 1986a; Greenstein 1984; Higgins & Gehring 1978; Kovacs, Griffin & Wilson 1983; Mainwaring and Irving 1973; Milgrom, Atger & Baulieu 1973; Moudgil et al 1985; Muller et al 1983; Raaka et al 1985; Renoir & Mester 1984; Sato, Ohara-Nemoto & Ota 1986),** however, the dissociation of the 9.6S glucocorticoid receptor (236000-daltons) to the 4.6S (95500-daltons) form was reported by Weatherill and Bell (1982) to preceed activation; limited proteolysis (Puca et al 1977),

The following references have been omitted from the text:
Schmidt et al 1975 (*)
Holbrook et al 1983a; Raaka & Samuels 1983; Vedeckis 1983 (**)

although the conversion of the 8S oestrogen-receptor complex from calf uterus by an endogenous endopeptidase yielded a modified 4S form that was distinct from the native receptor (Gregory and Notides 1982); the action of cytoslic factor(s)(Goidl et al 1977; Noma et al 1980; Sato et al 1979; Thrower et al 1976); and finally because molybdate stabilised the unactivated complex and inhibited activation in a concentration dependent manner (Kovacs et al 1983), this was taken as circumstantial evidence for dephosphorylation being involved in receptor activation.

Activation of the androgen receptor has been associated with: changes in sedimentation coefficient (deBoer et al 1986a; Kovacs et al 1983; Mainwaring and Irving 1973); a shift to a more basic isoelectric point (Greenstein 1984; Mainwaring and Irving 1973); increased affinity for DNA-cellulose (deBoer et al 1986; Kovacs et al 1983; Mainwaring and Irving 1973); and an increased affinity for steroid (deBoer et al 1986; Kaufman and Pinsky 1983; Kaufman et al 1982a,b): the last has also been descibed for the oestradiol-receptor complex (Muller et al 1984). In a recent study Keenan et al (1986) found that activation of the human androgen receptor, from cultured fibroblasts, was accompanied by a decrease in molecular radius and a loss of negative charge, with a possible loss of a 20000-dalton macromolecular component. However, in a recent study, Smith and co-workers (Smith, Elasser & Harmon 1986) showed that the alteration of surface charge accompanying activation of the glucocorticoid receptor (IM-9 cells) was the result of a conformational change rather than a covalent charge modification.

Kaufman and co-workers from kinetic studies with the androgen receptor from cultured GSF, proposed a model for activation invoving three conformational states (Fig.1.3; Kaufman and Pinsky 1983; Kaufman et al 1982a,b). Dissociation of the activated complexes was normally monophasic, however in the presence of sodium thiocyanate, purified, DHT-receptor complexes dissociated with complex kinetics: it was suggested that this chaotropic salt was affecting the van der Waals forces and hydrogen bonding within the receptor molecule resulting in deactivation of the activated complexes and the observed complex dissociation kinetics (Kaufman et al 1982a,b). Interestingly sodium thiocyanate has also been shown to affect the activation of the rat hepatic glucocorticoid receptor (Kalimi and Hubbard 1982).

The term "transformation" has also been used to describe the above process; however, work by different groups suggests this term should be restricted to describing the oestrogen receptor system. Activation has been described for all classes of steroid receptor, but to date only the nuclear form of the oestrogen-receptor complex shows an increase in sedimentation coefficient (4S to 5S)(Yamamoto 1974). Subsequent work has shown that this transition was distinct from receptor activation (Bailly et al 1980; Muller et al 1983; Muller et al 1984). Activation of the oestradiol-receptor complex was first-order, and was stimulated by an increase in temperature or ionic stength; subsequent transformation of the activated complex followed second order kinetics, and involved either receptor dimerization (Muller et al 1983, 1984) or the interaction of a cytosolic protein "X" (Bailly

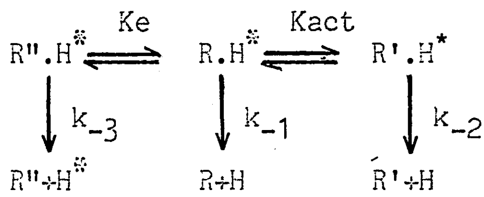


Figure 1.3 Postulated conformational status for the androgen receptor after binding steroid. Taken from Kaufman et al 1982b.

$R''.H^{*}$: dysactivated complex

$R.H^{*}$: preactivated complex

$R'.H^{*}$: activated complex

k_{-1}, k_{-2}, k_{-3}: dissociation rate constants

Kact: rate constant of activation

Ke: equlibrium constant

et al 1980; Gorski & Gannon 1976; Higgins & Gehring 1978; Yamomoto 1974).

Although the precise mechanism of receptor activation remains open to discussion, it is clear that it is an essential step leading to receptor-mediated changes in gene transcription. Transformation, on the other hand, has only been observed for the oestrogen receptor and the phyisiological significance remains unclear.

E. Steroid Receptor Interactions with Nulear Structures.

The main action of steroid hormones result from receptor mediated changes in gene transcription, in a tissue specific manner: regulation of gene expression by steroids can either be positive or negative (Chan & O'Mally 1976; Feigelson et al 1978; Jensen et al 1968; Jensen and deSombre 1973; Tymoczko et al 1978; Yamamoto 1985; Yamamoto and Alberts 1976).

The ability of steroids to act on the genome of target cells in this way, was elegantly demonstrated in the studies with the insect hormone ecdysone, reviewed recently by Dwarniczak et al (1983). This hormone controls insect growth and development; and effects were shown most dramatically on the giant polytene chromosomes from the salivary glands of Drosophilia larvae. Metabolic labelling experiments showed that the "chromosomal puffs" induced by hormone treatment were associated with gene transcription; it was subsequently demonstrated that this was a complex response, initially involving only a few genes (early puffs) and progressing, after a delay, to a much larger number

(late puffs); the latter appeared to be dependent upon the early puffs as shown by the use of inhibitors of protein synthesis (Dwarniczaak et al 1983).

The problem facing steroid-receptor complexes, and gene regulatory molecules in general, is how to find and interact with the appropriate target gene(s). In the field of steroid receptor research therehas been a considerable debate as to the existence and nature of nuclear acceptor sites for receptor complexes (Gorski and Gannon 1976; Tymoczko et al 1978; Yamamoto 1985; Yamamoto and Alberts 1976). A number of investigators have described the interaction of receptor complexes with nucleotide sequences (Birnbaun and Baxter 1986; Cato et al 1984; Chandler, Maler & Yamamoto 1983; Dean et al 1983, 1984; Eliard et al 1985; Giesse et al 1982; Groner et al 1984; Goner, Lakey & McBlain 1984; Jost, Seldran & Geiser 1984; Karin et al 1984; Kumar and Dikerman 1985; Lee et al 1984; Payvar at al 1983; Renkawitz et al 1982; Romanov et al 1984; Schreidereit et al 1983; von der Ahe et al 1985, 1986; Yamamoto 1985), chromosomal proteins (de Boer et al 1986b; Kaye et al 1986; Liao, LIang & Tymoczko 1972; Mainwaring, Syms & Higgins 1976; O'Mally et al 1972; Pratt et al 1984; Ruh et al 1986; Spelsberg et al 1983, 1984; Tanuma, Johnson & Johnson 1983; Webster, Pikler & Spelsberg 1976), the nuclear envelope (Jackson & Chalkley 1974; Lefebvre & Novosad 1980) and the nuclear matrix (Brown and Migeon 1986; Buttyan et al 1983; Gonor et al 1984), in an attempt to characterise possible nuclear acceptor sites.

DNA:recognition of specific nucleotide sequences.

Despite the experimental evidence that steroid receptors

could bind to DNA and polynucleotide sequences, the detection of specific DNA-binding sites was hindered by the masking effect of a large number of non-specific sites (Kumar and Dikerman 1985; Yamamoto 1985). This was resolved by enrichment of the putative (specific) binding sequences, initially by nitrocellulose filter binding (Riggs, Suzuki & Bourgeois 1970), and subsequently using competition assays, electron microscopy, immunoprecipitation and nuclease footprinting techniques (Yamamoto 1985).

Early studies focused on glucocorticoid control of mouse mammary tumor virus (MTV) gene expression as a model for receptor-DNA binding (Ringold et al 1983; Rousseau 1984). Payvar et al (1983) mapped five regions of MTV DNA that were bound specifically by purified glucocorticoid receptor; one site was upstream of the transcription start site, while the others were distributed within the transcribed sequence. Other studies have found that both the 40K and 90K molecular weight forms of the receptor bound restriction fragments including the right 400-500 nucleotides of the MTV-long terminal repeat (LTR)(Geisse et al 1982); a further two regions at positions -124 to -72 and -220 to -140 were shown to bind receptor, and were necessary for glucocorticoid control of transcription (Chandler et al 1983; Lee et al 1984; Scheidereit et al 1983; Rousseau 1984).

Receptor binding sites 5' to and within transcribed sequences of hormone regulated genes have subsequently been reported for the glucocorticoid receptor at the growth hormone, lysozyme, uteroglobin, metallothionein II_A and placental lactogen genes (Birnbaum and Baxter 1986; Cato et al 1984; Eliard et al 1985;

Karin et al 1984; Renkawitz et al 1982; von der Ahe et al 1985, 1986); for the progesterone receptor at the ovalbumin, transferrin, ovomucoid, lysozyme, and uteroglobin genes (Bailly et al 1983; Dean et al 1983, 1984; Renkawitz et al 1982; von der Ahe et al 1985, 1986; Yamamoto 1985); and for the oestrogen receptor at the ovalbumin, and vitellogenin genes (Dean et al 1984; Jost, Geiser & Seldran 1985; Jost et al 1984). Nuclease protection studies have identified directly the sequences bound by the glucocorticoid receptor within or near DNA encoding for MTV (Karin et al 1984; Payvar et al 1983; Scheidereit et al 1983; von der Ahe et al 1985), human metallothionein II_A (Karin et al 1984) and growth hormone, and chicken lysozyme (von der Ahe et al 1985) genes; from this work the consensus sequence 5'-$T/_{C}GGTA/_{T}CAA/_{T}TGTT/_{C}CT$-3' and related octanucleotide 5'-$AGAA/_{T}CAGA/_{T}$-3' and hexanucleotide 5'-TGTTCT-3' sequences have been described (Ringold et al 1983; Yamamoto 1985). However other sequences and/or factors must also play a part in receptor recognition since the above consensus sequence has been found in DNA not associated with receptor binding or steroid action (Yamamoto 1985). The functional and biological significance of the DNA-receptor binding sites observed *in vitro* was shown using gene fusion, the introduction of specific deletions, and gene transfection techniques. Putative hormone control sequences (with or without deletions) could be linked to selectable marker genes, not normally under hormonal control, and the hybrid gene(s) introduced into suitable host cell, where expression could be assayed under basal and hormone stimulation conditions. From such studies it became clear that the *in vivo*

"glucocorticoid response element"(GRE) and *in vitro* receptor binding sites were co-incident (Chandler et al 1983; Lee et al 1984; Payvar et al 1983; Romanov et al 1984). It was also found that the activity of the GRE was independent of distance and orientation from the transcription start site; this has led to the suggestion that receptor binding sites function as steroid-dependent enhancer elements, that act by providing a "bidirectional entry site" for the machinary of transcription (Karin et al 1984; Parker 1983; von der Ahe et al 1985; Yamamoto 1985). Enhancer elements were originally identified as short *cis*-acting regulatory elements that were capable of increasing the transcription efficiency of genes independently of their orientation and position relevant to the gene, in the DNA-tumor virus SV40 (Khoury & Gruss 1983). Enhancers have subsequently been found associated with a number of cellular genes in a tissue specific manner, the best characterised being the immunoglobulin gene enhancers (Boss 1983; Dunn and Gough 1984; Khoury and Gruss 1983; Voss, Scholkat & Gruss 1986). More recent research, reviewed by Voss et al (1986), has suggested that enhancer activity involves both *cis*- and *trans*-acting elements, which allows for the interaction of general and/or tissue specific factors.

Chromosomal proteins and alterations in chromatin structure.

In eukaryotes the genetic material is organised into chromatin, and as a result of several levels of packaging (10nm to 30nm fibres) with histones and non-histone proteins the DNA is inaccessible to the transcriptional apparatus, therefore the

structure of chromatin must differ at regions of gene activity. It was subsequently found that active genes are generally in regions of more open chromatin (euchromatin); and these regions were found to be preferentially digested with the endonuclease DNase I. This phenomenon was also found to be tissue specific since globin genes were nuclease-sensitive in erythroid tissue but not oviduct, and the reverse being true for the ovalbumin gene (Weisbrod 1982). Subsequently, Elgin and co-workers (Elgin 1981, 1983) showed that within regions of DNase sensitivity there were so called hypersensitive sites, thought to be generated by the binding of non-histone proteins; such sites at or near the 5' end of genes are beleived to indicate the potential for transcription of a particular locus.

Hypersensitive sites have also been correlated with steroid hormone regulated gene expression. Pratt et al (1984) found that oestrogen receptors bound to pre-existing nuclease-sensitive sites in uterus, lung, and kidney nuclei. Fritton et al (1984) reported that the pattern of hypersensitive sites upstream from the lysozyme promoter changed depending onwhether the gene was under constitutive or hormonal control. Furthermore, a recent report from Chambon and co-workers (Kaye et al 1986) described four regions of nuclease-hypersensitivity in the 5'-flanking region of the ovalbumin gene, that were also dependent upon steroid stimulation.

In addition to increased sensitvity to nuclease digestion, regions of active chromatin may contain non-histones proteins (especially "high mobility group" species HMG14 and HMG 17), modified histones and altered base structure (Weisbrod 1982).

HMG proteins are low molecular weight proteins with highly conserved and unusual amino acid sequences: evidence for their involvement with gene activity comes from fluorescent antibody studies, which showed "HMG-like" proteins associated with the chromosomal puffs of polytene chromosomes, and from the observation that erythrocyte chromatin depleted of HMG 14 and 17 was no longer preferentially sensitve to DNase-I digestion (Weisbrod 1982). HMG 14 and 17 are associated with active genes, at the end of the nucleosome core and internucleosome regions, where they are thought to replace histone H1 and thereby generate a more open structure (Weisbrod 1982). Tanuma et al (1983) observed that glucocorticoids caused a reduction in endogenous ADP-ribosylation of HMG 14 and 17; furthermore, they concluded that a loss of $(ADP\text{-}ribose)_n$ moieties from these proteins may play a role in MTV gene expression. Post-translational modification of histones (phosphorylation or acetlyation) may, similarly play a part in steroid action (Yamamoto 1985).

Although it is feasible that receptor complexes binding to "steroid responsive elements" upstream and/or within transcribed sequences could initiate the changes in chromatin structure discussed above, there is a strong opinion that non-histone proteins play a more direct role in receptor-acceptor interactions (de Boer et al 1986b; Gorski & Gannon 1976; Mainwaring et al 1976; O'Mally et al 1972; Pikler et al 1976; Ruh et al 1986; Spelsberg et al 1972, 1983, 1984; Webster et al 1976; Yamamoto & Alberts 1976). Extensive studies by Spelsberg and associates (O'Mally et al 1972; Pickler et al 1976; Spelsberg et

al 1972, 1983, 1984; Webster et al 1976) on a subfraction of nuclear acidic proteins (AP_3 or CP_3), extracted from avian oviduct nuclei, have concentrated on the role of this fraction in the nuclear binding of the progesterone receptor. Construction of "hybrid" chromatin with the acidic proteins from one tissue and the histones from another revealed that the ability to specifically bind receptor resided with the donor tissue of the acidic protein fraction. Furthermore, these acidic proteins were responsible for masking acceptor sites in non-target tissues and about 70% of the sites in target tissue as well. The reconstituted "native-like" acceptor sites also required specfic DNA sequences in addition to the nucleoacidic fraction (Spelsberg et al 1984). However, because of the technical difficulties inherent in this type of study, the above conclusions have been questioned (Yamamoto 1985; Yamamoto and Alberts 1976).

Mainwaring et al (1976) using a different approach to the above, immobilised nuclear components on a Sepharose 2B column, identified a non-histone, basic fraction showing apparent acceptor activity.

Finally, changes in chromatin structure have also been associated with modifications to the bases in DNA; in vertebrates the principal one being methylation of certain cytosine residues to 5-methylcytosine (Bird 1984,1986; Jahner et al 1982; Weisbrod 1982). Undermethylation at key CpG doublets has been associated with the 5'-end of a number of active genes (Bird 1984; Jahner et al 1982), and also with steroid stimulation of the ovalbumin (Mandel and Chambon 1979), vitellogenin (Burch and Wientraub 1983; Jost et al 1984, 1986; Saluz, Jiricny & Jost 1986; Wilks et

al 1982; Wilks, Seldran & Jost 1984), and prostatic steroid binding protein (component C3(1); Parker, Hurst & Page 1984)genes. This has led to the suggestion that demethylation may play a role in the control of gene expression. However transcription of X.laevis sperm rRNA was unaffected by heavy methylation of spacer and promoter regions (Bird 1984), and although the hypomethylation site at the 5' end of the chicken vitellogenin gene was co-incident with an oestrogen receptor binding site (Jost et al 1984), the demethylation of this site appeared to be an effect of gene transcription rather than the cause (Burch & Weintraub 1983; Wilks et al 1982,1984). The emerging picture, is that house-keeping genes are associated with clusters of CpG sequences in "C+G rich islands" that escape the normal suppression of this dinucleotide sequence by methylation as a result of bound "factors". De novo methylation of these regions occurs secondary to inactivation of the gene (loss of trans-acting factor(s) ?) and serves to reinforce the silence of the gene. These "G+C rich islands" are not associated with tissue specific genes, which it is suggested depend on tissue-specific factors to fufil a similar role, and the observed demethylation at such genes would be an effect of transcription and not the cause (Bird 1986; Mar 1984).

Nuclear matrix.

The nuclear matrix was first described by Berezney and Coffey (1974) as a residual protein skeleton after depletion of nuclear membrane phospholipids and chromatin from rat liver nuclei: it was 98.4% protein (5-10% of total nuclear protein)

consisting mainly of three acidic protein fractions, 0.1% DNA, and 0.5% phospholipid. It has been associated with DNA replication (Berezney & Coffey 1975; Pardoll, Vogelstein & Coffey 1980; Vogelstein, Pardoll & Coffey 1980), anchorage sites for DNA supercoiled loops (Vogelstein et al 1980), and actively transcribed genes (Ciejek, Tsai & O'Malley 1983; Robinson et al 1983). In view of the latter finding it is of interest that specific binding of oestrogens and androgens, which is tissue specific and sensitive to hormone manipulation, has been associated with this structure (Barrack & Coffey 1980). Furthermore, steroid-receptor complexes have also been isolated bound to this nuclear substructure (Brown & Migeon 1986; Buttyan et al 1983; Kaufman et al 1986; Rennie, Bruchovsky & Cheng 1983).

Androgen receptor-nuclear associations.

Androgen receptors have been found to bind to RNA (Gonor et al 1984; Mulder et al 1984), polynucleotides (Mulder et al 1984), DNA (Davies and Thomas 1984; Foekens et al 1985; Kandala, Kistler & Kistler 1985; Lin and Ohno 1981; Mulder et al 1984; Page and Parker 1983; Parker et al 1984), chromosomal proteins (Davies and Thomas 1984; Foekens et al 1985; Liao et al 1972; Mainwaring et al 1976), and the nuclear matrix (Brown and Migeon 1986; Buttyan et al 1983).

Clones of the gene(s) encoding the C3 component of the prostatic steroid binding protein and fusion genes containing the C3 promoter sequences have been successfully expressed in the androgen reponsive Shionogi 115 cell line (Page & Parker 1983; Parker et al 1984). However in a competition assay specfic

binding of the androgen receptor to C3 restriction fragments could not be demonstrated, and this was thought to be due to an absence of specfic binding sequences on the clones used and/or the loss of the DNA-binding domain from the receptor (Mulder et al 1984). Interestingly, the C3 gene and another androgen reponsive gene, encoding for seminal vesicle secretory protein IV, were found to share sequence homology from position -330 to -190 upstream of the main transcription start sites; the fuctional significance of this sequence for receptor binding and _in vivo_ expression of these genes was not determined (Kandala et al 1985).

The involvement of nuclear proteins in androgen-acceptor function was shown by the concomitant release of oligonucleosome fractions, RNA polymerase b and androgen receptors from the rat prostate nuclei; all three parameters were dependent upon steroid status (Davies & Thomas 1984). After _in situ_ chemical crosslinking of receptors to nuclear structures, with formaldeyde, Foekins and associates (Foekens et al 1985) observed that 18% of receptors could be released with micrococcal nuclease treatment, 74% with trypsin digestion and 97% when both were used together. The conclusion was that two classes of receptor binding site, involving either DNA or protein, were present in the rat prostate. However the significance of these putative acceptor sites to androgen action in this tissue was still to be investigated.

Conclusions.

In conclusion, this section has described the different interactions that have been observed between steroid-receptor

complexes and target cell nuclei. Yamamoto (1985) in an excellent review, has recently attempted to explain the many facets of steroid control of specfic genes, including the possible existance of gene domains (Schrader et al 1981),multi-factor control of a single gene, and tissue specfic gene expression. The model was based on receptor complexes binding to specfic steroid response elements ("modulatory" enhancer sequences), and the triggering of secondary _trans_-acting transcription factors which could act within gene-networks similar to those proposed by Britten and Davidson (1969). The role of specfic DNA sequences was central to this model, however the possible involvement of non-DNA component in receptor recognition was not ruled out, and it would seem prudent to assume that such structures do have a part to play in the steroid control of gene expresion. Finally, the studies of Jost and co-workers (Jost et al 1986) on the _in vitro_ secondary activation of the chicken vitellogenin gene ("memory effect") has suggested that other factors, in addition to receptor complexes, are necessary for gene transcription, since stimulation of the gene was only partly reduced by the addition of inhibitors of protein kinase II and calmodulin-dependent kinase (55%) or by removing oestrogen receptors (40%).

F. Control of Receptor Levels by Hormone.

The regulation of receptor concentration by the corresponding ligand could play an important role in controlling the cellular response to a given steroid. Glucocorticoids, oestrogens and progesterone have all been found to reduce

receptor levels by increasing the rate of receptor degradation. On binding glucocorticoids, the receptor from the GH_1 rat pituitary cell line dissociates from a 10S oligomeric structure to a 3-4S species, with a concomitant decrease in receptor half-life (19h to 9.5h) and a 50% reduction in receptor levels (McIntyre & Samuels 1985; Raaka & Samuels 1983). More recently, Okret et al (1986) using a cDNA clone of the rat glucocorticoid receptor, demonstrated that after treatment of rat hepatoma cells with steroid the receptor mRNA was reduced by 50-95%. This was independent of protein synthesis; and the levels of receptor message were restored, after 72 hours. Similarly a 50-70% reduction in oestrogen receptor levels has also been observed on steroid binding ("nuclear processing") (Eckert & Katzenellenbogen 1982; Horwitz & McGuire 1978); and the nuclear 5S receptor species was found to be rapidly turned over ($t_{1/2}$=2.25h)(Scholl & Lippman 1984), while dense amino acid and sedimentation analysis showed that receptor half-life was decreased in response to oestradiol (Eckert et al 1984). It was suggested that nuclear processing was necessary for oestogen induction of the progesterone receptor (Horwizt & McGuire 1978), however subsequent studies in MCF-7 cells (Eckert & Katzenellenbogen 1982) and rat uterus (Kassis, Walent & Gorski 1986) have shown that progesterone receptor levels can be stimulated in the absence of oestrogen receptor processing. Finally in the guinea pig uterus progesterone receptor levels were found to be under positive control by oestrogens and negative control by progesterone (Milgrom et al 1973).

In contrast to the above classes of steroid hormone, androgens have been found to increase the level of basal receptor

binding, without altering the affinity of binding (Kd) in cultured human GSF (Kaufman, Pinsky & Hollander 1981; Kaufman et al 1983; Ring & Hodgins 1984) and in the tumuor cell lines derived from rat prostate (DDT[1]MF-2) and hamster ductus deferens (R3327H-g8-A1)(Syms, Norris & Smith 1983; Smith, Syms & Norris 1984). In the tumuor cell lines the levels of receptor binding increased 2-fold within a 6 hour peroid. This increase was inhibited by glucocorticoids and apparently dependent upon protein synthesis (Smith et al 1984; Syms et al 1983). Using dense amino acid labelling to follow the degradation of existing receptor molecules, it was found that the receptor half-life was increased (3h to 6h) and that the rate of receptor synthesis was also increased (k:1.35 to 2.23fmoles/ug DNA/h)(Syms et al 1985). The increase in receptor binding in cultured GSF was also believed to be due to *de novo* synthesis of receptor protein (Kaufman et al 1981,1983). More recent studies by Ring and Hodgins (1984; Dr.Hodgins personal communication) support an alternative explanation for receptor "up-regulation", which can be explained solely on the basis of the increase in receptor half-life, such that on binding ligand, the receptor attains a more stable conformation.

1.2 Androgen Insensitivity

A. Hormone Resistance.

Hormone resistance or insensitivity has been defined as the inability of target tissues to respond to normal or elevated levels of circulating hormone. The first reported cases of end-organ resistance to a hormone were by Albright and associates for 1,25-dihydroxyvitamin D (Albright et al 1937) and the peptide parathyroid hormone (Albright et al 1942); the latter condition was described as pseudohypoparathyroidisim to distinguish it from hypoparathyroidism due to an absence of hormone.

End-organ insensitivity to steroid hormones has been describedfor nearly all classes: glucocorticoids (Lipsett et al 1985); mineralocorticoids (Cheek & Perry 1958; Oberfield et al 1979); vitamin D_3 (Marx et al 1984); progesterone (Keller et al 1979); and androgens (Wilson et al 1983). The absence of reported cases of inherited resistance to oestrogens is probably due to the essential role these hormones play in early foetal development, and any disruption of oestrogen action is therefore believed to be lethal. However, a special case of oestrogen resistance has been observed in certain breast tumours, whose growth becomes **independent** with respect to oestrogens and refractory to hormone therapy. It has been suggested that this may arise from an abnormality in oestrogen-receptor function (Romic-stojkovic & Gamulin 1980).

B. Male Sexual Differentiation and Andogen Insensitivity.

Resistance to androgens disrupts normal male sexual

development and results in the clinical condition of male pseudohermaphroditism; individuals with male genetic and gonadal sex differentiation who develop partially or completely as phenotypic females (Griffin & Wilson 1980; Griffin et al 1982; Hodgins 1983a; Wilson et al 1983). Insensitivity to androgens has also been described in normal appearing men with infertility (Amian et al 1979; Amian & Griffin 1982).

Mammalian embryos of both sexes differentiate in an identical fashion during the early stages of development (Wilson 1978). From animal experiments and genetic disorders (i.e. syndromes of androgen insensitivity) it was apparent that male differentiation had to be actively imposed on the indifferent gonads and urogenital tract at key stages of development to prevent passive differentiation of the female phenotype (Jost 1970, 1972; Wilson 1978). In the later stages of development, male differentiation of the indifferent urogenital tract (wolffian and mullerian ducts) was dependent upon two hormones secreted by the foetal testes (Jost 1970; Wilson 1978) (Fig. 1.4). The first, mullerian regression factor, a peptide hormone originating from the spermatogenic tubules, suppresses the development of the mullerian duct into the uterus and upper portion of the vagina (Wilson 1978). The second, testosterone, produced in the Leydig cells, acts indirectly as a prohormone and directly on the wolffian duct to give the epididymis, vas deferens and seminal vesicles (Siiteri & Wilson 1974; Wilson 1978). The conversion of testosterone to the 5α-reduced metabolite DHT was found to be a prerequisit for the development

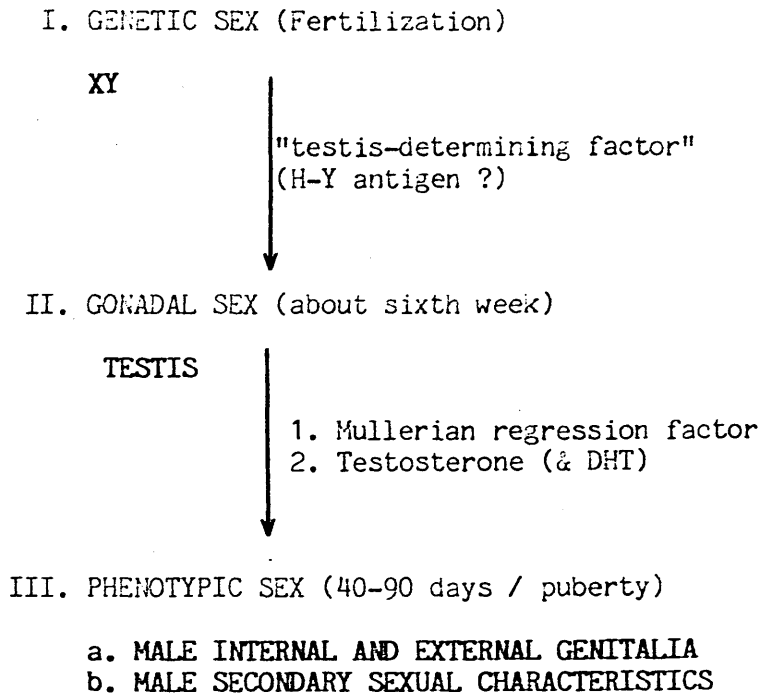


Figure 1.4 **Sexual differentiation in Man.** (Jost 1970,1972; Wilson 1978).

of the male external genitalia (Siiteri & Wilson 1974; Wilson 1978; Hodgins 1983b).

Therefore male sexual differentiation was an active process dependent upon the Y chromosome for gonadal differentiation, and on the hormonal secretions of the testes at subsequent stages.

Individuals with androgen insensitivity were characterised by the absence of mullerian duct structures, normal or elevated levels of plasma testosterone, and variable degrees of feminization at birth and puberty. The defect was associated with the target cell which was unable to respond to testosterone and/or DHT (Griffin & Wilson 1980; Griffin et al 1982; Pinsky 1978; Wilson et al 1983). The defects in target cells have been associated with mutations of the androgen receptor system or with the enzyme 5α-reductase.

C. Receptor Disorders.

Complete Androgen Insensitivity.

Complete testicular feminization has been described in Man, cattle, dogs, rats, and mice (Bardin et al 1970; Bullock & Bardin 1972; Chung et al 1983; McLean-Morris 1953; Ohno & Lyon 1970; Short 1967; Wieland & Fox 1979).

The disorder in man was first described by McLean-Morris (1953; McLean-Morris & Mahesh 1963), who used the term testicular feminization to describe patients with a female habitus and feminine breast development and body fat distribution. There were no internal genitalia except for undescended testes, the external genitalia were unambiguously female, and there was absent or scanty axillary and pubic hair growth in most cases. The testes

have been shown to respond to gonadotrophins and to synthesise steroids normally (Turksoy, Mitchell & Safaii 1976). Furthermore, 70% of 17β-oestradiol in normal males and 60% in patients with testicular feminization was found to be due to testicular secretion (McDonald et al 1980).

The aetiology of the disease was compatible with an X-linked recessive mutant allele or male sex limited autosomal dominant allele. The elegant studies of Migeon and co-workers (Migeon et al 1981), with human-mouse cell hybrids demonstrated that the locus for the androgen receptor was on the X chromosome, and that this was homologous to the Tfm locus in the mouse. Supporting evidence for the X-linked nature of the disorder comes from receptor studies on cultured genital skin fibroblasts from obligate heterozygotes, which showed receptor deficiency compatible with inactivation of the X-chromosome (Elawady et al 1983; Hodgins, Duke & Ring 1984; Meyer, Migeon & Migeon 1975).

Partial Androgen Insensitivity: Incomplete Testicular Feminization; Refenstein Syndrome; Infertile Male Syndrome.

The incomplete forms of androgen insensitivity have similar clinical and endocrine profiles to the complete syndrome, but can be distinguished from it and each other by the variable degrees of virilization seen at birth and puberty (Griffin et al 1984; Hodgins 1983a; Madden et al 1975; Wilson et al 1983).

D. 5α-Reductase Deficiency.

This condition (also refered to as incomplete male pseudo-hermaphroditism type 2 and pseudovaginal perineoscrotal

hypospadias: Pinsky 1978; Wilson et al 1983) was first described by Walsh et al (1974) and Imperato-McGinely and co-workers (Petterson et al 1977). The mutation affected the normal differentiation of the urogenital sinus and male external genitalia, resulting in ambiguity of the external genitalia although the general appearance was female at birth; wolffian structures differentiated normally and there were no Mullerian structures. At puberty there were varying degrees of virilization, which could lead to a male habitus and gender identity (Hodgins 1983a; Pinsky 1978; Wilson et al 1983).

The condition was inhereted as an autosomal recessive trait, and the primary defect was found to be in the conversion of testosterone to DHT (Griffin et al 1982; Hodgins 1983a; Lay, Pomberton & Hilton 1978; Moore, Griffin & Wilson 1975; Moore & Wilson 1976; Peterson et al 1977; Walsh et al 1974; Wilson et al 1983). The activity of the enzyme involved, 5α-reductase, has been studied in tissue biopsies (Hodgins 1983a) and in cultured GSF (Hodgins 1983a,b; Moore et al 1975; Wilson 1975). From such studies four classes of defect have been recognised: a reduction in the levels of the enzyme; decreased affinity for the substrate testosterone (Dallas/Dominican Rebublic); decreased affinity for the co-factor NADPH (Los Angeles); and finally a mutation affecting both testosterone and NADPH binding (Griffin et al 1982,1984; Wilson et al 1981).

E. Mutations of the Androgen Receptor.

Abnormalities of receptor function have been identified

across the whole spectrum of phenotypes described, reflecting the heterogeneous nature of androgen insensitivity in man (Amrhein et al 1976; Griffin et al 1984). The routine methods of investigating the stuctural and functional integrity of the androgen receptor protein have involved the measurement of whole cell and cell free binding of [^{3}H]steroid. The use of cultured human GSF has proved to be a useful model for studying androgen action and the mutations of androgen insensitivity, as these cells maintained their differentiated characteristics in culture and express high affinity (Kd=0.2-1.6nM) and low capacity (1250-18500 sites/cell) binding activity. The levels of binding were much lower in fibroblasts derived from non-genital skin biopsies, which made them unsuitable for binding studies (Brown & Migeon 1981; Griffin, Punyashiti & Wilson 1976; Griffin et al 1984; Hodgins 1983a; Hodgins et al 1984; Kaufman, Straisfeld & Pinsky 1977).

Using [^{3}H]androgen binding as a marker of receptor activity a number of defects have been identified and classified as quantitative, qualitative or receptor positive (Table 1.2). Quantitative defects have been further sub-divided into "absent", where the levels of binding are too low to be measured (Donti et al 1982; Evans, Jones & Hughes 1984; Griffin et al 1984; Keenan et al 1974; Sultan et al 1983), and "reduced" where receptor binding could be detected, but was significantly lower than control levels (Perieria et al 1984; Griffin et al 1984; Kaufman, Straisfeld & Pinsky 1976).

Qualitative defects have been associated with reduced and normal levels of receptor binding. Abnormalities in receptor

Table 1.2 Types of androgen receptor mutation recognised by steroid binding assays.

Type of Mutation	Description	References
QUANTITATIVE	Steroid binding absent	1-5
	Steroid binding reduced	1,6
QUALITATIVE	Thermolabile binding	7-9
	Instability of complexes	10-14
	Failure to activate*	15-18
	Failure to "up-regulate"	12,19,20
RECEPTOR POSITIVE	Apparantly normal receptor	1,21-23

References:

1. Griffin et al 1984
2. Donti et al 1982
3. Sultan et al 1983
4. Evans et al 1984
5. Keenan et al 1974
6. Periera et al 1984
7. Griffin 1979
8. Brown et al 1982
9. Coulam, Graham, & Spelsberg 1984
10. Griffin & Durrnt 1982
11. Wilson et al 1974
12. Jukier et al 1984
13. Pinsky et al 1984
14. Pinsky, Kaufman & Chadley 1985
15. Kaufman et al 1982a
16. Eil 1983
17. Kovacs et al 1984
18. Kovacs et al 1983
19. Kaufman et al 1981
20. Kaufman et al 1983
21. Collier, Griffin & Wilson 1978
22. Gyorki et al 1983
23. Amrhein et al 1976

*: failure to generate DNA-binding form _in vitro_.

Summary of the findings of receptor binding studies on intact cells or isolated receptor complexes. All studies used cultured human GSF, except Evans et al (1984) and Coulam et al (1984) who used dispersed fibroblasts and gonadal tissue respectively.

structure were subsequently revealed by kinetic and functional criteria, and have included: thermolability of ligand binding when the assay temperature was raised to 42°C (Brown et al 1982; Coulam et al 1984; Griffin 1979); general instability of receptor complexes, manifested as an increased Kd (decreased affinity) and/or dissociation rate, and failure to form an "8S" peak on sucrose density gradients in the presence of molybdate (Griffin & Durrant 1982; Jukier et al 1984; Pinsky et al 1984, 1985; Wilson et al 1974); failure to activate to the DNA-binding form (Eil 1983; Kaufman et al 1982a; Kovacs et al 1984); and finally failure to "up-regulate" basal receptor levels in response to steroid (Evans & Hughes 1985; Jukier et al 1984; Kaufman et al 1981, 1983,). A defect in one or more of the above parameters was taken as evidence for an underlying structural abnormality of the receptor molecule, as a result of a mutation at the X-linked receptor locus.

Recent studies by Kaufman and co-workers (Jukier et al 1984; Kaufman et al 1983, 1984; Pinsky et al 1984, 1985) investigating apparent binding affinity (Kd), dissociation rates of steroid-receptor complexes, and augmentation of receptor levels in response to steroid, deserve a special mention, since the receptor defect in different kindreds with partial androgen insensitivity was found to be apparently ligand specific. Abnormalities in all three of the above parameters of steroid binding were expressed with both DHT and the synthetic androgen methyltrienolone (R1881) (Kaufman et al 1984), or with DHT alone (Pinsky et al 1984, 1985). In one kindred only up-regulation with

DHT was impaired (Kaufman et al 1983). Finally the receptor from one individual had an elevated Kd, failed to up-regulate basal levels, but had normal rates of dissociation for both DHT and R1881 (Jukier et al 1984). The defects expressed with DHT alone were apparently not due to excessive metabolism of this ligand by cultured GSF. These findings were interpreted in terms of the kinetic model of receptor activation descirbed previously (Fig. 1.3, Introduction 1.1D). The mutation(s) was believed to affect the time and steroid concentration dependent transformation of low affinity complexes to high affinity state(s), which was necessary for mediating the up-regulation of recptor binding and presumably for the <u>in vivo</u> responses to androgens.

Despite the obvious heterogeneity seen between receptor binding activity and phenotype abnormality, it can be generalised that unmeasurable binding was associated mainly with the complete testicular feminization phenotype, while reduced binding and qualitative defects were found in a spectrum of phenotypes ranging from female to male (Griffin et al 1984).

F. Receptor Positive Resistance.

Receptor positive resistance has been associated with all abnormal phenotypes, and was characterised by apparently normal receptor binding activity (Griffin et al 1982, 1984; Hodgins 1983a; Wilson et al 1983). The condition was first described by Amrhein et al (1976) in certain patients with complete testicular feminization and apparently normal uptake and receptor binding of steroid. Collier et al (1978) described two unrelated patients with androgen insensitivity but normal 5α-reductase activity,

whole cell DHT binding, and normal nuclear up-take.

Although in most cases the nature of the mutation was unknown, it was assumed to occur at a post-receptor binding site (Griffin et al 1982; Hodgins 1983a). It is of interest therefore, that Funder and co-workers (Gyorki et al 1983) have described three cases of androgen insensitivity where the defect appeared to lie with the nuclear acceptor site. The three patients were described as "nuclear transfer deficient" on the basis of abnormal nuclear localization of complexes, despite normal whole cell receptor levels and normal intracellular distribution of glucocorticoid receptors in parallel experiments. The conclusion that the defect resided with the nucleus rather than with the receptor protein was based on the evidence of reconstitution experiments, where cytosol fractions (+labelled receptor) were mixed with "naive" nuclei, with only the combination of mutant cytosol/control nuclei giving a similar distribution to control combinations.

The exisitance of receptor positive mutations would be highly suggestive of the involvement of additional factors in androgen (steroid hormone) action; however the failure to detect abnormalities in receptor activity may simply reflect the limitation of steroid binding assays used.

G. Hormone Resistance to Other Classes of Steroid Hormone.

Defects in steroid receptor systems of other classes of steroid hormone have also been identified by ligand binding assays.

Vitamin D_3.

End-organ resistance to 1,25-dihydroxy vitamin D or vitamin D-dependent rickets is associated with hypocalcemia and secondary hypoparathyroidism, and more severly affected individuals also exhibit alopecia (Marx et al 1984). Since the classical target tissues for 1,25-dihydroxy vitamin D were inaccessible for *in vitro* studies, cultured skin fibroblasts were found to be a suitable model system for studing the action of 1,25-dihydroxy vitamin D in these patients (Eil & Marx 1981; Simpson & DeLuca 1980). As with the mutations of the androgen receptor described above, four classes of defect associated with the 1,25-dihydroxy vitamin D-receptor have been described: receptor negative (unmeasurable); receptor deficient; qualitative defects affecting the interaction of receptor complexes with the nucleus; and receptor positive (Castells et al 1986; Eil et al 1981; Hirst, Hochman & Feldman 1985; Liberman, Eil & Marx 1983; Liberman et al 1986; Marx et al 1984).

Interestingly, monoclonal antibodies raised against the chicken intestinal receptor have identified a 3.7S protein irrespective of the hormone binding status (Pike et al 1981). This is further evidence for the limitation of ligand binding studies in the identification of structural mutations of receptor molecules.

Glucocorticoids.

Resistance to glucocorticoids was described initially in certain mouse lymphoma cell lines, which were found to become refractory to the lethal effects of glucocorticoids. The

insensitivity phenotype was found to be associated with defects of the glucocorticoid receptor: "receptorless" (r^-); "nuclear transfer deficient" (nt^-); and "increased nuclear transfer" (nt^i) (Bourgeois & Gasson 1985; Gehring & Tomkins 1974; Sibley & Tomkins 1974; Yamamoto, Stampfer & Tomkins 1974).

Steroid binding, immunoprecipitation, and cloning studies have shown that the wild-type and nt^- phenotypes were associated with a 6kb transcript coding for the 94K receptor protein, while r^- and nt^i cells both contained reduced levels of this 6kb transcript thought to code for a non-functional 94K receptor protein. Inaddition the nt^i phenotype was associated with a 40K steroid binding protein believed to be coded by a specific 5kb transcript (Miesfeld et al 1984; Gehring & Tomkins 1974; Sibley & Tomkins 1974; Westphal et al 1984; Yamamoto et al 1974). Furthermore, recent work by Miesfeld and co-workers (Miesfeld et al 1986) demonstrated that sensitivity to glucocorticoids in r^- cells could be restored by transfecting cDNA coding for the glucocorticoid receptor.

Primary cortisol resistance has recently been reported in Man (Chrousos et al 1983a; Lipsett et al 1985). The condition was characterised by elevated plasma cortisol levels and the absence of the stigmata of Cushing's syndrome. Steroid binding studies in intact mononuclear leukocytes and cultured skin fibroblasts showed normal levels of glucocorticoid receptors with a reduced affinity for ligand, however in cytosol binding assays the levels of receptor were also reduced suggesting instability in ligand binding. Other parameters of receptor structure, such as thermal stability, heat activation, and molecular mass after affinity

labelling, all appeared normal (Chrousos et al 1983a,b; Lipsett et al 1985). In lymphocytes, from these same patients, transformed with Epstein-Barr virus the levels of induced receptor and affinity for steroid were both reduced compared to control cultures (Tomita et al 1986). In an other kindred, Iida et al (1985) reported a patient who had a 50-60% reduction in the levels of receptor in mononuclear cells, the affinity of the remaining binding sites was normal.

Progesterone.

In contrast to the above forms of hormone resistance, Keller et al (1979) reported a patient who presented with infertility apparently due to a localised resistance to progesterone. In vitro studies suggested that the underlying cause was a reduction in the number of progesterone receptors in the endometrium, the remaining sites had a similar affinity for steroid as controls.

H. Hormone Resistance in New World Primates.

New World Primates, such as Squirrel monkey and Common Marmoset, have relatively high levels of circulating steroid hormones compared to Old World Primates, such as Cynomologous, and Man; leading to the suggestion that the New World Primates have a generalised resistance to steroid hormones, and may therefore serve as a suitable model for studying steroid hormone insensitivity in Man (Lipsett et al 1985). Table 1.3 summarises the results of steroid binding and metabolism studies carried out on New World Primates compared to Old World species.

It has been suggested that the elevated levels of

Table 1.3 Receptor defects associated with hormone resistance in New World primates (comparison with Old World species).

Hormone	Receptor Levels	Binding Affinity	Other Defects	References
Aldosterone	Reduced	Similar		1
Androgens	Reduced?	Similar	5α-reductase activity reduced	2
Cortisol	Similar	Reduced		2
Oestradiol	Reduced	Similar		4
Progestins	Reduced	Similar		3,4,5
Vit.D	Reduced	Similar	Low DNA-binding*	6,7

(*, Binding of receptor complexes to DNA-cellulose)

References:

1. Chrousos et al 1984b
2. Lipsett et al 1985
3. Chrousos et al 1982
4. Chrousos et al 1984a
5. McClusky et al 1984
6. Shinki et al 1983
7. Takahahi et al 1985

circulating steroid hormones in these species was an evolutionary adaptation to changes in receptor function and/or steroid metabolism.

1.3 Aims

The broad aims of this work were to explore new methods of investigating the molecular mechanisms of androgen insensitivity, in the cultured human GSF model.

Androgen insensitivity has been shown to be associated with abnormalities of the androgen receptor by [^{3}H]steroid binding assays in GSF. There is, however, a need to study the receptor protein independently of steroid binding. Therefore in the absence of specfic antibodies to the androgen receptor and of cDNA probes for the receptor gene, variants of the androgen receptor have been searched for by combining two-dimensional gel electrophoresis with a dual-labelling technique and partial purification of the receptor.

Following the electrophoretic studies it was decided to attempt to covalently link a marker to the receptor, thereby allowing direct analysis of the receptor molecule (via the steroid-binding domain) under denaturing conditions. Studies were therefore undertaken with the conjugated synthetic androgen Methyltrienolone (R1881), which was deemed a suitable ligand for photoaffinity labelling of the fibroblast receptor.

Finally, since some degree of purification was essential to both the above approaches, extraction and partial purification of "functional" receptor complexes allowed further characterisation of the androgen receptor from control and androgen insensitive cell lines by sucrose density gradients and chromatographic techniques.

1.4 Introduction to Methods Used

This section describes the rationale behind the approaches taken during this project.

A. Double-label Autoradiography and Two-dimensional Gel Electrophoresis.

The use of the double-label autoradiography technique (Lecocq, Hepburn & Lamy 1982) allowed proteins metabolically labelled with either [^{35}S]methionine or [^{75}Se]selenomethionine to be mixed and resolved concomitantly by two-dimensional gel electrophoresis (2-DGE). This had the advantage that variations between gels and/or running conditions were avoided when comparing complex protein patterns from control and androgen insensitive fibroblast cultures.

Both sets of labelled protein were detected by fluorography (film exposed, -80°C) while only the [^{75}Se]selenomethionine labelled proteins were picked up by subsequent autoradiography (film exposed room temperature) of the same gel, the light and β-emmissions from ^{35}S being screened out. The strategy adopted involved labelling control cells with [^{35}S]methionine and androgen insensitive cells with [^{75}Se]selenomethionine; the optimum conditions for detecting possible mutant receptor proteins. A spot missing from the autoradiograph would suggest a lack of receptor synthesis, while a shifted spot due to a size and/or charge change would be indicative of a structurally abnormal receptor. This latter conclusion would have to be confirmed experimentally by reversing the labelling strategy

outlined above. Furthermore, the technique is also suitable for studying the effects of hormonal manipulation on newly synthesised fibroblast proteins, and it should therefore be possible to identify androgen dependent or regulated proteins by comparing normal and androgen insensitive cultured GSF.

2-DGE was the ideal technique for the type of study undertaken, as it exploits differences in charge (first dimension) and size (second dimension) to give high resolution of complex protein mixtures (O'Farrell 1975; O'Farrell, Goodman & O'Farrell 1977). The types of protein mutation that can be discriminated by this procedure fall into three groups:

1. No protein synthesised.
2. Protein synthesised in abnormal amounts.
3. Structurally abnormal protein:
   a) charge shift, b) size shift.

As was discussed in Section 1.2C, [^{3}H]ligand binding assays have highlighted abnormalities in receptor levels suggestive of 1 and 2 above. Although qualitatively or structurally abnormal receptor forms have also been identified by ligand binding studies, there has been no direct evidence to show that the mutation resulted in a charge or size variant. However, by considering the genetic code it has been estimated that one third of all point mutations, the most frequent type of mutation, will result in a charge change (Harris 1983). Furthermore, analysis of normal and variant forms of the glucocorticoid receptor has shown the potential for changes in steroid receptor size.

Gustaffson and co-workers (Wrange and Gustaffson 1978; Carlstedt-Duke et al 1982) using limited proteolysis showed that the glucocorticoid receptor could be selectively cleaved into

discrete domains (Fig.1.2a). It is therefore not difficult to imagine a mutation affecting an inter-domain region, rendering the receptor protein more susceptible to partial or complete proteolytic digestion, which in turn would give rise to an unstable and/or size variant of the normal protein.

Direct evidence for the existence of size variants of the glucocorticoid receptor came from studies on glucocorticoid-insensitivity clones of the mouse lymphoma cell line, S49.1 (TB4.1A). These hormone resistant variants fall into one of three phenotypes: "receptorless" (r^-); "nuclear transfer deficient" (nt^-); or "increased nuclear transfer" (nt^i) (Gehring & Tomkins 1974; Sibley and Tomkins 1974; Westphal et al 1984; Yamamoto et al 1974). The latter mutation has been found to contain a hormone-binding species (40000-daltons), which does not react with monoclonal antibodies raised against wild-type receptor (94000-daltons); however a 94K protein can be pulled out using an immuno-competition assay (Westphal et al 1984). This latter protein was believed to be a defective receptor protein, that was unable to bind hormone, common to the parent S49.1 wild-type cells which were known to be hemizygous for the glucocorticoid receptor (Westphal et al 1984). These findings were confirmed by the studies of Miesfeld et al (1984) and Northrop, Danielson and Ringold (1986). Characterization of the glucocorticoid receptor gene and mRNA in wild-type and mutant cells revealed that the receptor was encoded by a single-copy gene which specified a 6kb transcript in rat and mouse cells. Furthermore it was suggested that the 40K nt^i receptor was encoded for by a nt^i-specific

transcript, while reduced levels of the 6kb mRNA coded for the non-functional 94K protein.

It was concluded from the above that both charge and size mutations of the androgen receptor were likely to exist, and that the combination of dual-labelling and 2-DGE had the potential to discriminate between normal and variant receptor polypeptides.

Finally, it is of interest that apparent differences in the whole cell 2-D protein patterns from control and androgen insensitive cells have already been observed. Funder and co-workers (Risbridger et al 1982; Warne et al 1983) reported two proteins (45000- and 85000-daltons, pI ~5) that were apparently more prominant in control cultures, a subsequent study using non-equlibrium pH gradient electorphoresis (NEPHGE) in the first dimension, revealed a third protein (41000-daltons, pI ~6) which was again more prominant in contol cells.

The second study was initially concerned with identifing mutant proteins in Duchenne Muscular Dystrophy (DMD) fibroblasts (Rosenmann et al 1982). However a 56K dalton protein thought to be absent from DMD cells was subsequently shown to be specific for biopsy site, and was found only in GSF (Thompson et al 1983). Furthermore this protein was apparently absent from fibroblast cultures derived from androgen insensitve patients (Wrongeman et al 1984). The relationship between this protein and the androgen receptor remain to be determined, it may be the receptor, a pool of pro-receptor molecules, or a receptor mediated protein.

In conclusion, variants of the androgen receptor in androgen insensitive GSF have been searched for by combining 2-DGE with a dual-labelling technique, and partial purification of the

receptor. The latter was believed necessary because of the low abundance of the androgen receptors in GSF. By assuming a relative molecular weight of 100000-daltons and a basal level of 50fmoles/mg cell protein (34.0±10.1fmoles/mg protein (mean±SD, n=15; Hodgins et al 1984) it was esitmated that the receptor would represent only 0.0005% of the total cell protein at best.

B. Photoaffinity-labelling of the Fibroblast Androgen Receptor.

Photoaffinity labelling of steroid receptors has been successsfully achieved for the avian progesterone receptor (Dure, Schrader & O'Malley 1980; Horwitz & Alexander 1983), the glucocorticoid receptor (Nordeen et al 1981), and the androgen receptor (Brinkmann et al 1985b, 1986). In the absence of poly- or monoclonal-antisera to the receptor, the covalent linking of a radiolabelled ligand to the receptor would be of considerable advantage, allowing analysis of the receptor protein under denaturing conditions.

Although the use of the synthetic steroid methyltrienolone (R1881) as a photoactive ligand has been questioned, because of the inefficiency of the reaction (Mainwaring and Randall 1984) and the tendency of the ligand to self-polymerise on U.V.-irradiation (Williams et al 1986), Brinkmann and associates have been successful in covalently labelling the androgen receptor from a number of sources, including rat prostate (46K-daltons), a human prostatic carcinoma cell line (50K-daltons), and calf uterus (95K-daltons) (Brinkmann et al 1985b, 1986). The success of these studies can be attributed to partial purification of the

androgen receptor and to adequate control of non-receptor binding of [^{3}H]R1881 before attempting the irradiation and SDS-PAGE analysis.

The two strategies adopted in attempting to photoaffinity label the androgen receptor from human GSF were: U.V.-irradiation of [^{3}H]R1881-receptor complexes after partial purification, or _in situ_ U.V.-irradiation of receptor complexes followed by extraction and partial purification. Subsequent analysis involved either SDS-PAGE or High Performance Liquid Chromatography (HPLC)-gel filtration.

The main disadvatage of the photoaffinity labelling procedure was thought to be the inefficiency of the photo-linking reaction. Brinkmann et al (1985b) estimated an efficiency of about 0.2%, while Horwitz and Alexander (1983) reported an efficiency of 15% for the _in situ_ photo-labelling of the nuclear progesterone receptor; a ten-fold increase over the earlier study by O'Mally and co-workers (Dure et al 1980). It was essential therefore, that sufficient starting material was used and that some degree of purification was included in the protocol. Therefore, confluent cultures were incubated with [^{3}H]steroid for 24 hours prior to irradiation to stimulate the levels of androgen receptor (See Section 1.1F: Kaufman et al 1981; Ring and Hodgins 1984 & unpublished observations; Syms et al 1985), and receptor complexespartially purified by ammonium sulphate precipitation and/or anion exchange chromatography.

C. Miscellaneous.

Fast Protein Liquid Chromatography (FPLC) anion exchange on Mono Q column and 2'5'-ADP-Sepharose chromatography were used as possible purification steps. The latter was also used to compare receptors from a control and androgen insensitive cell line.

HPLC-size exclusion chromatography and sucrose density gradient analysis were used to determine some physical paramaters for the fibroblast androgen receptor; and as a possible means of detecting subtle differences between normal and variant forms of the receptor.

METHODS
&
MATERIALS

2.1 Chemicals.

All chemicals used were of AR or BIOCHEMICAL grade, except for acrylamide, agarose, NN'-methylenebisacrylamide (ELECTRAN), and urea (ARISTAR), and supplied by BDH chemicals unless otherwise stated; a full list of names and addresses of suppliers is given in Appendex 5.1.

2.2 Cell Culture.

Fibroblast cultures derived from genital skin biopsies (Hodgins 1982), were routinely grown as monolayers in bottles, flasks or petri dishes, in Eagles Minium Essential medium (Glasgow modified; MEM) supplemented with 10% newborn calf serum and penicillin (100units/ml) and streptomycin (0.1mg/ml): EC10 medium. Cells were kept at 37°C in a humidified, 5% CO_2/air environment.

When cultures reached confluence, a solution of trypsin(0.25%):EDTA (0.2g/l) (1:5, by volume) was used to detach cells from culture flasks. The action of the trypsin was subsequently stopped by the addition of EC10 medium, and the cells seeded in clean, sterile, culture dishes as required.

Medium, serum, antibiotics and other reagents were obtained from GIBCO; through the Biochemistry Department of Glasgow University. Plastic culture flasks (80cm^2), petri dishes (60mm, 100mm, 140mm diameter) and 24 well plates were supplied by NUNC (Inter Med), Falcon (Div. Becton Dickinson and Co.) and COSTAR.

2.3 Whole Cell Binding Studies.

A. Receptor concentration (Bmax) and Equilibrium dissociation constant (Kd) (Hodgins 1982; Hodgins et al 1984).

Whole cell binding studies wereunder taken as previously described. Briefly, $2x10^5$ cells were seeded in 60mm plastic dishes and grown to confluence. Cultures were then placed in MEM + 1% newborn calf serum (BC1) for 24 hours prior to incubation for 30 minutes at 37°C with 5ml of serum-free medium containing [^{3}H]-DHT or -mibolerone at concentrations ranging from 0.05 to 3.20nM (Total binding, B_T). A parallel set of cultures contained [^{3}H]steroid with an excess of unlabelled ligand, to give the level of non-specific binding (B_N). The cell monolayers were subsequently washed extensively with Dulbecco's phosphate buffered saline (PBS), harvested, centrifuged down and extracted with chloroform:methanol (1:1, by volume). Extracts were then assayed for cell bound radioactivity (supernatant) and for protein (pellet). From linear plots of specificly bound [^{3}H]steroid (B_S=B_T-B_N) versus Bs/free radioactvity (Scatchard 1949), the concentration of receptors (Bmax) and the equilibrium dissociation constant (Kd) were determined.

B. Rate of dissociation of androgen-receptor complexes (Hodgins 1982).

Dishes of cells were prepared and incubated at 37°C for 30 minutes with MEM containing 1nM [^{3}H]-DHT or -Mibolerone ± 1000-fold excess of cold ligand. The medium was then removed and replaced with MEM containing 1000nM-unlabelled steroid, and the incubation continued at 37°C; dishes were removed (in triplicate)

at different times for determination of cellular bound radioactivity. B_S was calculated as above. By plotting the logarithm of the ratio of B_S (time t)/B_S (time 0) against time, it was possible to calculate the half-life of steroid-receptor dissociation.

C. Augmentation of androgen receptor binding (Kaufman et al 1981; Ring & Hodgins 1983; Rowney & Hodgins 1985).

Dishes of cells were grown to confluence in EC10 medium and incubated at 37°C for 24 hours with EC1 medium containing 3nM [^{3}H]-DHT or -mibolerone ± an excess of unlabelled ligand. Cellular bound radioactivity was measured as described above, and B_S was calculated by subtracting B_N from B_T. The basal level of receptor binding was measured in parallel cultures incubated with 3nM [^{3}H]steroid ± unlabelled ligand for 30 minutes, with no previous exposure to androgens.

2.4 Receptor Preparation.

All preparative procedures were carried out at 0-4°C, unless otherwise stated. Extraction and partial purification of androgen receptor complexes was followed by labelling _in situ_ with a [^{3}H]steroid: 5α-dihydro[1,2,4,5,6,7,-^{3}H]testosterone (100-150Ci/mmol; Amersham) (DHT) or the synthetic androgens 7α[17α-^{3}H]-dimethyl-19-nortestosterone (70-85Ci/mmol; Amersham) (Mibolerone) or [^{3}H]17β-hydroxy-17α-methylestra-4,9,11-triene-3-one (86Ci/mmol; Du Pont)(Methyltrienolone or R1881). Confluent cultures were placed in EC1 medium for 24 hours, before being incubated with 1nM [^{3}H]steroid, for 30 to 40 minutes at 37°C. Cultures were then placed on ice and the cell monolayers washed

twice with PBS to remove free steroid. The cells were then scraped off in PBS and collected by centrifugation at 3000xg for 10 minutes (8x50ml fixed angle rotor, Hi-spin 21; MSE).

The pelleted cells were then disrupted by sonication (Ultrasonic Processor W-375) in PEM buffer [10mM-KH_2PO_4, 1mM EDTA, 12mM-monothioglycerol], pH7.4, containing 500mM-KCl. During sonication samples were placed in a Cup-Horn (431B) and kept on ice and subjected to 3x10-30 second bursts (at 50 cycles/full power) with 30 seconds cooling periods. Alternatively cells were broken up by 20 stokes with a hand-homogeniser (2ml Tissue grinder Dounce/Pestle) and osmotic shock (PEM buffer), and the salt concentration adjusted to 500mM-KCl. All buffers contained the protease inhibitors phenyl methyl sulphonyl fluoride (0.1mM; PMSF) and leupeptin (5uM). From the study of Kovacset al (1984) leupeptin seems to be particularly good for androgen receptor recovery. Nuclear bound receptors were extracted using high salt (500mM-KCl), and the 105000xg (1 hour:Type 65 rotor; Beckman L8-55 ultracentrifuge) salt extract prepared. In a preliminary experiment this extract was fractionated with solid ammonium sulphate as follows: 0-15%, 15-30% and 30-45% saturation (0°C). In all subsequent protocols this salt extract was brought to 35% saturation with 0.194g $(NH_4)_2SO_4$ per ml (Data for Biochemical Research), and left for 30 minutes on ice. The precipitated proteins were collected by centrifugation (50000xg for 10-15min.) and resuspended in the appropriate buffer.

Radioactivity remaining in the 105000xg cell pellet was investigated further for specfic binding that could be resistant

to salt extraction (Clark & Peck 1976; Davies 1983; Kaufman et al 1978). The pellet was resuspended in PEM buffer (+500mM-KCl) and re-sonicated as before, and centrifuged at 105000xg for 1 hour. This second pellet was resuspended in PEM buffer and extracted with 0.5%(v/v) Triton X-100 for 5-10 minutes, and the 105000xg supernatant prepared. These steps were repeated with DNAase I (50ug/ml) and trypsin (3mg/ml) digestions of successively pelleted material. The supernatant fractions were assayed for total and bound radioactivity recovered.

To give a quantitative estimate of non-specific binding the androgen precursor [^{14}C]dehydroepiandrosterone (57mCi/mmol; Amersham)(DHA) was added to the cell extract during sonication or homogenisation, to a final concentration of between 25-100nM. The rationale for using DHA as a means of showing the level of non-specfic binding was based on the following assumptions: 5α-DHT and DHA, because of similarities in structure and polarity (i.e. very similar elution characteristics on paper and thin layer partition chromatography (Hodgins 1971)), would show a similar degree of non-spcific binding; non-specfic binding would be a linear function of steroid concentration, therefore the fraction of DHA bound would be equivalent to the fraction of DHT non-specificly bound; and finally, DHA would lack competition for the androgen receptor (Shain & Boesel 1975). If the latter did not hold then the degree of non-specific binding would be over estimated. However, by following the proportions of ^{3}H and ^{14}C during the subcellular fractionation it was possible to determine the degree of specific binding in the 35% ammonium sulphate precipitate.

2.5 Sucrose Density Gradient Analysis.

Sucrose gradients were layered by hand from the bottom of the tube, starting with the lowest density of sucrose (5, 10, 15, 20%); the final gradient (4x1.6ml) approximately half-filled the centrifuge tube (Ultra-clear, 14x95mm; Beckman). The gradients were allowed to stand at room temperature for 2-3 hours to equilibrate, and then cooled (0-4°C). Just prior to sample loading the remainder of the tube was filled, carefully, with 5ml of PEM buffer (+500mM-KCl), lightly coloured with bromophenol blue to show the gradient/buffer interface. The sample (0.2-0.5ml) containing 0.5mg of a fluorescein isothiocyanate conjugated sheep antihuman IgG (Scottish Antibody Production Unit) (FITC-IgG) as an internal marker and 2-3%(w/v) sucrose was loaded at the interface between the gradient and the buffer overlay solution using a syringe with a piece of teflon tubing attached to the needle. A second gradient containing the marker proteins FITC-IgG (6-7S) and either bovine serum albumin (BSA)(4.6S) or ovalbumin (3.6S) was prepared in the same way. Gradients were routinely centrifuged at 30000rev/min (SW40 Ti rotor; Beckman) for 18 to 20 hours (4°C), and subsequently fractionated from the bottom (Beckman fraction recovery system); eight drop fractions were assayed directly for radioactivity or for protein markers.

The formation of a linear gradient at room temperature was confirmed by using the dye bromophenol blue. Proportionate amounts of the dye were added to the sucrose stock solutions prior to layering the gradient. The gradient was left for two

hours at room temperature and fractionated as above. The amount of dye in each fraction was measured at OD_{600nm} and related to the % of sucrose from standard readings (Appendix 5.2).

2.6 2'5'-ADP-Sepharose Chromatography.

Proteins precipitated by 35% ammonium sulphate were resuspended in 2ml of PEM buffer containing 10mM-KCl (low ionic strength buffer). 0.375g of 2'5'-ADP-sepharose 4B (Pharmacia fine chemicals) was reconstituted with the same buffer to give a 1.5ml slurry (1g reconstituted approximately 4ml gel; about 2 mol 2'5'-ADP/ml:Pharmacia). The resuspended ammonium sulphate fraction and the gel slurry were mixed and dialysed against PEM (+10mM KCl) buffer for 3 to 4 hours at 4°C, and then packed into a 1ml glass syringe column. The column was washed with about five column volumes of low ionic strength buffer before the receptor was eluted with a stepped salt gradient (0.1, 0.2, 0.3, 0.4, 0.5, and 1.0M-KCl). Either 1.0 or 2.0ml fractions were collected and 200ul and 50ul samples were removed for liquid scintillation counting and protein determination respectively.

The following modifications were introduced in later experiments: 400ug of BSA was added to the collected fractions to stabilise binding activity, and peak fractions were incubated with 0.1nM [^{3}H]DHT for 3 hours at 4°C and bound steroid assayed by the dextran coated charcoal (DCC; **Methods** 2.12) method.

2.7 FPLC-Ion Exchange Chromatography.

$2x10^6$ cells (RM & SW) were seeded in 140mm plastic dishes (4 or 6 per experiment), grown to confluence, and incubated at 37°C

with EC1 medium containing 2nM [^{3}H]mibolerone, in order to stimulate the levels of androgen receptor binding (Introduction 1.1F, Methods 2.3C).

The soluble salt extract and ammonium sulphate fractions were then prepared as described above, and chromatographed on a Mono Q (Pharmacia) anion exchange column, using the Pharmacia FPLC system. The pH of all buffers used was 7.7. The 105000xg salt extract was de-salted using centricon-10 microconcentrators (Amicon) and dilution, prior to FPLC. 1ml of extract together with 0.5ml of PEM buffer were mixed in the microconcentrator unit, and centrifuged at 5000xg for 30-60 minutes (8x50 Hi-spin 21); a volume of 0.5ml of sample was recovered, which was further diluted 1:2 with PEM buffer to give a final volume of 1.5ml. The ammonium sulphate precipitate was gently washed with PEM buffer and resuspended in 1.0 to 1.5ml of the same buffer, and chromatographed with or without prior de-salting. Samples were loaded on to the column via a 500ul sample loop and a manually operated valve. Initially 100% Buffer A (PEM) was pumped through the column (0-10min.), followed by increasing amounts of Buffer B (PEM +0.35M or 1.00M-KCl) to produce a linear salt gradient. 100% Buffer B was then maintained for five minutes , before returning to 100% Buffer A. FPLC was carried out at room temperature, at a flow rate of 1ml/minute, and 1ml fractions were collected and placed immediately on ice. Fractions were assayed for radioactivity, protein, and the linearity of the salt gradient was checked by measuring the conductivity of each fraction compared to solutions of known KCl concentration.

2.8 HPLC-Size Exclusion Chromatography.

High Performance Liquid Chromatography (HPLC) gel filtration separation of the androgen receptor from control and androgen insensitive GSF was carried out on the LKB HPLC-system, using a TSKG3000 SW (7.5x300mm) column, preceeded by TSKP SW (7.5x7.5mm) precolumn.

Cells (4x140mm dishes) were incubated for 24 hours in EC1 medium with either 2nM [^{3}H]mibolerone or [^{3}H]R1881. The ammonium sulphate fraction was prepared as above and resuspended in PEM buffer containing 500mM-KCl and 10%(v/v) glycerol (pH7.4). The sample was then centrifuged at 105000xg for 10 to 15 minutes to remove insoluble material before HPLC analysis. The samples were loaded on to the column(s) via a 200ul sample loop and a manually operated valve, and the receptor complexes eluted with PEM buffer (+500mM-KCl, 10% glycerol), at a maxium flow rate of 0.5ml/minute. The separation was carried out at room temperature and took between 30 and 60 minutes, and 0.5 and/or 1.0ml fractions were collected and assayed directly for radioactivity. Protein elution profiles were obtained by continuous monitoring of the eluate at 280nm (2151 variable wavelength monitor;LKB).

The elution of free steroid was determined in a separate experiment, by injecting 10000-15000d.p.m. of [^{3}H]mibolerone, in PEM buffer (+500mM-KCl, 10% glycerol): 0.5ml fractions were collected and assayed for radioactivity.

Calibration of the G3000 SW (7.5x300mm) column.

The column was calibrated by resolving mixtures of standard

proteins of known molecular weight and Stokes radius (Mr,Rs): Alcohol dehydrogenase (150K, 4.55nm), BSA (66K, 3.55nm), Ovalbumin (45K,2.9nm), Carbonic anhydrase (29K), Trypsin inhibitor (20.1K), and Cytochrome c (12.4K, 1.7nm). From these data, physical paramaters of the human GSF androgen receptor were calculated. The void volumn (Vo) and the total volumn (Vt) were measured using Blue dextran ($2x10^6$) and Phenol red (or [^{3}H]leucine) respectively.

All buffers and protein standards were passed through 0.2 micron membrane filters (Whatman) before chromatography, and all buffers were degassed under vacuum before use.

2.9 Photoaffinity Labelling Studies.

A. Rat Prostate Cytosol Androgen Receptor.

The procedure followed was a modification of the method of Brinkmann et al (1985b). All procedures were carried out at 0-4 °C, unless otherwise stated. The prostates from six rats, castrated 24 hours earlier, were dissected out, washed, and homogenised in 4ml of TEGM buffer [40mM-Tris-HCl, 1mM-EDTA, 10%(v/v) glycerol, 20mM-sodium molybdate; pH7.7], containing 0.1% monothioglycerol and 0.6mM PMSF: 3 x 10 second burst with a Ultra-turrax, with 30 second cooling intervals. After centrifugation at 105000xg for 1 hour (SW 60 rotor; Beckman), 3ml of cytosol were recovered. 1ml was incubated with 15nM [^{3}H]R1881 for 2 hours at 4 °C, while the remaining fraction was stored at -80 °C until required.

The labelled cytosol was then centrifuged in a Beckman Airfuge (at maximum 30 psi) for 15 minutes, prior to loading on

to a Mono Q anion exchange column via a 500ul sample loop and a manually operated valve. The column was washed with at least 10ml of TEGM buffer before elution of the receptor with a linear salt gradient (0-350mM-NaCl). Forty 1ml fractions were collected and 100ul removed from each for liquid scintillation counting.

The peak fractions, once identified, were pooled and irradiated using an Osram HBO 100 W/W-2 high pressure mercury lamp (Oriel Scintific Ltd.) for 10 minutes. The sample was placed on ice approximately 5cm from UV source, with a saturated solution of copper sulphate placed in between to filter out wavelengths below 300nm. The photolinked receptor complexes were subsequently precipitated overnight with trichloroacetic acid (10%w/v).

The remainder of the prostate cytosol was thawed and treated in the same way as above; the trichloroacetic acid precipitates were then pooled. The trichloroacetic acid insoluble material was washed with 10% trichloroacetic and extracted with ethylacetate, 70% ethanol and finally diethlyether. The precipitate was then dried and redissolved in 200ul of SDS-sample buffer (Methods 2.11B) at room temperature, and analysed on a 8% polyacrylamide gel by the method of Laemmlli (1970) (See Methods 2.11B for details). The sample was then loaded in a 2cm well, with 20ul mixture of high molecular weight standards (30000-200000 daltons; Sigma) in an adjacent well; the gel was run (in Bio-rad PROTEAN I electrophoresis tank) at 20mA/gel constant current after stacking at 10mA/gel.

On completion of electrophoresis the region of the gel

containing the molecular weight maker proteins was fixed and stained (0.025% commassie blue), while the track containing the sample was sliced into 2mm pieces which were incubated with 1ml of the following solution; diaminoheptane:Triton X-100:distilled water (1:1:10 by volume: Dr A.O.Brinkmann personal communication), in order to swell the gel and elute the protein. After an overnight incubation with this solution, at room temperature, 10ml of Pico-fluor (Packard Instument Company Inc.) scintillation cocktail was added and the samples counted for 30 minutes each.

B.Calf Uterus Androgen Receptor.

The method used to photoaffinity label the calf uterus androgen receptor was essentially identical to the one described by Brinkmann et al (1985b). Calf uterus tissue was stored at -80°C until required: 15g of tissue was thawed in 60ml of TEG buffer [40mM-Tris-Cl, 1mM-EDTA, 10%(w/v) glycerol; pH7.4], containing 10mM-sodium molybdate and 0.1mM-dipyridyldisulphide. Tissue was homogenised using an Ultra-turrax homogeniser: 3x10 second burst at maxium setting, with 30 second cooling peroids. After centrifugation at 10000xg (HB-4 rotor; Sorvall) for 10 minutes to remove cell debris and lipid material, the 105000xg (SW40 rotor, 1 hour;Beckman) cytosol fraction was prepared. The volume of the recovered cytosol was adjusted to 15ml and incubated with 7.5uM triamcinolone acetonide (Sigma)(TA) for 30 minutes at 4°C to block progesterone receptor binding sites (Asselin et al 1979; Wilbert, Griffin & Wilson 1983), followed by a 2 to 3 hour incubation with 10 to 15nM [^{3}H]R1881 ± 3.0uM cold DHT. The labelled cytosol was then brought to 40% saturation with ammonium

sulphate (left for 30 minutes), and the precipitated proteins collected (10000xg for 10 minutes) and stored at -80°C.

The precipitate was later thawed, washed with TEGD buffer [TEG + 10mM-dithiothretol; pH7.4] and solubilised in 6ml of the same buffer containg 7.5uM TA, and [^{3}H]R1881 ± 3.0uM DHT. The redissolved precipitate was then mixed with a slurry of DNA-cellulose [The DNA-cellulose had already been prepared by the method of Alberts & Herrick (1971), using calf thymus DNA(Sigma)](about 500fmoles of receptor per g DNA-cellulose) and the volume adjusted to 120ml with TEGD buffer. The mixture was left for 2 hours (4°C) with gentle mixing (Roto-rack) before being packed into a column (with a bed volume of 10-15ml), and washed with 50 to 100ml of TEGD buffer until the run through fraction contained less than 500c.p.m./ml. The androgen receptor was then eluted from the column with TEGD buffer containing 15mM $MgCl_2$. Thirty 1ml fractions were collected and 50ul was removed from each fraction for liquid scintillation counting.

The peak fractions from the "HOT" sample and the equivalent fractions from the "HOT+COLD" incubation were pooled separately and irradiated as described for the rat prostate receptor. The trichloroacetic acid insoluble material from the two incubations were treated as described above, and analysed on a 8% poly-acralyamide gel as above.

C. Human GSF Androgen Receptor.

Three methods were followed during photoaffinity labelling studies of the human fibroblast receptor (Fig.2.1).

In protocols I and IIa control cells were incubated with EC1

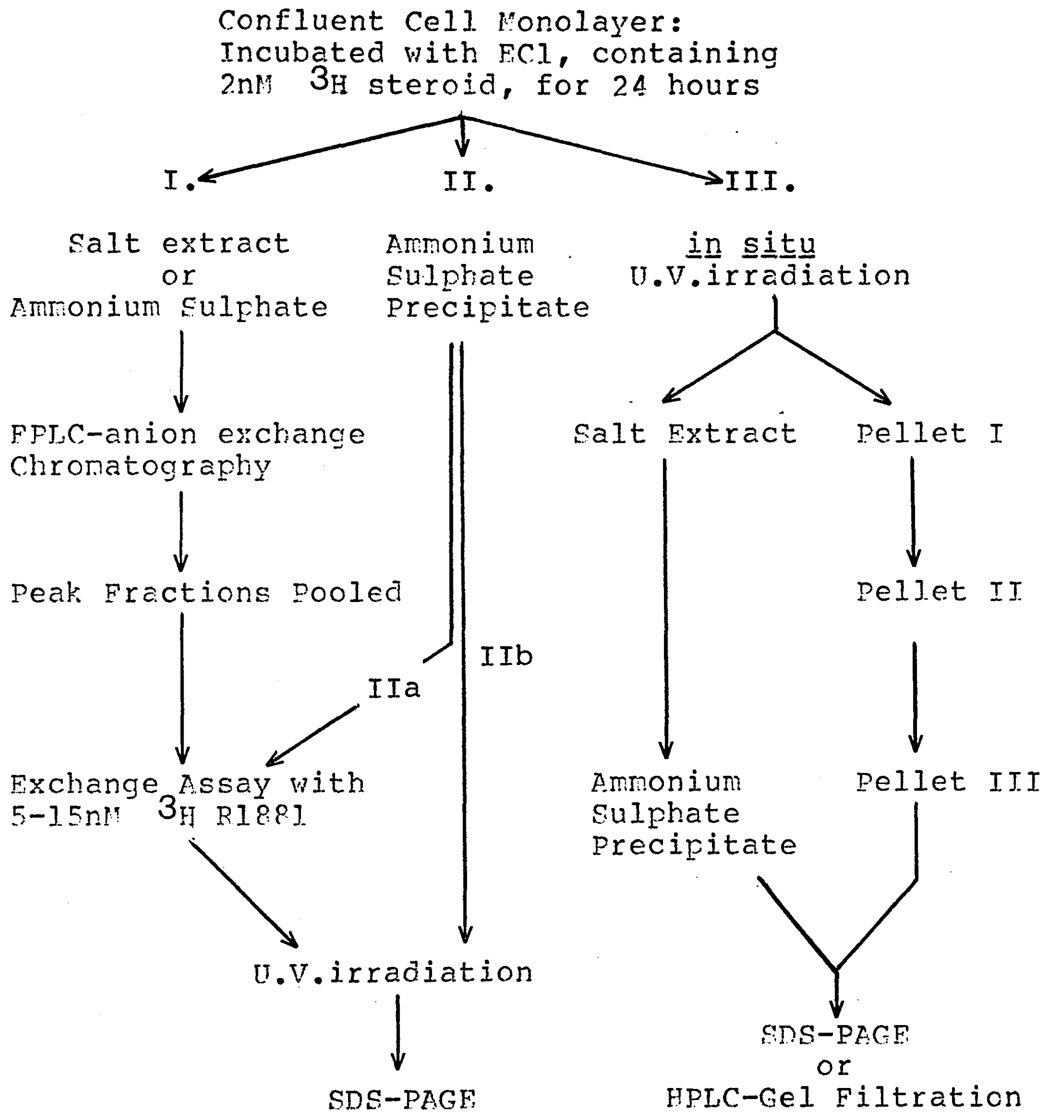


Fig.2.1 Photoaffinity labelling of the human GSF androgen receptor.

medium containing 2nM [^{3}H]mibolerone for 24 hours, prior to receptor extraction and partial purification. Mibolerone was the ligand of choice during the early stages of protocol I because the FPLC-anion exhange chromatograpny was at room temperature (Methods 2.7), and whole cell binding studies had shown that mibolerone-receptor complexes dissociated more slowly than DHT-recptor complexes ($t_{1/2}$ of 4h and 2h respectively at 37°C: Dr M.B.Hodgins personal communication), and the latter are known to dissociate more slowly than R1881-receptor complexes (Brown, Rothwell & Migeon 1981; Pinsky et al 1985; Traish, Muller & Wotiz 1984). It was therefore necessary to exchange the bound mibolerone for [^{3}H]R1881 before attempting the U.V.-irradiation: the pooled peak fractions from the Mono Q column (I) or the ammonium sulphate fraction (IIa) were incubated with 5 to 15nM [^{3}H]R1881 ± 100-fold excess of cold steroid for at least 21 hours (4°C). In protocols IIb and III cells were incubated for 24 hours with 2nM [^{3}H]R1881 and no exchange assay was required before U.V.-irradiation of R1881-androgen recptor complexes.

In protocols I and II, samples (either peak fractions from ion-exchange column or ammonium sulphate precipitates) were treated with DCC (Methods 2.13) prior to irradiation, to remove free steroid and reduce the opportunity for non-specific covalent binding. Samples, were kept on ice, and irradiated with 110W high pressure mercury lamp (Hanovia Slou; Kindly supplied by Dr Hill, Department of Organic Chemistry, University of Glasgow) for 10 to 15 minutes. The samples were approximately 5cm from the U.V. source and a saturated solution of copper sulphate was placed in between. In addition the lamp was enclosed in a quartz cooling

jacket (running tap water was used as coolant during irradiation). After irradiation proteins were precipitated with trichloroacetic acid (5-10%w/v) overnight (4°C), and treated as described above. The resolubilised samples were then analysed on 8% polyacrylamide gels.

The third method investigated was based on the _in situ_ irradiation procedure described by Horwitz and Alexander (1983), for photoaffinity labelling of the avian progesterone receptor. After incubating cells with EC1 containing 2nM [^{3}H]R1881 for 24 hours, the culture medium was removed and the cell monolayer washed 2 to 3 times with PBS (0°C). The culture dishes were then inverted on a U.V. transilluminator (TM36 series max.302nm; U.V. Products Inc.) for 2 minutes (room temperature) and then replaced on ice. The cells were then scraped off the dish into PBS (0°C), and the (0-35%) ammonium sulphate fraction prepared. In some experiments, label remaining in the 105000xg pellet after salt extraction was investigated (Fig.2.1). The pellet was resuspended in PEM buffer using a small Dounce hand homogeniser, and extracted with 1%(v/v) Triton X-100 for 30 minutes, followed by centrifugation at 105000xg. The resulting pellet was then digested with DNAase I (25ug/ml) for 60 minutes (on ice), and centrifuged at 105000xg. The Triton extract was treated with trichloroacetic acid prior to further analysis. All subcellular fractions to be analysed further were resolubilised in either PEM buffer (+500mM KCl, 10% glycerol) or SDS-sample buffer for HPLC-size exclusion chromatography or SDS-PAGE respectively.

2.10 Metabolic Ladelling of GSF Proteins.

Control and androgen insensitive cells were seeded at a density of $1x10^6$ to $2x10^6$ cells, in either 60mm or 100mm diameter plastic petri dishes and grown to confluence. The cultures were then incubated with either [^{35}S]methionine (>1000Ci/mmol; Amersham) or [^{75}Se]selenomethionine (30-50Ci/mmol; Amersham) for between 8 and 12 hours: isotopes were usually added to a final concentration of 50uCi/ml.

Incubation Medium:

MEM (w/o methionine and glutamine).....8.70ml
New born calf serum...................1.00ml
EC10 medium...........................0.10ml
Glutamine(x100)........................0.10ml
Penicillin and Streptomycin(x100)......0.10ml

Total=10.00ml

Time-course of isotope incorporation.

Cells were seeded in 24 well plates (22000 cells/well) and grown on coverslips; the latter were cleaned with sodium hydrochlorite ("CHLOROS", industrial grade) and 100% ethanol and sterilised before use. This procedure offered a fast and simple method for measuring the incorporation of labelled methionine or selenomethionine into total newly synthesised fibroblast protein. Cells were collected and washed with PBS (0°C:2x250ml), precipitated with 5%(w/v) trichloroacetic acid (0°C:2x250ml), and extracted with 95%(v/v) ethanol (room temperature:2x250ml). The trichloroacetic acid insoluble material was mixed with hyamine hydroxide (0.5ml/vial; Packard) prior to liquid scintillation counting.

During the time coarse study, cells were incubated with the above medium containing 10uCi of either [^{35}S]methionine or [^{75}Se]selenomethionine. Cells were harvested, in duplicate, after 4, 8, 12, 16, and 24 hours, and treated as described above.

The effect of cold methionine concentration on isotope incorporation.

The effect of cold methionine on the incorporation of labelled methionine into total cell protein was investigated in the same way as the above time-course. Cells were grown on cover-slips and incubated for 10 hours with 10uCi of [^{35}S]methionine or [^{75}Se]selenomethi onine in methionine-free medium containing: 0.744 (10%serum + 1%EC10), 0.260 (1%serum + 1%EC10), 0.207 (1%EC10), or 0.0 µmoles of cold methionine respectively. Cells were incubated for 10 hours at 37 °C, and subsequently collected and treated as in the time-course study.

2.11 Two-dimensional Gel Electrophoresis and Double-label Autoradiography.

Proteins from control and androgen insensitive GSF were metabolically labelled with [^{35}S]methionine and [^{75}Se]seleno-methionine respectively, as described in Methods 2.10: cell mono-layers were then washed 2-3 times with PBS (0°C), scraped off the dishes and collected by centrifugation (Methods 2.4). Carrier cells or protein was then added during sonication to ensure that there was sufficient protein in the 105000xg salt extract for ammonium sulphate precipitation. Ammonium sulphate, receptor enriched fractions were then mixed and resolved by 2-DGE.

Proteins from control (^{35}S) and androgen insensitive (^{75}Se) cells were then distinguished by fluorography (^{35}S and ^{75}Se) and subsequent autoradiography (^{75}Se) (Lecocq et al 1982)(Fig.2.2).

Ammonium sulphate precipitated proteins were resuspended in PEM buffer (and mixed with an equal volume of lysis buffer) or directly into lysis buffer [O'Farrell 1975; 9.5M-urea, 2%(w/v) NP-40, 2% Ampholines (1.6% pH5-7 + 0.4% pH3.5-10; LKB), 5% β-mercaptoehanol: stored at -20°C until required].

A.
First Dimension: Isoelectric Focusing.

IEF rod gels were cast in glass tubes (180 x2-3mm internal diameter) sealed at the base with parafilm; 125mm long gels were routinely prepared. Table 2.1 describes the composition of the gel mixture used. The solution was loaded into the gel tubes using a 146mm long steel syringe needle (0.5-1.0mm internal diameter), and overlayed with "Gel overlay solution" [O'Farrell 1975; 8M-urea: stored at -20°C], and left to polymerise for 1 to 2 hours; the overlay solution was then replaced with 20ul of lysis buffer and a similar volume of distilled water. The gels were left for at least two more hours before the base was unsealed and the rod gels placed in a standard tube gel electrophoresis apparatus (Shandon Tube-gel Electrophoresis Tank). 20ul of fresh Lysis buffer was added to the top of the gels, and the top buffer reservoir (cathode) filled with 0.02M-NaOH (degassed under vacuum) and the lower compartment (anode) with 0.01M-H_3PO_4. The gels were pre-run at: 200 volts for 15 minutes, then 300 volts for 30 minutes, and finally 400 volts for a further 30 minutes. Lysis buffer and NaOH were then removed and samples loaded. The samples were overlayed with 10ul of "Sample

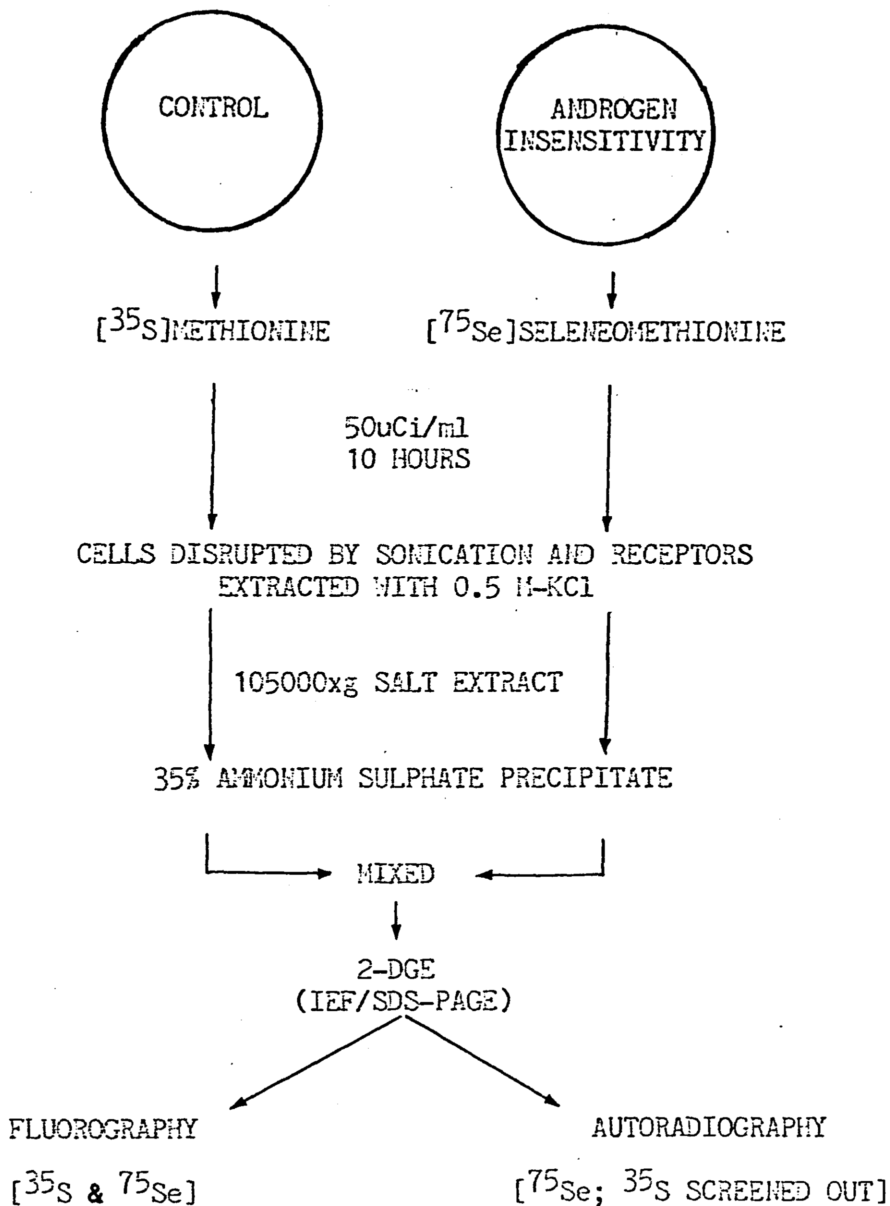


Figure 2.2 Double-label autoradiography and 2-DGE of fibroblast proteins. 1-2x10^6 cells were seeded in 60mm or 100mm plastic dishes, and grown to confluence in EC10 medium. Cells were then incubated with either [^{35}S]methionine or [^{75}Se]selenomethionine (50uCi/ml) for 10h at 37 C. Cell monolayers were then rinsed, and the cells collected: carrier cells or protein was added just prior to sonication. 35% ammonium sulphate precipitates were prepared, mixed and co-electrophores ed. Control and androgen insensitive proteins were subsequently distinguished by differential exposure of the gel with X-ray film. (See text for experimental details)

Table 2.1 First dimension (IEF) gel composition.

Component	Amount/10ml gel mixture.
Urea	5.5g
Acrylamide Stock*	1.33ml (4%)
Ampholines:	
pH range 5-7	0.40ml
pH range 3.5-10	0.10ml
NP-40 (10%)	2.00ml (2%)
Distilled Water	1.97ml
10%(w/v) ammonium persulphate	10ul
TEMED	7ul

*, 30% acrylamide stocksolution: 28.38%(w/v) acrylamide, 1.62%(w/v) NN'-methylenebisacrylamide. Stored in the dark, over mixed bed ion-exchange resin (Amberlite MB-3; Sigma) at 4 C.

Table 2.2 Second dimension (SDS-PAGE) gel composition.

Component	Amount	
	Running Gel/25ml	Stacking Gel/10ml
Acrylamide Stock*	5.83ml (7.0%)	1.50ml (4.75%)
	6.67ml (8.0%)	
	8.33ml (10%)	
Distilled Water	12.92ml (7.0%)	6.00ml
	12.08ml (8.0%)	
	10.42ml (10%)	
Lower Gel Buffer[ø]	6.25ml	-
Upper Gel Buffer	-	2.50ml
10% (w/v)ammonium persulphate	82.50ul	30.00ul
TEMED	12.50ul	10.00ul

*, 30% acrylamide stock: 29.2%(w/v) acrylamide, 0.8%(w/v) NN'-methylenebisacrylamide. Stored as above.
ø, Lower gel buffer: 1.5M-Tris-HCl (pH8.8), 0.4%(w/v)SDS
Upper gel buffer: 0.5M-Tris-HCl (pH6.8), 0.4%(w/v)SDS (O'Farrel).

overlay solution" [O'Farrell 1975; 9M-urea, 1% Ampholines] and the upper buffer compartment refilled with fresh 0.02M-NaOH. The gels were then run overnight at 400V constant voltage (or constant power) for a total of 7500 volthours. In some cases the voltage was increased to 1000V for the final hour to sharpen the resolution of the bands.

The gels were removed from the tubes using a disposable syringe (with 21G $1_{1/2}$, 0.8x40mm neddle) and water pressure, and placed directly into 5ml SDS-sample buffer [O'Farrell 1975; 10%(v/v) glycerol, 5%(v/v)β-mercaptoethanol, 2%(w/v) SDS, and 0.0625M-Tris-HCl, pH6.8]. The gels were then equilibrated for either a total of 30 minutes (15 minutes before and after strorage) or 2 hours; gels were kept at -20 or -70°C until required. The equilibration time of the gel was reduced inorder to minimize the loss of protein at this stage (Bravo 1984).

The pH gradient was measured by slicing a gel (run under identical conditions, minus sample) into 5mm segments and eluting the Ampholines in 1.5ml boiled distilled water in 1.5ml sealed plastic micro test tubes (Brand), at room temperature for about 2 hours. The pH was then measured using a micro-pH electrode (Type CMAWL; Russell) (See Appendex 5.3).

B.
Second Dimension:SDS-PAGE.

The second dimension was based on the Laemmli (1970) discontinuous SDS-gel system (O'Farrell 1975).

Table 2.2 describes the gel mixtures used: slab gels were cast in home-made cassettes consisting of two glass plates (200x175x3mm) kept apart with 0.8mm plastic spacers and sealed

with adhesive tape and clips. The running gel (150x155x0.8mm) was poured, overlayed with distilled water, and allowed to polymerise overnight. After removing the overlay solution, the Stacking gel (50x155x0.8mm) was cast on top and allowed to polymerise for one hour. The whole cassette was then placed in a Pharmacia gel electrophoresis apparatus (GE-2/4 LS); the base of the gel being unsealed beforehand. The IEF tube gel was then positioned on top of the Stacking gel and held in place with a plastic wedge (Pharmacia) or a 1%(w/v) agarose gel. Running buffer [O'Farrell 1975; 0.025M Tris base, 0.192M-glycine, and 0.1%(w/v) SDS] was added to both upper and lower reservoirs. Protein stacking was achieved at 15-20mA/gel, with subsequent separation at a constant current of 20mA/gel. Bromophenol blue was added as tracking dye, and 10ul of standard protein mixture (Dalton Mark VII, 14000-70000 daltons, or High Molecular Weight standards, 30000-200000 daltons; Sigma) were resolved concomitantly.

Electrophoresis was stopped when the dye front had travelled at least 100mm into the separating gel. The region containing the molecular weight markers was cut out and stained with 0.25% coomassie blue solution. The remainder of the gel was fixed in a solution of 50% methanol:7.5% acetic acid (overnight), and subsequently impregnated with a commercial fluorographic agent (Amplify; Amersham) for 20 to 30 minutes (room temperature) with constant agitation. The gel was then dried down on to 3mm chromatography paper (Whatman) using a Bio-Rad slab gel drier (Model 224) under vacuum; the dried gel was then exposed to X-AR5 medical X-ray film (Kodak), sandwiched between two glass plates, covered with tin foil, and placed in a light-proof box. After a

fluorographic image was obtained by exposing the gel at -70°C, the gel was re-exposed at room temperature, with a piece of blackened film placed between the gel and the x-ray film to screen out the light and β-emmissions from the ^{35}S-labelled proteins (autoradiograph). The "blackened film" was prepared by exposing a piece of XAR-5 film to the white light from an enlarger for approximately 30 seconds and then developing the film as normal. This method of screening-out the ^{35}S was found to be more effective than using tin-foil. All films were processed by standard procedures.

2.12 2-DGE of Whole Cell GSF Protein.

$0.5x10^6$-$1.0x10^6$ cells were seeded in 30mm plastic dishes, and incubated with [^{35}S]methionine (50uCi/ml) as previously described (Methods 2.10). Cultures were then rinsed three times with PBS (0°C) and scraped off in 100-200ul of lysis buffer (Methods 2.11), and stored at -70°C until required. Proteins were then resolved by 2-DGE as described in Section 2.11. In addition to analyse the more basic proteins, samples were resolved by NEPHGE (O'Farrell et al 1977) in the first dimension. The gel mixture for NEPHGE gels was similar to that described for IEF gels (Table 2.1), with the exception that Ampholines in the pH range 7-9 (0.25ml) and 8-9.5 (0.25ml) were used, because of the more basic Ampholines, these gels required douole the quntities of ammonium persulphate and TEMED inorder to polymerise. The gels were cast in same way as the IEF rod gels (Methods 2.11). Once polymerised, NEPHGE rod gels were placed in a tube-gel

electrophoresis tank (Shandon); the lower reservior was filled with 0.02M-NaOH (Cathode) and the upper reservior with 0.01M-H_3PO_4 (Anode), the reverse of IEF. There was no pre-focusing of the gels. Samples were loaded immediately, and resolved at constant voltage (400 volts) for a total of 1600Vhours (O'Farrell et al 1977). After electrophoresis the gels were treated as described for IEF first dimension gels. The second dimension, SDS-PAGE, was as described before (Methods 2.10), with the exception that proteins were resolved on 5-15% linear gradient polyacrylamide gels (Bio-Rad Gradient Former, model 385). Detection of the proteins by fluorography was as described previously (Methods 2.10).

2.13 Miscellaneous.

A. Liquid Scintillation Counting.

Radioactivity was routinely measured using a Packard Tri-carb 300 series scintillation counter. Samples were mixed with either 10ml of a toluene based scintillation cocktail [10% methanol, 0.5%(w/v) 2,5-diphenyloxazole (Sintran grade)] or 2.5 to 10ml of Ecoscint (National Diagnostics) for liquid scintillation counting.

^{3}H was determined using either a single nuclide or a dual nuclide program, with an efficiency of about 40% for both; ^{14}C was measured using a dual label program (with ^{3}H) with an efficiency of around 80%. The isotopes ^{35}S and ^{75}Se were measured independently using a single nuclide ^{14}C program (Lecoq et al 1882); counting efficiencies of 90% were obtained for both isotopes.

B. Protein Measurements.

Protein estimations were determined from duplicate or triplicate samples by the method of Bradford (1976). Samples were diluted to a final volume of 100ul with distilled water and either 1 or 3ml of "Bradford's regeant" [0.01%(w/v) coomassie blue G-250, 4.7%(v/v) ethanol, 8.5%(w/v) phosphoric acid] added. Samples were mixed vigorously and the absorbance measured at 595nm. A standard calibration curve was prepared in parallel using BSA. From this data it was possible to calculate the amount of protein present in whole cell and sub-cellular fractions.

C. Dextran Coated Charcoal Assay (DCC).

Samples were incubated with a charcoal suspension [1% Activated charcoal (Sigma), 0.1% dextran (Pharmacia); equilibrated in assay buffer 24 hours before use] at a ratio of 2:1 for 5 to 10 minutes (0 °C). The charcoal was then pelleted by centrifugation (2000xg, 10 minutes) and a sample removed from the supernatant for liquid scintillation counting.

D. Hydroxyapatite Assay.

An alternative to the DCC technique for measuring bound steroid involved absorption of receptor complexes by Hydroxyapatite (Williams & Gorski 1975; Clark & Peck 1976).

The hydroxyapatite (Type I; Sigma) was washed extensively with PEM buffer (+10%(v/v) glycerol) until the pH of the supernatant was 7.4. The volume of the slurry was then adjusted so that 0.5ml contained 0.3 to 0.35ml of packed hydroxyapatite, and stored at 4 °C.

Samples (50- 200ul) were incubated with 250ul of

hydroxyapatite slurry for 15 to 20 minutes (0°C), with mixing. PEM buffer (+10% glycerol) was then added (4ml) and the contents of the assay tube vortexed and centrifuged at 2000xg for 2 minutes. The pellet was mixed with 4ml of buffer, vortexed and centrifuged. This was repeated a total of four times, the supernatant fractions being discarded after each wash. The final hydroxyapaptite pellet was then extracted with 4ml of ethanol (room temperature) for 10 to 15 minutes, and the total alcohol extract assayed for radioactivity.

RESULTS

&

DISCUSSION

3.1 Cell Lines and Binding Studies.

Figure3.1a and b shows a representive Scatchard analysis and Dissociation time-course, respectively, for androgen receptor binding in intact human cultured GSF: cell lines RM and CD. From such data, the concentration of receptor (Bmax), the equilibrium dissociation constant (Kd), and the half-life ($t_{1/2}$) of steroid dissociation were calculated. Table 3.1 summarizes the findings of such whole cell assays for the cell lines used in this study; the data was taken from the relevant references or supplied by Dr.M.B.Hodgins (personal communication).

The control cell lines RM, SW, and CD all showed binding kinetics within the normal range (Bmax=34.0±10.1fmoles/mg protein, Kd=0.27±0.22nM:mean±S.D.;Hodgins et al 1984). A fourth cell line GR, also used as a control for androgen receptor binding, was from a patient with perineal hypospadias: cells were kindly supplied by Dr.P.Smial, Royal Aberdeen Sick Childrens Hospital).The patient was 46 X,Y, and a study of his family pedigree revealed his father was similarly affected, suggesting the hypospadias was paternally inherited, and arguing strongly against androgen resistance. Furthermore a number of other genetic abnormalities were present in this pedegree: polydactyly and epidermolysis bullosa.

The androgen insensitive cell lines T4, Matheson, and TCF also showed normal whole cell binding kinetics (Table 3.1). The cell line T4 (material supplied by Professors R.Scott and

Table 3.1 Whole cell androgen receptor binding studies.

Cell line	Diagnosis	5α-reductase Activity	Receptor Studies				Hormone Binding	References
			*Bmax	Kd	$t_{1/2}$	Augmentation		
RM	Normal	Normal	37.5	0.20	230	+ (>2x)	Normal	-
SW	5α-red.def.	Deficient	75.0	0.14	198	+ (>2x)	Normal	1,2,6
CD	HSDH def.	Normal	53	0.14	264	+ (>2x)	Normal	8
GR	Hypospadias	Normal	49	0.22	282	+ (>2x)	Normal	-
T4	CAI	Normal	54	0.34	96	+ (>2x)	Normal	7
Matheson	CAI	Normal	30	0.23	100	+ (<1.5x)	Normal	-
Ia(b)	PAI	Normal	6.7	0.26	230	+ (1.9x)	Deficient	6
TCF	PAI	–	40	–	–	- (0.87x)	Unstable[ø]	4,5
605	CAI	–	Unmeasurable Binding				Absent	
4479	CAI	Normal	Unmeasurable Binding				Absent	3

5α-red.def.= 5α-reductase deficiency.
HSDH def.= 17β-hydroxysteroid dehydrogenase deficiency.
CAI= complete androgen insensitivity.
PAI= partial androgen insensitivity.
ø= fast dissociation.

Controls: CD, GR, RM & SW.
Androgen Insensitive: Ia, Matheson, TCF, T4, 605 & 4479

References:
1. Corral et al 1984
2. Hodgins 1982
3. Kaufman et al 1976
4. Kaufman et al 1981
5. Kaufman et al 1982a
6. Pereira et al 1984
7. Rowney & Hodgins 1984
8. Wilson et al 1985

Bmax and Kd values given are for DHT, except RM and SW (Mibolerone).
$t_{1/2}$ values given are for Mibolerone, except T4 and Matheson (DHT).
Augmentation studies were with DHT (RM,CD,GR) and/or Mibolerone.

* **Bmax, fmoles/mg protein; Kd, nM; $t_{1/2}$, minutes.**

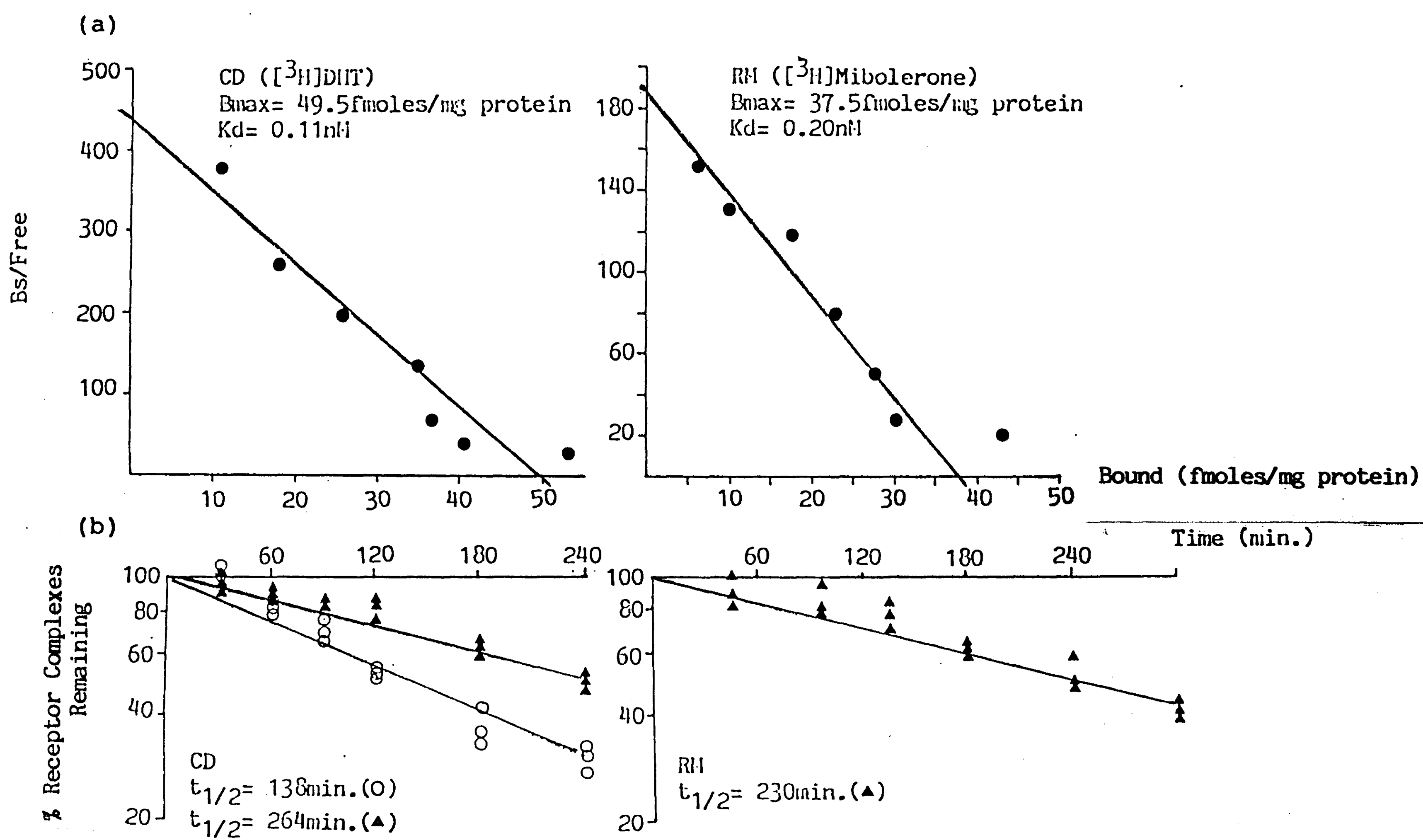


Figure 3.1. Scatchard plot (a), and steroid dissociation time course (b) for RM and CD cell lines. Results shown are for [^{3}H]DHT (O) and [^{3}H]mibolerone (▲) binding. Data kindly supplied by Dr.M.B.Hodgins.

R.Stiton, University of Leeds), was derived from a post pubertal patient with a female phenotype and complete androgen insensitivity. The X-linkage of the disorder was supported by the observation that a maternal half sister was found with androgen insensitivity. The Matneson cell line (cells supplied by Professor C.R.W.Edwards, University of Edinburgh), was from a patient with a male phenotype similar to that seen in the Refeinstein syndromes, and was diagnosed as having partial androgen insensitivity. No obvious defect in ligand binding or augmentation was seen for the receptor complexes from T4 or Matheson; however, the receptor complexes from TCF failed to "up-regulate" and showed abnormal dissociation kinetics in both whole cell and cell free extracts, suggesting an underlying structural defect in the receptor protein (Kaufman et al 1981, 1982a).

Quantitative binding defects were found for both the cell lines 4479 (unmeasurable), 605 (unmeasurable) and Ia (Deficient).

In all cell lines with measurable receptor binding, except TCF, the level of basal binding was found to increase in response to prolonged (24 hours) incubation with [^{3}H]androgen. The cell lines RM, CD, GR increased receptor binding with both DHT and mibolerone; however, with SW, T4, Matheson and Ia, augmentation was seen only with the synthetic androgen Mibolerone. In the case of SW and T4 this was shown to be due to the high rate of metabolism of DHT by these cells (Rowney and Hodgins 1985), and with the Ia cell line, the problem was the high level of non-specific binding. It is now widely accepted that [^{3}H]mibolerone is a very useful ligand for androgen receptor binding studies because of high affinity binding to the receptor, lower non-

specific binding and greater *in situ* stability (non-metabolizable) over [^{3}H]DHT (Evans & Hughes 1985; Rowney & Hodgins 1985; Dr.M.B.Hodgins personal communication).

3.2 Partial Purification of the Human Androgen Receptor.

Precipitation of a 0.5M-KCl extract of GSF with 15-30% ammonium sulphate resulted in a 3-4 fold enrichment of the recovered counts over the total cell sonicate fraction (Table 3.2); in all subsequent experiments the 105000xg salt extract was brought to 35% saturation with ammonium sulphate. A number of groups have suggested that the proportion of radioactive counts that are non-extractable in high salt (0.3-0.6M) buffers, may have a functional significance in terms of receptor-acceptor binding (Clark & Peck 1976; Davies 1983; Kaufman et al 1983), and more recently with prostatic disease (Kyprianou & Davies 1986; Kyprianou et al 1986). The present study does not attempt to address the above questions, as the methodology used, differs from those described above. However, further analysis of the 105000xg salt extracted pellet, showed that 69% of the radioactivity was recovered after repeated sonication in 0.5M-KCl buffer (19%) and subsequent Triton X-100 extraction (50%); this suggested that at least 75% of the salt extractable radioactivity was solubilised by a single round of sonication, and at least 50% of the 105000xg pellet d.p.m. were associated with membrane material (Table 3.3). A further 3% of the radioactivity was released by DNAase I and trypsin digestion; it should be noted that although only a small fraction of the pellet

Table 3.2 Ammonium sulphate fractionation of GSF salt extract.

Fraction	[^{3}H] d.p.m. Total	Bound	Protein (mg)	Specific Activity (dpm/mg protein)
Cell Sonicate	102360	ND	4.52	22600
Cell Pellet	58260	ND	3.50	16700
KCl Extract	44000	13000	ND	-
0-15% AS	1048	ND	*	-
15-30% AS	7000	ND	0.08	87500
30-45% AS	6354	ND	0.32	19900
Supernatant	12000	2400	0.62	19400

A confluent monolayer of SW cells was incubated with 1nM [^{3}H]DHT for 30-40min. at 37 °C. The cells were then harvested and disrupted by sonication and receptor complexes extracted with 0.5M-KCl in PEM buffer. The 105000xg salt extract was then brought to 15%, 30%, and 40% saturation with ammonium sulphate: precipitates were then collected and analysed for total and bound radioactivity (DCC assay, see Methods 2.13), and protein recovered.

AS=ammonium sulphate precipitate.
ND=Not determined.
* =To low for accurate measurement.

Table 3.3 Extraction of 105000xg pellet associated counts.

Treatment	Volume (ml)	Radioactivity Recovered (d.p.m.) Total	Bound
Pellet I	1.0	58350	-
Sonication/Salt	1.0	10932	560
Triton X-100	1.0	29182	22077*
DNAase I	1.0	1151	151
Trypsin	1.0	479	248
Pellet V	0.5	1423	231

Radioactivity associated with the 105000xg salt extracted pellet was investigated further by: re-sonication and 0.5M-KCl extraction; extraction with 0.5% (v/v) Triton X-100; digestion with DNAase I (50ug/ml), and finally trypsin (3mg/ml). After each treatment the 105000xg supernatant was assayed for total and bound d.p.m. recovered; as described in Methods 2.4.

(*=This figure was thought to be an over estimation of the bound d.p.m. in this fraction, as in the presence of detergent 20.1% of free steroid was found not to be precipitated by charcoal treatment.)

d.p.m. (0.8%) was released by trypsin digestion, 52% of these d.p.m. were bound (Table 3.3). Although the functional significance of this fraction, for androgen action was not determined in the present study, it is possible that this small fraction of salt and DNAase I resistant d.p.m. could represent receptor-acceptor interactions.

From these studies it was concluded that the combination of sonication in 0.5M-KCl PEM buffer was a fast and useful system for extracting nuclear bound fibroblast androgen receptor complexes. Table 3.4 summarizes the salt extraction and partial purification data for the androgen receptor from control and androgen insensitive cell lines. Enrichment of ^3H d.p.m.by 35% ammonium sulphate precipitation ranged from 0-4 fold, and reflected the binding characteristics of the cell lines studied. Furthermore analysis of the ^3H d.p.m. recovered after ammonium sulphate treatment, in the precipitate and supernatant fractions, shows that for the control cell lines (RM, SW & CD) and the androgen insensitive cell lines with normal or deficient binding (T4 & Ia): $34 \pm 13\%$ and $11.6 \pm 1.5\%$ (Mean±SD) of total cell sonicate d.p.m. was recovered in the precipitate and supernatant fractions respectively. For the other cell lines, 4479 (unmeasurable binding) and TCF (unstable binding) only 9% and 13% of the total sonicate d.p.m. was recovered in the precipitate, while 39% and 16% was found in the supernatant fraction respectively.

From the proportion of [^{14}C]DHA recovered after ammonium sulphate precipitation it was estimated that about 40-50% of the [^3H]DHT d.p.m. and 70-80% of the [^3H]mibolerone d.p.m. represented specific binding (Table 3.5); as expected a similar

Table 3.4 Extraction and partial purification of the human fibroblast androgen receptor.

Cell Line	Whole Cell Binding	Specific Activity (dpm*/mg protein)				
		Sonicate	Pellet	Salt Extract	AS	Supernatant
RM	Normal	16000	13300	21000	41300	7300
SW	Normal	26800	27800	55300	80200	16100
CD	Normal	19400	7900	26100	78900	4800
T4	Normal	17800	15300	20800	47000	7100
4479	Absent	6000	5400	7800	4300	8500
TCF	Unstable	11100	23400	22100	16000	11200
Ia/b	Deficient	10500	10500	10800	25900	3700

Cells were grown to confluence, and placed in EC1 medium for 24h, and then incubated with MEM containing 1nM [^{3}H]steroid for 30 to 40 minutes at 37 C. Cells were then washed with PBS and sonicated in PEM buffer containing 0.5M-KCl. After centifugation for 1h. at 105000xg, the supernatant (Salt Extract) was precipitated with 35% ammonium sulphate (AS). (See Methods 3.4 for details)

AI=Androgen insensitivity.
* =Total counts recovered.
All cell lines were incubated with [^{3}H]mibolerone except for SW and T4, which were incubated with [^{3}H]DHT.

Table 3.5 Measurement of non-specific binding.

(a) ^3H DHT; 30-40 minute incubation.

Fraction	Proportion of Radioactivity Recovered (d.p.m.)			
	DHT (SW,n=3)		DHT (T4,n=6)	
	^3H	^{14}C	^3H	^{14}C
Total Sonicate	1.00	1.00	1.00	1.00
Pellet	0.46±0.13	0.30±0.10	0.37±0.11	0.31±0.09
KCl Extract	0.49±0.10	0.60±0.08	0.49±0.04	0.54±0.08
A.S.^{Ø}	0.21±0.10	0.11±0.05	0.28±0.04	0.17±0.03
Supernatant	0.21±0.08	0.36±0.08	0.12±0.03	0.33±0.06
Specific Binding^Ø	48%		39%	

(b) ^3H Mibolerone.

Fraction	Proportion of Radioactivity Recovered (d.p.m.)			
	30-40 minute incubation		24 hour incubation	
	RM & CD, n=3		RM/SW, n=5	
	^3H	^{14}C	^3H	^{14}C
Total Extract	1.00	1.00	1.00	1.00
Pellet	0.31±0.02	0.47±0.16	0.38±0.08	0.58±0.17
KCl Extract	0.70±0.20	0.55±0.12	0.61±0.08	0.43±0.02
A.S.^{Ø}	0.42±0.16	0.12±0.01	0.35±0.18	0.08±0.02
Supernatant	0.15±0.03	0.34±0.02	0.12±0.04	0.30±0.05
Specific Binding^Ø	71%		77%	

The androgen precursor [4-^{14}C]DHA was added to the total cell extract, and the proportion recovered in each subsequent fraction determined. As non-specific binding will be linear with respect to steroid concentration the proportion of ^{14}C DHA was assumed to be equivalent to the proportion of ^{3}H- DHT or mibolerone bound non-specificly.

A.S.= 35% ammonium sulphate precipitate.

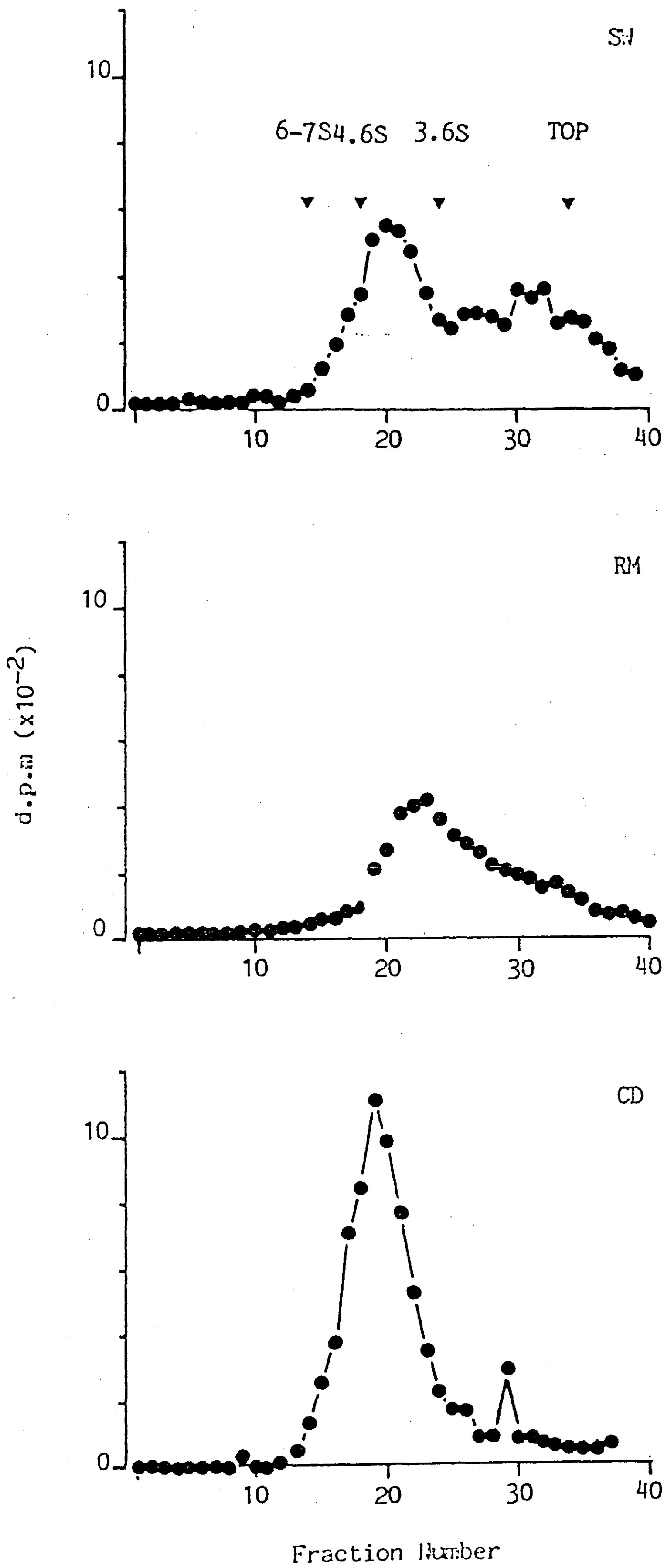
level of specific binding was seen after 24 hour incubation with 2nM-[^{3}H]mibolerone (Table 3.5). It is proposed, that the use of [^{14}C]DHA binding, probably gives a more realistic estimate of non-specific binding in sub-cellular extracts than the conventional method of adding cold steroid to the whole cells. After washing of cell monolayers very little radioactivity will be cellular bound, and will be reduced further during sub-cellular fractionation, thus underestimating the level of non-specific binding. The observed difference between the levels of DHT and mibolerone non-specific binding fits well with the findings of whole cell studies, where non-specific binding was measured by incubating cells with [^{3}H]steroid$\pm$100-fold excess unlabelled steroid (Evans & Hughes 1985; Dr.M.B.Hodgins personal communication); it is therefore clear, that mibolerone is a superior ligand to DHT for *in vitro* studies of the androgen receptor.

3.3 Sucrose Density Gradient Analysis.

In order to demonstrate that the ammonium sulphate fraction contained androgen receptor, and as way of comparing the receptor complexes from control and androgen insensitive cell lines, the re-suspended ammmonium sulphate precipitate was analysed on 5-20%(w/v) linear sucrose density gradients. In the presence of 0.5M-KCl the androgen receptor complex from the control cell lines (RM, SW, & CD) sedimented at about 4S (3.6-4.6S)(Fig.3.2), as did the receptor from the androgen insensitive cell line T4 (Fig.3.3). Under identical conditions, the corresponding peak was

Figure 3.2. Sucrose density gradient analysis of the androgen receptor from control GSF cell lines. 5-20%(w/w) linear sucrose density gradients were prepared in PEM buffer containing 0.5M-KCl. Ammonium sulphate precipitates were resuspended in 0.5ml of the same buffer containing 2-3%(w/v) sucrose and FITC-IgG as a marker: a second gradient containing the proteins FITC-IgG (6-7S) and either BSA (4.6S) or Ovalbumin (3.6S) was prepared in the same way. Gradients were centrifuged at 30000 rev./min. (SW40 rotor, Beckman) for 18-20h. at 4 C; eight drop fractions were then collected from the bottom of the gradient and assayed directly for radioactivity or protein. Arrows indicate the position of the marker proteins. (See Methods 2.5 for details)

(Note. All cells were incubated with ^{3}H mibolerone, except for SW T4 which were incubated with ^{3}H DHT.)



SW
10
6-7S
4.6S
3.6S
TOP
0
10
20
30
40
RM
10
d.p.m (x10^-2)
0
10
20
30
40
CD
10
0
10
20
30
40
Fraction Number

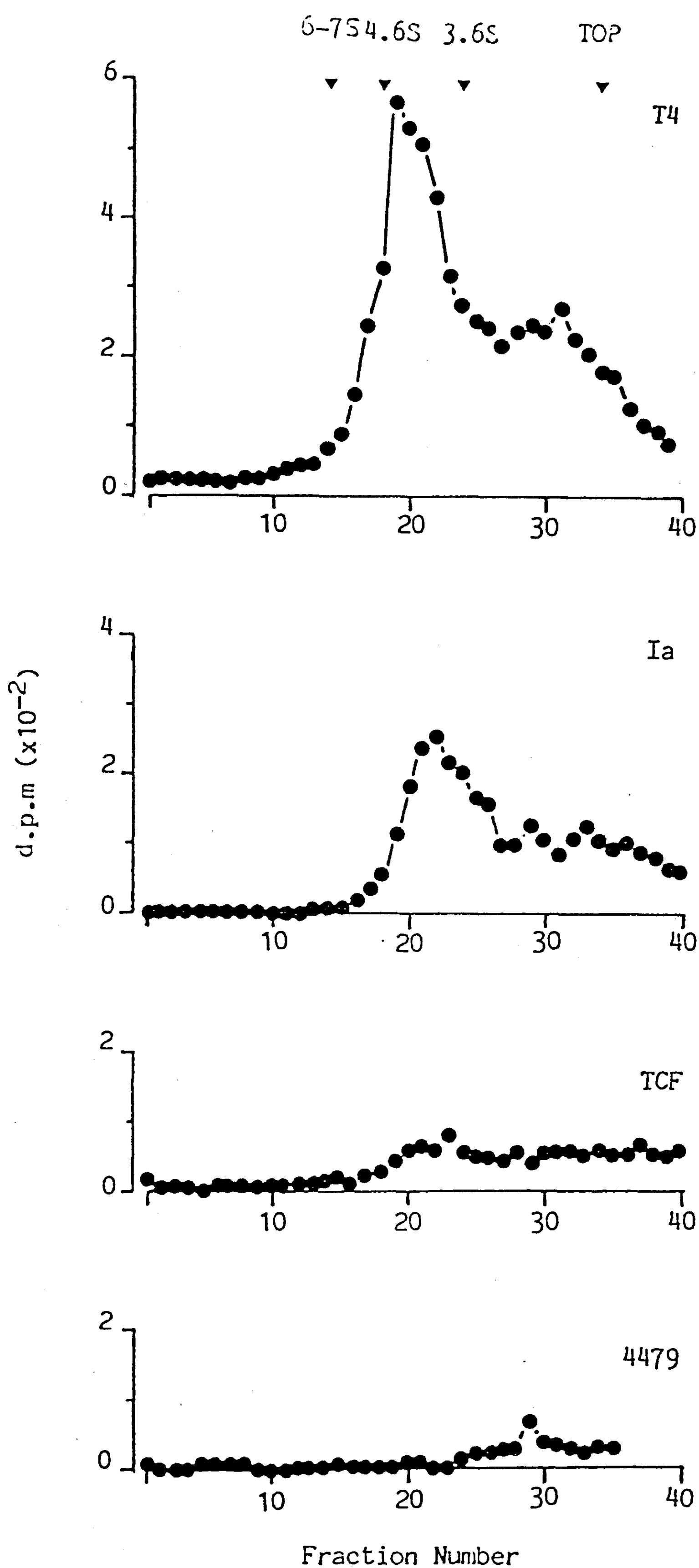


Figure 3.3. Sucrose density gradient analysis of the androgen receptor from androgen insensitive GSF cell lines. For details see the legend to Fig.3.2.

diminished or absent from the androgen insensitive cell lines Ia, TCF and 4479 respectively (Fig.3.3). It was concluded from these studies that the observed sedimentation profiles reflected the findings of whole cell binding assays (Table 3.1), with the exception of TCF, and also the levels of radioactivity recovered in the ammonium sulphate fractions (Table 3.4). It is believed that this is the first report of a correlation between the sedimentation pattern of partially purified receptor complexes and whole cell binding kinetics.

3.4 2'5'-ADP-Sepharose Chromatography.

Figure 3.4 (a & b) shows the elution of the SW and T4 receptor complexes from 2'5'-ADP-sepharose: both control and androgen insensitive complexes eluted at a peak between 0.5 and 1.0M-KCl (fractions 10 & 11). No enrichment of the counts in the peak fractions was seen, however in the fractions 9-12 the amount of protein recovered was 20-30-fold lower than in the total sonicate extract, and 2-4-fold lower than the 35% ammonium sulphate fraction.

This technique has been used successfully by Mulder et al (1984) to partially purify the androgen receptor from rat prostate tissue. In that study, washing the gel apparently removed more than 95% protein, and the receptor was purified 50-fold with a recovery of 70%. Studies with the human fibroblast receptor failed to show similar results, although both rat prostate cytosol and human fibroblast ammonium sulphate fractions gave qualitatively similar elution profiles for labelled receptor. Using the ammonium sulphate fraction from human

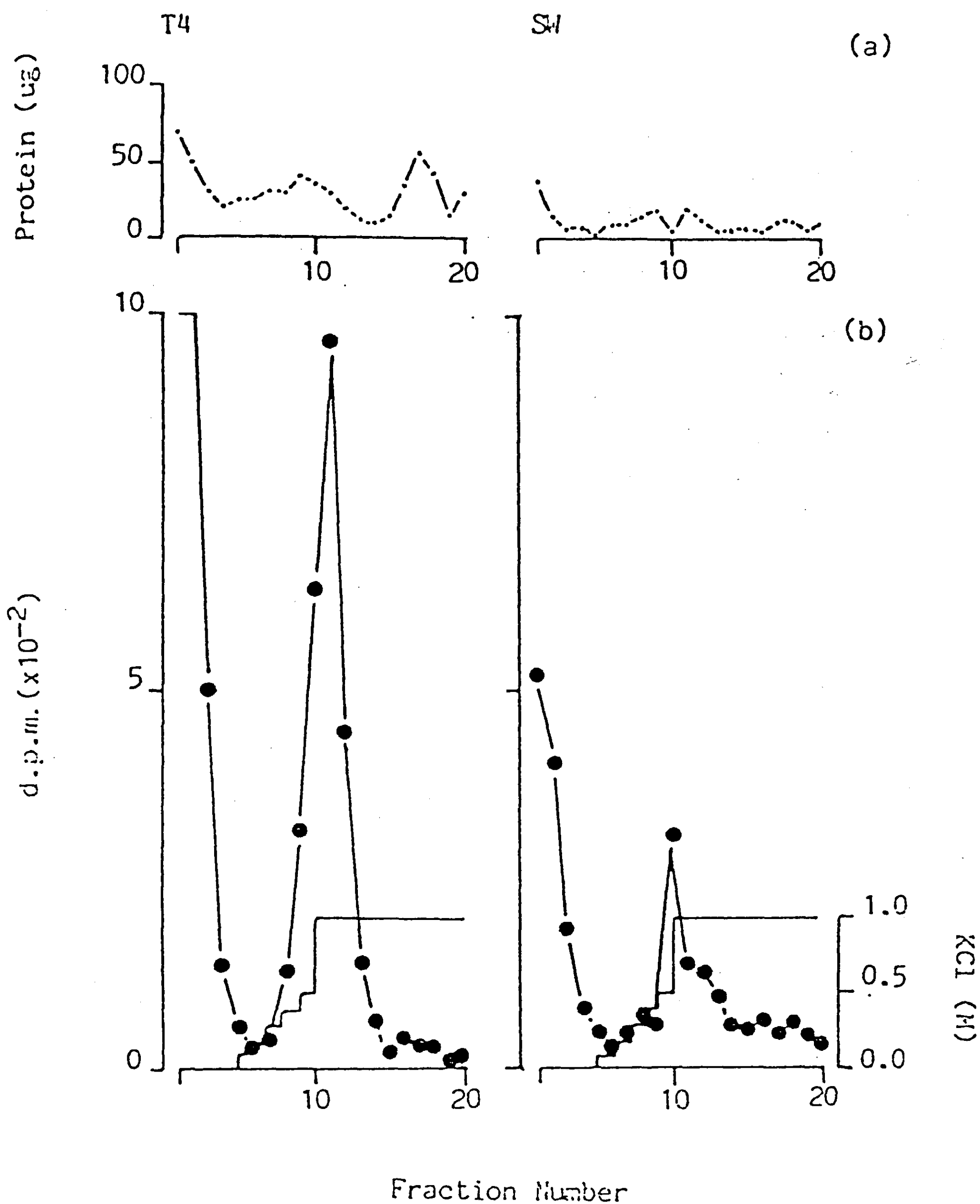


Figure 3.4. 2'5'-ADP-Sepharose chromatography of the GSF androgen receptor. Ammonium sulphate precipitates were resuspended in 2ml PEM buffer containing 10mM-KCl, mixed with a slurry of 2'5'-ADP-sepharose (1g/4ml) and dialysed against the above buffer for 3-4h. at 4 C. After washing the column with 5-10ml of the above bufferthe androgen receptor was eluted with a discontinuous salt gradient (0.1M, 0.2M, 0.3M, 0.4M, 0.5M & 1.0M-KCl); 2ml fractions were collected and assayed for protein (a) and radioactivity (b). The results shown are the mean of three (T4; peak fraction 11 each time) or two (SW; peak fraction 10) separate experiments. (See Methods 2.6 for details)

(Note. Cells were incubated with ^{3}H DHT)

fibroblasts, washing the gel removed between 19-64% of the total protein. Only 20-50% of the total d.p.m. incubated with the ADP-sepharose was recovered by elution with KCl. It was concluded from these data, that binding was lost during the dialysis incubation and/or radioactivity was remaining bound to the column. Therefore in an attempt to determine if more receptor was being eluted than suggested by the recovered d.p.m., fractions were incubated with 1nM [^{3}H]DHT for 3-4 hours at 4°C, and bound counts assayed by DCC (Methods 2.13). The "binding" observed was difficult to interpret, probabably due to non-specific binding. It was concluded from these studies that: the fibroblast receptor lost steroid binding activity, probably irreversibly, and therefore further purification of the receptor complexes by this protocol was not feasible; however this method was useful for comparing the receptor complexes from a control and androgen insensitive cell line, both showed similar binding and elution characteristics.

3.5 Augmentation of the GSF Androgen Receptor.

Table 3.6 shows the partial purification of the androgen receptor from control cultures (pooled SW and RM cells) after incubation of the cells with 2nM [^{3}H]mibolerone or [^{3}H]R1881 for 24 hours. There was no change in the proportion of ^{3}H d.p.m. recovered in the KCl extract (68%) or the ammonium sulphate precipitate (40%), however there was a 2-3-fold increase in the yield of radioactivity in these fractions (see Table 3.4 for comparison). It was therefore concluded that this was a superior

Table 3.6 Extraction of GSF androgen receptors after 24h incubation with 2nM [^{3}H]mibolerone **or Methyltrienolone (R1881).**

Fraction	Mean ± SD (n=7) d.p.m.	Protein (mg)	Specific activity (dpm/mg protein)
[^{3}H]Mibolerone:			
Cell Homogenate	259193±57082	5.42±1.76	49400± 8600
Pellet	97554±37991	2.32±0.72	42000±10500
Salt extract	176357±54520	2.94±1.21	63000±14400
AS	103193±55499	0.78±0.30	148000±71100
Supernatant	70464±71813	2.18±0.93	27500±16900
[^{3}H]R1881:			
Cell Homogenate	228533±46774	5.00±1.27	46100±2200
Pellet	93017±30816	2.20±0.62	42300±8900
Salt Extract	169367±67642	2.87±0.67	57600±9600
AS	91667±24384	0.67±0.23	143600±45800
Supernatant	72700±62253	2.33±0.71	30400±15200

$2x10^6$ cells (SW and RM cell lines) were seeded in 140mm plastic petri dishes (four/experiment), and grown to confluence. The cells were then incubated in EC1 medium containing 2nM [^{3}H]mibolerone for 24h at 37 C, prior to salt extraction and partial puification of the androgen receptor. See Methods 2.3 and 2.4 for details).

(The data represents the mean±standard deviation (SD): n=7, Mibolerone binding; n=3, R1881 binding.)

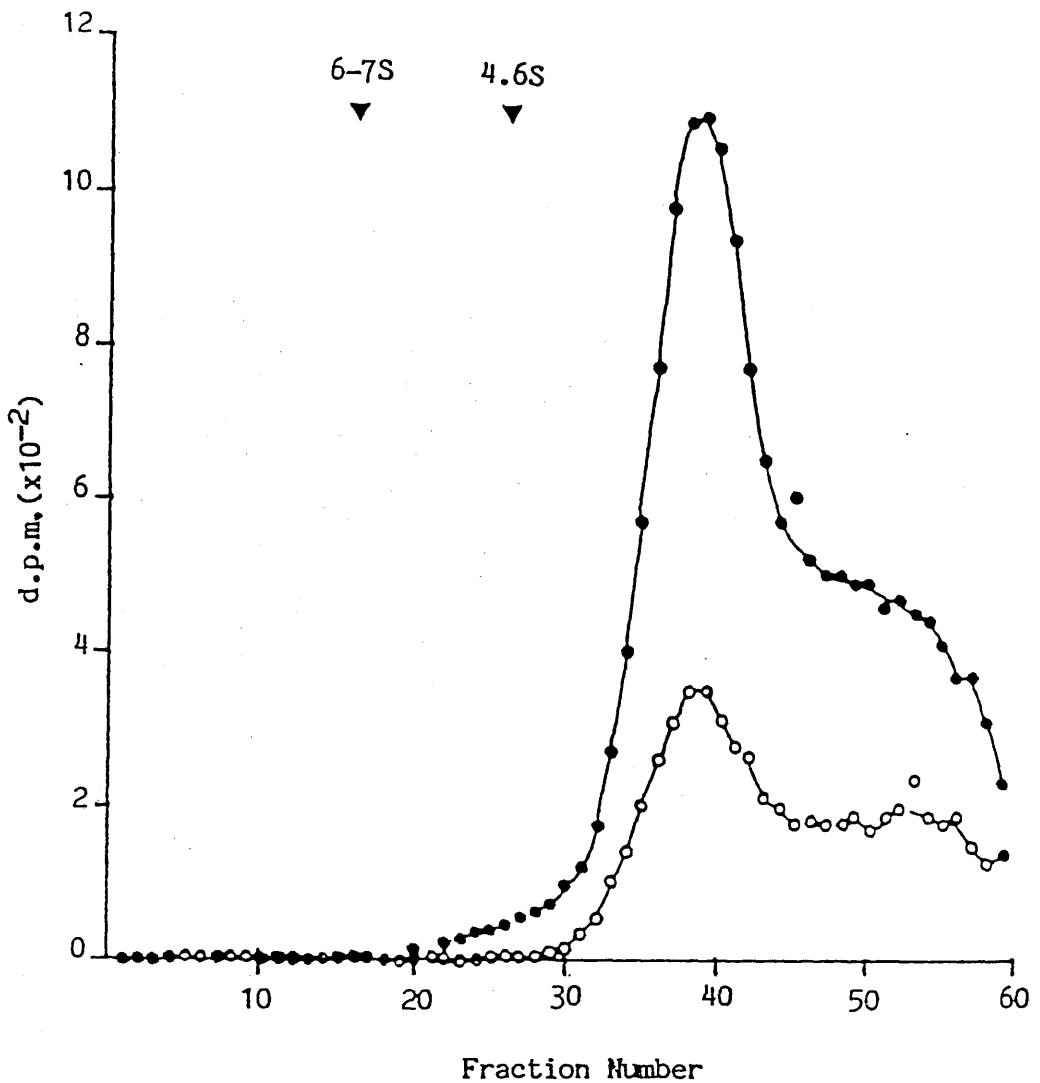


Figure 3.5 Sucrose density gradient analysis of the GSF androgen receptor after incubating cell for 24 hours with 2nM [^{3}H]mibolerone. Cells were collected and sonicated in PEM buffer containing 0.5M-KCl: the 105000xg salt extract was then layered on to a 5-20%(w/v) linear sucrose density gradient, and centrifuged overnight at 40000 rev./min. (SW40 rotor). Four drop fractions were collected from the bottom of the tube and assayed for radioactivity. ○ , 0.5-1.0 hour incubation; ●, 24 hour incubation. The marker proteins IgG (6-7S) and BSA (4.6S) were centrifuged in a parallel gradient. Data kindly supplied by Dr.M.B.Hodgins.

method for labelling receptors than the shorter 30-40 minute incubation. Ammonium sulphate precipitation resulted in a 3-fold enrichment of the ^{3}H counts; and Figure 3.5 shows a representive sucrose density gradient of the 105000xg KCl-extract before and after androgen receptor augmentation.

3.6 FPLC-Anion Exchange Chromatography of the Human Androgen Receptor.

After incubating control cultures (RM & SW) for 24 hours with 2nM [^{3}H]mibolerone, the receptor complexes from the 105000xg salt extract (desalted first; Methods 2.7) and the ammonium sulphate fraction, eluted from a Mono Q column as single peaks, at 0.13-0.18M-KCl (Fig.3.6 a,b,c). Of the loaded radioactivity 30-45% was recovered by eluting with KCl, the remaining d.p.m. could then be recovered by washing the column with 80%(v/v) methanol and 75%(v/v) acetic acid. These latter d.p.m. were thought to represent non-specific interaction between the ligand and the column, as extending the salt gradient to 1.0M-KCl failed to elute any other peaks of ^{3}H binding (Fig.3.6c).

The androgen receptor from rat prostate, epididymis and calf uterus cytosol have been partially purified by FPLC-anion exchange on a Mono Q column (Brinkmann et al 1985a). All three forms of the receptor eluted as sharp peaks at 0.32M-NaCl; this would suggest that there was a weaker interaction between the human fibroblast receptor (0.13-0.18M-KCl) and the anion exchange resin. This finding is in agreement with a recent study by Keenan et al (1986), who reported that the activation of the receptor

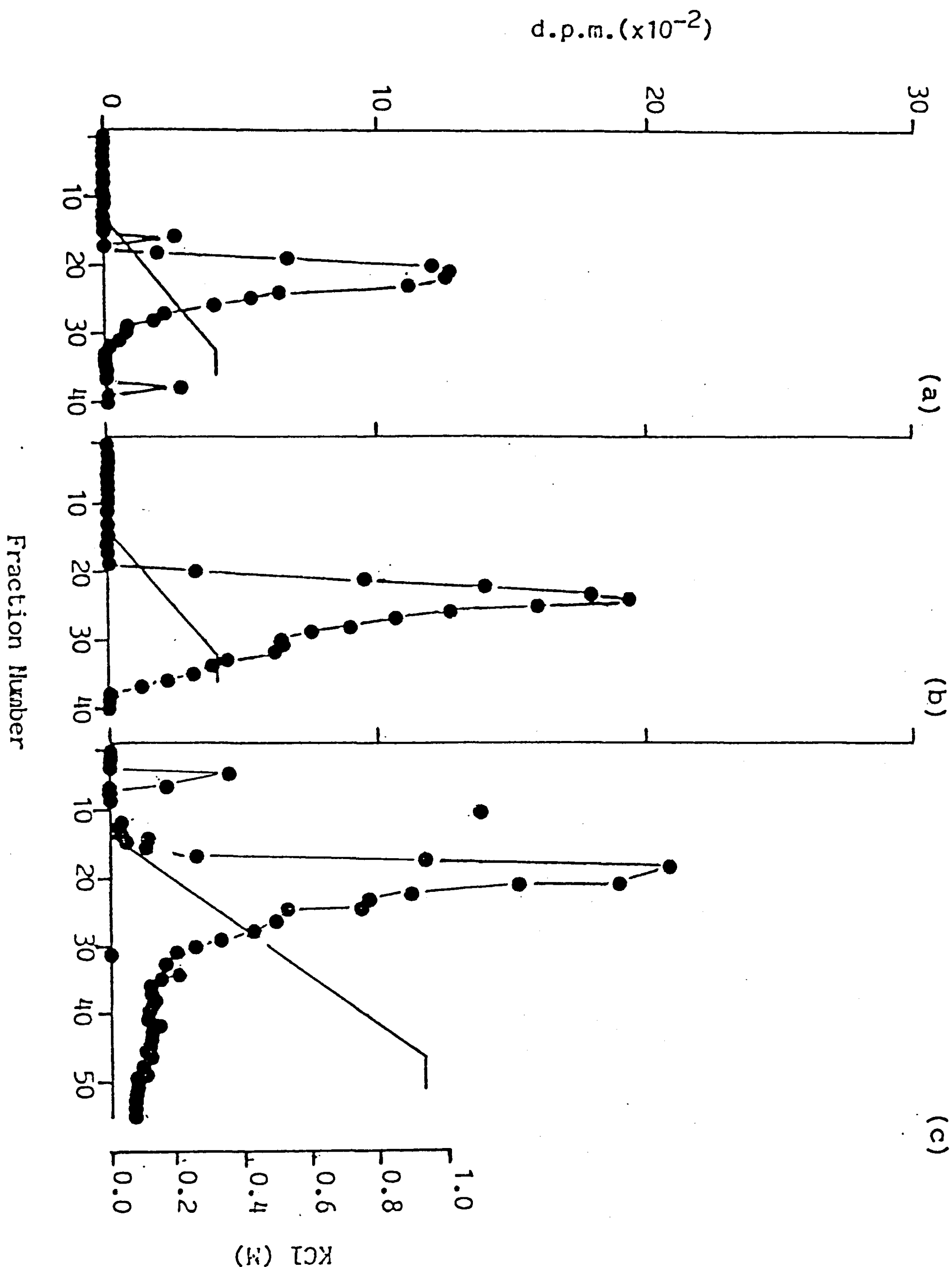


Figure 3.6 FPLC-anion exchange chromatography of the GSF androgen receptor. Confluent cultures of SW and RM cells were incubated with 2nM[^{3}H]mibolerone for 24h. and the 105000xg salt extract (a) or 35% ammonium sulphate fraction (b & c) chromatographed on a Mono Q anion exchange column. The androgen receptor was eluted with a linear salt gradient, 0-0.35M-KCl (a & b) or 0-1.0M-KCl (c), at a flow rate of 1ml/min.; 1ml fractions were collected and assayed for radioactivity. (See Methods 2.7 for details)

complex from human GSF was associated with, among other things, a loss of negative charge. The work of Brinkmann and assoaciates (Brinkmann et al 1985a) was carried out on the unactivated molybdate-stabilised receptor form, while the present studies were on the salt-extractable nuclear receptor form. It is possible therefore, that the difference in salt concentration required to elute the GSF androgen receptor may be due to receptor activation.

In the study of Brinkman et al (1985a) the rat prostate receptor was purified 75-fold with a recovery of 71%, while the receptors from rat epididymis and calf uterus had recoveries of 85%. Similar results could not be achieved with the human GSF receptor complexes. In the peak fraction, there was a 4-6-fold enrichment of d.p.m./mg protein over the total sonicate, with a recovery of 3-7% (of d.p.m. loaded onto the column): extending the analysis to cover fractions 18-28, the recovery of d.p.m. increases to 17-33% but the enrichment is decreased to 0.8-3.0-fold. Therefore, it would seem that under these experimental conditions (working at room temperature), receptor binding was impaired so that the degree of purification and yield of receptor complexes was much poorer than expected.

Finally, in one experiment, the amount of bound counts in the ammonium sulphate and FPLC peak fractions (19-23) was determined (hydroxapatite assay; Methods 2.13), and found to be 31.4% and 14.6% of the total d.p.m. recovered in these fractions respectively. It seemed likely that these were underestimates of the binding, possibly due to the interference of salt in the

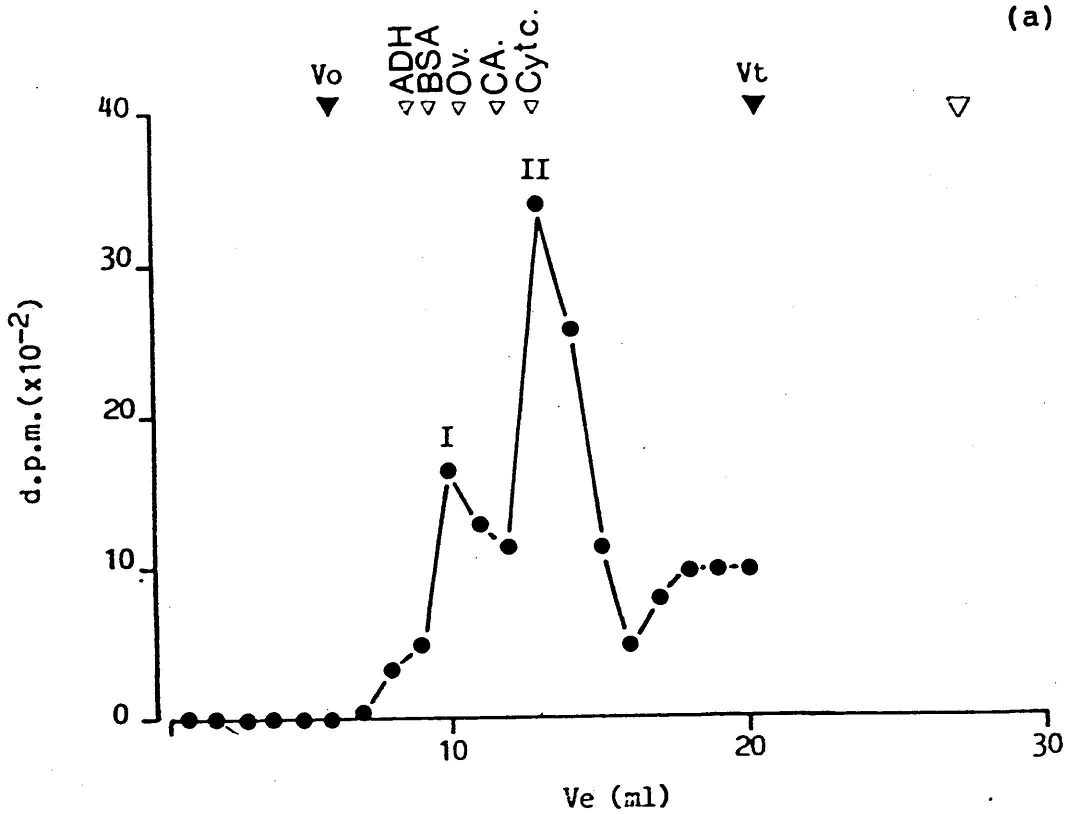
binding assay and/or the loss of bound radioactivity during the anion exchange chromatography.

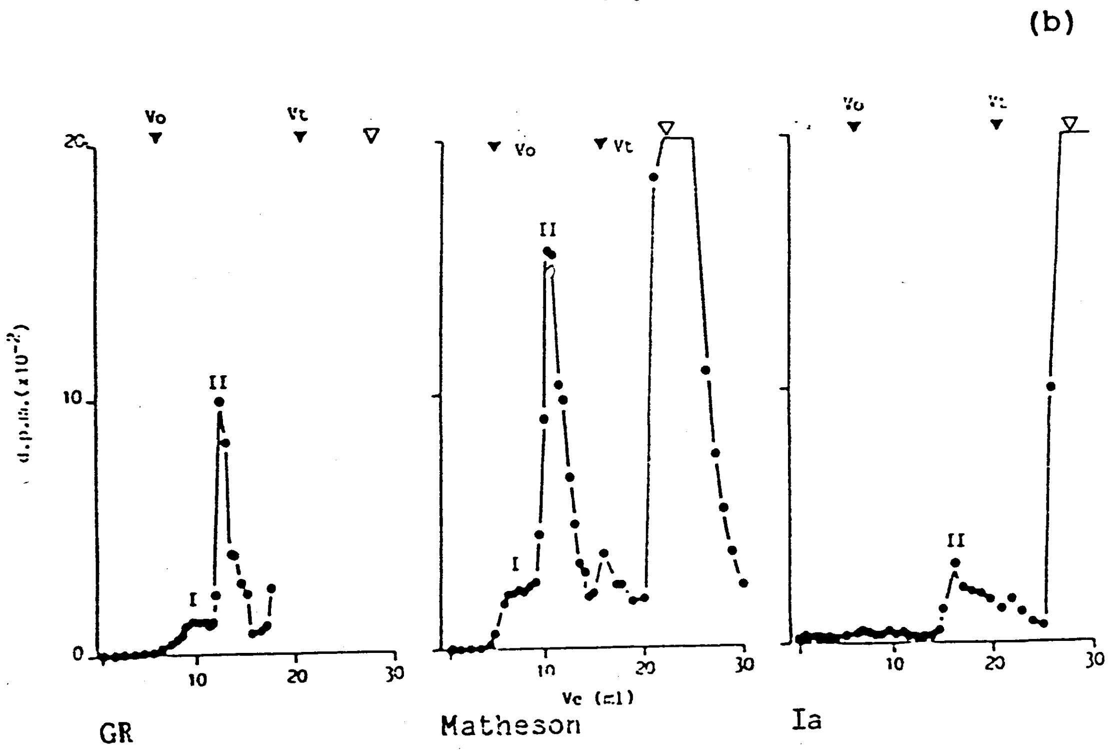
3.7 HPLC-Size Exclusion Chromatography.

The androgen receptor from control cells (pooled SW and RM cultures) eluted from a gel filtration column as two distinct peaks; with relative molecular masses (Mr.) of 63.1K (I) and 13K (II), and Stokes Radii (Rs) of 3.16nm and 1.58nm respectively (Fig.3.7a). The Mr. and Rs were calculated using standard proteins resolved under identical conditions (Fig.3.8). A smaller amount of the larger species was recovered. The receptor complexes from another control cell line, GR, also resolved into two "peaks" of radioactivity: at Mr. 63K and 15K, and Rs 3.16nm and 1.78nm respectively (Fig.3.7b). HPLC-gel filtration of the androgen receptor from two androgen insensitive cell lines gave quite different results. The receptor from Matheson showed a similar elution profile to that of the control cell lines; two "peaks" of activity were seen at Mr. 89.1K and 15.8K, and Rs 3.98nm and 1.78nm respectively. However, with the cell line Ia only a very small fragment, eluting close to the total volume of the column was seen (Fig.3.7). Table 3.7 summarizies this data, together with the Mr. and the f/f_o ratio calculated from the Stokes radius and sedimentation coefficient. The values obtained for sedimentation coefficient, Mr., Rs and f/f_o of the human GSF androgen receptor are in close agreement with those reported recently for the calf uterus androgen receptor (de Boer et al 1986). However the results of Keenan et al (1986) suggested that the human GSF androgen receptor was larger, with a calculated

Figure 3.7 HPLC-size exclusion chromatography of the androgen receptor from normal and androgen insensitive GSF. Confluent monolayer cultures of SW and RM cells were incubated with [^{3}H]R1881 for 24h.The 35% ammonium sulphate precipitate, of the salt extract, was re-suspended in 0.3-0.5ml of PEM buffer containing 0.5M-KCl and 10%(v/v) glycerol and resolved on a TSK G3000 SW column; 1ml fractions were collected and assayed for radioactivity (a). The androgen receptor complexes from the control (GR) and the androgen insensitive (Matheson and Ia) cell lines were also chromatographed on the TSK G3000 SW column, with the modifications that cells were incubated with 2nM [^{3}H] mibolerone and 0.5ml fractions were collected up to 15ml and thereafter 1ml fractions collected to the end (b). The elution of free steroid (▽) was determined in a separate experiment by loading 100000 d.p.m. of [^{3}H]mibolerone. (see Methods 2.8 for details)

Ve=Elution volume
Vo=Void volume; elution volume of Blue Dextran
Vt=Total volume; elution volume of Phenol Red
(See legend to Figure 3.8 for details on column calibration)


(a)
Vo
ADH
BSA
Ov.
CA.
Cytc.
Vt
40
30
20
10
0
d.p.m.(x10^{-2})
II
I
10
20
30
Ve (ml)


(b)
Vo
Vt
20
10
0
d.p.m.(x10^{-2})
II
I
10
20
30
Ve (ml)
GR
Matheson
Ia

(a)

Vo

ADH BSA CARBONIC ANHYDRASE CYT. C.

O.D. 280nm

0.0 2.0 4.0 6.0 8.0 10.0 12.0

Ve (ml)

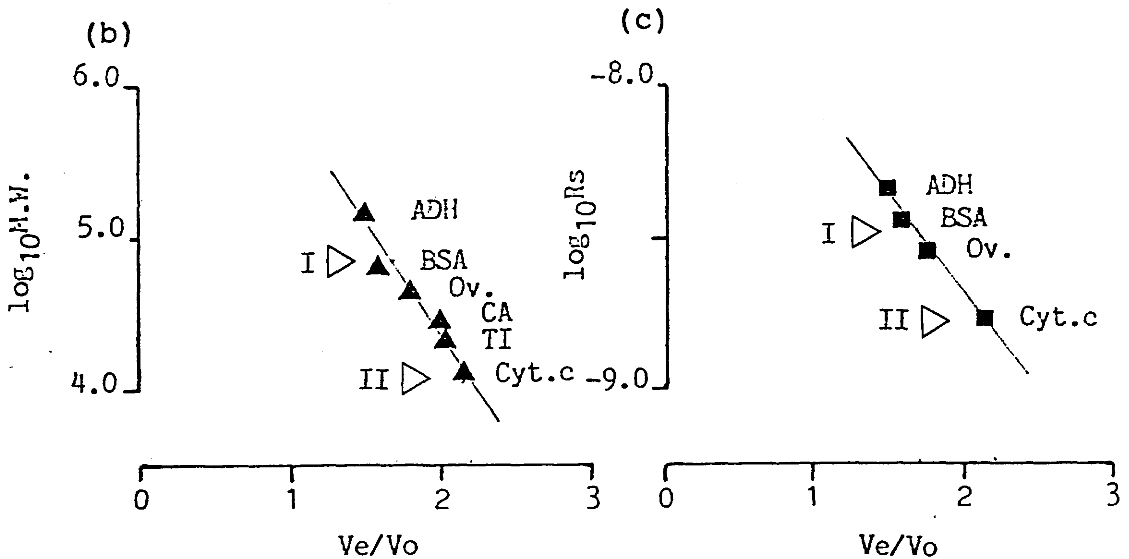


Figure 3.8 Calibration of the HPLC-gel filtration column (a). The column was calibrated using mixtures of proteins of known molecular weight (b) and Stokes radius (c), resolved under identical conditions to the ammonium sulphate fractions (See Methods 2.8 for details). The position of the peaks of bound radioactivity (I and II) has been superimposed on the standard curves (b & c).

Table 3.7 Physicochemical parameters of the human GSF androgen receptor.

Cell line	Sedimentation Coefficient (S)	Rs. (nm)	Mr. a.	Mr. b.	f/f_o	Axial Ratio*
RM/SW	4.4	3.16	63.1K	60K	1.21	3.00
		1.58	13.0K	-	-	-
GR	4.0	3.16	63.1K	54K	1.43	6.75
		1.78	15.0K	-	-	-
Matheson	4.0	3.98	89.1K	68.5K	1.46	6.75
		1.66	15.8K	-	-	-

Sedementation coefficients derived from sucrose density gradient centrifugation; except for Matheson, 4.0S assumed value. Stokes radius (Rs) measured from HPLC-gel filtration, as was Mr. (a); Mr. (b) was calculated using the the sedimentation coefficient and the Rs:

$$Mr = 6.\pi.\eta.N.Rs.S/(1-vp) \qquad - 1.$$

$$f/f_o = Rs/(3.v.M/4.\eta.N)^{1/3} \qquad - 2.$$

Where:

π= 3.14

η= viscosity= 0.914 (calculated using ovalbumin in equation 1)

S= sedimentation coefficient ($x10^{-13}$S)

v= partial specific volume= $0.74cm^3$

p= density of the medium= $1.0259g/cm^3$

M= Mr. and N= avagadro's Number ($6.02x10^{23}$)

* Axial ratio for prolate ellipsoid (a>b) was calculated from the frictional ratio (f/f_o), assuming solvation of 0.2g/g of protein, and published tables (Oncley 1941).

molecular weight of between 114300-134500-daltons.

The elution of free steroid (large open arrow), under identical conditions, occured after the elution of Phenol red (21ml) at between 26.5-30.5ml (peak at 27.5ml).

3.8 Photoaffinity Labelling Studies.

A. Rat prostate.

The rat prostate cytosol receptor was partially purified by FPLC-anion exchange chromatography; eluting as a single peak at 0.26M-NaCl (Fig.3.9a). After U.V. irradiation and SDS-PAGE a peak of bound radioactivity believed to be the androgen receptor was recovered at Mr. 56K (Fig.3.9b).

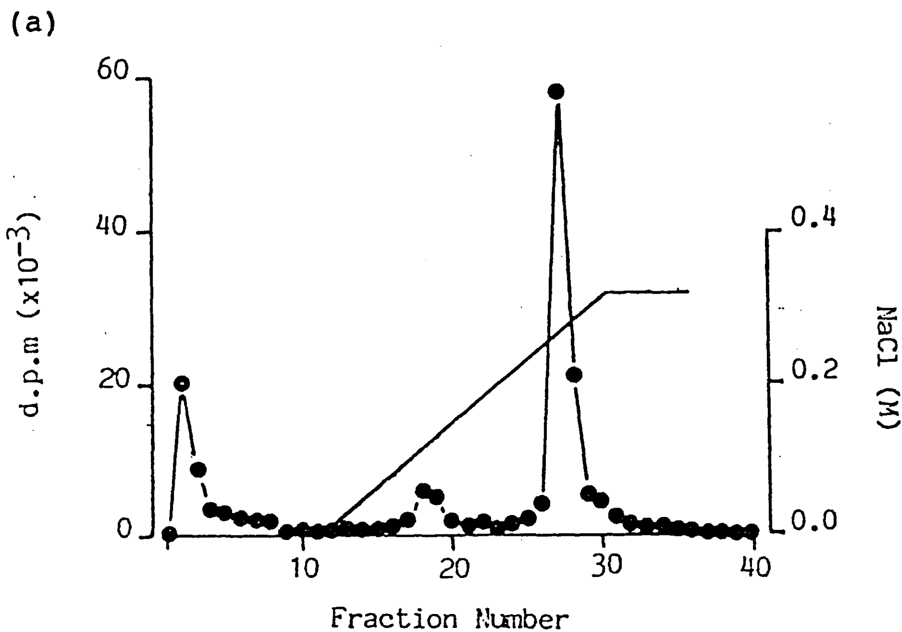
B. Calf uterus.

The calf uterus androgen receptor was partially purified by DNA-cellulose chromatography; eluting with a recovery of 10%, as a relatively broad peak (Fig.3.10a). After U.V. irradiation and SDS-PAGE a peak of bound radioactivity was seen at about 100K Mr. (Fig.3.10b). The peak from both the DNA-cellulose column and the polyacrylamide gel could be completely surpressed if excess cold steroid was present throughout the experiment.

These findings were in close agreement with those already reported by Brinkmann et al (1985a,b), and confirmed the usefulness of this protocol for studing the androgen receptor in different tissues.

C. Human GSF.

Control cultures, RM and SW, were incubated with either [^{3}H]mibolerone or [^{3}H]R1881 for 24 hours. Androgen receptor



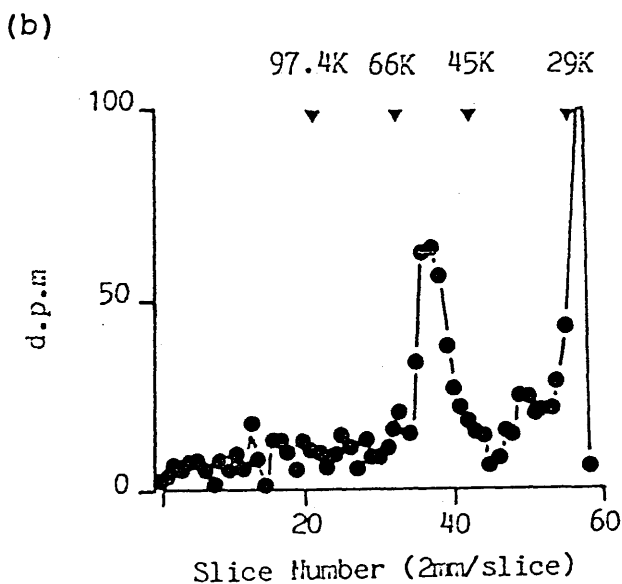
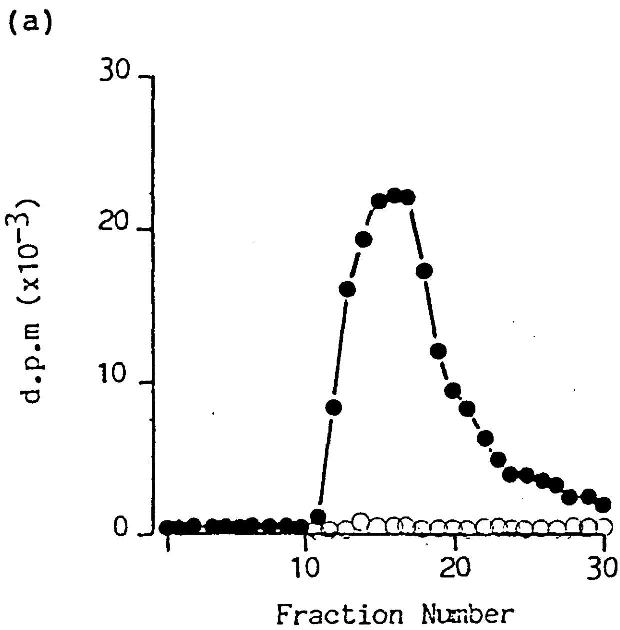


Figure 3.9 Photoaffinity labelling of the rat prostate androgen receptor. Prostate cytosol from 24h castrated rats was incubated with 15nM [^{3}H]R1881 for 2h and the labelled androgen receptor partially purified by FPLC-anion exchange chromatography. The receptor complexes were eluted with a linear salt gradient (0-0.35M-NaCl), and 1ml fractions collected and assayed for radioactivity: the result shown is the mean from three separate experiments (a). The peak fraction (fraction 27) was irradiated, and the photolinked [^{3}H]R1881-receptor complexes were then precipitated with 10%(w/v) trichloroacetic acid. The trichloroacetic acid insoluble material from three separate experiments was then pooled and analysed by SDS-PAGE (b). The arrows indicate the position of the marker proteins: Phosphorylase b (97.4K), BSA (66K), Ovalbumin (45K), and Carbonic anhydrase (29K). (See Methods 2.9A for details)

(a)



(b)

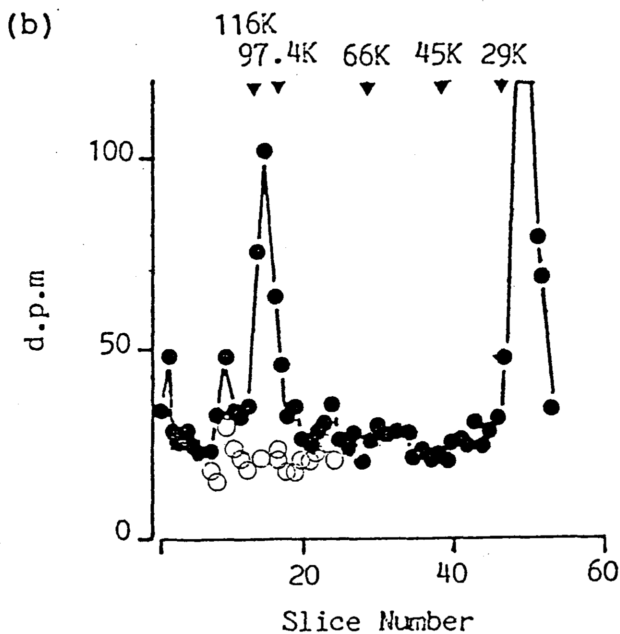


Figure 3.10 Photoaffinity labelling of the calf uterus androgen receptor. Labeled calf uterus cytosol ([^{3}H]R1881 with (○) or without (●) 3uM-DHT) was brought to 40% saturation with ammonium sulphate; and the receptor further purified by DNA-cellulose chromatography (a). Fractions 13-18 were pooled from both incubations (Hot ± Cold) and irradiated; the photolinked receptor complexes were then precipitated with 10%(w/v) trichloroacetic acid and analysed by SDS-PAGE (b). The arrows indicate the position of the marker proteins: -galactosidase (116K), Phosphorylase b (97.4K), BSA (66K), Ovalbumin (45K), and Carbonic anhydrase (19K). (See Methods 2.9B for details)

complexes were then partially purified by FPLC-anion exchange chromatography and/or 35% ammonium sulphate precipitation and irradiated with a high pressure mercury U.V. lamp for 10-15 minutes (bound mibolerone had to be exchanged for R1881 prior to irradiation) (Methods 2.9). Irrespective, of the protocol followed (I, II a or b; Methods 2.9C), after trichloroacetic acid precipitation and extraction with ethylacetate, no photolinked material was recovered on 8% polyacrylamide gels, except at the dye front (Fig.3.11 a & b). On this % gel it is difficult to resolve proteins of Mr. below 24-29K; therefore it is possible that a small fragment of the receptor, containing covalently linked [^{3}H]R1881 (Mereoreceptor ?), is running with the dye front. Furthermore, it would be interesting to see if the peak of radioactivity seen at 13-15K, after HPLC-gel filtration related to this material that runs coincident with the dye front on SDS-gels. However, it should be pointed out, that this material could simple be cross-linked steroid (William et al 1986) and/or non-extractable (in organic solvents), non-specifically associated steroid (Mainwaring & Randall 1984).

In situ U.V.-irradiation. Table 3.8 shows the extraction and partial purification of the androgen receptor after in situ U.V.-irradiation of human GSF cells (RM &SW): there was a reduction in the total number of d.p.m. recovered in the total cell homogenate (4-fold) and ammonium sulphate (about 20-fold) fractions, and no enrichment of counts in the latter (see Table 3.6 for comparison). Analysis of the ammonium sulphate precipitated material by SDS-PAGE (Fig.3.11c) and HPLC-gel filtration (Fig.3.12a) failed to detect a peak of photolinked receptor

Table 3.8 Extraction of the GSF androgen receptor after in situ U.V. irradiation.

Fraction	Mean±SD (n=3)		
	d.p.m.	Protein (mg)	Specific activity (dpm/mg protein)
Cell Homogenate	52610±1886	4.71±0.49	11400±3600
Salt extract	19691±7864	1.45±0.40	14900±4300
AS	2205± 227	0.37±0.18	7400±4300
Supernatant	14162±5594	1.17±0.18	12800±6000
Pellet I	37153±7377	3.43±0.45	10900±2200
Triton X-100	23661±1734	2.02±0.79	13400±6800
DNAase I	1880± 971	0.39±0.26	5100±1000
Pellet III	3528±2743	0.32±0.25	11300±4300

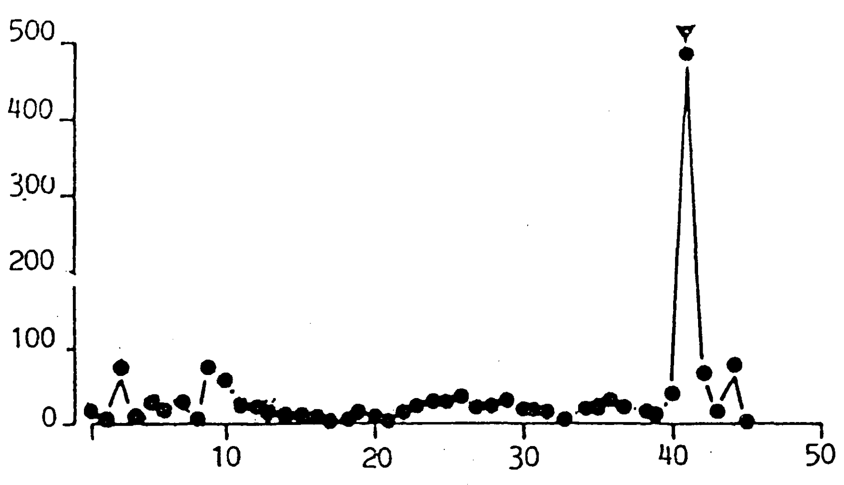
$2x10^6$ cells (SW and RM cell lines) were seeded in 140mm plastic petri dishes (four/experiment) and grown to confluence. The cells were then incubated in EC1 medium (15ml/dish) containing 2nM [^{3}H]R1881 for 24h. The cell monolayers were subsequently rinsed with ice cold PBS and the dishes inverted on a U.V.-transiluminator for 2 min. A salt extract and a 35% ammonium sulphate fraction were prepared. Label remaining in the 105000xg pellet I after salt extraction was further investigated by 1%(v/v) Triton X-100 extraction (30min.) and subsequent DNAase I (25ug/ml) digestion (60min.). See Methods 2.9 for details.

Figure 3.11 SDS-PAGE of the fibroblast androgen receptor after U.V. irradiation. Cells were incubated with 2 nM [^{3}H]mibolerone for 24h and the androgen receptor partially purified (FPLC-anion exchange and/or 35% ammonium sulphate precipitation). The sample was then incubated with 15nM [^{3}H]R1881 for 20h at 4 C, to exchange the bound mibolerone for the photoactive ligand R1881, and irradiated. Photolinked receptor complexes were recovered by 5-10% trichloroacetic acid precipitation, and resolved on an 8% polyacrylamide gel (a). Alternatively, cells were incubated with 2nM [^{3}H]R1881 for 24h. The ammonium sulphate fraction was then irradiated directly, and the trichloroacetic acid insoluble material resolved on an 8% polyacrylamide gel (b). Lastly, cells were incubated with 2nM [^{3}H]R1881 for 24h and irradiated _in situ_ using a U.V. transilluminator. After salt extraction the receptor complexes were precipitated with 35% ammonium sulphate and resolved on an 8% polyacrylamide gel (c).

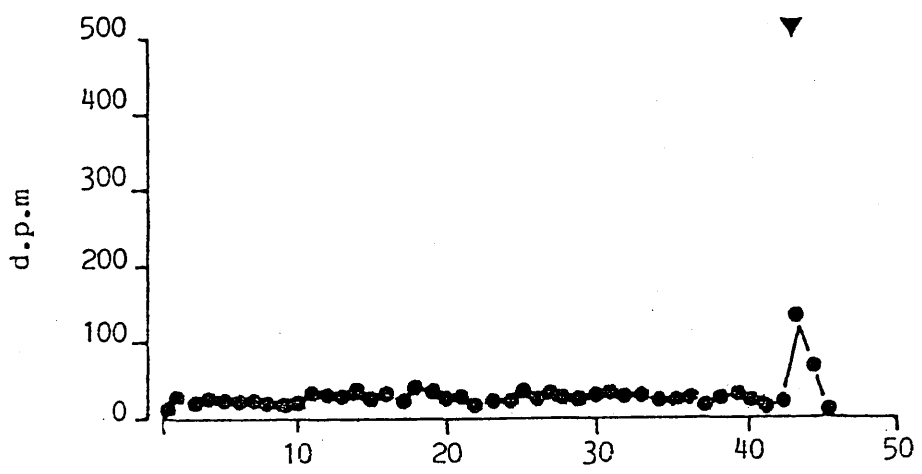
(a) cells labelled with [^{3}H]Mibolerone, and receptor complexes exchanged with [^{3}H]R1881 prior to irradiation.
(b) as a, except cells labelled with [^{3}H]R1881 (no exchange necessary).
(c) cells incubated with [^{3}H]R1881, and irradiated directly.

(See Methods 2.9C and Fig.2.1, for details)

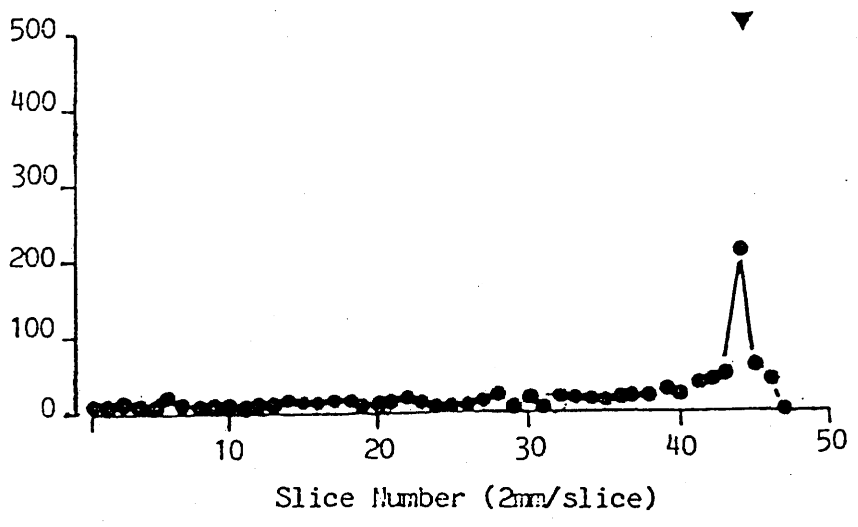
(a)



(b)



(c)



complexes; HPLC-gel filtration of the residual pellet (after Triton extraction and DNAase I digestion) gave a similar result (Fig.3.12c), (to that of the ammonium sulphate fraction). However gel filtration of the Triton X-100 extracted radioactivity (after trichloroacetic acid precipitation) revealed a peak of radioactivity at, or very close to the void volume, which would suggest an aggregate of Mr. >300K (Fig.3.12b). The significance of this species is not clear, and it may simply be an artefact of the experimental procedure. The lack of success with the *in situ* U.V.-irradiation protocol for the human fibroblast androgen receptor, as compared to the avian oviduct progesterone receptor (Horwizt & Alexander 1983), may reflect a general instability of the androgen receptor. It is of interest, therefore, that without *in situ* U.V.-irradiation, 50-60% of the salt extracted d.p.m. were precipitated and about 40% remained in the supernatant (Table 3.6), whereas after *in situ* U.V.-irradiation only 11.2% of salt extracted radioactivity was precipitated and 71.9% remained in the supernatant. It is possible therefore, that U.V. irradiation has resulted in receptor degradation to a fragment(s) that is(are) no longer precipitated by 35% ammonium sulphate. It would be of interest to analyse the ammonium sulphate supernatant fraction by SDS-PAGE and/or HPLC-gel filtration, to see if any peaks of radioactivity could be recovered. Comparison of Tables 3.6 and 3.8 suggested that *in situ* U.V.-irradiation of fibroblast cultures resulted directly or indirectly in a loss of receptor bound d.p.m. This was investigated by incubating the cells with [^{3}H]R1881$\pm$200-fold excess of cold steroid (Table 3.9).

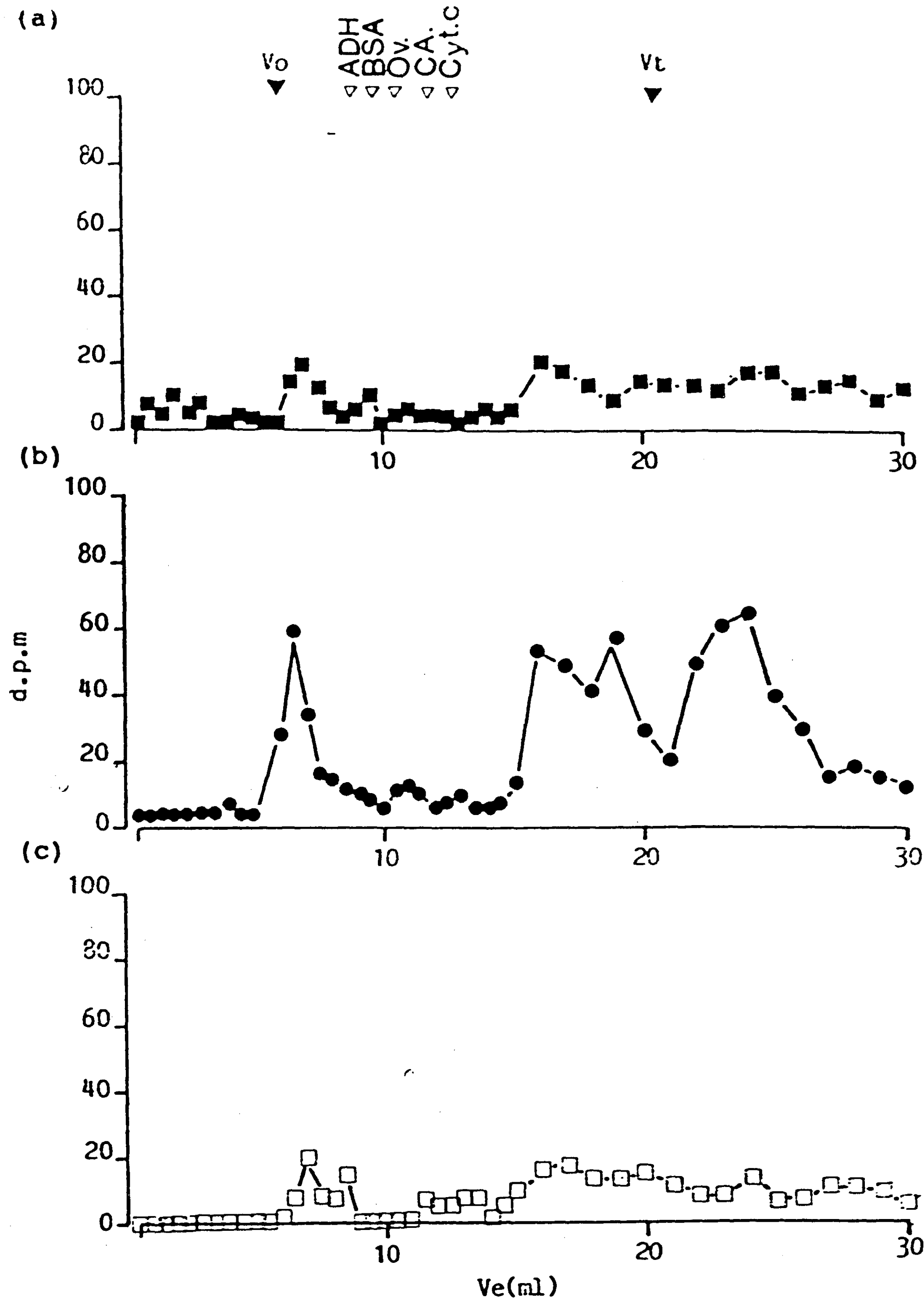


Figure 3.12 HPLC-size exclusion chromatography of the GSF androgen receptor after *in situ* U.V. irradiation. Cells were incubated with 2nM [^{3}H]R1881 for 24h, and irradiated *in situ*. The 35% ammonium sulphate precipitate was prepared, and label remaining in the 105000xg pellet after salt extraction further investigated by extraction with 1% Triton X-100 and subsequent DNAase I (25ug/ml) digestion. All samples were suspended in PEM buffer containing 0.5M-KCl and 10% (v/v) glycerol, and resolved on TSK G3000SW column; 0.5ml fractions (up to 15ml) and 1ml fractions were collected and assayed for radioactivity.

(a) Ammonium sulphate fraction.
(b) Triton extract } See Methods 2.9C
(after trichloroacetic acid precipitation) (Fig.2.1)
(c) Residual pellet.

Although saturable binding was demonstrated, there was no up-regulation of the androgen receptor and no enrichment of specific binding after 35% ammonium sulphate precipitation (actually a reduction, only 21% of the "specific binding" of the total cell homogenate was recovered in the precipitate). However, as was discussed previously, Methods 3.2, incubating whole cells with cold steroid probably underestimates the level of non-specific binding in sub-cellular fractions due to the redistribution of label, so that the apparent "specific binding" may be much lower than indicated (Table 3.9).

It can be concluded from these experiments, that the yield of receptor was reduced after in situ U.V.-irradiation, due directly or indirectly to the harmful affects of the U.V. light. This loss of receptor binding, coupled with the inefficiency of the photolinking reaction could explain the difficulty encountered in detecting photolinked receptor complexes after SDS-PAGE or HPLC-gel filtration.

3.9 2-D Gel Electrophoresis Studies.

A. The use of Dual-label autoradiography and 2-DGE to compare receptor enriched fractions, from control and androgen insensitive GSF.

i. Time course of isotope incorporation (Methods 2.10). The incorporation of [^{35}S]methionine and of the methionine analogue [^{75}Se]selenomethionine, into total newly synthesised protein, reached a maxium between 8 and 12 hours (Fig.3.13). About twice as much ^{35}S as ^{75}Se was incorporated at all the time points studied.

Table 3.9 Effect of in situ U.V.-irradiation on Specific Binding.

Fraction	Volume (ml)	d.p.m. Total	d.p.m. NS	Protein (mg) Total	Protein (mg) NS	"Specfic Binding"
Cell Homogenate	1.00	33760	16350	2.84	2.89	32.5
Salt Extract	1.00	12650	4150	1.34	1.42	34.0
AS	0.25	2338	1300	0.36	0.25	6.8
Supernatant	1.00	8650	3000	0.54	0.49	51.8
Pellet I	0.50	25225	12725	2.54	2.61	26.2
Triton X-100	0.50	14575	6225	0.92	0.94	48.2
DNAase I	0.50	1575	850	0.21	0.26	22.0
Pellet III	0.50	5675	3950	0.72	0.61	7.3

Cells from control cell lines (RM and SW) were seeded in 140mm dishes, and grown to confluence. Cells were then incubated with 2nM [^{3}H]R1881 ± 200-fold excess of cold R1881 for 24 hours, at 37 C, the cell monolayers were then rinsed three times with ice cold PBS and inverted on a U.V.-transilluminator for 2 min., and then scraped off in PBS. Cells were then fractionated as decribed in the legend to Table 3.8.

Specific Binding= Total-Non specific (NS): fmoles/mg protein.

ii. Composition of the labelling medium (Methods 2.10). The labelling medium was originally choosen to ensure that the level of methionine would not become a limiting factor during the metabolic labelling of fibroblast proteins; it was therefore necessary to check that the level of cold methionine used did not inhibit the incorporation of label into newly synthesised protein. Table 3.10 shows that altering the cocentration of cold methionine between 0 and 3.72uM did not significantly affect the recovery of labelled methionine or methionine analogue in the trichloroacetic acid insoluble material. However, slightly more ^{35}S was incorporated at the highest concentration of cold methionine, with the opposite being true for ^{75}Se.

Again, as was noted above, twice as much ^{35}S appeared to be incorporated; this was observed for all concentrations of cold methionine used. A similar trend was seen in all subsequent labelling experiments, and was not due to differences in protein content of the different fractions. The difference could not be accounted for soley in terms of the different specific acivities of the two isotopes (Methods 2.10), as in the labelling medium used there was at least a 1000-fold molar excess of unlabelled methionine. Therefore contrary to the findings of Lecocq et al (1982), [^{35}S]methionine and [^{75}Se]selenomethionine were not incorporated with equal efficiency, into newly synthesised proteins by human GSF.

The discrepancy in isotope incorporation did not affect subsequent 2-DGE studies directly, as the ammonium sulphate fractions were routinely mixed (Methods 2.11) at a ratio of 1:2

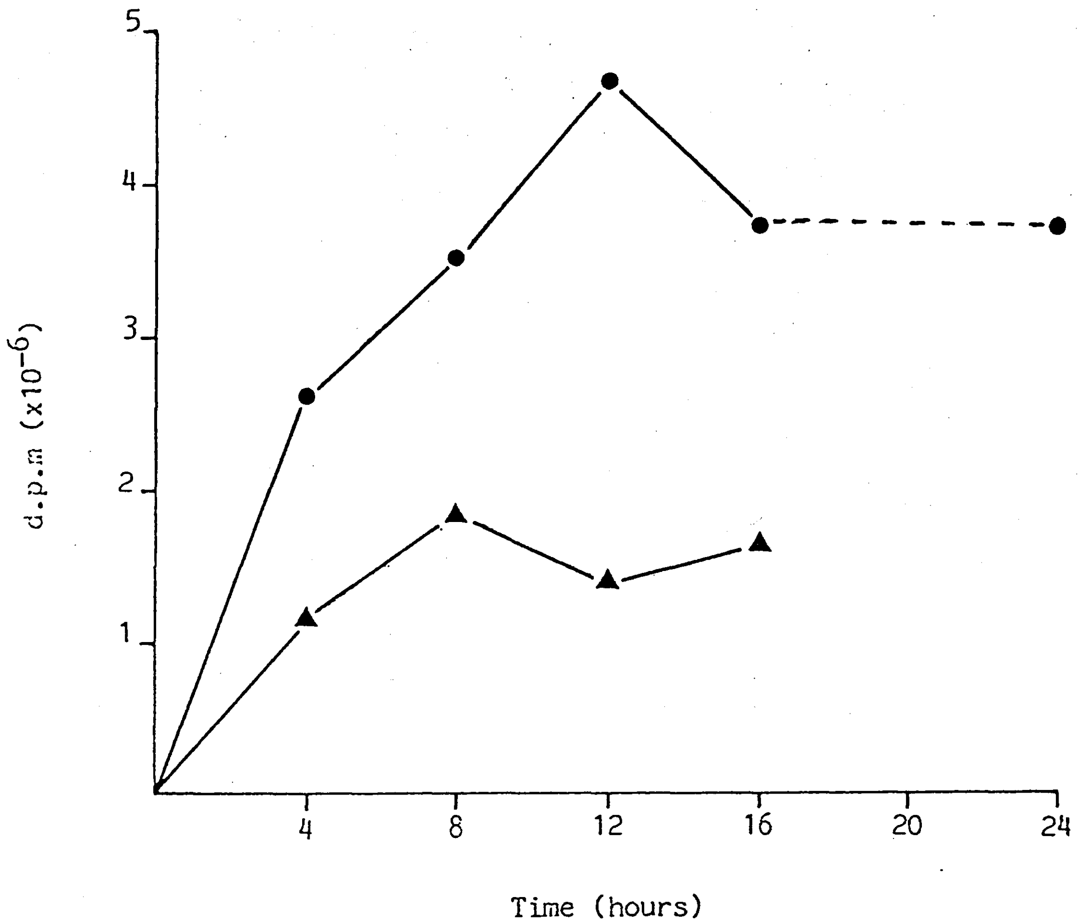


Figure 3.13 Time-course of [^{35}S]methionine and [^{75}Se]selenomethionine incorporation into newly synthesised protein by GSF cultures. About 20000 cells were seeded in each well of a 24 well plate and grown to confluence on coverslips. The cells were then incubated with either [^{35}S]methionine (●) or [^{75}Se]selenomethionine (▲) for 4, 8, 12, or 24h. Samples were then collected in duplicate and assayed, after rigorous washing in PBS (0 C) and ethanol (room temperature), for total radioactivity recovered after 5% trichloroacetic acid precipitation. (See Methods 2.10 for details)

Table 3.10 Effects of cold methionine on the incorporation of labeled methionine into newly synthesised GSF protein.

MEM (w/o met)*	[cold met] (uM)	Incorporated d.p.m. ($x10^{-6}$)	
		^{35}S	^{75}Se
+ 1% EC10, 10% CS	3.72	2.46	1.05
+ 1% EC10, 1% CS	1.30	2.24	0.78
+ 1% EC10	1.02	2.13	1.10
No addition	0	1.99	1.35

Cells were seeded in 24 well plates and grown to confluence on coverslips, in EC10 medium. The cells were then incubated with [^{35}S]methionine or [^{75}Se]seleneomethionine (50uCi/ml) for 10 hours, in 200ul of the methionine free medium with the above additions. Samples were collected in duplicate and after rigorous washing, assayed for total radioactivity incorporated into trichloroacetic acid insoluble material. The concentration of cold methionine was calculated assuming that newborn calf serum (CS) and GMEM contained 4mg/l (Documenta Geigy 7th edition) and 15mg/l (Gibco) respectively. See Methods 2.10 for details.

* = The basic medium was MEM (w/o methionine) supplemented with glutamine, and antibiotics (penicillin/streptomycin).

in favour of ^{75}Se d.p.m.

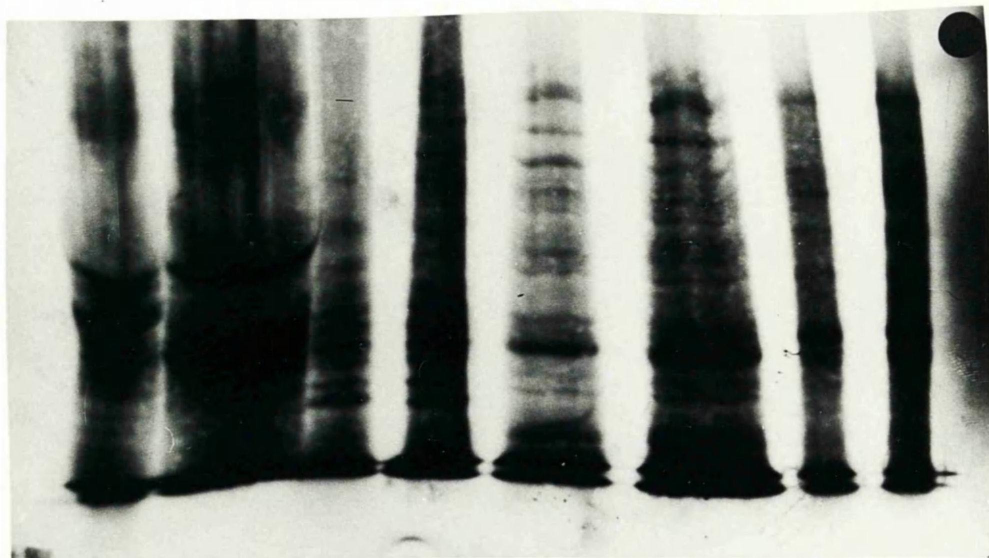
iii. Screening of ^{35}S emmissions for autoradiography (Methods 2.11). Plate 3.1(a & b) shows that the use of a blackened film was more efficient than a double layer of tin-foil at screening out the light and β-emmissions from ^{35}S, during the relatively long exposure times required (i.e. 1 to 3 months) for the detection of [^{75}Se]selenomethionine labelled proteins. Furthermore, the double layer of tin-foil caused a loss of definition(sharpness) in the final autoradiograph image (Plate 1.3b).

iv. 2-DGE of receptor enriched fractions (Methods 2.11). Control and androgen insensitive cells were incubated with [^{35}S]methionine and [^{75}Se]seleomethionine respectively: receptor enriched fractions were prepared, mixed, and resolved by 2-DGE. Plates 3.2 to 3.8 illustrate the results of seven separate comparisons between control and androgen insensitive cell lines.

Before considering the 2-D analysis in detail, it will perhaps be useful to briefly reconsider the rationale for using dual labelling autoradiography and 2-DGE to look for mutations of the androgen receptor. By differential labelling and subsequent detection, control and androgen insensitive proteins could be resolved on the same gel under identical experimental conditions (Fig 2.1). Therefore, any observed differences between the fluorograph and autoradiograph could be correlated with the underlying defect in the androgen insensitive cells. Figure 3.14a, illustrates what might be expected: from a consideration of labelling scheme 1, it can be seen that a mutation affecting receptor levels would result in the loss of a spot(s) from the

Plate 1. Screening out [^{35}S] light and β-emmissions. A single dimension SDS-gel with alternating tracks of [^{35}S] and [^{75}Se] labelled protein was impregnated with a fluorographic agent, dried down and exposed to XAR-5 X-ray film at -70 C: FLUOROGRAPH (A). Half the gel was then covered with a double layer of tin-foil and the other with a piece of blackened film, and the gel re-exposed with XAR-5 film at room temperature: AUTORADIOGRAPHY (B).

Plate 3.1



A: FLUOROGRAPH (1-2 Days exposure)

B: AUTORADIOGRAPH (1-2 Months exposure)

Tracks 1-4; Tin-foil used to screen out ^{35}S

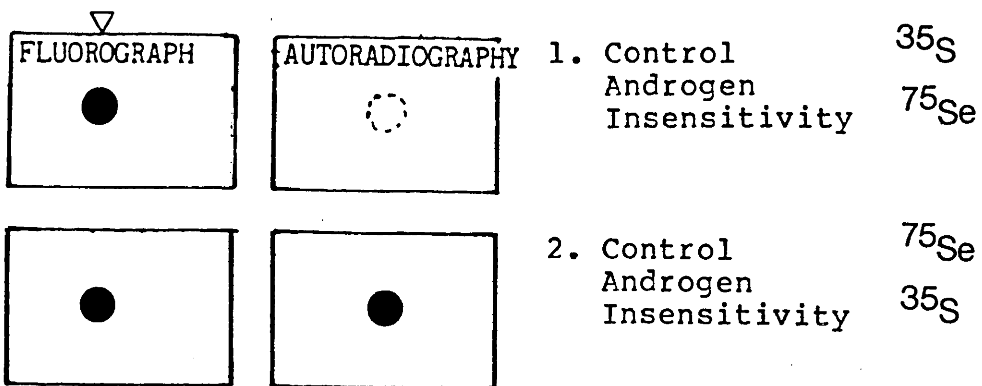
Tracks 5-8; Blackened film used to screen out ^{35}S

Tracks 1,3,5 & 7; [^{75}Se] labelled protein.

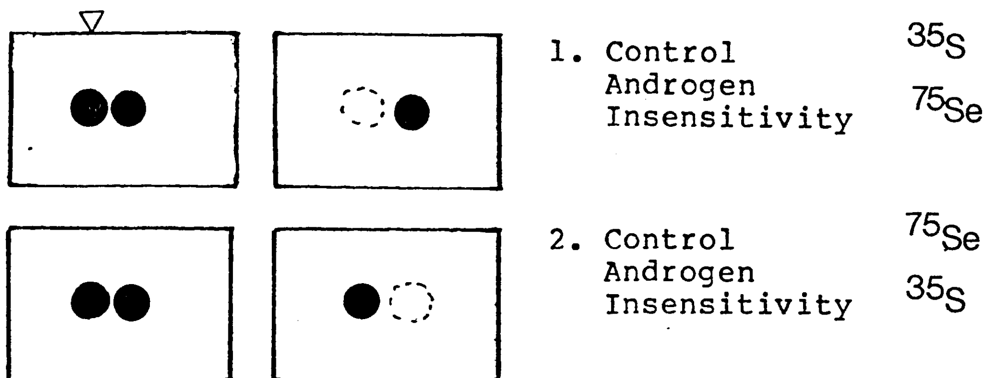
Tracks 2,4,6 & 8; [^{35}S] labelled protein.

(a)

A: Receptor Negative or
Receptor Deficient Resistance



B: Receptor Positive Resistance.



(b)

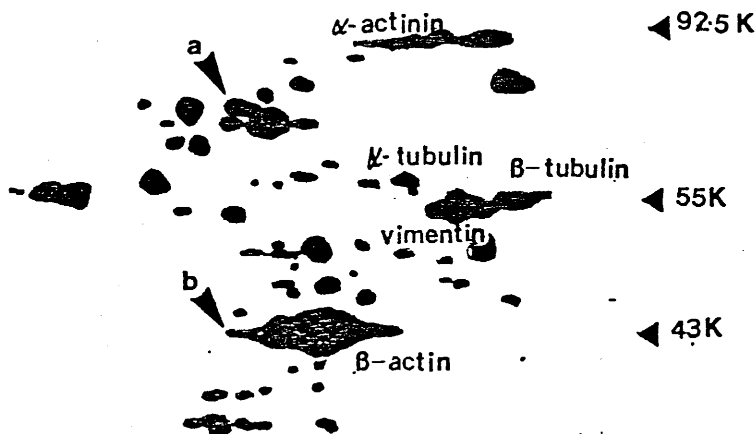


Figure 3.14. The principle behind using double-label autoradiography and 2-DGE to detect variant forms of the androgen receptor (a). Schematic representation of mouse kidney fibroblast cytoskeletal proteins,& the position of the 85K (arrow a) and 45K (arrow b) proteins reported to be diminished in the cultured GSF of patients with androgen insensitivity (b). See Text for discussion.

autoradiograph (Example A), while a structural defect (i.e. resulting from a charge change or increased susceptibility to protease activity) would produce two androgen receptor spots on the fluorograph but only one on the autoradiograph (Example B). However, in practice it would be very difficult to distinguish between mutations A and B on the evidence of a single experiment; it would therefore be necessary to repeat the comparison under labelling scheme 2, which would then allow the discrimination of a qualitative or quantitative defect. For simplicity, we looked initially for the loss or reduced intensity of spots on the autoradiograph under labelling scheme 1 (Methods 2.11).

Figure 3.14b shows a schematic representation of mouse skin fibroblast cytoskeleton proteins, resolved by 2-DGE (taken in part from Fey et al 1984): under the extraction and partial purification protocol described previously (Methods 2.4) it was estimated that actin, actinin and possibly small amounts of tubulin and vimentin would be present in the 35% ammonium sulphate precipitate (based on information given in Frederiksen and Cunningham, Methods in Enzymology vol.85). Since actin appeared to be a major constituent of all gels analysed (Plates 3.2-3.8), and was adjacent to the 45K protein (arrow b, Fig.3.14b) reported by Risbridger et al (1982) to be abnormal in GSF from androgen insensitive cells, it proved a useful internal reference point.

To facilitate the comparison of control and androgen insensitive proteins, the fluorograph and autoradiograph images were placed on a light box and copied onto acetate sheets. This

then allowed the two images to be superimposed and any differences in the protein patterns recorded. Figures 3.15 to 3.21 illustrate the comparisons for the seven separate experiments; the autoradiograph image has been photocopied for ease of presentation. Table 3.11 summarizes the differences observed in the protein patterns. One of the criteria laid down by Lecocq et al (1982) for the optium comparison of ^{35}S- and ^{75}Se-labelled proteins was that the intensity of spots on the autoradiograph equalled that of the corresponding spots on the fluorograph. It is of significance that for the three experiments (Fig.3.15, 3.16, & 3.21) where most (17-46) "differences" were seen, that the autoradiograph was underexposed relative to the fluorograph. It was therefore not possible to correlate the differences observed with the condition of androgen insensitivity. In the four remaining experiments the exposure of the autoradiograph was not considered to be a limiting factor, and the observed differences (2-4) in the protein patterns from androgen insensitive proteins were noted (Table 3.11 a&b). From Table 3.11b it is clear that although relatively few differences were seen for each comparison, there were no consistent changes between experiments. It therefore seemed unlikely that these differences were related to the androgen receptor or to androgen dependent proteins. However, it is also clear that for the RM/TCF (Fig.3.19) and SW/Matheson (Fig.3.20) comparisons that the resolution of proteins (in either IEF and/or SDS-PAGE dimensions) has been hampered. While this does not invalidate the comparison (both sets of proteins subjected to the same artefact) it does make it difficult to know the significance of the differences

Table 3.11 Summary of the differences seen in the protein patterns of ammonium sulphate fractions from Control and Androgen Insensitive GSF, after dual-labelling and 2-DGE.

(a).

Control/Androgen Insensitivity Comparison	Hormone Binding ø	Fig.	Differences Seen in ^{75}Se-labelled proteins (Autoradiograph Image)	Overall Intensity of spots on the Autoradiograph*
SM/4779	Absent	3.15	23 spots "absent"-all over	Less
SM/605	Absent	3.16	17 spots "absent"-all over	"Less"
RM/605	Absent	3.17	3 spots absent B12,B17,C8 1 spots intensity F12	Equal
SM/Ib	Deficient	3.18	2 spots absent D19	Equal
RM/TCF	Unstable	3.19	3 spots absent D4	Equal
SM/Matheson	Normal	3.20	4 spots absent C3,C11	Equal
SM/T4	Normal	3.21	46 spots "absent"-all over	Less

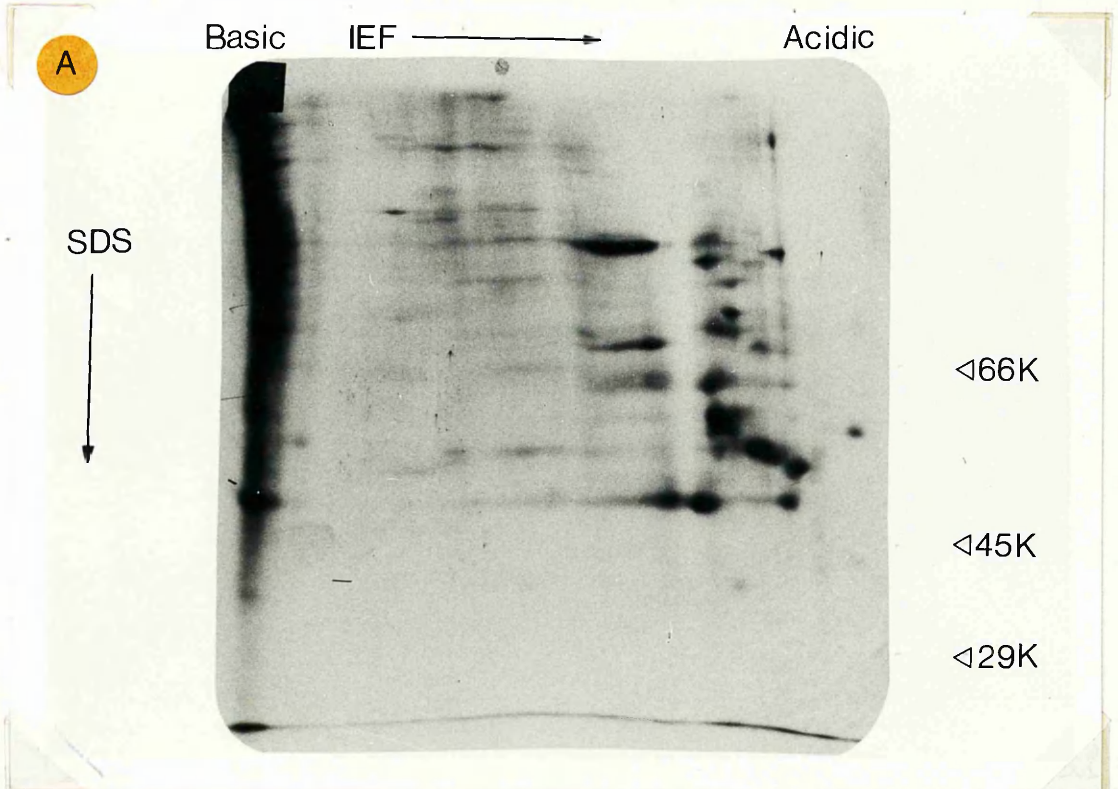
ø, Data from whole cell binding assays (Table 3.1).

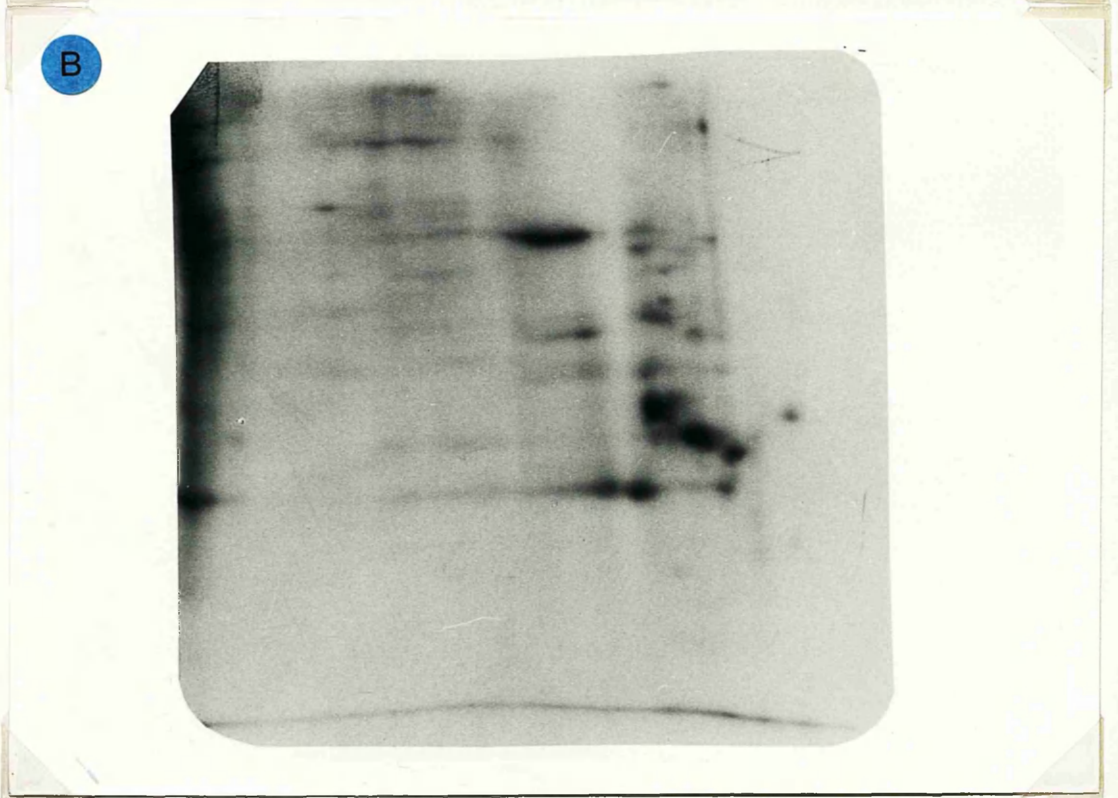
*, relative to the fluorograph image.

(b)

Comparison	Fig.	Position of Difference on 2-D Pattern Ref.	pH	Mr.	Description
RM/605	3.17	B17	Basic	30K	Absent
		C8	Basic/Acidic	70K	Absent
		B12	Basic	45K	Absent
		F12	Basic	40-50K	Intensity
SM/Ib	3.18	D19(2)	Acidic	30K	Absent
RM/TCF	3.19	D4(3)	Basic	100K	Absent
SM/Matheson	3.20	C3(4)	Acidic	>100K	Absent

Plate 3.2





A: FLUOROGRAPH, proteins from SW + 4479 cell lines,
B: AUTORADIOGRAPH, proteins from 4479 cell line only.

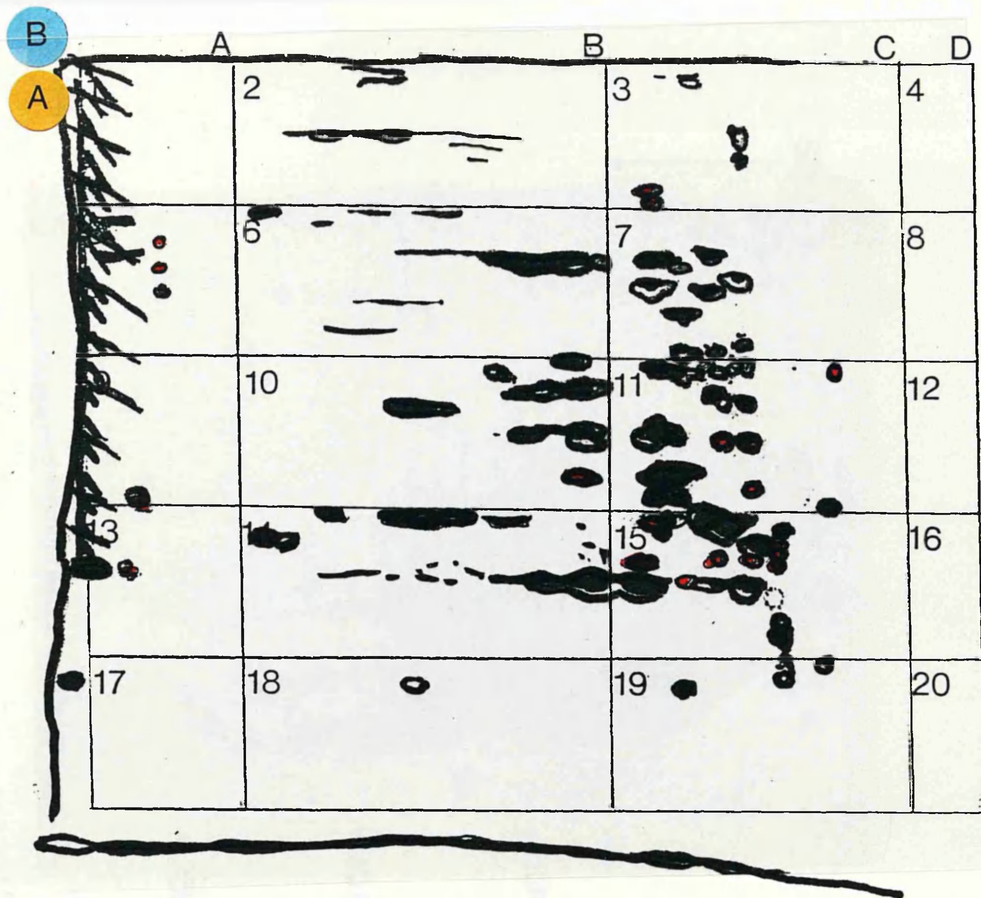


Figure 3.15. Comparison of ammonium sulphate fractions from SW and 4479 cell lines labelled with ^{35}S methionine and ^{75}Se selenomethionine respectively. The fluorograph image (control and androgen insensitive proteins) has been superimposed on the autoradiograph (androgen insensitive proteins only). (See Methods 2.11 for details.)

●, proteins missing from the autoradiograph.

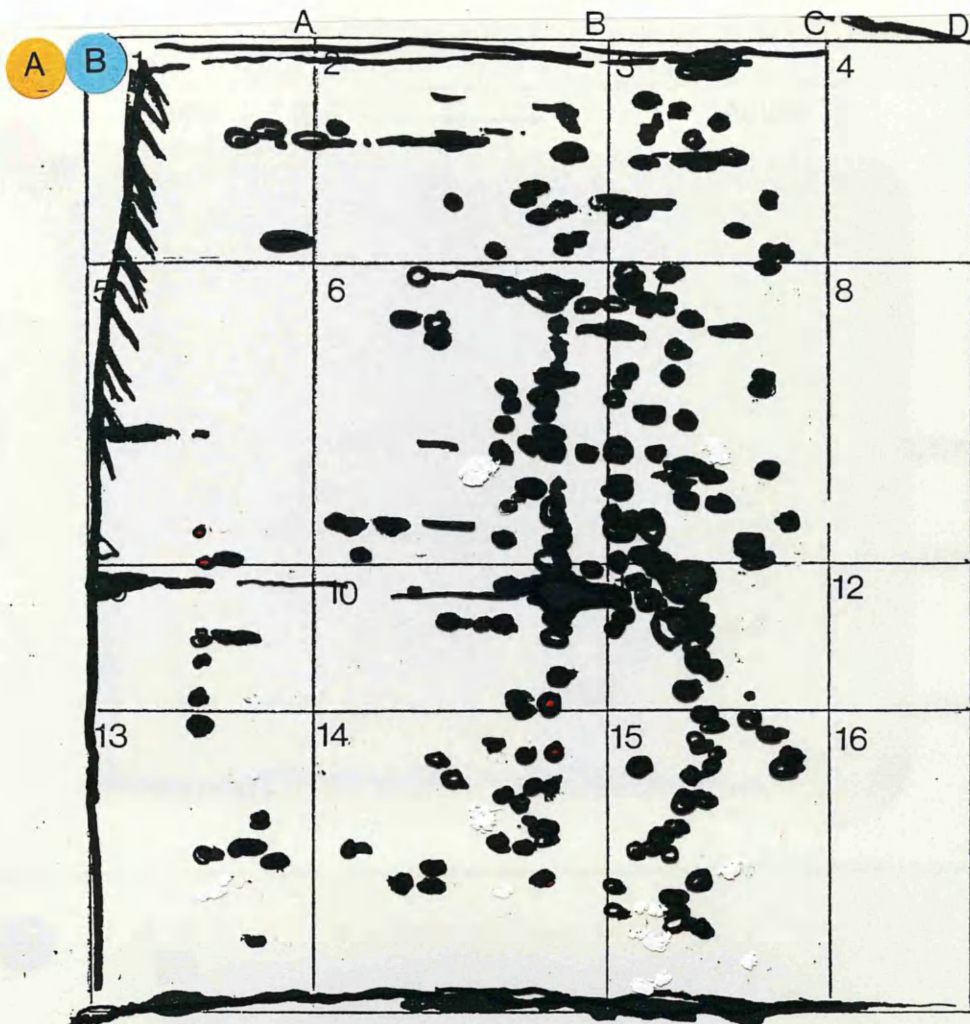
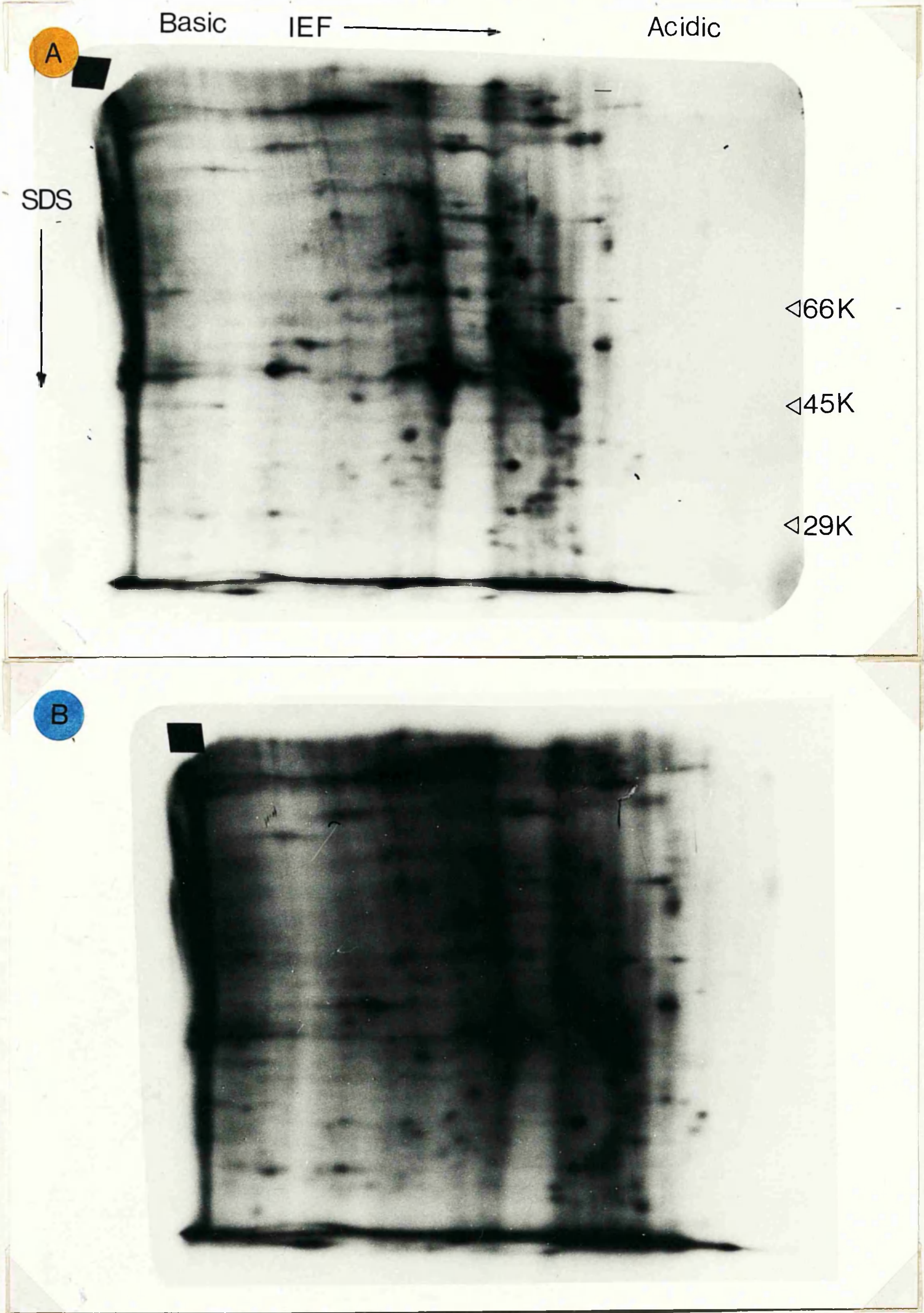


Figure 3.16. Comparison of ammonium sulphate fractions from SW and 605 cell lines labelled with ^{35}S methionine and ^{75}Se selenomethionine respectively. The fluorograph image (control and androgen insensitive proteins) has been superimposed on the autoradiograph (androgen insensitive proteins only). (See Methods 2.11 for details.)

●, proteins missing from the autoradiograph.

Plate 3.4



A: FLUOROGRAPH, proteins from RM + 605 cell lines,
B: AUTORADIOGRAPH, proteins from 605 cell line only.

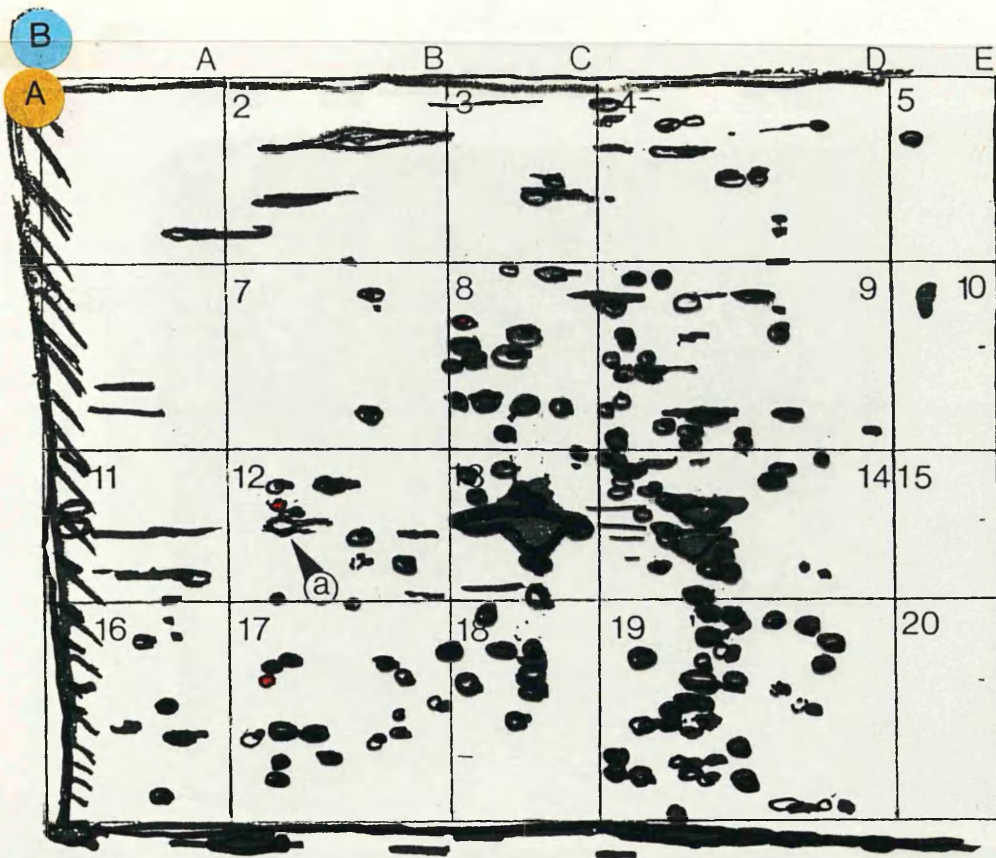
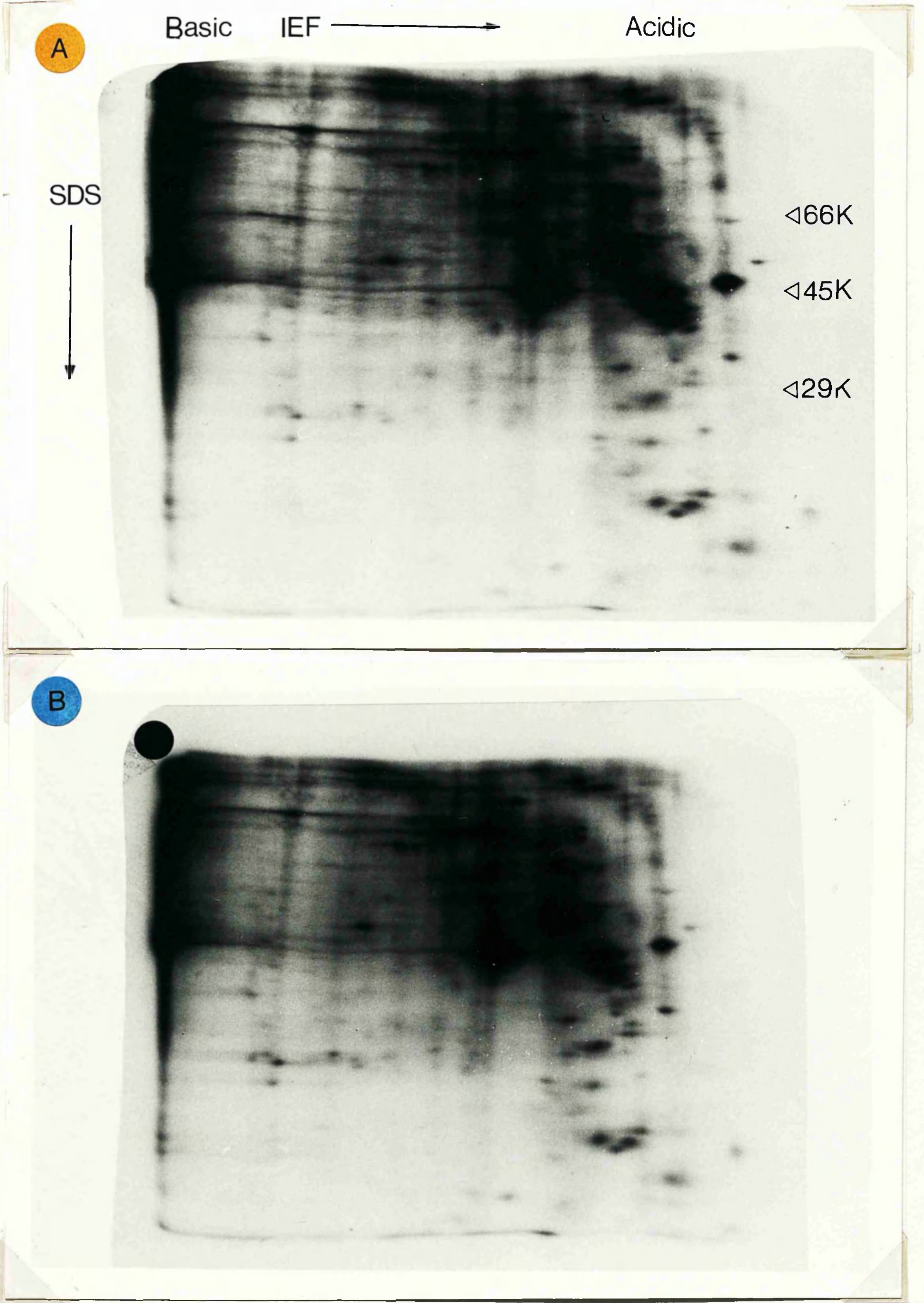


Figure 3.17. Comparison of ammonium sulphate fractions from RM and 605 cell lines labelled with ^{35}S methionine and ^{75}Se selenomethionine respectively. The fluorograph image (control and androgen insensitive proteins) has been superimposed on the autoradiograph (androgen insensitive proteins only). (See Methods 2.11 for details.)

●, proteins missing from the autoradiograph.
Arrow a : intensity of spot decreased on the autoradiograph.

Plate 3.5



A: FLUOROGRAPH, proteins from SW + Ib cell lines,
B: AUTORADIOGRAPH, proteins from Ib cell line only.

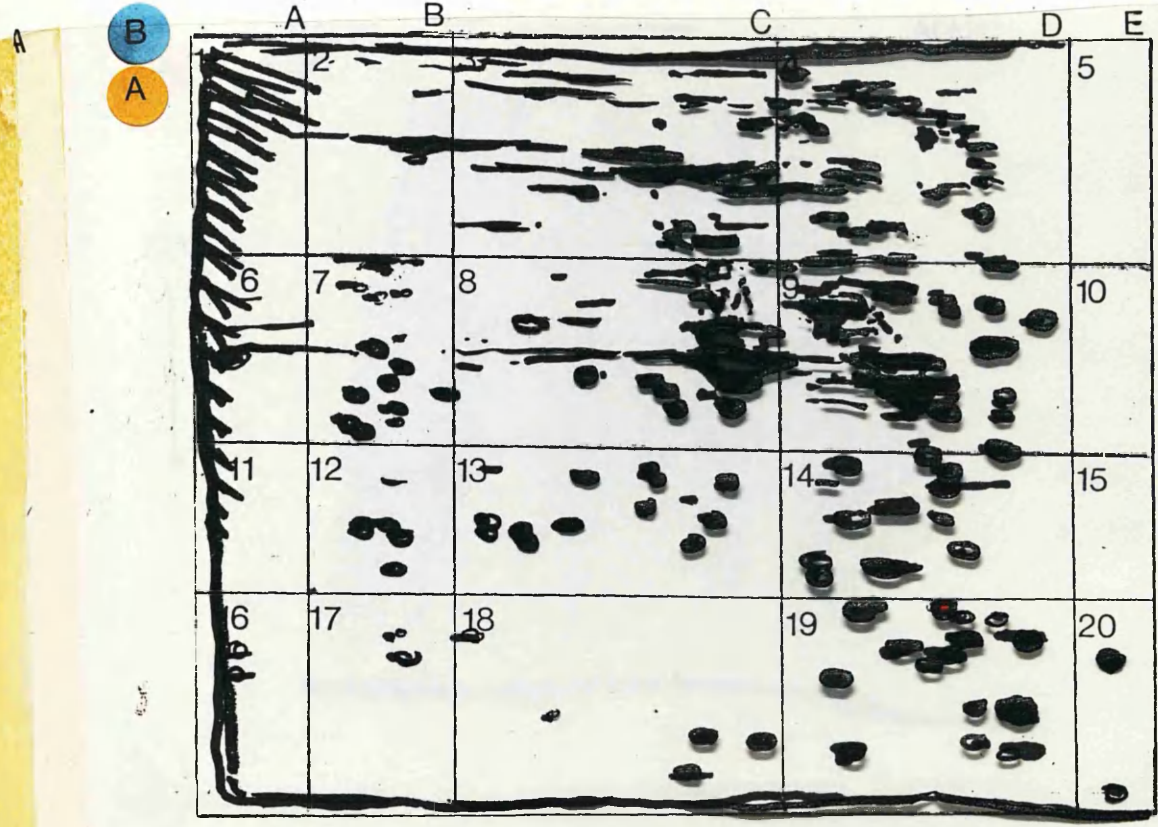
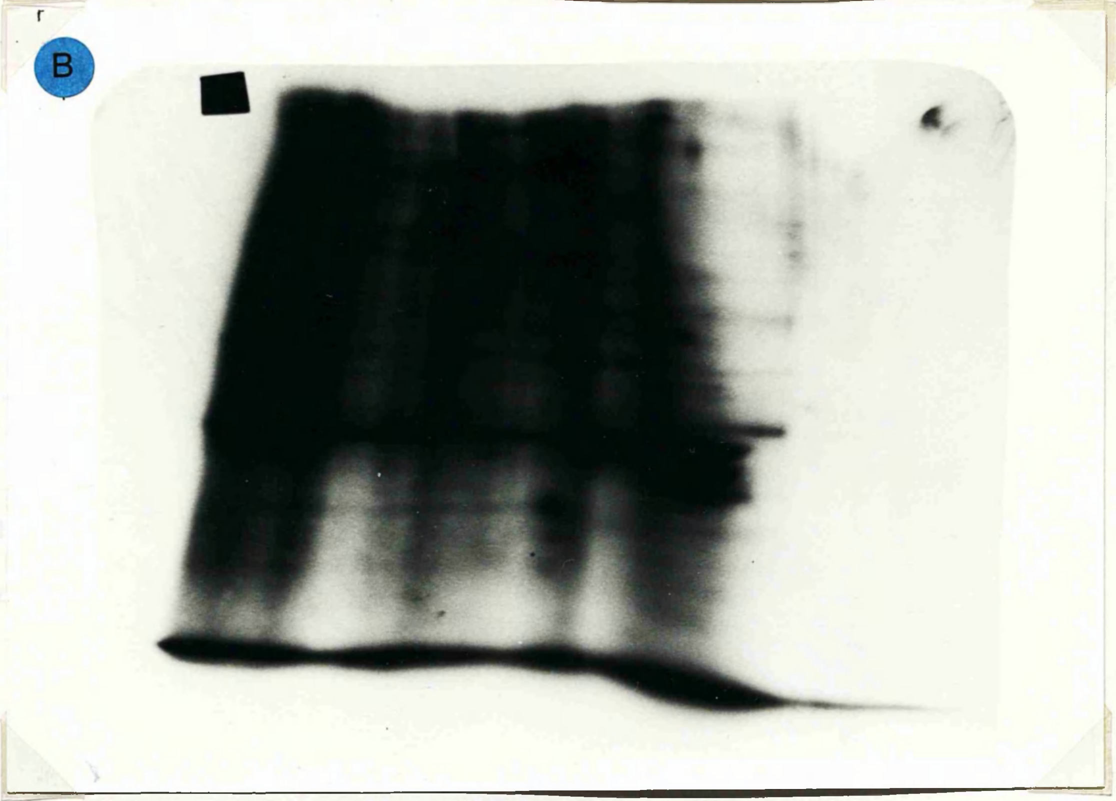


Figure 3.18. Comparison of ammonium sulphate fractions from SW and Ib cell lines labelled with ^{35}S methionine and ^{75}Se selenomethionine respectively. The fluorograph image (control and androgen insensitive proteins) has been superimposed on the autoradiograph (androgen insensitive proteins only). (See Methods 2.11 for details.)

o, proteins missing from the autoradiograph.

Plate 3.6



A: FLUOROGRAPH, proteins from RM + TCF cell lines,
B: AUTORADIOGRAPH, proteins from TCF cell line only.

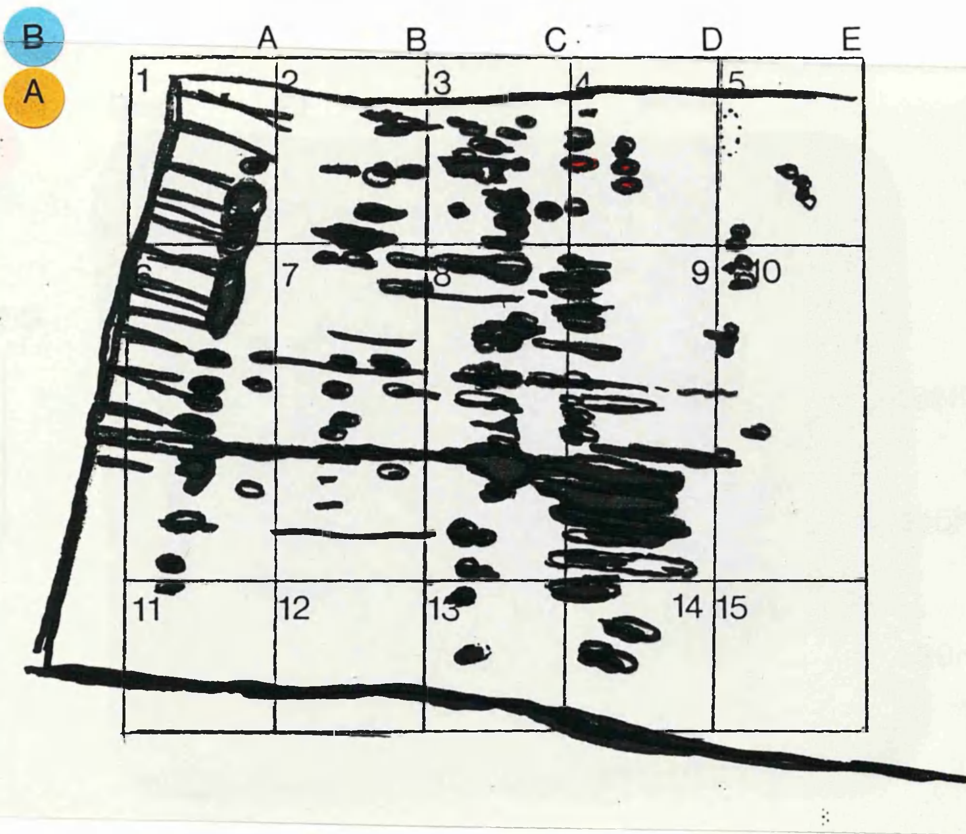
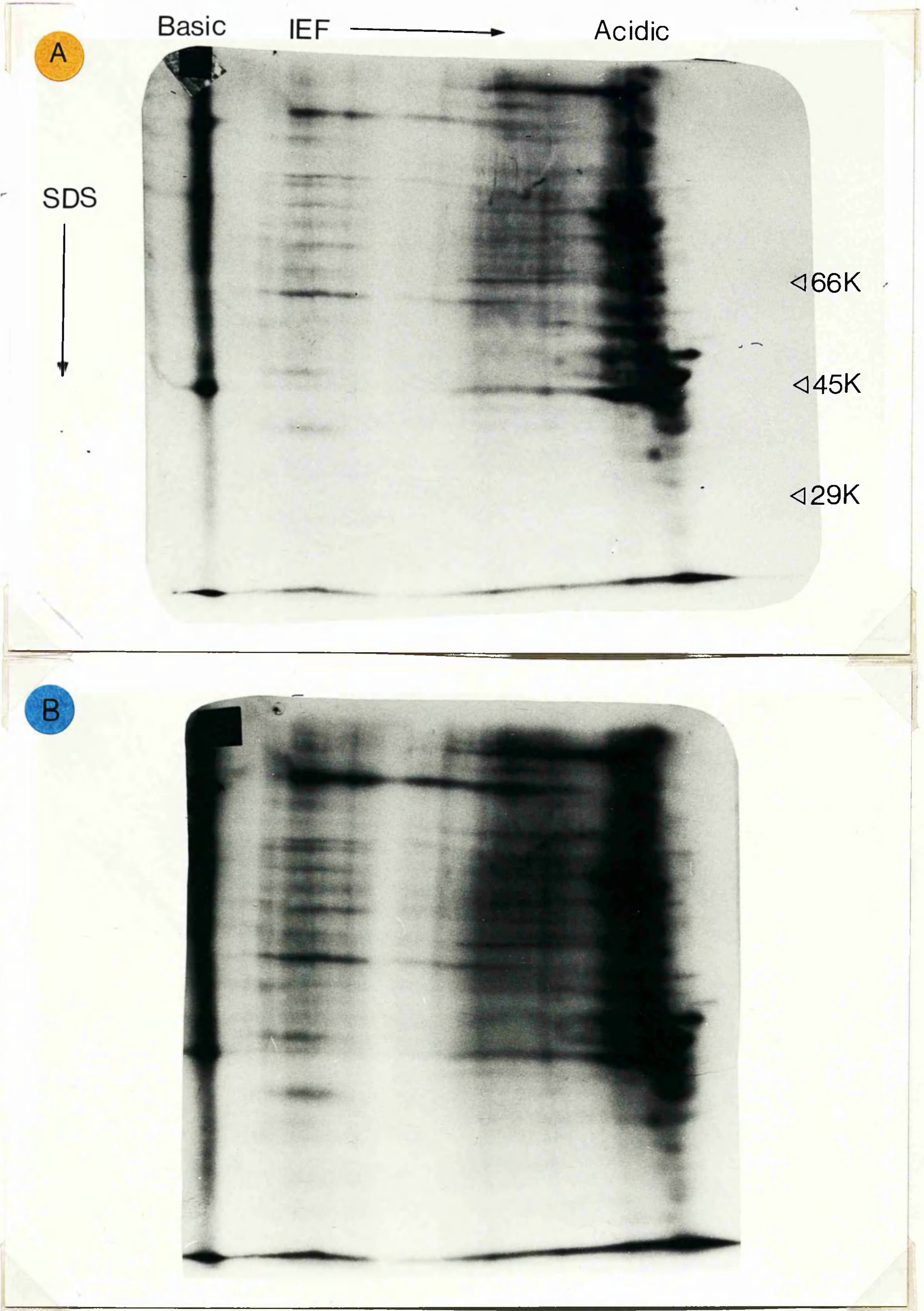


Figure 3.19. Comparison of ammonium sulphate fractions from RM and TCF cell lines labelled with ^{35}S methionine and ^{75}Se selenomethionine respectively. The fluorograph image (control and androgen insensitive proteins) has been superimposed on the autoradiograph (androgen insensitive proteins only). (See Methods 2.11 for details.)

●, proteins missing from the autoradiograph.

Plate 3.7



A: FLUOROGRAPH, proteins from SW + MATHESON cell lines,
B: AUTORADIOGRAPH, proteins from MATHESON cell line only.

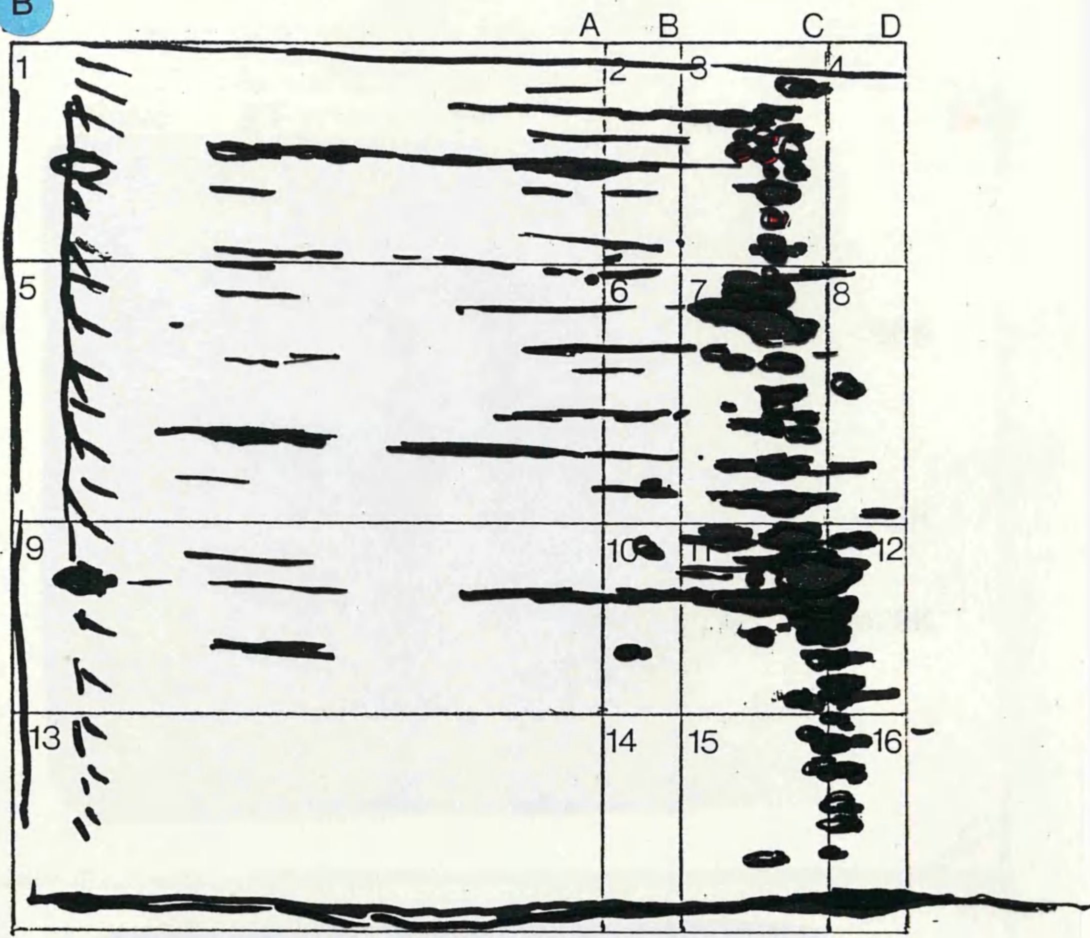
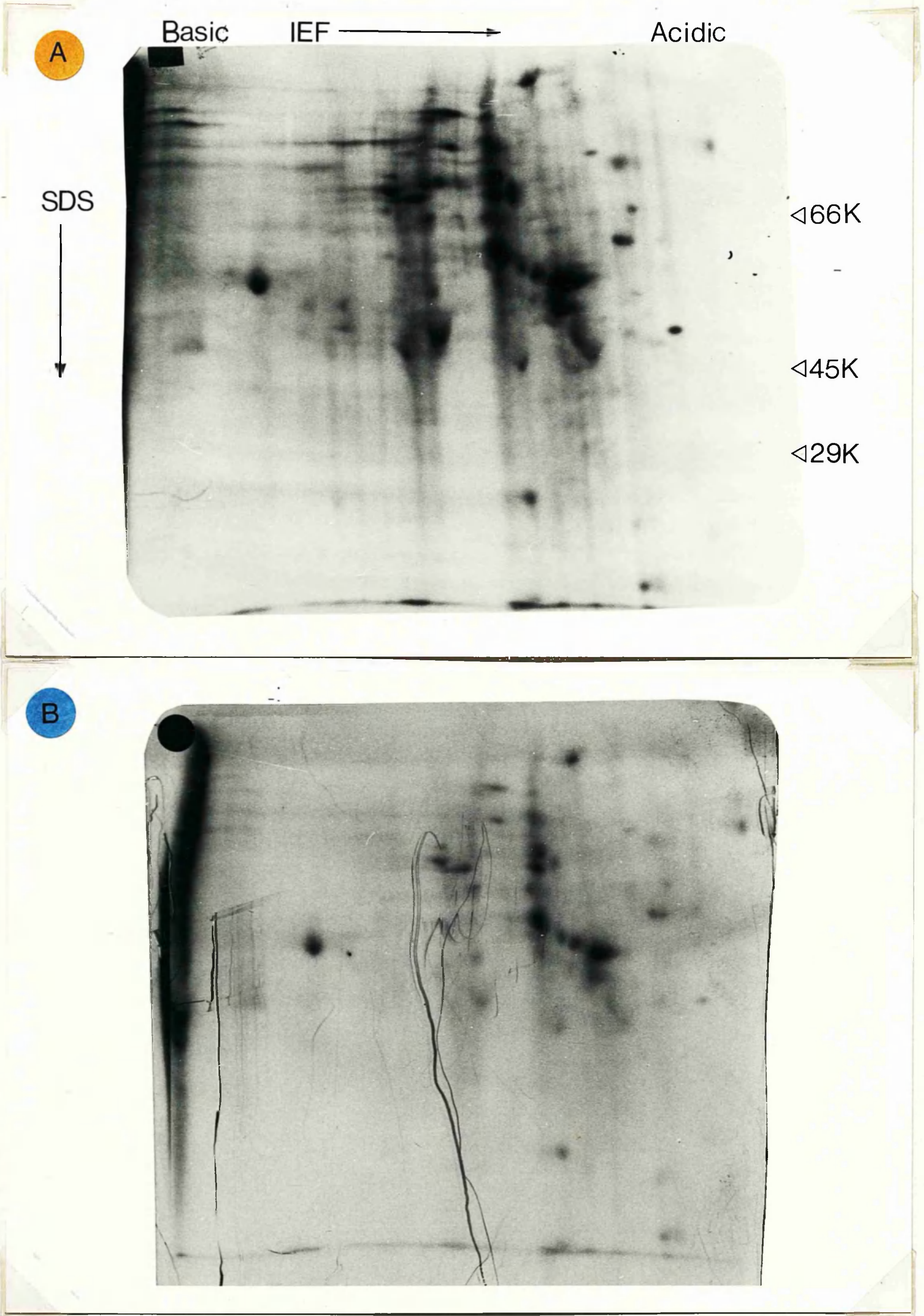


Figure 3.20. Comparison of ammonium sulphate fractions from SW and Matheson cell lines labelled with ^{35}S methionine and ^{35}Se selenomethionine respectively. The fluorograph image (control and androgen insensitive proteins) has been superimposed on the autoradiograph (androgen insensitive proteins only). (See Methods 2.11 for details.)

●, proteins missing from the autoradiograph.

Plate 3.8



A: FLUOROGRAPH, proteins from SW + T4 cell lines,
B: AUTORADIOGRAPH, proteins from T4 cell line only.

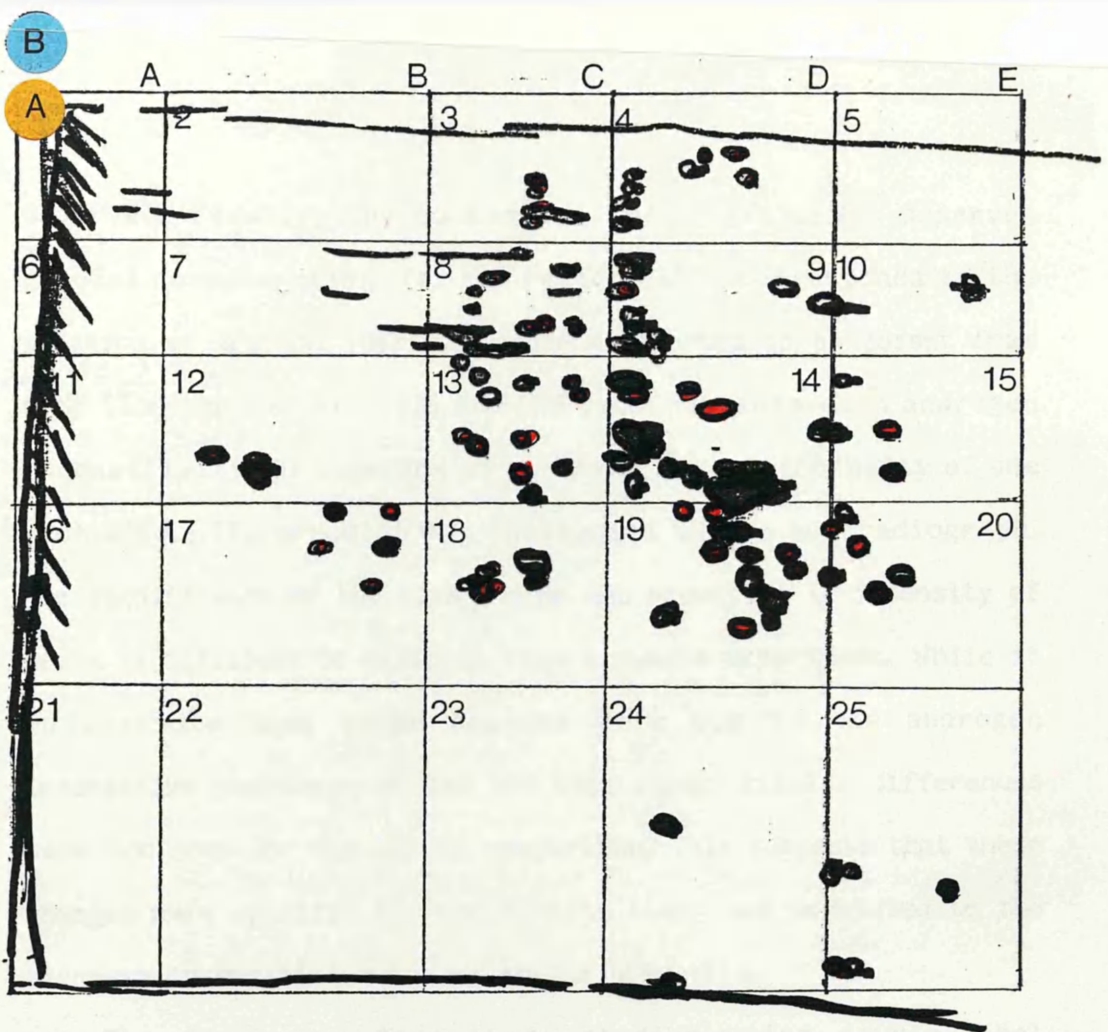


Figure 3.21. Comparison of ammonium sulphate fractions from SW and T4 cell lines labelled with ^{35}S methionine and ^{75}Se selenomethionine respectively. The fluorograph image (control and androgen insensitive proteins) has been superimposed on the autoradiograph (androgen insensitive proteins only). (See Methods 2.11 for details.)

●, proteins missing from the autoradiograph.

observed. Finally, the comparison RM/605 (Fig.3.17) deserves special consideration: (a) the region [B12] corresponds to the position of the 56K (pKa 6.7) protein reported to be absent from NGSF (Thompson et al 1983) and GSF from patients with androgen insensitivity (Wrongemann et al 1984); (b) the intensity of one spot (Fig.3.17, arrow a) was decreased on the autoradiograph. The significance of the loss and/or the reduction in intensity of spots is difficult to evaluate from a single experiment. While it is possible that these changes were due to the androgen insensitive phenotype of the 605 cell line, similar differences were not seen for the SW/605 comparison. This suggests that these changes were specific for the RM cell line, and unrelated to the androgen insensitive mutation in the 605 cells.

The conclusion from these studies using double-label autoradiography and 2-DGE, was that no consistent differences between control and androgen insensitive proteins in receptor enriched fractions could be found. It was therefore not possible to locate the position of the androgen receptor polypeptide(s) on 2-D protein patterns using these comparisons, or assign the observed differences to the androgen insensitive phenotype. Where underexposure of the autoradiograph and resolution artefacts could be eliminated, the differences seen between control and androgen insensitive cells may have been due to genetic variation in the human population.

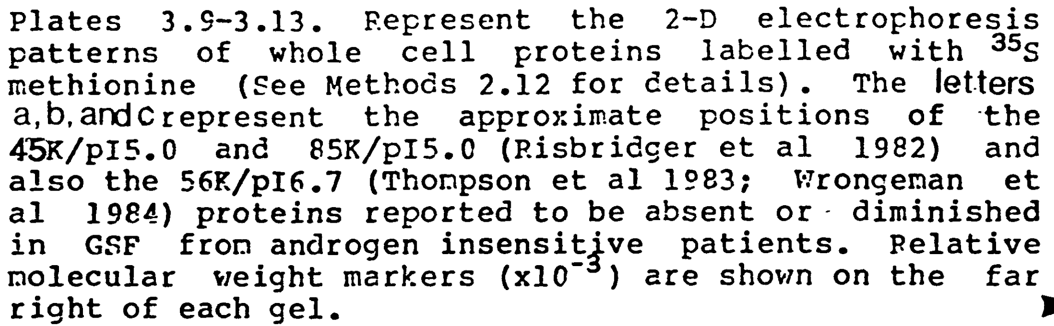
Finally, it was observed during these studies that ^{35}S-labelled proteins were detected more efficiently by fluorography than ^{75}Se-labelled proteins. This phenomena was seen most clearly

in Plate 3.1a, where equal amounts of radioactivity was loaded per track and the exposure time was not long enough for the half-life of the isotopesto have any significant influence.

B. 2-DGE of whole cell protein labelled with [^{35}S]methionine.

As the above studies, comparing receptor enriched fractions, failed to show reproducible differences (for different comparisons) of the type described by others, a retrospective study was under taken to compare the 2-DGE pattern of total ^{35}S-labelled fibroblast proteins from control (RM), androgen insensitive (Ia, TCF, & Mathesison) and a NGSF (HF/E,JP) cell lines (Methods 2.12). The rationale for this was that the 45K, 56K, and 85K proteins (Risbridger et al 1982; Thompsom et al 1983; Wrongemann et al 1984) could have been selected out during the salt extraction and/or ammonium sulphate steps.

A comparison of Plate 3.9 with Plates 3.10-3.13 showed that the resolution of labelled human fibroblast whole cell protein gave reproducible 2-D patterns. However, the relatively large number of apparent differences seen prevented the delination of specific changes, that could be correlated with the androgen insensitive mutation or with the body site of the skin biopsy (Areas a, b & c), suggested by others (Risbridger et al 1982; Wrongemann et al 1984). These studies did however serve to confirm that the double-label autoradiography and partial purification protocol (described above) was the best approach to detecting the androgen receptor and/or androgen dependent proteins by 2-DGE, as the complexity of the whole cell protein 2-D patterns and experimental variations made whole cell protein comparisons very difficult (at least for a small sample number).



Plates 3.9-3.13. Represent the 2-D electrophoresis patterns of whole cell proteins labelled with ^{35}S methionine (See Methods 2.12 for details). The letters a,b,and c represent the approximate positions of the 45K/pI5.0 and 85K/pI5.0 (Risbridger et al 1982) and also the 56K/pI6.7 (Thompson et al 1983; Wrongeman et al 1984) proteins reported to be absent or diminished in GSF from androgen insensitive patients. Relative molecular weight markers ($x10^{-3}$) are shown on the far right of each gel.

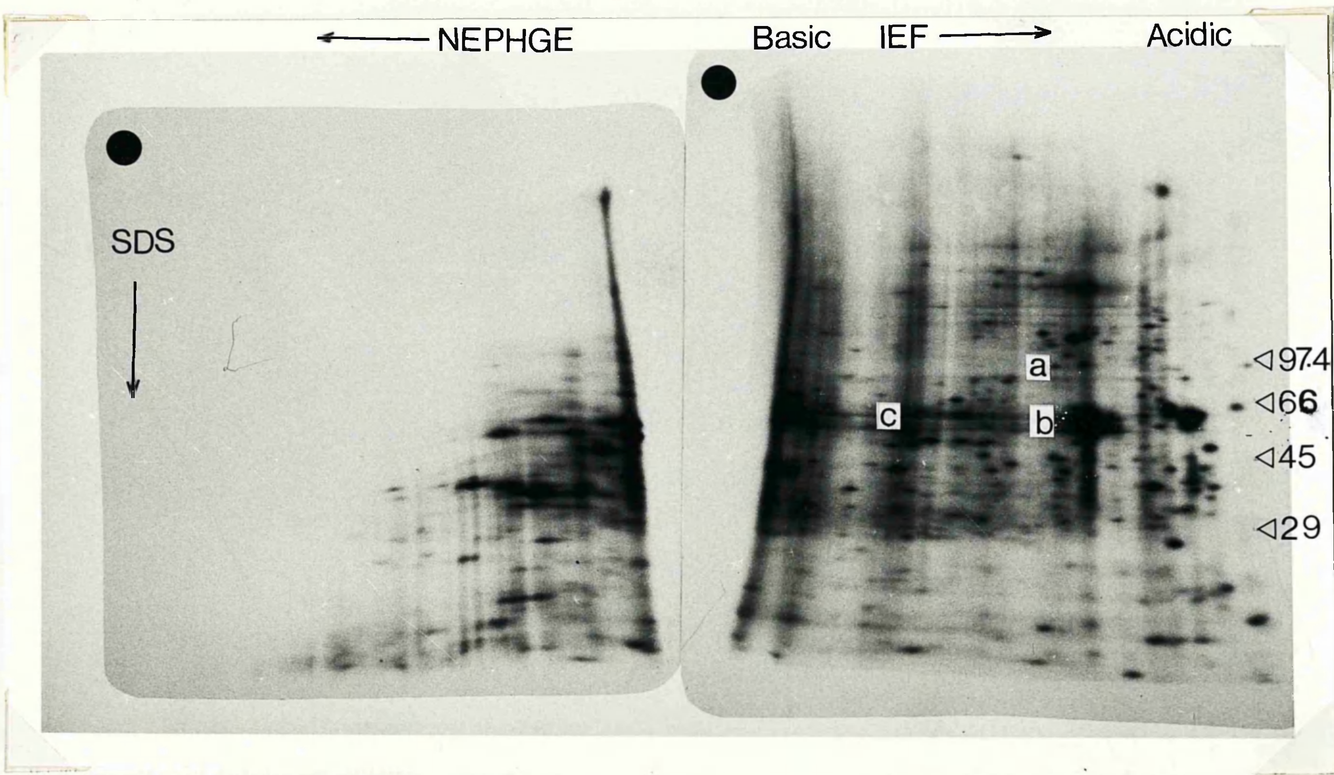
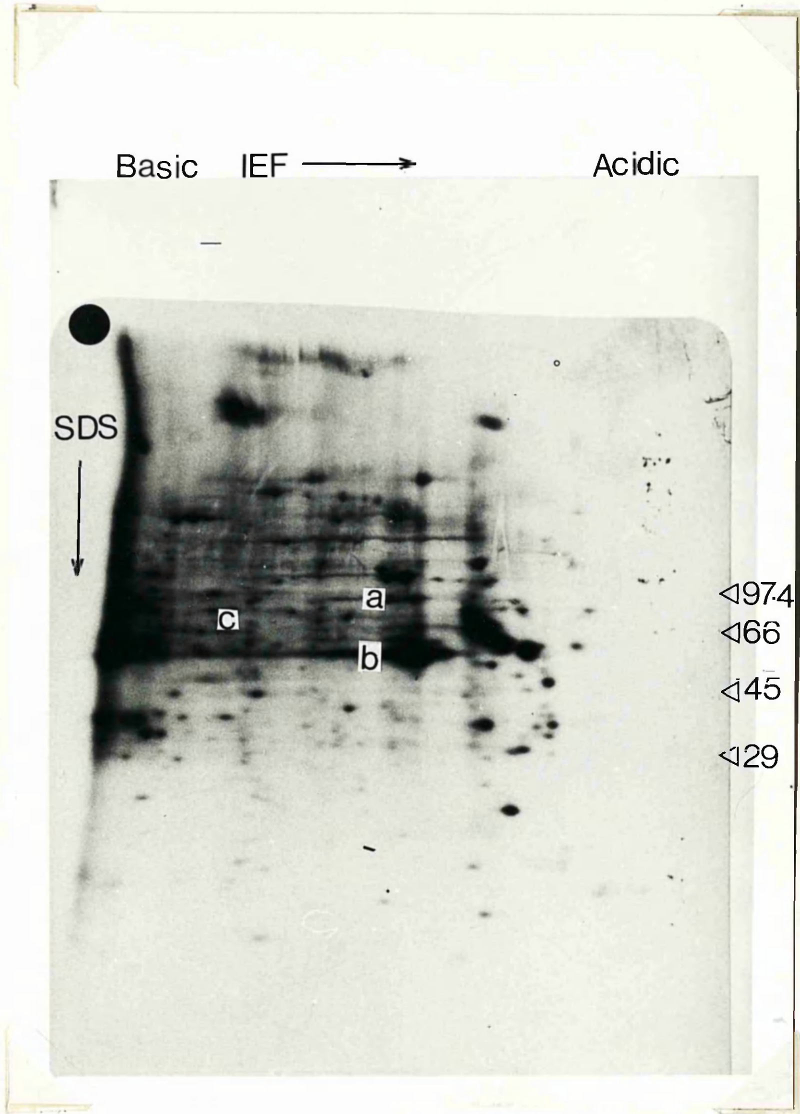


Plate 3.9. RM cell line (Control). In separate experiments proteins were resolved by IEF (Right side) and also by NEPHGE (Left side) in the first dimension; this gives the optium conditions for looking at whole cell labelled protein.



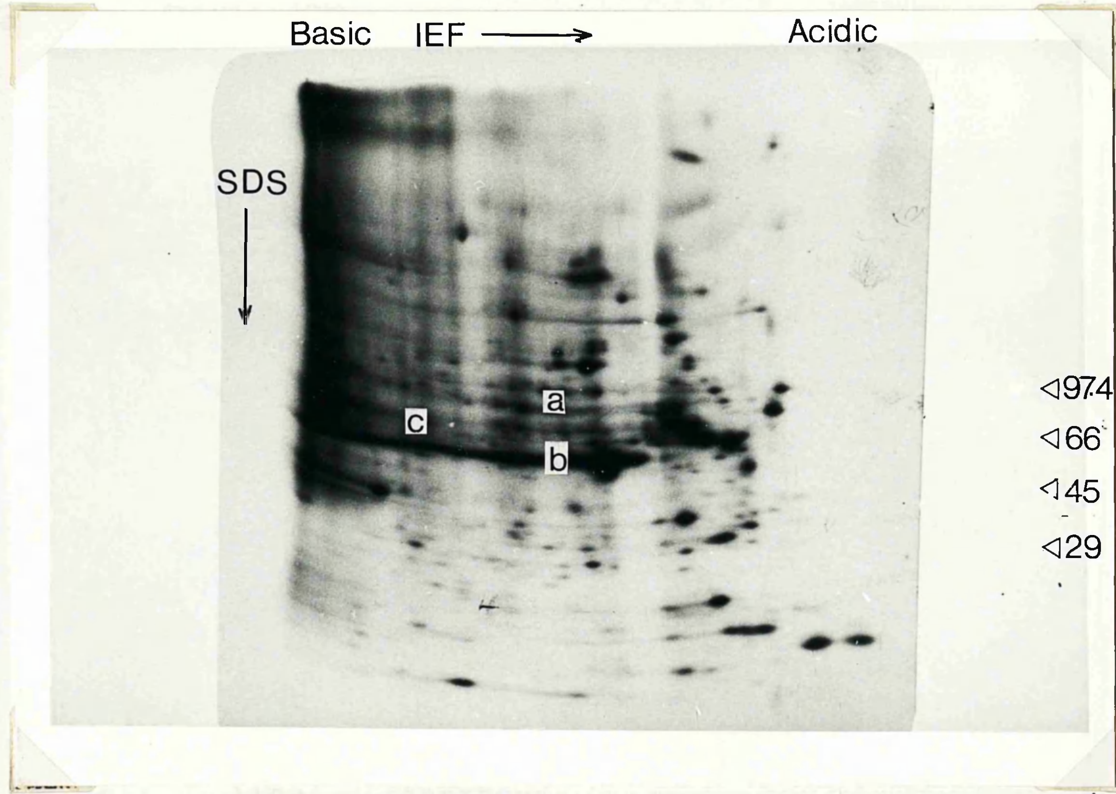


Plate 3.11. Ia cell line (Androgen Insensitivity: deficient receptor binding).

Plate 3.10. TCF cell line (Androgen Insensitivity: unstable receptor binding).

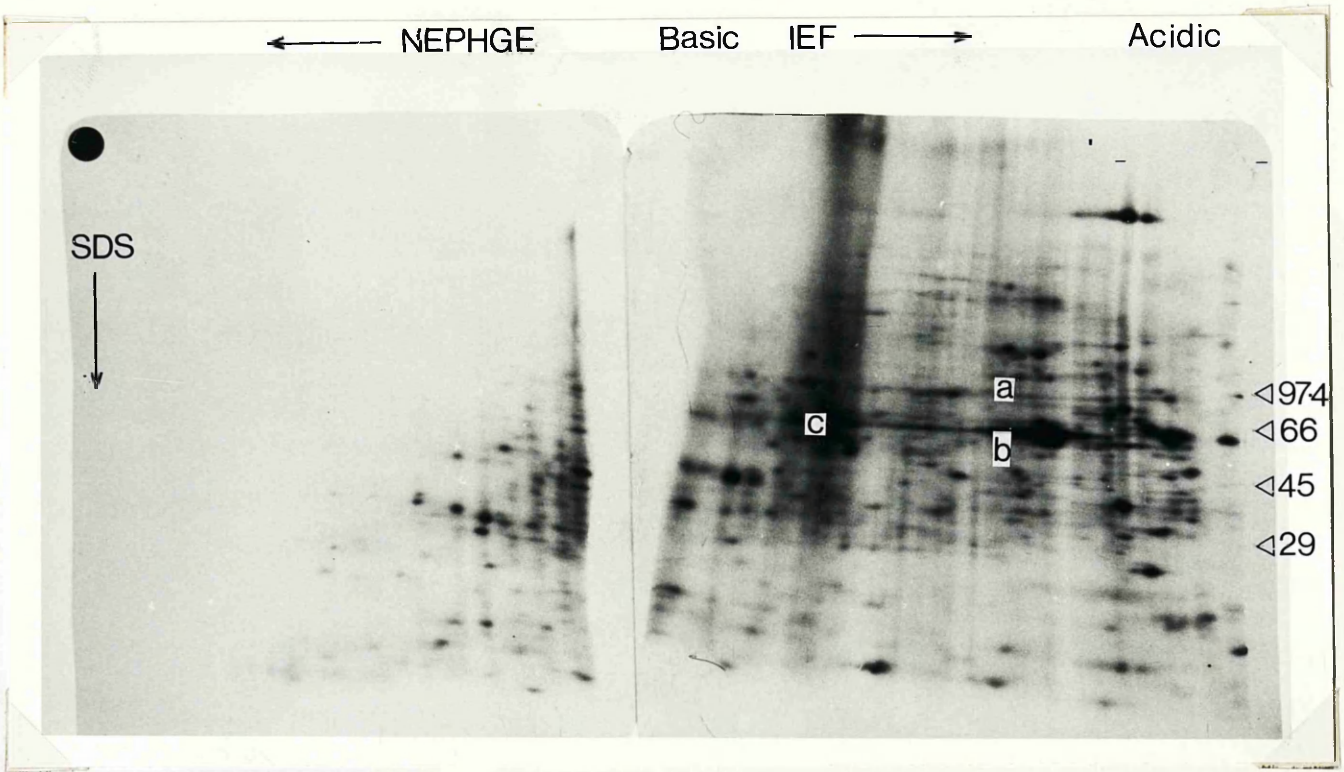


Plate 3.12. Matheson cell line (Androgen Insensitivity:normal receptor binding). In separate experiments proteins were resolved by IEF (Right side) and also by NEPHGE (Left side) in the first dimension; this gives the optium conditions for looking at whole cell labelled protein.

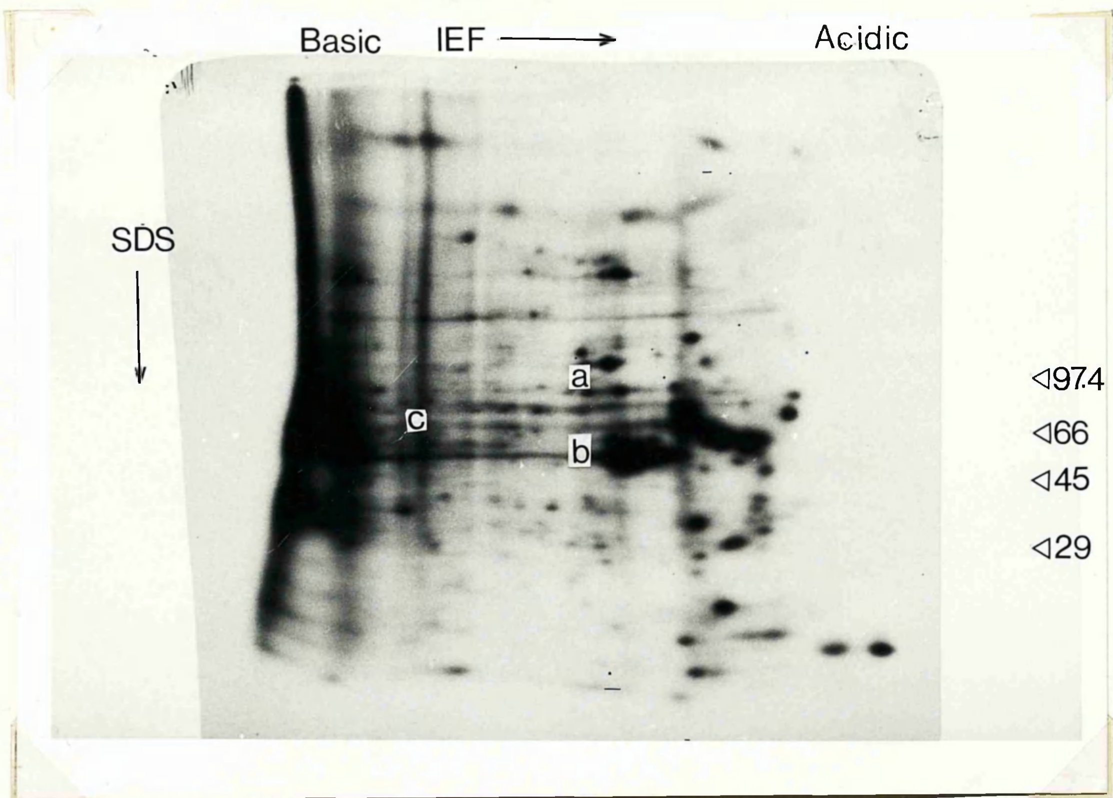


Plate 3.13. HF/E, JP cell line (Normal NGSF).

CONCLUSIONS

4.1 The Androgen Receptor from Control and Androgen Insensitive Cultured Human GSF.

After incubating confluent cell monolayers with 1nM [^{3}H] steroid (DHT or Mibolerone), for 30-40 minutes at 37°C, the ^{3}H steroid-receptor complexes were extracted by sonication in PEM buffer containing 0.5M-KCl, and partially purified by 35% ammonium sulphate precipitation of the 105000xg salt extract (Table 3.4). There has been considerable interest in the functional significance of salt extractable and salt resistant nuclear bound steroid (Brown et al 1981; Clark & Peck 1976; Davies 1983; Kaufman et al 1983; Mainwaring 1969a; Mainwaring & Irving 1973). In a recent study, Brown and Migeon (1986) compared the binding of the androgen receptor to the nuclear matrix fraction of human GSF, from controls and patients with androgen insensitivity. In normal human GSF about 50% of the total binding was found in the nucleus, and of this 28-49% was associated with the nuclear matrix. A similar distribution of androgen receptor binding was observed in two (unrelated) patients with receptor positive complete androgen insensitivity. However, the binding affinity of these receptor complexes to the nuclear matrix was three-fold lower than controls. It is of significance that in the present study, after sonication and salt extraction, between 30% & 50% of the total radioactivity was recovered in the 105000xg salt extracted pellet (see Table 3.5, the proportion of ^{3}H d.p.m. recovered in the Pellet and

Salt Extract fractions). Further studies showed that this was mainly nonspecific binding; 68.7% of the pellet associated radioactivity was recovered after repeated salt extraction and subsequent extraction of pelleted material (105000xg for 1h.) with 0.5% Triton X-100 (Table 3.3). Furthermore, a recent study by Kaufman et al (1986) has implicated the formation of disulphide bonds _in vitro_ (i.e. during nuclei manipulation) on rendering between 50-70% of the rat liver glucocorticoid receptors resistant to nuclease and salt (1.6M-NaCl) extraction in the absence of sulphydryl-blocking agents in the preparation buffer. In the present study 12mM-monothioglycerol was included at all stages of receptor preparation (Methods 2.4), which may have aided the recovery of soluble (salt extractable) receptor by protecting the free sulphydryl groups on the receptor molecule.

Qualitative defects of androgen receptor function (Introduction 1.2, Table 1.2) have been identified by several different methods, such as thermolability of steroid binding, an increased Kd (decreased affinity), and failure to up-regulate basal binding levels in response to hormone, using intact cultured GSF. However, very few studies have been undertaken to investigate receptor integrity in a cell free system.

In the present study, sucrose gradient analysis has been combined with a partial purification protocol (Methods 2.5) to compare the receptor complexes from control

(Fig.3.2) and androgen insensitive (Fig.3.3) cell lines. The observed sedimentation profiles were in good agreement with the data from whole cell binding assays (Table 3.1) and with amounts of radioactivity recovered after salt extraction and ammonium sulphate precipitation (Table 3.4). The value of the procedure was seen during the analysis of receptor complexes from the TCF cell line. In whole cell binding assays (Table 3.1) the levels of receptor were within the normal range, giving a diagnosis of receptor positive resistance. However, after partial purification and sucrose density gradient centrifugation, the profile observed resembled that of a receptor negative cell line (Fig.3.3). This procedure could be useful in determining or confirming quantitative and qualitative mutations of the androgen receptor complex. Interestingly, Griffin and Durrant (1982) described the use of sucrose density gradient centrifugation, and the failure of molybdate to stabilise the 7-8S form of the receptor complex, as a sensitive probe for qualitative defects. More recently, Hirst et al (1985) also used sucrose density gradient analysis in the study of a kindred with vitamin D resistance but normal 1,25-dihydroxyvitamin D binding: the complexes of two individuals failed to aggregate as 6S forms on low salt gradients.

A later modification to the above protocol was the incubation of confluent cultures for 24 hours with 2nM[^{3}H]-mibolerone or -R1881, prior to receptor extraction and partial purification, in order to exploit the phenomenon of "up-regulation" of androgen receptor binding. This proved to

be a successful way of increasing the yield of soluble receptor (Table 3.6) without the increase in time and expense of culturing more cells per experiment. Augmentation of receptor binding levels did not affect the dissociation constant (Kd)(Kaufman et al 1981, 1983; Syms et al 1983, 1984; Dr M.B.Hodgins unpublished data) or the sedimentation coefficient of the receptor on sucrose gradients (Fig.3.5, Dr.M.B.Hodgins personal communication).

In my hands, further purification (as shown by an increase in specific activity) of the receptor, beyond the ammonium sulphate step, using either 2',5'-ADP sepharose (Fig.3.4) or FPLC-anion exchange chromatography (Fig.3.6) proved unsuccessful. The most likely explanation for this seemed to be the loss of steroid binding activity during these manipulations. However, both techniques proved useful in further qualitative characterisation of the human GSF androgen receptor complex.

It is of interest that the human GSF androgen receptor complex (Fig.3.4) and the rat prostate receptor (Fig.3.10a; Brinkmann et al 1985a) exhibited apparently different affinities for the Mono Q anion exchange column, as shown by the concentrations of salt required to elute the receptors. This could reflect slight differences in the receptor preparations or possible species and/or tissue specific receptor forms. However, another possibility is that the weaker interaction of the GSF receptor complexes (0.13-0.18M-KCl v's 0.26M-NaCl) was as a result of receptor

activation. If this was the case, then the use of FPLC-anion exchange chromatography would be an alternative means of detecting mutations affecting receptor activation. At present, such qualitative defects have been suggested from DNA-cellulose binding (Kovacs et al 1983) and kinetic studies (Kaufman et al 1983). Interestingly, Mainwaring and Irving (1973) reported that the isoelectric point of the rat prostate receptor increased from 5.8 to 6.5 on activation (accompanied by 8S to 4.2S conversion). More recently Keenan et al (1986) have shown that activation of the human GSF androgen receptor was concomitant with the loss of negative charge (binding of the receptor to DEAE-sepharose and hydroxyapatite columns) and a reduction in the molecular radius. The activation of the glucocorticoid receptor has also been associated with alterations in chromatographic behaviour (DEAE-cellulose) and increases in pI, suggesting the unmasking of "+" or the loss of "-" charges (Ben-or & Chrambach 1983; Holbrook et al 1983a; Milgrom et al 1973; Munck & Foley 1979; Parchman & Litwack 1977; Wrange 1979). However, a recent study by Smith and co-workers (Smith,Elsasser and Harmon 1986), using 2-DGE (after immuno-purification & affinity labelling), found that activation did not affect the isoelectric point of two isoforms of the glucocorticoid receptor, and concluded that confromational change rather than covalent charge modification was involved in receptor activation.

The results from 2',5'-ADP sepharose chromatography were of interest since the complexes from a control (SW) and

a androgen insensitivity (T4) cell line gave very similar elution profiles (FIG.3.4a and b). While it may be reasonable to assume that this interaction involved the DNA/nuclear binding domain of the receptor complex (Fig.1.2b), there is no direct evidence to support this. However, it is of interest that Mulder and associates (Mulder et al 1983,84) found that a 3S androgen receptor species (from the rat prostate) bound to ADP-sepharose but not to DNA-sepharose, while a 4S receptor form bound to both. Furthermore, only the binding of the 4S species to ADP-sepharose could be competed out by double stranded DNA. These data would suggest, that the receptor nuclear binding site can have an indirect effect on the interaction of receptor complexes with ADP-sepharose.

The cell line T4, derived from a patient with complete testicular feminization (Results 3.1), deserves special consideration, since the findings of the whole cell (Table 3.1) and *in vitro* (Table 3.2, Figs.3.1,3.3,3.4) receptor studies were indistinguishable from controls (although a possible "ligand specific" defect was observed during the augmentation studies; Introduction 1.2, Rowney & Hodgins 1984). The implication of these results, was that the mutation in these cells occurred distal to steps involving the receptor (Receptor Positive Resistance). Alternatively, the methods used may not have been sensitive enough to probe for subtle defects in receptor structure. The androgen dependent expression of a reporter gene, linked to an

androgen responsive element, transfected into T4 (and control) cells may provide a more sensitive means of assaying for normal receptor function. Unfortunately such an experiment was not possible in the present study, as the cell line T4 was difficult to grow and maintain; making it impractical to pursue this line of research.

Table 3.7 summarizes the physical properties of the human GSF androgen receptor derived from sucrose density gradient centrifugation and HPLC-size exclusion chromatography. The 4S form of the receptor appeared to be a protein with a molecular weight of around 60000-daltons, a Stokes radius of 3.16nm, frictional ratio of between 1.21-1.43 which corresponded to an asymmetric protein with an axial ratio of 3.00-6.75. The significance of the apparent larger receptor form (90000-daltons) seen for the Mathesion cell line is not known. However, it could be due to the fact that this analysis was carried out without a Guard column preceeding the TSK3000 SW column, resulting in a more compact profile and possible alterations in the resolution obtained.

Mainwaring and Irving (1973), showed that there was no evidence for tissue or species -specific forms of the androgen receptor in the male accessory glands, although as was discussed earlier (Table 1.1) there is a degree of variation in the data reported from different groups. In the classical androgen target tissue of the rat ventral prostate, the size of the activated receptor complex has ranged from 4.0-4.5S with a relative molecular mass of 50K-

100K (Chang et al 1983; Goueli et al 1984; Mainwaring 1969a; Mainwaring & Irving 1973). The above data for the human GSF androgen receptor is in good agreement with that reported for the steer seminal vesicle receptor (a protein sedimenting at 3.8S, with Mr. and Rs of 57K and 3.50nm respectively and f/fo=1.42: Chang et al 1982) and also with the receptor from calf uterus (4.5S, Mr. and Rs of 85K and 4.40nm respectively and f/fo=1.39, axial ratio of 7.4: de Boer et al 1986). Most striking perhaps were the discrepancies with the recent findings of Keenan et al (1986). These workers described a 5.1S protein with Mr./Rs of 143K/6.00nm and 114K/4.80nm for the unactivated and activated forms of the human GSF androgen receptor respectively, in the presence of 0.5M-KCl. The finding of a larger receptor species by these workers may refect a difference in receptor preparation compared with the present study, as these workers routinely used 10mM sodium molybdate in their extraction buffers and gel filtration eluent. This compound has been found by a number of groups to stabilise receptors (usally unactivated form) as large oligomeric aggregates (Noma et al 1980; Rowley et al 1984; Wilbert et al 1983). In the present study, preparation of receptor complexes prior to HPLC-gel filtration was done at 0-4°C, in the presence of the protease inhibitors PMSF and Leupeptin (Methods 2.4). However the gel filtration step was done in the absence of protease inhibitors and at room temperature. It is possible therefore, that some degradation of the

receptor could occur at this point, and indeed in all experiments with control or a receptor positive mutant cell line a second peak of about 15K was seen (Fig.3.7). Although the relationship between peaks I and II was not examined further, it is interesting that for one androgen insensitive cell line (Ia, receptor deficient) only the second smaller peak was observed (Fig.3.7b). This suggests that the mutation in these cells affects the stability of the receptor comlexes and/or renders the receptor more susceptible to proteolytic degradation.

In conclusion the combination of sonication in 0.5M-KCl containing buffer followed by 35% ammonium sulphate precipitaion of the 105000xg salt extract was found to be a useful method for the extraction and partial purification of the human GSF androgen receptor. This protocol was subsequently used in all further studies of the receptor complexes: FPLC-anion exchange chromatography, HPLC-gel filtration, photoaffinity labelling, and 2-DGE stud ies. Furthermore, the use of sucrose gradient analysis of salt extracts (from human GSF) demonstrated a defect in the TCF receptor complex that was not apparent from whole cell binding studies of steroid binding levels. Finally, the use of FPLC-anion exchange chromatography to distinquish activated and unactivated receptor complexes from cultured GSF, may prove a quick and efficient method for detecting mutations affecting receptor activation.

4.2. Photoaffinity Labelling of the Androgen Receptor using

the Synthetic Steroid [^{3}H]R1881.

In a recent review Gromemeyer and Govindan (1986) summarised the main advantages of affinity labelling steroid hormone receptors, and also highlighted some of the problems that could be encountered. The advantages include: detection of the steroid binding domain, detection of receptors under denaturing conditions, allowing more versatility in receptor isolation and purification schemes (leading to the raising of antibodies against the receptor protein), comparison of different receptor forms, and finally, the identification of chromatin binding sites. It is obvious therefore, that affinity labelling of the human GSF androgen receptor complex would be a very useful tool in dissecting the molecular defects underlying androgen insensitivity: directly, by allowing the comparison of control and variant receptor complexes by high voltage IEF and SDS-PAGE, and indirectly by aiding in the purification of the receptor protein.

Tindall and coworkers (Chang et al 1982, 1983, 1984) have successfully covalently labelled the androgen receptor from rat ventral prostate and steer seminal vesicle with the affinity ligand 17β- (bromoacetyl)-oxy -5α-androstane-3-one, and the photoactivated ligand R1881. R1881 has also been used by Brinkmann and coworkers (Brinkmann et al 1985b, 1986) to characterise the receptor complexes from the rat prostate, calf uterus and a prostatic carcinoma cell line. In a recent study, Mainwaring and Randall (1984) highlighted the limitations of photoaffinity labelling receptor

complexes with R1881; the low level of covalent attatachment and non-specific binding of the steroid.

Using the photoactivated ligand, R1881 it was possible to covalently label the receptor proteins from rat prostate (Fig.3.9) and calf uterus (Fig.3.10); so confirming the findings of Brinkmann et al (1985b, 1986). The difference in size (50K vs 100K respectively) of the receptor from these tissues was thought to be the result of high levels of proteolytic activity present in prostate tissue (Brinkmann et al 1985b)

Attempts to repeat the above procedure with the human GSF androgen receptor from control cells (pooled cultures of RM and SW cells), were less successful (Figs.3.12 and 3.13). This may have been due to:

1. The low efficiency of the photoactivation reaction (i.e 0.2-8.0%: Brinkmann et al 1985b; Mainwaring & Randall 1984) meant that sufficient starting material and some purification procedure(s) were necessary. Therefore any loss of receptor binding activity during the pre-irradiation steps (i.e. FPLC-anion exchange chromatography) would lead to further reductions in the overall efficiency, and subsequent chances of detecting specific receptor binding above background (non-specific) levels.

2. Inefficient exchange of [^{3}H]mibolerone for [^{3}H]R1881, in earlier experiments (Fig.2.2, Scheme I), may have adversely affected the procedure. Subsequent studies

suggested that exchange assay conditions were not optium (Appendex 5.4).

3. The U.V. source and the period of irradiation used for Schemes I and II(a & b)(Fig.2.2) may also have affected the efficiency of the covalent linking of R1881-receptor complexes. Although the lamp was used successfully to covalently link a diazo-steroid derivative to the rat liver 5α-reductase enzyme (Beattie, Hodgins & Nimmo 1986).

Attempts to photoaffinity label the human GSF androgen receptor using the _in situ_ U.V. irradiation procedure (Fig.2.2, Scheme III) described for the chick oviduct progesterone receptor (Horwitz and Alexander 1983) were also unsuccessful (Figs.3.12, 3.13). Since these workers estimated the efficiency of this technique to be about 15%, the observed results may reflect an increased sensitivity of the R1881-androgen receptor complex to damage by U.V. energy, compared to the R5020-progesterone receptor complex. Another possibility is that the nature of the receptor complex was so altered by the irradiation that it was no longer precipitable by 35% ammonium sulphate (Tables 3.8, 3.9: counts remaining in the supernatant fraction).

Before leaving these studies it is intriguing to speculate on the peak of radioactivity that was consistently observed at the dye front on 8% SDS-polyacrylamide gels (Fig.3.12). Since it is difficult to resolve proteins below 30K on this percentage of gel and the trichloroacetic acid precipitated material was washed and extracted thoroughly

(Methods 2.9) before electrophoresis, it is conceivable that this material represents a fragment of the receptor containing the steroid binding domain (a similar "peak" was observed during the studies on the rat prostate and calf uterus receptor (Figs.3.10 & 3.11; and Brinkmann et al 1985a,1986), but it's significance was not determined). However, from the work of Mainwaring and Randall (1985), it is possible that this simply represents steroid that was trapped in the protein structure and only released under the denaturing conditions of SDS-PAGE.

The conclusion from the above studies was that the loss of receptor binding activity prior to irradiation, together with the inefficiency of the photolinking reaction made it impossible to gain any consistent results with the human GSF androgen receptor. Recently Gyorki et al (1986) claim to have covalently labelled the androgen receptor from normal foreskin tissue and from cultured fibroblasts. Two peaks of binding were observed under denaturing conditions, at 40K- and 85K-daltons. However, despite the use of an affinity chromatography step to partially purify the receptor complexes, the peaks shown were not as convincing as those for the rat prostate or calf uterus (Fig. 3.10 & 3.11; Brinkmann et al 1985b,1986), due mainly to the high levels of non specific binding.

4.3 Double-label Autoradiography and 2-DGE Studies.

The high resolution of proteins by 2-DGE (O'Farrell 1975; O'Farrell et al 1977) is a very powerful tool for comparing different populations of proteins, and has been used in a wide number of applications: identifying changes in protein synthesis in disease, after neoplastic transformation and during differentiation;

studying the heat

shock response in humans cells; identification of primary defects in inborn errors of metabolism; measuring the degree of genetic polymorphism in the human population ("Two-dimensional Gel Electrophoresis of Proteins, Methods and applications", Celis & Bravo 1984; and Clinical Chemistry (1982) volume 28, part 4). Although there are now a number of systems (i.e GELLAB, TYCHO) for computerised densitometry, these may not be readily available, and the comparison of complex 2-D protein patterns can be a time consuming exercise subject to errors. An added complication is the occurrence of non-reproducible differences between samples. These difficulties can be overcome by double-label autoradiography (Lecocq et al 1982; McConkey 1979), where both sets of proteins are coelectrophoresed on one gel. Two independent autoradiographic images of the final gel are then produced, specific for one or both sets of proteins, which can then be superimposed for easy comparison. Another problem with the type of study being undertaken, is the possiblity that differences unrelated to the problem being addressed could be observed due to genetic polymorphism in the human population. However,

two independent groups (McConkey, Taylor & DucPhan 1979; Walton, Styer & Gruenstein 1979) found that the average difference in whole cell labelled protein from normal individuals was less than or equal to 1%, using 2-DGE. This was a much lower figure than had been expected, based on the data from enzyme studies (i.e. 6%). Both groups concluded

that this was because using 2-DGE, a different subpopulation of cellular proteins was being studied, namely the more abdundant and conserved structural proteins. Therefore, because of the relatively low % of differences due to genetic variations between normal individuals seen on 2-D gel patterns, this is a powerful technique for identifying specific differences due to point mutations.

Therefore, using a modification of the procedure of Lecocq et al (1982), control and androgen insensitive GSF proteins were labelled with [^{35}S] methionine and [^{75}Se] selenomethionine respectively and receptor enriched fractions resolved by 2-DGE (O'Farrell 1975)(Methods 2.11). Using this method it was believed that differences in the autoradiograph (androgen insensitive proteins) could be correlated with the androgen insensitive mutation. Furthermore, it was thought that the procedure could be sensitive enough to pick up normal and variant forms of the androgen receptor protein.

The main problem encountered in this study was the large number of differences that were seen between control and androgen insensitive cells, which were not consistent between the different comparisons (Table 3.11a). In the earlier studies (Plates 3.2, 3.3, 3.8; Figs.3.15, 3.16, 3.21) the problem was a technical one in that insuffícent ^{75}Se d.p.m. were loaded on the first dimension gel to allow for decay and the relatively long exposure times required for the autoradiograph of the final gel. In the later experiments (Plates 3.4-3.7; Figs.3.17-3.20) the ratio of

^{75}Se to ^{35}S d.p.m. was increased. However, although fewer differences were seen (2-4 spots), these still varied considerably between the different comparisons (Table 3.11b). It was concluded that these changes were unlikely to be related to the androgen receptor or the primary androgen insensitivity mutation. Other explanations for the observed differences include:

1. Selective loss of ^{75}Se-labelled proteins during the preparative steps up to IEF.
2. Differences in the incorporation of ^{75}Se selenomethionine into newly synthesised protein by GSF.
3. Differences could be related to cell age and/or passage number.
4. Differences may be due to genetic polymorphism in the human population.

Selective loss of ^{75}Se-labelled proteins seems unlikely, since both control and androgen insensitive samples were treated in exactly the same manner (in parallel,Fig.2.2), and no changes were seen in the fluorograph image to suggest loss of control proteins. Similarly, the second possibility seems unlikely, as such an effect might be expected to be more specific and/or to affect all proteins. Point "3", remains a possibility, since these factors are known to affect protein synthesis in cultured cells. However, all cultures were labelled at the same stage of growth (on reaching confluence) and cells were

used at equivalent passages as far as possible. The last possiblity, that these difference were due to genetic variations in the human population, seems most likely. The observed difference of 2-4 spots out of 100-150 may seem rather high (an average of 3%) compared to the above studies of McConkey et al (1979) and Walton et al (1979)(average 0.5-1.2%). However these gels represent a specific subfraction (35% ammonium sulphate precipitate) of the total cell protein, and so genetic differences may have been preferentially selected for during the preparative stages and/or at the detection levels (i.e. less abundant proteins should be detected more readily in the present study).

Since these comparisons were between receptor enriched fractions (Table 3.4) the proteins of 45K and 85K (Risbridger et al 1982) and 56K (Wrongemann et al 1984), which were apparently less abundant in androgen insensitive cells, were unlikely to represent the androgen receptor as was suggested. The possibility that these were androgen dependent or regulated proteins, which were selected out during the salt extraction and partial purification steps in the present study, was tested by looking at whole cell protein labelled only with [^{35}S]methionine. The complexity of the patterns, and again the apparent variable differences between the 2-D protein patterns (Plates 3.9-3.13), made it difficult to assign a given difference to the androgen insensitive phenotype. Futhermore it was not possible to identify the three proteins (of 45K, 56K and 85K) that were apparently absent from androgen insensitive cell lines and

non genital skin fibroblasts (Pisbridger et al 1982; Thompson et al 1983; Wrongemann etal 1984).

Summary.

1. Properties of the human GSF androgen receptor complexes:

   Control Cells.

   - -extracted by sonication and 0.5M-KCl
   - -precipitated by 35% saturated ammonium sulphate
   - -sedimented at 4S (5-20% linear sucrose gradients)
   - -Mr. of about 60K (HPLC-gel filtration)
   - -Eluted from ADP-sepharose at 0.5-1.0M-KCl
   - -Eluted from FPLC, Mono Q at 0.13-0.18M-KCl

   Androgen Insensitive Cells.

   - -receptor from a receptor positive cell line sedimented at 4S (T4 cell line)
   - -receptor from receptor negative and receptor deficient cells, showed quantitatively abnormal sedimentation profiles
   - -apparent Mr. of 90K (Matheson cell line)
   - -eluted from ADP-sepharose at 0.5-1.0M-KCl (T4 cell line)

2. Photoaffinity Labelling of the androgen receptor with $[^3H]$ R1881

   Rat Prostate.

   - -Mr. of 50K (SDS-PAGE)

   Calf Uterus.

   - -Mr. of 100K (SDS-PAGE)

3. 2-DGE Studies:

Receptor enriched fractions (Dual labelling of control and androgen insensitive cells).

- -no differences were found that could be directly correlated with the androgen receptor or the androgen insensitive phenotype

Whole cell studies.

-again the differences seen could not be related to the androgen insensitive mutation or the anatomical origin of the cells. However, these studies did show the advantage of the dual-labelling technique for comparing complex protein samples by 2-DGE.

4.4 Prospects for future research into the molecular defect of androgen insensitvity.

A. Direct follow up to the above study.

Since the rationale for using double-label autoradiography and 2-DGE to search for the androgen receptor remains valid, a possible improvement to the protocol used above (Fig.2.2) would be to attempt to purify the receptor protein further prior to 2-DGE. Control and androgen insensitive proteins could be mixed after ammonium sulphate precipitation, prior to additional purification (i.e. FPLC-anion exchange, affinity chromatography) which would reduce the work involved and also avoid the preferential loss of material from either sample.

The search for androgen dependent proteins could also be pursued further by analysis of the other fractions, which was not carried out in the present study; 105000xg salt extracted pellet, the supernatant fraction after 35% ammonium sulphate preceipitation and culture medium after labelling incubation (secreted proteins). An interesting facet of this work would be to identify a possible physiological response of fibroblasts to androgens. That such a reponse(s) exists, has been suggested by the work of

Ozasa et al (1981), who found that protein synthesis and collagen production by cultured human fibroblasts were both slightly elevated in response to DHT.

B. Sequence data from the oestrogen, glucocorticoid, progesterone, and vitamin D recptors.

As was discussed earlier (Introdution1.1) comparison of the amino acid sequences of the oestrogen and glucocorticoid receptors from human and other sources, has revealed three domains which have a relatively high degree of homology. Using site directed mutagenesis, Kumar et al (1986) have confirmed that two of these domains at the C-terminus are involved in steroid and nuclear binding. It could be speculated therefore that using this tool of site directed mutagenesis specific mutations could be introduced into the oestrogen and/or glucocorticoid sequences that would mimic the findings of androgen receptor binding studies (Table 1.2), and thus suggest the type of mutation that occurs _in vivo_.

Furthermore, as was discussed earlier (Introduction 1.2) variant forms of the glucocorticoid receptor have been associated with abnormalities in receptor message (Miesfeld et al 1985; Northrop et al 1986). It is of considerable interest therefore, that a recent study by Danielsen et al (1986), mapping functional domains of the mouse glucocorticoid receptor, found that two receptor cDNA clones could be isolated from the nt- phenotype: one coded for a protein which was deficient in steroid binding and the

other, a protein with steroid binding activity but reduced affinity for nuclear structures. The lesions in these two variant receptors were mapped to the replacement of glu_{545} with gly, and arg_{458} with his respectively. It is possible that similar mutations could account for the receptor negative (no steroid binding) and deficient nuclear binding reported for the androgen receptor (Table 2.2 and 3.1).

Finally, the sequence of the vitamin D receptor is awaited with keen interest (Haussler et al 1987), since a number of receptor defects associated with vitamin D dependent rickets (Introduction 1.2) have been reported which appear (from steroid binding studies) to be very similar to the types of mutation seen in androgen insensitivity. This might provided the best model for site directed mutagenesis studies.

C. Isolation of the androgen receptor gene.

The best way to probe the molecular defects responsible for androgen insensitivity, would be to study the receptor gene and gene product directly. It is the aim of several groups to purify the receptor protein, raise poly and/or monoclonal antisera and isolate the receptor message and ultimately the gene. Since attempts to purify the androgen receptor, using conventional procedures have had mixed success, an alternative approach is to use the information from the steroid and/or the nuclear domains of the oestrogen and glucocorticoid receptors to synthesis short oligonuceotides, which could then be used to "fish out" the

receptor message directly without the need for a pure receptor preparation (Evans et al 1987). However, it should be stressed, that identifying point mutations that give rise to abnormal receptor function is only part of the answer. Further studies would be required to relate these changes with the phenotype of the individual, and so obtain a clearer understanding of structure-function relationships of steroid receptors.

In conclusion, it is believed that future developments in the above areas (B and C) will have important implications for steroid hormone action, and for gene regulation and cell differentiation in general, while a better understanding of androgen action and androgen receptor function (A and C) could aid in the diagnosis and early managment of patients with androgen insensitivity.

APPENDIX 5.1

Names & Addresses of Suppliers.

Appendix 5.1 Names and Addresses of Suppliers.

A.

A/S NUNC
Kamstrupvej 90,
Kamstrup,
DK-4000 Roskilde.
Denmark.

Aldrich Chemical Co.Ltd.
The Old Brickyard-New Road,
Gillingham-Dorset,
SP8 4JL.
England.

Amersham International plc.
White Lion Road, Amersham,
Buckinghamshire HP7 9LL.
England.

Amicon Ltd.
Upper Mill Stonehouse,
Glos. GL10 2BJ.
England.

B.

BDH Chemicals Ltd.
Poole,
Dorset.
England.

Beckman-RIIC Ltd.
Turnpike Road,
Cressex Industrial Estate,
High Wycombe HP12 3NR.
Bucks.
England.

Bio-Rad Laboratories Ltd.
Caxton Way,
Watford Business Park,
Watford,
Hertfordshire WD1 8RP.
England.

C.

Costar
205 Broadway,
Cambride MA02139.

D.

Du Pont
New England Nuclear (UK)
Du Pont (UK) Ltd.
2 New Road,
Southhampton,
Hampshire SO2 0AA.
England.

F.

Fissons plc.
(Hi Spin-21 MSE)
Gatwick Road,
Crawley,
Sussex RH10 2UL.
England.

G.

GIBCO Europe Ltd.
P.O. Box 35,
Trident House,
Renfrew Road,
Paisley PA3 4EF.
Scotland.

H.

Hanovia
(High Pressue Hg-lamp),
Slough.
England.

Heat Systems-Ultrasonics Inc.
(see Life Science Laboratories).

K.

Kodak
Eastman Kodak Company,
Rochester, N.Y. 14650.
England.

Kontes
(Small Dounce homogenizer)
Glass Company,
Vine land.
New Jersey.

L.

Life Science Laboratories
(Model W-375 Sonicator, with cup horn 1431A).
Biotechnology Division,
Sorum Road,
Leagrave, Luton,
Beds. LU3 2RA.
England.

LKB Instuments Ltd.
232 Addington Road,
South Croydon.
Surrey CR2 8YD.
England.

M.

May & Baker (M & B) Ltd.
Dagenham,
England.

N.

National Diagnostics
Unit 3,
Chamberlain Road,
Aylesbury,
Bucks. HP19 3DY.
England.

P.

Packard
(see United Technologies Packard).

Pharmacia (GB) Ltd.
Pharmacia House,
Midsummer Boulevard,
Central Milton Keynes,
Bucks. MK9 3HP.
England.

S.

Scottish Antibody Production Unit (SAPU)
Glasgow & West of Scotland Blood Transfusion Service.
Law Hospital,
Carluke, ML8 5ES
Lanarkshire.
Scotland.

Sigma Chemical Co.Ltd.
Fancy Road,
Dorset, BH17 7NH.
England.

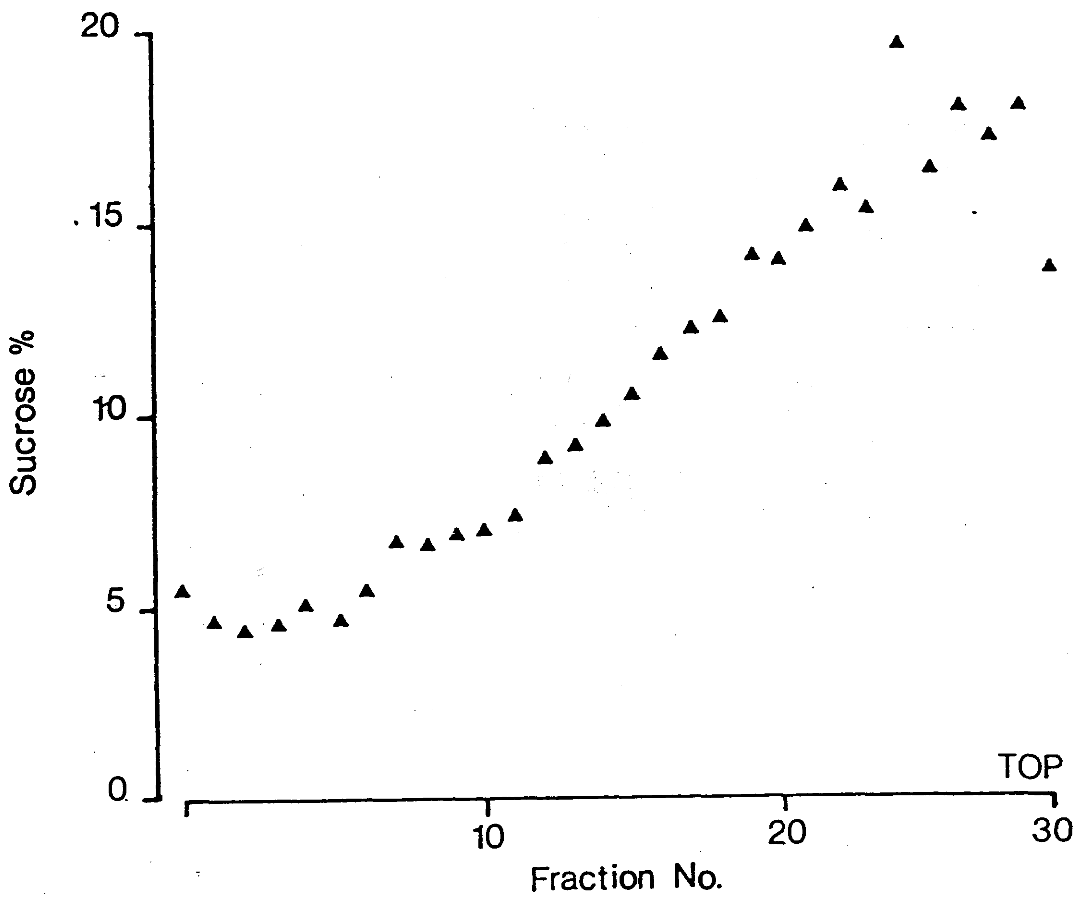
U.

United Technologies Pakard
Parkard Instrument Co.Ltd.
2200 Warrenville Road,
Downers Grove Ill.60515.
U.S.A.

U.V. Products Inc.
(U.V. Transilluminator)
5100 Walnut Grove,
San Gabriel.
U.S.A.

APPENDIX 5.2

Formation of a Linear Sucrose Gradient.

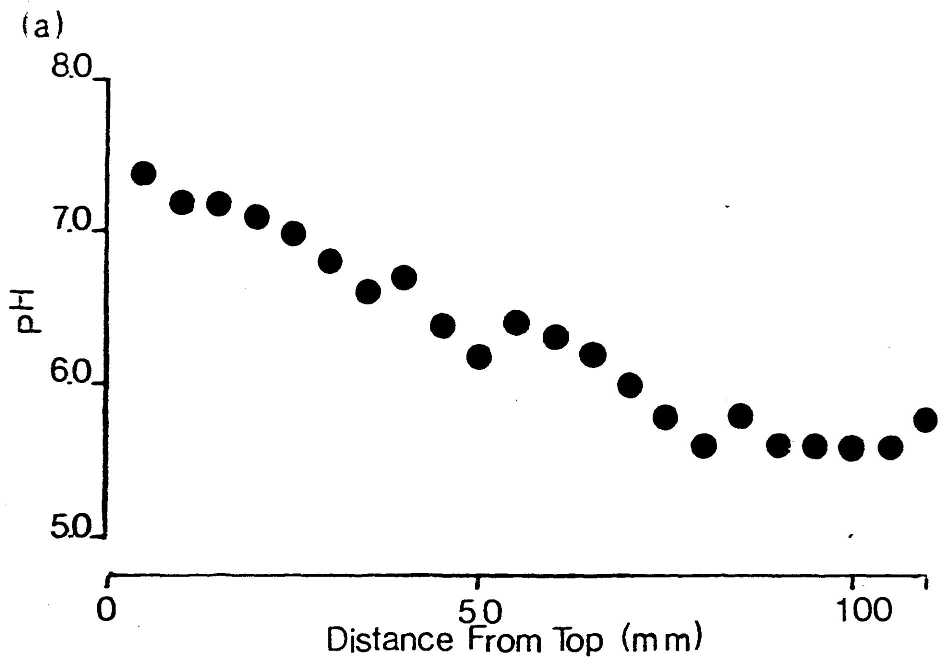


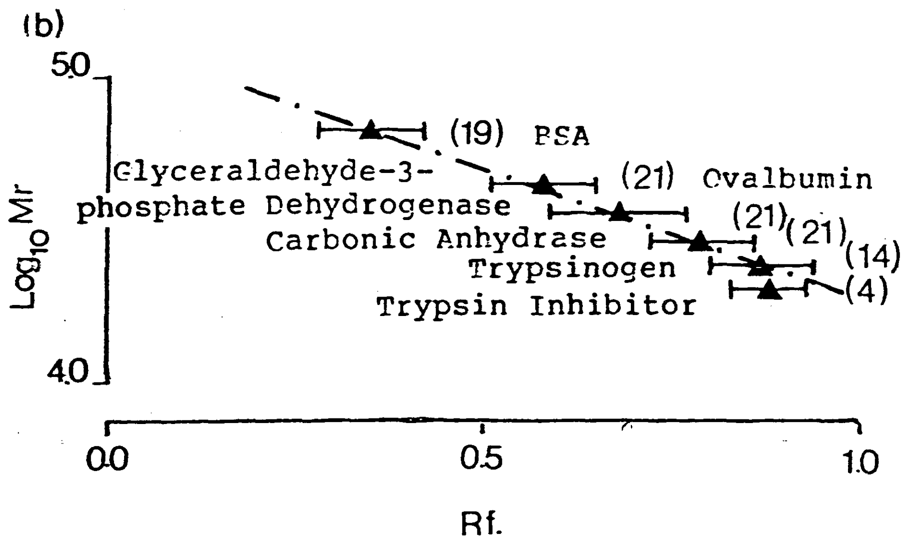
Appendix 5.2 The Formation of a Linear Sucrose Gradient.

This was checked by adding proportionate amounts of the dye bromophenol blue to the sucrose stock solutions*, prior to layering the gradient. The gradient was then left for two hours at room temperature, and fractionated as described previously (Methods 2.5). The amount of dye in each fraction was determined from the measured O.D.600nm, and the percentage of sucrose calculated from standard data.
(*, 10,20,30 and 40ul of 0.05% bromophenol blue solution was added to the 5,10,15 and 20% (w/w) sucrose stocks respectively.)

APPENDIX 5.3

Electrophoresis Standards.





Appendix 5.3

(a) The pH gradient was measured by slicing a gel (run under identical conditions, without sample) into 5mm segments, and eluting the Ampholines in 1.5ml of boiled distilled water. This was done in a sealed plastic micro testube at room temperature, for at least two hours. The pH of each sample was measured using a micro-pH electrode.

(b) Molecular weight size markers (Dalton Mark VII, Sigma) resolved on an 8% polyacralamide gel; data shown is the Mean ± the standard deviation (number of observations).

APPENDIX 5.4

[^{3}H]R1881 Exchange Assay.

Appendix 5.4 Exchange Assay. (0-4°C)

Control cells (**RM** & **SW**) were seeded in 140mm diameter petri dishes, and grown to confluence. After incubating the cells with 2nM (cold) **M**ibolerone in EC1 medium for 24 hours, the cells were collected and the 35% ammonium sulphate fraction was prepared (Methods 2.4). The ammonium sulphate precipitate was resuspended in 0.5ml PEG buffer (10mM-KH_2PO_4, 1mM-EDTA, 10%(v/v) Glycerol) containing 15nM [^{3}H]R1881, and divided into two equal amounts (A & B). Monothioglycerol (final concentration 12mM) and mersalyl acid (final concentration 1mM) was added to **A** and **B** respectively. The mercurial sulphydryl blocking agent, mersalyl acid, has been used to reversibly dissociate steroid from the progesterone and vitamin D receptors (Coty 1980). After a 30 minute incubation, the inhibitory effects of the mersaly acid were overcome by the addition of monothioglycerol (final concentration 24mM) (Coty 1980). From samples A and B 50ul was removed at 0, 2, 8, 21 and 30 hours, and the amount of bound radioactivity determined by hydroxyapatite (Methods 2.10).

Results:

Time (h)	Sample Volume (ul)	d.p.m./500ul* A	d.p.m./500ul* B
0	250	2980	4130
2	200	1520	7030
8	150	2700	-
21	100	4180	4270
30	50	3080	2740

(*Expected value for a 100% exchange> 15000 d.p.m.)

Conclusions:

1. Exchange of bound Mibolerone for [3H]R1881 was not optium under the conditions choosen. This could be due to the loss of receptor binding activity during the exchange assay, and/or the length of time allowed (maxium 30 hours) was insufficent to allow dissociation of the tightly bound Mibolerone.

2. The dissociation of androgen receptor complexes under the influence of mersalyl acid was not reversible (i.e sample B) under the above assay conditions.

As this was a single experiment it is felt that further studies would be required to determine the optimal conditions for exchanging Mibolerone for R1881. For example, if the receptors were labeled <u>in situ</u> with [3H]Mibolerone and then incubating with or without cold R1881, it would be possible to check if receptor binding was stable for the time course being used, aswell as determining the optimal conditions for R1881 exchange. The effects of mersalyl acid on androgen receptor binding could best be studied in a separate seres of experiments.

REFERENCES

Aiman,J. & Griffin,J.E. (1982) The frequency of androgen receptor deficiency in infertile men. The Journal of Clinical Endocrinology & Metabolism, 54, 725-732.

Aiman,J.,Griffin,J.E.,Gazak,J.M.,Wilson,J.D. & MacDonald,P. (1979) Androgen insensitivity as a cause of infertility in otherwise normal men. New England Journal of Medicine, **300**, 223-227.

Alberts,B. & Herrick,G. (1971) DNA cellulose chromatography. Methods in Enzymology, **21**, 198-220.

Albright,F.,Butler,A.M. & Bloomberg,E. (1937) Rickets resistant to vitamin D therapy. American Journal of Disease of Children **54**, 527-547.

Albright,F.,Burnett,C.H.,Smith,P.H.,Parson,W. (1942) Pseudo-hypoparathyrodism: An example of "Seabright-Bantam" syndrome. Endocrinology, 30, 922-932.

Amrhein,J.A.,Meyer,W.J.,Jones,H.W. & Migeon,C.J. (1976) Androgen insensitivity in man: evidence for genetic heterogeneity. Proceedings of the National Academy of Science-USA, **73**, 891-894.

Anderson,D.C. (1974) Sex-hormone binding globulin. Clinical Endocrinology, 3, 69-96.

Anderson,K.M. & Liao,S. (1968) Selective retention of DHT by prostatic nuclei. Nature, **219**, 277-279.

Antakly,T. & Eisen,H.J. (1984) Immunocytochemical localization of glucocorticoid receptor in target cells. Endocrinology, **115**, 1984-1989.

Asselin,J.,Melangon,R.,Gourdeau,Y.,Labrie,F.,Bonne,C. & Raynaud, J.P. (1979) Specific binding of [^{3}H] methyltrienolone to both progestin and androgen binding components in human benign

prostatic hypertrophy (BPH). Journal of Steroid Biochemistry, 10, 483-486.

Auricchio,F.,Migliaccio,A.,Castoria,G.,Lastoria,S. & Shiavone,E. (1981) ATP-dependent enzyme activating hormone binding of the oestrogen receptor. Biochemical & Biophysical Research Communication, 101, 1171-1178.

Auricchio,F.,Migliaccio,A.,Castoria,G.,Rotondi,A. & Lastoria,S. (1984) Direct evidence of in vitro phosphorylation of the oestradiol-17β receptor. Role of Ca^{2+}-calmodulin in the activation of hormone binding sites. The Journal of Steroid Biochemistry, 20, 31-35.

Auricchio,F.,Migliaccio,A.,Rotondi,A. & Castoria,G. (1985) In Molecular Mechanism of Steroid Hormone Action, ed. Moudgil,V.K., Walter deGryter & Co., Berlin-New York.

Bailly,A.,LeFevre,B.,Savouret,J. & Milgrom,E. (1980) Activation and changes in sedimentation properties of steroid receptors. The Journal of Biological Chemistry, 255, 2729-2734.

Bannister,P.,Sheridan,P. & Losowsky,M.S. (1985a) Identification and characterization of the human hepatic androgen receptor. Clinical Endocrinology, 23, 495-502.

Bannister,P.,Sheridan,P. & Losowsky,M.S. (1985b) Use of a new radioactive ligand 7α,17α-dimethyl 17β-methyl [^{3}H] 19-nortestosterone for the estimation of androgen receptor in rat liver cytosol. The Journal of Steroid Biochemistry, 23, 121-123.

Bardin,C.W.,Bullock,L.,Schneider,G.,Allison,J.E. & Stanley,A.J. (1970) Pseudohermaphrodite rat: End organ insensitivity to testosterone. Science, 167, 1136-1137.

Barrack,E.R. & Coffey,D.S. (1980) The specific binding of estrogens and androgens to the nuclear matrix of sex hormone responsive tissues. Journal of Biological Chemistry, 255, 7265-7275.

Baulieu,E.E. & Jung,I. (1970) A prostatic cytosol receptor. Biochemical & Biophysical Research Commumication, 38, 599-606.

Beattie,J.,Hodgins,M.B. & Nimmo,H. (1986) Solubilization and partial characterization of rat hepatic testosterone 5α-reductase. Journal of Steroid Biochemistry, 25 (suppl.), abs. 180, pp 66S.

Ben-or,S. & Chrambach,A. (1983) Heterogeneity of the glucocorticoid receptors: Molecular transformations during activation, detected by electrofocusing. Archives of Biochemistry and Biophysics, 221, 343-543.

Berezney,R. & Coffey,D.S. (1974) Identification of a nuclear protein matrix. Biochemical & Biophysical Research Communication, 60, 1410-1417.

Berezney,R. & Coffey,D.S. (1975) Nuclear protein matrix: associated with newly synthesised DNA. Science, 189, 291-293.

Berg,J.M. (1986) More metal-binding fingers. Nature, 319,

Bird,A.P. (1984) DNA methylation-how important in gene control ? Nature, 307, 503-504.

Bird,A.P. (1986) CpG-rich islands and the function of DNA methylation. Nature, 321, 209-213.

Birnbaum,M.J. & Baxter,J.D. (1986) Glucocorticoids regulate the expression of a rat growth hormone gene lacking 5' flanking sequences. The Journal of Biological Chemistry, 261, 291-297.

Boss,M.A. (1983) Enhancer elements in immunoglobulin genes. M.

Nature, 303, 281-282.

Bradford,M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principal of protein-dye binding. Analytical Biochemistry, 72, 248-254.

Bravo,R. (1984) In Two-dimensional gel electrophoresis of proteins. Methods and Applications. Chapter 1, eds. Celis,J.E. & Bravo,R., Academic Press, London.

Brinkmann,A.O.,Bolt-de vries,J.,Lindh,M.,de Boer,W.,Mulder,E. & van der Molen,H. (1985a) Characterization of steroid hormone receptors with ion exchange Fast Protein Liquid Chromatography. The Journal of Steroid Biochemistry, 22, 85-90.

Brinkmann,A.O.,Kuiper,G.,de Boer,W. Mulder,E.,Bolt,J.,Van Steenbrugge,G.J. & Van der Molen,H.J. (1986) Characterization of androgen receptors after photoaffinity labelling with methyltrienolone (R1881). The Journal of Steroid Biochemistry, 24, 245-249.

Brinkmann,A.O.,Kuiper,G.,de Boer,W.,Mulder,E. & van der Molen,H. (1985b) Photoaffinity labelling of the androgen receptor with R1881. Biochemical & Biophysical Research Communication, 126, 163-169.

Britten,R.J. & Davidson,E.H. (1969) Gene regulation for higher cells: A theory. Science, 165, 349-357.

Brown,T.R.,Maes,M.,Rowthwell,S.W. & Migeon,C.J. (1982) Human complete androgen insensitivity with normal DHT receptor binding capacity in cultured genital skin fibroblasts, evidence for a qualitative abnormality of the receptor. The Journal of Clinical

Endocrinology & Metabolism, 55, 61-69.

Brown,T.R. & Migeon,C.J. (1981) Cultured human skin fibroblasts: a model for the study of androgen action. Molecular & Cellular Biochemistry, 36, 3-22.

Brown,T.R. & Migeon,C.J. (1986) Androgen binding in nuclear matrix of human genital skin fibroblasts from patients with androgen insensitivity syndrome. The Journal of Clinical Endocrinology & Metabolism, 62, 542-550.

Brown,T.R.,Rothwell,S.W. & Migeon,C.J. (1981) Comparison of methyltrienolone and DHT binding and metabolism in human genital skin fibroblasts. Journal of Steroid Biochemistry, 14, 1013-1022.

Bruchovsky,N. & Wilson,J.D. (1968) The conversion of testosterone to 5α-androstan-17β-ol-3-one by rat prostate in vivo and in vitro. The Journal of Biological Chemistry, 243, 2012-2021.

Bullock,L.P. & Bardin,C.W. (1974) The androgen receptor in mouse kidney: A study of male, female and androgen insensitive (tfm/Y) mice. Endocrinology, 94, 746-756.

Burch,J.B.E. & Weintraub,H. (1983) Temporal order of chromatin structural changes associated with the activation of the major chicken vitellogenin gene. Cell, 33, 65-67.

Buttyan,R.Olsson,C.A.,Sheard,B. & Kallos,J. (1983) Steroid receptor-nuclear matrix interactions: The role of DNA. The Journal of Biological Chemistry, 258, 14366-14370.

Carlstedt-Duke,J.,Okret,S.,Wrange,O. & Gustafsson,J.A. (1982) Immunochemical analysis of the glucocorticoid receptor: Identification of a third domain separate from the steroid-binding and DNA-binding domains. Proceedings of the National Academy of Sciences-USA, 79, 4260-4264.

Castells,S.,Greig,F.,Fusi,M.A.,Finberg,L., Yasumura,S.,Liberman, U.A.,Eil,C. & Marx,S.J. (1986) Severly deficient binding of 1,25-vitamin D_3 to its receptor in a patient responsive to high doses of this hormone. Journal of Clinical Endocrinology & Metabolism, **63**, 252-256.

Cato,A.C.B.,Geisse,S.,Wrnz,M.,Westphal,H.M. & Beato,M. (1984) The nucleotide sequences recognized by the glucocorticoid receptor in the rabbit uteroglobin gene region are located far upstream from the initiation of transcription. The EMBO Journal, **3**, 2771-2778.

Ciejek,E.M.,Tsai,M.J. & O'Malley,B.W. (1983) Actively transcribed genes are associated with the nuclear matrix. Nature, **306**, 607-609.

Chan,L. & O'Malley,B.W. (1976) Mechanism of action of the sex steroid hormones. New England Journal of Medicine, **294**, 1322-1328, 1372-1381, 1430-1437 (3 parts).

Chandler,V.L.,Maler,B.A. & Yamamoto,K.R. (1983) DNA sequences bound specifically by glucocorticoid receptor in vitro render a heterologous promoter hormone responsive in vivo. Cell, **33**, 489-499.

Chang,C.H.,Lobl,T.J.,Rowley,D.R. & Tindal,D.J. (1984) Affinity labelling of the androgen receptor in rat prostate cytosol with 17β-[(Bromoacetyl)oxy]-5α-androstan-3-one. Biochemistry, **23**, 2527-2533.

Chang,C.H.,Rowley,D.R.,Lobl,T.J. & Tindall,D.J. (1982) Purification and characterization of the androgen receptor from steer seminal vesicle. Biochemistry, **21**, 4102-4109.

Chang,C.H. & Tindall,D.J. (1983) Physicochemical characterization

of the androgen receptor in rat uterine cytosol. Endocrinology, 113, 1486-1493.

Cheek,D.B. & Peey,J.W. (1958) A salt wasting syndrome in infancy, Archives of Diseases in Childhood, 33, 252-256.

Chrousos,G.P., Brandon,D., Requeist, D.M., Tomita,M., Johnson,E., Loriaux,D.L. & Lipsett,M.B. (1984a) Uterine estrogen and progesterone receptors in an estrogen-and progesterone-"resistant" primate. Journal of Clinical Endocrinology & Metabolism, 58, 516- 520.

Chrousos,G.P.,Loriaxux,D.L.,Brandon,D.,Shull,D.,Renquist,D.,Hogan ,W.,Tomita,M. & Lipsett,M.B. (1984b) Adaptation of the mineralocorticoid target tissues to the high circulating cortisol and progesterone plasma levels in the Squirrel monkey. Endocrinology, 115, 25-32.

Chrousos,G.P.,Vingerhoeds,A.C.M.,Loriaux,D.L. & Lipsett,M.B. (1983a) Primary cortisol resistance: A family study. Journal of Clinical Endocrinology & Metabolism, 56, 1243-1245.

Chrousos,G.P.,Loriaux,D.L.,Brandon,Tomita,M.,Vingerhoeds,A.C.M.,M erriam,G., Johnstone,E.O.,Lipsett,M.B. (1983b) Primary cortisol resistance: A familial syndrome and an animal model. Journal Steroid Biochemistry, 19, 567-575.

Chung,K.W.,Chan,W.,Dressler,J.B.,Allison,J.E. & Rennert,O.E. (1983) Androgen receptors in the brain of neonatal normal male and androgen insensitive rats. Biochemical & Biophysical Research Communication, 111, 717-722.

Clark,J.H. & Peck,E.J. (1976) Nuclear retention of receptor-oestrogen complex and nuclear acceptor sites. Nature, 260, 635-637.

Collier,M.E.,Griffin,J.E. & Wilson,J.D. (1978) Intranuclear binding of [^{3}H] DHT by cultured human fibroblasts. Endocrinology, 103, 1499-1505.

Corrall,R.J.M.,Wakelin,K.,O'Hare,J.P.,O'Brien,A.D.,Ishmail,A. & Honour,J. (1984) 5-α reductase deficiency: Diagnosis via abnormal plasma levels of reduced testosterone derivatives. Acta Endocrinologica, 107, 538-543.

Coty,W.A. (1980) Reversible dissociation of steroid hormone receptor complexes by mercurial reagents. The Journal of Biological Chemistry, 255, 8035-8037.

Coulam,C.B.,Gragham,M.L. & Spelsberg,T.C. (1984) Androgen insensitivity syndrome: gonadal androgen receptor activity. American Journal of Obstetrics & Gynecology, 150, 531-533.

Cunningham,G.R.,Lobl,T.J.,Cockrell,C.,Shao,T.C. & Tindall,D.J. (1983) Characterization of steroid binding specificity of the androgen receptor in human foreskin fibroblasts. Steroids, 41, 617-626.

Danielsen,M.,Northrop,J.P. & Ringold,G.M. (1986) The mouse glucocorticoid receptor: Mapping of functional domains by cloning, sequencing and expression of wild-type and mutant receptor proteins. The EMBO Journal, 5, 2513-2522.

Davies,P. (1983) Extraction of androgen receptor complexes from regions of rat ventral prostate nuclei sensitive or resistant to nucleases. Journal of Endocrinology, 99, 51-61.

Davies,P. & Griffiths,K. (1974) Further studies on the stimulation of prostatic ribonucleic acid polymerase by 5α-DHT-receptor complexes. Journal of Endocrinology, 62, 385-400.

Davies,P. & Thomas,P. (1984) Interaction of androgen receptors with chromatin and DNA. <u>Journal Steroid Biochemistry</u>, **20**, 57-65.

Davies,P.,Thomas,P.,Borthwick,N.M. & Giles,M.G. (1980) Distribution of acceptor sites for androgen receptor complexes between transcriptionally active and inactive fractions of rat ventral prostate chromatin. <u>Journal of Endocrinology</u>, **87**, 225-240.

Davies,P.,Thomas,P. & Griffith,K. (1976) The influence of steroid-receptor complexes on the transcription of target-tissue chromatin. <u>Journal of Steroid Biochemistry</u>, **7**, 993-1000.

Dean,D.C.,Gnope,R.,Knoll,B.J.,Riser,M.E. & O'Malley,B.W. (1984) A similar 5'-flanking region is required for estrogen and progesterone induction of ovalbumin gene expression. <u>The Journal of Biological Chemistry</u>, **259**, 9967-9970.

Dean,D.C.,Knoll,B.J.,Riser,M.E. & O'Malley,B.W. (1983) A 5'-flanking sequence essential for progesterone regulation of an ovalbumin fusion gene. <u>Nature</u>, **305**, 551-554.

de Boer, W., Lindh, M., Bolt, J., Brinkmann, A.O. & Mulder, E. (1986a) Characterization of the calf uterus androgen receptor and its activation to the DNA-binding state. <u>Endocrinology</u>, **118**, 851-861.

deBoer,W.,Snippe,L.,Ab,G. Gruber,M. (1986b) Interaction of the calf uterus oestrogen receptor with acceptor sites in heterologous chicken target cell nuclei. <u>Journal Steroid Biochemistry</u>, **24**, 825-833.

deLarminat,M.,Bruchovsky,N.,Rennie,P.S.,Ping Lee,S. & Tertzakian,G. (1984) Synthesis and evaluation of immobilised androgens for affinity chromatography in the purification of

nuclear androgen receptor. The Prostate, 5, 123-140.

Donti,E.,Nicoletti,I.,Filipponi,P.,Venti,G. & Bocchini,V. (1982) DHT-receptors in cultured human fibroblasts: Binding study in a family with androgen insensitivity (complete Testicular Feminization). Journal of Medical Genetics, 19, 349-353.

Dougherty,J. (1985) Phosphorylation of the progesterone receptor In Molecular Mechanism of Steroid Hormone Action, ed. Moudgil,V.K., Walter deGryter & Co., Berlin-New York.

Dougherty,J.J.,Puri,R.K. & Toft,D.O. (1985) Phosphorylation of steroid receptors Trends in Bichemical Sciences, 6, 83-85.

Dunn,A. & Gough,N. (1984) Tissue-specific enhancers. Trends in Biochemical Sciences, 9, 81-83.

Dure,L.S.,Schrader,W.T. & O'Malley,B.W. (1980) Covalent attachment of a progestational steroid to chick oviduct progesterone receptor by photo-affinity labelling. Nature, 283, 784-786.

Dworniczak,B.,Kobus,S.,Schaltmann-Eiteljorge,K. & Pongs,O. (1983) In Gene Regulation by Steroid Hormones II, Chapter 4., eds. Roy,A.K. & Clark,J.H., Springer, New York.

Eckert,R.L. & Katzenellenbogen,B.S. (1982) Effects of oestrogens and antioestrogens on oestrogen receptor dynamics and the induction of progesterone receptor in MCF-7 breast cancer cells. Cancer Research, 42, 139-144.

Eckert,R.L.,Mullick,A.,Rorke,E.A. & Katzenellenbogen,B. (1984) Oestrogen receptor synthesis and turnover in MCF-7 breast cancer cells measured by a density shift technique. Endocrinology, 114, 629-637.

Edwards,D.P.,Weigel,N.L.,Schrader,W.T.,O'Malley,B.W. & McGuire, W.L. (1984) Structural analysis of chicken oviduct progesterone receptor using monoclonal antibodies to the subunit B protein. Biochemistry, 23, 4427-4435.

Eil,C. (1983) Familial incomplete male pseudohermaphroditism associated with impaired nuclear androgen retention. Journal of Clinical Investigation, 71, 853-858.

Eil,C.,Liberman,U.A.,Rosen,J.F. & Marx,S.J. (1981) A cellular defect in heriditary vitamin D-dependent rickets type II, Defective nuclear uptake of 1,25-dihydroxy-vitamin D in GSF. New England Journal of Medicine, 304, 1588-1591.

Eil,C. & Marx,S.J. (1981) Nuclear uptake of 1,25-dihydroxy-[^{3}H]-cholecalciferol in dispersed fibroblasts cultured from normal human skin. Proceedings of the National Academy of Sciences-USA,78, 2562-2566.

Elawady,M.K.,Allman,D.R.,Griffin,J.E. & Wilson,J.D. (1983) Expression of a mutant androgen receptor in cloned fibroblasts from a heterozygous carrier for the syndrome of testicular feminization. American Journal of Human Genetics, 35, 376-384.

Elgin,S.C.R. (1981) DNase I-hypersensitivity sites of chromatin. Cell, 27, 413-415.

Elgin,S.C.R. (1983) Anatomy of hypersensitive sites. Nature, 309, 213-214.

Eliard,P.H.,Morchand,M.J.,Rousseau,G.G.,Formstecher,P.,Mathy-Hartert,M. ,Belayew,A. & Martial,J.A. (1985) Binding of the human glucocorticoid receptor to defined regions in the human growth hormone and placental lactogen genes. DNA, 4, 409-417.

Evans,B.A.J. & Hughes,I.A. (1985) Augmentation of androgen

receptor binding _in vitro_: Studies in normals and patients with androgen insensitivity. _Clinical Endocrinology,_ **23**, 567-577.

Evans,B.A.J.,Jones,T.R. & Hughes,I.A. (1984) Studies of the androgen receptor in dispersed fibroblasts: Investigation of a patient with androgen insensitivty. _Clinical Endocrinology,_ **20**, 93-105.

Evans,R.M.,Weinberger,C.,Giguere,V.,Hollenberg,S.,Thompson,C., Arriza,J. & Yan,N. (1987) Steroid receptor gene structure and fuction. _Journal of Cellure Biochemistry,_ **Suppl.11A**, abs. B002, pp87.

Fang,S.,Anderson,K.M. & Liao,S. (1969) Receptor proteins for androgens. _The Journal of Biological Chemistry,_ **244**, 6584-6595.

Fang,S. & Liao,S. (1971) Androgen receptors: Steroid and tissue-specific retention of a 17β-hydroxy-5α-androstane-3-one-protein complex by the cell nuclei of ventral prostate. _The Journal of Biological Chemistry,_ **246**, 16-24.

Feigelson,P.,Ramanarayanan-Murthy,L. & Colman,P.D. (1979) In _Receptors and Hormane Action Vol.II_, 225-249, ed. O'Malley,B.W. & Birnbaumer,L., Academic Press, New York-london.

Feit,E.I. & Muldoon,T.G. (1983) Differences in androgen binding properties of the two molecular forms of androgen receptor in rat ventral prostate cytosol. _Endocrinology,_ **112**, 592-600.

Fey,S.J.,Bravo,R.,Larsen,P.M. & Celis,J.E. (1984) In _Two-dimensional gel electrophoresis of proteins. Methods and Applications,_ Chapter 6, eds. Celis,J.E. & Bravo,R., Academic Press, London.

Fleming,H.,Blumenthal,R. & Gurpide,E. (1983) Rapid changes in

specific estrogen binding elicited by cGMP or cAMP in cytosol from human endometrial cells. Proceedings of the National Academy of Sciences-USA, 80, 2486-2490.

Foekens,J.A.,Mulder,E.,Vrij,L. & van der Molen,H.J. (1982) Purification of the androgen receptor of sheep seminal vesicles. Biochemical & Biophysical Research Communication, 104, 1279-1286.

Foekens,J.A.,Rennie,P.S.,Gheng,H. & Bruchovsky,N. (1985) In situ cross-linking of androgen receptor to nuclear acceptor sites of rat prostate with formaldehyde. The Journal of Biological Chemistry, 260, 10093-10098.

Fritton,H.P.,Igo-Kemenes,T.,Nowock,J.,Strech-Jurk,U.,Theisen,M. & Sippel,A.E. (1984) Alternative sets of DNase I-hypersensitive sites characterize the various functional states of the chicken lysozyme gene. Nature, 311, 163-165.

Gehring,U. & Tomkins,G.M. (1974) A new mechanism for steroid unresponsiveness: loss of nuclear binding activity of a steroid hormone receptor. Cell, 3, 301-306.

Geisse,S.,Scheidereit,C.,Westphal,H.M.,Hynes,N.E.,Gröner,B. & Beato,M. (1982) Glucocorticoid receptors recognise DNA sequences in and around Mammary Tumor Virus DNA. The EMBO Journal, 1, 1613-1619.

Ghosh-dastidar,P.,Coty,W.A.,Griest,R.E.,Woo,D.D.L. & Fox,C.F. (1984) Progesterone receptor subunits are high-affinity substrates for phosphorylation by epidermal growth factor receptor. Proceedings of the National Academy of Sciences-USA, 81, 1654-1658.

Giorgi,E.P. (1980) The transport of steroid hormones into animal cells. International Review of Cytology, 65, 49-115.

Giorgi,E.P. & Stein,W.D. (1981) The transport of steroids into animal cells in culture. Endocrinology, 108, 688-697.

Goidl,J.A.,Cake,M.H.,Dolan,K.P.,Parchman,L.G. & Liwack,G. (1977) Activation of the rat liver glucocorticoid-receptor complex. Biochemistry, 16, 2125-2130.

Gonor,S.E.,Lakey,W.H. & McBain,W.A. (1984) Relationship between concentrations of extractable and matrix bound nuclear androgen receptor and clinical response to endocrine therapy for prostatic adenocarcinoma. The Journal of Urology, 131, 1196-1201.

Gorski,J. & Gannon,F. (1976) Current models of steroid hormone action. Annual Review of Physiology, 38, 425-450.

Goueli,S.A.,Holtzman,J.L. & Ahmed,K. (1984) Phosphorylation of the androgen receptor by a nuclear cAMP-independent protein kinase. Biochemical & Biophysical Research Communication, 123, 778-784.

Gower,D.B. (1979) In Steriod Hormones, Croom Helm Ltd., London.

Graham,M.L.,Razel,A.J.,Spelsberg,T.C. & Coulam,C.B. (1984) Evidence for an androgen receptor in human testis. American Journal of Obstetrics & Gynecology, 150, 534-541.

Grandics,P.,Miller,A.,Schmidt,T.J. & Litwack,G. (1984) Phosphorylation in vivo of the rat hepatic glucocorticoid receptor. Biochemical & Biophysical Research Communication, 120, 59-65.

Green,S.,Walter,P.,Kumar,V.,Krust,A.,Bornert,J.M.,Argos,P. & Chambon,P. (1986) Human oestrogen receptor cDNA: Sequence, expression and homology to v-erbA. Nature, 320, 134-139.

Greene,G.L.,Sobel,N.B.,King,W.J. & Jensen,E.V. (1984)

Immunochemical studies of oestrogen receptors. The Journal of Steroid Biochemistry, 20, 51-56.

Greenstein,B.D. (1984) Analysis of activated androgen receptors in rat brain and anterior pituitary and ventral prostate glands, Nuclear binding and RNA polymerase activity. Journal of Endocrinology, 102, 181-188.

Gregory,M.R. & Notides,A.C. (1982) Characterization of two uterine proteases and their actions on the estrogen receptor. Biochemistry, 21, 6452-6458.

Griffin,J.E. (1979) Testicular feminization associated with a thermolabile androgen receptor in cultured human fibroblasts. Journal of Clinical Investigation, 64, 1624-1631.

Griffin,J.E. & Durrant,J.L. (1982) Qualitative receptor defects in families with androgen resistance: Failure of stabilization of the fibroblast cytosol androgen receptor. Journal of Clinical Endocrinology & Metabolism, 55, 465-474.

Griffin,J.E.,Leshin,M. & Wilson,J.D. (1982) Androgen resistance syndromes. In Receptors & Endocrine Disease: American Physiology Society., E81-E87.

Griffin,J.E.,Kovacs,W.J. & Wilson,J.D. (1984) In Regulation of Androgen Action, ed. Bruchovsky,N.,Chapdelaine,A. & Neumann,F., The Proceedings of an International Symposium, Montreal.

Griffin,J.E.,Punyashyhiti,K. & Wilson,J.D. (1976) DHT binding by cultured human fibroblasts, Comparison of cells from control subjects and from patients with hereditary male pseudohermaphroditism due to androgen insensitivity. Journal of Clinical Investigation, 57, 1342-1351.

Griffin,J.E. & Wilson,J.D. (1980) The syndromes of androgen

resistance. New England Journal of Medicine, 302, 198-209.

Gronemyer,H. & Govindan,M.V. (1986) Affinity labelling of steroid receptors. Molecular & Cellular Endocrinology, 46, 1-19.

Gronemyer,H.,Govindan,M.V. & Chambon,P. (1985) Immunological similarity between the chicken oviduct progesterone receptor forms A and B. Journal of Biological Chemistry, 260, 6916-6925.

Groner,B.,Kennedy,S.,Kroch,P.,Hynes,N.E. & Ponta,H. (1984) DNA sequences involved in the regulation of gene expression by glucocorticoid hormones. Biochimica et Biophysica Acta, 781, 1-6.

Gyorki,S.,James,R.,Warne,G.L. & Funder,J.W. (1986) Photoaffinity labelling of androgen receptors from human foreskin and cultured genital fibroblasts with ^{3}H methlytrienolone. Journal of Steroid Biochemistry, 25, 355-358.

Gyorki,S.,Warne,G.L.,Khalid,B.A.K. & Funder,J.W. (1983) Defective nuclear accumulation of androgen receptor in disorders of sexual differentiation. Journal of Clinical Investigation, 72, 819-825.

Harrison,S.C. (1986) Fingers and DNA half-turns. Nature, 322, 597-598.

Harris,H. (1980) In The Principles of Human Biochemical Genetics, 3rd. rev.edition, Amsterdam/Oxford.

Hartshorne,T.A.,Blumberg,H. & Young,E.T. (1986) Sequence homology of the yeast regulatory protein ADRI with xenopus transcription factor TFIIIA. Nature, 320, 283-287.

Haussler,M.R.,Mangelsdorf,D.J.,Komm,B.S.,Allegretto,E.A., Yamaoka,K.,Pike,J.,McDonnell,D. & O'Malley,B.W. (1987) Receptor meated action of vitamin D hormone. Journal of Cellular Biochemistry, Suppl. 11A, abs.B008, pp90.

Higgins,S.J. & Gehring,U. (1978) Molecular mechanisms of steroid hormone action. Advances in Cancer Research, 28, 313-384.

Hirst,M.A.,Hochman,H.I. & Feldman,D. (1985) Vitamin D resistance and Alopecia: A kindred with normal 1,25-dihydroxyvitamin D binding, but decreased receptor affinity for DNA. Journal of Clinical Endocrinology & Metabolism, 60, 490-495.

Hodgins,M.B. (1971) In vitro metabolism of Dehydroepiandrosterone and Dehydroepiandrosterone-sulphate in breast skin of woman. Steroids, 18, 11-23.

Hodgins,M.B. (1982) Binding of androgens in 5α-reductase-deficient human genital skin fibrblasts: Inhibition by progesterone and its metabolites. Journal of Endocrinology, 94, 415-427.

Hodgins,M.B. (1983a) The role of receptors and metabolism in androgen action: Studies in cultured cells and isolated tissues from male pseudhermaphrodites. In Hormones in Normal & Abnormal Human Tissue vol.III, eds. Fotherby,K. & Pal,B.S., Walter de Gruyter & Co., Berlin-New York.

Hodgins,M.B. (1983b) Possible mechanisms of androgen resistance in 5α-reductase deficiency: Implications for the physiological roles of 5α-reductase. Journal Steroid Biochemistry, 19, 555-559.

Hodgins,M.B.,Duke,E.M.C. & Ring,D. (1984) Carrier detection in the testicular feminization syndrome: Deficient 5 -DHT binding in cultured skin fibroblasts from the mothers of patients with complete androgen insensitivity. Journal of Medical Genetics, 21, 178-181.

Holbrook,N.J.,Bodwell,J.E.,Jeffries,M. & Muncl,A. (1983a) Characterisation of nonactivated and activated glucocorticoid-

receptor complexes from intact rat thymus cells. The Journal of Biological Chemistry, 258, 6477-6485.

Holbrook,N.J.,Bodwell,J.E. & Munck,A. (1983b) Effects of ATP and pyrophosphate on properties of glucocorticoid-receptor complexes from rat thymus cells. Journal of Biological Chemistry, 258, 14885-14894.

Hollenberg,S.M.,Weinberger,C.,Ong,E.S.,Cerelli,G.,Oro,A.,Lebo,R., Thompson,E.B.,Rosenfeld,M.G. & Evans,R.M. (1985) Primary structure and expression of a functional human glucocorticoid receptor cDNA.Nature, 318, 635-641.

Horwitz,K.B. & Alexander,P.S. (1983) In situ photolinked nuclear progesterone receptor of human breast cancer cells: Subunit molecular weights after transformation. Endocrinology, 113, 2195-2201.

Horwitz,K.B. & McGuire,W.L. (1978) Nuclear mechanisms of estrogen action: Effects of estradiol and antiestrogens on estrogen receptor. The Journal of Biological Chemistry, 253, 8185-8191

Housley,P.R. & Pratt,W.B. (1983) Direct demonstration of glucocorticoid receptor phosphorylation by intact L-cells. The Journal of Biological Chemistry, 258, 4630-4635.

Iida,S., Gomi,M., Moriwaki,K., Itoh,Y., Hirobe,K., Matsuzawa,Y., Katagiri,S.,Yonezawa,T. & Tarui,S. (1985) Primary cortisol resistance accompanied by a reduction in glucocorticoid receptors in two members of the same family. Journal of Clinical Endocrinology & Metabolism, 60, 967-971.

Jackson,V. & Chalkley,R. (1974) The binding of estradiol-17 to the bovine endometrial nuclear membrane. The Journal of

Biological Chemistry, 249, 1615-1626.

Jahner,D.,Stuhlmann,H.,Stewart,C.L.,Harbers,K.,Lohler,J., Simon,I.,Jaenisch, R. (1982) De novo methylation and expression of retroviral genomes during mouse embryogenesis. Nature, 298, 623-628.

Jensen,E.V. (1984) Location of unoccupied receptor and the validity of the two-step model. Laboratory Investigation, 51, 487-488.

Jensen,E.V. & deSombre,E.R. (1973) Estrogen-receptor interactions. Science, 182, 126-134.

Jensen,E.V.,Suruki,Kawashima,T,Stumpf,W.E.,Jungblunt,P.W. & deSombre, E.R. (1968) A two step mechanism for the interaction of estradiol with rat uterus. Proceedings of the National Academy of Sciences-USA, 59, 632-638.

Joab,I.,Radanyi,C.,Renoir,M.,Buchou,T.,Catelli,M.G.,Binart,N., Mester, J. & Baulieu,E.E. (1984) Common non-hormone binding component in non-transformed chick oviduct receptors of four steroid hormones. Nature, 308, 850-853.

Jost,A. (1970) Hormonal factors in the sex determination of the mammalian foetus. Philisophical Transactions of the Royal Society, London B, 259, 119-130.

Jost,A. (1972) A new look at the mechanisms controlling sex differentiation in mammals. John Hopkins Medical Journal, 130, 38-53.

Jost,J.P.,Geiser,M. & Seldran,M. (1985) Specific modulation of the transcription of cloned avian vitellogenin II gene by estradiol-receptor complex in vitro. Proceedings of the National Academy of Sciences, 82, 988-991.

Jost,J.P.,Moncharmont,B.,Jiricny,J.,Saluz,H. & Hertner,T. (1986) In vitro secondary activation (memory effect) of avian vitellogenin II gene in isolated liver nuclei. Proceedings of the National Academy of Science-USA, 83,43-47.

Jost,J.P.,Seldran,M. & Geiser,M. (1984) Preferential binding of the estrogen-receptor complex to a region containing the estrogen-dependent hypomethylation site preceeding the chicken vitellogenin II gene. Proceedings of the National Academy of Sciences-USA, 81, 429-433.

Jukier,L.,Kaufman,M.,Pinsky,L. & Peterson,R.E. (1984) Partial androgen resistance associated with secondary 5α-reductase deficiency: Identification of a novel qualitative androgen receptor defect and clinical implications. Journal of Clinical Endocrinology & Metabolism, 59, 679-688.

Kalimi,M. & Hubbard,J.H. (1982) Interaction of sodium thiocyanate with rat hepatic glucocorticoid-receptor complexes. Biochimica et Biophysica Acta, 719, 488-494.

Kandala,J.C.,Kistler,M.K. & Kistler,W.S. (1985) Androgen regulated genes from prostate and seminal vesicle share upstream sequence homologies. Biochemical & Biophysical Research Communication, 126, 948-952.

Karin,M.,Haslinger,A.,Holtgreve,H.,Richards,R,I.,Krauter,P., Westphal,H. & Beato,M. (1984) Characterization of DNA sequences through which cadmium and glucocorticoid hormones induce metallothionein IIA gene. Nature, 308, 513-519.

Kassis,J.A.,Walent,J.H.,Gorski,J. (1986) Estrogen receptor in cultured rat uterine cells: Induction of progesterone receptor in

the absence of estrogen receptor processing. Endocrinology, 118, 603-608.

Katsumata,M. & Goldman,A.S. (1974) Separation of multiple DHT-receptors in rat ventral prostate by a novel micro-method of electrofocusing. Blocking action of cyproterone acetate and uptake by nuclear chromatin. Biochimica et Biophysica Acta, 359, 112-129.

Katzenellenbogen,B.S. (1980) Dynamics of steroid hormone receptor action. Annual Review of Physiology, 42, 17-35.

Kaufman,M. & Pinsky,L. (1983) The dissociation of testosterone- and 5α-DHT-receptor complexes formed within cultured human genital skin fibroblasts. The Journal of Steroid Biochemistry, 18, 121-125.

Kaufman,M.,Pinsky,L.,Bowin,A. & Au,M. (1984) Familial external genital ambiguity due to a transformation defect of androgen receptor complexes that is expressed with 5α-DHT and the synthetic androgen methyltrienolone. American Journal Medical Genetics, 18, 493-507.

Kaufman,M.,Pinsky,L. & Hollander,R. (1981) Defective up-regulation of the androgen receptor in human androgen insensitvity. Nature, 293, 735-737.

Kaufman,M.,Pinsky,L.,Hollander,R. & Baily,J.D. (1983) Regulation of the androgen receptor by androgen in normal and androgen-resistant genital skin fibroblasts. The Journal of Steroid Biochemistry, 18, 383-390.

Kaufman,M.,Pinsky,L.,Simard,L. & Wong,S.C. (1982a) Defective activation of androgen-receptor complexes: A marker of androgen insensitivity. Molecular & Cellular Endocrinology, 25, 151-162.

Kaufman,M.,Pinsky,L.,Straisfeld,C.,Dobrenis,K.,Shiroky,J.,Chan,T. & MacGibbon,J. (1978) Some properties of the specific androgen binding activities in cultured human GSF. Journal of Clinical Endocrinology Metabolism, 47, 738-745.

Kaufman,M.,Pinsky,L.,Wong,S.C.,Simard,L. & Dorato,A. (1982b) Sodium thiocyanate: A probe for the conformations of the androgen receptor complex. Molecular & Cellular Endocrinology, 27, 121-128.

Kaufman,M.,Straisfeld,C. & Pinsky,L. (1976) Male pseudohermaphroditism presumably due to target organ unresponsiveness to androgens. Journal of Clinical Investigation, 58, 345-350.

Kaufman,M.,Straisfeld,C. & Pinsky,L. (1977) Expression of androgen-responsive properties in human skin fibroblast strains of genital and non-genital origins. Somatic Cell Genetics, 3, 17-35.

Kaufman,S.H.,Okret,S.,Wilkstrom,A.C.,Gustafsson,J.A. & Shaper, J.H. (1986) Binding of the glucocorticoid receptor to the rat liver nuclear matrix. The role of disulphide bond formation. The Journal of Biolgical Chemistry, 261, 11962-11967.

Kaye,J.S.,Pratt-Kaye,S.,Bellard,M.,Dretzen,G.,Bellard,F. & Chambon,P. (1986) Steroid hormone dependence of 4 DNaseI-hypersensitive regions located within the 7000-bp 5'-flanking segment of the ovalbumin gene. The EMBO Journal, 5, 277-285.

Keenan,B.S.,Greger,N.C. & Hedge,A.M. (1986) Studies of molecular species of the human androgen receptor: Comparison of the physicochemical properties of [^{3}H]R1881-androgen receptor complex

formed in cytosol to complex produced in intact genital skin fibroblasts. Journal of Clinical Endocrinology & Metabolism, 63, 222-230.

Keenan,B.S.,Meyer,W.J.,Hadjian,A.J. & Migeon,C.J. (1975) Androgen receptor in human skin fibroblasts characterization of a specific 17β-hydroxy-5α-androstan-3-one-protein complex in cell sonicates and nuclei. Steroids, 25, 535-552.

Keenan,B.S.,Meyer,W.J.,Hadjian,A.J.,Jones,H.W. & Migeon,C.J. (1974) Syndrome of androgen insensitivity in man: Absence of 5 - DHT binding protein in skin fibroblasts. Journal of Clinical Endocrinology & Metabolism, 38, 1143-1146.

Keller,D.W.,Wiest,W.G.,Askin,F.B.,Johnson,L.W. & Strickler,R.C. (1979) Pseudocorpus luteum insufficiency: A local defect of progesterone action on endometrial stroma. Journal of Clinical Endocrinology & Metabolism, 48, 127-132.

King,R.J.B. & Gordon,J. (1966) The localisation of [6,7-^{3}H]oestradiol-17β in rat uterus. Journal of Endocrinology, 34, 431-440.

King,W.J. & Greene,G.L. (1984) Monoclonal antibodies localise oestrogen receptor in nuclei of target cells. Nature, 307, 745-747.

Khoury,G & Gruss,P. (1983) Enhancer elements. Cell, 33, 313-314.

Kovacs,W.J.,Griffin,J.E.,Weaver,D.D.,Carlson,B.R. & Wilson,J.D. (1984) A mutation that causes lability of the androgen receptor under conditions that normally promote transformation to the DNA-binding state. Journal of Clinical Investigation, 73, 1095-1104.

Kovacs,W.J.,Griffin,J.E. & Wilson,J.D. (1983) Transformation of human androgen receptors to the deoxyribonucleic acid-binding state. Endocrinology, 113, 1574-1581.

Krust,A.,Green,S.,Argos,P.,Kumar,V.,Walter,P.,Bornert,J.M. & Chambon,P. (1986) The chicken oestrogen receptor sequence: Homology with v-erbA and the human oestrogen and glucocorticoid receptors. The EMBO Journal, 5, 891-897.

Kumar,S.A. & Dickerman,H.W. (1985) In Molecular Mechanism of Steroid Hormone Action, , 505-538, ed. Moudgil,V.M., Walter de Gruyter, Berlin-New York.

Kumar,V.,Green,S.Staub,A. & Chambon,P. (1986) Localisation of the oestradiol-binding and putative DNA-bind domains of the human oestrogen receptor. The EMBO Journal, 5, 2231-2236.

Kurl,R.N. & Jacob,S.T. (1984) Phosphorylation of purified glucocorticoid receptor from rat liver by an endogenous protein kinase. Biochemical & Biophysical Research Communication, 119, 700-705.

Kyakumoto,R.,Ohara-Nemoto,Y. & Ota,M. (1986) Sex differences in the cytosolic and nuclear distribution of androgen receptor in mouse submandibular gland. Journal of Endocrinology, 108, 267-273.

Kyprianou & Davies (1986) Association states of the androgen receptor in nuclei of human Benign Hypertrophic Prostate. The Prostate, 8, 363-368.

Kyprianou, N., Gingell, J.C. & Davies, P. (1987) Intranuclear distribution of androgen receptors in human prostate carcinoma. Endocrinology, 112, 161-169.

Lan,N.C.,Karin,M.,Nguyen,T.,Weisz,A.,Birnbaum,M.J.,Eberhardt,N.L. & Baxter,J.D. (1984) Mechanisms of glucocorticoid action. Journal of Biochemistry, 20, 77-88.

LaPointe,M.C.,Chang,C.H. & Vedeckis (1986) Structure of the mouse

glucocorticoid receptor: Rapid analysis by size-exclusion HPLC. Biochemistry, 25, 2094-2101.

Lay,H.N.,Pomberton,P.J. & Hilton,H.B. (1978) 5α-reductase deficiency causing male pseudohermaphroditism. Archives of Diseases of Childhood, 53, 751-753.

Lecocq,R.E.,Hepburn,A. & Lamy,F. (1982) The use of L-^{35}S-methionine and L-^{75}Se-selenomethionine for double label autoradiography of complex protein patterns by two-dimensional gels: A drastic shortening of the exposure time. Analytical Biochemistry, 127, 293-299.

Laemmli,U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227, 680-685.

Lee,F.,Hall,C.V.,Ringold,G.M.,Dobson,D.E.,Luh,J. & Jacob,P.E. (1984) Functional analysis of steroid hormone control regions of Mouse Mammary Tumor Virus. Nucleic Acid Research, 12, 4191-4206.

Lefebvre,Y.A. & Novosad,Z. (1980) Binding of androgens to a nuclear-envelope fraction from the rat ventral prostate. Biochemical Journal, 186, 641-647.

Lehoux,J.G.,Benard,B. & Elhilali,M. (1985) DHT-receptor in the human prostate: Characterization by chromatography, electrophoresis and IEF. Archives of Andrology, 15, 33-39.

Liao,S.,Liang,T.,Fang,S.,Castaneda,E. & Shao,T.C. (1973) Steroid structure and androgenic activity. The Journal of Biological Chemistry, 248, 6154-6162.

Liao,S.,Liang,T. & Tymoczko,J.L. (1972) Structural recognitions in the interactions of androgens and receptor proteins and their association with nuclear acceptor components. Journal of Steroid Biochemistry, 3, 401-408.

Liberman,U.A.,Eil,C. & Marx,S.J. (1983) Resistance to 1,25-dihydroxyvitamin D. Association with heterogeneous defects in cultured skin fibroblats. Journal of Clinical Investigation, **71**, 192-200.

Liberman,U.A.,Eil,C. & Marx,S.J. (1986) Receptor-positive hereditary resistance to 1,25-dihydroxyvitamin D: Chromatography of hormone-receptor complexes on DNA-cellulose shows two classes of mutation. Journal of Clinical Endocrinology & Metabolism, **62**, 122-126.

Lin,S. & Ohno,S. (1981) The binding of androgen receptor to DNA and RNA. Biochimica et Biophysica Acta, **654**, 181-186.

Lipsett,M.B.,Chrousos,G.P.,Tomita,M.,Brandon,D.D. & Loriaux,D.L. (1985) Defective glucocorticoid receptor in man and nonhuman primates. Recent Progress in Hormone Research, **41**, 199-241.

Logeat,F.,Pamphile,R.,Loosfelt,H.,Jolivet,A.,Fournier,A. & Milgrom,E. (1985) One-step immunoaffinity purification of active progesterone receptor.Further evidence in favor of the existence of a single steroid binding subunit. Biochemistry, **24**, 1029-1035

Loosfelt,H.,Atger,M.,Misrahi,M.,Guiochon-mantel,A.,Meriel,C., Logeat,F.,Benarous,R. & Milgrom,E. (1986) Cloning and sequence analysis of rabbit progesterone receptor cDNA. Proceedings of the National Academy of Sciences-USA, **83**, 9045-9049.

Loosfelt,H.,Logeat,F.,ThuVuHai,M. & Milgrom,E. (1984) The rabbit progesterone receptor: Evidence for a single steroid-binding subunit and characterization of receptor mRNA. Journal of Biological Chemistry, **259**, 14196-14202.

Madden,J.D.,Walsh,P.C.,MacDonald,P.C. & Wilson,J.D. (1975) Clinical and endocrinological characterization of a patient with

the syndrome of incomplete Testicular Feminization. Journal of Clinical Endocrinology & Metabolism, 41, 751-760.

Mainwaring,W.I.P. (1969a) The binding of 1,2,-[^{3}H] Testosterone within nuclei of the rat prostate. Journal of Endocrinology, 44, 323-333.

Mainwaring,W.I.P. (1969b) A soluble androgen receptor in cytoplasm of rat prostate. Journal of Endocrinology, 45, 531-541.

Mainwaring,W.I.P. (1977) The Mechanism of Action of Androgen. In, Monographys on Endocrinology vol.10, Springer-Verlag, New York-Heidelberg-Berlin.

Mainwaring,W.I.P. & Irving,R. (1973) The Use of DNA-cellulose chromatography and IEF for the characterization and partial purification of steroid-receptor complexes. Biochemical Journal, 134, 113-117.

Mainwaring,W.I.P. & Randall,V.A. (1984) Limitations in the use of ^{3}H methyltrienolone for the photoaffinity labelling of androgen receptor protein. Journal of Steroid Biochemistry, 21, 209-216.

Mainwaring,W.I.P.,Symes,E.K. & Higgins,S.J. (1976) Nuclear components responsible for the retention of steroid complexes, especially from thestandpoint of the specificity of hormonal responses. Biochemical Journal, 156, 129-144.

Mandel,J.L. & Chambon,P. (1979) DNA methylation: organ specific variations in the methylation pattern within and around ovalbumin and other chicken genes. Nucleic Acid Research, 7, 2081-2103.

Marx,S.J.,Liberman,U.A.,Eil,C.,Gamblin,G.T.,deGrange,D.A. & Balsan,S. (1984) Hereditary resistance to 1,25-dihydroxyvitamin D. Recent Progress in Hormone Research, 40, 589-615.

Max,E.E. (1984) New twist to DNA methylation. Nature, **310**, pp100.

Mellon,W.S. (1985) Analysis of hormone- and polynucleotide/histone-binding sites of the chicken intestinal 1,25-dihydroxyvitamin D_3 receptor by means of proteolysis. Endocrinology, **116**, 1408-1417.

Meyer,W.J.,Migeon,B.R. & Migeon,C.J. (1975) Locus on human X chromosome for DHT receptor and androgen insensitivity. Proceedings of the National Academy of Sciences, **72**, 1469-1472.

Miesfeld,R.,Okret,S.,Wilstrom,Wrange,O.,Gustafsson,J.A. & Yamamoto,K.R. (1984) Characterisation of a steroid hormone reeeptor gene and mRNA in wild-type and mutant cells. Nature, **312**, 779-781.

Miesfeld,R.,Rusconi,S.,Godowski,P.J.,Maler,B.A.,Okert,S.,Wilkstrom, A.C.,Gustafsson,J.A. & Yamamoto,K.R. (1986) Genetic complementation of a glucocorticoid deficiency by expression of cloned receptor cDNA. Cell, **46**, 389-399.

Milgrom,E.,Atger,M. & Baulieu,E. (1973) Acidophilic activation of steroid hormone receptors. Biochemistry, **12**, 5198-5205.

Milgrom,E.,Thi,L.,Atger,M. & Baulieu,E.E. (1973) Mechanisms regulating the concentration and the conformation of progesterone receptor(s) in the uterus. The Journal of Biological Chemistry, **248**, 6366-6374.

Migeon,B.R.,Brown,T.R.,Axelman,J. & Migeon,C.L. (1981) Studies of the locus for the androgen receptor: Localiization on the human X chromosome and evidence for homology with the Tfm locus in the mouse. Proceedings of the National Academy of Sciences-USA, **78**, 6339-6343.

Migliaccio,A.,Lastoria,S.,Moncharmont,B.,Rotondi,A. & Auricchio,

F. (1982) Phosphorylation of calf uterus 17β-oestradiol receptor by endogenous Ca^{2+}-stimulated kinase activating the hormone binding of the receptor. Biochemical & Biophysical Research Communications, **109**, 1002-1010.

Miller,M.A.,Mullick,A.,Greene,G.L. & Katzenellenbogen,B.S. (1985) Characterization of the subunit nature of nuclear estrogen receptor by chemical cross-linking and dense amino acid labelling. Endocrinology, **117**, 515-522.

Moore,R.J.,Griffin,J.E. & Wilson,J.D. (1975) Diminished5α-reductase activity in extracts of fibroblasts cultured from a patient with familial incomplete male psedohermaphroditism, TypeII. The Journal of Biological Chemistry, **250**, 7168-7172.

Moore,R.J. & Wilson,J.D. (1976) Steroid 5α-reductase in cultured human fibroblasts. Biochemical and genetic evidence for two distinct enzyme activities. The Journal of Biological Chemistry, **251**, 5895-5900.

Moudgil,V.K.,Buchou,T.E.,Renoir,J.M.,Mester,J. & Baulieu,E.E. (1985) Transformation of chick oviduct progesterone receptor in vitro: Effects of hormone, salt, heat, and ATP. Endocrinology, **116**, 1267-1274.

Mulder,E.,Vrij,A.A. & Brinkmann,A.O. (1983) DNA and ribonucleotide binding characteristics of two forms of the androgen receptor from rat prostate. Biochimical and Biophysical Research Communications, **114**, 1147-1153.

Mulder,E.,Vrij,A.A.,Brinkmann,A.O.,van der Molen,H.J. & Parker,M.G. (1984) Interaction of rat prostate androgen receptors with polynucleotides, RNA, DNA, and cloned DNA fragments. Biochemica et Biophysica Acta, **781**, 121-129.

Muller,R.E.,Traish,A.M.,Hirota,T.,Bercel,E. & Wotiz,H.H. (1984) Conversion of estrogen receptor from a state with low affinity for estradiol into a state of higher affinity does not require 4S to 5S dimerization. Endocrinology, **116**, 337-345.

Muller,R.,Traish,A. & Wotiz,H.H. (1983) Estrogen receptor activation preceedes transformation. Effects of ionic strengh, temperature, and molybdate. The Journal of Biological Chemistry, **258**, 9227-9236.

Munk,A. & Brink-Johnson,T. (1968) Specific and non-specific physicochemical interactions of glucocorticoids and related steroids with rat thymus cells *in vitro*. The Journal of Biological Chemistry, **243**, 5556-5565.

Munk,A. & Foley,R. (1979) Activation of steroid-receptor complexes in intact target cells in physiological conditions. Nature, **278**, 752-754.

Munk,A. & Holbrook (1984) Glucocorticoid-receptor complexes in rat thymus cells. Rapid kinetic behavior and cyclic model. The Journal of Biological Chemistry, **259**, 820-831.

MacDonald,P.C.,Madden,J.D.,Brenner,P.F.,Wilson,J.D. & Siiteri, P.K. (1980) Origin of estrogen in normal men and in women with testicular feminization. Journal of Clinical Endocrinology Metabolism, **49**, 905-919.

MacLusky,N.J.,Chrousos,G.P.,Brabdon,D.,Requist,D.M.,Loriaux,D.I., Lipsett,M.B. & Naftolin,F. (1984) Progestin receptors in the brain and pituitary of a progesterone "resistant" primate. Abs.1325 7th International congress of Endocrinology, Quebec, Canada.

McConkey,E.H. (1979) Double-label autoradiography for comparison of complex protein mixtures after gel electrophoresis. Analytical Biochemistry, 96, 39-44.

McConkey,E.H.,Taylor,B.J. & Phan,D. (1979) Human heterozygosity: A new estimate. Proceedings of the National Academy of Sciences-USA, 76, 6500-6504.

McIntyre,W.R. & Samuels,H.H. (1985) Triamcinolone acetonide regulates glucocorticoid receptor levels by decreasing the half-life of the nuclear form. The Journal of Biological Chemistry, 260, 418-427.

McLean-Morris,J. (1953) The syndrome of testicular feminization in male pseudohermaphrodites. American Journal of Obstetrics & Gynecology, 65, 1192-1211.

McLean-Morris,J. & Mahesh,V.B. (1963) Further observation on the syndrome "Testicular Feminization". American Journal of Obstetrics & Gynecology, 87, 731-745.

McNaught,R.W. & Smith,R.G. (1986) Characterization of a steroid estrogen receptor species in chick oviduct. Biochemistry, 25, 2073-2081.

Nielsen,C.J.,Sando,J.J. & Pratt,W.B. (1977) Evidence that dephosphorylation inactivates glucocorticoid receptor. Proceedings of the National Academy of Sciences-USA, 74, 1398-1402.

Noma,K.,Nakao,K.,Sata,B.,Nishizawa,Y.,Matsumaoto,K. & Yamamura,Y. (1980) Effect of molybdate on activation and stabilization of steroid receptors. Endocrinology, 107, 1205-1211.

Nordeen,S.K.,Lan,N.C.,Showers,M.O. & Baxter,J.D. (1981) Photoaffinity labelling of glucocorticoid receptors. The Journal

of Biological Chemistry, 256, 10503-10508.

Northrop,J.P.,Danielsen,M. & Ringold,G.M. (1986) Analysis of glucocorticoid unresponsive cell variants using a mouse glucocorticoid cDNA clone. The Journal of Biological Chemistry, 261, 11064-11070.

Oberfield,S.,Levine,L.S.,Carey,R.M.,Bejar,R. & New,M.I. (1979) Pseudohypoaldosteronism: Multiple target organ unresponsiveness to mineralocorticoid hormones. Journal of Clinical Endocrinology Metabolism, 48, 228-234.

Ohno,S. & Lyon,M. (1970) X-linked Testicular Feminization in the mouse as a non-inducible regulatory mutation of the Jacob-Monod type. Clinical Genetics, 1, 121-127.

Okret,S.,Poellinger,L.,Dong,Y. & Gustafsson,J.A. (1986) Down-regulation of glucocorticoid receptormRNA by glucocorticoid hormones and recognition by the receptor of a specific binding sequence within a receptor cDNA clone. Proceedings of the National Academy of Sciences-USA, 83, 5899-5903.

Oncley,J.L. (1941) Ann als of New York Academy of Sciences, 41, 121: see Introduction to Biophysical Chemistry, Martin,R.B., McGraw-Hills Book Co., New York-London.

Ozasa,H.,Tominaga,T.,Nishimura,T. & Takeda,T. (1981) Evidence for receptor-dependent response to DHT in cultured human fibroblasts. Endokrinologie, 77, 129-136.

O'Farrell,P.H. (1975) High resolution two-dimensional electrophoresis of proteins. The Journal Biological Chemistry, 250, 4007-4021.

O'Farrell,P.Z.,Goodman,H.W. & O'Farrell,P.H. (1977) High

resolution two-dimensional electrophoresis of basic as well as acidic proteins. Cell, 12, 1133-1142.

O'Malley,B.W.,Spelsberg,T.C.,Schrader,W.T.,Chytil,F. & Steggles, A.W. (1972) Mechanism of interaction of a hormone-receptor complex with the genome of eukaryotic target cell. Nature, 235, 141-144.

Page,M.J. & Parker,M.G. (1983) Androgen-regulated expression of a cloned rat prostatic C3 gene transfected into Mouse Mammary Tumor cells. Cell, 32, 495-502.

Parchman,L.G. & Litwack,G. (1977) Resolution of activated and unactivated forms of the purified rat hepatic glucocorticoid receptor. The Journal of Biological Chemistry, 183, 374-382.

Pardoll,D.,Vogelstein,B. & Coffey,D.S. (1980) A fixed site for DNA replication in eukaryotic cells. Cell, 19, 527-536.

Parker,M. (1983) Enhancer elements activated by steroid hormones? Nature, 304, 687-688.

Parker,M.,Hurst,H. & Page,M. (1984) Organization and expression of prostatic steroid binding protein genes. Journal of Steroid Biochemistry, 20, 67-71.

Payvar,F.,deFranco,D.,Firestone,G.L.,Edgar,B.,Wrange,O.,Okret, S.,Gustafsson,J.A. & Yamamoto,K.R. (1983) Sequence-specific binding of glucocorticoid receptor to Mouse Mammary Tumor Virus DNA at sites within and upstream of the transcribed region. Cell, 35, 381-392.

Pereira,R.,Brinkmann,A.O.,Ring,D. & Hodgins,M.B. (1984) Partial androgen insensitivity as a cause of genital maldevelopment. Helvetia Paediatrica Acta, 39, 255-259.

Peterson,R.E.,Imperato-McGinley,J.,Gautier,T. & Sturla,E. (1977)

Male pseudohermaphroditism due to steroid 5 -reductase deficiency. American Journal of Medicine, **62**, 170-191.

Pike,J.W.,Dokoh,S.,Haussler,M.R.,Liberman,U.A.,Marx,S.J. & Eil,C. (1984) Vitamin D_3-resistant fibroblasts have immunoassayable 1,25-dihydroxyvitamin D_3 receptors. Science, **224**, 879-881.

Pikler,G.M.,Webster,R.A. & Spelsberg,T.W. (1976) Nuclear binding of progesterone in hen oviduct. Binding to multiple sites in vitro. Biochemical Journal, **156**, 399-408.

Pinsky,L. (1978) The nosology of male pseudohermaphroditism due to androgen insensitivity. In Birth Defects: Original Article Seris **XIV (6C)**, 73-95.

Pinsky,L.,Kaufman,M. & Chudley,A.E. (1985) Reduced affinity of the androgen receptor for 5 -DHT but not methlytrinolone in a form of partial androgen resistance. Journal of Clinical Investigation, 75, 1291-1296.

Pinsky,L.,Kaufman,M.,Killinger,D.W.,Burko,B.,Shatz,D. & Volpe,R. (1984) Human minimal androgen insensitivity with normal DHT-binding capacity in cultured genital skin fibroblasts: evidence for an androgen-selective qualitative abnormality of the receptor. American Journal of Human Genetics, **36**, 965-968.

Pratt,K.,Wierowski,J.V.,Hilf,R. & Bambara,R.A. (1984) Bovine estrogen receptor binds chromatin at pre-existing nuclease hypersensitive sites. Molecular & Cellular Endocrinology, **35**, 205-214.

Puca,G.,Nola,E.,Sica,V. & Bresciani,F. (1977) Estrogen binding proteins of calf uterus. Molecular and fuctional characterization of the receptor transforming factor: A Ca^{2+}-activated protease.

The Journal of Biological Chemistry, 252, 1358-1366.

Puri,R.K.,Dougherty,J.J. & Toft,D.O. (1984) The avian progesterone receptor: Isolation and characterization of phosphorylated forms. Journal of Steroid Biochemistry, 20, 23-29.

Raaka,B.M.,Finnerty,M.,Sun,E. & Samuels,H.H. (1985) Effect of molybdate on steroid receptors in intact GH_1 cells. Evidence for the dissociation of an intracellular 10S receptor oligomer prior to nuclear accumulation. The Journal of Biological Chemistry, 260, 14009-104015.

Raaka,B.M. & Samuels,H.H. (1983) The glucocorticoid receptor in GH_1 cells. The Journal of Biological Chemistry, 258, 417-425.

Raymoure,W.J.,McNaught,R.W. & Smith,R.G. (1985) Reversible activation of non-steroid binding oestrogen receptor. Nature, 314, 745-747.

Razel,A.J.,Svensson,J.,Spelsberg,T.C. & Coulam,C.B. (1985) The androgen receptor in normal human foreskin. American Journal of Obstetrics & Gynecology, 153, 410-416.

Renkawitz,R.,Beag,H.,Graf,T.,Mathias,P.,Grez,M. & Shütz,G. (1982) Expression of a chicken lysozyme recombinant gene is regulated by progesterone and dexamethasone after microinjection into oviduct cells. Cell, 31, 167-176.

Rennie,P.S.,Bruchovsky,N. & Cheng,H. (1983) Isolation of 3S androgen receptors from salt-resistant fractions and nuclear matrices of prostatic nuclei after mild trypsin digestion. The Journal of Biological Chemistry, 258, 7623-7630.

Renoir,J.M. & Mester,J. (1984) Chicken oviduct progesterone receptor: Structure, immunology, and function. Molecular & Cellular Endocrinology, 37, 1-13.

Riggs,A.D.,Suzuki,H. & Bourgeois,S. (1970) lac repressor-operator interaction. I. Equilibrium studies. Journal of Molecular Biology, 48, 67-83.

Ring,D. & Hodgins,M.B. (1983) The regulation of androgen receptor turnover in human genital skin fibroblasts by 5 -DHT. Biochemical Society Transactions, 12, 658-659.

Ringold,G.M.,Dobson,D.E.,Grove,J.R.,Hall,C.V.,Lee,F. & Vannice, J.L. (1983) Glucocorticoid regulation of gene expression: Mouse Mammary Tumor Virus as a model system. Recent Progress in Hormone Research, 39,387-425.

Risbridger,G.P.,Khalid,B.A.K.,Warne,G.L. & Funder,J.W. (1982) Differences in proteins synthesised by fibroblasts from normal individuals and patients with Complete Testicular Feminization. Journal of Cinical Investigation, 69, 99-103.

Robinson,S.I.,Small,D.,Idzerda,R.,McKnight,G.S. & Vogelstein,B. (1983) The association of transcriptionally active genes with the nuclear matrix of the chicken oviduct. Nucleic Acid Research, 11, 5113-5120.

Romanov,G.A.,Kuzmenko,A.P.,Dashkevich,V.S.,Salganik,R.I. & Vanyushin,B.F. (1984) Purified glucocorticoid-receptor complexes from rat liver cytosol preferential binding in vitro to a homologous DNA fraction whose transcription is activated by cortisol. FEBS Letters, 165, 35-38.

Romic-Stojkovic,R. & Gamulin,S. (1980) Relationship of cytoplasmic and nuclear estrogen receptors in human breast cancer. Cancer Research, 40, 4821-4825.

Rosenmann,E.,Kreis,C.,Thompson,R,G.,Dobbs,M.,Hamerton,J.L. &

Wrongemann,K. (1982) Analysis of fibroblast proteins from patients with Duchenne muscular dystrophy by two-dimensional gel electrophoresis. Nature, **298**, 563-565.

Rousseau,G.G. (1984) Control of gene expression by glucocorticoid hormones. Biochemical Journal, **224**, 1-12.

Rowley,D.R.,Chang,C.H. & Tindall,D.J. (1984) Effects of sodium molybdate on the androgen receptor from the R3327 prostatic tumor. Endocrinology, **114**, 1776-1783

Rowney,D. & Hodgins,M.B. (1985) Androgen metabolism causing apparent androgen receptor defects in skin fibroblasts: Comparison of 5α-DHT and mibolerone. Journal of Endocrinology, **104** (Suppl.), pp30.

Ruh,M.F.,Singh,R.K.,Mak,P. & Callard,G.V. (1986) Tissue and species specificity of unmasked nuclear acceptor sites for the estrogen receptor of Squalus testes. Endocrinology, **118**, 811-818.

Saluz,H.P.,Jiricny,J. & Jost,J.P. (1986) Genomic sequencing reveals a positive correlation between the kinetics of strand-specific DNA demethylation of the overlapping estradiol/glucocorticoid-receptor binding sites and the rate of avian vitellogenin mRNA synthesis. Proceedings of the National Academy of Sciences-USA, **83**, 7167-7171.

Sando,J.J.,Hammond,N.D.,Stratford,C.A. & Pratt,W.B. (1979) Activation of thymocyte glucocorticoid receptor to the steroid binding form.The roles of reducing agents, ATP, and heat stable factors. The Journal of Biological Chemistry, **254**, 4779-4789.

Sato,B.,Nishizawa,Y.,Noma,K.,Matsumoto,K. & Yamamura,Y. (1979) Estrogen-independent nuclear binding of receptor protein of rat uterine cytosol by removal of low molecular weight inhibitor.

Endocrinology, **104**, 1474-1479.

Sato,N.,Ohara-Nemoto,Y. & Ota,M. (1986) Transformation of androgen receptors from mouse submandibular glands by gel chromatography. *Journal of Endocrinology*, **108**, 123-127.

Scatchard,G. (1949) The attraction of proteins for small molecules and ions. *Annals of New York Academy of Sciences*, **51**, 660-666.

Schmidt,T.J.,Miller-Diener,A.,Webb,M.L. & Litwack,G. (1985) Thermal activation of the purified rat hepatic glucocorticoid receptor. *The Journal of Biological Chemistry*, **260**, 16255-16262.

Schreidereit,C.,Geisee,S.,Westphal,H.M. & Beato,M. (1983) The glucocorticoid receptor binds to defined nucleotide sequences near the promoter of Mouse Mammary Tumaor Virus. *Nature*, **304**, 749-752.

Scholl,S. & Lippman,M.E. (1984) The estrogen receptor in MCF-7 turnover and a dimeric model of activated nuclear receptor. *Endocrinology*, **115**, 1295-1301.

Schrader,W.T. (1984) New model for steroid hormone receptors ? *Nature*, **308**, 18-19.

Schrader,W.T.,Birnbaumer,M.E.,Hughes,M.R.,Weigel,N.L.,Grody,W.W. & O'malley,B.W. (1981) Studies on the structure and function of the chicken progesterone receptor. *Recent Progress in Hormone Research*, **37**, 583-629.

Schuh,S.,Yonemoto,W.,Brugge,J.,Bauer,V.J.,Riehl,R.M.,Sullivan, W.P. & Toft,D.O. (1985) A 90K-dalton binding protein common to both steroid receptors and the Rous Sarcoma Virus transforming protein, $pp60^{v-scr}$. *The Journal of Biological Chemistry*, **260**,

14292-14296.

Shain,S.A. & Boesel,R.W. (1975) Saturation analysis of the binding of androgens, antiandrogens and oestrogens by the cytoplasmic high affinity androgen receptor of the rat ventral prostate. Journal of Steroid Biochemistry, 6, 43-50.

Shinki,T.,Trakahashi,N.,Tanioka,Y.,Koizumi,H. & Suda,T. (1983) Extremely high circulating levels of 1,25-dihydroxyvitamin D_3 in the Marmoset, a new world monkey. Biochemical & Biophysical Research Communications, 114, 452-457.

Short,R.V. (1967) Reproduction. Annual Review of Physiology, 29, 373-400.

Sibley,C.H. & Tomkins,G.M. (1974) Mechanisms of steroid hormone resistance. Cell 2, 221-227.

Siiteri,P. & Wilson,J.D. (1974) Testosterone formation and metabolism during male sexual differentiation in the human embryo. Journal of Clinical Endocrinology Metabolism, 38, 113-125.

Simpson,R.U. & deLuca,H.F. (1980) Characterization of a receptor-like protein for 1,25-dihydroxyvitamin D_3 in rat skin. Proceedings of the National Academy of Sciences-USA, 77, 5822-5826.

Smith,A.C.,Elsasser,M.S. & Harmon,J.M. (1986) Analysis of glucocorticoid receptor activation by high resolution two-dimensional electrophoresis of affinity labelled receptor. The Journal of Biological Chemistry, 261, 13285-13292.

Smith,A.C. & Harmon,J.M. (1985) Multiple forms of the glucocorticoid receptor steroid binding protein identified by affinity labelling and high resolution two-dimensional

electrophoresis. Biochemistry, 24, 4946-4951.

Smith,D.F.,Skipper,J.K.,Davidson,F.I. & Hamilton,T.H. (1986) Identification of two forms of progesterone receptor from chicken oviduct using non-denaturing gel electrophoresis. Journal of Steroid Biochemistry, 24, 787-793.

Smith,R.G.,Syms,A.J. & Norris,J.S. (1984) Differential effects of androgens and glucocorticoids on regulation of androgen receptor concentrations and cell growth. Journal of Steroid Biochemistry, 20, 277-281.

Spelsberg,T.C.,Littlefield,B.A.,Seelke,R.,Dani,G.M.,Toyoda,H.,Boyd-Leinen,P.,Thrall,C. & Kon,O.L. (1983) Role of specific chromosomal proteins and DNA sequences in the nuclear binding sites for steroid receptors. Recent Progress in Hormone Research, 39, 463-480.

Spelsberg,T.C.,Gosse,B.J.,Littlefield,B.A.,Toyoda,H. & Seelke,R. (1984) Reconstitution of native like nuclear acceptor sites of the avian oviduct progesterone receptor: Evidence for the involvement of specific chromatin proteins and specific DNA sequences. Biochemistry, 23, 5103-5113.

Spelsberg,T.C.,Streggle,A.W.,Chytyil,F. & O'Malley,B.W. (1972) Progesterone binding components of chicken oviduct. Exchange of progesterone-binding capacity from target to nontarget tissue chromatin. The Journal of Biological Chemistry, 247, 1368-1374.

Stumpf,W.E. & Madhabananda,S. (1975) Autoradiographic techniques for localizing steroid hormones. Methods in Enzymology, 36, 135-156.

Sultan,C.,Picard,J.Y.,Josso,N. & Migeon,C.J. (1983) Incomplete

androgen insensitivity syndrome: Partially masculinised genitalia in two patients with absence of androgen receptor in cultured fibroblasts. Clinical Endocrinology, 19, 565-574.

Syms,A.J.,Norris,J.S.,Panko,W.B. & Smith,R.G. (1985) Mechanism of androgen receptor augmentation. Analysis of receptor synthesis and degradation by the density-shift technique. The Journal of Biological Chemistry, 260, 455-461.

Syms,A.J.,Norris,J.S. & Smith,R.G. (1983) Androgen stimulated elevation in androgen receptor levels is inhibited by the synthetic glucocortiocoid triamcinolone acetonide. Biochemical & Biophyscal Research Communications, 116, 1020-1025.

Takahashi,N.,Suda,S.,Shinki,T.,Horiuchi,N.,Shina,Y.,Tanioka,Y., Koizumi,H. & Suda,T. (1985) The mechanism of end-organ resistance to 1,25-dihydroxycholecalciferol in the common marmoset. Biochemical Journal, 227, 555-563.

Tanuma,S.,Johnson,L.D. & Johnson,G.S. (1983) ADP-ribosylation of chromosmal proteins and Mouse Mammary Tumor Virus expression. The Journal of Biological Chemistry, 258, 15371-15375.

Thompson,R.G.,Nickel,B.,Finlayson,S.,Meuser,R.,Hamerton,J.L. & Wrongemann,K. (1983) 56K fibroblast protein not specific for Duchenne muscular dystrophy but for skin biopsy site. Nature, 304,

Thrower,S.,Hall,C.,Lim,L. & Davison,A.N. (1976) The selective isolation of the uterine oestradiol-receptor complex by binding to oligo(dT)-cellulose. The mediation of an essential activator in the transformation of cytosol receptor. Biochemical Journal, 160, 271-280.

Toft,D. & Gorski,J. (1966) A receptor molecule for estrogens:

Isolation from the rat uterus and preliminary characterization. Proceedings of the National Academy of Sciences, 55, 1574-1580.

Tomita,M.,Brandon,D.D.,Chrousos,G.P.,Vingerhoeds,A.C.M., Fester, C.M.,Fowler,D.,Loriaux,D.L. & Lipsett,M.B. (1986) Glucocorticoid receptors in Epstein-Barr transformed lymphocytes from patients with glucocorticoid resistance and a glucocorticoid-resistant new world primate species. Journal of Clinical Endocrinology Metabolism, 62, 1145-1154.

Traish,A.M.,Muller,R.E. & Wotiz,H.H. (1986) Differences in the physicochemical characteristics of androgen receptor complexes formed in vivo and in vitro. Endocrinology, 114, 1761-1769.

Traish,A.M.,Muller,R.E. & Wotiz,H.H. (1986) Binding of 7 ,17 - dimethly-19-nortestosterone (Mibolerone) to androgen and progesterone receptors in human and animal tissue. Endocrinology, 118, 1327-1333.

Turksoy,R.N.,Mitchell,G.W. & Safaii,H.S. (1976) Testicular response to exogenous gonadotropins in the syndrome of feminizing testes. Fertility & Sterility, 27, 670-676.

Tveter,K.J. & Attramadal,A. (1968) Selective uptake of radioactivity in rat ventral prostate following administration of testosterone-1,2-^{3}H. Acta Endocrinologica, 59, 218-226.

Tymoczko,J.L.,Liang,T. & Liao,S. (1978) In Receptors and Hormone Action vol.II, 121-156, eds. O'Malley,B.W. & Birnbaumer,L., Academic Press, New York-London.

Unhjem,O.,Tveter,K.J. & Aakvaag,A. (1969) Preliminary characterization of an androgen-macromolecular complex from the rat ventral prostate. Acta Endocrinologica, 62, 153-164.

Valladres,L. & Minguell,J. (1975) Characterization of a nuclear receptor for testosterone in rat bone marrow. Steroids, 25, 13-21.

Vedeckis,W.V. (1983) Subunit dissociation as a possible mechanism of glucocorticoid receptor activation. Biochemistry, 22, 1983-1989.

Vogelstein,B.,Pardoll,D.M. & Coffey,D.S. (1980) Supercciled loops and eukaryotic DNA replication. Cell, 22, 79-85.

von der Ahe,D.,Janich,S.,Scheideriet,C.,Renkawitz,R.,Schutz,G. & Beato,M. (1985) Glucocorticoid and progesterone receptors binding to the same sites in two hormonally regulated promoters. Nature, 313, 706-709.

von der Ahe,D.,Renoir,J.M.,Buchou,T.,Baulieu,E.E. & Beato,M. (1986) Receptors for glucocorticoid and progesterone recognise distinct features of a DNA regulatory element. Proceedings of the National Academy of Sciences-USA, 83, 2817-2821.

Voss,S.D.,Schlokat,U. & Gruss,P. (1986) The role of enhancers in the regulation of cell type specific transcription controls. Trends in Biochemical Sciences, 11, 287-290.

Walsh,P.C.,Madden,J.D.,Harrod,M.J.,Goldstein,J.L.,MacDonald,P. & Wilson,J.D. (1974) Familial incomplete male pseudohermaphroditism type 2. Decreased DHT formation in pereosrotal hyposadias. New England Journal of Medicine, 291, 944-949.

Walter,P.,Green,S.,Greene,G.,Krust,A.,Bornrt,J.M.,Jetsch,J.M., Staub,A.,Jensen,E.,Svrace,G.,Waterfield,M. & Chambon,P. (1985) Cloning of the human estrogen receptor cDNA. Proceedings of the National Academy of Sciences-USA, 82, 7889-7893.

Walton,K.E.,Styer,D. & Greenstein,E.I. (1979) Genetic

polymorphism in normal human fibroblasts as analysed by two-dimensional polyacrylamide gel electrophoresis. The Journal of Biological Chemistry, **254**, 7951-7960.

Warne,G.L.,Risbridger,G.P.,Khalid,B. & Funder,J.W. (1983) Fibroblast studies on clinical androgen insensitivity. Journal of Steroid Biochemistry, **19**, 583-586.

Weatherill,P.J. & Bell,P.A. (1982) Characterization of the molybdate-stabilized glucocorticoid receptor from rat thymus. Biochemical Journal, **206**, 633-640.

Webster,R.A.,Pickler,G.M. & Spelsberg,T.C. (1976) Nuclear binding of progesterone in the hen oviduct. Role of acidic chromatin proteins in high-affinity binding. Biochemical Journal, **156**, 409-418.

Weigel,N.L.,Tash,J.S.,Means,A.R.,Schrader,W.T. & O'Malley,B.W. (1981) Phosphorylation of hen progesterone receptor by cAMP dependent protein kinase. Biochemical & Biophysical Research Communications, **102**, 513-519.

Weinberger,G.,Hollenberg,S.M.,Rosenfeld,M.G. & Evans,R.M. (1985) Domain structure fo human glucocorticoid receptor and its relationship to the v-erb-A oncogene product. Nature, **318**, 670-672.

Weisbrod,S. (1982) Active chromatin. Nature, **297**, 289-295.

Welshons,W.V.,Lieberman,M.E. & Gorski,J. (1984) Nuclear localization of unoccupied oestrogen receptor. Nature, **307**, 747-749.

Westphal,H.M.,Mugele,K.,Beato,M. & Gehring,U. (1984) Immunochemical characterization of wild-type and variant

glucocorticoid receptors by monoclonal antibodies. The EMBO Journal, 3, 1493-1498.

Wieland,S.J. & Fox,T.O. (1979) Putative androgen receptors distinguished in wild-type and testicular feminized (tfm) mice. Cell, 17, 781-787.

Wilbert,D.M.,Griffin,J.E. & Wilson,J.D. (1983) Characterization of the cytosol androgen receptor of the human prostate. Journal of Clinical Endocrinology & Metabolism, 56, 113-120.

Wilks,A.F.,Cozens,P.J.,Mattaj,I.W. & Jost,J.P. (1982) Estrogen induces a demethylation at the 5' end region of the chicken vitellogenin gene. Proceedings of the National Academy of Sciences-USA, 79, 4252-4255.

Wilks,A.F.,Seldran,M. & Jost,J.P. (1984) An oestrogen-dependent demethylation of the 5'-end of the chicken vitellogenin gene is independent of DNA synthesis. Nucleic Acid Research, 12, 1163-1177.

Williams,D. & Gorski,J. (1975) In Methods in Enymology, 36, 275-283.

Williams,J.R.,Mattei,P.L.,Abdel-Magrid & Blout,J.F. (1986) Photochemistry of estr-4-en-3-ones: 17β-Hydroxy-4-estren-3-one, 17β-acetoxy-4,9-estradien-3-one, 17β-hydroxy-4,9,11-estratrien-3-one, and norgestrel. Photochemistry of 5α-estran-3-ones. The Journal of Organic Chemistry, 51, 769-773.

Wilson,J.D. (1974) Familial incomplete male pseudohermaphroditism type 2. Decreased DHT formation in pseudovaginal perineoscrotal hypospadias. New England Journal of Medicine, 291, 944-949.

Wilson,J.D. (1975) DHT formation in cultured human fibroblasts. Comparison of cells from normal subjects and patients with

familial incomplete male pseudohermaphroditism typeII. The Journal of Biological Chemistry, 250, 3498-3504.

Wilson,J.D. (1978) Sexual Differentiation. Annual Review of Physiology, 40, 279-306.

Wilson,E.M. & French,F.S. (1976) Binding properties of androgen receptors: evidence for identical receptors in rat testes, epididymis and prostate. The Journal of Biological Chemistry, 251, 5620-5629.

Wilson,J.D. & Gloyna,S. (1970) The intranuclear metabolism of testosterone in the accesory organs of reproduction. Recent Progress in Hormone Research, 26, 309-336.

Wilson,J.D.,Griffin,J.E.,Leshin,M. & MacDonald,P.C. (1983) In The Metabolic Basis of Inherited Disease, 5th edition. 1001-1026, eds. Stanbury,J.B.,Wyngaarden,J.B.,Freickson,D.S.,Goldstein,J.L., & Brown,M.S. McGraw-Hill, New York.

Wilson,J.D.,Harrod,M.J.,Goldstein,J.L.,Hemsell,D.L. & MacDonald, P.C. (1974) Familial incomplete male pseudohermaphroditism, Type I. Evidence for androgen resistance and variable clinical manifestations in a family with the Refenstein syndrome. The New England Journal of Medicine, 290, 1097-1103.

Wilson,J.D.,Griffin,J.E.,George,F.W. & Leshin,M. (1981) The role of gonadal steroids in sexual differentiation. Recent Progress in Hormone Research, 37, 1-39.

Wrange,O. (1979) A comparison of the glucocorticoid receptor in cytosol from rat liver and hippocampus. Biochimica et Biophysica Acta, 582, 346-357.

Wrange,O. & Gustafsson,J.A. (1978) Separation of the hormone- and

DNA-binding sites of the hepatic glucocorticoid receptor by means of proteolysis. The Journal of Biological Chemistry, 253, 856-865.

Wrange,O.,Okret,S.,Radojcic,M.,Carlstedt-Duke,J. & Gustafsson, J.A. (1984) Characterization of the purified activated glucocorticoid receptor from rat liver cytosol. The Journal of Biological Chemistry, 259, 4534-4541.

Wrongemann,B.,Nickel,A.,Schwartz,A. & Pinsky,L. (1984) The

Wrongemann et al (1984) 56K Polypeptide correlates with androgen binding in genital skin fibroblasts.

Yamamoto,K.R. (1974) Characterization of the 4S and 5S forms of the estradiol receptor protein and their interaction with deoxy-ribonucleic acid. The Journal of Biological Chemistry, 249, 7068-7075.

Yamamoto,K.R. (1985) Steroid receptor regulated transcription of specific genes and gene networks. Annual Review of Genetics, 19, 209-252.

Yamamoto,K.R. & Alberts,B.M. (1976) Steroid receptors: Elements for modulating eukaryotic transcription. Annual Review of Biochemistry, 45, 721-746.

Yamamoto,K.R.,Stampfer,M.R. & Tomkins,G.M. (1974) Receptors from glucocorticoid-sensitive lymphoma cells and two classes of insensitive clones: Physical and DNA-binding properties. Proceedings of the National Academy of Sciences-USA, 71, 3901-3905.

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