Analysis of Androgen-independence in Cell Models of Advanced Prostate Cancer

Ewan Stewart Grant

Thesis submitted for the degree of Doctor of Philosophy
Edinburgh, 1994
ABSTRACT OF THESIS

The expression of the androgen receptor gene in cell models of prostatic epithelia and its relationship with the apoptosis inhibiting oncogene bcl-2 was examined.

The inability of the metastatic prostate cancer cell lines DU145 and PC3 to respond to androgens is well documented. However, the LNCaP cell line displays marked growth stimulation in the presence of the androgen dihydrotestosterone (DHT) at concentrations between 0.001nM and 10.0nM. Paradoxically, LNCaP demonstrate growth stimulation in the presence of the non-steroidal anti-androgen hydroxy-flutamide with significant (p<0.05) effects being observed at concentrations of 1.0nM and 10.0nM 4 days post-administration. Primary cultures of prostatic epithelial cells derived from both benign hyperplastic (BPH) prostates and from prostate cancer (CaP) tissues, in common with DU145 and PC3, do not exhibit growth response to either DHT or to hydroxy-flutamide at any of the concentrations employed.

Northern analysis of total RNA samples, using a 1kb PCR probe complementary to the 3' portion of the androgen receptor (AR) cDNA, demonstrates that of all the cells examined only the LNCaP cell line displays detectable AR gene transcripts. Increasing the sensitivity of AR mRNA detection through the use of reverse transcription-polymerase chain reaction (RT-PCR) enables detection of AR gene expression in LNCaP, PC3 and both BPH-derived and CaP-derived primary epithelial cells. In contrast RT-PCR, coupled with Southern blotting, was unable to identify AR mRNA in the DU145 cell line. This apparent complete down-regulation of AR gene expression cannot be blamed on any gross rearrangements or deletions within the gene since all 8 exons were detectable by PCR. Indeed the only variation in the AR gene between the established cell lines was in the CAG repeat region of exon1, and since in all cells the size of this repeat was observed to fall within what is generally accepted as the normal range, it was deemed irrelevant in terms of AR expression and function.

Transfection with the steroid receptor reporter plasmid pMMTV/SPAP demonstrated that the extent of AR gene expression in the primary epithelia and PC3 is insufficient to furnish these cells with a functionally-significant level of receptor protein. When supplemented with either DHT or hydroxy-flutamide, pMMTV/SPAP-transfected PC3 and primary epithelial cells do not exhibit significant alterations (p>0.05) in the extent of alkaline phosphatase secreted compared with cells transfected with the control vector pRc/CMV. pMMTV/SPAP-transfected
LNCaP cells exhibit an approximately 200% increase in SPAP production compared with control cells when supplemented with 10.0nM DHT. Similar levels of SPAP production are observed following the administration of 10.0nM hydroxy-flutamide to pMMTV/SPAP-transfected LNCaP. Furthermore androgens and anti-androgens are observed to act synergistically to activate AR-driven gene transcription in this cell line. Such unusual co-operation was abolished upon the introduction of normal androgen receptor into LNCaP cells via the expression vector pCMV/ARcom.

The bcl-2 oncprotein has been implicated in the transition of prostatic adenocarcinomas from a state of androgen-dependence to one of androgen-independence during androgen ablation therapy. RT-PCR indicated that the three prostate cancer cell lines and all of the primary epithelial cell cultures examined contain measurable levels of both the bcl-2α and bcl-2β mRNA transcripts. Furthermore, Western blotting using an anti-bcl-2 monoclonal antibody illustrated that of the three cell lines, PC3 express the highest level of bcl-2, with conservative densitometric estimates placing this level of oncprotein at ~2.7 times that observable in the LNCaP model. bcl-2 Expression in primary epithelial cells is equivalent to that in the PC3 cell line.

The possibility of a relationship between AR expression and that of bcl-2 was examined through transfection of PC3 with pCMV/ARcom. Levels of bcl-2 oncprotein are not reduced in AR-positive PC3 cells and indeed DHT administration induces a pronounced increase in oncogene expression. It is therefore unlikely that AR loss and bcl-2 elevation are concomitant events in the acquisition of an androgen-independent phenotype.
DEDICATION

I dedicate this thesis to my parents, Ruth and Stewart Grant.
ACKNOWLEDGEMENTS

Firstly, I would like to thank Dr. Fouad K. Habib for giving me the opportunity to work in his laboratory. His excellent supervision combined with remarkable patience - despite some stern tests - made my time in Edinburgh extremely enjoyable and rewarding. I would also like to extend my gratitude to my co-supervisor, Dr. Ken W. Batchelor, who despite a move from Greenford to Glaxo Inc. in the U.S.A. retained a keen interest in the project and provided valuable input throughout.

I am also extremely grateful to Dr. Paul Kelly for his assistance with the statistics, to Dr. Hillary Critchley for her immaculate immunohistochemistry and to all the clinical staff within the hospital who provided me with prostate tissues.

I have many friends, both in Edinburgh and in London, who I must thank for their moral support and physical support when called upon. In Edinburgh I refer to Margaret, Roth, Alan and Shona, and in Greenford, all the lab boys i.e. Nick, Walis, Steve, Martin etc.. I would also like to thank Rob, Jenny, Nicola, Danni and Charlotte Fenton in Ealing for making me feel so welcome in their home.

Finally, I would like to pay tribute to Dr. Mark Edbrooke who became both mentor and friend without a single thought for his own personal safety and gave me all the right advice at all the right times. I owe him one.
DECLARATION

I, Ewan Stewart Grant, hereby declare, that unless otherwise stated the work embodied in this thesis is the result of my own independent investigation. This is in accordance with rule 3.4.7 of the University of Edinburgh Postgraduate Study Programme.
INTRODUCTION

1.1 The Prostate

1.1.1 Anatomy

1.1.2 Function

1.2 Endocrinology of the Prostate

1.2.1 The Hypothalmo-Pituitary-Gonadal Axis

1.2.1.1 The Hypothalmo-Pituitary Unit

1.2.1.2 The Adrenal-Testis-Prostate Network

1.2.2 Androgens and the Prostate

1.3 Carcinoma of the Prostate

1.3.1 Treatment of Prostatic Carcinoma

1.3.2 Principles of Endocrine Therapy

1.3.3 Effects of Androgen Ablation upon the Benign and Malignant Prostate

1.4 Programmed Cell Death (Apoptosis) in the Prostate

1.5 The Androgen Receptor

1.5.1 The Nuclear Receptor (NR) Superfamily

1.5.2 The Roles of the Functional Domains in the NR Superfamily

1.5.3 Androgen Receptor - Structure and Function

1.5.4 Anti-androgens - flutamide

1.5.5 The Androgen Receptor in the Benign and Malignant Prostate

1.6 bcl-2

1.7 In vitro Models of Prostatic Epithelia

1.7.1 Prostatic Carcinoma Cell Lines

1.7.1.1 The LNCaP Cell Line

1.7.1.2 The DU145 Cell Line

1.7.1.3 The PC3 Cell Line

1.7.2 Primary Prostatic Cell Cultures

1.8 Aims and Objectives

MATERIALS AND METHODS

2.1 Materials

2.1.1 Cell Lines

2.1.2 Cell Culture

2.1.2.1 General Consumables

2.1.2.2 Cell Lines - media/supplements

2.1.2.3 Epithelial and Fibroblast Primary Culture - media/supplements

2.1.2.4 Cell Proliferation

2.1.3 Molecular Biology

2.1.3.1 General Consumables

2.1.3.2 Plasmid Constructs

2.1.3.3 Isolation and Analysis of Nucleic Acids

2.1.3.4 Analysis of Proteins

2.2 Methods

2.2.1 Cell Culture

2.2.1.1 Cell Lines
2.2.1.2 Culture of Epithelial Cells and Fibroblasts from Human Prostatic Tissue................. 52
2.2.1.3 Spectrophotometric Determination of Viable Cell Numbers............................ 54
2.2.1.4 Measurement of the Androgenic Responses of Cultured Cells......................... 56
2.2.2 Extraction and Purification of Plasmid DNA.............................................. 58
  2.2.2.1 Preparation of Competent E.coli using Calcium Chloride......................... 58
  2.2.2.2 Transformation of Competent E.coli.................................................. 58
  2.2.2.3 Large-scale Preparation of Plasmid DNA......................................... 59
2.2.3 Transfection of Plasmid DNA into Prostatic Epithelia.................................. 62
  2.2.3.1 Transfection of pMMTV/SPAP into LNCaP, DU145, PC3 and Primary Epithelial Cells............. 63
  2.2.3.2 Co-transfection of pCMV/ARcom and pMMTV/SPAP into LNCaP and PC3 Cells.......... 64
  2.2.3.3 Assay of Secreted Placental Alkaline Phosphatase (SPAP)..................... 64
  2.2.3.4 bcl-2 Expression in LNCaP and PC3 Cells Transfected with pCMV/ARcom.......... 65
2.2.4 Isolation of Nucleic Acids............................................................................ 65
  2.2.4.1 Isolation of Total RNA from Cultured Cells......................................... 65
  2.2.4.2 Isolation of Total RNA from Snap-frozen Tissue.................................. 67
  2.2.4.3 Isolation of Genomic DNA from Cultured Cells.................................... 67
2.2.5 Analysis of Nucleic Acids.............................................................................. 68
  2.2.5.1 Reverse Transcription of Total RNA.................................................... 68
  2.2.5.2 Analysis of Genomic DNA and cDNA by the Polymerase Chain Reaction (PCR)... 69
  2.2.5.3 Northern Analysis.................................................................................. 77
  2.2.5.4 Southern Analysis................................................................................... 80
  2.2.5.5 Preparation of Radio-labelled Probes.................................................... 80
  2.2.5.6 Hybridisation of Radio-labelled Probes to Nucleic Acids Immobilised on Nitrocellulose Filters................. 84
2.2.6 Analysis of Proteins by Western Blotting....................................................... 86
  2.2.6.1 Isolation of Proteins from Cultured Cells............................................. 86
  2.2.6.2 Isolation of Proteins from Frozen Tissue.............................................. 87
  2.2.6.3 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Proteins............... 88
  2.2.6.4 Transfer of Proteins from SDS-Polyacrylamide Gels to Nitrocellulose........... 89
  2.2.6.5 Immuno-detection of the bcl-2 Oncoprotein Immobilised on Nitrocellulose...... 90
2.2.7 Immunohistochemical Analysis of Androgen Receptor Expression in Prostatic and Endometrial Tissues................. 91
2.2.8 Statistical Analysis......................................................................................... 91

RESULTS......................................................................................................................... 93
  3.1 The Effects of Dihydrotestosterone (DHT) and Hydroxyflutamide (HO-F) on the Growth of Cultured Prostate Cancer Cell Lines and Primary Epithelial Cell Cultures.................. 94
  3.1.1 Growth Response of the LNCaP, DU145 and PC3 Cell Lines to DHT and HO-F....... 95
    3.1.1.1 LNCaP............................................................................................. 95
    3.1.1.2 DU145............................................................................................ 95
    3.1.1.3 PC3............................................................................................... 98
  3.1.2 Growth Response of Primary Epithelial Cells to DHT and HO-F................. 98
  3.2 Androgen Receptor (AR) Expression in Cultured Prostate Cancer
Cell Lines and Primary Epithelial Cells - Analysis by Northern Blotting. 98
3.2.1 Synthesis and Analysis of 1.1ARp. 103

3.2.2 Detection of Androgen Receptor mRNA in Prostatic and Endometrial Tissues by Northern Blotting. 103
3.2.3 AR Gene Expression in Human Prostatic Carcinoma Cell Lines. 109
3.2.4 AR Gene Expression in Human Primary Epithelial Cell Cultures. 112

3.3 Androgen Receptor (AR) Expression in Cultured Prostate Cancer Cell Lines and Primary Epithelial Cells - Analysis by Reverse Transcription-Polymerase Chain Reaction (RT-PCR). 112
3.3.1 Analysis of Androgen Receptor-positive and -negative Tissues for Androgen Receptor mRNA. 116
3.3.2 AR Gene Expression in Human Prostatic Carcinoma Cell Lines. 118
3.3.3 AR Gene Expression in Human Primary Epithelial Cells. 121

3.4 Androgen Receptor (AR) Activity in Cultured Prostate Cancer Cell Lines and Primary Epithelial Cells. 121
3.4.1 Detection of AR Function in the LNCaP, DU145 and PC3 Cell Lines. 124
3.4.2 Co-transfection of the LNCaP and PC3 Cell Lines with pMMTV/SPAP and pCMV/ARcom. 127
3.4.3 Detection of AR Function in Primary Epithelial Cells. 129
3.4.4 Summary of the pMMTV/SPAP Transfection Studies. 131

3.5 Analysis of the Androgen Receptor Gene in the LNCaP, DU145 and PC3 Cell Lines. 131

3.6 Overview of the Androgen Receptor Studies. 135

3.7 bcl-2 Expression in Cultured Prostate Cancer Cell Lines and Primary Epithelial Cells. 136
3.7.1 Detection of bcl-2 mRNA in LNCaP, DU145, PC3 and Primary Epithelial Cells by Reverse Transcription-Polymerase Chain Reaction (RT-PCR). 136
3.7.2 Detection of the bcl-2 Oncoprotein in LNCaP, DU145, PC3 and Primary Epithelial Cells by Western Blotting. 138
3.7.3 bcl-2 Expression in pCMV/ARcom-transfected PC3 Cells. 141

3.8 Summary of Results. 141

DISCUSSION. 146
4.1 In vitro Responses to Androgens and Anti-androgens. 147
4.2 Androgen Receptor Expression and Function in Prostatic Epithelial Cells. 148
4.3 bcl-2 Expression in vitro. 152
4.4 Implications for Primary Epithelial Culture of the Prostate. 154
4.5 Hormone Escape in vitro and in vivo. 157
4.6 Future Studies. 161

REFERENCES CITED. 165
# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AR</td>
<td>Androgen Receptor</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BPH</td>
<td>Benign Prostatic Hyperplasia</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CaP</td>
<td>Carcinoma of the Prostate</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3'-Diaminobenzidine Tetrahydrochloride</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethyl Pyrocarbonate</td>
</tr>
<tr>
<td>DHT</td>
<td>Dihydrotestosterone</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>FCM</td>
<td>Fibroblast Conditioned Medium</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
</tr>
<tr>
<td>HGPRT</td>
<td>Hypoxanthine-Guanine Phosphoribosyl Transferase</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like Growth Factor</td>
</tr>
<tr>
<td>kDa</td>
<td>kiloDaltons</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal Antibody</td>
</tr>
<tr>
<td>ml</td>
<td>millilitre</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>mwt</td>
<td>molecular weight</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PNPP</td>
<td>p-nitrophenyl phosphate</td>
</tr>
<tr>
<td>RNase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse Transcription</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulphate</td>
</tr>
<tr>
<td>SPAP</td>
<td>Secreted Placental Alkaline Phosphatase</td>
</tr>
<tr>
<td>T</td>
<td>Testosterone</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-Borate-EDTA</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

Carcinoma of the prostate (CaP) is currently the most commonly diagnosed cancer in men in the U.S.A., excluding skin cancer, and is the second leading cause of cancer death. Data compiled by the American Cancer Society in 1992 predicts that approximately 1 of every 11 men will develop CaP during their lifetime and that 25.7% of this population will die as a consequence (Coffey, 1992). Translated, this data indicates that the United States possesses an age-adjusted death rate for CaP per 100,000 population of 15.7. World-wide the death rate ranges from 22.0 per 100,000 population in Switzerland to 0.5 per 100,000 population in Korea. If one considers, that of all cancers, the prevalence of CaP increases the most rapidly with age, such figures must surely increase, with continuing shifts in the world-wide demographic patterns toward an older-aged population. Indeed, in the United States it has been estimated, that during the years from 1985 to 2000, there will be a 37% increase in the number of prostate cancer deaths per year and a 90% increase in CaP cases diagnosed.

1.1 The Prostate

The prostate is a fibromuscular, glandular organ that surrounds the prostatic urethra, and is located between the neck of the bladder superiorly, and the urogenital diaphragm inferiorly. It is composed of acinar units and ducts, lined with epithelial cells, arranged within fibromuscular connective tissue and enclosed within a dense, fibrous capsule. The entire organ is sheathed in a heavily vascularised cover derived from pelvic fascia. It functions to secrete a multifaceted alkaline liquid into the seminal fluid at the time of ejaculation, in order to optimise the environment of the spermatozoa for their potentially hazardous journey to the vagina. The two major diseases of the prostate are benign prostatic hyperplasia (BPH) and
carcinoma (CaP), the former characterised by enlargement of the gland leading to the obstruction of urethral outflow.

1.1.1 Anatomy

McNeal's model of the prostate demonstrates four distinct zones that differ markedly in their tissue composition and highly significant differences in their susceptibility to the different pathologic conditions (Figure 1.01a.) (McNeal, 1981). It should be noted, that despite obvious histological differences, these various zones do not represent discrete compartments.

The peripheral zone constitutes almost 75% of the glandular tissue of the adult prostate and forms the postero-inferior portion of the prostate. It possesses long, branched glands, whose ducts curve posteriorly to open mainly into the prostatic sinuses, although a number open on the lateral urethral walls. In a study of 104 prostate glands obtained at radical prostatectomy for adenocarcinoma, of 88 cancers whose probable zone of origin could be identified, 68% were seen to arise in the peripheral zone (McNeal et al., 1988).

Those ducts that branch proximally into the mesenchyme surrounding and following the ejaculatory ducts are referred to as the central zone. Their orifices arise on the convexity of the verumontanum immediately surrounding the ejaculatory ducts. This region accounts for approximately 25% of the prostatic volume and presents with a mere 8% of adenocarcinomas.

In addition to the dramatic anatomic delineation that exists between the peripheral and central zones, striking histologic differences are observed between their glandular tissues which might suggest important differences in biological function. Such dissimilarities might explain why carcinoma commonly arises in the peripheral zone, while the central zone exhibits a degree of resistance to tumorigenesis. The acinar tissue of the central zone consists of large, irregularly shaped spaces into which numerous septa project. In contrast, the peripheral zone possesses long narrow ducts that branch into small, regular acini that are round in appearance and exhibit smooth non-septate walls. The epithelial cells resident within the glandular tissues of the peripheral zones are columnar in appearance, with readily distinguishable cell membranes and basally located small, dark nuclei. In contrast, the cells of the central zone exhibit variable lengths and appear more opaque, with a granular cytoplasm and less well-defined borders. They exist at a significantly higher cell density than the cells of the peripheral zone and possess large nuclei displaced to variable levels from the basement membrane.
Figure 1.01  

a. The anatomic zones of the prostate, showing the central, transitional, peripheral and anterior fibromuscular zones in sagittal section. The position of the verumontanum is also indicated. (adapted from Algaba, 1992).

b. The normal architecture of prostatic ducts. (taken from Tenniswood, 1993)
The transitional zone constitutes a small group of ducts, comprising a mere 5-10% of the total prostatic volume, arising at the junction point of the proximal and distal urethral segments. It exhibits two lobes, positioned on each side of the urethra, in the angle between the peripheral and central zones, with ducts opening onto the lateral urethral wall. Compared with that of the other main glandular regions of the prostate, the ductal branching of the transitional zone is observed to be considerably simpler, which may underlie the inability to assign any functional significance to this region. It does, nevertheless, have great relevance in adult pathology, presenting with 24% of prostatic adenocarcinomas and in combination with the other peri-urethral glands, being the exclusive site of origin of benign prostatic hyperplasia.

The fourth main anatomic region of the adult prostate is entirely fibromuscular, with no glandular structures. The anterior fibromuscular zone constitutes approximately 1/3 of the total prostatic volume, and forms the entire anterior surface of the prostate. The full significance of this region in prostate function and pathology remains to be ascertained.

Prostatic acini drain via the ductal systems into the prostatic urethra. Microdissection of the acinar units indicates the presence of three separate components - the proximal region (secretory), the distal region (non-secretory) and the proliferative distal tip (Tenniswood, 1993)(Figure 1.01b.). The glands are lined by tall, columnar epithelia separated from the basement membrane by a layer of basal or reserve cells. The ducts are lined by more cuboidal cells again with a basal cell layer. The basement membrane isolates the epithelia from the prostatic stromal tissue which is composed of fibroblasts, smooth muscle cells, endothelial cells, nerve cells and assorted infiltrating cells including mast cells and lymphocytes. Clearly, the proximity of the stromal compartment allows alteration of the epithelial micro-environment and it is evident that the basal and glandular cells receive growth factors, trophic nerve factors and androgens that have traversed the basement membrane.

1.1.2 Function.

At the moment of ejaculation, sperm which entered the prostate via the vas deferens and through the seminal vesicles into the central zone, are expelled into the urethra at the verumontanum. At this point the sperm is exposed to the combined secretions of the seminal vesicles and central zone. They continue along
the distal prostatic urethra, where they are combined with the secretions of the peripheral zone, prior to moving through the phallic urethra and into the vagina. The secretions of the central and peripheral zones, which represent approximately 15-30% of ejaculate volume (Mann and Lutwak-Mann, 1981) contain a multitude of both protein and non-protein components, many of which, have as yet to be assigned an irrefutable function.

The epithelia of the normal adult prostate produce a fluid of approximate pH 7.28, that is rich in electrolytes, notably zinc believed to be involved in the stabilisation of sperm chromatin. Other non-protein constituents, include citric acid, cholesterol and the polyamines; spermine, spermidine and putrescine. The protein components of the prostatic secretions are many and varied. Growth factors (e.g. epidermal growth factor), immunoglobulins, transferrin, prostatic acid phosphatase (PAP) and the exclusively prostatic in origin, prostate specific antigen (PSA) are all detectable. The prostate also express very high levels of annexins 1, 4 and 5 (Haigler and Christmas, 1990). Some of the more interesting facets of prostatic function arising from contemporary research will be discussed.

The annexins, of which there are 8 different forms in mammalian cells (Crumpton and Dedman, 1990), are a family of structurally related proteins that are known to bind certain intracellular phospholipids in a Ca²⁺-dependent manner. It has been proposed that annexins also have an extracellular site of action, due in part to their detection in peritoneal exudates. However cDNA sequence analysis would seem to indicate the lack of a hydrophobic signal sequence directing secretion (Pepinsky et al, 1988), and in addition, the N-termini of mammalian annexin proteins are observed to be blocked by acetylation. These observations are consistent with those of other intrinsically intracellular proteins.

Haigler and Christmas (1990, 1991) have demonstrated the presence of annexins 1 and 5 in seminal plasma. Analysis by immunoblotting demonstrated that annexin 5 and annexins 1 & 4 locate exclusively to seminal vesicle tissue and prostatic epithelia respectively. Thus, it would appear that although annexin 1 and annexin 4 both localise to the ductal epithelia of the prostate, only the former is secreted as part of the prostatic fluid.

The existence of annexins 1 and 5 in the seminal plasma, indicates that proteins without recognised secretory signalling can function extracellularly. Furthermore, the secretion of annexin 1 by prostatic epithelia suggests the presence of a selective pathway for its release from the cell, and potentially, a specific requirement for this protein within the prostatic exudate. As yet, roles for
annexins 1 and 5 in the seminal plasma have not been suggested, but the high concentration of calcium within the prostatic fluid might indicate that these proteins will, at least, be functional within the semen.

Spermine, spermidine and putrescine have been postulated to induce the formation of the seminal clot, following ejaculation, catalysed, potentially, by transglutaminase. The clot, principally composed of a polymerised gel formed from seminogelin and fibronectin, is broken down 5-20 minutes post-ejaculation through the activity of PSA, secreted into the prostatic component of the seminal plasma.

PSA is a 34kDalton glycoprotein, functioning as a kallikrein-type serine protease, that is expressed specifically by prostatic tissue. The concentrations of PSA in blood serum are commonly used as diagnostic and prognostic indicators in prostate cancer. Serum PSA levels are known to be proportional to the volume of intra- and extra-capsular prostate cancer (Stamey et al, 1987 & 1989) and are also used to follow prostate cancer patients after radical prostatectomy, radiotherapy and anti-androgen therapy.

Lee et al (1989) analysed the composition of the seminal clot and the involvement of PSA in its disruption. Using 2D gel electrophoresis a group of proteins, ranging from 28kD to 68kD, designated seminal vesicle-specific antigen has been identified in both seminal vesicle fluid and freshly ejaculated semen. The addition of PSA or prostatic fluid to seminal vesicle fluid completely removes seminal vesicle-specific antigen from the 2D profile. It is proposed that the structural component of the seminal coagulum is seminal vesicle-specific antigen, and that the digestion of this complex by PSA leads to liquefaction of the clot.

The primary function of the seminal plasma is to sustain the spermatozoa and to maximise the chances of fertilisation. To achieve this, the immune system of the female reproductive tract must be impeded.

The levels of antibodies in women directed against sperm is low (Bronson et al, 1984), due, in no small part, to the presence of powerful immunosuppressive agents arising within human seminal plasma. Among these agents are the prostaglandins; PGE₁, PGE₂, 19-hydroxy PGE₁ and 19-hydroxy PGE₂ (Templeton et al, 1978). In addition, Kelly et al (1991) have demonstrated the immunosuppressive capacity of microparticulate prostasomes derived from seminal plasma, and suggest that they cooperate with the prostaglandins in this function.
Prostasomes are submicrometre organelles that locate to membrane-bound storage vesicles within acinar epithelial cells of the prostate. They are released into the glandular lumen, either by translocation of the storage vesicle across the plasma membrane, or exocytically, through fusion of the storage vesicle membrane and plasma membrane (Ronquist and Brody, 1985). Ronquist and Brody (1982) were the first to isolate prostasomes from seminal plasma using a combination of ultracentrifugation and chromatography, and proposed that they function to promote motility of spermatozoa.

Kelly and colleagues (1991) have demonstrated the inhibition of phytohaemagglutinin (PHA)-induced proliferation of human T cells and the arrest of phagocytic activity in a mouse macrophage cell line by pure preparations of prostasomes. The mechanism of the observed immunosuppression is not fully understood, but it has been suggested that it arises from phagocytosis of the particles themselves. It could be envisaged that prostasomes, which are observed to associate with spermatozoa (Ronquist et al, 1990), are preferentially phagocytosed, and subsequently release inhibitory factors within the macrophage. In this way, a degree of protection against macrophage action can be offered to the spermatozoa.

1.2 The Endocrinology of the Prostate.

A number of steroid and peptide hormones exert influences upon the prostate. Figures 1.02 and 1.03 summarise the hormonal network that exists between the endocrine organs and the prostate. Androgens e.g. testosterone and dihydrotestosterone play a key role in the growth and development of the prostate from embryogenesis to puberty and on through adulthood. The synthesis and release of androgens from both the testis and adrenal cortex relies upon the input of the gonadotrophins and adrenocorticotropic hormone respectively, the production of which, within the pituitary, is regulated by hypothalamic releasing hormones. In addition, under the control of the hypothalamus, the pituitary releases prolactin, a relative of human growth hormone, which acts upon the prostate in an as yet undefined manner.

1.2.1 The Hypothalamo-Pituitary-Gonadal Axis.

In response to neural inputs, the median eminence of the hypothalamus liberates a number of factors that exert both stimulatory and inhibitory influences
upon the cells of the anterior pituitary. In response the anterior pituitary produces and secretes tropic hormones that target the adrenal cortex and the Leydig cells of the testis with consequent liberation of androgens and other steroid hormones into the bloodstream.

1.2.1.1 The Hypothalamo-Pituitary Unit (Figure 1.02).

Peptide hormones and neurotransmitters are secreted into the median eminence via peptidergic and bioaminergic tuberohypophyseal neurones respectively. These factors enter the bloodstream via capillary beds within the median eminence, and are subsequently delivered to the anterior pituitary (adenohypophysis) along short and long portal vessels. Hypothalamic products reaching the anterior pituitary in this way include; dopamine, $\gamma$-aminobutyric acid (GABA), vasopressin and the releasing hormones, luteinising hormone-releasing hormone (LHRH), corticotropin-releasing hormone (CRH), growth hormone-releasing hormone (GHRH) and thyrotropin-releasing hormone (TRH). In terms of the activity of the prostate and its dependence on androgenic input, the most significant of the releasing hormones is LHRH.

LHRH, released by the hypothalamus in a pulsatile fashion, binds to specific cell surface receptors on gonadotrope cells within the anterior pituitary, inducing 2nd messenger responses which ultimately result in the release of the trophic hormones; luteinising hormone (LH) and follicle-stimulating hormone (FSH). Similarly, CRH in combination with vasopressin etc. stimulates the release of adrenocorticotropic hormone (ACTH) and an ill-defined peptide from corticotrope cells which target the adrenal cortex.

In contrast to the gonadotrophins, corticotrophins and growth hormone the hypothalamic secretions predominantly inhibit the secretion of prolactin. Dopamine and GABA are mainly responsible for this suppression and are consequently referred to as prolactin inhibiting factors (PIF’s). However it has been proposed that several factors of hypothalamic origin can act as prolactin releasing factors (PRF’s). These include; TRH, vasopressin and oxytocin.

1.2.1.2 The Adrenal-Testis-Prostate Network (Figure 1.03).

The factors liberated by the pituitary gland exert their influence upon the prostate in both direct and indirect fashions (Figure 1.03).

LH, FSH and ACTH stimulate the release of androgenic steroids from the Leydig cells of the testis and adrenal cortex which subsequently target the prostate
Figure 1.02 The Hypothalamo-Pituitary Axis - Factors Relevant to Prostatic Metabolism.

Abbreviations: Prolactin Releasing Factors (PRF’s) e.g. thyrotropin releasing hormone, vasopressin, oxytocin, peptide-histidine-isoleucine-27 and neurointermediate lobe factor.
gland. In contrast to this characteristically indirect action of the trophic hormones, growth hormone (GH) is believed to act directly upon the prostate, owing to the detection of endogenous GH in BPH and CaP (El Etreby and Mahrous, 1979) and the identification of cell-surface GH receptors in prostatic tissue (Sibley et al, 1984; Prieto and Carmena, 1987). The exact function of GH in the prostate remains to be elucidated. However, Reiter et al (1992) have demonstrated that the responses of the rat prostate to GH are analogous to those observed with prolactin. These responses are manifest as up-regulation of AR and insulin-like growth factor-I (IGF-I) and IGF-I receptor.

Prolactin, in the context of the prostate, is perhaps the most intriguing of the pituitary factors in that it elicits both direct and indirect effects upon prostatic metabolism. Direct action is mediated by a specific cell surface receptor - cloned in the late 1980's by Boutin et al (1988 & 1989) - with the indirect effects being manifest as an elevation of testosterone synthesis in the testis (Hafiez et al, 1972).

Human prostate membranes are rich in receptors for prolactin (Leake et al, 1983). More recently prolactin receptor mRNA transcripts have been visualised in epithelial cells by in situ hybridisation techniques (Ouhtit et al, 1993). It has long been known that prolactin, via this cell surface receptor, dramatically increases the uptake of testosterone by prostatic tissue (Jacobi et al, 1978; Farnsworth, 1981). In addition to these observations, it has been demonstrated that there is a strong correlation between plasma prolactin and androgen receptor (AR) expression in patients suffering from BPH (Odoma et al, 1985). Reiter et al (1992) have shown such upregulation of AR protein in hypophysectomised rats in response to prolactin and determined that it correlates well with increases in AR mRNA. It would appear, in the face of such evidence, that prolactin has some direct role in the regulation of AR expression in vivo. Outwith the androgenic network, prolactin has, in addition, been observed to have upregulating influences upon the production of both citrate and mitochondrial aspartate aminotransferase by prostatic epithelia, independently of androgenic inputs (Franklin et al, 1990 & 1992).

Oestrogens too have been proposed to have both direct and indirect effects upon the prostate. However if one examines Figure 1.03 closely there is a question mark included alongside the input of oestrogen to the prostate. The reason for this being that the role of oestrogen within the human gland is very unclear despite a certain amount of evidence in animals. Oestrogens derived from dietary sources or from the action of the enzyme aromatase - a protein structurally typical of a p450 enzyme (Corbin et al, 1988) - has been postulated to have a role in the pathogenesis of BPH, synergising with DHT to enhance prostatic growth.
Figure 1.03 Endocrine Inputs to the Prostate.

Abbreviations: 5α-Reductase (5αR); Dihydrotestosterone (DHT).
in the dog (Walsh and Wilson, 1976; Aumuller et al, 1982). Such synergy is believed to result from upregulation of the androgen receptor (Moore et al, 1979) resulting in prostatic epithelial hyperplasia. There is, however, little evidence to suggest that these effects occur in the human prostate where stromal hyperplasia predominates. Indeed, the hyperplastic human prostate expresses significantly less oestrogen receptor than does the normal prostate (Robel et al, 1985).

If direct action within the prostate is debatable, there is little doubt concerning the indirect effects of oestrogen on the human prostate. Figure 1.03 demonstrates that both testosterone, released by the testis and derived from the metabolism of adrenal androgens, and oestrogenic compounds act upon the pituitary and hypothalamus to inhibit the synthesis and secretion of the trophic and releasing hormones with concomitant down-regulation of androgen synthesis. Such oestrogen-induced negative feedback is hugely significant in the context of the treatment of androgen-dependent prostatic neoplasms.

1.2.2 Androgens and the Prostate.

As long ago as 1792, John Hunter established the influence of testicular secretions on the growth of the prostate. Subsequent to this, following the isolation of testosterone by David et al in the mid 1930's, the further involvement of testicular testosterone in the progression of prostatic carcinoma was demonstrated by the trailblazing work of Huggins and Hodges (1941). These pioneering observations have spawned a profusion of treatment protocols based upon androgen ablation.

Adrenal androgens have also been demonstrated to have an effect on prostatic metabolism. Davidson and Moon (1936) observed growth of the rat ventral prostate following treatment of castrated rats with ACTH. However it was subsequently shown that removal of the adrenal has no significant effect on the prostate in non-castrated individuals (Mobbs et al, 1973) and indeed adrenalectomy merely offers palliation in the treatment of CaP following castration (Huggins, 1945).

The major sites of androgen biosynthesis are the testis and adrenal cortex, the latter accounting for approximately 10% of the 3-10mgml⁻¹ of plasma testosterone in the adult male (Williams, 1992). The synthesis of androgens in the testis and adrenal cortex, illustrated in Figure 1.04, is based upon the conversion of cholesterol and occurs through a series of enzymatic pathways (Ghanadian,
Figure 1.04 Pathways of Androgen Synthesis. (Adapted from Ghanadian, 1982).

a. Testis.

Cholesterol

\[ \rightarrow \]

Pregnenolone \[ \rightarrow \] Progesterone

17\(\alpha\)-hydroxypregnenolone \[ \rightarrow \] 17\(\alpha\)-hydroxyprogesterone

Dehydroepiandrosterone \[ \rightarrow \] Androstenedione

\(\Delta 5\alpha\)-androstenediol \[ \rightarrow \] Testosterone

b. Adrenal.

Cholesterol \[ \rightarrow \] Pregnenolone \[ \rightarrow \] 17\(\alpha\)-hydroxypregnenolone

Progesterone \[ \rightarrow \] Androstenedione \[ \rightarrow \] Dehydroepiandrosterone

17\(\alpha\)-hydroxyprogesterone

Dehydroepiandrosterone Sulphate
1982). Stimulation of the Leydig cells of the testis with LH initiates the conversion of cholesterol to testosterone via pregnenolone, progesterone, hydroxy-pregnenolone, hydroxyprogesterone, dehydroepiandrosterone (DHEA), androstenedione and androstenediol (Figure 1.04a.). The testes also secrete low levels of DHT. The major androgenic products of the adrenals are DHEA and its sulphated conjugate DHEAS (Figure 1.04b.). The adrenal cortex is also the main source of circulating androstenedione. DHEA and DHEAS influence prostatic growth through conversion to androstenedione and subsequently testosterone in peripheral tissue e.g. adipose tissue, the adrenals and the prostate itself.

98% of plasma testosterone and DHT are bound to plasma proteins (Vermeulen, 1977; Dunn et al, 1981). The literature suggests that there is variability in the percentage testosterone bound by each protein. Vermeulen (1977) reports that the binding distribution of testosterone per protein is as follows: testosterone binding globulin, TeBG, (56%); albumin (40%); transcortin or corticosteroid binding globulin, CBG, (2%). Free testosterone, therefore, represents only 2% of circulating androgen. For many years it was this free testosterone fraction that was regarded as the biologically active portion (Vermeulen, 1977), however, it became clear during the 1980's that the active fraction is larger than the free fraction owing to the dissociation of protein-bound testosterone within capillary beds (Pardridge and Landaw, 1985). Subsequently, Pardridge et al (1986) demonstrated in vivo, that almost all albumin-bound testosterone was available for tissue uptake and that consequently the levels of bioavailable testosterone was equivalent to the sum of free plus albumin-bound i.e. ~50% of the total circulating hormone. The levels of testosterone available to the prostate may well be higher owing to the presence of specific membrane TeBG receptors (Hryb et al, 1989).

Testosterone released into the prostate is converted to DHT through the activity of the enzyme 5α-reductase (Δ4-3-ketosteroid-5α-reductase), an enzyme believed to locate exclusively to the nucleus (Houston et al, 1985) and of which there are 2 known isoforms (Habib, 1994). It is DHT that is thought to be the biologically active androgen within the prostate and indeed the importance of DHT in prostatic development is borne out by the lack of a functional prostate in individuals with 5α-reductase deficiency syndrome (Imperato-McGinley et al, 1992).

The activity of 5α-reductase does not represent the end-point of androgen metabolism within the prostate since DHT is subsequently further metabolised to androstanediol or to the 5α-androstanediols, as outlined in Figure 1.05. These enzyme-catalysed reactions are reversible but it is clear that the diols in the normal prostate are significantly in excess of DHT (Geller et al, 1976). Isaacs et al (1983)
Figure 1.05 Androgen Metabolism in the Prostate Gland. (Adapted from Habib, 1990)
showed the importance of the DHT metabolising enzymes in prostatic pathogenesis by demonstrating that the elevation of intra-prostatic DHT associated with BPH (Geller et al, 1976; Habib et al, 1976) - a finding refuted by Walsh et al (1983) who did not observe any difference between the T/DHT ratios in normal and BPH tissue - arises not only from an increase in 5α-reductase activity (Bruchovsky et al, 1979) but also from a reduction of 3β-hydroxysteroid oxidoreductase and 17β-hydroxysteroid dehydrogenase (Isaacs et al, 1983).

The metabolism of androgens within the prostate is obviously well understood, nevertheless, the question still remains as to how the intraprostatic androgen pools control the growth, development and function of the prostate gland. A strongly held opinion is that DHT and, to a lesser extent, testosterone act mitogenically within the adult prostate, regulating the expression of growth factors, growth factor receptors and other prostatic proteins. And indeed, there is some evidence to support this theory.

A number of groups have demonstrated upregulation of the epidermal growth factor receptor (EGFr) in response to androgenic stimuli (Schuurmans et al, 1988; Wilding et al, 1989). Wilding et al (1989) also established that the production of TGFα is increased by androgens in the hormone-sensitive prostate cancer cell line LNCaP, and postulated that concomitant increases in EGFr may arise from such elevation of TGFα or indeed EGF, as is observed in other systems (Kudlow et al, 1986). It is worth noting however, that such up-regulation of the EGF receptor with androgen stimulation has not been observed by other groups using androgen-sensitive human prostate cell lines (MacDonald et al, 1992), and furthermore, in the rat, EGF receptor is markedly reduced in response to androgens (Traish et al, 1987). Clearly, the relationships between androgens, EGF and its receptor is a complex, perhaps even species-specific, one and requires further investigation.

Fibroblast Growth Factor (FGF) in its basic form is known to stimulate the growth of both prostatic fibroblasts and epithelial cells (Story et al, 1989; Zuck et al, 1992). Androgens administered to cultured LNCaP cells induce an upregulation of bFGF acting in a paracrine fashion to enhance the growth of a FGF-dependent cell line (Zuck et al, 1992). Clearly androgens promote the production of autocrine and paracrine acting growth factors leading to enhanced prostatic cell growth.

In addition to these influences upon growth factor networks, androgens also govern the expression of the epithelially produced proteins PSA and prostate-specific membrane antigen (PSM), the latter, recently cloned by Israeli et al (1993), displaying negative responses to DHT and testosterone (Israeli et al, 1994). In LNCaP, PSA is upregulated by androgens at both the RNA and protein levels.
(Young et al, 1991; Montgomery et al, 1992). Interestingly, the PSA gene has in its 5' region a putative androgen receptor-binding sequence or androgen response element (ARE) which is believed to confer androgen-responsive gene transcription by granting direct interaction of liganded androgen receptor (Riegman et al, 1991; Young et al, 1991; Murtha et al, 1993).

1.3 Carcinoma of the Prostate.

According to the Scottish Health Statistics (1993) supplied by the National Health Service in Scotland, the number of deaths resulting from carcinoma of the prostate (CaP) in 1992 in all age groups in Scotland was 693. This equates to a death-rate per 100,000 population of 28 and demonstrates an approximately 100% increase in CaP related mortality since 1975. Indeed, CaP is the second most prevalent malignancy in males in Scotland with only lung cancer exhibiting a higher incidence. Prostatic disease is relatively uncommon in young males but there is a dramatic increase in incidence with age. The Scottish Health Statistics bear out the age-relatedness of CaP with only one case being diagnosed in individuals below 45. However, between ages 65 and 84 years old there is a dramatic increase in CaP sufferers. Consequently, and as previously eluded to, shifts to an older-aged population in Scotland, will necessarily involve rapid increases in CaP-related death.

The vast majority of malignant prostatic tumours are adenocarcinomas arising from acinar epithelial cells within the peripheral zone of the prostate. Ductal epithelia give rise to ~10% of carcinoma (adenocarcinoma and transitional cell carcinoma)(Busuttil, 1990). Other malignancies observed include non-epithelial carcinosarcomas and sarcomas (Rhabdomyosarcoma and leiomyosarcoma) derived from the fibromuscular stroma, both of which occur very rarely.

There is wide variation in the clinical aggressiveness of prostate tumours and it is evident that the behaviour of the neoplasm relates to stage and grade at diagnosis. The generally accepted staging protocol is the TNM system proposed by the UICC (1978) which can be summarised as follows : T0 - no palpable tumour; T1 - incidental tumour; T2 - organ confined tumour; T3 + T4 - locally invasive tumour; N0-3 - regional lymph node metastasis; M0-1 - distant metastasis. Several systems for the classification of histological grade have been recognised notably those devised by Mostofi (1975) and Gleason (1966). Gleason's grading technique, which assesses the pattern of tumour growth as the relative state of differentiation based
on its ability to form glandlike structures, scores the tumour from 1-5 as it becomes less differentiated. As long ago as 1963, Vickery and Kerr observed that well differentiated tumours have a better prognosis than tumours of poor differentiation which exhibit a rapid course. Gleason’s system results in a histological score that correlates well with patient survival.

Prostatic carcinoma presents with an inordinately high incidence of latent or dormant cancer, which will, in all probability, never become clinically manifest (Franks, 1954). Approximately 10% of all men 50-59 years old and 50% of those 70-80 years old have histological cancers in their prostates, however, the majority of these histologically localised cancers will remain sub-clinical and never require treatment (Issacs, 1991). The greater excess of CaP patients already present with the symptoms of metastasis at the time of diagnosis (~2/3) and this relates to a 5 year survival rate of ~50% in Scotland. In view of this, there is a strong case for aggressive screening of men as they age such that tumours can be diagnosed at a curable phase of their natural history. However, with such a high prevalence of incidental prostatic cancers there is an obvious requirement for a consistent predictor of which localised tumours will progress and therefore require treatment. Such a prognostic indicator is as yet unavailable.

1.3.1 Treatment of Prostatic Carcinoma.

Radical prostatectomy and radiotherapy are commonly employed in the treatment of organ-confined prostate tumours. Lepor et al (1989) demonstrated a 15 year disease-specific survival rate of 86% following radical prostatectomy with a mere 14% of patients dying of metastatic tumour in the first 15 years post-operatively. The prognosis of the disease following radiotherapy is poorer with a 15 year survival rates of 66% observed after combined external beam and interstitial radiotherapy (Lerner et al, 1988). However, as previously discussed, the majority of CaP patients present with symptoms of metastatic disease and hence the above treatments are inappropriate. These patients consequently undergo androgen ablation therapy.

The concept of hormonal therapy for prostatic cancer was introduced in 1941 by Huggins and Hodges who reasoned that since normal prostatic epithelium is androgen-dependent, prostate tumours should be similarly dependent. They, and other groups subsequently, established that human prostate cancer could be induced to regress through the administration of pharmacological doses of
oestrogen or by castration (Huggins et al, 1941; Spirnak and Resnick, 1983). Regression of tumours was associated with impressive palliative effects in patients with symptoms, and it was assumed that this excellent palliation was also concomitant with increased survival. However, the Veterans Administration Cooperative Urological Research Group (VACURG)(1967) failed to establish any survival benefit in terms of pain reduction, urethral obstruction and neurological symptoms. In spite of these obviously disappointing results, hormonal therapy continues to play an important role in the treatment of advanced prostatic carcinoma.

1.3.2 Principles of Endocrine Therapy.

All types of endocrine management of prostate cancer utilise mechanisms that are related either to hypothalamic-pituitary-testicular feedback mechanisms or to the ability of anti-androgens to counteract the effects of androgens on prostatic epithelia.

Surgical castration (orchiectomy) can probably be considered the standard treatment for prostatic carcinoma. Ghanadian et al (1979) demonstrated a reduction of serum testosterone from 645ng/100ml to 11ng/100ml following primary orchiectomy. These findings are very much in keeping with those of other groups. In general, such dramatic reductions in circulating testosterone are observed within 24 hours post-operatively and prompt significant diminution of prostatic size and palpable tumour mass within 3 months, in almost 100% of cases (Carpentier et al, 1986).

Interference with hypothalmo-pituitary feedback mechanisms offers a less radical approach for the suppression of circulating androgens. As previously discussed, LHRH secretion is dependent on negative feedback with androgenic and oestrogenic hormones. Consequently, the commonest and certainly the oldest method of quenching LHRH secretion by the hypothalamus is through the use of exogenous oestrogens. This approach was first employed in the successful treatment of prostate cancer by Herbst in 1942. Synthetic oestrogens, of which there are a number, notably diethylstilboestrol (DES) and estramustine (estracyt - an oestrogen-mustard complex), are commonly used, with the former reducing serum testosterone to castrate levels at a dosage of 3mg daily (Shearer et al, 1973). The use of such oestrogenic compounds, in particular DES, is, however, associated with cardiovascular thromboembolic side effects and significantly increase the death rate from such diseases (VACURG, 1967; de Voogt et al,
Reduction of testicular testosterone synthesis without the side-effects characteristic of oestrogen administration can be achieved using LHRH agonists. These agents e.g. leuprolide and buserelin, were first described in the early 1970's, and offer more selective suppression of LH secretion.

The suppression of serum testosterone using androgen withdrawal regimes results in loss of libido and often impotence in the patient. Significant reductions of these side-effects and those associated with oestrogen treatment have been observed through the use of anti-androgens that counteract androgen action at the cellular target and latterly with 5α-reductase inhibitors e.g. finasteride, that block the conversion of testosterone to DHT in prostatic epithelia. The most widely used anti-androgens are the steroidal compound cyproterone acetate and the non-steroidal androgen antagonist flutamide. The latter binds to the androgen receptor competitively as an activated α-hydroxy metabolite (Neri, 1977; Simard et al, 1986). Subjective improvements have been observed in 90% of patients given flutamide (Sogani et al, 1984), however, resulting inhibition of androgen feedback induces a secondary increase in plasma testosterone (Knuth et al, 1984) and has discouraged many clinicians from using flutamide and other anti-androgens as monotherapy. Consequently, non-steroidal anti-androgens are commonly used in combination with castration or LHRH agonists to achieve what is termed "total androgen blockade" allowing simultaneous obstruction of both testicular and adrenal androgens. Debate continues as to the benefits in terms of duration of response and survival of such combination therapy (for review see Labrie, 1991). In view of this, and the fact all treatments necessarily involve a certain degree of side-effects, endocrine therapy is in general limited to patients with metastatic disease, usually associated with symptoms.

Owing to the age-group involved, a large proportion of the patients with prostate cancer will die of unrelated, concomitant disease during androgen ablation therapy. Those surviving patients, in virtually all cases, will have a relapse due to tumour progression that can no longer be influenced by endocrine manipulations (Sinha et al, 1977). The mechanisms that underlie this switch to hormone-independent growth or "hormone escape" are ill understood.

1.3.3 Effects of Androgen Ablation upon the Benign and Malignant Prostate.

After ~20 years of age, growth of the prostate ceases and a period of maintenance ensues, in which the rate of prostatic proliferation is in equilibrium
with the rate of prostatic cell death (Isaacs, 1987). In the adult male rat this rate of death/proliferation is in the order of 2% per day. Androgens play a pivotal role in prostate maintenance, acting both agonistically and antagonistically on prostatic cells through simultaneous stimulation and inhibition of the rate of proliferation and death respectively (Bruchovsky et al, 1975; Isaacs, 1984). Consequently, the rate of cell death is promoted under the androgen limiting conditions resulting from castration, and culminates in the involution of the prostate.

It has been demonstrated that it is the secretory epithelia not the basal epithelia or stromal fibroblasts that are lost during androgen ablation (English et al, 1987 and 1989). Indeed a 66% decrease in glandular epithelia has been observed following castration. The death of normal androgen-dependent non-malignant epithelia is not believed to be necrotic, but rather, is brought about through the initiation of programmed cell death (apoptosis) (Isaacs, 1984; Kyprianou and Isaacs, 1988a). Programmed cell death is also typical in malignant prostatic epithelia. Kyprianou and co-workers (1990) observed that in the androgen-responsive prostate tumour, PC82, grown as a xenograft in nude mice, androgen deprivation leads to an alteration of cell proliferation and death rates from 3.5% to 0.5% and from 0.5% to 4.7% respectively.

So if androgen ablation successfully induces programmed cell death in malignant epithelial cells, why in nearly all cases do prostatic tumours relapse and escape from androgenic control? Debate continues as to the answer to what is arguably the biggest question in prostate cancer research.

There is evidence to suggest that metastatic prostate cancer is a heterogeneous tumour comprising clones of both androgen-dependent and androgen-independent cells (Schulze et al, 1987). Controversy surrounds the potential mechanisms underlying this heterogeneity, nevertheless, what is clear is that once hormone-independent cancer cells are present within individual patients, these patients cannot be cured by endocrine management alone. This regrettable situation arises because partial or total androgen blockade only induces cell death in the androgen-dependent epithelia such that there is outgrowth of androgen-independent clones (Isaacs and Coffey, 1981; Schulze et al, 1987). Such clonal selection may well be the critical feature of hormone escape in prostatic carcinoma.

Rennie et al (1990) in their analysis of tumour recurrence in the androgen-dependent Shionogi mouse mammary carcinoma observed a proportionate increase in the number of tumourigenic stem cells from 1 per 4000 progeny (end) cells to 1 per 200 progeny cells - a 20-fold increase in the size of the stem cell population. This was concurrent with the complete elimination of nearly all
androgen-dependent progeny cells and an approximately 500-fold increase in the number of androgen-independent (AI) stem cells. These AI stem cells, which necessarily give rise to AI progeny, Rennie believes, arise from adaptation - not selection, as proposed by Isaacs and Coffey - of initially androgen-dependent cells, owing to their extremely low incidence in regressing parent tumours.

Cell-cell interactions have also been implicated in the transition from androgen-dependent to androgen-independent epithelial cell growth. Tenniswood (1993) suggests that the proximity of stromal cells attenuates the programmed cell death of prostatic epithelia following castration. In support of this claim he reminds us that in the normal adult prostate, it is only the epithelial cells at the distal tip (see Figure 1.01b.) of the acinar unit, which are separated from the stromal compartment only by sparsely distributed basal epithelia, that are induced to undergo apoptosis. In the proximal region basal cells form a continuous layer that it is intercalated between the stroma and the luminal epithelial cells.

Despite these theoretical differences, in all cases the end-product is an androgen-independent, apoptosis-resistant and potentially lethal epithelial cell. Undeniably, in terms of effective treatment protocols for advanced metastatic tumour, there is a desperate requirement for therapies that target both the androgen-dependent and androgen-independent epithelia. This will involve the use of total androgen blockade coupled with chemotherapeutic agents which will target and induce cell death in the androgen-insensitive cells. As yet, there are no such agents with the ability to control the growth of hormone escaped prostate cancer cells.

1.4 Programmed Cell Death (Apoptosis) in the Prostate.

Necrotic cell death is a passive cellular process, characterised by permeabilisation of the plasma membrane leading to osmotic lysis of the cell and its internal membranes. In contrast, programmed cell death involves active participation by the cell in its own death. The initiation of this energy-dependent process leads to a number of biochemical and morphologic alterations notably DNA fragmentation that precedes eventual breakdown of cellular membranes (Kerr et al, 1972; Wyllie, 1980). Fragmentation of genomic DNA is an early event in the apoptotic pathway that necessarily commits the cell to die. It is believed to result from the activation of Ca²⁺/Mg²⁺-dependent nuclear endonucleases. Cleavage of the DNA initially releases 50bp chromatin loop domains (Oberhammer et al, 1993) prior to liberation of stereotypic 180-200bp internucleosomal DNA fragments.
Nuclease activation is triggered by sustained elevation of intracellular Ca^{2+} concentration initiated early in programmed cell death (McConkey et al., 1989a. and b.). The increase in intracellular Ca^{2+} is derived from the extracellular Ca^{2+} pool (Furuya and Isaacs, 1993).

These events are characteristic of a number of different cell types, including the prostate, which undergo programmed cell death, however the precise cellular mechanisms which underlie not only prostatic epithelial apoptosis, are only now becoming clear.

12 to 24 hours post-castration the rat intra-prostatic DHT level plummets to ~5% of that observed in intact animals, with concomitant reduction of androgen receptor retained in isolated ventral prostatic nuclei (Kyprianou and Isaacs, 1988a.). These changes in the level of nuclear androgen receptor lead to expression of a number of genes which are normally repressed in the prostate. Notable amongst these genes are those for testosterone repressed prostate message-2 (TRPM-2; Montpetit et al., 1986), glutathione S-transferase (Chang et al., 1987), TGFβ1 & the TGFβ1 receptor (Kyprianou and Isaacs, 1989a. and 1988b.), C-CAM (Hsieh and Lin, 1994) and c-myc, hsp70 & Ca^{2+}-responsive c-fos (Buttyan et al., 1988). This epigenetic reprogramming is believed to induce initiation of the programmed death pathway with consequent DNA fragmentation and cell breakdown into apoptotic bodies (Kyprianou and Isaacs, 1988a.).

TRPM-2 [also termed sulphated glycoprotein-2 (SGP-2), clusterin, complement cytolytic inhibitor and SP-40/40] is expressed by a number of tissues - including the prostate during androgen ablation - undergoing programmed cell death and although as yet not fully characterised, provides a useful early marker for apoptosis (Buttyan et al., 1989). [It is worth noting that the homology of TRPM-2 to a factor whose function is to inhibit complement-induced cytolysis of epithelial cells (Jenne D.E. and Tschopp J., 1989) may suggest a protective role in surviving cells]. Additionally, both c-myc and TGFβ1 have been demonstrated to induce programmed death, the latter in rat ventral prostatic epithelia (Bissonnette et al., 1992; Fanidi et al., 1992; Martikainen et al., 1990). Kyprianou et al. (1989) observed that 4 days after castration both the expression of TRPM-2 and TGFβ1 reaches a maximum in the rat ventral prostate. The suggestion that TGFβ1 expression parallels the onset of programmed death lead to the demonstration that apoptosis can be induced in the rat ventral prostate by this growth factor, even in the presence of physiological levels of testosterone (Martikainen et al., 1990). In human tissues similar results have been obtained. Muir et al. (1994) have shown that prostatic TGFβ1 is highly expressed in patients receiving androgen ablation.
therapy and that the increased staining localises to the extracellular compartment of the stroma adjacent to the tumour. These findings suggest that stromal-epithelial relationships have a vital role in the programmed cell death of secretory epithelia. The precise role of the elevated TGFβ1 expression following androgen ablation is not clear, however, there are indications of involvement in the raising of intracellular free Ca\(^{2+}\) (Furuya and Isaacs, 1993).

Furuya and Isaacs (1993) have recently analysed the expression of a number of genes potentially involved in the biochemical steps of prostatic epithelial cell death. Perhaps most significant among these are the gene for the Ca\(^{2+}\)-binding protein, calmodulin, which demonstrates post-castration increases in expression and the genes encoding p53, ornithine decarboxylase, c-fos and H4-Histone whose expression is unchanged after androgen ablation but rises dramatically after testosterone replacement. It has also been demonstrated that c-myc is up-regulated in both cell death and cell proliferation.

Calmodulin is involved in the calcium-dependent regulation of a number of enzymes (perhaps nuclear endonucleases) and interestingly is implicated in dexamethasone-induced cell death in lymphocytes where its expression is increased ~10 fold after dexamethasone treatment (Dowd et al, 1991).

Cell cycle events have been suggested to regulate prostate cell apoptosis. Colombel et al (1992) at the 9th congress of the European Society for Urological Oncology and Endocrinology (E.S.U.O.E.) proposed that castration induces re-entry of epithelia onto a defective cell cycle in which they never reach mitosis. These cells have been proposed to leave the normal cell cycle onto this defective cycle either in the S-phase or G\(_2\)-phase. They base this theory upon observed increases in bromodeoxyuridine into epithelial genomic DNA and increases in the expression of proliferating cell nuclear antigen (PCNA) and the G\(_1\) proteins c-fos and c-myc. In addition, Colombel claims an up-regulation of the p53 tumour suppressor following castration and postulates that this may lead to initiation of apoptosis. These observations are in conflict with those of Furuya and co-workers who exhibited no increased expression of genes required for entrance of cells into the S-phase of the proliferative cell cycle (i.e. p53, histone-H4 and ornithine decarboxylase) following androgen ablation (Furuya et al, 1993; Berges et al, 1993). Further to this, Berges et al (1993) demonstrated that programmed cell death consequent of endocrine management does not require entry onto the cell cycle with cells dying out of cycle in G\(_0\) (interphase).

Programmed cell death in malignant epithelial cells would appear to be the major ally of the clinician in the control of prostatic neoplasia. However, it could be
argued that apoptosis in androgen-dependent cells merely allows unhindered progression of the tumour to a more aggressive state ultimately fatal to the patient. As discussed in the previous section, androgen ablation therapy leads to the outgrowth of epithelial cell clones which are resistant to androgen ablation therapies and therefore do not undergo apoptosis under such circumstances. It has been proposed that it is the proliferation of these cells that leads to tumour relapse and eventually death from the disease. This does not mean that the situation is completely hopeless, and indeed it has been demonstrated that androgen-independent tumour cells can be induced to undergo programmed cell death through administration of cytotoxic drugs (Kyprianou and Isaacs, 1989b). This group identified the major problem associated with this form of therapy as being a requirement for a relatively high rate of cell proliferation. A high proportion of prostatic tumours are slow growing and contain a high population of non-proliferative androgen-independent cells. In respect of this, Martikainen et al (1991) have analysed the response of the hormone-independent rat prostate cancer cells, AT3, to compounds that increase the intracellular Ca\(^{2+}\), since they have proposed that the lack of apoptosis in androgen-independent cells following androgen ablation is due to a repression of changes in Ca\(^{2+}\). Using the calcium ionophore ionomycin, elevation of intracellular Ca\(^{2+}\) 3-6 fold above basal levels was observed in AT3 cells \textit{in vitro}. This led to programmed cell death in these cells after 48-72 hours. Clearly Ca\(^{2+}\) offers an exciting new alternative target in the treatment of androgen-independent CaP.

1.5 The Androgen Receptor.

In the human male, sexual differentiation and development is under the direct regulation of androgens, specifically testosterone and its more biologically active metabolite 5\(\alpha\)-dihydrotestosterone (DHT). The influences of both testosterone and DHT are believed to be mediated by the same androgen receptor protein (Evans, 1988) functioning as a transcription factor within the nucleus (Rundlett \textit{et al}, 1990). This androgen receptor exists as a member of a superfamily of nuclear regulatory proteins, thought to have evolved from a common ancestral gene, that exert control over the expression of specific eukaryotic genes (for further review see Beato, 1989; O'Malley, 1990). Amongst this family of nuclear receptors (NRs), the androgen receptor is relatively under-studied and much information about its function has been obtained by extrapolation from its co-members.
1.5.1 The Nuclear Receptor (NR) Superfamily.

This group of phospho-proteins that includes, amongst others, all the steroid hormones in addition to the thyroid hormone, retinoic acid and vitamin D3 receptors, all direct the expression of target genes mostly in a ligand-dependent fashion, through direct contact with upstream DNA sequences termed hormone response elements (HRE's). The activated receptor binds to its HRE either as a homodimer (e.g. the steroid receptors), a heterodimer (e.g. the retinoid and thyroid hormone receptors which form dimers with the retinoid X receptor) or even perhaps as a monomer (e.g. NGFI-B). In addition to the hormone receptors, the NR superfamily contains a subset of, as yet, poorly characterised proteins termed Orphan receptors (for review O'Malley, 1992). Orphan receptors - which now constitute the vast majority of NR's - clearly belong to the superfamily, due to the presence of distinctive amino acid homologies, but in the main have no assigned function or indeed ligand specificity.

The members of the vertebrate nuclear receptor superfamily are listed in Table 1.01.

<table>
<thead>
<tr>
<th>Hormone Receptors</th>
<th>Orphan Receptors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucocorticoid</td>
<td>COUP</td>
</tr>
<tr>
<td>Mineralocorticoid</td>
<td>ARP-1</td>
</tr>
<tr>
<td>Oestrogen</td>
<td>EAR-1</td>
</tr>
<tr>
<td>Progesterone</td>
<td>EAR-2</td>
</tr>
<tr>
<td>Androgen</td>
<td>PPAR1</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>HNF-4</td>
</tr>
<tr>
<td>Thyroid hormone</td>
<td>ERR-1</td>
</tr>
<tr>
<td>Retinoic acid</td>
<td>ERR-2</td>
</tr>
<tr>
<td>Retinoid X</td>
<td>NGFI-B/Nur77</td>
</tr>
<tr>
<td></td>
<td>ELP/SF-1</td>
</tr>
</tbody>
</table>

(Parker, 1993)

Through their DNA binding properties the NR's provide direct links between extracellular signals and transcriptional responses, manifest as both up- and down-regulations of gene expression. The precise mechanisms underlying such alterations in gene transcription are not fully understood, however, four discrete steps are believed to be involved: 1. ligand induced allosteric activation of the receptor; 2. specific binding to the HRE; 3. stable complex formation at the HRE;
4. recruitment of other transcription factors and RNA polymerase II for transcription initiation (O'Malley, 1990). The exact role of the NR in the receptor-transcription factor-RNA polymerase II is, unsurprisingly, not yet fully understood, although theories range from stabilisation activity to stimulation of the assembly of the preinitiation complex composed of general transcription factors. The latter function may necessarily involve the alteration of the chromatin structure adjacent to the HRE such that the binding sites for other co-operating transcription factors are exposed.

The cloning and sequencing of the genes and cDNA's for a number of NR's during the 1980's, notably, the oestrogen, progesterone and glucocorticoid receptors, led to the disclosure of common functional domains shared by all members of the superfamily (Krust et al, 1986; Kumar et al, 1987). The domain structure of the NR superfamily, comprising 6 separate units lettered A-F, is represented below (Figure 1.06).

Figure 1.06 The Domain Structure of the Nuclear Receptor Superfamily.

This model is merely an approximation of the typical structure of a member of the NR superfamily, since each individual protein possesses its own unique structural characteristics. However, it does demonstrate the major functional domains (domains B,C and E) common to each receptor and summarises their roles within the molecule. It is evident that the receptor molecules demonstrate a hierarchy of conservation among their functional units, with the DNA-binding domain exhibiting the least amount of variability in length and amino acid sequence between NR's. It is the conserved nature of the DNA- and Ligand-binding domains that led to the supposition that, in vertebrates at least, these units derived from a common ancestral gene possessing these functions (Laudet et al, 1992).
1.5.2 The Roles of the Functional Domains in the NR Superfamily.

N-Terminus (regions A & B)

The N-terminal domain exhibits a degree of variability amongst the NR's and is believed to participate directly - in conjunction with the DNA-binding domain and sub-domains within the C-terminal region - in so-called "trans-activation" (the regulation of target gene expression by a protein expressed by a different gene). These regions of the receptor are proposed to interact, via protein-protein interactions, with other components of the transcriptional machinery (either other transcription factors or RNA polymerase II directly) in the formation of the pre-initiation complex. Indeed, members of the NR superfamily, for example COUP-TF and the oestrogen receptor, are known to make direct and specific contacts with the general transcription factor TFIIB (Baniahmad and Tsai, 1993).

How trans-activation domains direct protein inter-relationships and what features direct their function is as yet uncharacterised. What is becoming apparent is that transcriptional activation domains appear to be conserved within the NR superfamily much in keeping with the other major domain structures. Consider the oestrogen receptor which possesses two trans-activation domains termed TAF-1 (constitutively active) and TAF-2 (ligand-dependent) locating to the N- and C-termini respectively (Lees et al, 1989). Sequence alignment studies have demonstrated conservation of certain residues in TAF-2 amongst a number of nuclear receptors, notably the glucocorticoid, progesterone and androgen receptors (Danielian et al, 1992). These residues are believed to form an amphipathic α-helix that may directly contact other transcription factors.

C-Terminus (regions C,D & E)

The C-terminal portion of the nuclear receptors participates in many and varied activities including ligand-binding, nuclear localisation and dimerisation.

The ligand binding domain (LBD) on average encompasses around 210 amino acids and binds the ligand in a hydrophobic pocket, potentially via multiple specific contacts. For example, within the glucocorticoid receptor at least three different amino acids within a ~150 amino acid region come into close proximity with the ligand molecule. Furthermore, Forman and Samuels (1990) have identified two regions within the LBDs of the retinoic acid and thyroid hormone receptors.
termed Ligand 1 and Ligand 2 which they believe direct ligand binding. These two domains are almost identical among receptors of the same binding specificity. Two further domains have been identified by Forman and Samuels. These locate between Ligand 1 and Ligand 2, one being involved in receptor dimerisation and the other, a domain conserved amongst all the NRs, termed \( \tau_r \), is believed to be a ligand-sensitive transcriptional inactivating domain. The dimerisation domain consists of nine heptad repeats of hydrophobic amino acids that form into a coiled-coil \( \alpha \)-helix possessing a dimerisation interface along one surface. The juxtaposition of these repeats and \( \tau_r \) with Ligand 1 and Ligand 2 ensures that receptor dimerisation and suppression of transcriptional inactivation are inextricably linked to ligand binding.

The C-terminus is also implicated in the binding of heat-shock proteins (hsp's) and a number of other so-called receptor associated proteins (RAPs) to hormone-free receptors in the inactive state (for review see Smith and Toft, 1993). These include hsp90, hsp70, hsp56, p60, p54, p50 and p23. The ligand-free steroid hormone receptors exist in a 8-10s complex with hsp90, hsp70 and hsp56 (Banaihamad and Tsai, 1993). Binding of the ligand transforms the receptor into a transcriptionally active state through release of the hsp's from the receptor molecule. It is clear however that removal of the hsp's does not render the receptor constitutively active and hormone-binding is still required for full function (Bagchi et al, 1990). Although the fate of the hsp's seems fairly clear, the exact roles of these proteins within the inactive receptor is highly controversial. Theories range from molecular chaperones to inhibitors of unliganded receptor degradation and it is often proposed that hsp90 interacts with receptor molecules such that transcriptional activation domains e.g. the DNA-binding domain are masked (Smith and Toft, 1993). Hormone-binding is postulated to alter the conformation of the receptor such that the hsp's dissociate.

DNA-binding Domain (Region C)

The DNA-binding domain of the NR superfamily comprises a highly conserved 66 amino acid stretch of high cysteine content within the interior of the protein sequence. This region contains 9 cysteine residues, 8 of which have been demonstrated to form 2 zinc fingers akin to those first observed in Xenopus laevis transcription factor IIIA (for review see Schwabe and Rhodes, 1991; Freedman and Luisi, 1993). 4 cysteine residues associated with each zinc finger give rise to a
C2-C2 motif consisting of a stretch of α-helix and an N-terminal loop stabilised by a tetrahedrally co-ordinated zinc ion located toward the N-terminus of the helix. The N-terminal zinc finger is believed to contact the DNA in the major groove via specific amino acid residues on the lower surface of its α-helix (recognition helix). The second zinc finger determines the orientation of the recognition helix through protein-protein interactions (Figure 1.07 a.). The recognition helix possesses only three discriminatory amino acids that recognise one half of a palendromic cis-acting consensus sequence termed the Hormone Response Element (HRE) (Figure 1.07 b.). Shortcomings in terms of specificity offered by so few protein-DNA contacts are overcome through the correct orientation of the recognition helix in the major groove by the second C2-C2 motif.

It is clear that many questions relating to the activity of the NR superfamily remain as yet unanswered, with much emphasis being placed on the role of protein phosphorylation and the involvement of putative nuclear localisation domains in receptor activity. As long as such insights are denied to us, the precise mechanisms underlying NR-regulated gene transcription will remain unclear.

1.5.3 Androgen Receptor - Structure and Function.

The androgen receptor cDNA, cloned in the late 1980's by a number of groups (Trapman et al, 1988; Lubahn et al, 1988; Chang et al, 1988; Tillet et al, 1989), demonstrates an open reading frame of 2751 nucleotides encoding a protein of 917 amino acid residues with a calculated molecular weight of 98,845 Da. The androgen receptor gene is located on the X chromosome in the Xq11-12 region (Brown et al, 1989) and is of a size in excess of 90kb (Kuiper et al, 1989). Kuiper demonstrated that the information for the AR is divided over 8 exons. The first exon, which contains the ATG translation start codon, encodes the entire N-terminal domain of the receptor protein (1586bp)(Faber et al, 1989). The first zinc finger is encoded by exon 2 (152bp) while exon 3 (117bp) encodes the second of the two motifs. The ligand-binding domain coding region is divided over exons 4-8 (289bp, 145bp, 131bp, 158bp and 153bp respectively). The sizes of exons 1 and 8 are considerably greater than those quoted due to the large 5' (1.1kb) and 3' (6.8kb) untranslated regions (UTRs).

The promoter for the AR gene is unusual in that it lacks the typical "TATA" and "CAAT" sequence motifs. Transcription initiation occurs at 2 sites within a 13bp GC-rich region, ~1.1kb upstream of the initiator methionine of the AR open reading
b. Receptors: Glucocorticoid  
Mineralocorticoid  
Progesterone  
Androgen  
Discriminatory Amino Acids: cGS ckV  
HRE consensus: 5'AGAACA nnn TGTCT3'  
Note: the discriminatory amino acids on the recognition helix are written in bold text.

Figure 1.07 DNA-binding by Nuclear Receptors.

a. A schematic representation of the protein-DNA complex formed between the DNA-binding domain of the two receptor molecules and their palendromic recognition sequences. (taken from Schwabe and Rhodes, 1991).

b. The symmetrised DNA response element and contacting amino acids of the glucocorticoid-like nuclear receptors.
frame (Tilley et al., 1990; Faber et al., 1991). These two sites have been termed AR-TIS I (+1/2/3) and II (+12/13). The promoter is characterised by a short GC-box (-59/-32) and a longer homopurine region (-117/-60), the former directing transcription from AR-TIS II exclusively (Faber et al., 1993). Interestingly, the GC-box contains a single binding site for the transcription factor Sp1, mutation of which abolishes initiation from AR-TIS II. It would appear that the initiation of AR mRNA is somewhat idiosyncratic, nevertheless, it is clear from the work of Tilley that this promoter region is preferentially used for AR gene expression in many and varied tissue types.

Transcription initiated at either AR-TIS I or II yields two major RNA transcripts of approximate sizes 10kb (9614 bases) and 7kb (6682 bases) (Tilley et al., 1990a.). These two RNA species are believed to have the same hnRNA progenitor, however, an alternative splice event occurring in the 3' UTR removing approximately 3.3kb gives rise to the smaller transcript (Faber et al, 1991). A third AR mRNA, 4361 bases in length, is also commonly detectable in the LNCaP cell line and prostatic tissue (Tilley et al, 1990a.; Bonnet et al., 1993), although it would appear that this transcript is merely a degradation product of the larger mRNAs (Blok et al, 1991).

The protein product arising from translation of the AR mRNA transcripts exists as two different isoforms which migrate as a closely spaced doublet of 110-112 kDa on SDS-PAGE (Kuiper et al, 1991). Most receptor is present in the larger of the two forms although it is evident that both will bind hormone and undergo transformation to a nuclear binding form. Kuiper et al (1991 and 1992) demonstrated that the AR is synthesised as the 110kDa protein but is rapidly converted to the larger isoform in a hormone-independent phosphorylation step. Further receptor phosphorylation occurs in presence of androgens, with over 90% occurring in the N-terminal transactivation domain (Kuiper et al, 1993). The role of this hormone-dependent phosphorylation remains to be elucidated.

Testosterone and dihydrotestosterone exert different actions during the embryogenesis and post-natal life of the male but it is evident that their separate influences are mediated by the same androgen receptor. Although testosterone and DHT both bind to the AR, they differ markedly in their androgenicity. Deslypere et al (1992) demonstrated that DHT/AR complex is ~10 times more powerful as a transcriptional activator than the testosterone/AR complex. Such amplification of the androgenic signal results from a significantly higher affinity of the AR for DHT (Grino et al, 1990). Clearly, the action of 5α-reductase allows full androgenic potential within the prostate.
Sequence alignment coupled with mutational analysis demonstrates that the functional domain structure of the AR is comparable to that of the glucocorticoid and progesterone receptors (Jenster et al, 1992). Clearly the AR possesses domains for steroid- and DNA-binding in addition to sequences that direct transcriptional activation and nuclear import (Simental et al, 1991). It has recently been shown that nuclear localisation (determined by residues 608-625 within the DNA-binding domain) of the AR in addition to receptor dimerisation and binding to the androgen response element (ARE) are androgen-dependent (Jenster et al, 1993; Wong et al, 1993). Such hormone-induced transformations may relate to the dissociation of heat shock proteins, including hsp90, 70 and 56, from the receptor complex following androgen-binding (Veldscholte et al, 1992a).

Only a small number of cellular genes have thus far been characterised which are regulated by androgens through direct interaction of the AR with a localised ARE. Amongst these are the genes for human glandular kallikrein-2 (hKLK2), PSA (hKLK3), the components C1, C2 & C3 of rat prostatic binding protein and latterly the gene for rat probasin (Riezman et al, 1991; Murtha et al, 1993; Claessens et al, 1993; Rennie et al, 1993). Two response elements have been identified in the PSA and C3 gene with the sequences AGAACAgcaAGTGCT and AGTACGtgaTGTTCT respectively. These sequences are extremely similar to the glucocorticoid response element (GRE) consensus (Figure 1.07b) and indeed it has been demonstrated that the GRE can mediate induction by androgens in the mouse mammary tumour virus long terminal repeat (MMTV-LTR)(Darbre et al, 1986; Ham et al, 1988). Despite Roche et al (1992) having described a highly specific ARE consensus sequence, it is becoming clear that AR specificity is provided not only by the palindromic consensus but also by other juxtaposed sequences within the enhancer (Adler et al, 1993). It is such combinatorial function which may allow specific gene regulation by androgens in tissues containing a full range of steroid hormone receptors.

1.5.4 Anti-androgens - Flutamide.

Anti-androgens are used to block the action of testosterone and DHT at the target tissues through direct interaction with the androgen receptor such that formation of the activated receptor complex is abolished. The most widely used non-steroidal anti-androgen is flutamide (α-α-α-trifluoro-2-methyl-4'-nitro-m-propionotoluidine) marketed by Schering Plough (Sch-13521) as Drogenil. Flutamide is metabolised within the cell to an activated α-hydroxy metabolite,
hydroxyflutamide (Sch-16423; a-a-a-trifluoro-2-methyl-4'-nitro-m-lactotoluidide), which interacts with the AR such that the receptor complex cannot bind to the ARE and influence the expression of target genes (Neri, 1977; Simard et al, 1986; Wong et al, 1993; Veldscholte et al, 1992b). Symes et al (1978) demonstrated that hydroxyflutamide will displace testosterone at a 1000 to 10000 molar excess. These findings are in keeping with the observation that DHT-induced transcription from the MMTV-LTR promoter is inhibited by a 100-1000 molar excess of anti-androgen (Warriar et al, 1993).

The exact mechanism underlying the inhibition of DNA-binding by the AR following interaction with hydroxyflutamide is not yet clear, however, such information is only now becoming available from studies performed with other receptor systems and alternative anti-androgens. Banahmad and Tsai (1993) in a review of recent literature support the theory that differences in transcription activation potential of hormone/receptor and anti-hormone/receptor arise because of varying protein conformations. Such differences have been observed in the progesterone receptor, and the suggestion is that anti-progesteres act antagonistically because of a different conformation at the C-terminus of the protein, which may mask transactivation domains or even the DNA-binding domain. A similar situation appears in the AR, where a 30kDa protease-resistant fragment observed upon androgen binding does not arise in the presence of either steroidal or non-steroidal anti-androgens (Kallio et al, 1994).

1.5.5 The Androgen Receptor in the Benign and Malignant Prostate.

The androgen receptor is essentially an epithelial phenomenon. Immunohistochemical and steroid-binding analyses demonstrate that in the normal and hyperplastic prostate, glandular epithelia demonstrate high levels of nuclear AR, with basal epithelial cells and stromal exhibiting similar but lower intensities of staining (Hulka et al, 1987; Masai et al, 1990; Chodak et al, 1992; Miyamoto et al, 1993). In carcinoma, it appears that the extent of nuclear AR is lower than in hyperplasia and that the staining decreases with the increasing histological grade of the tumour. Well differentiated tumours (Gleason scores 1 and 2) generally have a higher number of AR-positive than poorly differentiated tumours (Gleason scores 4 and 5). It is also apparent that AR staining exhibits greater heterogeneity in cancer specimens compared with BPH. Indeed, Sadi and Barrack (1993) have correlated this variation in staining intensity with response to endocrine therapy and demonstrated that poor responders (<20 months to tumour progression) exhibit
greater variance in intensity than good responders (>20 months to tumour progression).

In endocrine management of metastatic prostate cancer, it is aimed to prevent AR activation by androgens and/or to block its function as a transcriptional activator. As discussed previously, such therapies rarely cure the disease with the response generally lasting for 2-3 years prior to relapse of the tumour to androgen-independent growth. In the rat Dunning model of prostate cancer it has been demonstrated that tumour progression into hormone escape is associated with the outgrowth of androgen-independent cells from an initially heterogeneous mixture of cells (Isaacs and Coffey, 1981). The nature of this heterogeneity and the intrinsic features of the androgen-independent cells are still controversial. Quarmby et al (1990) demonstrated that progression to hormone escape in the Dunning R-3327 tumour is associated with alteration of the level of expression of the AR. These findings are very much in keeping with those of Diamond and Barrack (1984) who demonstrated that the androgen-independent sublines of the Dunning tumour have low or undetectable levels of AR. In addition to these observations in the Dunning tumours, Rennie et al (1990) observed that the androgen-independent stem cells and their progeny obtained in the Shionogi model after androgen ablation also do not express detectable AR. Masai et al (1990) observed that in untreated human prostate adenocarcinomas, clusters of cancer cells contain both receptor-positive and receptor-negative clones. This group observed that endocrine management induced a lowering of the percentage of AR-positive cells to levels below, but not significantly below, those exhibited by poorly differentiated, untreated prostate neoplasms. The suggestion is that the transition to hormone-independence is coupled with the preferential growth of AR-negative epithelia. This view of hormone escape in advanced CaP is extremely contentious. Furuya et al (1992) have demonstrated that an androgen-independent subline of the androgen-independent mouse mammary adenocarcinoma Shionogi carcinoma-115 (SC-115) possesses a fully functional androgen receptor identical to that in the parent subline. In addition to these findings, van der Kwast et al (1991) have observed that in the majority of human adenocarcinoma under endocrine management, there is no preferential increase in AR-negative tumour. In these instances there is a possibility that the AR detected has altered function resulting from mutation and indeed only now are such mutations becoming apparent in androgen-deprived prostate tumours.

Partial and complete androgen insensitivity syndrome (AIS) is an X chromosome-linked disorder preventing normal development of the male phenotype in 46,XY individuals. AIS arises from defects in the AR. Mutations have
been observed in all 8 exons of the AR gene (Brinkmann and Trapman, 1992) and indeed in one published case, deletion of the entire AR gene has been demonstrated (Quigley et al, 1992). A number of groups have analysed relapsed prostate tumours in an attempt to identify AR mutations that may account for the androgen independence.

The LNCaP model of hormone-sensitive advanced metastatic CaP expresses high levels of AR (Horoszewicz et al, 1983; Tilley et al, 1990a. and b.). It is well documented that the LNCaP AR carries a point mutation (A to G; thr868 to ala) in its ligand-binding domain that alters the steroid specificity such that it demonstrates higher affinity for progestagenic and oestrogenic steroids and altered response to the anti-androgen flutamide/hydroxyflutamide (Brinkmann et al, 1991; Veldscholte et al, 1990a. and b.; Culig et al, 1993; Veldscholte et al, 1992b.). In prostatic tumours very few such mutations have been detected.

In early, organ-confined prostatic tumours, mutations have been observed in the ligand binding domain coding regions of the AR gene and in exon 1. In 1 of 40 tumour specimens examined, Schoenberg et al (1994) recognised an alteration in the number of CAG repeats in exon 1 from 24 down to 18. The consequence of this microsatellite mutation is an apparent agonist response to flutamide, however, it remains to be seen whether this alteration of the AR gene or another coincident mutation are responsible for the observed activity. Substitution of a valine residue with a methionine residue, resulting from a G to A exchange in codon 730, has been demonstrated in an untreated prostate adenocarcinoma (Newmark et al, 1992). This mutation was present in approximately 50% of the DNA in the specimen, indicating a possible relationship with cells exhibiting growth advantage. Perhaps more significantly, analysis of the AR gene in endocrine therapy-resistant tumours has also revealed somatic mutation (Culig et al, 1993; Suzuki et al, 1993). Culig et al (1993) detected a G to A substitution at codon 715 in a patient who had undergone orchiectomy. This mutation correlates with enhanced receptor function, illustrated by activation, not only by testicular androgens, but also by the adrenal androgens, DHEA and androstenedione, and by progesterone. It is therefore conceivable that this mutant AR will be functionally active in the androgen ablated patient. The AR mutation found by Suzuki et al (1993) in metastatic tissue is identical to that in the LNCaP cell line and must presumably demonstrate activation by progestagens, oestrogens and indeed anti-androgens.

Rundlett et al (1990) demonstrated that constitutive activity could be conferred upon the AR through truncation of the receptor protein in the carboxy terminus by 201-234 amino acids (removing ~50% of the ligand binding domain). In
theory, nonsense mutations which insert aberrant termination codons in the open reading frame of the gene and thus induce abnormal translation termination could give rise to such a shortened AR. No such mutations have been observed in human prostate cancer to date.

It would appear that, if anything, mutations in regressing CaP have a predisposition for altering the binding specificity of the AR leading to increased activation by alternative steroid hormones and by anti-androgens.

It should be borne in mind that mutation of the AR occurs very rarely in human prostate tumours. Those alterations of the AR gene established by Culig et al (1993) and Suzuki et al (1993) were observed in 1 of 4 and 1 of 8 hormone refractory tumours respectively. Clearly, if it is possible to question the validity of the no receptor theory on the grounds of a lack of conclusive evidence, by the same token there must be an element of doubt concerning the involvement of AR gene mutation. In van der Kwast's studies (1991) on AR expression in tumours subject to endocrine manipulation, 1 patient exhibited almost no positive AR staining, with a further 3 displaying considerable heterogeneity of staining. Therefore, it would not seem unreasonable to suppose that both AR down-regulation and receptor mutation may both result in regression of prostatic tumours.

Since evidence for alterations in the AR gene and AR gene expression is to say the least limited, and certainly inconclusive, attention has recently turned to other gene products which may be involved in epithelial cell death or survival under androgen limiting conditions. Notably much interest has been focused on the bcl-2 oncoprotein whose overexpression has been demonstrated to provide protection from apoptotic stimuli and which may, as a consequence, operate to prevent programmed cell death in prostatic epithelia.

1.6 bcl-2.

The bcl-2 oncoprotein first rose to prominence owing to its involvement in human follicular B-cell lymphomas (Tsujimoto et al, 1985). In this disease the bcl-2 gene on chromosome 18 recombines with a region on chromosomes 14 such that it is juxtaposed to the immunoglobulin heavy chain locus. The net effect of this t(14;18) translocation is the creation of a bcl-2/lgH fusion gene that demonstrates marked deregulation, resulting in the overproduction of bcl-2 mRNA and protein in malignant B-lymphocytes (Bakhshi et al, 1985; Cleary and Sklar, 1985). Such dramatic upregulation of gene expression is believed to arise from the proximity of enhancer sequences after recombination.
The t(14;18) translocation is diagnostic of B-cell lymphomas and may well upregulate bcl-2 expression, however, it has more recently and significantly been demonstrated that this genomic reorganisation is not essential for detectable bcl-2 expression (Pezzella et al, 1990).

The bcl-2 gene consists of three exons and is transcribed into 3 overlapping mRNA transcripts of sizes 8.5kb, 5.5kb and 3.5kb (McDonnell et al, 1993; Tsujimoto and Croce, 1986). The 5.5kb and 3.5kb mRNAs give rise to two very similar proteins bcl-2α (~26kDa) and bcl-2β (~21kDa), the former, which represents the major isoform, differing from its counterpart in the C-terminal portion. The oncogene products function to block the pathways of apoptosis in a number of cell types (Hockenbery et al, 1990; Sentman et al, 1991; Jacobson et al, 1993) and indeed their expression appears to be restricted to tissues characterised by apoptotic cell death (Hockenbery et al, 1991).

Hockenbery et al (1991) initially demonstrated that the anti-apoptotic oncoprotein bcl-2 locates to the mitochondrion and although this still appears to be its primary residence, further studies have shown that it also occupies the nuclear envelope and endoplasmic reticulum (ER) (Jacobson et al, 1993; Krajewski et al, 1993). This sub-cellular localisation is certainly unusual for an oncoprotein and has subsequently spawned considerable debate as to its mode of action. Perhaps the most intriguing of these is a potential role in the regulation of the Ca²⁺-dependent endonucleases whose activation is implicated in the early commitment to apoptotic death. Richter (1993) and Krajewski et al (1993) both support this theory and suggest that bcl-2 may inhibit endonuclease activity and consequently prevent the onset of apoptosis through obstruction of Ca²⁺ release from the mitochondria and endoplasmic reticulum (the lumen of the ER being the major site of intracellular Ca²⁺ storage) into the cytoplasm. This function may be mediated by direct action on Ca²⁺ pumps or channels.

bcl-2 is highly expressed in the prostate and locates exclusively to the basal epithelial cell layer (Hockenbery et al, 1991; McDonnell et al, 1992; Colombel et al, 1993). Both McDonnell et al (1992) and Colombel et al (1993) have analysed bcl-2 expression immunohistochemically in primary tumours, metastases and regressed neoplasms in order to establish any correlation with progression of the disease. Their findings are extremely similar and imply that androgen ablation therapy leads to an increased number of bcl-2-positive cancer cells. Indeed in the rat, McDonnell et al (1992) have demonstrated upregulation of bcl-2 mRNA in the ventral prostate as a consequence of androgen ablation, reaching a maximum 10 days post-
castration; and this effect could be reversed through subsequent testosterone administration. It is also apparent that androgen-independent prostatic tumours exhibit a significantly higher percentage of bcl-2 positivity compared to androgen-dependent neoplasms.

The observed bcl-2 staining patterns in CaP tend to suggest that its expression is not a primary molecular event in the tumourigenic process but rather associates with tumour recurrence associated with endocrine management. In this respect, expression of bcl-2 may be central to the mechanisms of hormone escape, through the provision of apoptosis-resistance for the malignant cell population such that they are able to survive under androgen limiting conditions. This hypothesis is supported by work previously eluded to, which indicates that bcl-2 expressing basal epithelia are unaffected by androgen withdrawal (English et al, 1987 and 1989). Therefore, the malignant epithelial cells with high bcl-2 content may represent the sub-population of androgen-independent cells originally observed by Isaacs and Coffey (1981). However, the extent of the bcl-2 requirement of such androgen-independent prostatic epithelia remains to be assessed.

1.7 in vitro Models of Prostatic Epithelia.

Analysis of tissues using techniques such as immuno-histochemistry, in situ hybridisation provides the researcher with a "snap-shot" of in vivo cellular behaviour in terms of gene expression, cell growth and cell differentiation. In vitro cell models, although perhaps not fully representative of the in vivo situation (a consequence of the difficulty involved in accurately reproducing the complex molecular and cellular environment of a functional whole body), provide a means of studying cellular action. It is their manipulable nature coupled with a general ease of use that makes the use of such model systems so appealing.

A number of in vitro approaches are available for the study of the human prostate including the use of cultured established cell lines, organ explant cultures and short term primary cultures.

1.7.1 Prostatic Carcinoma Cell Lines.

A number of human prostatic cancer cell lines are available including EB-33, PC-82, Honda, PC-EW, LNCaP, DU145 and PC3. The origins and growth characteristics in culture of the most commonly used prostate cancer cell lines and
those which are dealt with in the thesis, namely LNCaP, DU145 and PC3, are summarised below.

1.7.1.1 The LNCaP Cell Line.

The LNCaP (Lymph Node Carcinoma of the Prostate) established by Horoszewicz et al (1983) from needle biopsy from a supracavicular lymph node of a 50 year old patient with metastatic stage D2 CaP (stage M1a). Androgens stimulate the growth of LNCaP and induce the secretion of acid phosphatase and PSA via a high affinity receptor (Horoszewicz et al, 1983; Wilding et al, 1989; Schuurmans et al, 1988; Young et al, 1991; Montgomery et al, 1992; Tilley et al, 1990b.). It should be noted that LNCaP cells exhibit sensitivity to androgens but are not truly androgen-dependent. As previously discussed the LNCaP AR possesses a mutation in its ligand-binding domain that dramatically alters its ligand specificity (Veldscholte et al, 1990a. and b.).

The majority of LNCaP cells exhibit a pseudodiploid karyotype with a modal chromosome number of 46, however, it is apparent that a small sub-population are near-tetraploid in nature (Gibas et al, 1984). 7 marker chromosomes (m1-7) arising from 5 separate chromosomal translocation events are also detectable. Similar markers are also observable in the DU145 and PC3 cell lines.

A number of sub-clones of the LNCaP have been established, notably LNCaP-FGC (fast growing colony) and the LNCaPr (resistant). The LNCaP-FGC is very similar to the parent cell line apart from increased growth rate. The LNCaPr sub-clone was first characterised by Hasenson et al (1985), who demonstrated unresponsiveness to androgens, oestradiol and flutamide despite the apparent presence of an androgen receptor.

1.7.1.2 The DU145 Cell Line.

The DU145 cell line was established from a metastatic brain lesion derived by craniotomy from a patient who had undergone oestrogen treatment (Stone et al, 1978; Mickey et al, 1980). Cell proliferation studies have demonstrated that this cell line is neither androgen-sensitive or androgen-dependent; a finding that correlates with the inability to detect AR expression at either the mRNA or protein level (Tilley et al, 1990b.). Additionally, only very weak staining for prostatic acid phosphatase is observed (Mickey et al, 1980).
Karyotypic analysis indicates that the DU145 cell line is aneuploid i.e. it possesses a modal chromosome number of 64 (Mickey et al, 1980).

1.7.1.3 The PC3 Cell Line.

As with the DU145 cell line, PC3 cells, which originate from a bone metastasis of prostatic adenocarcinoma treated by means of castration and oestrogen therapy, are karyotypically aneuploid (modal chromosome number between 55 and 62) and do not display androgen-sensitivity or -dependence (Kaighn et al, 1979 and 1980). Unsurprisingly, since PC3 was established from an androgen-independent tumour, and displays such characteristics in culture, no androgen receptors in addition to 5α-reductase and acid phosphatase activities are detectable (Tilley et al, 1990b.; Kaighn et al, 1979).

1.7.2 Primary Prostatic Cell Cultures.

Established cell lines offer many advantages to the researcher yet cells cultured long-term are prone to phenotypic/genotypic alterations and one must surely question how representative they are of the in vivo situation. In theory, short-term primary cell cultures should not be subject to such changes and will subsequently have greater resemblance to their tissue progenitors.

Organ culture is the maintenance or growth of organ primordia or whole parts of an organ in vitro. This approach is useful for the study of interrelationships between different cell types in the same organ through preservations of a degree of tissue architecture, however, it is not fully indicative of some of the more complex interactions occurring in vivo. Primary cultures occupy a position intermediate between organ culture and a true replicative tissue culture and refers to the initial outgrowth of cells from tissue fragments or monolayer cultures of cells derived by tissue dispersion. The clear advantages of this approach are not only the similarity of the resulting cells to the tissue of origin but also the potential homogeneity of the cell population and the ease of study of responses to altered environments. Nevertheless, it should be noted that the necessary loss of extracellular matrix favours decreased opportunity for resynthesis and organisation in addition to possible selection of cell types perhaps not representative of the composition of the tissue.

The establishment of prostatic primary cultures has been reported by many groups since the first demonstration of epithelial cell outgrowth from
adenocarcinoma by Burrows et al in 1917. The various protocols for autonomous culture of prostatic epithelia and fibroblasts are in general merely variations on a theme and it is apparent that there are only two real considerations: 1. The use of mechanical or enzymatic separation of tissues; 2. The choice of medium for sustained growth of the different cell types.

There is no real evidence as to whether mechanical or enzymatic dispersion improves the outcome of the consequent cell culture or indeed alters its characteristics, however, it would appear that there is a tendency, from studies of the literature, for the seemingly less aggressive and potentially less damaging use of collagenase (Webber et al, 1980; Chevalier et al, 1981). In addition to this there is some evidence to support enhanced epithelial cell growth and/or suppression of fibroblasts using collagenase to prepare essentially fibroblast-free acini (Kaighn et al, 1975).

It is generally believed that cell culture media supplemented with foetal calf serum (FCS) will support both prostatic epithelial and fibroblastic growth and subsequently the use of such media necessarily leads to overgrowth of epithelia by fibroblasts (Webber, 1980). Although this phenomenon is certainly not true in all cases, a great deal of emphasis has been placed upon the development of serum-free media containing defined components that will only support epithelial cell growth. The pursuit of such culture conditions lead to the development of a serum-free medium by Chaproniere and McKeehan (1986), notably containing cholera toxin and bovine pituitary extract, both of which stimulate epithelial cell growth, the former probably via elevation of intracellular cAMP.

In contrast to the observed stimulation of primary epithelial cells by human fibroblasts, of both prostatic and non-prostatic origin (Kabalin et al, 1989), efforts to demonstrate a response to androgens have yielded equivocal results. Schroeder et al (1974) showed that both BPH- and CaP-derived epithelial cells do not respond favourably to testosterone and DHT. These findings are very much in keeping with those of McKeehan et al (1984) and Merchant (1990), however, conflicting data has been presented by both Syms et al (1982) and Hallowes et al (1991). The latter group not only demonstrated stimulation of prostatic organoid-derived epithelia by testosterone, but also a dose-dependent inhibition of cell growth by flutamide and hydroxyflutamide.
1.8 Aims and Objectives.

The mechanisms underlying hormone escape in human prostatic adenocarcinoma are very poorly understood. In animal models and humans, it would appear that the most credible theory of tumour relapse following androgen ablation is the outgrowth of androgen-independent cancer cells. However, if we are to believe this hypothesis, the nature of these androgen-independent cells must be ascertained.

The available in vitro models of benign and malignant prostatic epithelia display marked differences in their ability to respond to androgens and anti-androgens. The aim of this work was to analyse the characteristics of established prostatic carcinoma cell lines and primary epithelial cell cultures with respect to their responsiveness to androgenic stimuli. Consequently, the expression of the androgen receptor and the anti-apoptosis oncogene bcl-2, both of which have been implicated in the switch to androgen-independence, were characterised in detail.
CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Cell lines.

The LNCaP cell line [clone FGC (fast growing colony)] was obtained from the American Type Culture Collection (A.T.C.C.), Rockville, U.S.A.. This cell line was established from a metastatic lesion of human adenocarcinoma and presents many of the malignant properties characteristic of neoplastic epithelial cells in CaP (Horoszewicz et al., 1980 & 1883). Cells between passage numbers 80-85 were utilised for all experiments.

The cell line DU145 was kindly donated by Dr. D.D.Mickey, Department of Urology, University of North Carolina, Chapel Hill, U.S.A.. DU145 was originally derived from a brain metastasis identified as a moderately differentiated prostatic adenocarcinoma (Mickey et al, 1980). In all experiments, cells between passage numbers 80-90 were used.

The PC3 cell line was isolated from a poorly differentiated bone metastasis of prostatic adenocarcinoma (Kaighn et al, 1979), and was obtained from the European Collection of Animal Cell Cultures, PHLS Centre for Applied Microbiology and Research, Porton, Salisbury, England. The passage number of the cells was unknown, but for the purpose of our experiments, only cells passaged up to 10 times were employed.

Note: The cell lines were regularly screened for mycoplasma contamination using a mycoplasma detection kit supplied by Boehringer Mannheim UK. (Diagnostics and Biochemicals) Ltd., Lewes, East Sussex, UK.
2.1.2 Cell Culture.

2.1.2.1 General Consumables.

All plasticware, including tissue culture flasks, Universal flasks, Erlenmeyer flasks and pipettes, was obtained from Bibby Sterlin Ltd., Staffordshire, UK. All media and solutions were sterile-filtered before use, with 0.2μm bottle-top filters and 0.2μm sterile Acrodiscs supplied by the Sigma Chemical Company Ltd., Poole, Dorset, UK. and Gelman Sciences, Northampton, UK. respectively.

Oxoid Dulbecco 'A' phosphate buffered saline was purchased from Unipath Ltd., Basingstoke, Hampshire, UK. 10xTrypsin-EDTA (0.5%w/v trypsin; 0.2%w/v EDTA; 0.85%w/v NaCl) was purchased from Gibco BRL, Paisley, Renfrewshire, U.K..

Unless otherwise stated, all chemicals used in tissue culture procedures were obtained from the Sigma Chemical Company Ltd..

2.1.2.2 Cell Lines - media/supplements.

RPMI 1640 medium was obtained from Gibco BRL, Paisley, Renfrewshire, UK.. Dulbecco's Modified Eagles Medium and Ham's F12 medium were purchased from ICN Biomedicals Ltd., High Wycombe, UK.. All media were supplemented with foetal calf serum (FCS), L-glutamine (200mM), penicillin (10,000units ml⁻¹) and streptomycin (10,000μg/ml⁻¹), acquired from Gibco BRL.

2.1.2.3 Epithelial and Fibroblast Primary Culture - media/supplements.

Collagenase was obtained from Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A..

WAJC 404 medium was acquired from Kyokuto Pharmaceutical Industrial Co. Ltd., Tokyo, Japan. HEPES, zinc-stabilised insulin, cholera toxin and dexamethasone were all purchased from Sigma. Sodium hydrogen carbonate and epidermal growth factor were obtained from Fisons Scientific Equipment, Loughborough, Leicestershire, UK. and Universal Biologicals Ltd., London, UK. respectively. Fungizone was procured from Gibco BRL.
2.1.2.4 Cell Proliferation.

Phenol red-free versions of the cell culture media used were obtained from the suppliers listed in 2.1.2.2. [Note: Phenol red-free Ham's F12 is not normally supplied by ICN Biomedicals Ltd. and must be requested]

The MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) and dimethylsulphoxide were purchased from Sigma and the Aldrich Chemical Company Ltd., Gillingham, Dorset, UK. respectively.

The activated charcoal and Dextran T70 used in the removal of endogenous steroids from foetal calf serum were respectively obtained from Sigma and Pharmacia Biotech Ltd., Milton Keynes, UK. Dihydrotestosterone (5α-androstan-17β-ol-3-one) was purchased "cell culture tested" from Sigma.

Hydroxyflutamide (α-α-α-trifluoro-2-methyl-4'-nitro-m-lactotoluidide; SCH 16423) was kindly donated by the Schering Corporation, Bloomfield, New Jersey, U.S.A..

2.1.3 Molecular Biology.

2.1.3.1 General Consumables.

The majority of chemicals employed in molecular biology procedures were obtained from the Sigma Chemical Company Ltd., and were, wherever possible of molecular biology grade.

NaCl, tri-sodium citrate, NaOH, 37% formaldehyde (12.3M, pH>4.0), ethanol, Tris-saturated phenol (20mM, pH8.0) and glacial acetic acid were all purchased from Fisons Scientific Equipment.

Isopropanol (2-propanol), isoamylalcohol (3-methyl-1-butanol) and formamide were obtained from the Aldrich Chemical Company Ltd..

Agarose NA and DNA gel loading buffer (Bio 101 Inc.) were purchased from Pharmacia Biotech Ltd. and Stratech Scientific Ltd., Luton, Bedfordshire, UK. respectively.

Nuclease-free water was acquired from the Promega Corporation, Southampton, Hampshire, UK.

Sterile screw-cap Eppendorf tubes were purchased from Sarstedt Ltd., Beaumont Leys, Leicester, UK. Sterile pastettes were obtained Alpha Laboratories
Whatman 3MM paper was acquired from Whatman International Ltd., Maidstone, UK.

Hybond C extra (nylon supported nitrocellulose) was obtained from Amersham International plc., Aylesbury, UK. The Fastrad Autoradiography cassettes (Hoefer Scientific Instruments) and accompanying intensifying screens were acquired from Scottish Biotechnology Instrumentation, Auchterarder, Perthshire, UK.

2.1.3.2 Plasmid Constructs.

pCMV/ARcom and pMMTV/SPAP were kindly provided by Dr. Don Wallace, Biochemical Targets, Glaxo Research and Development, Greenford, Middlesex. pRc/CMV (Invitrogen Corporation) was purchased from British Biotechnology Ltd., Abingdon, Oxfordshire, UK. The three plasmid constructs used are illustrated in Figure 2.01.

Note: All three plasmids carry a beta-lactamase gene for ampicillin selection.

pAM41 carrying the mouse non-muscle β-actin cDNA was kindly donated by Professor A.H. Wyllie, Department of Pathology, University of Edinburgh.

pCMV/ARcom.

Expression of the androgen receptor cDNA is controlled by upstream promoter/enhancer sequences from the immediate early gene of human cytomegalovirus, ensuring high level constitutive transcription in mammalian cells. Polyadenylation and transcription termination sequences are provided by the human growth hormone gene.

pMMTV/SPAP.

Transcription of the secreted placental alkaline phosphatase (SPAP) cDNA is directed by a portion of the mouse mammary tumour virus long terminal repeat (MMTV-LTR). The MMTV-LTR weakly promotes transcription, but contains an androgen response element that on binding functional androgen receptor, significantly increases the promoter activity (Cato et al, 1987; Ham et al, 1988).
Figure 2.01 Plasmid Constructs.


pMMTV/SPAP.

pRC/CMV. (from the Invitrogen Corporation catalogue 1992)
SV40 provides polyadenylation and transcription termination sequences.

MAX Efficiency DH5α™ competent E.coli were obtained from Gibco BRL. The TOP10f strain was supplied from the Invitrogen Corporation with the pRC/CMV vector as a stab culture. Ampicillin an tetracycline were both purchased from the Sigma Chemical Company Ltd..

DOTAP transfection reagent was acquired from Boehringer Mannheim UK (Diagnostics and Biochemicals) Ltd., Lewes, East Sussex, UK.. Sigma 104 phosphatase substrate was obtained from the Sigma Chemical Company Ltd..

2.1.3.3. Isolation and Analysis of Nucleic Acids.

Guanidine isothiocyanate was purchased from Northumbria Biologicals Ltd., Cramlington, Northumberland, UK..

All materials employed in first strand cDNA synthesis were obtained from the Promega Corporation. PCR reactions were carried out using Promega Taq DNA polymerase and Taq 10xbuffer, with dNTP's and mineral oil supplied by Pharmacia Biotech Ltd. and the Sigma Chemical Company Ltd. respectively.

All primers were synthesised on an Applied Biosystems PCR Mate DNA synthesiser within the Department of Molecular Science, Glaxo Research and Development.

The de-ionised formamide (Clontech Laboratories) for denaturing RNA gel electrophoresis was obtained from Cambridge Bioscience, Cambridge, UK..

The Megaprime™ DNA labelling system (RPN 1606), [α-32P]dCTP (~3000Ci/mmol) and Hyperfilm™-MP were purchased from Amersham International plc.. NICK™ columns were obtained from Pharmacia Biotech Ltd..

49
The mouse monoclonal anti-human androgen receptor antibody was purchased from Novocastra Laboratories, Newcastle-upon-Tyne, U.K.
2.2 METHODS

2.2.1 Cell Culture.

2.2.1.1 Cell lines.

2.2.1.1a. Cell Culture Media.

The LNCaP and DU145 cell lines were routinely cultured in RPMI 1640 Medium and Dulbecco's Modified Eagles Medium respectively. The media were supplemented with 10% v/v foetal calf serum (FCS), L-glutamine (2mM) and with penicillin (100 units ml$^{-1}$) & streptomycin (100 $\mu$g ml$^{-1}$).

PC3 cells were maintained in Ham's F12 containing 7% v/v FCS, L-glutamine (2mM), penicillin (100 units ml$^{-1}$) and streptomycin (100 $\mu$g ml$^{-1}$).

2.2.1.1b. Culture of Cell Lines.

The cell lines were grown in monolayer culture, in 75cm$^2$ tissue culture flasks, in a humidified incubator (T305GF Assab; Kebo Assab AB, Solna, Sweden.) supplied with an atmosphere of 95% air and 5% CO$_2$ at 37°C. All tissue culture procedures were carried out aseptically in a laminar flow cabinet (Microbiological Class II; Howarth Air Engineering Ltd., Farnworth, Bolton, UK.) using sterile disposable plastic pipettes and pipette tips. To ensure that a sterile environment was preserved, the laminar flow cabinet was thoroughly cleaned before, during and after all operations with a solution of 70% ethanol. In addition, all handling of cell lines was performed using disposable latex gloves.

Continuous cell growth was sustained through regular subculture. Upon achieving approximately 90% confluence, cells were harvested through trypsinisation, diluted 1:3 in medium and delivered into 3 fresh flasks. The process of subculture involved the aspiration of spent medium from the cells, followed by 2 washes with Dulbecco 'A' phosphate buffered saline and a 5 minute incubation at 37°C in 5ml 1xTrypsin-EDTA (0.5ml of 10xTrypsin in 4.5ml of Dulbecco 'A' PBS). The cells were detached through gentle agitation and 15mls of the appropriate medium added to the subsequent cell suspension. Following centrifugation at 1500 r.p.m. for 5 minutes and resuspension in 36 ml of medium, repeated pipetting
ensured a single cell suspension, 12 ml of which were delivered to each flask. The cells were allowed to adhere over a 24 hour period before being supplemented with fresh medium. Thereafter the medium was changed every 48 hours.

2.2.1.2 Culture of Epithelial Cells and Fibroblasts from Human Prostatic Tissue.

The primary culture of epithelial cells from prostatic acini in serum-free medium was first described by Chaproniere et al (1986). The protocol described here for the autonomous culture of epithelial and fibroblast cells is based on that developed by Chaproniere.

Prostate tissue was obtained from patients undergoing transurethral resection of the prostate (TURP) to treat the symptoms of BPH. The prostate chips were immediately placed in 20ml of transport medium [ RPMI 1640 medium supplemented with 5% v/v FCS ], and could be stored at 4°C for up to 5 days before use. Pathology was determined by routine examination of representative samples of excised tissue by the Department of Pathology, Western General Hospital, Edinburgh.

Note: The handling of epithelial and fibroblast cell cultures was essentially the same as that for the immortalised cell lines, as described in section 2.2.1.1b.

2.2.1.2a. Enzymatic Dissociation of Epithelial and Stromal Cells.

The prostatic chips were washed twice in 25ml of Dulbecco 'A' PBS, weighed and cut into approximately 3mm² cubes using scissors and forceps sterilised in 70% ethanol. [ Note: At all stages of the procedure, the tissue was kept moist under transport medium.]. The tissue was subsequently transferred to a sterile Erlenmeyer flask, washed with 20ml of transport medium and finally suspended in 5ml of transport medium per gram of tissue. A sterile-filtered solution of collagenase (600unitsml⁻¹) in transport medium, equivalent to 2.5ml per gram of tissue was mixed with the tissue, and the flask placed in a 37°C incubator/shaker (Luckham R300; Luckham Ltd., Burgess Hill, UK.). The tissue was incubated for 20hours, with gentle shaking to maintain the integrity of the acini. The digest was triturated by repeated pipetting, decanted into sterile Universal flasks and centrifuged at 2,000r.p.m. for 10minutes. The supernatant was decanted and the cells resuspended by inversion in 25ml of Dulbecco 'A' PBS to wash out the collagenase. The cells were again centrifuged at 2000r.p.m. for 10minutes. The washing and centrifugation step was repeated a further twice, prior to resuspension
in 10ml of transport medium. The acini, containing the epithelial cells, were
sedimented by centrifugation at 800 r.p.m. for 20 seconds. The supernatant contains
aggregates of fibroblasts. The acini deposits were carefully aspirated using a sterile
Pasteur pipette and transferred to a fresh Universal. The acini were spun down
twice more and collected.

2.2.1.2b. Primary/Secondary Epithelial Cell Culture.

The acinar deposits were resuspended in 6mls of epithelial growth medium
(EGM) per gram of the original tissue and plated out in 75 cm² tissue culture flasks.
One flask was used for every 2 grams of tissue. The cultures were incubated at
37°C for 48 hours and then supplemented with 9mls of EGM. After a further 48 hour
incubation the epithelial cells could be supplied with fresh medium.

Epithelial growth medium (EGM) : 1.104%w/v WAJC 404 medium.
(1 litre) 0.67%w/v HEPES.
0.12%w/v sodium hydrogen carbonate.
2mls of 0.25mgml⁻¹ zinc-stabilised insulin.
2mls of 10μg/ml⁻¹ cholera toxin.
100μl of 3.92mgml⁻¹ dexamethasone in
ethanol.
1ml of 10μg/ml⁻¹ epidermal growth factor.
0.5%v/v foetal calf serum.
10mls of penicillin/streptomycin
(10,000unitsml⁻¹/10,000μgml⁻¹).
10mls of fungizone (250μgml⁻¹), pH7.6.

The epithelial cell cultures were subcultured as per the immortalised cell
lines. Following the detachment of cells from flask, the 0.1% trypsin solution was
neutralised using LNCaP growth medium. Following centrifugation at 1,500 r.p.m.
for 5 minutes and resuspension in 24mls of EGM, the cell suspension could be split
into two 75cm² flasks. In general, it was possible to passage the cells twice.
Beyond passage number 2 the cultures exhibited a rapid decline in growth rate.
2.2.1.2c. Primary/Secondary Fibroblast Culture.

The supernatant from 2.2.1.2b. was centrifuged at 2,000 r.p.m. for 10 minutes and the resulting cell pellet resuspended in 12mls of LNCaP growth medium, supplemented with 2.5μg/ml-1 fungizone, per gram of tissue. The fibroblastic suspension was seeded into one 75cm² flask per gram of tissue and grown in monolayer culture at 37°C for 48 hours. Subsequent to this incubation period, the attached cells were washed with 12mls of Dulbecco 'A' PBS and supplemented with fresh medium until 90% confluent. Sub-culture was performed as described in 2.2.1.1b.

2.2.1.3 Spectrophotometric Determination of Viable Cell Numbers.

The proliferative rates of cultured cell lines and primary epithelial cells was determined using the MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay.

Cells were grown in 96 well plates, supplemented with 100μl of the appropriate medium per well. 50μl of MTT solution at a concentration of 1.5mg per ml of culture medium, was aliquoted into each test well using a multi-channel pipette (Titertek plus; ICN Biomedicals Ltd., and the cells incubated at 37°C for 2 hours. Subsequently, 130μl of the assay mixture could be carefully aspirated from each well. The formazan crystals, produced as a result of the activity of intracellular dehydrogenases, could be solubilised by suspension in 150μl of dimethylsulphoxide containing 0.5%v/v FCS. The plates were shaken at room temperature on an orbital shaker for 30 minutes to ensure complete dissolution of the crystals and the absorbance of resulting solution measured at 540nm on a microplate reader (model 450; Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, UK.).

The viability of the MTT assay as a determinant of prostatic epithelial cell number was assessed using the DU145 cell line.

DU145 cells were plated in 96 well tissue culture plates at an initial plating density of 1000 cells per well. 5 plates, corresponding to days 0-4, were used for both the MTT assay and cell counts, with 8 wells employed in each. 48 hours post-seeding, the medium was removed and replaced with 100μl of fresh medium. At this time, and at 24 hour intervals subsequently, the cell number was assessed using the MTT assay or by counting using a haemocytometer.
Figure 2.02 Measurement of the Proliferation of DU145 Cells by Cell Counting and MTT Assay.

Cells were plated in DMEM supplemented with 10% FCS at an initial density of 1000 cells/well in 96 well plates. 48 hours post-seeding (day 0) the medium was replaced and the cell numbers determined using a haemocytometer with concomitant performance of an MTT assay. Subsequently, cell numbers were assessed in this manner at 24-hour intervals up to day 4. Each data-point represents the mean of 8 wells +/- SEM.
Cells to be counted were washed twice in PBS 'A', incubated with 20μl of 1xtrypsin-EDTA for 2 minutes at 37°C, mixed with 80μl of DU145 growth medium and cell numbers determined using both chambers of a haemocytometer (five 1mm squares in each chamber).

Figure 2.02 exhibits the growth pattern of DU145 cells as assessed by both cell counting and by the MTT assay. Clearly both approaches demonstrate similar trends in DU145 growth, however, it is also apparent that the experimental error associated with cell counting performed using the haemocytometer is considerably greater than those with the MTT assay. These findings would tend to support the use of the MTT assay for the assessment of cell proliferation.

2.2.1.4 Measurement of the Androgenic Responses of Cultured Cells.

The responses of LNCaP, DU145, PC3 and primary epithelial cells to dihydrotestosterone (DHT) and hydroxyflutamide were determined as outlined in 2.2.1.3.. All media employed were purchased phenol red-free and the FCS supplements were rendered steroid hormone-free through treatment with Dextran Coated Charcoal (DCC).

All cells were grown in 96well tissue culture plates.

2.2.1.4a. Preparation of DCC-Stripped FCS.

50ml of DCC suspension was pelleted by splitting into two 25ml aliquots and centrifuging at 4000r.p.m. for 20minutes (4°C) in a Denley BS400 centrifuge.

DCC suspension (50ml) : 1%w/v activated charcoal.
1ml of 5mgml⁻¹ Dextran T70.
0.5ml of 1M tris-HCl, pH7.5.
0.5ml of 0.1M EDTA.

The charcoal pellets were mixed with 100ml of FCS pre-warmed to 37°C in an Erlenmeyer flask, and the mixture shaken in a 37°C incubator for 1hour. Subsequent to this period of incubation, the charcoal was removed by centrifugation as described previously, and the serum sterile filtered through a 0.2μm bottle-top filter.
2.2.1.4b. Cell Lines.

The growth of LNCaP, DU145 and PC3 cells over a 4 day period was assessed after exposure to a range of concentrations of DHT and hydroxyflutamide. The concentrations employed were 10nM, 1nM, 0.1nM, 0.01nM, 0.001nM and 0nM (control). 1mM stock solutions of DHT and hydroxyflutamide in ethanol were prepared and diluted down to 0.21μM. The addition of 5μl of 0.21μM DHT/hydroxyflutamide to 100μls medium gave a final concentration of 10nM. The 0.21μM solution was serially diluted 10 fold to achieve the desired well concentrations. The control wells were supplemented with 5μls of ethanol.

Five 96well plates corresponding to days 0-4 were employed. A total of 8 wells per plate were used for each androgen/anti-androgen concentration, with an initial plate density of 1000cells per well for DU145 & PC3 and 4000cells per well for LNCaP.

Cells grown in monolayer culture in 75cm² tissue culture flasks were harvested through trypsinisation, as described in 2.2.1.1b., and resuspended in 10mls of their normal growth medium. The cell number was determined using a haemocytometer and the cells diluted to the desired final concentration per 100μl of medium in a total volume of 25mls. 100μls of the cell suspension was delivered to each test well and the cells allowed to adhere to the plastic over a 24hour period in a 37°C incubator. [ Note : 100μl of medium was aliquoted into all unused wells to limit the loss of medium by evaporation from the test wells. ]. The medium was subsequently aspirated from the test wells and replaced with 100μl of phenol red-free medium containing DCC-treated FCS. The cells were incubated for a further 48hours at 37°C prior to the addition of DHT or hydroxyflutamide to the desired concentration in 5μl ethanol. [ Note : DU145 and PC3 cells had their spent medium replaced with 100μl of fresh medium prior to the addition of the androgen/anti-androgen ]. The cell numbers were determined on Day0 using the MTT assay described previously (2.2.1.3.) and subsequently measured every 24hours up to 96hours.

2.2.1.4c. Primary Epithelial Cells.

The protocol for measurement of epithelial cell proliferation in response to DHT and hydroxyflutamide was essentially the same as that described for the cell lines (2.2.1.4b.). 4 wells were used for each concentration of drug per plate and the cells were plated at a density of 5,000 cells per well in EGM. After 24hours at
37°C, the media was replaced with 100μl of phenol-red free LNCaP medium supplemented with 10%v/v DCC-stripped FCS. 48 hours later the medium was replaced with 100μl of fresh medium and the androgenic responses measured as per 2.2.1.4.b..

2.2.2 Extraction and Purification of Plasmid DNA.

2.2.2.1 Preparation of Competent E. coli Using Calcium Chloride.

Using a sterile inoculating loop (Sigma Chemical Company LTD.) the TOP10F' strain was streaked onto an LB agar plate supplemented with 15mgml⁻¹ tetracycline, and subsequently grown for 16hours in a 37°C incubator.

LB medium (Luria-Bertani medium) : 1% w/v tryptone; 0.5% w/v select yeast extract. 0.17M NaCl. pH 7.0.

Note: The LB medium was autoclaved for 20minutes at 15lb/sq.in. on liquid cycle before use.

LB agar was prepared by the addition of select agar to LB medium to a final concentration of 15gL⁻¹, and autoclaving at 15lb/sq.in. for 20minutes.

A single colony was picked from the plate and transferred to a sterile Falcon tube containing 2ml of LB medium, and the tube shaken at 225r.p.m. in a 37°C incubator/shaker (Model 625; New Brunswick Scientific Co. Inc., Edison, New Jersey, U.S.A.) for 16hours overnight. 1ml of the overnight culture was diluted into 40ml of LB medium and grown in the 37°C in an incubator/shaker until it achieves an A₆₀₀ of between 0.4 and 0.8. The bacterial culture was pelleted through centrifugation at 6,000r.p.m. for 5minutes (4°C) and subsequently resuspended gently in 10ml of ice-cold 0.1M CaCl₂. The cells were incubated on ice for 1hour and then spun for a further 5minutes at 6,000r.p.m. (4°C). The pellet was resuspended in 2ml of 0.1M CaCl₂. After 2hours on ice, the cells were ready for transformation with pRc/CMV.

2.2.2.2 Transformation of Competent E. coli.

MAX Efficiency DH5α™ competent E. coli were transformed with both
pCMV/ARcom and MMTV/SPAP. TOP10F' were transformed with pRc/CMV (Invitrogen Corporation). The transformation protocol adopted was identical for both strains of E.coli.

The DH5α cells should be stored at -70°C and prior to transformation must be thawed slowly on ice.

Competent cells were mixed gently by hand and two 100μl aliquots transferred to pre-chilled 15ml Falcon tubes. 1.7μl of a solution of 1.5M β-mercaptoethanol was delivered to each aliquot, the cells swirled gently and incubated on ice for 10minutes. The cells were swirled gently every 2minutes. Subsequently 50ng of plasmid DNA was aliquoted into one Falcon tube and an equivalent volume of autoclaved deionised water placed in the other to serve as a "no DNA" control. The cells were swirled gently and left on ice for 30minutes. The transformations were then heat pulsed at 42°C for 45seconds, followed by a 2minute incubation on ice. 0.9ml of SOC medium, preheated to 42°C, was added to each tube. The tubes could then be shaken at 225 r.p.m. for 1 hour in a 37°C incubator/shaker.

SOB medium: 2% tryptone.  
0.5% select yeast extract.  
8.56mM NaCl.  
2.5mM KCl. pH 7.0.

The medium was autoclaved at 15lb/sq. in. for 20minutes and immediately prior to use, sterile MgCl₂ added to a final concentration of 10mM. SOC medium was prepared through the mixing of glucose, sterilised by filtration, with SOB medium, to 20mM.

200μl of both transformation mixtures were plated onto LB agar plates supplemented with 50μgml⁻¹ ampicillin, and the plates incubated at 37°C for 16 hours.

2.2.2.3 Large-Scale Preparations of Plasmid DNA.

2.2.2.3a. Growth of Bacterial Cultures.

Note: All bacterial cultures were supplemented with ampicillin at a concentration of 50μgml⁻¹ to maintain the selection for transformed bacteria.
A single bacterial colony from an ampicillin selection plate was used to inoculate 5ml of ampicillin-supplemented LB medium in a sterile 15ml Falcon tube. The culture was then incubated overnight at 37°C in a shaking incubator set at 225r.p.m.. 4ml of the overnight culture was delivered to a further 50ml of LB medium and grown as before until an OD500 of ~0.6 (corresponding to late log phase) was achieved. Subsequently, 500ml of LB medium in a 2L flask was incubated with the entire late-log-phase culture, and shaken at 225r.p.m. in the 37°C incubator/shaker for 16hours. The bacteria were harvested from the culture by splitting the culture into two 250ml Nalgene centrifuge pots and centrifuging at 5000r.p.m. for 5minutes in a Sorvall GS3 rotor. The spent medium could then be decanted and treated with one 2.5g Presept disinfectant tablet (Surgikos LTD., Livingstone, UK.) for 1hour prior to disposal. Any residual medium in the pots was removed using tissue paper.

The remaining 1ml of the overnight culture was used to prepare a stock culture of bacteria, containing glycerol, that could be stored at -70°C indefinitely. 0.15ml of sterile glycerol was added to 0.85ml of the bacterial culture and dispersed evenly by vortexing. The culture was transferred to a screw-cap Eppendorf tube, snap frozen in liquid nitrogen and then stored.

The bacteria could be recovered, when needed, by scraping the surface of the frozen culture using a sterile inoculating needle and streaking the adhering bacteria onto an ampicillin-containing LB agar plate. The plate could then be incubated at 37°C overnight.

2.2.2.3b. Lysis of Bacterial Cultures by Alkali.

The protocol described here, for the alkaline lysis of large scale bacterial cultures, is a modification of that described in Maniatis et al (1989; 1.38-1.39).

The bacterial pellets from 2.2.2.3a. were completely resuspended in 5ml of sterile, ice-cold solution 1, each, by stirring with a sterile, plastic 100ml pipette. The 5ml suspensions were pooled, delivered to a fresh centrifuge pot and mixed with 1ml of a freshly prepared solution of 10mg/ml lysozyme in 10mM Tris.Cl. Subsequently a 20ml aliquot of freshly prepared solution 2, was added and the bacteria lysed through repeated inversion of the pot. The preparation was stored on ice for 5minutes and then mixed, by shaking, with 15ml of ice-cold solution 3, forming a flocculent white precipitate. Following a 10minute incubation on ice, the bacterial lysate could be centrifuged at 5000r.p.m. for 10minutes, and the resulting supernatant decanted into a fresh centrifuge pot through four layers of
The supernatant was mixed with 0.6 volume of isopropanol, stored at room temperature for 30 minutes and centrifuged at 10,000 r.p.m. for 5 minutes. The supernatant was discarded, and the pellet dried in a 37°C for 20 minutes before resuspension in 2 ml of Tris-EDTA buffer (10 mM Tris, 1 mM EDTA; pH 8.0).

Solution 1.: 50 mM glucose;
25 mM Tris-Cl, pH 8.0;
10 mM EDTA, pH 8.0.

Solution 2.: 0.2 N NaOH;
1% w/v sodium dodecyl sulphate.

Solution 3.: 3 M potassium acetate;
11.5% v/v glacial acetic acid.

2.2.2.3c. Purification of Plasmid DNA by Equilibrium Centrifugation in CsCl-Ethidium Bromide Gradients.

2.5 g of CsCl was added to the plasmid preparation (2.2.2.3b.) and the solution warmed to 30°C to facilitate complete dissolution of the salt. A 0.2 ml aliquot of 10 mg/ml-1 ethidium bromide was mixed with the solution, which could subsequently be placed in a Beckmann Quickseal tube (13 x 32 mm), the tube topped up with 1 mg/ml-1 CsCl, sealed and spun at 100,000 r.p.m. for 4 hours at 22°C in a Beckmann Optima Ultracentrifuge TLX (rotor TLA 100-3). Following the insertion of a 21-gauge hypodermic needle into the top of the tube, a syringe fitted with an 18-gauge needle was used to remove, firstly, the upper band of nicked circular or linear DNA followed by the lower band of intact plasmid DNA. The linearised DNA could be discarded and the plasmid DNA transferred to a 15 ml Falcon tube.

2.2.2.3d. Removal of Ethidium Bromide and CsCl from Purified Plasmid DNA.

An equivalent volume of isoamyl alcohol was mixed with the solution of plasmid DNA (2.2.2.3c.) by vortexing, and the resulting mixture centrifuged at 1,500 r.p.m. for 3 minutes at room temperature. The lower aqueous phase, containing the plasmid DNA, was removed using a DNase-free Pastette to a fresh Falcon tube. This extraction process was repeated until the pink colouration was
removed from both the aqueous and organic phases.

The DNA was purified from the CsCl by dialysis for 48 hours against 3 changes of 2L of Tris-EDTA buffer (pH 8.0) [Note: The dialysis was performed using Spectra-Por Molecularporous dialysis membrane (molecular weight cut-off 3,500; Spectrum, Houston, Texas, U.S.A.)].

A 5µl aliquot of the plasmid DNA preparation was diluted in 2.995ml of Tris-EDTA buffer (pH 8.0) and the concentration determined spectrophotometrically at 260nm. An OD$_{260}$ of 1 corresponds to a plasmid concentration of approximately 50µgml$^{-1}$ (Maniatis et al, 1989). The plasmid solution was diluted to a final concentration of 1mgml$^{-1}$ in Tris-EDTA buffer.

2.2.2.3e. Analysis of Plasmid DNA by Agarose Gel Electrophoresis.

100ng of plasmid DNA was separated electrophoretically on 0.8% agarose gels (50ml) containing 1xTris-Borate-EDTA (TBE) buffer. Electrophoresis was performed using submarine mini-gel apparatus supplied by Hoefer Scientific Instruments (Scottish Biotechnology Instrumentation, Auchterarder, Perthshire, UK.).

The 0.8% agarose gel was prepared by melting 0.4g of Agarose NA in 50ml of 1xTBE using a microwave oven. The molten agarose was cooled to ~40°C before adding 1µl of 10mgml$^{-1}$ of ethidium bromide and subsequently pouring into a level gel tray carrying a comb of well dimensions 1.5x2.6mm.

5xTBE : 5.4% w/v Tris-base.
   2.75% w/v boric acid.
   10mM EDTA (pH 8.0).

9µl of the plasmid preparation, at a concentration of 10ngµl$^{-1}$ was mixed with 1µl of DNA gel loading buffer.

The gel tray carrying the 0.7% agarose gel was placed in the pre-chilled gel apparatus, covered with 250ml of 1xTBE and the plasmid sample loaded alongside 10µl of Lambda DNA HindIII fragments (~0.1mgml$^{-1}$; Gibco BRL). The gel was run at 80volts for 1hour prior to examination with a U.V. transilluminator.

2.2.3 Transfection of Plasmid DNA into Prostatic Epithelia.

All transfections were performed with the DOTAP (N-[1-(2,3-
DOTAP is a cationic lipid that, after sonication, forms liposomes which interact with DNA, establishing stable complexes. These complexes can adhere to the cell surface, fuse with the cell membrane and release the DNA into the cytoplasm.

2.2.3.1 Transfection of pMMTV/SPAP into LNCaP, DU145, PC3 and Primary Epithelial Cells.

Cells were transfected with pMMTV/SPAP and the control vector pRc/CMV. Cells were grown in monolayer culture in eight 25cm² tissue culture flasks until approximately 60% confluence was achieved. 30μl of the transfection reagent was diluted up to 100μl with HBS in a bijou bottle. 5μg of plasmid DNA was separately diluted up to 100μl with HBS.

HBS : 20mM HEPES;
      0.15M NaCl. pH7.4.
      Sterilised by autoclaving for 15minutes at 15lb/sq.in. on liquid cycle.

Both solutions were mixed by repeated pipetting and incubated at room temperature for 10minutes, prior to the addition of 6mls of the appropriate cell culture medium. The medium was mixed with the DNA by gentle pipetting. After aspiration of the old cell culture medium, the cells could be incubated with the mixture for 16hours in a 37°C incubator.

The response of the transiently transfected cell lines to DHT (10nM) and hydroxyflutamide (10nM) in terms of their production and secretion of SPAP was assessed. SPAP transfected cells and control transfections were given 6mls of phenol red-free medium containing DCC-stripped FCS and the following supplements:

- Control : 12μl ethanol;
- DHT : 6μl 10μM DHT and 6μl ethanol;
- Hydroxyflutamide : 6μl 10μM hydroxyflutamide and 6μl ethanol;
- DHT/hydroxyflutamide : 6μl 10μM DHT and 6μl 10μM hydroxyflutamide.
The cells were incubated in a 37°C incubator for 48 hours before harvesting the medium for immediate assay or storage at -20°C.

2.2.3.2 Co-transfection of pCMV/ARcom and pMMTV/SPAP into LNCaP and PC3 Cells.

LNCaP and PC3 cells were transfected with pCMV/ARcom plus pMMTV/SPAP or pRc/CMV plus pMMTV/SPAP.

The cell lines were grown in eight 25cm² flasks until 60% confluence was achieved. The plasmids were complexed with the DOTAP reagent as outlined in 2.2.3.1. and mixed with 6mls of cell culture medium. The plasmid mixtures were combined with their transfection partners and added to the cultured cells. The cells were incubated in a 37°C incubator for 16 hours. The cotransfected cells could subsequently be supplemented as described in 2.2.3.1. and incubated at 37°C. The medium was harvested after 48 hours.

2.2.3.3 Assay of Secreted Placental Alkaline Phosphatase (SPAP).

Each medium sample was assayed in quadruplicate. Immediately prior to assay, 1xPNPP (p-nitrophenylphosphate) was prepared through dilution of 20xPNPP in DEA buffer.

The medium was mixed through brief vortexing and 50μls aliquoted into a 96well plate and heat-inactivated by incubation at 65°C for 1 hour. 200μl of 1xPNPP was delivered to each well and the plate incubated at 37°C for 30 minutes prior to measurement of the absorbance at 415 nm on a microplate reader. The absorbance of each medium sample was blanked against 4 wells containing 200μl of 1xPNPP only.

**DEA buffer:** 10.72mls 98% w/v diethanolamine; (100ml) 1.636g NaCl; 50μls 1M MgCl₂, pH9.85. Stored at 4°C.

**20xPNPP:** 371mg Sigma 104 phosphatase substrate in 10mls of DEA buffer. Store at -20°C in a glass universal shielded from light by wrapping in foil.
The concentration of SPAP in each sample in units ml\(^{-1}\) was calculated using the equation below.

\[
[\text{SPAP}] \text{ in medium} = \frac{\text{OD}_{415\text{nm}}}{t \times 18.5 \times v} \text{ units ml}^{-1}
\]

where 
- \(t\) = time of reaction (minutes),
- \(v\) = volume of medium assayed (ml).

2.2.3.4 bcl-2 Expression in LNCaP and PC3 Cells Transfected with pCMV/ARcom.

The cell lines were grown to 60% confluence in three 25cm\(^2\) tissue culture flasks and transfected with pCMV/ARcom as outlined for pMMTV/SPAP and pRc/CMV in 2.2.3.1.

Two flasks of cells were transfected with pCMV/ARcom, with the remaining flask providing a control for normal cellular expression of bcl-2. The cells were transfected for 16 hours, after which the medium from the three flasks was replaced with phenol red-free medium containing DCC-stripped FCS. One flask of transfected cells was supplemented with 10nm DHT and the cells incubated at 37\(^\circ\)C for 24 hours. Protein was isolated from the cultured cells as outlined in 2.2.6.1.

2.2.4 Isolation of Nucleic Acids

2.2.4.1 Isolation of Total RNA from Cultured Cells.

Total RNA was extracted from cultured cells using a modification of the AGPC (acid-guanidinium-phenol-chloroform) method of Chomczynski and Sacchi (1987).

In order to minimize RNAase contamination of the RNA preparation, all solutions were prepared using RNAase-free glassware & plasticware, water and chemicals. Wherever possible, the solutions were treated with 0.1% DEPC for 24 hours at room temperature and subsequently autoclaved for 15 minutes at 15lb/sq.in. on liquid cycle.

Solution D was prepared through the addition of 2-Mercaptoethanol to the GTC solution to a final concentration of 0.1M.
GTC solution: 4M guanidine isothiocyanate; 25mM sodium citrate, pH 7.0. 0.5% N-lauroylsarcosine.

Cells were grown in monolayer culture to 80% confluency in 75cm² tissue culture flasks, washed in DEPC-treated phosphate buffered saline and lysed in 2.5mls of solution D per flask. The tissue culture flask was shaken, at 4°C, for 10 minutes to ensure complete cell lysis. 500μl Aliquots of the cell lysate were placed in Eppendorf tubes and 50μl of 2M sodium acetate (pH4.0) delivered to each. The tubes were vortexed for 10 seconds prior to the addition of 500μl of water saturated phenol (pH4.0) plus 100μl of chloroform-isoamylalcohol (49:1). The resulting suspension was vortexed for 10 seconds and then incubated on ice for 15 minutes. The samples were subsequently centrifuged at 13,000 r.p.m. for 20 minutes at 4°C in a microcentrifuge and the upper, aqueous phase transferred to a fresh Eppendorf tube where it was mixed with 1 volume of isopropanol. The RNA was precipitated overnight at -20°C. The RNA precipitate was sedimented, through further centrifugation, at 13,000 r.p.m. for 20 minutes (4°C). The pelleted RNA was resuspended in 60μl of solution D, the samples pooled and precipitated with 1 volume of isopropanol at -20°C for 2 hours. The RNA sample was centrifuged at 13,000 r.p.m. for 10 minutes (4°C) and the resulting pellet washed in 200μl of 75% ethanol prior to a further spin for 5 minutes. The supernatant was carefully aspirated and the RNA air dried for 30 minutes at room temperature before resuspension in 50μl of nuclease-free water.

A 5μl aliquot of the RNA solution was diluted in 2.995ml of DEPC treated water and the concentration and purity of the preparation determined spectrophotometrically. In order to quantify the amount of RNA, readings were taken at 260nm and 280nm. An OD260 of 1 corresponds to an RNA concentration of approximately 40μg/ml (Maniatis et al). The ratio between the readings at 260nm and 280nm (OD260/OD280) provides an estimate of the purity of the RNA preparation, with a ratio of 2 corresponding to a pure sample. If the OD260/OD280 ratio was less than 2, the RNA sample was extracted again with phenol-chloroform.

45μl of phenol : chloroform (1:1) was added to the RNA solution, the suspension vortexed and spun at 13,000 r.p.m. for 5 minutes (4°C). The aqueous phase was transferred to a fresh Eppendorf tube, and mixed with 45μl of chloroform prior to a further spin at 13,000 r.p.m. for 5 minutes (4°C). The aqueous phase was removed and the RNA precipitated by addition of 3 volumes of ethanol and 3M sodium acetate to 0.3M. The RNA precipitate was pelleted and
subsequently resuspended in 50\mu l of nuclease-free water. The concentration and purity of the prepared RNA could be reassessed, spectrophotometrically, as before.

2.2.4.2 Isolation of Total RNA from Snap-Frozen Tissue.

Prostatic tissue obtained by TURP was promptly snap frozen in liquid nitrogen, and either used immediately in the preparation of RNA or stored at -70°C for use within 7 days of resection. Up to 1 gram of frozen tissue could be used per preparation.

The frozen tissue was disaggregated using a Mikro-Dismembrator II (B. Braun Biotech International GmbH, Melsungen, Germany.) fitted with a 7 ml teflon shaking flask and accompanying stainless steel grinding ball, both of which were pre-chilled in liquid nitrogen. The Mikro-Dismembrator II was operated at full power for 15 seconds, and the resulting tissue powder scraped into 5 mls of solution D (2.2.4.1.) using a pre-chilled micro-spatula. The stainless steel grinding ball was also placed into the aliquot of solution D, since a significant amount of the powdered tissue was seen to adhere to the ball. The RNA preparation was continued as outlined in 2.2.4.1.

2.2.4.3 Isolation of Genomic DNA from Cultured Cells.

The cells in two 75cm² flasks were washed once in PBS 'A', subsequently scraped into 10 mls of PBS 'A' and pelleted by centrifugation at 1500 r.p.m. for 5 minutes. The cell pellet was resuspended in 1 ml of lysis buffer and incubated on ice for 20 minutes with occasional swirling.

Lysis buffer: 0.32M sucrose;
10mM tris-HCl, pH7.5;
5mM MgCl₂;
1% Triton X-100.

The resulting lysate was spun for 20 minutes at 2500 r.p.m. at 4°C to sediment the nuclei. The nuclear pellet was resuspended in 4.5 ml of resuspension buffer, mixed immediately with 0.5 ml of a solution of 5% SDS and 2 mg ml⁻¹ proteinase K and incubated at 37°C for 2-12 hours.
Resuspension buffer: 0.075M NaCl; 0.024M EDTA, pH 8.0.

5ml of phenol saturated with 20mM tris (pH 8.0) was added to the preparation and mixed gently by inversion. The mixture was spun at 2,000 r.p.m. for 10 minutes and the upper aqueous phase transferred to a fresh tube using a wide-bore Pasteur pipette. 5ml of chloroform-isoamylalcohol (24:1) was mixed with the aqueous phase and the suspension spun as before. The aqueous phase was extracted once more with phenol and chloroform-isoamylalcohol, prior to the addition of 0.5ml 3M sodium acetate and 11ml ethanol at room temperature. After mixing, the DNA precipitate was lifted out using a bent Pasteur pipette and delivered to 500ml of Tris-EDTA buffer (pH 8.0). The DNA was stored at 4°C and allowed to dissolve over 48 hours with occasional stirring.

A 5μl aliquot of the DNA solution was diluted in 2.995ml of Tris-EDTA buffer and the concentration of the preparation determined spectrophotometrically at 260nm. An OD\textsubscript{260} of 1 corresponds to a double-stranded DNA concentration of approximately 50μg/ml\textsuperscript{-1}.

2.2.5 Analysis of Nucleic Acids.

2.2.5.1 Reverse Transcription of Total RNA.

The protocol for first strand cDNA synthesis from samples of total RNA is a modification of that described in the Promega Protocols and Applications Guide (2nd Edition, 1991). 5μg of total RNA was diluted in nuclease-free water up to 49.5μl and aliquoted into a 500μl micro-test tube. The tube was heated to 85°C for 5 minutes and subsequently cooled on ice for 3 minutes. The tube was pulsed in a microfuge and the following ingredients added to the RNA solution:

- 2.5μl of rRNasin ribonuclease inhibitor (40unitsμl\textsuperscript{-1});
- 20μl of 25mM MgCl\textsubscript{2};
- 10μl of 10x reverse transcription buffer;
- 10μl of 10mM dNTP mixture;
- 5.0μl of oligo(dT)\textsubscript{15} (0.5μgμl\textsuperscript{-1});
- 3.0μl of AMV reverse transcriptase (HC; 25unitsμl\textsuperscript{-1}).

The tube was vortexed, pulsed in a microfuge and then incubated at 42°C for
60 minutes. Following the polymerisation step, the reverse transcription mixture was heated to 99°C for 5 minutes followed by a 5 minute incubation on ice.

When using random primers for first strand cDNA synthesis, the reaction was first incubated at room temperature for 10 minutes prior to the 60 minutes at 42°C. This pre-incubation ensures that the primers remain hybridised when the temperature is raised to 42°C.

2.2.5.2 Analysis of Genomic DNA and cDNA by the Polymerase Chain Reaction (PCR).

2.2.5.2a. Amplification of Genomic Androgen Receptor DNA Sequences.

The androgen receptor gene was analysed using a series of primers that span each of its 8 exons. All primers except 1.CAGs and 1.CAGas were described in Marcelli et al (1990). The positions of the primers on the androgen receptor gene are indicated in Figure 2.03.

Primers.

Note: The primer sequences are written 5’ to 3’ with their locations on the sequenced regions of the androgen receptor gene bracketed. The primers were designed using sequence information obtained from Genebank EMBL. The accession numbers for each sequence are quoted. Primers that locate to intron sequences are indicated with an asterix.

Exon 1 (AC. No. : M35844)

Region 1 (AR1.1 product size 354bp)

AR1.1s  5' TGG AAG ATT CAG CCA AGC TCA AG3' (139-162)
AR1.1as TTC CTC ATC CAG GAC CAG GTA GCC T (492-468)

Region 2 (AR1.2 product size 534bp)

AR1.2s GCG CAG CAC CTC CCG GCG CCA GTT T (299-323)
AR1.2as CTA AGT AAT TGT CCT TGG AGG AAG T (832-808)

Region 3 (AR1.3 product size 325bp)

AR1.3s GCA GCA GCT GCC AGC ACC TCC GGA C (591-615)
AR1.3as CAA CGC CTC CAC ACC CAG GCC CAT G (915-891)
Region 4 (AR1.4 product size 710bp)

AR1.4s  TCC TTC AGC AAC AGC AGG AAG C (731-755)  
AR1.4as  GGC TGA GGG TGA CCC AGA ACC GGG T (1440-1416)

Region 5 (AR1.5 product size 233bp)

AR1.5s  AGC CTG CAT GGC GCG GGT GCA GCG G (1390-1414)  
AR1.5as  AGC CCC TGA GGG GGC CGA GTG TAG (1622-1599)

Region 6 (AR1.6 product size 153bp)

AR1.6s  CAC CTG ATG TGT GGT ACC CTG GCG G (1390-1414)  
*AR1.6as  CGA AAG CGA CAT TTC TGG AAG GAA A (1801-1777)

CAG Repeat (1.CAG product size 198bp)

1.CAGs  TGC GCG AAG TGA TCC AGA AC (251-270)  
1.CAGas  CTT GGGGAG AAC CAT CCT CA (429-448)

Exon 2 (AC. No.: M35845) (AR2 product size 269bp)

*AR2s  CAT TCA GTG ACA TGT GTT GCA TTG G (1-25)  
*AR2as  TGA AAG GTT AGT GTC TCT CTC TGG AA (269-244)

Exon 3 (AC. No.: M35847) (AR3 product size 185bp)

*AR3s  AAC TTA TTA TCA GGT CAA TCA ACT C (1-25)  
*AR3as  AGG AAG AGA AAA AGT ATC TTA C (185-161)

Exon 4 (AC. No.: M35847) (AR4 product size 371bp)

*AR4s  TGA TAA ATT CAA GTC TCT CTT CCT T (8-32)  
*AR4as  CAC TAA ATA TGA TCC CCC TTA TCT C (378-354)

Exon 5 (AC. No.: M35848) (AR5 product size 284bp)

*AR5s  CCC AAC AGG GAG TCA GAC TTA GCT C (3-27)  
*AR5as  CCA ACC AGG TCT GGC CAA GCT GGT GT (266-261)

Exon 6 (AC. No.: M35849) (AR6 product size 245bp)

*AR6s  TAT TGT AAA CTT CCC CTC ATT CCT T (1-25)  
*AR6as  AAT GGC AAA AGT GGT CCT TGA A (245-221)

Exon 7 (AC. No.: M35850) (AR7 product size 266bp)

*AR7s  TCT AAT GCT CCT TCG TGG GCA TGC T (1-25)  
*AR7as  GCT CTA TCA GGC TGT TCT CCC TGA T (266-242)
**PCR Reaction.**

Genomic DNA isolated from the LNCaP, Du145 and PC3 cell lines and male human placental DNA were analysed. DNA was diluted to a concentration of 100ngμl⁻¹.

All PCR reactions were carried out in 500μl micro-test tubes on a Hybaid thermal reactor. [Note: In general, if multiple samples were to be analysed bulk solutions containing the Taq DNA polymerase, reaction buffer, dNTPs and water would be prepared. 79μl of this mixture could then be delivered to the DNA and primers. In this way, a degree of consistency between tubes could be maintained.]

**PCR reaction mix:**
- 1μl of genomic DNA (100ngμl⁻¹);
- 10μl of sense primer (50μgml⁻¹);
- 10μl of antisense primer (50μgml⁻¹);
- 10μl of 10x Reaction Buffer (containing 1.5mM MgCl₂);
- 16μl of dNTP mix (1.25mM of each dNTP);
- 0.2μl of Taq DNA polymerase (5unitsμl⁻¹);
- 52.8μl of nuclease-free water.

The components of the PCR reaction were thoroughly mixed through brief vortexing, the mixture overlaid with 50μls of mineral oil and the tube subsequently pulsed in a microfuge. Note: The tubes should be evenly distributed over the block of the thermal reactor.

In all PCR reactions 35 amplification cycles were employed.

**PCR cycles:**
- a. Exons 1 (except the CAG repeat), 2, 5, 6, 7 and 8.

  Denaturation    95°C, 1 minute.
  Annealing &
  Polymerisation 68°C, 6 minutes.
Figure 2.03 Locations of the Primer Pairs for the Analysis of Androgen Receptor Genomic DNA and cDNA.
b. Exons 3 & 4 and the CAG repeat region.

Denaturation 95°C, 1 minute.
Annealing 55°C, 2 minutes.
Polymerisation 72°C, 2 minutes.

The PCR products were analysed by electrophoresis in 2% agarose gels.

75mls of 2% agarose was prepared using 1xTBE buffer as outlined in 2.2.4.5. 1μl of 10mgml⁻¹ ethidium bromide was added prior to pouring the agarose into a sealed, level midi-gel tray carrying a 5.5x1.5mm 16 well comb. The gel was run in 750ml of 1xTBE in midi-gel apparatus (Northumbria Biologicals Ltd.). 18μl of each PCR reaction with 2μl of DNA loading buffer could be loaded per well, alongside 20μl of 100bp ladder (~0.1mgml⁻¹). [Note: After removing aliquots of the PCR reaction from the reaction tube, the pipette tip was wiped on the rim of the tube to limit carry-over of mineral oil]. Electrophoresis was performed for 4 hours at 50volts, after which the gel was examined and photographed under U.V. transillumination.

2.2.5.2b. Amplification of Androgen Receptor and bcl-2 cDNA Sequences.

cDNA was prepared as described in 2.2.5.1.

Primers.

Note: The primer sequences are written 5' to 3' with their locations on the gene bracketed. All sequence information was obtained from Genebank EMBL. The accession numbers for the bcl-2 sequences are quoted.

a. Androgen Receptor.

Two sets of primers were used to analyse the androgen receptor cDNA locating to exons 2 & 3 and exons 7 & 8. The former set span intron 2 (>9kb) and the latter set intron 7 (~13kb). [Note: The accession numbers for exons 2, 3, 7 and 8 of the androgen receptor gene are quoted in 2.4.2.1..]. The locations of the primers for the analysis of androgen receptor cDNA are shown in Figure 2.03.
2.3 (AR2.3 product size 265bp)

AR2.3s (Exon 2) \[5'GGA GAC TGC CAG GGA CCA TGT \] (48-68)
AR2.3as (Exon 3) \[TCC CAG AGT CAT CCC TGC TTC \] (159-139)

7.8 (AR7.8 product size 228bp)

AR7.8s (Exon 7) \[GAT GAA CTT CGA ATG AAC TAC \] (94-114)
AR7.8as (Exon 8) \[CAC TTG CAC AGA GAT GAT CTC \] (159-139)

b. bcl-2

The bcl-2 gene is believed to be transcribed into at least 2 mRNA's (Tsujimoto and Croce, 1986). Sets of primers specific to the 5.5kb (bcl-2α) and 3.3kb (bcl-2β) transcripts of the bcl-2 gene and which span a putative intron located at positions 2043bp and 732bp respectively were employed. The primer sets share the same sense oligonucleotide. The positions of each primer pair on their respective transcripts are indicated in Figure 2.04.

bcl-2α (AC. No. N81292) (5.5 product size 247bp)

5.5s \[CAC ACC TGG ATC CAG GAT AAC \] (2014-2034)
5.5as \[ATG GTA CAT CAC TGA CAA TGC A \] (2260-2239)

bcl-2β (AC. No. 81293) (3.5 product size 199bp)

3.5s \[CAC ACC TGG ATC CAG GAT AAC \] (702-722)
3.5as \[AGT GAA CGC TTT GTC CAG AGG \] (900-880)

c. HGPRT

Primers directed against the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) gene were used in the control PCR reactions. HGPRT is a housekeeping protein that catalyses the conversion of the free purines, hypoxanthine and guanine to IMP and GMP respectively. For each cDNA sample examined, no DNA and HGPRT control PCR reactions were run in parallel. HGPRT primers were used in both controls.

HGPRT (product size ~310bp)

HGPRTs \[CTT GCT CGA GAT GTG ATG AAG \]
HGPRTas \[GTC TGC ATT GTT TTG CCA GTG \]
Figures 2.04 Locations of the Primer Pairs for the Analysis of Bcl-2 cDNA.

a. Bcl2α

![Diagram of Bcl2α](image)

b. Bcl2β

![Diagram of Bcl2β](image)
PCR Reaction.

The procedure for the amplification of cDNA sequences was essentially the same as that described for genomic DNA (2.4.2.1), with the reaction mixture being described below.

**PCR reaction mix:**
- 20μl of reverse transcription reaction;
- 10μl of *sense* primer (50μg/ml⁻¹);
- 10μl of *antisense* primer (50μg/ml⁻¹);
- 8μl of 10x reaction buffer (containing 1.5mM MgCl₂);
- 16μl of dNTP mix (each dNTP at 1.25mM);
- 0.2μl of Taq DNA polymerase (5unitsμl⁻¹);
- 35.8μl of nuclease-free water.

35 amplification cycles were used in all PCR reactions.

**PCR cycles:**

- **a. Androgen Receptor - AR2.3 and AR7.8.**
  - Denaturation: 94°C, 1 minute.
  - Annealing: 55°C, 1 minute.
  - Polymerisation: 72°C, 2 minutes.

- **b. bcl-2α - 5.5.**
  - Denaturation: 94°C, 1 minute.
  - Annealing: 58°C, 1 minute.
  - Polymerisation: 72°C, 2 minutes.

- **c. bcl-2β - 3.5.**
  - Denaturation: 94°C, 1 minute.
  - Annealing: 60°C, 1 minute.
  - Polymerisation: 72°C, 2 minutes.

The PCR products were analysed by agarose gel electrophoresis as outlined in 2.2.5.2a.
2.2.5.2c. Restriction Endonuclease Digestion of Amplified Androgen Receptor and bcl-2 cDNA.

The products of the PCR reactions form 2.4.2.2. were cut with appropriate restriction enzymes to determine the efficacy of the amplification.

AR2.3, AR7.8, 5.5 and 3.5 were digested with Hind III (restriction site - A/AGCTT), Dra III (restriction site CACN\textsubscript{3}/GTG), Alu I (restriction site - AG/CT) and EcoRII (restriction site - /CC(\textup{A}T)GG) respectively. The restriction sites and digest product sizes are listed below.

AR2.3 (Hind III) : restriction site - base 125 (exon2).
product sizes - 187bp and 78bp.

AR7.8 (Dra III) : restriction site - base 108 (exon8)
product sizes - 177bp and 51bp.

5.5 (Alu I) : restriction site - base 2140.
product sizes - 127bp and 120bp.

3.5 (EcoRII) : restriction site - base 805.
restriction site - 104bp and 95bp.

**Restriction Digest** : 10µl of PCR reaction;
2.5µl of 10x Palette\textsuperscript{TM} reaction buffer;
0.5µl of restriction endonuclease;
12µl of nuclease buffer.

Note: Blue, yellow and green Palette\textsuperscript{TM} buffers were used with Hind III, Alu I and EcoRII respectively.

The digestion products were analysed by electrophoresis through 2% agarose gels as outlined as in 2.2.5.2a..

2.2.5.3 Northern Analysis.

The protocol described here for the analysis of RNA by electrophoresis through formaldehyde gels followed by Northern transfer, is an adaptation of that
described in Maniatis (1989; 7.43-7.48).

All solutions were prepared using RNAase-free glassware & plastic ware, water and chemicals. Wherever possible, the solutions were treated with 0.1% DEPC for 24 hours at room temperature and subsequently autoclaved for 15 minutes at 15lb/sq.in. on liquid cycle.

2.2.5.3a. Denaturing RNA Gel Electrophoresis.

Up to 20μg of each RNA sample were separated electrophoretically on 1% agarose gels (75ml) containing 2.2M formaldehyde and 1x gel buffer. Electrophoresis was performed using midi-gel apparatus supplied by Northumbria Biologicals LTD.. [ Note : The electrophoresis tank and gel tray were cleaned thoroughly with a weak detergent solution, rinsed in water and soaked in a solution of 3% hydrogen peroxide for 10 minutes. The apparatus was then rinsed exhaustively in DEPC-treated water. ].

The gel was prepared by melting 0.75g of Agarose NA (Pharmacia Biotech LTD., Milton Keynes, UK.) in 54ml of DEPC-treated water using a microwave oven. The molten agarose was allowed to cool to approximately 60°C prior to the addition of 1μl of 10mgml⁻¹ ethidium bromide, 7.5ml of 10x gel buffer and 13.5ml of 37% formaldehyde (12.3M, pH>4.0). Following thorough mixing of the gel components, the agarose could be poured into a sealed, level gel tray carrying a comb of well dimensions 2 x 1.5mm thick.

10x gel buffer: 0.2M MOPS, pH7.0.
0.05M sodium acetate.
0.01M EDTA, pH8.0.

Note : The gel buffer was autoclaved before use and should achieve a pH of between 5.5 and 7.0.

An aliquot of each RNA sample corresponding to 20μg of total RNA was dried down in a vacuum oven at 60°C and the pellet resuspended in 4.5μl of nuclease-free water. The samples were incubated on ice for 30 minutes with occasional vortexing to ensure complete dissolution of the RNA and subsequently mixed with 2.0μl of 10x gel buffer, 3.5μl of 37% formaldehyde and 10.0μl of deionised formamide (Clontech Laboratories) The specimens could then be
incubated at 55°C for 15 minutes, cooled on ice for a further 5 minutes and pulsed in a microfuge, ready to be loaded onto the gel immediately.

The gel tray carrying the formaldehyde gel was placed in the electrophoresis apparatus, covered with ~800ml of 1x gel buffer and the total RNA samples loaded alongside 20μl of undenatured 1kb DNA ladder (~0.1mgml⁻¹; Gibco BRL, Paisley, UK). [Note that the RNA samples were run without the addition of loading buffer]. The gel was run at 18volts for 16 hours, after which it could be examined with a U.V. transilluminator and photographed under illumination.

2.2.5.3b. Northern Blotting.

RNA separated on formaldehyde gels was transferred immediately to nitrocellulose filters by capillary elution using 20xSSC as the transfer buffer.

The blotting apparatus consists of a basin straddled by a glass plate, both of which should be thoroughly cleaned with detergent and rinsed with de-ionised water prior to use. A piece of Whatman 3MM paper, of sufficient length to reach the bottom of the basin when suspended over the glass support, was pre-wet in 20xSSC for use as a wick.

20xSSC : 3M NaCl.
0.3M tri-sodium citrate. pH7.0.

Sufficient 20xSSC must be placed in the basin to maintain a supply to the wick over a 24 hour period.

After trimming off the unused portions and cutting off one corner to facilitate orientation, the gel was soaked twice in 500ml of DEPC-treated water for 5 minutes each, to remove excess formaldehyde that might otherwise interfere with the transfer. The gel was placed on the wick with its upper surface facing downward, and surrounded completely with Saran wrap. A piece of nylon-supported nitrocellulose (Hybond C extra), ~1mm larger than the gel in both dimensions, initially wetted with DEPC-treated water and then soaked in 20xSSC for 5 minutes, was placed directly on top of the gel and the appropriate corner removed to orient. Two pieces of 3MM paper, cut to the same dimensions as the nitrocellulose filter and soaked in 2xSSC, were then positioned above the membrane. At each stage, air bubbles were excluded by gently rolling each layer flat. A stack of tissues ~8cm
high were placed above the 3MM papers, a glass plate located on top and the entire stack weighed down with a 500g weight. As the tissues became, wet they were replaced. The transfer of RNA was allowed to proceed for 24hours, after which the apparatus was dismantled, the nitrocellulose filter soaked in 6xSSC for 5minutes-to remove any adherent agarose-and then dried at room temperature for 30minutes on a piece of 3MM paper. Finally, the filter was placed between two fresh pieces of 3MM paper, and baked for 1hour at 80°C in a vacuum oven in order to irreversibly bind the RNA to the nitrocellulose. Filters were stored sealed in Saran wrap at room temperature until use.

2.2.5.4 Southern Analysis.

cDNA prepared using LNCaP, DU145 and PC3 mRNA and amplified, by PCR, with the AR7.8 primer pair was examined.

The gel used to analyse the products of the PCR reaction was trimmed, one corner removed to aid orientation and subsequently immersed in 500ml of a solution of 1.5M NaCl, 0.5N NaOH to denature the DNA. The gel was gently agitated on an orbital shaker for 45minutes. Following a brief rinse in deionised water the gel could be neutralised by soaking for 30minutes in 500ml of 1M Tris (pH7.4), 1.5M NaCl at room temperature, with gentle agitation. The neutralisation solution was replaced with another 500ml volume and the gel soaked for a further 15minutes. The DNA was blotted onto Hybond C extra as described in 2.2.5.3b..

2.2.5.5 Preparation of Radiolabelled Probes.

2.2.5.5a. Production of Androgen Receptor Probes.

Two probes directed against androgen receptor sequences were synthesised by PCR. These are referred to as 1.1ARp and AR7.8p, and were respectively used in the analysis of Northern blotted RNA, and 7.8 PCR products blotted onto nitrocellulose as outlined in 2.2.5.3b.. The locations of the probes on the androgen receptor cDNA are indicated in Figure 2.05.

Note: The PCR reactions for the synthesis of both probes were performed as outlined in 2.2.5.2a. and used pCMV/ARcom (1ng/µl) as template DNA. 35 cycles were used both reactions.
1.1ARp. (product size 1093bp)

Primers: AR2.3s and AR7.8as.

PCR cycle:  
- Denaturation: 94°C, 1 minute.  
- Annealing: 50°C, 1 minute.  
- Polymerisation: 72°C, 4 minutes.

AR7.8p. (product size 166bp)

Primers:  
- AR7.8ps: 5'CAA GGA ACTCGA TCG TAT CA3' (117-136)  
- AR7.8as: GTC CACGCT CAC CAT GTG (120-183)

PCR cycle:  
- Denaturation: 95°C, 1 minute.  
- Annealing: 55°C, 1 minute.  
- Polymerisation: 72°C, 2 minutes.

2.2.5.5b. β–Actin.

pAM41 (0.1µgµl-1) was linearised through EcoRI digestion.

Restriction Digest:  
- 10µl of pAM41;  
- 2.5µl of 10xSuRE/Cut buffer H;  
- 0.5µl of EcoRI;  
- 12µl of nuclease-free water.

2.2.5.5c. Purification and Analysis of Probes.

The androgen receptor probes were purified from the PCR reaction components by electrophoresis through 2% agarose gels prepared using low melting point agarose and 1xTBE (2.2.4.5.) Electrophoresis was performed using submarine mini-gel apparatus and the gel was formed with a preparative comb of dimensions 1.5mmx47mm.

60µl of the PCR reaction was mixed with 10µl of DNA gel loading buffer and subsequently loaded along the entire length of the preparative well. 15µl of either a 1kb DNA ladder (~0.1mgml⁻¹; 1.1ARp) or a 100bp ladder (~0.1mgml⁻¹; 7.8ARp) was loaded alongside the PCR reaction. The gel was run at 80volts for 1 hour prior to examination under U.V. illumination.

The appropriate band was excised from the gel using a clean scalpel, the agarose slice cut into three equally sized fragments and transferred into three...
Figure 2.05 The Positions of the Androgen Receptor Probes, 1.1ARp and AR7.8p, on the Androgen Receptor cDNA.
Eppendorf tubes. The agarose was melted at 70°C and subsequently mixed with 100μl of tris-saturated phenol (pH8.0) per tube. The tubes were incubated at -70°C for 10 minutes, thawed and then centrifuged at 13,000 r.p.m. for 5 minutes in a microfuge. The preparation was removed, transferred to fresh tubes and extracted sequentially with 100μl of tris-saturated phenol, 100μl of tris-saturated phenol + 100μl of chloroform-isoamylalcohol and finally 100μl of chloroform-isoamylalcohol per tube. After each extraction step the tubes were centrifuged at 13,000 r.p.m. for 5 minutes. The DNA could be precipitated at the conclusion of the extractions through mixing with 0.15 volumes of NaCl and 2.5 volumes of 100% ethanol. The DNA precipitate was pelleted by a further spin at 13,000 r.p.m. for 10 minutes, dried at room temperature for 30 minutes and resuspended in 20μl of nuclease-free water per tube. The resuspended DNA was pooled and mixed thoroughly by vortexing.

The probes were analysed by restriction endonuclease digestion followed by electrophoresis through 2% agarose gels, as outlined in 2.4.2.3.

1.1.ARp and AR7.8p were digested with EcoRI (restriction site - G/AATTC) and EcoRII (restriction site - /CC(Ap)GG) respectively. EcoRI (SuRE/Cut buffer - H) cuts 1.1ARP at base 141 of exon 6, yielding two products of sizes 791bp and 302bp. Digestion of AR7.8p with EcoRII produces 2 bands on an agarose gel at 81bp and 85bp. [Note: Only 5μl of the PCR reaction was digested, and the digestion reaction, as described in 2.2.5.2c., was altered accordingly.]

The digestion products were run alongside 5μl of uncut probe DNA (containing 1μl of DNA loading buffer).

Linearised pAM41 was purified as described for the androgen receptor probes, with the entire digestion reaction (2.2.5.5b.) being loaded onto the preparative gel.

2.2.5.5d. 32P-Labelling of cDNA.

The androgen receptor and β -actin probes were 32P-labelled using the Megaprime™ DNA labelling system (RPN 1606) with [α-32P]dCTP. The DNA synthesis was primed using random sequence hexanucleotides as first described by Feinberg and Vogelstein (1983 and 1984).

10μl of purified probe (2.2.5.5c.) was diluted into 18μl of nuclease-free water and subsequently mixed 5μl of primer solution. The DNA was denatured by incubation at 95°C for 5 minutes prior to the addition of 10μl of Megaprime reaction buffer, 5μl of [α-32P]dCTP and 2μl of "Klenow" DNA polymerase 1. The components of the reaction were thoroughly mixed by repeated pipetting before
incubating at 37°C for 1 hour.

2.2.5.5e. Separation of Radio-labelled Probes from Unincorporated [α-32P]dCTP.

Labelled probes were purified chromatographically using NICK™ column packed with Sephadex G-50 DNA Grade F.

The top cap of the column was removed, the liquid decanted and the column then rinsed with 3ml of equilibration buffer. The bottom cap was discarded and the Sephadex gel subsequently equilibrated with 3ml of equilibration buffer. The buffer was allowed to completely enter the gel prior to loading the entire labelling reaction (2.2.5.5d.). 400μl of equilibration buffer was added to the column and permitted to enter the gel. The purified sample was eluted through the addition of a further 400μl of equilibration buffer and collected in a sterile Eppendorf tube.

Equilibration buffer: 10mM Tris-Cl, pH7.5; 1mM EDTA.

2.2.5.6 Hybridisation of Radio-labelled Probes to Nucleic Acids Immobilised on Nitrocellulose Filters.

2.2.5.6a. Prehybridisation and Hybridisation.

Note: The procedures described apply to both Northern and Southern blots.

The filters were prehybridised in a Hybritube 15 (Gibco BRL) containing 12.5ml of prehybridisation fluid at 42°C for 24hours.

Prehybridisation fluid: 6.25ml formamide; 3.125ml 20xSSPE; 0.5ml 50xDenhardt’s reagent; 0.125ml 10% SDS; 2.5ml DEPC-treated water; 0.125ml sonicated, denatured salmon testes DNA (10mgml⁻¹).
20xSSPE: 3M NaCl;
0.23M Monosodium phosphate (NaH$_2$PO$_4$);
25mM EDTA, pH7.4.
(Sterilised by autoclaving for 30 minutes at 15lb/sq.in. on liquid cycle)

50xDenhardt’s: 1% Ficoll;
Reagent 1% polyvinylpyrrolidone;
1% bovine serum albumin.
(Sterilised by filtration through a 0.2µm bottle-top filter).

The purified radio-labelled probe was denatured through heating to 95°C for 5 minutes and immediately cooling on ice. It could then be added directly to the prehybridisation fluid. Hybridisation was performed for 24 hours at 42°C, after which the filter could be removed from the hybridisation tube and washed sequentially in increasingly stringent washing buffers (2.2.5.6b-c).

Washed membranes were rinsed briefly in 0.1xSSC, drained and wrapped in Saran wrap. The blots could be placed in a Fastrad Autoradiography cassette with intensifying screens and the filter covered with a sheet of Hyperfilm™MP. The autoradiography cassette could be placed in a -70°C freezer for 24-48 hours after which the film could be developed in an RP X-Omat developer.

2.2.5.6b. Detection of Androgen Receptor mRNA using 1.1ARp.

Northern blotted total RNA was probed with labelled 1.1ARp for androgen receptor gene expression, as outlined in 2.2.5.6a. Probed blots were washed at room temperature as described below.

Wash 1: 3xSSC, 0.1%SDS. 20 minutes.
Wash 2: 2xSSC, 0.1%SDS. 20 minutes.
Wash 3: 1xSSC, 0.1%SDS. 20 minutes.
Wash 4: 0.2xSSC, 0.1%SDS. 10 minutes.
Wash 5: 0.1xSSC, 0.1%SDS. 10 minutes.

Note: After each washing step the blot was monitored to determine the level of radioactivity remaining hybridised.
2.2.5.6c. Detection of AR7.8 PCR Products using AR7.8p.

Southern blotted AR7.8 products were probed with labelled AR7.8p, as described in 2.2.5.6a. The blot was washed under the following conditions:

- **Wash 1**: 1xSSC, 0.1%SDS. 15 minutes. Room temperature.
- **Wash 2**: 0.1xSSC, 0.1%SDS. 15 minutes. Room temperature.
- **Wash 3**: 0.1xSSC, 0.1%SDS. 15 minutes. 65°C.

2.2.5.6d. Detection of β-Actin.

Northern blots probed with 1.1ARp were stripped using the procedure described in 2.2.5.6e, and re-probed with labelled mouse β-actin cDNA. The blot was washed as described for AR7.8p with an extra 15 minute wash in 0.1xSSC and 0.1%SDS at 65°C.

2.2.5.6e. Removal of Radio-labelled Probes from Nitrocellulose Filters.

500ml of a solution of 0.05xSSC and 0.01m EDTA (pH8.0) was heated to boiling, removed from the heat and 5ml of 10% SDS added. The nitrocellulose filters were immersed in the hot stripping solution for 15 minutes. The procedure was repeated with a further 500ml of hot stripping solution. The filters could be rinsed briefly in 500ml of 0.01xSSC, the majority of the liquid removed by placing on a pad of paper towels and the blot re-applied to a sheet of film for 48 hours (2.2.5.6a). If all of the probe was seen to be removed, the blot could be dried at room temperature for 30 minutes and stored. Re-stripping could be performed if necessary.

2.2.6 Analysis of Proteins by Western Blotting.

2.2.6.1 Isolation of Protein from Cultured Cells.

Cells were grown in monolayer culture to ~80% confluency in 75cm² (25cm²) tissue culture flasks. The cells were washed twice in 12ml of Dulbecco 'A' PBS, drained completely and subsequently lysed in 300µl (100µl) of 1xSDS gel-loading buffer pre-heated to 85°C.
1x SDS gel-loading buffer: 50mM Tris-HCl (pH6.8); 100mM dithiothreitol; 2% w/v SDS; 0.1% w/v bromophenol blue; 10% v/v glycerol.

The lysates were transferred to Eppendorf tubes and boiled in a water-bath for 10 minutes. The tubes were placed on ice and sonicated in a Soniprep 150 (MSE; Fisons Scientific Equipment Ltd., Loughborough, Leics., UK.) set at full power for 10 seconds. The tubes could then be centrifuged in a microfuge at 13,000 r.p.m. for 10 minutes at room temperature and the resulting supernatants transferred to fresh tubes.

20μl of the protein preparation was mixed with 80μl of 100% ethanol and subsequently boiled in a water-bath for 1 minute. The resulting precipitate was pelleted through centrifugation at 13,000 r.p.m for 5 minutes in a microfuge, dried at room temperature for 5 minutes and resuspended in 20μl of autoclaved water. The protein was precipitated as before and finally resuspended in 80μl of autoclaved water. The protein content of this 80μl aliquot was determined colourimetrically by the method of Bradford (1976) using Bio-Rad protein assay reagent. 20μl of reagent was added to the resuspended protein and mixed by vortexing. The assay mixture was transferred to a 96well plate, the plate read at 595nm on a microplate reader and the protein content assessed against bovine serum albumin (BSA) standards ranging from 40μg/ml⁻¹ to 1μg/ml⁻¹ of protein.

The protein samples were either used immediately for SDS-PAGE or stored at -20°C.

2.2.6.2 Isolation of Protein from Frozen Tissue.

0.5g of human tonsil was snap-frozen in liquid nitrogen and disaggregated using the Mikro-Dismembrator II as described in section 2.2.4.2. The powdered tissue was immediately scraped into 200μl of ice-cold suspension buffer using a pre-chilled micro-spatula and subsequently mixed with 200μl of 2x SDS gel-loading buffer. The protein preparation was continued as outlined in section 2.2.6.1.
Suspension buffer: 0.1M NaCl;
0.01M Tris.HCl (pH 7.6);
0.001M EDTA (pH 8.0);
1μgml⁻¹ aprotinin;
100μgml⁻¹ PMSF.

2.2.6.3 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Proteins.

The procedure detailed is based on the discontinuous buffer system described by Laemmli (1970). Electrophoresis was performed using the Protean II vertical electrophoresis system (16cm configuration [spacers 18.3cm long and 1.5mm thick]; Bio-Rad Laboratories Ltd.).

The electrophoresis plates were washed in a weak detergent solution, rinsed thoroughly and the side of each plate coming into contact with the gel cleaned with 100% ethanol. The gel plates were assembled according to Bio-Rad instructions. The resolving gel was prepared by pouring 30mls of a 15% polyacrylamide solution into the gap between the plates. [Note: The polyacrylamide solutions were prepared through the addition of each component in order, with thorough mixing ensured through swirling immediately after the TEMED was included.] The resolving gel was overlaid with isobutanol to a depth of ~0.5cm and polymerisation allowed to proceed for 30 minutes.

15% Polyacrylamide : 10.65mls autoclaved water;
Resolving Gel (30ml): 11.25mls 40% w/v acrylamide/bis-acrylamide;
7.5mls 1.5M Tris-base (pH 8.8);
0.3ml 10% w/v SDS;
0.3ml 10% w/v ammonium persulphate;
12μl TEMED.

The isobutanol was removed using a paper towel and the top of the gel washed with deionised water to remove any unpolymerised polyacrylamide. 10mls of a 5% stacking gel was poured onto the surface of the resolving gel and a 10 well Teflon comb (10mmx1.5mm) inserted, ensuring that no air bubbles were trapped. The stacking gel was allowed to set for 30 minutes at room temperature.
5% Polyacrylamide: 7.25mls autoclaved water;
Stacking Gel (10ml): 1.275mls 40% w/v acrylamide/bis-acrylamide;
1.25mls 1M Tris-base (pH6.8);
0.1ml 10% w/v SDS;
0.1ml 10% w/v ammonium persulphate;
10μl TEMED.

After the completion of polymerisation the comb was removed and the wells washed with de-ionised water to remove unpolymerised acrylamide. The gel could then be mounted into the gel apparatus and Tris-glycine electrophoresis buffer placed in the top and bottom reservoirs. Air bubbles trapped at the bottom of the gel between the glass plates were removed.

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-glycine</td>
<td>9.09g Tris-base;</td>
</tr>
<tr>
<td>electrophoresis buffer</td>
<td>43.23g glycine;</td>
</tr>
<tr>
<td>(3 litres)</td>
<td>3g SDS.</td>
</tr>
</tbody>
</table>

The protein samples were boiled in a water-bath for 10 minutes and incubated on ice for 5 minutes prior to loading into the bottom of the wells using Prot/Elec tips (Bio-Rad Laboratories Ltd.). The samples were loaded alongside 15μl of Rainbow™ molecular weight markers (Amersham International plc.; molecular weight range 14,300-200,000). The "Rainbow markers" (15μl) were mixed with 15μl of 1x SDS gel-loading buffer, boiled for 10 minutes and loaded onto the gel.

The gel was initially run at 20mA until the bromophenol blue reached the interface between the stacking and resolving gels. Subsequently the gel was run at 40mA until the bromophenol was seen to run off the bottom of the resolving gel.

2.2.6.4 Transfer of Proteins from SDS-Polyacrylamide Gels to Nitrocellulose.

The SDS-polyacrylamide gel was placed in 1L of transfer buffer and soaked for 30 minutes. The gel could then be measured and nitrocellulose (Hybond C extra) plus 4 pieces of Whatman 3MM paper cut to the same dimensions. The gel, nitrocellulose and 3MM paper was equilibrated in transfer buffer for 30 minutes.
Transfer buffer: 5.82g Tris-base; 
(1 Litre) 2.93g glycine; 
200ml methanol; 
3.75mls 10% SDS. pH9.2.

Blotting was performed with Transblot SD semi-dry transfer cell (Bio-Rad Laboratories Ltd.). The electrodes were carefully cleaned with 100% ethanol prior to the blotting procedures.

2 pieces of 3MM paper were placed on top of each other on the anode. The nitrocellulose could then be positioned above the 3MM paper with the gel placed exactly above the membrane. The remaining pieces of 3MM paper were stacked on top of the gel. [Note: At each stage, air bubbles were excluded by gently rolling each layer flat.] After carefully placing the cathode atop the stack, transfer was permitted for 16hours at 5volts.

2.2.6.5 Immuno-detection of the bcl-2 Oncoprotein Immobilised on Nitrocellulose.

Proteins detection was carried out using the ECL Western blotting detection system.

The nitrocellulose membrane was washed in 500mls of washing buffer for 10minutes and subsequently incubated in blocking solution (0.1ml solution per cm²) for 2hours in a shallow tray. [Note: All incubations and washing steps were performed at room temperature on an orbital shaker.] The membrane was subsequently washed for 5minutes in 500mls of washing buffer at room temperature, placed in a heat-sealable bag and then incubated with the monoclonal anti-human bcl-2 antibody diluted 1:200 in blocking solution (0.1ml solution per cm²). The membrane was incubated with the primary antibody for 2hours.

Washing buffer: 24g Tris-base; 
(4 Litres) 35.2g NaCl; 
2.96g EDTA. pH7.5.

Blocking Solution: 5g dried skimmed milk; 
(100ml) 2g bovine serum albumin.
The components of the blocking solution were dissolved in washing solution.
At the conclusion of this 2-hour incubation the membrane was washed in 500 mls of washing buffer for 15 minutes followed by two further 5-minute washes. The membrane was then placed in a fresh heat-sealable bag and incubated with the secondary antibody (mouse Ig, horseradish peroxidase-linked whole antibody) diluted 1:1000 in blocking solution (0.1 ml solution per cm²) for 2 hours. Subsequently, the membrane was washed as before, once for 15 minutes and then twice for 5 minutes. The membrane was drained completely and incubated for 1 minute with the two ECL detection reagents (0.125 mls per cm²) previously mixed in equal quantities. The membrane was drained, wrapped in Saran wrap and placed in an autoradiography cassette fitted with intensifying screens. Hyperfilm™ECL was exposed to the membrane for 30 seconds, 1 minute, 2 minutes and 4 minutes to ensure optimum exposure.

Densitometric analysis of exposed autoradiographs was performed with a Quantimet 970 supplied by Cambridge Instruments. The optical densities of each band are based on that of the darkest band in a particular group and are thus quoted in arbitrary units.

2.2.7 Immunohistochemical Analysis of Androgen Receptor Expression in Prostatic and Endometrial Tissues.

All immunohistochemistry was performed by Dr. H. O. Critchley, Department of Obstetrics and Gynaecology, Centre for Reproductive Biology, University of Edinburgh.

Paraffin sections of BPH and endometrial tissues were examined using a mouse monoclonal antibody raised against a site of high antigenicity on the human androgen receptor. Negative controls were treated with the anti-mouse IgG secondary antibody alone. Detection of the antigen was improved through the use of microwave antigen retrieval.

The biotinylated secondary antibody was detected using a streptavidin-horse radish peroxidase (HRP) conjugate with 3,3'-Diaminobenzidine Tetrahydrochloride (DAB) as the enzyme substrate. Consequently, positively stained nuclei appeared dark brown in colour. The negative controls were counter-stained with haematoxylin.

2.2.8 Statistical Analysis.

Statistical significance was determined using Student's t-test for comparison.
of 2 means and a modification of the t-statistic termed the Bonferroni method for the comparison of a number of means against a control mean (Wallenstein et al., 1980). Using the Bonferroni method the critical value is obtainable from a standard table of the t-distribution using a significance level of $P/n$, where $n$ is the number of pairwise comparisons e.g. for 5 comparisons, the $P$ value of 0.05 would be replaced by $0.05/5 = 0.01$. 
At the time of diagnosis, the majority of prostatic adenocarcinomas have already progressed to a state where radical surgical approaches to treatment are insufficient for complete tumour management. The logical course of action is then to deny the inherently androgen-dependent tumour cells androgenic stimulation either by chemical or surgical castration. The subsequent induction of apoptosis in the majority of tumour cells leads to dramatic initial reductions in tumour mass with corresponding diminution of symptoms. However, these extremely favourable responses are only temporary and in nearly all cases there is a recurrence of tumourigenic symptoms leading ultimately to the death of the patient.

Patient relapse results from progression of the tumour from a state of androgen-dependent growth to androgen-independence. Such "hormone escape" has been postulated to occur by a number mechanisms (see Introduction), however, the net effects are essentially identical i.e. the development of a carcinoma containing cells which will not undergo programmed cell death in an androgen-free environment. In order to develop effective therapies which target both androgen-dependent and androgen-independent prostate cancer cells effectively it is preferable that the phenotype of both cell types be fully characterised. In this respect, the available in vitro models of prostate cancer provide ideal systems for the analysis of hormone escape owing to their inherent ease of study.

The expression of the androgen receptor and the anti-apoptosis oncoprotein bcl-2 is extremely cell-specific within the prostate, the former generally
being restricted to the secretory epithelia and the latter arising almost exclusively in the basal epithelial cells. Additionally, altered expression of both these proteins has been implicated in the development of androgen-independent prostate cancer. Subsequently, it was the aim of the thesis to determine the relationship between hormone-independence in vitro and the expression of bcl-2 and the androgen receptor. Furthermore, the possibility of liaison between the androgen signalling networks and the bcl-2 oncoprotein was also investigated. The approach used is summarised below:

1. Assessment of the growth responses of prostate cancer cell lines and primary epithelial cell cultures to androgens and anti-androgens.

2. Determination of the expression of the androgen receptor in cell lines and primary cultures at the transcriptional level.


4. Determination of the expression of the bcl-2 oncoprotein in the cell models and its relationship with androgen responsiveness and the androgen receptor.

3.1 The Effects of Dihydrotestosterone (DHT) and Hydroxyflutamide (HO-F) on the Growth of Cultured Prostate Cancer Cell Lines and Primary Epithelial Cell Cultures.

The effects of androgenic stimuli on the LNCaP, DU145 and PC3 cell lines is well documented (Horoszewicz et al, 1983; Wilding et al, 1989; Mickey et al, 1980; Kaighn et al, 1979; Schulz et al, 1990). It was felt necessary to determine the growth characteristics of our cell lines in order to ensure the appropriate responses to DHT and hydroxyflutamide. This was felt to be of particular importance in LNCaP in view of the subclones of androgen-insensitive cells (LNCaPr) known to contaminate this cell line (Hasenson et al, 1985).

A number of groups have analysed the response of primary prostatic epithelial cell cultures to androgens and indeed to anti-androgens (Schroeder et al, 1974; McKeehan et al, 1984; Hallowes et al, 1991). Their findings differ greatly and it was intended, in these studies, to determine conclusively, whether or not primary cultures retain any of the in vivo growth characteristics of secretory epithelia.
The growth response of cultured cells to both dihydrotestosterone and also to the non-steroidal anti-androgen hydroxyflutamide was assessed. DHT, being the most potent and certainly the most physiologically significant androgen within the prostate, was employed to determine hormone-responsiveness. Hydroxyflutamide was included in the study since truly androgen-dependent cells should not exhibit any response to such an androgen receptor antagonist.

3.1.1 Growth Response of the LNCaP, DU145 and PC3 Cell Lines to DHT and HO-F.

3.1.1.1 LNCaP.

Figures 3.01 a. and 3.01 b. demonstrate the impact of increasing concentrations of DHT and HO-F on the growth of the LNCaP cell line over a 4 day period.

LNCaP cells exhibit significant (p<0.05) growth stimulation at all concentrations of DHT used by day 2 compared to control (Figure 3.01 a.). There is no significant difference in cell numbers between all DHT concentrations at day 2. [ Note: the tailing-off of the growth curves for the control and the majority of the tests after day 3 is presumably either due to medium exhaustion or due to cell confluence ].

After 4 days, LNCaP cells supplemented with 0.001nM, 0.01nM and 0.1nM HO-F show no significant (p>0.05) increases in cell number compared to control (Figure 3.01 b.). At day 3, the cells exhibit significant (p<0.05) growth stimulation in the presence of 10.0nM HO-F but not 1.0nM HO-F. Both 10.0nM and 1.0nM HO-F stimulate LNCaP proliferation significantly (p<0.05) by day 4. There is a significant difference between cell numbers in the 10.0nM HO-F treatment compared to the 1.0nM HO-F treatment, at day 4. Such variation is not observed at day 3.

The responses of the LNCaP cell line to DHT and the androgen antagonist Hydroxyflutamide are very much in keeping with those observed by other groups (Olea et al, 1990; Wilding et al, 1989).

3.1.1.2 DU145.

As Figures 3.02 a. and 3.02 b. indicate, DHT and HO-F in the range 0.001nM to 10.0nM, have no significant (p>0.05) influence on the rate of proliferation of DU145 cells compared to control.
Figure 3.01 Dose Response of LNCaP Cells to Dihydrotestosterone and Hydroxyflutamide.

Cells were plated in RPMI 1640 medium supplemented with 10% FCS at a density of 4000 cells/well in 96 well plates. After 24 hours the medium was aspirated and replaced with phenol red-free RPMI 1640 containing 10% DCC-stripped FCS. Subsequent to a further 48 hour incubation, DHT [Figure 3.01 a.] or HO-F [Figure 3.01 b.] was added to a final concentration of 0.001, 0.01, 0.1, 1.0 and 10.0 nM. Control wells were supplemented with an aliquot of ethanol equivalent to the volume given to the test wells. 8 wells were used for each dilution. Cell numbers were assessed spectrophotometrically (540 nm) using the MTT assay at days 0, 1, 2, 3 and 4. Each data point represents the mean +/- SEM. Statistical significance (p<0.05) over control values is indicated by an asterisk.
Cells were plated in DMEM supplemented with 10% FCS at a density of 1000 cells/well in 96 well plates. After 24 hours the medium was aspirated and replaced with phenol red-free medium containing 10% DCC-stripped FCS. 48 hours later, after replacement with fresh medium (100 μl), DHT [Figure 3.02 a.] or HO-F [Figure 3.02 b.] was added to a final concentration of 0.001, 0.01, 0.1, 1.0 and 10.0 nM. Control wells were supplemented with an aliquot of ethanol equivalent to the volume delivered to the test wells. 8 wells were used for each drug concentration. Cell numbers were assessed spectrophotometrically (540nm) using the MTT assay at days 0, 1, 2, 3 and 4. Each data-point represents the mean +/- SEM.
3.1.1.3 PC3.

PC3 cells exhibit similar responses to androgens/anti-androgens as the DU145 cell line (Figure 3.03 a. and 3.03 b.). Over a 4 day period, there is no significant (p>0.05) increase in the proliferative rate of cells supplemented with DHT (0.001nM - 10.0nM) or HO-F (0.001nM - 10.0nM) compared to control. [Note: No obvious explanation can be offered for the observed "lag" in growth up to day 2, but since it occurs in both control and test wells, it may indicative of adverse effects of ethanol on this particular cell line.]

3.1.2 Growth Response of Primary Epithelial Cells to DHT and HO-F.

Primary epithelial cells derived from specimens obtained by TUR of both benign and malignant prostates were analysed for their response to androgen and anti-androgen. Primary cells were used in this study since they exhibit significant growth advantage over secondary/tertiary cultures.

Cultures derived from 2 BPH (Figures 3.04 and 3.05) samples and 1 poorly differentiated adenocarcinoma of the prostate (Figure 3.06) were studied. It is evident from the data that there is no significant growth stimulation of either BPH or CaP derived epithelial cells in the presence of DHT or HO-F at concentrations of between 0.001nM and 10.0nM.

3.2 Androgen Receptor (AR) Expression in Cultured Prostate Cancer Cell Lines and Primary Epithelial Cells - Analysis by Northern Blotting.

Having established that the DU145 and PC3 cell lines plus all of the primary epithelial cell cultures do not respond to either androgens or anti-androgens the logical progression was to establish the extent of androgen receptor gene expression, the rationale being that an inability to respond to hormonal stimuli may be due to a lack of functional receptor.

Total cellular RNA was prepared from tissues, prostatic carcinoma cell lines and primary epithelial cells using the approach described by Chomczynski and Saachi (1987). 20μg of total RNA was separated electrophoretically on 1% agarose formaldehyde gels and blotted by capillary transfer to nylon-supported nitrocellulose. Blots were analysed for AR mRNA transcripts using a 1093bp PCR probe (1.1ARp) labelled with 32P and hybridisation assessed qualitatively through exposure to Hyperfilm™.MP.
Figure 3.03 Dose Response of PC3 Cells to Dihydrotestosterone and Hydroxyflutamide.

Cells were plated in HAM'S F12 medium supplemented with 7% FCS at a density of 1000 cells/well in 96 well plates. Following a 24-hour incubation the medium was aspirated and replaced with phenol red-free medium containing 7% DCC-stripped FCS. After a further 48 hours the spent medium was replaced with 100μl of fresh medium and DHT [Figure 3.03 a.] or HO-F [Figure 3.03 b.] added to a final concentration of 0.001, 0.01, 0.1, 1.0 and 10.0nM. Ethanol was delivered to control wells. 8 wells were used for each concentration of androgen/anti-androgen. Cell numbers were assessed spectrophotometrically (540nm) using the MTT assay at days 0, 1, 2, 3 and 4. Each data-point represents the mean +/- SEM.
Figure 3.04 Dose Response of BPH-derived Primary Epithelial Cells to Dihydrotestosterone and Hydroxyflutamide.

Cells (BPH 1) were plated in EGM at a density of 5000 cells/well in 96well plates. Following a 24 hour incubation the medium was aspirated and replaced with phenol red-free RPMI 1640 medium supplemented with 10% DCC-stripped FCS. After a 48 hour incubation the spent medium was replaced with 100μl of fresh medium and DHT [Figure 3.04 a.] or HO-F [Figure 3.04 b.] added to a final concentration of 0.001, 0.01, 0.1, 1.0 and 10.0nM. Ethanol was added to control wells. 4 wells were used with each concentration of androgen/anti-androgen. Cells numbers were assessed spectrophotometrically (540nm) using the MTT assay at days 0, 1, 2, 3 and 4. Each data-point represents the mean +/- SEM.
Figure 3.05 Dose Response of BPH-derived Primary Epithelial Cells to Dihydrotestosterone and Hydroxyflutamide.

Cells (BPH 2) were plated in EGM at a density of 5000 cells/well in 96 well plates. After a 24 hour incubation the medium was aspirated and replaced with phenol red-free RPMI 1640 medium supplemented with 10% DCC-stripped FCS. After a further 48 hour period the spent medium was replaced with 100μl of fresh medium and DHT [Figure 3.05 a.] or HO-F [Figure 3.05 b.] added to a final concentration of 0.001, 0.01, 0.1, 1.0 and 10.0 nM. Ethanol was added to control wells. 4 wells were used with each concentration of androgen/anti-androgen. Cell numbers were assessed spectrophotometrically (540nm) using the MTT assay at days 0, 1, 2, 3 and 4. Each data-point represents the mean +/- SEM.
Figure 3.06 Growth Response of CaP-derived Primary Epithelial Cells to Dihydrotestosterone and Hydroxyflutamide.

CaP derived epithelial cells were plated at a density of 5000 cells/well in EGM. Following a 24 hour incubation the medium was aspirated and replaced with phenol red-free RPMI 1640 medium containing 10% DCC-stripped FCS. 48 hours later, after replacing the spent medium with fresh medium (100μl), DHT [Figure 3.06 a.] or HO-F [Figure 3.06 b.] was added to a final concentration of 0.001, 0.01, 0.1, 1.0 and 10.0nM. Ethanol was delivered to control wells. 4 wells were used for each concentration of androgen/antiandrogen. Cell numbers were assessed spectrophotometrically (540nm) using the MTT assay at days 0, 1, 2, 3 and 4. Each data-point represents the mean +/- SEM.
3.2.1 Synthesis and Analysis of 1.1ARp.

The complete human AR cDNA, purified from pCMV/ARcom, was used in initial attempts to detect Northern blotted AR transcripts. However a high degree of non-specific binding, in particular to the 28s ribosomal RNA band, was observed after moderately stringent washes and attempts to remove this through increasing stringency resulted in the loss of all hybridisation. In attempts to overcome these specificity problems a probe of smaller size was prepared spanning the AR cDNA from the coding region for the DNA-binding domain to 3' of the Ligand-binding domain code (Figure 2.05). The new probe, termed 1.1ARp (1093bp), was prepared by PCR using pCMV/ARcom as template DNA and the primers AR2.3s and AR7.8as (Section 2.2.5.2b.). Two restriction fragments of sizes 791bp and 302bp are diagnostic of EcoRI digestion of 1.1ARp. Prior to use, 1.1ARp was digested with EcoRI to ensure complementarity with AR cDNA.

Digestion of 1.1ARp with EcoRI yields two products of approximate size 800bp and 300bp as indicated in Figure 3.07. It is evident that undigested 1.1ARp (Lane 2) is retarded in the gel relative to purified 1.1ARp (Lane 1). This phenomenon may be due to the effects of the restriction buffer or the restriction enzyme itself on the mobility of the fragments.

The ability of 1.1ARp to hybridise specifically with receptor sequences was analysed using Northern blotted RNA derived from tissue samples of known androgen receptor status. For the purpose of this study prostate tissue was used as a positive and endometrium provided a receptor-negative control.

3.2.2 Detection of Androgen Receptor mRNA in Prostatic and Endometrial Tissues by Northern Blotting.

The androgen receptor status of prostatic and endometrial tissues was determined immunohistochemically using an anti-AR monoclonal antibody raised against a site of high antigenicity.

Immunohistochemical analysis of tissue from a benign hyperplastic prostate demonstrates the localisation of the majority of androgen receptors to the nuclei of secretory epithelial cells within the glandular structures (Figure 3.08). In addition, receptor-positive nuclei are also evident diffusely spread throughout the fibromuscular stroma immediately surrounding the gland.

No androgen receptor-specific staining within either epithelial or stromal
Figure 3.07 Digestion of 1.1ARp with EcoRI.

1.1ARp was prepared in a 35cycle PCR reaction using the primers 2.3s and 7.8as and AR cDNA as template. The PCR product was purified from a 2% agarose preparative gel by phenol-chloroform extraction and 10μl of the isolate digested with EcoRI at 37°C for 60minutes.

Lane 1 : 1kb DNA ladder. Lane 2 : 1.1ARp. Lane 3 : 1.1ARp/EcoRI.
Figure 3.08: Androgen Receptor Immunostaining of BPH Tissue.

Paraffin sections of benign prostatic hyperplasia tissue were immunostained with AR mAb. x25 magnification.

SE - secretory epithelial cell; S - fibromuscular stroma; L - glandular lumen.
Figure 3.09 Blank Immunostain of BPH Tissue.

Paraffin sections of benign prostatic hyperplasia tissue were immunostained without the addition of primary antibody and were counterstained with Mayer's haematoxylin. x25 magnification.

SE - secretory epithelial cell; S - fibromuscular stroma; L - glandular lumen.
Figure 3.10  *Androgen Receptor Immunostaining of Endometrial Tissue.*

Paraffin sections of endometrium were immunostained with AR mAb. x25 magnification.

E - epithelia; S - stroma.
Figure 3.11 Blank Immunostain of Endometrial Tissue.

Paraffin sections of endometrial tissue were immunostained without the addition of AR mAb and were counterstained with Mayer's haematoxylin. x25 magnification.

E - epithelia; S - stroma.
cells of the endometrium was evident (Figure 3.10).

Northern blot analysis using 1.1ARp as probe DNA indicates that prostatic tissue contains high levels of AR mRNA as opposed to the endometrium which contains little or no AR transcripts (Figure 3.12 b. & c.). Exposure of probed blots for 24 hours demonstrates the presence of the 4361 base AR transcript in both malignant and benign prostates (Figure 3.12 b.). This transcript is proposed to be a degradation product of larger AR mRNAs (Blok et al, 1991). At this exposure time the ~4kb AR mRNA is undetectable in endometrial tissues. Autoradiography over a 72 hour period again indicates the presence of the 4kb transcript in BPH and CaP, but there is also evidence to suggest the presence of higher molecular weight transcripts, potentially the 6682 base and 9614 base species observed by Tilley et al (1990). The endometrium possesses very low levels of 4kb AR transcript, as indicated by the 72 hour exposure, and indeed there appears to be a faint band on the autoradiograph of a size greater than 10kb. [ Note : The presence of blotted RNA in each sample lane is indicated by the hybridisation of β-actin probe. ]

Clearly 1.1ARp is specific for AR transcripts since little hybridisation is observed with Northern blotted RNA derived from AR-deficient tissue. Similarly, the weak signal obtained with endometrial RNA would support the claim that the detectable 4kb band does not merely represent hybridisation with the 28s ribosomal RNA. In addition, it would appear that using total RNA it is possible to predict the receptor status of heterogeneous tissues such as the prostate and, by extrapolation, cultured cells, without the need for purification of mRNA.

3.2.3 AR Gene Expression in Human Prostatic Carcinoma Cell Lines.

The extent of androgen receptor expression in the LNCaP, DU145 and PC3 cell lines, which display marked differences in their responses to androgenic inputs as previously described, was examined by Northern hybridisation with 1.1ARp. Figure 3.13 B. demonstrates the expression of high levels of AR mRNA in LNCaP, a feature which correlates well with the observed androgen-sensitivity of this cell line. 3 AR mRNA species are detectable in LNCaP : 2 major transcripts of sizes 6682 bases and 9614 bases and the 4kb transcript, previously described in 3.2.2., which represents the minor AR gene product (Tilley et al, 1990; Brown et al, 1988). Both the DU145 and PC3 cells express little or no detectable AR mRNA as indicated by the 24 hour and 72 hour autoradiographs and certainly appear to contain manifestly less transcript than the LNCaP cell line.
Figure 3.12 Androgen Receptor Gene Expression in Prostatic and Endometrial Tissue.

20mg of total RNA isolated from BPH, CaP and endometrium was electrophoresed on 1% agarose gels containing 2.2M formaldehyde (A.) and transferred to Hybond™-C extra transfer membrane. AR mRNA was detected using $^{32}$P-labelled 1.1ARp. Autoradiography was performed, with intensifying screens, for 24 (B.) and 72 hours (C.).

A. 1% Denaturing Agarose Gel. Lane 1 - BPH. Lane 2 - CaP. Lane 3 - endometrium.

B. Autoradiograph - AR. 24 hour exposure. Lane 1 - BPH. Lane 2 - CaP. Lane 3 - endometrium.

C. Autoradiograph - AR. 72 hour exposure.

D. Autoradiograph - β-Actin. Lane 1 - BPH. Lane 2 - CaP. Lane 3 - endometrium.
Figure 3.13 Androgen Receptor Gene Expression in the LNCaP, DU145 and PC3 Cell Lines.

20µg of total RNA isolated from LNCaP, DU145 and PC3 cells in addition to 20µg of endometrial RNA (negative control) was electrophoresed on a 1% agarose gel containing 2.2M formaldehyde and transferred to Hybond™-C extra. $^{32}$P-labelled 1.1ARP was used in the detection of AR mRNA transcripts. Autoradiography was performed, under intensification, for 24 hours (B.) and 72 hours (C.).

A. 1% Denaturing Agarose Gel. Lane 1 - 1º Epi. culture (CaP). Lane 2 - LNCaP. Lane 3 - DU145. Lane 4 - PC3.

B. Autoradiograph - AR. 24 hour exposure. Lane 1 - 1º Epi. culture (CaP). Lane 2 - LNCaP. Lane 3 - DU145. Lane 4 - PC3.

C. Autoradiograph - AR. 72 hour exposure.

D. Autoradiograph - $\beta$-Actin. Lane 1 - 1º Epi. culture (CaP). Lane 2 - LNCaP. Lane 3 - DU145. Lane 4 - PC3.
3.2.4 AR Gene Expression in Human Primary Epithelial Cell Cultures.

Five primary epithelial cultures, 4 of which were BPH-derived (BPH 1, 2, 3 and 4) and 1 CaP-derived (CaP 1), were examined for AR mRNA. [ Note : BPH 1 and BPH 2 are the same BPH-derived cultures whose dose response to DHT and HO-F was examined in section 3.1.2. ] LNCaP total RNA, serving as a positive control, was run in parallel to the primary culture samples.

It is evident from the results shown in Figure 3.14 B., that none of the primary epithelial cultures investigated express sufficient AR mRNA to be detectable in samples of total RNA analysed by Northern Blotting. [ Note : The poor quality of the β-actin autoradiograph is an unfortunate feature of the stripping and reprobing of Hybond™-C extra membranes. It appears that the harsh conditions required to strip the membranes often causes irreversible damage leading to high levels of background hybridisation. ].

From both the assessment of the growth responsiveness of primary epithelial cells and the measurement of androgen receptor gene expression it would appear that the cells obtained from cultured prostatic acini are not ideal models of secretory epithelia in vivo. This is probably a feature of epithelial cell growth in serum-free conditions. Therefore the influence of plating the acini in an environment that retains some of the attributes common to the prostate gland was briefly examined.

Prostatic acini were plated and the cells grown in EGM supplemented with either 1nM DHT, an equal volume of medium conditioned by BPH-derived prostate fibroblasts for 48 hours or a combination of 1nM DHT plus fibroblast conditioned medium. Total RNA isolated from these cells was subsequently examined for AR mRNA by Northern blotting. Figure 3.15 demonstrates that none of these treatments gives rise to sufficient AR mRNA to be detectable by Northern blotting. Clearly to obtain a truly representative model of prostatic epithelia requires other factors, the exact nature of which are not entirely obvious at this juncture, and therefore it was decided not to attempt optimisation of the culture conditions.

3.3 Androgen Receptor (AR) Expression in Cultured Prostate Cancer Cell Lines and Primary Epithelial Cells - Analysis by Reverse Transcription-Polymerase Chain Reaction (RT-PCR).

Northern blotting offers limited sensitivity for the detection of mRNA transcripts. Abundant RNAs (0.1% or more of the mRNA population) can usually be
20μg of total RNA isolated from BPH- and CaP-derived primary epithelial cell cultures was electrophoresed on a 1% formaldehyde agarose gel (A.) and transferred to Hybond™-C extra. AR mRNA was detected using 32P-labelled 1.1ARp. Autoradiography was performed with intensifying screens for 24 hours (B.).

A. **1% Denaturing Agarose Gel.** Lane 1 - LNCaP. Lane 2 - BPH 1. Lane 3 - BPH 2. Lane 4 - CaP 1. Lane 5 - BPH 3. Lane 6 - BPH 4.

B. **Autoradiograph - AR.** 24 hour exposure. Lane 1 - LNCaP. Lane 2 - BPH 1. Lane 3 - BPH 2. Lane 4 - CaP 1. Lane 5 - BPH 3. Lane 6 - BPH 4.

C. **Autoradiograph - β-Actin.** Lane 1 - LNCaP. Lane 2 - BPH 1. Lane 3 - BPH 2. Lane 4 - CaP 1. Lane 5 - BPH 3. Lane 6 - BPH 4.
Acini obtained from TUR specimens from a hyperplastic prostate were seeded in 75cm$^2$ tissue culture flasks and epithelial cells grown in either of the following: 1. 12ml EGM + 1nM DHT; 2. 6ml EGM + 6ml FCM; 3. 6ml EGM + 6ml FCM + 1nM DHT. [Note: The FCM was removed from BPH-derived fibroblast cultures after 48 hours.] Total RNA was isolated from sub-confluent cells and 20μg electrophoresed on a 1% formaldehyde agarose gel (A.) and transferred to Hybond™-C extra. $^{32}$P-labelled 1.1ARp was used to detect AR mRNA. Autoradiography was performed with intensifying screens for 24 hours (B.).

A. **1% Denaturing Agarose Gel.** Lane 1 - LNCaP. Lane 3 - 10nM DHT. Lane 4 - FCM. Lane 5 - 10nM DHT + FCM.

B. **Autoradiograph - AR.** 24 hour exposure. Lane 1 - LNCaP. Lane 3 - 10nM DHT. Lane 4 - FCM. Lane 5 - 10nM DHT + FCM.

C. **Autoradiograph - β-Actin.** 24 hour exposure. Lane 1 - LNCaP. Lane 3 - 10nM DHT. Lane 4 - FCM. Lane 5 - 10nM DHT + FCM.
detected by Northern analysis of 10-20μg of total cellular RNA. RT-PCR overcomes these limitations and allows low copy-number transcripts to be observed through amplification of specific RNA sequences.

In samples of reverse transcribed RNA, the presence of AR cDNA was determined using 2 sets of intron-spanning primer pairs locating to exons 2 & 3 (AR2.3s and AR2.3as) and exons 7 & 8 (AR7.8s and AR7.8as) respectively. [ Note: The use of intron-spanning primer pairs generates discrimination between cDNA and genomic DNA contaminants ]. These primers will amplify cDNA corresponding to the DNA-binding domains and C-terminus of the AR, allowing detection of both full-length AR mRNA, and potentially, truncated transcripts known to give rise to constitutively active receptors (Rundlett et al, 1990).

Control primers specific to the cDNA of the house-keeping protein hypoxanthine-guanine phosphoribosyl transferase (HGPRT) were used with all samples to determine the efficiency of the reverse transcription reaction. These primers, denoted HGPRTs and HGPRTas, give rise to a product of size ~310bp.

All PCR products were visualised on 2% agarose gels.

3.3.1 Analysis of AR-Positive and AR-Negative Tissues for Androgen Receptor mRNA.

Reverse transcribed RNA from prostatic and endometrial tissues was examined for AR sequences. Figure 3.16 A. and B. displays the production of AR2.3 and AR7.8, not only from BPH and CaP samples but also from endometrial cDNA. Clearly the AR gene is transcribed in the endometrium, however, the mRNA product would appear to be strikingly lower in abundance than in prostatic epithelial cells in view of the data obtained by Northern analysis (section 3.2.2.). HGPRT is present in all three cDNA samples. The mechanism underlying the observed down-regulation of AR mRNA in the endometrium is unclear, although there may be a role for either decreased transcription of the AR gene (arising from alterations in the transcriptional control elements of the AR gene) or perhaps a change in the half-life of the gene transcript.

The specificity of AR2.3s/AR2.3as and AR7.8s/AR7.8as for AR sequences was assessed through restriction digestion of the AR 2.3 and AR7.8 PCR products. Digestion of AR2.3 and AR7.8 with HindIII and DraIII respectively should yield products of sizes 187bp and 78bp from the former and 177bp and 51bp fragments in the latter case.

Figure 3.16 D. demonstrates the results of the digestion of AR2.3., with two
Figure 3.16 Androgen Receptor Gene Expression in Prostatic and Endometrial Tissue.

Total RNA (1μg per PCR reaction) isolated from BPH (A.), CaP (B.) and endometrium (C.) was reverse transcribed and amplified by PCR for 35 cycles using HGPRTs/as, AR2.3s/as and AR7.8s/as. The PCR reactions for the "no DNA" controls were performed without cDNA. 10μl of AR2.3 and AR7.8 from endometrium were digested at 37°C for 60 minutes with HindIII (D.) and DraIII (E.) respectively. The RT-PCR products and restriction fragments were analysed by electrophoresis through 2% agarose gels containing ~0.1μg ml⁻¹ ethidium bromide. 100bp molecular weight markers were electrophoresed alongside the RT-PCR products. The gels were photographed under transillumination.

A. 2% Agarose Gel - RT-PCR BPH tissue. Lane 1 - 100bp ladder. Lane 2 - No DNA. Lane 3 - HGPRT. Lane 4 - AR2.3. Lane 5 - AR7.8.

B. 2% Agarose Gel - RT-PCR CaP tissue. Lane 1 - 100bp ladder. Lane 2 - No DNA. Lane 3 - HGPRT. Lane 4 - AR 2.3. Lane 5 - AR7.8.

C. 2% Agarose Gel - RT-PCR Endometrium. Lane 1 - 100bp ladder. Lane 2 - No DNA. Lane 3 - HGPRT. Lane 4 - AR2.3. Lane 5 - AR7.8.

D. Restriction Digest (AR2.3/HindIII). Lane 1 - 100bp ladder. Lane 2 - AR2.3. Lane 3 - AR2.3/HindIII.

E. Restriction Digest (AR7.8/DraIII). Lane 1 - 100bp ladder. Lane 2 - AR7.8. Lane 3 - AR7.8/HindIII.
bands clearly evident. The largest fragment corresponds to undigested AR2.3, with the band at ~190-200bp representing the larger of the two restriction fragments. The 78bp restriction product cannot be visualised. Digestion of 7.8 with DraIII produces a single restriction fragment observable on an agarose gel (Figure 3.16 E.). The fragments appears to be running at ~190-200bp. It is evident that restriction analysis of AR2.3 and AR7.8 results in fragments of sizes marginally greater than that expected, as indicated by gel electrophoresis.

Both DraIII and HindIII are "rare-cutter" restriction enzymes in eukaryotic systems. This being the case, it would seem unlikely that mis-priming of the primer pairs used to analyse AR cDNA would yield PCR products, not only of the expected size but also that exhibit digestion by these restriction endonucleases. It can be concluded, therefore, that the digestion observed with both AR2.3 and AR7.8 derived from endometrium is indicative of the specificity of AR2.3s/AR2.3as and AR7.8s/AR7.8as for their respective regions of the AR cDNA. The inappropriate sizes of the restriction fragments observed in 3.13 D. and E. may merely represent artefactual retardation as observed in section 3.2.1.

3.3.2 AR Gene Expression in Human Prostatic Carcinoma Cell Lines.

Figures 3.17 A. and 3.17 B. exhibit the results of PCR reactions using cDNA derived from LNCaP, DU145 and PC3 cells with the AR primers. AR2.3 and AR7.8 sequences are both detectable in LNCaP and PC3 cDNA, although undoubtedly to a lesser extent in the latter. AR2.3 and AR7.8 sequences cannot be detected in the DU145 cell line.

The gel in Figure 3.17 B. was blotted onto Hybond™-C extra using the technique of Southern and subsequently probed for AR7.8 using radiolabelled AR7.8p, a PCR probe spanning a 166bp stretch of the AR cDNA located within AR7.8. Thus it was possible not only to analyse the specificity of AR7.8s/as for AR cDNA once more, but also, potentially, to detect trace amounts of AR7.8 produced from DU145 cDNA by PCR.

Figure 3.18 A. demonstrates the results of digestion of AR7.8p with EcoRII which cuts the probe diagnostically liberating 2 fragments of sizes 81bp and 85bp. These restriction fragments cannot be visualised on the gel, however, since identical amounts of DNA were applied to both the "no digest" and "digest" lanes of the gel, the lower intensity of the band corresponding to undigested probe in the EcoRII digest suggests the presence of the appropriate restriction site in the PCR product.
Figure 3.17 Androgen Receptor Gene Expression in the LNCaP, DU145 and PC3 Cell Lines.

Total RNA was isolated from duplicate cultures of LNCaP, DU145 and PC3 cells. Total RNA (1 µg per PCR reaction) was subjected to reverse transcription followed by PCR for 35 cycles using HGPRTs/As (A.), AR2.3s/As (A.) and AR7.8s/As (B.). The PCR reactions for the "No DNA" controls were performed without cDNA. The RT-PCR products were electrophoresed, alongside 100bp molecular weight markers, through 2% agarose gels containing ~0.1 µg/ml ethidium bromide. The gels were photographed under transillumination.

A. 2% Agarose Gel - RT-PCR. Lane 1 - 100bp ladder. Lane 2 - No DNA. Lane 3 - HGPRT/ BPH tissue.
   Lane 4 - HGPRT/ LNCaP a.. Lane 5 - HGPRT/ LNCaP b..
   Lane 6 - HGPRT/ DU145 a.. Lane 7 - HGPRT/ DU145 b.. Lane 8 - HGPRT/ PC3 a..
   Lane 9 - HGPRT/ PC3 b.. Lane 10 - AR2.3/ LNCaP a.. Lane 11 - AR2.3/ LNCaP b..
   Lane 12 - AR2.3/ DU145 a.. Lane 13 - AR2.3/ DU145 b.. Lane 14 - AR2.3/ PC3 a..
   Lane 15 - AR2.3/ PC3 b.. Lane 16 - 100bp ladder.

B. 2% Agarose Gel - RT-PCR. Lane 1 - 100bp ladder. Lane 2 - AR7.8/ LNCaP a.. Lane 3 - AR7.8/ LNCaP b.
   Lane 4 - AR7.8/ DU145 a.. Lane 5 - AR7.8/ DU145 b.. Lane 6 - AR7.8/ PC3 a..
   Lane 7 - AR7.8/ PC3 b..
Southern blot analysis yields the same findings as before, i.e. strong signals are obtained for LNCaP and PC3, however, no bands are visible on the autoradiograph corresponding to the DU145 lanes of the gel [Figure 3.18 B.].

3.3.3 AR Gene Expression in Human Primary Epithelial Cell Cultures.

Three of the primary epithelial cell cultures examined for AR mRNA by Northern blotting (section 3.2.4.), i.e. BPH 1 & 2 and CaP 1, were analysed for receptor transcripts through RT-PCR [Figure 3.19]. AR2.3 and AR7.8 are detectable in the cDNAs of all three primary cultures examined. It would appear that in terms of AR gene expression, primary epithelial cells strongly resemble the PC3 cell line in that receptor transcripts are detectable by RT-PCR but not by Northern blotting.

3.4 Androgen Receptor (AR) Activity in Cultured Prostate Cancer Cell Lines and Primary Epithelial Cells.

The established prostate cancer cell lines and primary epithelial cells exhibit striking differences in terms of their responsiveness to androgens and the extent of androgen receptor gene transcription. Furthermore, two of the in vitro models of prostatic epithelia which do not exhibit androgen responsive growth display detectable androgen receptor gene expression. This prompts the question as to whether the levels of AR mRNA revealed by RT-PCR actually give rise to functional receptor?

It was intended, not just to demonstrate the presence or absence of receptor protein, but to assess whether or not AR function - the binding of ligand leading to alterations of target gene expression through protein-DNA interactions - could be demonstrated in the presence of its normal ligand. Receptor activity was assayed through transient transfection of cells with the plasmid construct pMMTV/SPAP, which carries the cDNA for secreted placental alkaline phosphatase (SPAP) downstream of the mouse mammary tumour virus - long terminal repeat (MMTV-LTR). The MMTV-LTR contains a promoter region that directs the transcription of 3' sequences upon induction by adjacent hormone response elements (HREs) e.g. the response elements for the androgen, glucocorticoid and progesterone receptors. Binding of liganded receptor is required for the activation of these HREs. Therefore, if a pMMTV/SPAP transfected cell contains a functional AR, added DHT will bind to the receptor, the receptor will then dimerise and bind to
Figure 3.18 Southern Blot Analysis of AR7.8 Derived from LNCaP, DU145 and PC3.

AR7.8p was prepared in a 35 cycle PCR reaction using the primers AR7.8ps and AR7.8pas with AR cDNA as template. The PCR product was purified from a 2% agarose preparative gel by phenol-chloroform extraction and 10μl of the isolate digested with EcoRII at 37°C for 60 minutes. The digest was analysed by electrophoresis through a 2% agarose gel containing ~0.1μg/ml ethidium bromide.

AR7.8 produced by PCR using cDNA obtained from LNCaP, DU145 and PC3 was electrophoresed on a 2% agarose gel and capillary-transferred to Hybond™-C extra. AR7.8 was detected using 32P-labelled 1.1ARp. Hybridisation was assessed qualitatively through autoradiography under intensification for 24 hours.

A. 2% Agarose Gel - AR7.8p digest. Lane 1 - 100bp ladder. Lane 2 - AR7.8p. Lane 3 - AR7.8p/EcoRII.
B. Autoradiograph - AR7.8. Lane 1 - LNCaP a.. Lane 2 - LNCaP b.. Lane 3 - DU145 a.. Lane 4 - DU145 b..
Lane 5 - PC3 a.. Lane 6 - PC3 b..
Figure 3.19 Androgen Receptor Gene Expression in Primary Epithelial Cells.

Total RNA (1μg per PCR reaction) isolated from 2 BPH-derived (BPH 1 and 2) and 1 CaP-derived primary epithelial cell culture was reverse transcribed and amplified by PCR for 35 cycles using HGPRTs/as, AR2.3s/as and AR7.8s/as. The PCR reactions for the "no DNA" controls were performed without cDNA. The RT-PCR products were electrophoresed through 2% agarose gels containing ~0.1μg/ml ethidium bromide. The gels were photographed under transillumination.

Lane 1 - 100bp ladder. Lane 2 - No DNA. Lane 3 - HGPRT/ BPH 1. Lane 4 - AR2.3/ BPH 1. Lane 5 - AR7.8/ BPH 1. Lane 6 - HGPRT/ BPH 2. Lane 7 - AR2.3/ BPH 2. Lane 8 - AR7.8/ BPH 2. Lane 9 - HGPRT/ CaP 1. Lane 10 - AR2.3/ CaP 1. Lane 11 - AR7.8/ CaP 1.
the androgen response element (ARE) within the MMTV-LTR. This interaction will switch on the neighbouring promoter, resulting in transcription of the SPAP cDNA. Alkaline phosphatase activity secreted into the cell culture medium is assayable colourimetrically.

The SPAP activity secreted by pMMTV/SPAP transfected prostatic epithelial cells in response to treatment with DHT, the androgen antagonist HO-F or combined DHT & HO-F was determined with a view to assessing endogenous AR content. Cells possessing a fully functional androgen receptor should display clear stimulation of SPAP synthesis in the presence of the androgen and corresponding antagonism of MMTV activation with the anti-androgen.

Cells were grown in monolayer to ~60% confluence before transfecting, by means of the DOTAP transfection reagent (Boehringer Mannheim), with either pMMTV/SPAP or the control vector pRc/CMV for 16 hours. Subsequently, the medium was replaced with medium containing DCC-stripped FCS and supplemented with either 10nM DHT, 10nM HO-F or 10nM DHT plus 10nM HO-F. 48 hours later, the medium was removed, heat inactivated to denature endogenous alkaline phosphatase, and subsequently assayed for SPAP activity through spectrophotometric measurement (415nm) of the conversion of p-Nitrophenyl phosphate (phosphatase substrate) to p-Nitrophenol.

3.4.1 Detection of AR Function in the LNCaP, DU145 and PC3 Cell Lines.

Figure 3.20 demonstrates the SPAP profiles obtained from the prostate cancer cell lines.

Transfection of LNCaP cells with control plasmid (pRc/CMV) does not result in a significant increase in the levels of secreted alkaline phosphatase activity over control, following administration of either DHT or the androgen antagonist HO-F at 10nM (3.20 a.). However these cells do respond to the addition of 10nM DHT plus 10nM HO-F with a significant (p<0.05) rise in the levels of alkaline phosphatase secreted. There is apparently no difference between the abilities of DHT and HO-F, at the concentrations employed, to direct MMTV-LTR-mediated transcription in pMMTV/SPAP-transfected LNCaP. Similarly, HO-F, when given to cells in conjunction with DHT does not antagonise the effects of the androgen, but rather acts synergistically with it, to significantly (p<0.05) increase the rate of MMTV-LTR-driven SPAP transcription, not only over control but also over that observed with DHT or indeed HO-F alone. These observations support the findings of the analysis of the growth responsiveness of the LNCaP cell line to DHT and
Figure 3.20 SPAP Activity Secreted by pMMTV/SPAP Transfected Prostate Cancer Cell Lines in Response to DHT and HO-F.

LNCaP, DU145 and PC3 cell lines were grown to ~60% confluence in 25cm² tissue culture flasks prior to transfection with 5μg of either pMMTV/SPAP or the control vector pRc/CMV. The transfections were allowed to proceed for 16 hours before aspirating the medium and replacing with medium containing DCC-stripped FCS and supplemented with 10nM DHT, 10nM HO-F or 10nM DHT + 10nM HO-F. Control cells were given medium containing ethanol only. After a 48 hour incubation the cell culture medium was harvested, heat inactivated (65°C for 1 hour) and the SPAP content determined through spectrophotometric assessment (415nm) of the conversion of p-Nitrophenyl phosphate to p-Nitrophenol over a 30 minute period at 37°C. The SPAP activities are given in Units/ml. Each bar represents the mean of 4 separate SPAP assays +/- SEM. Statistical significance (p<0.05) over control values is indicated by an asterisk.

a. LNCaP, b. DU145, c. PC3.
HO-F (section 3.1.1.1) in which it was observed that growth is significantly stimulated by 10nM HO-F. pMMTV/SPAP transfected cells display significantly (p<0.05) higher levels of secreted alkaline phosphatase than pRc/CMV transfectants in all treatment groups and additionally in the control group. The levels of SPAP produced by untreated pMMTV/SPAP transfectants is presumably a consequence of endogenous steroids present within the cells prior to treatment. Ideally, the cells would have been rendered steroid hormone-free before experimentation. However, it was found that the rate of transfection was drastically reduced in LNCaP using medium containing DCC-stripped FCS and it was not possible to give the cells 48 hours in steroid hormone-free medium post-transfection due to the finite duration of transient transfectants and the limitations imposed by the time to 100% confluence.

The significantly increased levels of alkaline phosphatase produced by pRcCMV-transfected LNCaP in response to DHT plus HO-F may be representative of high levels of endogenous alkaline phosphatase synthesised in response to androgenic stimuli.

As Figure 3.20 b. indicates, in both pMMTV/SPAP- and pRc/CMV-transfected DU145 cells, the addition of DHT, HO-F or DHT plus HO-F does not significantly (p>0.05) increase the alkaline phosphatase released by this cell line compared to controls.

As was observed with the DU145 cell line, PC3 cells transfected with pMMTV-SPAP do not exhibit significant (p>0.05) increases in SPAP secretion in any of the treatment groups compared with control (Figure 3.20 c.). Similar responses are observed in cells carrying pRcCMV. However, it is noticeable that within treatment groups the general trend is for the pMMTV/SPAP-transfected cells to liberate higher levels of alkaline phosphatase than their counterparts. Indeed, in 3 of the 4 groups - the control, hydroxyflutamide and combined DHT & hydroxyflutamide groups - there was significantly (p<0.05) higher levels of alkaline phosphatase in pMMTV/SPAP-transfected cells [ Note : The critical values of the 4 groups are very close to that quoted for the 95% confidence interval, indicating only marginal significance in the relevant groups ]. This phenomenon may arise due to the presence of basal levels of AR, or more likely, either due to basal promoter activity or the function of other steroid hormone receptors (This being indicative of the promiscuity of these proteins for other ligands.) In respect of the latter speculation, it should be borne in mind that the MMTV-LTR also possesses HREs for the progesterone and glucocorticoid receptors.
Comparison of the SPAP activities released by the pMMTV/SPAP-transfected cell lines would seem to indicate that of the three it is only the LNCaP cell line which contains sufficient functional receptor to activate the MMTV-LTR promoter.

It is unusual that the androgen antagonist hydroxyflutamide given to LNCaP cells not only promotes cell growth but also induces upregulation of the transcription rate from the MMTV-LTR in pMMTV/SPAP transfectants. Does this arise due to interaction with other members of the nuclear hormone receptors or is it a function of the mutation known to exist within the ligand-binding domain coding region of the LNCaP AR gene (Brinkmann et al, 1991)?

3.4.2 Co-transfection of the LNCaP and PC3 Cell Lines with pMMTV/SPAP and pCMV/ARcom.

In order to establish whether the observed response of pMMTV/SPAP-transfected LNCaP to hydroxyflutamide was due to the mutated AR endogenous to this cell line, normal AR was introduced into cells via pCMV/ARcom and the ability to activate the MMTV-LTR promoter through treatment with DHT and HO-F reassessed. It was hoped that the AR constitutively produced by pCMV/ARcom would swamp the mutated LNCaP receptor and downregulate its expression. pCMV/ARcom was also transfected into PC3 cells in order to assess the SPAP profiles from a cell in which there appears to be little or no functional receptor protein.

Figure 3.21 a. demonstrates that LNCaP cells transfected with pRc/CMV and pMMTV/SPAP display identical responses to DHT and hydroxyflutamide as illustrated previously (Figure 3.20). DHT and HO-F at 10nM, stimulate a significant (p<0.05) rise in alkaline phosphatase synthesis over control cells. These responses are clearly not as pronounced as those observed in cells transfected with pMMTV/SPAP alone. This is presumably a feature of transfection with more than one plasmid, with cells less likely to accept pMMTV/SPAP in the presence of the control vector. DHT and HO-F together significantly (p<0.05) increases SPAP production over control levels and over that observed with DHT and HO-F given separately. LNCaP co-transfected with pCMV/ARcom and pMMTV/SPAP exhibit altered profiles of SPAP production to the pRc/CMV + pMMTV/SPAP transfections. 10nM DHT significantly (p<0.05) increases SPAP production compared to control cells. However cells incubated with 10nM HO-F for 48 hours do not secrete SPAP to a significantly greater extent than the controls. SPAP synthesis is significantly
Figure 3.21 SPAP Activity Secreted by LNCaP and PC3 Cells Co-transfected with pCMV/ARcom & pMMTV/SPAP in Response to DHT and HO-F.

LNCaP and PC3 cells were grown to ~60% confluence in 25cm² tissue culture flasks prior to transfection with 5mg each of either pRcCMV & pMMTV/SPAP or pCMV/ARcom & pMMTV/SPAP. Transfection was carried out for 16 hours before replacing the medium with fresh medium containing DCC-stripped FCS and supplemented with 10nM DHT, 10nM HO-F or 10nM DHT + 10nM HO-F. Control cells were given medium containing ethanol alone. After 48 hours the cell culture medium was harvested, heat inactivated (65°C for 1 hour) and the SPAP content determined through spectrophotometric determination of the conversion of p-Nitrophenyl phosphate to p-Nitrophenol over a 30 minute period at 37°C. The SPAP activities are given in Units/ml. Each bar represents the mean of 4 separate SPAP assays ± SEM. Statistical significance (p<0.05) over control values is indicated by an asterisk.

a. LNCaP, b. PC3.
enhanced in cells given DHT and HO-F together. It is also noteworthy that in the control and 10nM DHT treatment groups, the pCMV/ARcom transfectants secrete significantly greater quantities of alkaline phosphatase than their pRc/CMV-transfected counterparts.

The results obtained with the PC3 cell line are in good agreement with the observations from LNCaP. Again DHT and DHT + HO-F supplements significantly (p<0.05) increase SPAP release by pCMV/ARcom + pMMTV/SPAP cotransfectants. Such a response is not exhibited by cells given HO-F alone. In contrast to LNCaP, pCMV/ARcom transfectants display significantly increased SPAP production compared to pRc/CMV transfectants in all treatment groups.

In both the LNCaP and PC3 cell lines we might have anticipated that, with the introduction of normal AR, repression of DHT induced SPAP synthesis by simultaneous addition of HO-F would eventuate. However, this outcome is quite evidently not furnished by either cell line. The only explanation that can be offered in this respect is that the concentration of HO-F employed is insufficient to antagonise the DHT. In competitive binding studies testosterone and DHT displace labelled testosterone from the AR maximally in 100-fold excess (Symes et al., 1978). In contrast, maximal displacement by hydroxyflutamide is achieved at 1,000 to 10,000-fold excess. Clearly, with DHT and HO-F combined and at the same concentration, the DHT is 10-100 times more likely to occupy the ligand binding domain of the AR.

It can be concluded from these co-transfection studies that the effects of hydroxyflutamide on MMTV-LTR-mediated gene transcription in LNCaP is a function of the mutated AR endogenous to this cell line.

3.4.3 Detection of AR Function in Primary Epithelial Cells.

The primary epithelial culture BPH 4, which was examined for AR mRNA by Northern blotting in section 3.2.4., was transfected with pMMTV/SPAP and subsequent responses to DHT and HO-F assessed. Figure 3.22 demonstrates that the SPAP profile obtained using this BPH-derived culture is similar to that of the PC3 cell line.

Cells transfected with pRc/CMV exhibit no significant (p>0.05) increases in alkaline phosphatase synthesis over controls when grown in the presence of either DHT, HO-F or combined DHT and HO-F. Identical responses to these treatments are demonstrated by cells transfected with pMMTV/SPAP. The suggestion is ultimately that primary epithelial cells, as was observed in the DU145 and PC3 cell
Figure 3.22 SPAP Activity Secreted by pMMTV/SPAP Transfected Primary Epithelial Cells in Response to DHT and HO-F.

BPH-derived primary epithelial cells (BPH 4) were grown to ~60% confluence in 25cm² tissue culture flasks prior to transfection with 5µg of either pMMTV/SPAP or pRc/CMV in epidermal growth medium (EGM). Transfection was performed for 16 hours before aspirating the medium and replacing with EGM without dexamethasone and supplemented with 10nM DHT, 10nM HO-F or 10nM DHT + 10nM HO-F. Control cells were given medium containing ethanol only. After a 48 hour incubation period the cell culture medium was harvested, heat inactivated (65°C for 1 hour) and the SPAP content determined through spectrophotometric assessment (415nm) of the conversion of p-Nitrophenyl phosphate to p-Nitrophenol over a 30 minute period at 37°C. The SPAP activities are given in Units/ml⁻¹. Each bar represents the mean of 4 separate assays +/- SEM.
3.4.4 Summary of the pMMTV/SPAP Transfection Studies.

Androgen receptor function is detectable using the SPAP system only in the LNCaP cell line and is not detectable in either DU145, PC3 or primary epithelial cell cultures. AR mRNA is undetectable in DU145 cells by Northern blotting or indeed by RT-PCR and therefore it is unsurprising that AR function cannot be discerned through transfection with pMMTV/SPAP. AR mRNA is detectable in PC3 and primary epithelial cells by RT-PCR, however, the extent of AR gene expression in these cells is clearly insufficient to have any significant influence on the transcription rate of androgen responsive genes.

3.5 Analysis of the Androgen Receptor Gene in the LNCaP, DU145 and PC3 Cell Lines.

The established prostate cancer cell lines exhibit marked differences in their AR mRNA levels and it is evident that 3 tiers of AR gene expression exist. In the LNCaP cell line AR mRNA is detectable by both Northern blotting and RT-PCR. AR transcripts can be identified by RT-PCR but not by Northern blotting in PC3 cells. AR gene expression is undetectable by both Northern blotting and RT-PCR in the DU145 cell line. In an attempt to determine the underlying features of these observed variations, the AR gene was examined by PCR using primers spanning each of its 8 exons to determine whether or not there are any gross differences in gene structure between the 3 cell lines. Male genomic DNA isolated from human placenta was analysed in parallel.

Figure 3.23 demonstrates that exons 2-8 of the AR gene are present in samples of genomic DNA isolated from LNCaP, DU145 and PC3 cells. AR1.1, AR1.2, and AR1.3 are also detectable in the cell lines. In all the samples analysed AR1.4, AR1.5 and AR1.6 could not be amplified using the primers quoted in Marcelli et al. (1990). Altering the conditions of the PCR cycle used did not increase the specificity of the PCR reaction such that the appropriate fragment could be visualised. Such difficulties were experienced by Marcelli and it was felt inappropriate to attempt to further optimise the PCR reactions for AR1.4s/as, AR1.5s/as and AR1.6s/as.

Clearly, AR1.1 and AR1.2 [Figures 3.23 a. and 3.23 b.] demonstrate variability in size amongst the three cell lines and indeed the human placental DNA.
Figure 3.23 Amplification of Exons 1-8 of the Androgen Receptor Gene using the Polymerase Chain Reaction.

100ng of genomic DNA from LNCaP, DU145, PC3 and human placenta (male) was analysed for AR sequences using primers spanning each of the 8 exons of the AR gene (section 2.2.5.2.a.). The PCR reactions were carried out for 35 cycles. The PCR products were examined electrophoretically on 2% agarose gels containing ~0.1µg/ml ethidium bromide. 100bp molecular weight markers were electrophoresed in parallel. The gels were photographed under transillumination. A. AR1.1 (354bp). Lane 1 - 100bp ladder. Lane 2 - placenta. Lane 3 - LNCaP. Lane 4 - DU145. Lane 5 - PC3. B. AR1.2 (534bp). Lane 1 - placenta. Lane 2 - LNCaP. Lane 3 - DU145. Lane 4 - PC3. Lane 5 - 100bp ladder. C. AR1.3 (325bp). Lane 1 - 100bp ladder. Lane 2 - placenta. Lane 3 - LNCaP. Lane 4 - DU145. Lane 5 - 100bp ladder. D. AR2 (269bp) : Lane 1 - 100bp ladder; Lane 2 - placenta; Lane 3 - LNCaP; Lane 4 - DU145; Lane 5 - PC3. AR3 (185bp) : Lane 6 - placenta; Lane 7 - LNCaP; Lane 8 - DU145; Lane 9 - PC3; Lane 10 - 100bp ladder. E. AR4 (371bp) : Lane 1 - 100bp ladder; Lane 3 - placenta; Lane 4 - LNCaP; Lane 5 - DU145; Lane 6 - PC3. AR5 (284bp) : Lane 7 - placenta; Lane 8 - LNCaP; Lane 9 - DU145; Lane 10 - PC3. F. AR6 (245bp) : Lane 1 - 100bp ladder; Lane 2 - placenta; Lane 3 - LNCaP; Lane 4 - DU145; Lane 5 - PC3. AR7 (266bp) : Lane 6 - placenta; Lane 7 - LNCaP; Lane 8 - DU145; Lane 9 - PC3; Lane 10 - 100bp ladder. G. AR8 (294bp) : Lane 1 - 100bp ladder. Lane 2 - placenta. Lane 3 - LNCaP. Lane 4 - DU145. Lane 5 - PC3.
Figure 3.23 continued overleaf >
Both these regions encapsulate the longest of two CAG repeat regions (nucleotides 333 to 426) within exon1 of the AR gene which gives rise to a polyglutamine stretch in the receptor protein. AR1.1 possesses an AvaI restriction site 5' of the CAG repeat region which will allow determination of whether it is variation in the sizes of the repeat region that accounts for the observed differences in the PCR products. From the published sequence of the AR gene, digestion with AvaI yields 2 fragments of sizes 132bp and 222bp. The latter fragment contains the CAG repeat which spans a 94bp portion. Digestion of AR1.1 from the four genomic DNA samples with AvaI yields in all cases the 132bp fragment [Figure 3.24 A.]. The larger fragment indicates variability amongst the samples, similar to that observed in AR1.1. This would suggest that the differences in AR1.1 from the LNCaP, DU145 and PC3 is due to disparity in the numbers of CAG triplets. Amplification of the CAG repeat using flanking primers (1.CAGs/as) supports the findings of the AvaI restriction digest [Figure 3.24 B.]. In normal individuals the length of the polyglutamine region varies from 17 to 26 residues with an average repeat number of 21 (La Spada et al, 1991). Variations in the numbers of glutamine residues from the normal range have been demonstrated to be associated with AR-linked disorders e.g. increased thermolability associated with partial androgen insensitivity syndrome (12 glutamine residues) and X-linked spinal and bulbular muscular atrophy (La Spada et al, 1991; McPhaul et al, 1991). From the published sequence, 1.CAGs and 1.CAGas will produce a 198bp PCR fragment with 19 CAG triplets. Genomic DNA from human placenta and DU145 cells yields PCR products of the expected size (~200bp). Clearly the products obtained from the LNCaP and PC3 cell lines are larger in size but would appear to lie within the accepted normal range i.e. a normal product may be up to a size of 219bp. Further the PC3 cell line has an approximately equivalent number of repeats to LNCaP which contains a fully functional AR, despite having a mutation in its ligand binding that alters its binding characteristics.

3.6 Overview of the Androgen Receptor Studies.

The data presented supports the idea that the hormone-independence characteristic of relapsed prostate cancers is associated with a down-regulation of the androgen receptor. All of the cells which exhibit androgen-independent growth also display a level of receptor protein which is insufficient to significantly influence the transcription of target genes. Furthermore, the alteration of androgen receptor
gene expression is clearly manifest at the DNA level where three tiers of gene transcription are in operation.

Whether these observations on the androgen receptor are truly representative of the in vivo situation in humans is at best extremely debateable judging by the contradictory results obtained in immunohistochemical analyses. Less controversy surrounds the relationship between the bcl-2 oncoprotein and hormone-independence in vivo. There is little doubt that a strong correlation exists between the extent of cellular expression of this oncogene and resistance to androgen ablation therapy. Indeed, in the normal prostate bcl-2 is exclusively localised to the basal epithelial cells which are resistant to androgen deprivation and which coincidentally do not generally exhibit androgen receptor expression. Therefore the expression of bcl-2 in the cell models was examined to ascertain whether there is any relationship with androgen responsiveness, and thus by association, the androgen receptor.

3.7 bcl-2 Expression in Cultured Prostate Cancer Cell Lines and Primary Epithelial Cells.

The bcl-2 oncoprotein acts to block the pathways of apoptosis in many tissues, including prostatic epithelia, which characteristically undergo programmed cell death. Epithelial cells in the normal prostate will suffer apoptosis under the androgen limiting conditions consequent of androgen ablation therapy. The hormone escape characteristic of the prostate during such therapy may result from promotion of a subset of epithelial cells resistant to apoptosis perhaps due to upregulated bcl-2 oncoprotein. Indeed it has been demonstrated in the rat ventral prostate that following castration, bcl-2 mRNA levels rapidly increase reaching a maximum at around 10 days (McDonell et al, 1992).

The prostate cancer cell lines and primary epithelial cells were examined for bcl-2 expression both at the RNA and protein levels.

3.7.1 Detection of bcl-2 mRNA in LNCaP, DU145, PC3 and Primary Epithelial Cells by Reverse Transcription-Polymerase Chain Reaction (RT-PCR).

bcl-2α (5.5kb) and β (3.5kb) mRNA was detected by RT-PCR using intron-spanning primers specific to each transcript, namely 5.5s/as and 3.5s/as respectively. These primers share the same 5' primer, however bcl-2α and β exhibit different sequences 3' of the putative intron and therefore discrimination between
AR1.1 from human placenta plus the LNCaP, DU145 and PC3 cell lines was digested with Aval at 37°C for 60 minutes (A.). The CAG repeat of the AR gene from LNCaP, DU145, PC3 and human placenta (male) was amplified by PCR for 35 cycles using 1.CAGs and 1.CAGas (B.). Digested AR1.1 and the PCR products were analysed by electrophoresis through 2% agarose gels containing ~0.1µg/ml−1 ethidium bromide. The gels were photographed under transillumination.

A. 2% Agarose Gel - AR1.1 : Lane 1 - placenta; Lane 2 - LNCaP; Lane 3 - DU145; Lane 4 - PC3.
AR1.1/Aval : Lane 5 - placenta; Lane 6 - LNCaP; Lane 7 - DU145; Lane 8 - PC3; Lane 9 - 100bp ladder.
B. 2% Agarose Gel - CAG repeat. Lane 1 - 100 bp ladder. Lane 2 - placenta. Lane 3 - LNCaP. Lane 4 - DU145. Lane 5 - PC3.
the two transcripts is provided by the 3' primer. 5.5s/as and 3.5s/as yield PCR products of sizes 247bp and 199bp respectively.

Figure 3.25 demonstrates the outcome of the analysis of reverse transcribed mRNA from the prostate cancer cell lines and the primary epithelial cultures BPH 1, BPH 2 and CaP 1 examined in section 3.2.4.. bcl-2α and β sequences are detectable in all three of the cell lines and indeed in all the primary epithelial cells examined.

5.5 and 3.5 amplified from CaP 1 mRNA were analysed by restriction digestion with Alul and EcoRII respectively to determine the specificity of the primer pairs employed. From the published sequences for 5.5 and 3.5, the respective digests should generate fragments of sizes 127bp & 120bp and 104bp & 95bp. Figure 3.26 A. indicates the results of the digestion of 5.5 with Alul which clearly demonstrates the production of a fragment running at approximately 120-130bp. Digestion of 3.5 with EcoRII produces a fragment of approximately 110bp (Figure 3.26 B.). It would appear that the PCR products generated using 5.5s/as and 3.5s/as are complementary to bcl-2 cDNA and that these primer pairs are specific for bcl-2 sequences under the reaction conditions employed.

3.7.2 Detection of the bcl-2 Oncoprotein in LNCaP, DU145, PC3 and Primary Epithelial Cells by Western Blotting.

Lysates from LNCaP, DU145 and PC3 cells all demonstrate bcl-2 expression by Western blotting (Figure 3.27 A.). In equivalent 10μg loadings of lysates from the three cell lines the relative levels of bcl-2 expression as determined by densitometry were as follows : PC3 (0.792) > DU145 (0.443) > LNCaP (0.296). Evidently the androgen-independent cell lines express more bcl-2 oncoprotein than the hormone-sensitive cell line LNCaP. From the densitometric analysis, it would appear that the PC3 cell line can be conservatively estimated to express ~2.7 times more bcl-2 than LNCaP.

Primary epithelial cells express approximately equivalent amounts of bcl-2 to the PC3 cell line (Figure 3.27 B.).

It is worth noting that PC3 and primary epithelial cells are similar in terms of their androgen receptor expression and responses to androgenic hormones. It may be, that the unresponsiveness of these cells to androgens arising from insufficient expression of a functional AR underlies the elevated expression of bcl-2 relative to LNCaP. Could bcl-2 be an androgen-repressed gene? Secretory epithelial cells in the normal prostate possess an androgen receptor but do not express bcl-2 to any
Figure 3.25 Detection of bcl-2α and β mRNA Transcripts in Prostatic Carcinoma Cell Lines and Primary Epithelial Cell Cultures.

Total RNA (1μg per PCR reaction) isolated from the LNCaP, DU145 & PC3 cell lines and from the primary epithelial cultures BPH1, BPH2 and CaP1 was reverse transcribed and amplified in a 35 cycle PCR using the primers HGPRTs/as, 5.5s/as and 3.5s/as. "No DNA" control PCRs were performed without cDNA. The RT-PCR products were analysed by electrophoresis through 2% agarose gels containing ~0.1μg ml⁻¹ ethidium bromide. The gels were photographed under transillumination.

A. 2% Agarose Gel - RT-PCR. Lane 1 - 100bp ladder. Lane 2 - No DNA. Lane 3 - HGPRT/ LNCaP. Lane 4 - HGPRT/ DU145. Lane 5 - HGPRT/ PC3. Lane 6 - bcl-2α/ LNCaP. Lane 7 - bcl-2α/ DU145. Lane 8 - bcl-2α/ PC3.

B. 2% Agarose Gel - RT-PCR. Lane 1 - 100bp ladder. Lane 2 - No DNA. Lane 3 - HGPRT/ LNCaP. Lane 4 - HGPRT/ DU145. Lane 5 - HGPRT/ PC3. Lane 6 - bcl-2β/ LNCaP. Lane 7 - bcl-2β/ DU145. Lane 8 - bcl-2β/ PC3.

C. 2% Agarose Gel - RT-PCR. Lane 1 - 100bp ladder. Lane 2 - No DNA. Lane 3 - HGPRT/ BPH1. Lane 4 - bcl-2α/ BPH1. Lane 5 - HGPRT/ BPH2. Lane 6 - bcl-2α/ BPH2. Lane 7 - HGPRT/ CaP1. Lane 8 - bcl-2α/ CaP1.

D. 2% Agarose Gel - RT-PCR. Lane 1 - 100bp ladder. Lane 2 - No DNA. Lane 3 - HGPRT/ BPH1. Lane 4 - bcl-2β/ BPH1. Lane 5 - HGPRT/ BPH2. Lane 6 - bcl-2β/ BPH2. Lane 7 - HGPRT/ CaP1. Lane 8 - bcl-2β/ CaP1.
Figure 3.26 Analysis of 5.5 and 3.5 Produced by RT-PCR from CaP 1 Total RNA.

10μl of 5.5 (A.) and 3.5 (B.) synthesised by PCR from reverse transcribed total RNA isolated from CaP1 were digested with AluI and EcoRII respectively. Digestion was carried out for 60 minutes at 37°C. The digestion products were electrophoresed through 2% agarose gels alongside 10μl of undigested PCR product.

A. Restriction Digest (5.5/AluI). Lane 1 - 100bp ladder. Lane 2 - 5.5. Lane 3 - 5.5/AluI.

B. Restriction Digest (3.5/EcoRII). Lane 1 - 100bp ladder. Lane 2 - 3.5. Lane 3 - 3.5/EcoRII.
great extent. Bearing this in mind, it was intended to determine whether by introduction of the AR back into PC3 would similarly reduce bcl-2 expression.

3.7.3 bcl-2 Expression in pCMV/ARcom-Transfected PC3 Cells.

In LNCaP cells rendered steroid-free through growth in steroid-depleted medium for 48 hours the addition of fresh medium supplemented with 10nM DHT for 24 hours induces a reduction in bcl-2 protein content (Figure 3.28). Densitometry expresses this down-regulation as lowering of the optical density of the protein band from 0.783 to 0.661.

It appears that the bcl-2 oncoprotein is down-regulated in hormone-sensitive cells and indeed bcl-2 gene expression exhibits a degree of hormone sensitivity. pCMV/ARcom was transfected into PC3 cells and the cells subsequently treated with or without 10nM DHT. Analysis of these cells for bcl-2 by Western blotting indicates that compared to normal PC3, pCMV/ARcom-transfected cells do not demonstrate any change in bcl-2 expression in the absence of DHT (Figure 3.29). However, transfected cells given 10nM DHT exhibit a marked increase in bcl-2 expression.

These observations tend to suggest that the increased expression of bcl-2 in relapsed prostate cancer does not necessarily go hand in hand with the loss of androgen receptor gene expression. However, it is clear that bcl-2 expression can be influenced by androgens in both hormone-dependent cells and hormone-independent cells expressing the androgen receptor although in a paradoxical fashion.

3.8 Summary of Results.

The data presented can be summarised as follows:

1. Of all the in vitro models of prostatic epithelia examined, LNCaP, DU145 PC3 and primary epithelial cells, only the LNCaP cell line exhibits any response to androgens or anti-androgens. Furthermore, LNCaP display anomalous growth responses to 1.0 nM and 10.0nM hydroxyflutamide.

2. Northern blotting demonstrates that androgen receptor mRNA is only detectable in LNCaP with little or no transcripts exhibited by any of the androgen-independent cell models.
Figure 3.27 Determination of bcl-2 Expression in LNCaP, DU145, PC3 and Primary Epithelial Cells by Western Blotting.

The prostate cancer cell lines, LNCaP, DU145 and PC3 plus the primary epithelial cultures, BPH1, BPH2 and CaP 1 were analysed. Human tonsil was included as a positive control. Cells grown to ~80% confluence in 75cm² tissue culture flasks were lysed in 1xSDS gel loading buffer, the protein content assessed using the method of Bradford and appropriate volumes of the cell lysates subsequently electrophoresed in 15% denaturing polyacrylamide gels. The proteins were transferred to Hybond™-C extra by electroblotting and the blot probed with an anti-bcl-2 monoclonal antibody. The protein bands were visualised using the ECL system. Autoradiography was performed for 1 minute.

A. Autoradiograph - Bcl-2. (10 μg of protein per well.) Lane 1 - LNCaP. Lane 2 - DU145. Lane 3 - PC3. Lane 4 - Human tonsil.

B. Autoradiograph - Bcl-2. (20 μg of protein per well.) Lane 1 - PC3. Lane 2 - BPH1. Lane 3 - BPH2. Lane 4 - CaP1.
LNCaP cells were grown to ~60% confluence in 75cm² tissue culture flasks prior to replacing the medium with RPMI 1640 containing 10% DCC-stripped FCS. The cells were incubated for 48 hours before aspirating the medium and replacing with fresh medium containing either 10nM DHT or ethanol. 24 hours later the cells could be washed in PBS and subsequently lysed in 300µl of 1xSDS gel loading buffer. The protein contents of both cell lysates were determined and 40µg of protein per sample electrophoresed through a 15% polyacrylamide gel. The proteins were blotted onto Hybond™-C extra and the blot probed with an anti-bcl-2 monoclonal antibody. The bcl-2 band was visualised using the ECL protein detection system. Autoradiography was performed for 1 minute with intensifying screens.

Lane 1 - LNCaP +10nM DHT (24 hours). Lane 2 - LNCaP -10nM DHT (24 hours).
Figure 3.29 bcl-2 Expression in PC3 Cells Transfected with pCMV/ARcom.

PC3 cells were grown to ~60% confluence in 25cm² tissue culture flasks before transfecting with 5µg of either pCMV/ARcom or pRC/CMV. Transfection was performed for 16 hours prior to aspirating the medium and replacing with Ham's F12 containing 7% DCC-stripped FCS supplemented with either 10nM DHT or ethanol. Following a further 24 hour incubation, the cells were washed in PBS and subsequently lysed in 1xSDS gel loading buffer. The protein contents of the cell lysates were determined and 20µg of protein per sample electrophoresed on a 15% polyacrylamide gel. The proteins were blotted onto Hybond™-C extra and the blots probed with an anti-bcl-2 antibody. The protein band was detected using the ECL protein detection system. Autoradiography was performed for 1 minute with intensifying screens.

Lane 1 - untransfected PC3 -10nM DHT. Lane 2 - pCMV/ARcom -10nM DHT.
Lane 3 - pCMV/ARcom +10nM DHT.
3. Using the more sensitive detection method of RT-PCR, androgen receptor transcripts could be visualised in the LNCaP, PC3 and primary epithelial cells but not in the DU145 cell line.

4. Transfection with the steroid receptor reporter plasmid pMMTV/SPAP indicated that only LNCaP cells possess a functioning androgen receptor and that this receptor is activated by both DHT and hydroxyflutamide.

5. The lack of any detectable androgen receptor mRNA in the DU145 cell line is not due to any gross deletions in the receptor gene. The only variation between the cell types was in the CAG repeat region of the gene which in all cases was of a size well within what is accepted as the normal range.

6. The bcl-2 oncoprotein is detectable in all four cell types, however, it is markedly up-regulated in the androgen-independent species. This increase in oncogene expression does not appear to correlate with androgen receptor loss.
CHAPTER 4

DISCUSSION

In order to establish the phenotype of the androgen-independent epithelial clones residing within relapsed prostatic adenocarcinoma, cell lines and primary cultures of prostatic epithelia provide suitable, if not ideal, model systems. The results presented in this thesis demonstrate striking differences between androgen-sensitive and androgen-insensitive cells in terms of the expression of the androgen receptor gene and the oncogene bcl-2. Although these findings are reasonably conclusive, attempts to reconcile this data with those observations in vivo, would tend to indicate that great care must be taken in the conclusions drawn.

The three cell lines studied, LNCaP, DU145 and PC3, are all models of advanced metastatic prostate cancer with marked differences in their sensitivity to androgenic inputs. Before consideration of the features underlying the extent of their androgen responsiveness it is worth reflecting upon the lesions from which they were derived and the treatment these lesions had been subjected to. The LNCaP cell line, as discussed in section 1.7.1., was derived from a lymph node of a patient with stage D2 (stage M1a) who had undergone 6 months of treatment with estracyt followed by a short period of chemotherapy (Horoszewicz et al, 1983). Similarly the patients from which the DU145 and PC3 cells were obtained had undergone oestrogen treatment and combined orchietomy plus oestrogen treatment respectively (Stone et al, 1978; Kaighn et al, 1979 and 1980). It is noteworthy that all of these cell lines were obtained from patients undergoing endocrine therapy.

Recently, attempts have been made to determine the expression of the androgen receptor in the LNCaP, DU145 and PC3 cell lines (Tilley et al, 1990a &
The extent of bcl-2 gene expression in these cell lines has, however, not been ascertained.

In primary cultures of the prostate the responsiveness of epithelial cells to androgens is less clear-cut. A number of groups have analysed the hormone-responsiveness of such cultures without establishing an unequivocal answer. Furthermore, it is clear from the literature that little or no attempt - at least by molecular biological approaches - has been made to establish the expression of the androgen receptor and bcl-2 genes in primary epithelial cells.

The approach used in the study of the available in vitro models can be summarised as follows: 1. measurement of cell growth in the presence of a range of concentrations of androgens and anti-androgens; 2. assessment of androgen receptor and bcl-2 expression by Northern analysis, RT-PCR and Western blotting; 3. measurement of androgen receptor function through transfection with the reporter plasmid pMMTV/SPAP; 4. analysis of the androgen receptor gene in the prostate cancer cell lines.

4.1 In vitro Responses to Androgens and Anti-androgens.

It is clear from the observed effects of androgens and anti-androgens upon their growth, that all the cell lines and primary cultures examined display either aberrant responses or indeed no response at all. At concentrations of between 0.001nM and 10.0 nM, DHT and the flutamide metabolite, hydroxyflutamide, have no significant influence upon the growth of either PC3 or DU145 cells. The LNCaP cell line however demonstrates significantly increased growth in the presence of DHT in the range 0.001nM-10.0nM, two days post-administration. Sonnenschein et al (1989) observed that the addition of DHT to LNCaP generates a biphasic proliferative response with maximal stimulation at ~3x10^{-10}M and marked inhibition of growth at <3x10^{-12}M and >3x10^{-7}M. Such a biphasic response is not exhibited in the range of concentrations employed by the LNCaP cells used in this study. Despite the pronounced increase in growth rate consequent of androgen administration, and as has been previously demonstrated by other workers, the LNCaP cell line does not exhibit corresponding growth inhibition in the presence of the anti-androgen hydroxyflutamide. After a four day period, the cells exhibit significant increases in growth rate in the presence of 1.0nM and 10.0nM hydroxyflutamide. It is noteworthy that the concentration of the anti-androgen and indeed the time taken to elicit a significant response are considerably greater than
in the case of DHT. This may be a feature of the greater affinity for DHT displayed by the LNCaP androgen receptor.

The growth stimulatory action of hydroxyflutamide on LNCaP cells raises the question of whether or not this model can be used as a model of hormone-dependent malignant prostatic epithelia. Quite clearly it cannot.

All the primary epithelial cells behave in an androgen-independent manner in culture. Using concentrations of androgen and anti-androgen which elicit a growth response in the LNCaP cell line, no effect is observable in either BPH- or CaP-derived epithelial cultures. These findings are very much in agreement with those of McKeehan et al (1984) and Merchant (1990), the former group consequently speculating that the epithelial cells obtained through culture of prostatic acini are either undifferentiated basal cells or dedifferentiated secretory epithelia. This theory is supported by observed androgen-independence in primary cultures from rat ventral prostate coupled with a lack of expression of the prostatic binding protein genes which are markers of secretory epithelium in this tissue source (Montpetit et al, 1988).

4.2 Androgen Receptor Expression and Function in Prostatic Epithelial Cells.

A number of groups have demonstrated high levels of androgen receptor gene expression in the LNCaP cell line, both at the mRNA and protein levels (Tilley et al, 1990a.; Faber et al, 1991; Horoszewicz et al, 1983). Tilley et al (1990b.) analysed the expression of the androgen receptor gene, not only in LNCaP, but also in the androgen-insensitive cell lines DU145 and PC3. Examination of samples of total RNA by Northern blotting with a 3' prime probe similar to that used in the thesis, demonstrated that AR mRNA is undetectable in the DU145 and PC3 cell lines. These observations are supported by steroid-binding and Western blot analysis. In addition, attempts by Tilley et al (1990b.) to increase the sensitivity of AR mRNA detection using S1 nuclease protection did not aid in the detection of receptor transcripts in either DU145 or PC3.

RT-PCR performed with intron-spanning primer pairs demonstrates that the androgen receptor gene is expressed in both the LNCaP and PC3 cell lines but not in DU145. Southern blot analysis, used to improve the detection of low levels of PCR product, fails to demonstrate the presence of AR transcripts in DU145. These findings have recently been corroborated by Culig et al (1993). Furthermore, it is also apparent that the transcripts detectable in LNCaP and PC3 appear to be of
full length owing to the detection of both DNA- and ligand-binding domain coding regions.

In primary epithelial cell cultures of both benign and malignant origin, which have been demonstrated to be androgen-independent, androgen receptor mRNA is undetectable by Northern blotting but can be measured by RT-PCR.

Clearly three tiers of androgen receptor gene expression exist in the in vitro models studied. In this respect LNCaP and DU145 are polar, with the latter apparently expressing no receptor message at all. Such a lack of AR mRNA does not arise from any gross alterations or deletions of the receptor gene in DU145 since all 8 exons are detectable by PCR. The PC3 cell line and primary epithelial cells fall some way between LNCaP and DU145, expressing sufficient AR mRNA to be detectable by RT-PCR but not enough to overcome the detection limits of Northern blotting. In view of such findings the obvious question to be addressed is whether or not the levels of AR mRNA in the PC3 and primary epithelial cells are sufficient to generate significant quantities of functional receptor protein.

Transient transfection of cells with the steroid receptor reporter plasmid pMMTV/SPAP indicates that only LNCaP of the three cell lines studied possesses significant androgen receptor function. Administration of 10nM DHT to pMMTV/SPAP-transfected DU145, PC3 and LNCaP cells induces an increase in the secreted alkaline phosphatase activity only in the latter case. No significantly higher levels of SPAP activity are observed in either DHT-treated DU145 or PC3 cells over control cells. Similarly transfection with pMMTV/SPAP followed by DHT treatment does not elevate the levels of alkaline phosphatase secreted by primary epithelial cells. Thus it would appear that under the experimental conditions used there is insufficient androgen receptor in either DU145, PC3 or primary epithelia to raise the rate of transcription from the MMTV promoter. Therefore, it is reasonable to propose that the extent of AR gene expression observed in the PC3 cell line and primary epithelial cells does not generate, in terms of transcriptional control, a cellularly-significant level of receptor protein. This is further borne out by the observation that the PSA gene, which has been postulated to be under the direct control of androgens owing to the close proximity of an androgen response element, which is not expressed at all in the DU145 cell line, is basally expressed in PC3 cells and primary epithelial cells (Meyer et al, 1993; Fong et al, 1991). Additionally, Fong et al (1991) could not detect any significant increase in PSA release from primary cultures following DHT administration.

So why is the androgen receptor mRNA down-regulated in the DU145, PC3 and primary epithelial cells compared to LNCaP? The two potential reasons for
these dramatic alterations are either reductions in the rate of transcription of the AR gene or changes in the half-life of the receptor message itself. In the case of the DU145 cell line we can probably discount decreased half-life of the receptor message since it would surely be possible to detect some AR gene transcripts by RT-PCR. More likely is that alteration of the promoter is responsible for the complete shut-off of the AR gene in DU145. This alteration may take the form of a direct mutation within a transcription factor binding site for example or perhaps hypermethylation of the promoter DNA. Such site-specific methylation of cytosine has been demonstrated to induce transcriptional inactivation in a number of eukaryotic genes (for review see Strobl, 1990) and it is worth noting that the AR gene promoter is very C-rich.

Whatever the mechanisms involved, it would appear at first glance that the hormone-independence which characterises the DU145 and PC3 cell lines in addition to the primary epithelial cells has its origins in the lack of significant levels of fully functional androgen receptor. However the re-introduction of AR into these cells produces unexpected results following androgen administration. Yuan et al (1993) in their analysis of the behaviour of PC3 cells stably transfected with AR cDNA established that the addition of DHT at a range of concentrations from 0.1nM to 10μM decreased the rate of proliferation by ~40-50% compared to untransfected and mock transfected cells. It is certainly surprising that at all of the test concentrations examined their response should be negative, bearing in mind the response of LNCaP to DHT. In addition to this, it is worth noting that the arrest of cell growth associated with LNCaP maintained in steroid depleted medium is not a feature of AR-positive PC3 cells. The transfectants demonstrate only slight and almost certainly insignificant decreases in growth rate under these circumstances. In view of these observations it would appear that in terms of the regulation of cell growth at least, the AR acts as both a stimulatory and inhibitory transcription factor in prostatic tumour cells. As a consequence, the loss of the AR in androgen-independent cells represents the removal of a transcriptional repressor not a transcriptional enhancer.

Using immunohistochemistry, Sica et al (1994) have recently demonstrated that the PC3 cell line, in common with some androgen-independent tumours, displays heterogeneous staining for the AR with a low percentage of strongly positive cells. Interestingly, treatment with β-interferon (β-IFN) increases the number of receptor-positive cells with concomitant decreases in growth rate. Furthermore, the increased expression of the androgen receptor results in an increased growth rate following the administration of the anti-androgen
hydroxyflutamide. Evidently the paradoxical action of DHT in AR-positive PC3 cells is paralleled by unusual responses to androgen antagonists.

These observations in the PC3 cell line raise a number of interesting questions regarding the nature of androgen-independent prostate cancer cells. It is likely that the AR mRNA detectable by RT-PCR originates from the small number of AR expressing cells that contaminate PC3. The receptor protein encoded by this mRNA is structurally normal judging by the sequence information provided by Culig et al (1993) and therefore it must be concluded, bearing in mind the results of Yuan et al (1993), that in AR-positive PC3 cells different genes are being regulated or are regulated in a different manner than in ordinary receptor-positive prostate cancer cells. In this respect it might prove interesting to determine whether there is any differences in growth factor expression between LNCaP and PC3 and how, if at all, this relates to androgen inputs. What is clear if one considers the apparent basal expression of PSA in PC3, is that the changes in androgen-regulated gene expression apparently characteristic of androgen-independent prostate cells are extremely specific and do not necessarily involve all AR-dependent genes.

Unusual features of androgenic control are not only confined to hormone-independent cells. The transfection studies demonstrate that the growth stimulatory effect of hydroxyflutamide on the LNCaP cell line is paralleled by agonistic effects upon the endogenous AR. pMMTV/SPAP-transfected LNCaP respond to the administration of 10nM hydroxyflutamide with an increase in alkaline phosphatase synthesis and secretion equivalent to that invoked by DHT. In addition, the anti-androgen does not antagonise the action of DHT at this concentration but rather acts in synergy with it to enhance the MMTV promoter activity. Veldscholte et al (1992b), amongst others, have published similar data to that presented here using a reporter plasmid containing the cDNA for chloramphenicol acetyltransferase (CAT) downstream of the MMTV-LTR. With this system Veldscholte et al (1992b) demonstrated that the LNCaP AR could induce CAT production when binding both hydroxyflutamide and the synthetic androgen/progesterone receptor ligand methyltrienolone (R1881), the latter proving to be only ~1.5-2 times more potent agonistically.

The apparently paradoxical effects of hydroxyflutamide have been blamed upon the thr868 to ala point mutation present within ligand binding domain of the LNCaP androgen receptor. Certainly the introduction of wild-type AR into LNCaP via the plasmid construct pCMV/ARcom dramatically alters the outcome of co-transfection studies with the pMMTV/SPAP reporter. Co-transfectants exhibit significant increases in alkaline phosphatase activity when supplied with DHT,
however in contrast to previous findings, hydroxyflutamide cannot enhance the rate of transcription of the SPAP gene. This profile of AR function is identical to that obtained in the AR-negative cell line PC3. The effects of the AR mutation in LNCaP is quite startling, however, quite why a single somatic alteration should impel a receptor protein to completely reverse one its functional characteristics is unclear. It seems unlikely that altered binding kinetics are relevant in this context since the LNCaP receptor displays only a marginally higher affinity for hydroxyflutamide than its wild-type counterpart (Veldscholte et al, 1992b.). What seems more plausible is that the mutation somehow dictates an altered conformational response to hydroxyflutamide binding, allowing transformation of the receptor into a DNA-binding state. Although evidence in support of this claim is limited, normal pathways of receptor activation appear to prevail. In particular, the shedding of the heat shock proteins hsp90, hsp70 and hsp56, which predisposes the AR to transcriptional activation following ligand-binding, also occurs upon interaction with hydroxyflutamide (Veldscholte et al, 1992a.).

4.3 bcl-2 Expression in vitro.

Since its initial discovery in human B-cell lymphomas, the bcl-2 oncoprotein has spawned much interest amongst researchers. Cell death currently being a very fashionable topic in biological research, it is of no great surprise that an oncogene product whose expression is so tightly restricted to tissue types characterised by programmed cell death and which has the ability to block this form of cell death has gained so much attention.

In the prostate bcl-2 expression, which is essentially a basal epithelial phenomenon, has been implicated in the progression of prostate tumours to androgen-independent growth. The rational of this hypothesis being that bcl-2 expressing cells are able to survive in circumstances which would normally initiate apoptosis in malignant prostatic epithelia e.g. under conditions of androgen deprivation. Data presented by McDonnell et al (1992) and Colombel et al (1993) would tend to support such an idea, both groups having observed that the expression of the bcl-2 oncoprotein is elevated in hormone refractory prostatic tumours compared to androgen-dependent neoplasms.

The results presented in the thesis demonstrate that expression of the bcl-2 oncogene, assessed at both the protein and mRNA levels, is detectable in the three cell lines and all of the primary epithelial cell cultures examined. Western blotting has shown that the PC3 cell line in addition to the primary epithelial cells
contain high levels of bcl-2 protein, conservatively estimated by densitometric analysis to be approximately 2-3 times greater than in LNCaP. Expression of bcl-2 in the DU145 cell line falls some way between LNCaP and PC3. It would therefore appear that the bcl-2 oncoprotein is up-regulated in the hormone-independent prostate cells compared to the androgen-dependent epithelia.

The full significance of this altered bcl-2 gene expression is not entirely clear, however, there is strong evidence to suggest that susceptibilities to programmed cell death are altered concurrently. Buttyan et al (1993) demonstrated in their analysis of the behaviour of LNCaP cells stably transfected with bcl-2 cDNA that the levels of bcl-2 oncoprotein normally present in this cell line are insufficient to prevent cell death inducible either by culture in serum-free medium or treatment with phorbol esters. Apoptosis cannot be induced as easily in androgen-independent cells as confirmed by the primary epithelial cells used in the thesis which are generated and maintained under serum-free conditions. Furthermore, Yuan et al (1993) have demonstrated that the PC3 cell line is highly resistant to apoptosis-inducing agents such as the calcium ionophore ionomycin in high concentration. It may well be, in face of such evidence, that the elevated bcl-2 expression in PC3 and primary epithelia suppresses or at least contributes to the suppression of apoptosis in these cells.

The fact that the androgen-sensitive cell line LNCaP expresses significantly less bcl-2 than its hormone-independent counterparts, lead us to consider whether some relationship exists between AR expression and that of bcl-2. In LNCaP the levels of oncoprotein are modulated by DHT such that it increases in an androgen-deprived environment. However, re-introduction of the androgen receptor into the PC3 cell line does not result in any dramatic reduction of bcl-2 expression in these cells, on the contrary, administration of DHT produces a marked up-regulation. The suggestion is that receptor loss and bcl-2 up-regulation are purely coincidental.

The paradoxical effects of DHT administration upon the levels of bcl-2 in the AR-positive LNCaP and PC3 cells are perhaps unsurprising in light of the observations of Yuan et al (1993) and may either represent altered gene expression or a change in the proportion of cells expressing the oncogene. In this context it might have proved useful to examine both cell lines immunohistochemically to ascertain the staining patterns for bcl-2 and how these are altered in response to androgens. It might be envisaged, for example, that LNCaP and PC3 cells are composed of a mixture of bcl-2-positive and bcl-2-negative cells and that cells expressing the oncogene display growth advantage in unfavourable androgen environments and subsequently increase proportionately in
the population. This situation may well mimic that observed by McDonnell et al. (1992) in the rat ventral prostate following androgen ablation in which increased bcl-2 expression parallels the outgrowth of apoptosis-resistant cells.

If the cells in a hormone-independent tumour contain high levels of bcl-2 oncoprotein it may prove difficult to induce cell death through the use of non-mitosis-dependent chemotherapeutic agents such as ionomycin or the endoplasmic reticulum Ca\(^{2+}\)-ATPase pump inhibitor thapsigargin (Thastrup et al., 1990). Subsequently, specific inhibition of the bcl-2 oncoprotein or other anti-apoptosis proteins may be necessary in order to induce programmed cell death in androgen-independent prostate cancer cells.

Reed et al. (1990) demonstrated that antisense oligonucleotides targeted against the predicted translation-initiation site of the bcl-2 oncogene could specifically inhibit the growth of the leukaemic cell line 697 in a dose dependent fashion. This growth inhibition followed the near complete removal of the bcl-2 oncoprotein from the 697 cells. In theory, the cellular significance of bcl-2 in prostate cancer cells could be assessed through its antisense-mediated eradication.

Preliminary experiments with the bcl-2 phosphorothioate antisense oligonucleotide 5'CAGCGTGCGCCATCCTTCCC'3' - a sequence which does not cross-react with any other human genes - and the PC3 cell line have yielded disappointing results. After supplementing with antisense oligonucleotides at concentrations of 50 to 300\(\mu\)M, Western blotting demonstrated that the levels of bcl-2 oncoprotein was not altered over a four day period. Either the oligonucleotides were unable to enter the cell or could not target the bcl-2 mRNA efficiently. It is noteworthy in this respect that phosphorothioate oligonucleotides despite offering the benefit of a degree of nuclease resistance are notoriously difficult to transport across the plasma membrane, and to date, attempts at improving delivery through the use of lipofection has only led to cytotoxicity problems. If these membrane transport problems can be overcome it will be possible to properly assess the influence of the bcl-2 oncogene in androgen-independent prostate malignant epithelial cells.

4.4 Implications for Primary Epithelial Culture of the Prostate.

The prime concern in establishing a primary prostatic culture is that the population of cells obtained should be a pure population of either epithelial or fibroblast cells with little or no cross-contamination. It is generally held that the
plating of prostatic organoids or fragments of prostatic tissue in media containing high percentages of foetal calf serum necessarily leads to the overgrowth of the cultures by fibroblasts (Webber, 1980). Therefore, in attempts to develop epithelial cell cultures which are fibroblast-free, much emphasis has been placed upon the use of serum free conditions similar to those defined by Chaproniere and MCKeehan in 1986. This group successfully cultured pure prostatic epithelia from acinar deposits - derived from collagenase treated prostatic tissues - in a serum-free medium which will not support fibroblastic growth. However, since at least three varieties of cell in the prostate are epithelial in nature, there is an obvious necessity once a successful and reproducible method of culture has been established to determine the exact cell type. Clearly in terms of the study of prostatic neoplasia it is the secretory epithelial cell which is of greatest interest and consequently the ability to efficiently culture this cell type would be of immense value. Unfortunately, as the results presented in the thesis would lay testimony to, the cells yielded under the culture conditions of Chaproniere and MCKeehan are in no way representative of a secretory epithelial cell in vivo.

The secretory epithelial cells within a prostatic acinus are without doubt the most significant cell type in terms of prostatic physiology and the pathogenesis of neoplasia. They are inherently androgen-dependent, expressing high levels of androgen receptor and displaying dramatic alterations in gene expression dependent upon the hormonal environment. The epithelial cells obtained using the methods of Chaproniere and MCKeehan display markedly less androgen receptor gene expression than the LNCaP cell line and do not exhibit androgen-sensitive growth characteristics. Furthermore, previous studies in this laboratory have demonstrated that primary cells do not secrete PSA.

The androgen-independence characteristic of the cells employed in this study has also been observed in primary epithelial cells derived from the rat ventral prostate and has prompted conjecture that these cultures are derived from either basal cells or de-differentiated secretory epithelia (McKeehan et al, 1984; Montpetit et al, 1988). Certainly in common with the primary cultures the basal epithelial cells of the human prostate are essentially androgen receptor-negative and express high levels of the bcl-2 oncoprotein.

We can only speculate as to the reasons why such a cell type should predominate in primary culture. As previously eluded to, hormone-sensitive prostate epithelia such as the LNCaP model are prone to programmed cell death when grown in serum-free medium (Buttyan et al, 1993) and it is entirely likely that the conditions in which the primary cultures are maintained are unsuitable for
secretory cell propagation. It could be envisaged that when prostatic acini are plated in serum-free medium the basal epithelia are able to survive and multiply whereas the secretory cells are induced to undergo programmed cell death in much the same way as they would under conditions of androgen-deprivation in vivo. Presumably since fibroblasts are unable to survive under these circumstances they are similarly pre-disposed to cell death. Why the basal epithelia are able to survive when the secretory epithelia and fibroblasts do not is also unclear, however, it is possible that the overexpression of the bcl-2 oncoprotein is involved.

Obviously primary epithelial cell culture serum-free medium requires some refining in order to permit secretory cell outgrowth. Initial attempts to promote secretory cell propagation by creating a culture environment that retains some characteristics of the prostate, i.e. high levels of DHT and fibroblast-derived growth factors, has failed to generate a cell which expresses the androgen receptor and is therefore responsive to androgens. Fong et al (1991) have demonstrated that epithelial cells grown in serum-free medium can be induced to secrete high levels of PSA when grown on reconstituted basement membranes. This PSA expression is significantly increased in the presence of DHT, fibroblast conditioned medium and a combination of DHT plus fibroblast conditioned medium. It is unknown at this stage whether or not this PSA secreting cell exhibits androgen-sensitive growth and if, as suspected, it expresses the androgen receptor.

There is a school of thought that basal epithelial cells are the progenitors of secretory epithelia. This theory propounds that basal epithelia are acting as so-called "reserve cells" able to replace their luminal counterparts via one or more stages of differentiation (Trump et al, 1981). It is extremely interesting that the provision of an extracellular matrix has the potential to allow cellular progression through such differentiation in vitro, however, further characterisation must surely be carried out before this approach can be considered standard protocol for primary culture of secretory prostatic epithelial cells.

Despite the fact that the primary epithelial cells used in the thesis are non-secretory by nature, it is not necessarily the case that they are worthless as an in vitro model in prostate research. On the contrary there are recognisable similarities between these cells and the hormone-independent prostate cancer cell line PC3 which might be usefully exploited in the study of potential therapeutic agents for the treatment of relapsed prostatic tumours. A good deal of emphasis has been placed upon the development of such chemotherapeutic drugs with the ability to induce cell death in hormone-independent prostate cancer cells. In this respect primary
epithelial cells may provide a more in vivo-like alternative to the established cell lines for in vitro studies.

4.5 Hormone Escape in vitro and in vivo.

How do the underlying features of hormone-independence in the cell models compare with what is observed in the relapsed prostate tumour?

Both the DU145 and PC3 cell lines do not express functionally significant levels of androgen receptor. However, immunohistochemical analysis of escaped prostatic adenocarcinomas for androgen receptor staining has failed to demonstrate that the receptor-negative phenotype characteristic of DU145 and PC3 is typical of all androgen-independent cells in vivo. Different groups using assorted anti-AR antibodies have demonstrated that the progression of prostatic adenocarcinomas to androgen independence correlates well with a reduction in the numbers of AR-positive cells (Quarmby et al, 1990; Masai et al, 1990). A similar number of research groups have observed no alteration in receptor expression following tumour relapse (van der Kwast et al, 1990; Furuya et al, 1992)). These divergent findings may merely represent differences in the specificity of the receptor antibodies, however, it is entirely likely that relapsed prostatic adenocarcinomas display phenotypic variation. Potentially androgen-independent tumours cells may express normal or mutated androgen receptor or indeed no receptor protein at all. What dictates this AR status is unclear.

It is now known that PC3 cells expressing the androgen receptor do not behave like normal prostatic epithelial cells in an androgen-deprived environment. On the contrary, such PC3 cells are unaffected by androgen ablation but are growth inhibited in the presence of DHT. If AR-positive cells such as this population of PC3 existed within a prostatic tumour it might be expected that their propagation would be suppressed in a normal androgenic environment but they would be allowed to grow as normal at the expense of androgen-dependent cells following androgen ablation therapy.

Since PC3 cells will grow in an androgen-deprived environment irrespective of whether they express the androgen receptor or not, the question must be asked as to why the AR is down-regulated. This may well be the final step in the pathways of differentiation in androgen-independent prostate cancer cells in which they isolate themselves completely from any androgenic influence. Such an event is likely to be a late event in the natural history of prostate tumours. For instance, it is
hard to envisage that AR-negative PC3 cells would be present in a pre-treatment tumour as they would undoubtedly grow uncontrolled in any hormonal environment.

It is clear from the data presented in the thesis and from other studies that great care must be taken when describing the LNCaP model of advanced prostate cancer. This cell line may exhibit positive responses to androgens in terms of propagation and transcription activation via the androgen receptor, but is no more a model androgen-dependent CaP than the PC3 or DU145 lines. Far from it, there is strong evidence to suggest that LNCaP cells mimic the malignant epithelial cells predominating in a number of androgen-independent tumours.

In addition to DHT, the growth rate of LNCaP cells is increased in response to the anti-androgens flutamide, hydroxyflutamide and cypoterone acetate plus oestrogenic and progestagenic compounds. These unusual growth characteristics are mediated by an androgen receptor protein mutated in the ligand-binding domain (thr<sup>868</sup> to ala) such that its ligand specificity is altered to accommodate these additional ligands. The only documented example of a thr<sup>868</sup> to ala AR mutation within a prostatic tumour was until recently in a single metastatic lesion analysed by Suzuki et al in 1993. Gaddipati et al (1994) have subsequently demonstrated that the LNCaP mutation is far more wide-spread than previous studies would suggest. Sequence analysis of archival tumour specimens from 24 patients with advanced disease (stage C to D<sub>2</sub>) demonstrates that 6 individuals (25% of patients) carry the thr<sup>868</sup> to ala mutation. Undoubtedly, if these tumours were treated in either monotherapy with oestrogens or in combination therapies involving flutamide the cells carrying the mutated androgen receptor would have growth advantage over normal hormone-dependent malignant epithelial cells. It may even be the case that treatment with either oestrogens or flutamide induces hormone escape in prostatic adenocarcinomas through promotion of LNCaP-like cells. Unfortunately Gaddipati et al (1994) do not include the treatment protocols used with each individual patient studied otherwise it might be possible to validate this theory, however, it is perhaps noteworthy that almost all the advanced prostate tumours which were observed to be AR-positive by van der Kwast et al (1991) had been treated with oestrogens.

All of the recent observations in the PC3 and LNCaP cell lines have forced us to consider hormone escape in advanced prostate tumours in a completely new light. Mechanistically it is clear that there are at present at least two distinct possibilities for tumour relapse i.e. the complete or partial hormone-independence characteristic of PC3 and the "hormone-promiscuity" of the LNCaP cell line. The
ability of the PC3 cell and of potential PC3-like cells in vivo to prosper in an androgen-free environment is proposed to demand an alteration in the control of androgen-regulated genes rather than any alteration in the AR. In contrast, the AR itself is central to the survivability of LNCaP-like cells through its inherent ability to scavenge for alternative ligands. Whether different genes are targeted by the androgen receptor in LNCaP than in truly hormone-dependent tumour cells is unknown, although it is probably unlikely owing to the similarity of response to ligand deprivation.

Is the phenotype of the hormone escaped prostatic adenocarcinoma treatment-related? Evidence to support the claim that treatment protocols involving the use of oestrogens, flutamide and cyproterone acetate pre-dispose tumours to relapse through promotion of cells with androgen receptors carrying the thr$^{868}$ to ala mutation is at best very limited. Furthermore, whether orchiectomy induces tumour relapse through an alternative pathway e.g. the outgrowth of PC3-like cells is equally unclear. If a strong link between treatment type and tumour progression could be established then it might be possible to tailor therapies such that a more effective management protocol could be offered. Endocrine manipulation of prostatic adenocarcinoma would subsequently take account of the potential for PC3 and LNCaP cell outgrowth.

Akakura et al (1993) have published a treatment regime based upon the intermittent suppression of circulating androgens. Androgen ablation therapy was performed until such times as the serum PSA achieved normal levels before cessation of the treatment. Thereafter, upon recovery of testicular function, androgen-withdrawal was resumed when the serum PSA rose to ~20mgL$^{-1}$. This cycle was repeated sequentially over treatment periods of 21 to 47 months with no loss of androgen dependence. The rationale behind this approach to tumour management is that androgen replacement following a period of apoptotic regression might result in the regeneration of cells with further apoptotic potential rather than allowing therapy-resistant cells to become established. Furthermore it is now apparent that if AR-positive PC3-like cells form part of the tumour cell population they will be growth inhibited upon re-introduction of androgens.

Whether this novel treatment regime offers any significant benefits in terms of survival is unclear at this juncture owing to the small numbers of patients involved and the obvious limitations of a relatively short follow-up period. Nevertheless, the delayed onset of hormone escape associated with this approach is very heartening and with slight adjustment may offer the way forward for endocrine management of prostatic adenocarcinoma.
Akakura et al. (1993) achieved complete androgen blockade using combinations of the non-steroidal anti-androgen cyproterone acetate and diethylstilbestrol or cyproterone acetate plus a LHRH agonist goserelin. Considering the potential for the outgrowth of LNCaP-like cells, this may not be the most advisable drug cocktail. Veldscholte et al. (1992) demonstrated that the LNCaP androgen receptor is activated by cyproterone acetate in a similar fashion to hydroxyflutamide, reaching a maximum at concentrations above $10^{-7}$ M. Beyond doubt, if cyproterone acetate or indeed diethylstilbestrol induces growth of LNCaP-like cells in the tumour the recovery of normal androgen levels will only serve to further promote the proliferation of these cells along with the remaining androgen-dependent cells. The effects of subsequent administration of cyproterone acetate and diethylstilbestrol are obvious.

The effectiveness of the intermittent suppression of circulating androgens or indeed standard androgen ablation protocols in the treatment of advanced may be improved by taking account of LNCaP-like cell propagation and using drugs that do not activate the mutated androgen receptor. Oestrogens are therefore not advisable and emphasis must be placed on the use of LHRH agonists. Of the commonly used anti-androgens only Casodex marketed by Zeneca Ltd. efficiently blocks the transcription activation function of the LNCaP androgen receptor (Veldscholte et al., 1992).

Whether or not such tailoring of endocrine management protocols will provide any long-term benefits for CaP sufferers remains to be seen. What is apparent at this stage is that the new approaches to treatment cannot offer complete immunity from hormone escape. Akakura et al. (1993) observed in the androgen-dependent Shionogi carcinoma that despite favourable initial responses to intermittent androgen suppression the tumour would progress to an androgen-independent state after the fifth treatment cycle. At this point the use of chemotherapy represents the only course of action.

A number of chemotherapeutic agents which target mitotically active cells have been used in the treatment of relapsed prostatic adenocarcinomas with little benefit in terms of patient survival. The reasons underlying the failure of these cytostatic/cytotoxic drugs is that more than 90% prostate cancer cells are in interphase at any particular moment in time and are therefore insensitive to the effects of chemotherapy. However, there is light at the end of the tunnel. Human androgen-independent prostate cancer cells can undergo programmed cell death. Kyprianou et al. (1994) have recently demonstrated that the depletion of intracellular thymidine-5-phosphate using 5-fluoro-2-deoxyuridine or
trifluorothymidine can induce apoptosis in the highly proliferative PC3 and DU145 cell lines. Whether or not other apoptosis-inducing agents which do not rely on cell division, such as calcium ionophores, will have similar influences on human prostatic cells in vivo and in vitro remains to be fully elucidated. The effects of ionomycin with hormone-independent rat prostate cancer cells are extremely encouraging (Martikainen et al, 1991) and although the results from studies involving such Ca\(^{2+}\) ionophores and human cells are to date slightly less heartening (Yuan et al, 1993), it is easy to envisage that these drugs or other apoptosis-targetting agents could represent the future for the treatment of advanced prostate cancer.

4.6 Future Studies.

The cellular mechanisms underlying the hormone-independence of relapsed prostatic adenocarcinomas are so inadequately defined that there remains a wealth of topics for future investigation.

The loss of a functionally significant level of androgen receptor in the hormone-independent cell lines DU145 and PC3, although probably not the step that initially commits the prostatic epithelial cell to androgen unresponsiveness, certainly requires further investigation. This AR down-regulation may occur by one of two routes; either a decrease in the half-life of the androgen receptor mRNA or an alteration of the androgen receptor promoter.

The LNCaP androgen receptor gene promoter was initially cloned and sequenced by Tilley et al in 1990, but is only now becoming fully characterised. Using the cloned LNCaP androgen receptor promoter it should be possible to determine whether the DU145 and PC3 cell lines have the necessary transcriptional machinery to allow androgen receptor gene expression. Transfection of the hormone-independent cells with a plasmid construct containing the SPAP gene under direct transcriptional control by the AR promoter would allow determination of whether AR down-regulation in these cells is a consequence of the loss of one or more transcription factors.

Assuming that the AR promoter is inactive in either the DU145 or PC3 cell lines and the SPAP gene is not transcribed, then logically the next step must surely be to determine the missing transcription factor or factors. In this respect, it is essential that sequences important in transcriptional control of the receptor gene be determined. Such information may be obtained through mutation or deletion of
potential transcription factor binding sites in the promoter region of the AR gene. Using band-shift assays it may then be possible to screen nuclear extracts derived from the DU145 and PC3 cell lines with relevant sequences in an attempt to determine the transcription factor deficiencies.

If the LNCaP AR promoter is fully functional in the hormone-independent cell lines then the endogenous promoter regions must be examined.

There may be a case for sequencing of the androgen receptor promoter in DU145 and PC3 cells in order to ascertain the potential disruption of transcription factor binding sites or other translation initiation sequences. Of particular interest in this respect is the binding site for the transcription factor SP1 residing within the GC-box of the AR gene promoter, mutation of which results in the abolition of mRNA synthesis from at least one of two potential transcription initiation sites (Faber et al, 1993).

Additionally it might be possible to determine whether the AR promoter is hypermethylated in DU145 and PC3 through the analysis of restriction fragments obtained using restriction enzymes sensitive to CpG methylation. There are a number of restriction endonucleases, for example BclI and MboII, which are not sensitive to certain site-specific methylations and can therefore be used to achieve complete digestion of modified DNA. However, almost all enzymes with CpG in the recognition sequence fail to cleave m5CpG-methylated DNA (Nelson and McLelland, 1987). Consequently, such methylation-sensitive endonucleases will generate aberrant restriction fragments on Southern blots if the target DNA displays hypermethylation.

The findings of Yuan et al (1993) concerning the behaviour of the PC3 cell upon reintroduction of the androgen receptor are extremely important in terms of the study of hormone escape. The implications of this work are that the switch from androgen-dependent growth to androgen-independent growth may be associated with a change in the control of gene expression and not necessarily with a change in genes expressed. Could there be a hormone-independent transcription factor for instance?

In an androgen-dependent epithelial cell a fine balance is maintained between cell propagation and cell death. DHT is central to the maintenance of this equilibrium and carries out two basic functions within the epithelial cell i.e. to enhance the expression of growth promoting genes and to suppress the transcription of cell death genes. Androgen ablation therefore has rather obvious and dire consequences. Yuan's observations imply that the essential feature of
androgen-independent prostate cancer cells is that androgen regulated gene expression exhibits paradoxical responses to androgen ablation such that, for example, cell death genes are not activated following androgen deprivation but may well be upon administration of DHT. Evidence to support this theory is exceptionally thin on the ground mainly due to the fact that only one or two genes in humans are known to be truly androgen-responsive. Nevertheless, the elevated levels of serum PSA in the vast majority of orchiectomised patients with relapsed prostatic adenocarcinomas (Fossa et al, 1992) would tend to reinforce belief in a change in the control of gene expression in androgen-independent cells.

The obvious question arising from Yuan's work is whether or not the effects of androgens on AR-positive androgen-independent prostate cancer cells is merely a peculiarity of the PC3 cell line. It is therefore essential the responses to androgens of DU145 cells, stably transfected with androgen receptor cDNA, be assessed. It might also prove useful, in view of the apparent differences in PSA production in vivo and in vitro, to re-examine the expression of PSA in DU145 - perhaps at the transcriptional level - and PC3 and its relationship with the androgen receptor. At the present time this is about as far as such a study could be carried through owing to, as previously eluded to, the lack of recognised androgen-responsive genes and our minimal understanding of the mechanisms underlying androgen-dependent gene expression.

There is a strong case for using the expression of the bcl-2 oncogene prognostically as an indicator of patient performance and the time between primary hormone treatment and relapse due to its strong correlation with the onset of androgen-independence. However the question still remains as to the significance of bcl-2 in androgen-independent cells. Antisense oligonucleotides offer a viable means of ablatting the bcl-2 oncogene function such that the susceptibilities to cell death-inducing agents could be re-assessed. Either phosphorothioate or phosphodiester oligonucleotides could be used, the former offering the advantage of greater nuclease resistance but also displaying poorer membrane transport properties. Lipofection or electroporation could be used to get the antisense into the cells, however, if these approaches proved unsuccessful, it might be possible to transfect the cells with a plasmid construct carrying the antisense sequence.

Primary epithelial cell cultures are without doubt the most desirable in vitro model for prostate research, offering everything that the established cell lines have in terms of ease of study but retaining more of the in vivo characteristics often lost
through differentiation in the cell lines. A pure secretory epithelial cell population is truly the holy grail of prostatic cell culture and all work carried out in this field is geared toward the establishment of this cell type \textit{in vitro}. Unfortunately the primary cells used in the thesis are phenotypically more akin to basal epithelial cells. However, some encouragement has been provided by Fong \textit{et al} (1991) who have demonstrated that the presence of an extracellular matrix allows these basal cells to progress onto a state where they exhibit secretory attributes, notably the synthesis and secretion of PSA. It is surely necessary to determine whether or not these differentiated cells also express the androgen receptor and exhibit androgen-dependence before accepting them as secretory epithelia. Establishment of androgen-dependence \textit{in vitro} would represent a monumental advance.
REFERENCES CITED


