ANDROGEN-DEPENDENT GROWTH SUPPRESSION AND EVENTUAL DEATH IN PC-3 PROSTATIC CELL CULTURES EXPRESSING A FULL-LENGTH HUMAN ANDROGEN RECEPTOR

by

Andreas Evangelou

A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Zoology
University of Toronto

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Masters of Science
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 Andreas Evangelou
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ABSTRACT

Several clonal lines of PC-3 cells (human androgen-independent prostate cancer cells) transfected with an episomal vector (pCEP4) containing a full-length human androgen receptor (AR) cDNA were developed (PC-3(AR) cells). These clonal lines expressed varying levels of a 110 kDa AR that bracketed the concentration measured in androgen-sensitive LNCaP human prostate cancer cells. The expressed ectopic receptor was viable and capable of inducing the expression of the androgen-sensitive MMTV-luciferase but not the hormone-insensitive RSV-luciferase. This activation of luciferase activity by androgen was inhibited by the AR antagonist hydroxyflutamide. Treatment of these cells with androgen produced a paradoxical growth inhibition response and that appeared to culminate in cell death. It is hoped that the PC-3(AR) clonal cell lines established and characterized in this thesis will serve as useful models to assess AR action in prostate cancer. Work in these cell lines may establish a viable approach to the treatment of prostate cancer.
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<tbody>
<tr>
<td>5α-DHT</td>
<td>5α-Dihydrotestosterone</td>
</tr>
<tr>
<td>AcP</td>
<td>Acid Phosphatase</td>
</tr>
<tr>
<td>AF</td>
<td>Autonomous Activation Function</td>
</tr>
<tr>
<td>AIS</td>
<td>Androgen Insensitivity Syndrome</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen Receptor</td>
</tr>
<tr>
<td>ARE</td>
<td>Androgen Response Element</td>
</tr>
<tr>
<td>BPH</td>
<td>Benign Prostatic Hyperplasia</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CAIS</td>
<td>Complete Androgen Insensitivity Syndrome</td>
</tr>
<tr>
<td>CAT</td>
<td>Chloramphenicol Acetyl Transferase</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>dsDNA</td>
<td>double stranded DNA</td>
</tr>
<tr>
<td>EBNA-1</td>
<td>Ebstein-Barr Virus Nuclear Antigens-1</td>
</tr>
<tr>
<td>EBV</td>
<td>Ebstein-Barr Virus</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>EGF-R</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen Receptor</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid Receptor</td>
</tr>
<tr>
<td>HBS</td>
<td>Hepes-Buffered Saline</td>
</tr>
<tr>
<td>HRE</td>
<td>Hormone Response Element</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IGF-I</td>
<td>Insulin-like Growth Factor-I</td>
</tr>
<tr>
<td>IGF-II</td>
<td>Insulin-like Growth Factor-II</td>
</tr>
<tr>
<td>IGFBP</td>
<td>Insulin-like Growth Factor Binding Protein</td>
</tr>
<tr>
<td>LHRH</td>
<td>Luteinizing Hormone Releasing Hormone</td>
</tr>
<tr>
<td>LNCaP</td>
<td>Lymph Node Carcinoma of the Prostate</td>
</tr>
<tr>
<td>MES</td>
<td>2-N-[morpholino]ethanesulfonic Acid</td>
</tr>
<tr>
<td>MR</td>
<td>Mineralocorticoid Receptor</td>
</tr>
</tbody>
</table>
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CHAPTER I

Introduction

1-1. PROSTATE CANCER

(i.) Overview.

Carcinoma of the prostate is one of the most common tumors in American males, and the number one cause of cancer-related death among elderly men in the United States. In the U.S. alone prostate cancer accounts for about 38,000 deaths per year and approximately 200,000 new cases are expected to be diagnosed in 1995.\textsuperscript{133} It is now the most frequently diagnosed non-skin cancer in North American males. The risk of developing prostate cancer increases with age; within the population of 50-yr old American men, it is projected that at least one-third will develop prostate cancer in the course of their life span.\textsuperscript{29,232} Because the prostate is an androgen-dependent organ,\textsuperscript{81} most prostate carcinomas respond to androgen ablation therapy. Following removal of androgen by medical (antiandrogens, estrogens, or LHRH agonists) or surgical (orchidectomy) methods, androgen-dependent prostate tumours undergo regression; however, most prostate carcinomas progress to an androgen-independent state within a few years from the start of treatment.\textsuperscript{82}

The development of androgen-independent prostate cancer is a poorly understood process. Androgen-independent cells may have been present since the early formation of the primary tumor and simply overtake androgen-dependent cells in an androgen starved environment. Alternatively androgen-independent cells may arise from androgen-dependent cells through genetic mutations or adaptation to the changed environment. In any event, androgen-independent prostate cancer ceils are difficult to treat as they typically exhibit slow growth and are thus resistant to conventional chemotherapeutic approaches. At present, advanced prostate cancer is without a cure or even a viable treatment. Unfortunately, about half of all men with newly diagnosed prostate cancer are present with metastatic disease, which is associated with an exceedingly high mortality rate.
(ii.) Failure of Androgen Ablation Therapy.

In its early stages, prostate cancer can be effectively treated by radical surgery and/or radiation therapy.77 The aim of the options available for treating advanced prostate cancer (metastatic stage D or for selected cases of stage C) are basically to interrupt the biological pathways that lead to the synthesis of testosterone and its action in prostate cells.77 Such a blockade (usually performed by administering such drugs as leuprolide, flutamide and finasteride, alone or in combination) prevents testosterone from inducing the growth of tumors derived from cancerous prostate cells.255 The goal is to prevent testosterone, or its more potent 5α-reductase metabolite, 5α-dihydrotestosterone, from interacting with and exerting actions through the androgen receptor (AR) present in androgen-dependent prostate cancer cells. However, testosterone can also be metabolized to estradiol and exert effects through the estrogen receptor (ER). More than 70% of patients experience some form of tumor regression, but eventually the disease progresses to an androgen-independent state. Thus, these treatments are largely only palliative as marginal and somewhat equivocal results are realized in terms of life expectancy. Because of the invariant progression of androgen-independent tumor growth, relapses ultimately resulting in death are an almost certainty.77

(iii.) Emergence of Androgen-Independent Disease.

The loss of androgen dependency could occur through several basic processes, some of which include a possible direct mutation in the DNA allowing the cell to become independent of androgenic stimulation for growth. Hormone-resistant prostate cancer may be generated through the expression of an altered AR that could occur through common genetic and biological mechanisms such as point mutations, truncation or alternate splicing, altered post-translational processing of the receptor itself, and differential expression of naturally-occurring receptor isoforms. Alternatively, cells may simply adapt to a changed environment, that is, in the absence of androgen, cells may exploit alternate growth stimulatory signals available to them. In this latter process, normal AR expression is still possible but may include an altered response. A primary tumor may include androgen-independent cells from the
very start of its development. There cells could be present in small numbers and emerge as the dominant
cell type as androgen treatment effectively removes androgen-dependent cells. Advanced prostate
cancer tumors often have a heterogeneous distribution of AR-containing cells that may vary in sensitivity
to androgen.38

One group of such cells are the neuroendocrine cells, which are known to constitute, in addition
to the basal and secretory exocrine cells (epithelial cells), a third population of highly specialized
epithelial cells in the prostate gland.63 In prostatic adenocarcinomas with extensive neuroendocrine
differentiation, a subpopulation of AR-negative neuroendocrine cells has been demonstrated.181,184 It has
been shown that these cells can secrete neuropeptides that may be able to act as growth factors in an
autocrine-paracrine fashion or activate AR responses in neighboring non-neuroendocrine cells by a
ligand-independent mechanism.184 The presence of neuroendocrine cells in prostate tumors has been
associated with poor prognosis.

Usually prostate cancer spreads first to the lymph nodes that are immediately downstream from
the prostate gland, then to bone and other organs,77,104 where androgen-independent prostate cancer cells
continue to proliferate. The possibility that a primary tumor may not include androgen-independent cells
from the start of its development could still, in theory, be possible. Androgen ablation treatment may
initially delete androgen-dependent cells, but with time some cells may become resistant to this
environment (i.e., lose their ability to express AR or evolve an AR gene mutation) and hence progress to
a metastatic stage as they no longer require androgen for growth. With the loss of androgen dependence,
cells can migrate to other regions of the body as androgen-independent cells (e.g. PC-3 or DU-145 cells).
This does not necessarily mean that they must lose AR prior to metastasizing. All metastatic tumors
become resistant to hormonal therapy at some point and then progress rapidly.

(iv.) Benign Prostatic Hyperplasia (BPH).

Two predominant and clinically important forms of abnormal prostate growth are benign
prostatic hyperplasia (BPH) and prostatic adenocarcinoma.194 Homeostasis is maintained in the prostate
by complex interactions between activators of cell proliferation and cell death.\textsuperscript{229} Growth factors that can either stimulate or inhibit proliferation and death of cells in the prostate have been identified, and are associated with the interactions taking place between prostatic stroma and epithelial cells.\textsuperscript{229} The abnormal growth of the prostate leading to BPH may arise as a lack of balance between cellular proliferation and cell death.\textsuperscript{229}

BPH affects two out of three males over the age of 50.\textsuperscript{28,29} Unlike the normal prostate, there is a heterogeneous distribution of AR in BPH and prostate cancer.\textsuperscript{38} Androgens have an important supportive role in establishing BPH and confirm the concept that BPH is under endocrine control.\textsuperscript{194} With age, patients with larger volumes of BPH have higher serum androgen and estrogen levels.\textsuperscript{194} This suggests that these two hormones may be involved in the progression of BPH in men. The androgen 5α-dihydrotestosterone (DHT) is the major intracellular androgenic metabolite within the prostate. However, androgens are not alone in influencing the growth and regulation of the prostate. In BPH, epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) are expressed at comparable levels to that found in prostate carcinomas, however, the levels of EGF are on average twice those of TGF-α.\textsuperscript{281} A small positive correlation was observed between testosterone and EGF, and testosterone and TGF-α in prostate carcinomas, while there was no such correlation in BPH where DHT becomes the predominant hormone.\textsuperscript{281} These findings suggest an indirect role for testosterone in the regulation of growth factor production.\textsuperscript{281} Perhaps through the ER pathway following aromatization to estrogen.

Recently, ER mRNA has been demonstrated to be expressed in human BPH tissue and this expression is upregulated when patients are treated with a long-acting gonadotropin releasing hormone agonist.\textsuperscript{115} Furthermore, two estradiol binding proteins have been shown to exist in BPH tissue: (1) true ER binding protein (high affinity, low capacity), and (2) a protein with lower affinity and higher capacity.\textsuperscript{183} The incidence of BPH increases with age.\textsuperscript{87} Aging also results in a shift of the androgen:estrogen ratio in favor of estrogens, with respect to the levels in serum and in the prostate organ.\textsuperscript{87} Thus, estrogens may play an important role in the development of BPH.
Prostatic Acid Phosphatase

Prostatic Acid Phosphatase (PAcP) is the "prostate epithelium-specific differentiation antigen". It is the major acid phosphatase (AcP) in normal, differentiated prostate epithelial cells (PEC). In post-pubertal and adult males, PAcP is expressed in high levels in normal, well-differentiated PEC. There are two species of PAcP in the human prostate: a major secreted form and a minor intracellular form. Huggins and Hodges (1941) have shown that the growth of prostate cancer cells was stimulated in prostate cancer patients when given androgens and that this was followed with a concomitant increase in serum PAcP activity. Significant levels of PAcP measured in the circulation have been reported in patients treated for advanced prostate cancer with androgen deprivation, suggesting that, at least in this disease state, PAcP expression may not require androgen. Serum AcP activity has been noted to often increase in these patients because of the increased bulk of tissue and metastatic foci involving blood vessels and lymphatic channels in the neoplastic prostate, resulting in leakage of enzymes into the circulation of the patient. Secretion of PAcP has been shown however, to be stimulated by androgens in prostate carcinoma cell lines.

The prostate cancer cell line LNCaP expresses endogenous PAcP and its expression and secretion are androgen-responsive but not dependent processes. These cells express high levels of PAcP when grown in serum-free medium. Androgen regulation of PAcP expression is modulated by cultured cell density. DHT can stimulate PAcP mRNA levels in low-density LNCaP cells and causes a decrease in high-density cells. In these cells secretory PAcP activity was increased following treatment with DHT in a dose-dependent fashion at concentrations of up to 1 µM. Furthermore, its secretion was stimulated 150% over that of control cells, after 5 days treatment with 10 nM DHT in media stripped of steroids. However, PAcP was also secreted from cells grown in the absence of added DHT. PAcP activity increases in confluent cultures of LNCaP cells; however, their growth in fresh cultured media or in the presence of DHT yielded a decrease in PAcP activity. Therefore in
LNCaP cells, cell density could modulate PAcP expression at the mRNA level including androgen regulation.

Regulation of PAcP expression in cultured LNCaP cells can be achieved by other steroid hormones besides the two natural androgens testosterone and DHT. A 100-fold lower concentration of the synthetic androgen R1881 (methyltrienolone) relative to DHT induced an almost five-fold increase in the expression of PAcP.\(^{222}\) Stimulation of PAcP expression by androgens and estradiol was found to be dose-dependent.\(^{222}\) In the presence of the synthetic glucocorticoid triamcinolone acetonide (TA), either alone or with R1881, there was no regulation of PAcP expression in LNCaP cells.\(^{222}\)

Garcia-Arenas \textit{et al.}\(^{76}\) have shown by southern blot analysis the presence of PAcP gene in three prostate carcinoma cell lines PC-3, DU-145, and LNCaP. Northern blot analysis and reverse transcriptase polymerase chain reaction (RT-PCR) detected showed PAcP mRNA in LNCaP cells but not in PC-3 or DU-145 cells.\(^{76}\) Furthermore, in LNCaP cells, the level of PAcP mRNA diminished with pasaging number, while the level of AR did not change appreciably.\(^{76}\)

The prostate adenocarcinoma cell line PC-3 does not express endogenous PAcP.\(^{76,141}\) However, when these cells were transfected with a cDNA coding the full-length human PAcP, diminished cellular growth rate was observed.\(^{141}\) This shows an inverse relationship between cellular levels of PAcP and cell growth rate, and suggests that PAcP may be involved in the regulation of growth in human prostate carcinoma cells.\(^{141}\) Furthermore, transfection of the human AR cDNA into PC-3 cells has resulted in no effect on the expression of PAcP in these cells.\(^{76}\) Therefore it has been concluded that the variable expression of PAcP found in different prostate carcinoma cells, may be regulated at the transcriptional level but with little significance to the expression or the presence of ARs.\(^{76}\)

PAcP has phosphotyrosyl protein phosphatase activity and has been shown to dephosphorylate three different phosphotyrosine-containing protein substrates.\(^{138}\) It is composed of a group of acid phosphomonoesterases where the major isoenzyme (Mr 100,000) is a glycoprotein which hydrolyzes phosphomonoesters.\(^{131,280}\) Hence, PAcP may play a role in determining the phosphorylation state of
phosphotyrosine-containing proteins, such as epidermal growth factor receptor (EGF-R) or TGF-α receptor. There is evidence that PAcP dephosphorylates the EGF-R from prostate carcinoma cells.\textsuperscript{142}

\textit{(vi.) Prostate Specific Antigen.}

Prostate-specific antigen (PSA) has become an important biological marker in clinical medicine over the last decade, for prostate disease, specifically for prostatic adenocarcinoma.\textsuperscript{32,52} PSA, a serine protease enzyme with its gene locus on chromosome 19, is a glycoprotein product synthesized by the prostate epithelium.\textsuperscript{211} It is secreted as a normal constituent of seminal fluid;\textsuperscript{136,237} small amounts are released into the blood.\textsuperscript{237} Because adenocarcinoma of the prostate originates from the glandular epithelium, it also produces PSA.\textsuperscript{32} In general, serum PSA concentration is proportional to the amount of cancer tissue present.\textsuperscript{32} Thus, serum PSA levels are considered a useful means for monitoring prostate cancer for treatment.\textsuperscript{112}

The mechanisms that regulate PSA expression in prostate cancer cells are unclear. But PSA expression is known to be under direct hormonal influence via the AR.\textsuperscript{210} Treatments that reduce the number of prostate tumor cells or remove or antagonize androgen action can cause a decrease in serum PSA levels.\textsuperscript{238-240} PSA is upregulated by androgen but is not affected by either EGF or basic fibroblast growth factor in LNCaP cells.\textsuperscript{169}

1-2. THE HUMAN ANDROGEN RECEPTOR AND ITS BIOLOGICAL ACTIONS

\textit{(i.) Discovery, Identification, and Localization of the Human AR Gene.}

The complementary DNA (cDNA) encoding the human AR was first identified in 1988\textsuperscript{34,35,152,153,254} and was the last classical steroid receptor to be cloned. Lubahn et al.\textsuperscript{153} localized the AR gene to the long arm of the X chromosome at Xq11-12 (Figure 1). In 1989, Lubahn et al.\textsuperscript{151} reported the sequence of the AR intron-exon boundaries (Figure 1). This discovery became critical in the understanding of the molecular basis of AR action involved in such diseases as Androgen Insensitivity Syndrome (AIS) and Prostate Cancer.
The AR gene encodes for a 110 kDa protein\(^{257}\) that is structurally and functionally similar to other steroid receptors. The gene itself spans 75-90 kilobases (kb) of genomic DNA\(^{15,124,152}\). The region of this gene that codes for the AR protein is approximately 2757 base pairs of open reading frame and is comprised of 8 exons\(^{156}\) and separated by introns of up to 26 kb in size\(^{124}\) (Figure 1).

(ii.) Molecular Biology of the AR

The AR is a nuclear transcription factor that belongs to a superfamily of hormone receptors referred to as the Steroid/Thyroid Hormone Receptor Superfamily\(^{68}\) that all share a number of common domains. This includes a hormone binding domain, which gives these receptors specificity for a particular ligand. Furthermore, the AR is a member of a group of four closely related steroid receptors that are sometimes referred to as the Glucocorticoid Receptor (GR)-like receptors, the other members of which are the GR, mineralocorticoid receptor (MR), and progesterone receptor (PR)\(^{27,73}\). This group of receptors are related by their sequence homology and their ability to stimulate transcription of a target gene by utilizing the same hormone response element (HRE)\(^{6}\). They also comprise a subfamily in a larger and more diverse group of nuclear transcription factors that includes ER, thyroid hormone receptor (TR\(_{\alpha}\) and TR\(_{\beta}\)), vitamin D receptor, and retinoic acid receptor (RAR), and a number of other related receptors for which ligands have been only recently or not yet identified\(^{53,64}\). Like other members of this superfamily of receptors, the AR, as a hormone-receptor complex, interacts directly with its target genes to regulate their transcription\(^{2,40,60,94,208,249}\). If a receptor fails to activate its target gene, either in the presence or absence of its ligand or hormone, the result is resistance to hormone action in the target organ.

(iii.) Structural and Functional Domains of the AR Protein

The AR gene has been shown to encode a receptor protein of apparent molecular weight ranging between 110 to 114 kDa\(^{107,205,274}\) and comprising 910-919 amino acids\(^{35,151,152,252,254}\). Recently, an 87 kDa form of the AR lacking approximately 188 amino acids from the amino terminus was demonstrated in genital skin fibroblasts\(^{275}\). This truncated receptor (AR-A) represents about 10% of total AR and its in
vivo function is presently unknown.\cite{275} AR-A is thought to result from an alternative translation-initiation methionine codon (met-188)\cite{275} located within exon 1 and cannot be immuno-detected with the PG-21 polyclonal antibody.\cite{302} which can bind to the first 21 amino acids of the normal AR (AR-B or just AR in this thesis). AR-A has been reported to bind androgens with the same affinity and specificity as the full-length wild-type receptor.\cite{245}

The AR, like other steroid receptors, is a single polypeptide comprised of four relative and discrete functional domains: a transcription-regulating amino-terminal domain, a DNA-binding domain, a hinge region, and a steroid binding domain. The amino-terminal domain, the largest domain, is encoded by exon 1 of the AR gene (see Figure 1), and comprises more than half of the AR protein (residues 1-537).\cite{35} This domain is the least homologous in sequence and most variable in size between members of the steroid receptor family.\cite{15,174} It contains a transactivation (transcription activation) region located between amino acids 141 and 338,\cite{234} and possibly also other subregions involved in the regulation of target gene transcription.\cite{108,192,215,234}

The DNA-binding domain, encoded by exons 2 and 3, is a centrally located cysteine-rich region of the AR (amino acids 538-627) with the DNA binding region lying between amino acid residues 559 and 564.\cite{35,152} This region is thought to be arranged as a pair of loop structures folded to form a single structural unit made up of two zinc-binding motifs.\cite{72,227} Four cysteine residues, invariably present in all steroid receptors, coordinately bind a zinc ion in each of the two motifs (commonly referred to as zinc fingers).\cite{72} The first zinc finger (residues 559-579) is encoded by exon 2 and the second (residues 595-619) by exon 3.\cite{72} The amino acid sequence of this domain is the most highly conserved region among members of the steroid receptor family.\cite{15} As it is with other steroid receptors, the DNA-binding domain determines the specificity of AR interaction with DNA.\cite{6,54,72,83} The first zinc finger contains a group of amino acids, which is identical to that of GR, PR, and MR, and appear to be important in that they interact with transcriptional enhancer nucleotide sequences referred to as HREs (hormone response elements),\cite{162} that are present in or near target genes, usually in the 5'-'flanking region. DNA receptor
interaction is stabilized by the second zinc finger, which is highly basic and makes contact with the DNA phosphate backbone, thereby mediating receptor dimerization.6,83,155,278

Between the DNA-binding domain and the steroid-binding domain lies the hinge region of the AR protein. This region is of low sequence homology between the AR and other steroid receptors.15 It is encoded by the 5'-portion of exon 4 and contains a major part of the AR nuclear targeting signal (a cluster of basic residues at positions 629-633: lys, leu, lys, lys), which mediates the transfer of AR from the cytoplasm to its site of action in the nucleus.108,235,287

The carboxy-terminal third of the AR contains the steroid-binding domain, and is encoded by the 3'-portion of exon 4 and exons 5-8, making up amino acid residues 670-917.35 The amino acid sequence of this region displays about 50% homology with the corresponding residues in GR, MR, and PR. This region is known to have specific, high-affinity binding to androgen and is also thought to be the binding site for inhibitory proteins such as the 90-kDa heatshock protein.99,236 Recent studies have suggested the possible presence of a transactivation region within this domain.108,234
FIGURE 1: Structural Organization of the AR Gene and Protein. The AR gene has been localized to the long arm of the X-chromosome at the Xq11-12 position.20,153 The AR complementary DNA (cDNA) comprises of eight coding exons (numbered boxes). Exons 1, 2-3, and 4-8 encode for the amino terminal, DNA-binding, and steroid binding domains, respectively. These 3 domains are common to all members of the nuclear receptor family. (Adopted from Sultan et al.144 and reprinted by permission.)
(iv.) The Molecular Basis of Androgen Action.

Binding of androgen induces a conformational change in the AR that results in receptor dimerization, nuclear transport, and interaction with DNA by allowing binding of the DNA-binding domain to a specific HRE of a target gene. This results in the regulation of target gene transcription via interaction of the N-terminal transactivating domain, with the target gene, as well as other transcription factors, resulting in gene activation.

A variety of androgens and other steroids can bind to the AR. The AR however, has highest affinity for DHT and testosterone, primarily towards the former because the latter hormone dissociates from AR more rapidly. However, the AR has a relatively low affinity for adrenal androgens such as dehydroepiandrosterone and androstenedione and for non-androgenic steroids such as progesterone and estradiol.

When androgen binds to the AR, its macromolecular and three-dimensional structure is induced to change by converting from its inactive state into its active DNA-binding state. As is the case with other steroid receptors, hormone binding results in the removal of receptor-associated proteins, such as the 90-kDa heatshock ("docking") protein. This exposes its functional domains and allows for the necessary conformational changes to occur, for nuclear transport, dimerization, and DNA binding.

The functional activity of the AR would then correlate with the specific binding of ligand that stabilizes and increases its immunoreactivity and DNA-binding state. The AR is also phosphorylated both in the presence and absence of hormone. In the presence of androgen however, there is an increase in receptor phosphorylation, a posttranslational process that is common to steroid receptors and other transcriptionally active proteins. Both the phosphorylated and unphosphorylated forms of the AR are capable of binding androgen with high affinity. Therefore the role of AR phosphorylation is presently unknown. Nevertheless, binding of androgen is necessary for subsequent DNA binding and transcriptional activity of the wild type receptor, although in vitro studies have
demonstrated that a mutant AR with deletion of its steroid-binding domain induces transcription in the absence of hormone.\textsuperscript{108,234}

Once it is synthesized in the cytoplasm of living cells, the unliganded AR is predominantly translocated and retained in the nucleus, its primary intracellular location, by binding to components of the chromatin.\textsuperscript{156} Immunohistochemical studies have localized the AR in the nucleus of prostatic epithelial cells in both intact and castrated rats.\textsuperscript{230} It is speculated that the affinity of this nuclear AR for its DNA AREs (androgen response elements) in the absence of androgen may be lower than that of the ligand-bound AR complex, but not negligible.\textsuperscript{156} Other interactions with either or both DNA and chromatin proteins within the nucleus, however, may be necessary to localize a great majority of ARs to appropriate genomic AREs.\textsuperscript{156} Once bound by androgen in the nucleus, the AR is capable of acting as a nuclear transcription regulator\textsuperscript{234} by binding with high affinity to AREs and engaging transcription factors.

Regulation of the AR, both at the transcriptional and at the translational level, is very complex. AR protein and mRNA expression have been shown to have differing responses to androgens. In the prostate cancer cell line LNCaP and in the breast cancer cell line T47D, treatment with the synthetic androgen mibolerone resulted in down-regulation of AR mRNA within 48 hours, while there was no response in normal skin fibroblasts.\textsuperscript{277} Incubation of LNCaP cells with androgens (10 nM DHT or 10 nM mibolerone) caused a decrease in AR mRNA expression in a concentration- and time-dependent fashion, with maximal suppression occurring after 48 hours.\textsuperscript{123} In contrast, incubation of LNCaP cells with these androgens resulted in a 2-fold increase in AR protein within 24 hours.\textsuperscript{123} With prolonged incubation (49 hours) AR protein level began to decrease.\textsuperscript{123} In the male rat ventral prostate and seminal vesicles, AR mRNA levels increased about 2-fold within 24 hours after castration and continued to rise within the next 48 hours.\textsuperscript{230} When 1-day castrated rats were administered pharmacological doses of testosterone (400 \(\mu\)g/day) for 24-48 hours there was a decrease in AR mRNA levels in accessory sex organs that approached that of intact control animals.\textsuperscript{230} In these studies the withdrawal of androgen by
castration resulted in an increase in rat AR mRNA, which was reversed by androgen replacement. Furthermore, this suppression of AR mRNA and protein was shown to be reversible upon removal of ligand.\textsuperscript{230} Down-regulation of the AR mRNA by androgen is a receptor-mediated process \textit{in vivo} and \textit{in vitro} in cultured cells.\textsuperscript{123,230} However, factors other than androgens have also been found to regulate AR. For example, EGF has been reported to down-regulate AR mRNA in LNCaP prostate cancer cells:\textsuperscript{93} however, this action may be mediated through the AR in a ligand-independent manner.\textsuperscript{289}

(v.) \textit{Pathological Conditions Associated with Abnormal AR}

At least three pathological situations have been shown to be associated with abnormal AR function and structure: Androgen Insensitivity Syndrome (AIS), Spinal and Bulbar Muscular Atrophy (SBMA) and Prostate Cancer.

1) ANDROGEN INSENSITIVITY SYNDROME (AIS)

Characterization of the molecular defects associated with androgen sensitivity were demonstrated after the cloning of the AR gene. Androgen Insensitivity Syndrome (AIS) is an X-linked disorder (46,XY) caused by mutations of the AR gene resulting in a variety of sex phenotypes that range from complete female (complete AIS or CAIS) to nearly normal male (partial AIS or PAIS).\textsuperscript{85} That is, from a female phenotype (testicular feminization) to minor degrees of undervirilization or infertility.\textsuperscript{85} Therefore defects in the AR gene can prevent the normal development of both internal and external male structures in 46,XY individuals. Demonstration of AR impaired ligand binding in cultured genital skin fibroblasts has been shown to be the most reliable means of diagnosing AIS.\textsuperscript{243}

It has been shown that androgen resistance can be caused by mutations within the AR gene that can result in: 1. an overall change in structure of the AR gene or mRNA; 2. an alteration of the primary structure of the AR; and/or 3. an alteration in the level of AR mRNA.\textsuperscript{166} Gene deletions however, have been demonstrated to be very rare in patients with CAIS or PAIS.\textsuperscript{244} Furthermore, insertion of premature termination codons by nucleotide substitutions in the androgen-binding domain or the N-terminal region resulted in a failure to form a functional protein.\textsuperscript{211,244} A decrease or absence of AR binding activity was
demonstrated by single amino acid substitutions within the ligand-binding domain.\textsuperscript{181,203,244} Such mutations within the DNA-binding domain resulted in the production of a receptor that was capable of ligand binding but still exhibited androgen insensitivity.\textsuperscript{110,244,288} For example, a point mutation in the second zinc finger of the DNA binding domain of the human AR cDNA, converting Arg 614 (a highly conserved residue) to His, was found to be responsible for causing CAIS in two patients with receptor-positive androgen resistance.\textsuperscript{172} Point mutations at several different sites in exons 2-8 encoding the DNA and androgen-binding domain, have been reported for PAIS and CAIS.\textsuperscript{111,181,203,244,288} The number of mutations in exon 1 has been shown to be extremely low and no mutations have been reported in the hinge region.\textsuperscript{17}

de Bellis \textit{et al.}\textsuperscript{57} have reported a mutation in exon 3 of the AR, in a 46.XY patient with PAIS, by using the polymerase chain reaction and denaturing gradient gel electrophoresis. This mutation in the region encoding the second zinc finger, converted a leucine residue at position 616 to arginine resulting in a decreased binding of AR to an ARE DNA sequence. However, this mutant AR was still capable of some low level of transcriptional activity in the presence of physiological levels of androgen, hence PAIS rather than CAIS. In another patient, de Bellis \textit{et al.}\textsuperscript{57} identified a single base mutation in exon 7, that resulted in substitution of histidine for arginine at position 840 and decreased AR binding affinity in genital skin fibroblasts from the patient. Furthermore, this mutant receptor also exhibited a reduction in transcriptional activity. In a third patient, the valine residue at position 889 was replaced by methionine resulting in a mutant receptor with apparent normal androgen-binding affinity but reduced androgen binding capacity.\textsuperscript{57}

Using Single-Strand Conformation Polymorphism Analysis and sequencing, Lobaccaro \textit{et al.}\textsuperscript{150} identified a 13 base pair deletion within exon 4 of the AR. This deletion resulted in a frameshift of the open reading frame that produced a premature stop codon at position 783 instead of 919. This deletion was reproduced in AR wild type cDNA and transfected into mammalian cells.\textsuperscript{150} Western blot analysis indicated a smaller AR of 94 kDa for the transfected mutated cDNA instead of 110 kDa. Androgen-
binding assays demonstrated that this mutated AR lacked binding of androgen. Lobaccaro et al.\textsuperscript{150} further demonstrated, by gel retardation assay, that this mutant AR was still capable of binding target DNA; however, it was unable to transactivate a reporter gene.

(2) BULBAR AND SPINAL MUSCLE ATROPHY (SBMA)

SBMA is an X-linked disease (also known as Kennedy's Disease) that is associated with an expanded length of 40 or more residues being added to one of the polyglutamine stretches in the N-terminal domain of the AR.\textsuperscript{17} It is a motor neuron disease that is characterized by the progression of spinal and bulbar muscular atrophy in men between the ages of 30 and 50 and is associated with androgen insensitivity and infertility.\textsuperscript{269} In experiments performed by Brinkmann et al.,\textsuperscript{17} complete deletion of the polyglutamine stretch resulted in a significant increase in the transcriptional regulation of CAT reporter activity linked to the promoter of the PSA gene but not to a MMTV promoter. There was a significant reduction in transcription activation of CAT activity with both reporter constructs, when an AR with a 48 glutamine stretch in its N-terminal was used.\textsuperscript{17} These findings confirm the fact that the N-terminal domain exhibits transactivation activity that is promoter specific. Deletion of the polyglutamine stretch of this domain, results in the inability of the AR to recognize AREs. Furthermore, expanding the length of the polyglutamine stretch prevents or inhibits the transcriptional activity of the N-terminal and hence androgen cannot exert its normal biological action. That is, the increase in the polyglutamine region changes the receptors function.

(3) ANDROGEN RECEPTOR AND PROSTATE CANCER

Cancer of the prostate develops from genetic changes that allows neoplastically transformed prostatic epithelial cells to escape from their normal conditions of proliferation. Mutations in tumor suppressor or transcription-regulating genes, including the AR gene, could alter the control of their proliferation or cell death. One important feature of prostate cancer is that although medical or surgical castration initially induces remission of cancer growth, most prostatic carcinomas subsequently become resistant to androgen ablation therapy (i.e., they become androgen-independent). It has been theorized
by many researchers that mutations in the AR gene could contribute to a selective advantage of prostate cancer cells by abolishing their normal ligand response and control of AR transcriptional activity.

Growth of most early forms of prostatic carcinomas requires normal activity of AR. It is necessary to translate the well known effects of androgens on prostate cells, both normal and malignant. The critical function of AR is to activate target genes. Transactivation activity resides in the N-terminal domain encoded by exon 1 and contains polymorphic CAG and GGC repeats (microsatellites).

The AR can play a critical role in the regulation of growth and differentiation of the prostate. Effects of androgen in the early form of prostate cancer is an important component in the development of this disease. Although the AR is required to translate the androgen response, it has not been implicated in any direct way in the etiology of prostate cancer. AR gene mutations in prostate cancer are very rare and have thus far been reported only in exons 4-8.

Somatic mutations in AR have been reported in advanced prostate cancer tumors. AR gene mutations occur in relation to endocrine therapy-resistance. Some prostatic tissue specimens of patients with metastatic cancer reveal genetic alteration in the hormone-binding domain of the AR gene such as codon 877 mutation (ACT to GCT or Thr to Ala). In the prostate metastatic cell line LNCaP, this type of mutation conferred upon the AR an altered ligand-binding specificity which was stimulated by estrogen, progestagens, and antiandrogens, thus providing an alternative growth route for prostate cancer cells during androgen ablation therapy. Hence AR mutations in prostate cancer cells may be an important factor in determining the appropriate treatment for prostate cancer patients in terms of hormonal therapy. Antiandrogens for example, are frequently used during hormonal therapy for patients with advanced prostate cancer.

There is increasing evidence that the course of prostatic carcinoma is determined by a complex interplay between genetic events, paracrine interactions, and hormonal and dietary factors (androgens, vitamin D3, retinoids, and thyroid hormones). These factors may influence the growth and
differentiation of prostatic tumor cells that express a mutated AR or lose their ability to express AR, and contribute to the understanding of the complex pathways that are involved in the progression of androgen-independent prostatic carcinoma and metastasis.

1-3. HUMAN PROSTATE CANCER CELLS

In the development of prostate cancer, there is an important transition from androgen-dependent growth (which can be treated) to androgen-independent growth (which is at present beyond medical control). This transition may be associated with genetic changes, resulting in the activation of oncogenes or the inactivation of tumor suppressor genes. Among androgen-dependent cells in a prostate carcinoma, there are also androgen-independent cells.\textsuperscript{102} These cells do not respond to androgen ablation therapy and continue to divide, contributing to the formation of an androgen-independent prostate carcinoma.\textsuperscript{207}

The human prostate is generally regarded as the main organ in the development of androgen-independent tissue during cancer progression.\textsuperscript{217} Presently, three different human prostate cancer cell lines are widely used in studying the differentiation state of prostatic carcinomas, LNCaP, DU-145, and PC-3. PC-3 cells quickly degrade testosterone and exhibit a high formation rate of androstenedione whereas both DU-145 and LNCaP cells mostly retain high levels of unconverted testosterone, with a limited production of androstenedione and its derivatives.\textsuperscript{30} The conversion of testosterone to its more potent derivative 5α-DHT via 5α-reductase activity is a critical event in the action of androgen in many tissues, that contain this enzyme.\textsuperscript{30} It has been demonstrated that both LNCaP and DU-145 cells can generate relatively high amounts of DHT, whereas in PC-3 cells no DHT or its main metabolites were detected.\textsuperscript{30} Therefore these human prostate cancer cells have different enzymes available for metabolizing androgens.\textsuperscript{30}

Elevated levels of EGF and EGF-R have been demonstrated in prostate cancer cell lines.\textsuperscript{44,170,223} The growth and metastasis of prostate tumor cells has been suggested to be under control by polypeptide growth factors.\textsuperscript{56,71,170} Estrogens have been demonstrated to upregulate the production and secretion of
growth factors, in human breast cancer cells, that are capable of acting in an autocrine and paracrine fashion.\textsuperscript{148} Such mechanisms may also be involved in human prostate cancer cells in the presence of androgens.\textsuperscript{117} Therefore, enhanced expression of EGF-R gene may play a role in the growth of androgen-independent prostate tumors, possibly by an autocrine pathway.\textsuperscript{117} Highest levels of EGF-R mRNA have been found in the human prostatic carcinoma cell lines PC-3 and DU-145, with prostate carcinomas exhibiting a higher expression of EGF-R mRNA than BPH.\textsuperscript{170}

Several studies have demonstrated androgen responsive human normal prostate cells undergo programmed cell death after androgen ablation.\textsuperscript{127,128} However, it has been shown that the AR-positive LNCaP cells do not undergo apoptosis in the absence of androgen.\textsuperscript{4} Similarly, androgen-independent human prostate cancer cells do not activate this apoptotic pathway in response to androgen ablation.\textsuperscript{163} However, androgen-independent human prostate cancer cells (PC-3 and DU-145) have been shown to undergo apoptosis after treatment with cytotoxic drugs that induce a "thymineless" state.\textsuperscript{126}

The availability of a number of transformed cell lines has enhanced the study of the human prostate and prostate cancer. These cell lines have been cloned from metastatic human prostate cancers and are briefly described here:

(i.) \textit{PC-3 Cells.}

In 1979 M.E. Kaighn et al.\textsuperscript{110} established an epithelial cell line, PC-3, from a human prostatic adenocarcinoma metastatic to bone. These cells exhibited anchorage-independent growth in both monolayer and soft agar suspension cultures.\textsuperscript{110} When transplanted into "nude" mice, they produced subcutaneous tumors.\textsuperscript{110} PC-3 cells are non-responsive to androgens, glucocorticoids, EGF, or FGF and have a reduced dependence on serum for their growth.\textsuperscript{110} These cells have also been found to be completely aneuploid with modal chromosome number in the hypotriploid range.\textsuperscript{110} Furthermore, PC-3 cells have been shown to have many features in common with other epithelial neoplastic cells, such as numerous microvilli, gap junction complexes, and abnormal nuclei and nucleoli.\textsuperscript{110} However, the PC-3 cell line maintains a higher degree of differentiation than DU-145 cells.\textsuperscript{23}
PC-3 cells are generally classified as androgen-insensitive and lack expression of the AR. However, two different strains of PC-3 cells have been reported in the literature: (i) PC-3 cells that seem to express very low levels of normal AR mRNA and protein (PC-3$^{AR^+}$)$^{50,251}$, and (ii) PC-3 cells that have undetectable levels of AR mRNA or protein (PC-3$^{AR^-}$)$^{251,253}$ In PC-3$^{AR^-}$, the loss of AR expression may be due to diminished AR mRNA levels.$^{253}$ Genomic Southern analysis has shown that the absence of AR expression in this PC-3 cell group is not associated with AR gene mutations.$^{253}$ In the PC-3$^{AR^+}$ strain, using both PCR and DNA sequencing, no mutations were detected in the AR gene or protein.$^{251}$ Furthermore, both strains of PC-3 cells exhibited a complete absence of specific androgen binding to cytosolic extracts, and their proliferation was unaffected by androgen treatment.$^{251}$

Injection of PC-3 cells into various sites in athymic nude mice resulted in different patterns of metastasis, but did not constitute an entirely suitable animal model of human prostate cancer due to the lack of metastasis to the skeleton of these animals.$^{233}$ Currently there are only 3 well established and well characterized human prostate cancer cell lines: PC-3, LNCaP, and DU-145. Although all have been shown to be tumorigenic in mice, only the PC-3 line has been reported to result in metastasis to other organs.$^{122,267,268}$

It has been recently shown that estradiol induces inhibition of PC-3 cell proliferation presumably by acting upon a specific ($\Delta 4$ exon deleted) ER isoform.$^{26}$ High affinity binding sites for estrogen have been localized to the nuclear compartment of these cells by radioligand-binding assays.$^{26}$ The use of anti-TGF-β$_1$ monoclonal antibodies resulted in increased growth proliferation of PC-3 cells that was almost entirely reversed in the presence of 100 nM estradiol.$^{26}$

(ii.) **DU-145 Cells.**

In 1978, K.R. Stone et al.$^{241}$ isolated a long-term tissue culture cell line derived from a human prostate adenocarcinoma brain metastasis. DU-145 cells are epithelial and grow in isolated colonies on the bottom of plastic petri dishes, and also form colonies in soft agar suspension culture.$^{241}$ They are characterized by a non-euploid human karyotype with a modal chromosome number of 64.$^{241}$ It is a
poorly differentiated cell line that lacks the presence of AR, and is somewhat less aggressive than PC-3 cells.\textsuperscript{50,122,248} It has been demonstrated that DU-145 cells secrete EGF-like polypeptides in serum-free culture media that may specifically bind human EGF, with both high- \( (K_d = 1.8 \times 10^{-10} \text{ M}) \) and low- \( (K_d = 1.1 \times 10^{-9} \text{ M}) \) affinity.\textsuperscript{44} Furthermore, DU-145 cells may be stimulated to grow by its own production of EGF and related polypeptides.\textsuperscript{44}

(iii.) LNCaP Cells.

LNCaP cells were established in 1983 by J.S. Horoszewicz \textit{et al.}.\textsuperscript{97} from a metastatic lesion derived from a lymph node carcinoma of the human prostate. They have been shown to grow readily \textit{in vitro} at 800,000 cells/cm\(^2\) with a doubling time of 60 hours.\textsuperscript{97} They form clones in semisolid media, are highly resistant to human fibroblast interferon (IFN), and have been karyotyped as aneuploid with a modal number of chromosomes (76 to 91).\textsuperscript{97} These cells produce tumors in athymic nude mice.\textsuperscript{97} In both cell cultures and tumors, LNCaP cells produce AcP and have been shown to contain high-affinity ARs that are present in both the cytosol and nuclear fractions.\textsuperscript{97} The AR having a binding capacity for the androgen DHT, of 920 fmol/mg cytosol protein and a binding affinity of 0.4 nM.\textsuperscript{26} Therefore LNCaP cells are hormonally responsive to DHT \textit{in vitro}, which also modulates their growth and stimulates AcP production.

LNCaP cells contain a modified AR with respect to both steroid specificity and antiandrogen sensitivity: stimulatory effects are due to a mutated amino acid in the steroid binding domain of the AR.\textsuperscript{261-263} A single point mutation from a threonine to alanine substitution has been reported to occur at amino acid position 868 of the steroid binding domain.\textsuperscript{263} Therefore, comparable changes in the specificity of the AR in prostate cancer cells may give these cells an advantage in growth rate and may contribute to development of tumors characterized as hormone independent. It has been reported that progestins, estradiol and several antiandrogens can compete with androgens for binding to the AR in LNCaP cells to a greater degree than in other androgen sensitive systems.\textsuperscript{262} These agents, however, did
not inhibit the growth of LNCaP cells in the presence of androgen, but rather stimulated growth by increasing EGF receptor level and AcP secretion.\textsuperscript{262}

Furthermore, cotransfection of PC-3 cells with the LNCaP AR and a vector carrying a chloramphenicol acetyltransferase (CAT) reporter gene linked to the mouse mammary tumor virus (MMTV) promoter, resulted in the induction of CAT activity by either androgens, progestins or the antiandrogen hydroxyflutamide.\textsuperscript{120} In cells containing the normal AR, CAT activity was induced only by androgens.\textsuperscript{120} These findings indicate that the inability of LNCaP cells to discriminate between different steroid hormones or antagonists is specifically linked to the expression of its mutated AR.

Montgomery \textit{et al.},\textsuperscript{169} have also reported that the receptor in LNCaP cells had altered affinity for a number of steroids or analogs such as the synthetic progestin R5020, the GR antagonist RU486, two antiandrogens (cyperoterone acetate and hydroxyflutamide), and the androgen metabolite epitestosterone. However, the affinity of the LNCaP AR for androgens (mibolerone, DHT, and testosterone) was reported to be similar to that of the wild AR.\textsuperscript{169} The different ligand specificities for the AR in LNCaP cells has also resulted in other steroids, besides androgens, in influencing expression of PSA.\textsuperscript{169}

Horoszewicz \textit{et al.}\textsuperscript{97} first reported the presence of low affinity and low capacity ERs in LNCaP cells. However, this finding was demonstrated before the discovery of the mutated AR in LNCaP cells, and therefore it is probable that their binding data included estradiol binding to this AR. Nevertheless, the growth of LNCaP cells has recently been reported to be significantly stimulated in the presence of physiological levels of estradiol acting through ERs.\textsuperscript{31} This growth increase was comparable to that of testosterone or DHT treatment.\textsuperscript{37} Furthermore, the detected ER in LNCaP cells from this latter study\textsuperscript{31} was measured in the presence of a constant excess (10^{-7} M) of R1881 to displace any possible binding of estradiol to the AR in these cells.

A recent study by Limonta \textit{et al.}\textsuperscript{137} had shown that all the major key enzymes required for the metabolism of androgens (5α-reductase, 17β-hydroxysteroid-oxidoreductase, 3α- and 3β-
hydroxysteroid-oxidoreductase) are present and active in LNCaP cells. Using RT-PCR they were able to obtain a 228 base pair cDNA band from LNCaP cells that hybridized with a $^{32}$P-labeled LHRH oligonucleotide probe. This finding indicated that a mRNA for LHRH or a LHRH-like peptide is expressed in these cells. Upon culturing LNCaP cells in media stripped of steroids and treated with a LHRH antagonist, there was a significant increase in cell proliferation. However, there was a significant decrease in proliferation when they were treated with either a monoclonal antibody against the EGF-R or with immunoneutralizing antibodies against EGF and TGF-α. It was concluded that a local LHRH (or a LHRH-like) inhibitory loop is expressed in LNCaP cells, that is capable of inhibiting their growth by interrupting a growth stimulatory pathway such as the EGF/TGF-α pathway to the EGF-R. It is therefore suggested by Limonta et al., that this LHRH (or LHRH-like) inhibitory system is activated when steroids (i.e., androgens) are not present in the culture medium. Further, androgens such as testosterone can stimulate the proliferation of LNCaP cells, at least in part, by inhibiting the activity of this local LHRH (or LHRH-like) inhibitory loop, and at the same time activate a stimulatory pathway.

(iv.) EB-33 Cells

This cell line was established from primary cultures of a moderately differentiated human prostatic adenocarcinoma. Phase contrast photomicrographs of these cells after their 4th passage showed that they were polygonal in shape and had varying numbers of prominent nucleoli. In 1976 Okada et al. noted that the growth rate of the original EB-33 heterogeneous population was not influenced by withdrawal or addition of androgenic hormones. They therefore established from a single plating, 111 clones of EB-33 cells in which 23 clonal lines responded to steroid-extracted media with at least a 50% decrease in growth. In the presence of testosterone and (DHT) there was a slight increase in growth, but not enough to make up for the number of cells they observed in the presence of media stripped of steroids. It was concluded that these findings were consistent with hormone-dependent growth. However, there was no hormone-dependence of EB-33 cells transplanted in “nude” mice. Furthermore, EB-33 cells were initially biochemically characterized as AR-negative using AR low
affinity fluorescein ligands that do not relate to AR binding sites.\textsuperscript{8} The question of AR presence in these cells thus requires reexamination. PAcP has been immunochemically identified in this cell line.\textsuperscript{201}

Chromosomal analysis performed on this permanent epithelial cell line, indicated hypotriploid to tetraploid chromosome numbers in cells continuously cultured for more than 1 year.\textsuperscript{106} Chromosome counts revealed that most cells of the 7th passage of EB-33 had 65 chromosomes.\textsuperscript{189} The range of chromosome numbers increased with time in culture and was reduced to the original model number of 64 by only one animal passage in "nude" mice, suggesting that animal passage increased the state of differentiation of EB-33 cells.\textsuperscript{106}

(v.) JCA-1 Cells.

An androgen-independent prostatic cancer cell line derived from a primary, poor to moderately differentiated prostatic adenocarcinoma tumor by Muraki \textit{et al.} in 1990.\textsuperscript{76} These cells grow easily in culture and also form colonies in soft agar suspension cultures.\textsuperscript{176} When transplanted into nude mice they formed poorly differentiated tumors that exhibited PSA expression.\textsuperscript{176} Unlike DU-145 cells. karyotype analysis of these cells has demonstrated that they are aneuploid with a modal chromosome number of 69 and six marker chromosomes.\textsuperscript{176}

JCA-1 cells secrete a growth factor (prostate cell derived growth factor or PRGF) that can promote the proliferation of murine fibroblast 3T3 cells\textsuperscript{177}. Cell cycle analysis revealed a mechanism by which PRGF is able to induce entry of these cells into the S and G\textsubscript{2}M replicating phases of the cell cycle while at the same time decreasing the number of cells entering the G\textsubscript{1} phase\textsuperscript{177}. These results show that JCA-1 prostate cancer cells are able to secrete autocrine or paracrine factors affecting proliferation and growth of other cell types.

JCA-1 cells have been shown to be growth-inhibited by IFN-\textgamma\textsuperscript{180} and by the phorbol ester 12-O-tetradecanoyl phorbol-13-acetate (TPA).\textsuperscript{266} This growth inhibition by TPA was attributed to cell differentiation by Novichenko \textit{et al.}\textsuperscript{185} Exposure to TPA reduced cell proliferation by almost 80\% after 3 days but the cells remained viable.\textsuperscript{185} Interestingly, cell cycle analysis revealed that these cells were
arrested in $G_1$ phase and blocked from entering into the replicating $S$ and $G_2M$ phases. Upon analysis of chromosomal DNA by agarose gel electrophoresis, TPA-induced cells had no fragmented DNA, in which Novichenko et al. excluded the possibility of apoptosis. However, they did note that TPA-induced cells became larger in size by swelling and showed dendritic-like cytoplasmic extensions. Furthermore, cells treated with TPA acquired the expression of cytokeratin 18 and increased their expression of actin and vimentin, three well-known biological markers cellular differentiation.

(vi.) **PC-82 Cells**

PC-82 is a hormone-dependent transplantable human prostatic tumor line. It is a serially transplantable tumor cell line derived from human prostatic carcinoma tissue, that remained moderately differentiated after 2.5 years in nude mice. This tumor cell line contains large amounts of PAeP that does not appear to be regulated by androgen. PC-82 cells have been shown to have a slow growth rate, and regress after castration and estrogen treatment when implanted into nude mice. This regression of PC-82 cells has been shown to be due to a sequence of biochemical and morphological events leading to cessation of cell proliferation and to the activation of programmed cell death, or apoptosis.

PC-82 cells have also been used as a model system to investigate the effect of various levels of androgen on the growth of prostatic tumor tissue. The level of testosterone in the plasma of mice transplanted with PC-82 tumor cells was found to correlate with the size of the tumor and was associated with increasing concentrations of testosterone and DHT within the tumor. PC-82 tumor growth was stable at a plasma testosterone concentration of 0.8 nM and increased in size when this concentration was elevated and decreased in size when this concentration was lowered.

The AR from PC-82 tumor tissue grown in athymic nude mice exhibited a binding affinity ($K_d$) of 0.1 nM for R1881 and a binding capacity ($B_{max}$) of 120 fmol/mg cytosol protein. The receptor showed high affinity for R1881, testosterone, and 5α-DHT, respectively, whereas little or no affinity was found for progesterone and estradiol. Polyacrylamide gel electrophoresis under denaturing conditions
showed that the DNA-binding form of the AR is a protein with a molecular mass of approximately 95 kDa. This latter value is comparable to those reported for the DNA-binding forms of the progesterone and the GR.

(vii.) **PC-93 Cells.**

This permanent tumor cell line, initiated in 1978 from a moderately differentiated primary adenocarcinoma of the prostate, is androgen-independent. This cell line was initially biochemically characterized as AR-positive by fluorescent androgen derivatives (testosterone-17β-hemisuccinate-BSA-fluorescein isothiocyanate, testosterone-17β-hemisuccinate-fluoresceinamine, and 5α-DHT-17β-hemisuccinate-fluoresceinamine). However these fluorescent ligands have a low AR binding affinity and were not displaced with excess 5α-DHT or R1881. Thus it remains somewhat equivocal as to whether PC-93 cells express AR. The AR cannot be detected using these fluorescent ligands.

PC-93 cells have an epithelial character in culture. Their doubling time is 20 hours in the exponential phase of growth. Immunohistochemically PC-93 cells stained positively for human PAcP, although this enzyme was hardly detectable after 35 or more passages.

(viii.) **ALVA-101 and ALVA-41 Cells.**

These two human prostate cancer cell lines were initiated by Drs. S. Loop and R. Ostenson at American Lake VA Medical Centre, Tacoma, WA, USA. Both were obtained from a bony metastasis in men with prostatic carcinoma. Although until recently only the LNCaP cell line was used as a model to study androgen action in prostate carcinomas, both of these cell lines have now been shown to express ARs and demonstrate 5α-reductase activity. For the present thesis however, only LNCaP cells were available. ALVA-41 cells have demonstrated prostate specific antigen (PSA) by immunohistochemical staining, however they do not secrete this protein. ALVA-101 cells demonstrated PSA mRNA expression following hybridization with an oligonucleotide probe for PSA detected by Northern blot analysis.
The ALVA-41 cell line grows rapidly and adheres readily and firmly to culture vessels. Unlike LNCaP cells, it contains high affinity receptors for androgens (K_d = 0.46 ± 0.11 nM; B_max = 955 ± 213 fmol/mg cytosol protein) and is androgen responsive. This is comparable to that of the AR characterized by Schuurmans et al. in LNCaP Cells, in both binding affinity (0.4 nM) and binding capacity (920.0 fmol/mg cytosol protein), respectively. It appears that the AR in ALVA-41 cells mediates an increase in growth and an increase in secretion of PAcP in response to DHT. These cells also contain a GR with some characteristics typical of this receptor, but they lack a receptor for estrogens.

In both the ALVA-101 and ALVA-41 cells, Sex Hormone Binding Globulin (SHBG) inhibited the uptake of [^3H]DHT, _in vitro_. This action is thought to occur through high affinity cell membrane receptors for SHBG, that may be involved in inhibiting the uptake of steroids into the prostate, but may also act to stimulate growth via an autocrine pathway from SHBG or a SHBG-related peptide.

(ix.) **NCI-H660 (Human Prostate Small Cell Carcinoma).**

NCI-H660 is an extrapulmonary established small cell carcinoma cell line of the prostate, derived by A.F. Gazdar et al. from a lymph node metastasis to the lung of a 63-year-old male Caucasian prior to treatment. These cells express elevated levels of L-dopa carboxylase and possess bombesin-like immunoreactivity. They also express functional atrial natriuretic peptide (ANP) receptors but their cell growth is non-responsive to treatment with this peptide.

Of the five widely used cell lines (EB-33, DU-145, PC-3, PC-82, and LNCaP), only the LNCaP line increases its rate of growth and alters secretion of PAcP in response to androgens. However, this cell line is difficult to work with, because it grows slowly and attaches poorly to culture vessels. Furthermore, the presence of a mutated form of the AR renders this cell line as a somewhat questionable control in many studies.
<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Origin</th>
<th>Growth Response to Androgens</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EB-33</td>
<td>Adenocarcinoma of the Prostate</td>
<td>Insensitive</td>
<td>Okada, K. and Schroeder F.H., Urological Research 2(3): 111-121, 1974</td>
</tr>
<tr>
<td>PC-3</td>
<td>Bone Metastasis</td>
<td>Insensitive</td>
<td>Kaighn et al., Invest. Urol. 17(16): 1979</td>
</tr>
<tr>
<td>PC-82</td>
<td>Adenocarcinoma of the Prostate</td>
<td>Stimulatory</td>
<td>Hoehn et al., The Prostate 1: 95, 1980</td>
</tr>
<tr>
<td>LNCaP</td>
<td>Lymph Node Metastasis</td>
<td>Stimulatory</td>
<td>Horoszewicz et al., Cancer Res 43: 1809, 1983</td>
</tr>
<tr>
<td>JCA-1</td>
<td>Primary prostatic Tumor</td>
<td>Insensitive</td>
<td>Muraki et al., Urology 36(1): 79-84, 1990</td>
</tr>
</tbody>
</table>
1-4. TRANSFECTION STUDIES

One approach to address the issue of hormone insensitivity as well as to examine the action of receptor isoforms is to transfec hormone insensitive cells with cDNA encoding the receptor. This approach has been used extensively in breast cancer cell studies and to a much more limited extent in studies with prostate cancer cells.

(i.) Estrogen Receptor Transfections in Breast Cancer Cells.

Breast cancer is somewhat analogous to prostate cancer in that some forms are hormone (estrogen) dependent. Treatment with antiestrogen provides some relief but the tumors ultimately progress to an estrogen-independent state. To examine the effect of ER expression in previously ER-negative breast cancer cells, ER cDNA has been transfected into several cell lines resulting in an overexpression of ER. Upon transfection of these cells they regain hormonal responsiveness; but paradoxically estradiol inhibits, rather than stimulates, cell proliferation. Jiang and Jordan hypothesized that by reactivating ER expression in these cells, it is possible to develop some therapeutic approach (e.g., gene therapy) for controlling the growth of ER-negative tumors during hormonal therapy. The inhibitory growth of ER-negative cell lines after stable expression of transfected ER and treatment with estradiol, has been attributed to a disturbance in the cell cycle phases of the ER transfectants.

In breast cancer there also exists a heterogeneous distribution of cell lines expressing AR. Birrell et al. have shown that the AR expressing breast cancer cell lines T47-D and ZR-75-1 are growth inhibited in the presence of androgen. While other AR expressing breast cancer cell lines, MCF-7 and MDA-MB-453 were growth stimulated. The antiandrogen hydroxyflutamide reversed these inhibitory or stimulatory effects of androgen (1 nM) when used in a 100-fold excess. It has been demonstrated that the AR is expressed by breast cancer cell lines that are positive for both ER and PR. In these cells AR expression is regulated by ligands also known to regulate these receptors, including progestins and retinoids.

Several hypotheses have been formed to explain the paradoxical finding of estrogen-induced decreased growth of breast cancer cells transfected with ER cDNA.
1. Transfection has caused genetic rearrangements resulting in the expression of cytotoxic or growth inhibitory genes. This is based on the possibility that the transfected genetic cDNA insert may become incorporated into the genome, disrupting the expression of genes important to cell survival. One approach to reduce this argument would be to use episomal vectors which are thought to be maintained extrachromosomally.

2. The constitutive expression of high levels of ER produces a "squelching" or interference with other transcriptional factors. Jiang and Jordan\textsuperscript{107} postulate that by engaging other transcription factors, high expression of ERs could lessen the availability of these factors that are necessary for expression of normal growth-promoting genes. An approach to diminish this argument would be to express receptor at lower, physiological levels.

3. Estrogen-insensitive cells constitutively express previously estrogen sensitive growth promoting genes and upon reinstatement of ER expression, ER actually slows the expression to a more moderate level.

4. The exogenous receptor may not be fully functional despite the lack of structural mutations. Perhaps expression of a key kinase is lacking resulting in only partial phosphorylation of the receptor. The result might be altered transcriptional regulation.

5. Estrogen is reported to have a biphasic effect on tumour growth, being inhibitory at high dose levels. Expression of high levels of receptor might shift that response such that inhibitory effects might be seen at lower dose levels.

6. Overexpressed receptor bound to estradiol may bind widely to DNA and physically interfere with some fundamental process, such as DNA replication.

(ii.) Androgen Receptor Transfections in Prostate Cancer Cells.

Previously it was reported that transfection of the androgen-insensitive prostate cancer cell line PC-3, with a plasmid containing a human AR cDNA\textsuperscript{33,34} resulted in an androgen-sensitive cell line PC-3(T).\textsuperscript{284} In that study, the full-length AR cDNA was inserted into the plasmid pSG5 by ligation at EcoRI (5') and BamHI (3') restriction sites with expression controlled by an SV40 promoter. The resulting vector, pSGAR was cotransfected with pSR\textsubscript{1}neo into PC-3 cells and transformants (PC-3(T) cells) were selected by resistance to genticin. PC-3(T) cells exhibited a decreased rate of proliferation by 72 hours and a reduction in characteristics associated with the malignant state in response to concentrations of DHT as low as 1 nM.

The initial approach of this thesis was to study androgen responses in the PC-3(T) cell line. It was believed that comparison of this cell line with both the parental line and the androgen-sensitive cell line (LNCaP) might reveal critical aspects of androgen action important in the progression of prostate
cancer. Specifically, it was of critical importance to look at the impact of AR on growth factor effects in PC-3 and PC-3(T) cells with respect to EGF, TGF-α and TGF-β, and the role these growth factors play in androgen-induced alterations in prostate growth. Unfortunately, results of cytogenetic studies performed on PC-3(T) cells were inconsistent with the cell line being of strictly human origin. One possibility was that the transfected cell line had suffered extensive chromosomal aberrations perhaps as a result of incorporation of the transfected material into the cellular genome. It was decided at this point to develop a better and more valid AR transfection system with which to generate several PC-3 cell lines capable of responding to androgen without any changes to their overall phenotype.

Other studies have also examined the effect of transfecting some form of AR into PC-3 cells as well as a rat prostatic cancer cell line, CUB-II. Using the same pSG5 vector as Yuan et al., Hansen et al. demonstrated that stable transfection of the hAR into the PC-3 cell line resulted in cell death upon androgen removal from media. In a subsequent study using this cell line (PC3-hAR), Brass et al. demonstrated that androgen up-regulated EGF-R expression and increased binding affinity in these cells.

Suzuki et al. transfected the AR-negative rat prostatic cancer cell line CUB-II with the full-length human AR cDNA contained in the expression vector pSG5. In culture containing testosterone, the growth of AR-transfectant cells (CUB-II/AR) was inhibited. This effect was reduced when flutamide was added to the culture media along with testosterone. Moreover, when CUB-II/AR cells were transplanted into BALB/cAJcl strain of athymic male mice, their growth was much slower than those in female mice. Furthermore, in culture, the addition of testosterone also increased AcP or PAcP activity of CUB-II/AR cells. Suzuki et al. concluded from their findings that the resumption of androgen-dependent processes in CUB-II cells resulted in a reduction of growth rate which was accompanied by changes in phenotype.
It was demonstrated by Garcia-Arenas et al.\textsuperscript{76} that PC-3 cells exhibited low level of AcP activity in their conditioned media with no PAcP mRNA detected by either northern blot analysis or RT-PCR assay. Transfection of human AR cDNA\textsuperscript{33,34} into PC-3 cells did not affect expression of PAcP.\textsuperscript{76}

Marcelli et al.\textsuperscript{158} stably transfected PC-3 prostate carcinoma cells with the non-episomal expression plasmid CMV3 containing a constitutively active AR construct that was truncated at its hormone-binding domain (CMV-ARCA). This cell line exhibited a growth rate approximately 35\% slower than that of the mock-transfected control cell line (PC3-Neo). The presence of this constitutively active (mutated) AR resulted in the modulation of growth of these cells. Furthermore, this regulation was shown to correlate with the down-regulation of a insulin-like growth factor binding protein (IGFBP-3) from PC3-ARCA cells but not from PC3-Neo cells. Several IGF-binding proteins (IGFBPs) have been shown to be abnormally produced by prostate cancer.\textsuperscript{41,45,103,198} Moreover, IGF-I and IGF-II stimulated the proliferation of PC3-ARCA cells but not PC3-Neo. Administration of IGFBP-3 alone had no effect when given alone. However, when IGFBP-3 was administered together with IGF-I or IGF-II, there was a further increase in the stimulation of growth observed in PC3-ARCA cells, but no effect was detected in PC3-Neo cells.\textsuperscript{158}

The exact mechanism leading to a slower growth of cells after transfection with AR or ER to respective hormone insensitive cells is very unclear. However, Suzuki et al.,\textsuperscript{246} have hypothesized that it is reasonable and possible that activation of sex hormone-dependent processes changes the cellular metabolism to a more differentiated state, resulting in a decrease in the fraction of proliferating cells. This may be true, with the one possible exception being the findings demonstrated by Hansen et al.\textsuperscript{90} and in the subsequent study by Brass et al.\textsuperscript{13}

\textbf{1-6. GROWTH FACTORS AND ONCOGENES INVOLVED IN ANDROGEN ACTION}

Growth factors have been proposed to be involved in neoplastic development. The AR is the main intracellular mediator of androgen action. Androgens affect growth of the prostate gland and many
prostate cancers. Cells can respond to certain factors that bind to cell surface receptors possessing intrinsic tyrosine kinase activity. Androgens can mediate their mitogenic effects on prostate cells by utilizing autocrine loops via AR that lead to the recruitment of other growth factors and pathways within the cell. Overexpression of these molecules has been associated with tumor progression. Enhanced prostatic cancer cell growth in vitro has been reported in the presence of certain growth factors.

(i.) Epidermal Growth Factor.

The development of prostate cancer to a hormone-independent state may involve EGF or EGF-like proteins that may become important in regulating the growth of prostate cancer cells. EGF can increase the tissue invasive properties of PC-3 cells. This has been shown to occur, in part through the overproduction of urokinase plasminogen activator, an extracellular protease. It has also been demonstrated that DU-145 cells can secrete EGF-like polypeptides into their media. These findings can lead to the postulation that androgen-independent cells may be autologously stimulated by endogenously produced EGF and related polypeptides. A plausible autocrine production of EGF may potentiate tumor cell invasion when androgen ablation therapy is employed as a means against androgen-dependent prostate cancer tumors.

In LNCaP cells stimulation of proliferation by the androgen R1881 (0.1 nM) was accompanied by a concomitant increase in the number of EGF receptors from 11500 to 28500 sites/cell. This leads to the idea that one of the mechanisms involved in androgen action in these cells is the increased sensitivity to EGF. Therefore, while in the absence of DHT, EGF has been shown to downregulate the level of AR mRNA and thereby the level of ARs, EGF can still stimulate the proliferation of LNCaP cells through a pathway different from that of DHT.

Amphiregulin is a heparin-binding EGF-related peptide that has a high affinity for EGF receptor. It has been demonstrated that in LNCaP cells, the expression of this peptide is regulated by androgen stimulation. Elevated levels of amphiregulin in the presence of DHT, for example, can autologously lead to its binding to EGF-R that can mediate its action through the EGF/EGF-R pathway leading to
stimulation of growth of LNCaP cells. Studies with the estrogen-responsive breast carcinoma cell line, MCF-7, suggest that regulation of amphiregulin by estrogen may also be mediated via an EGF-R pathway.228

(ii.) Transforming Growth Factor-α and Transforming Growth Factor-β.

Transforming growth factor-alpha (TGF-α) is a polypeptide whose mitogenic activity is mediated through its binding to EGF-R, in much the same way as amphiregulin. This ligand/receptor combination has been shown to be important in the development and maintenance of function of a variety of organs, and is involved in tumorigenic and tumor progression events in several human neoplasms.146,154,273 TGF-α has been proposed as an autocrine growth factor and a mediator of the mitogenic effect of steroid hormones in several types of tumor cells.117,147 Androgens may stimulate the proliferation of prostate cancer cells by up-regulating an autocrine growth loop involving EGF/TGF-α receptor and its ligand, TGF-α.148

Recently it has been demonstrated that the growth of PC-3 cells can be stimulated by TGF-α and inhibited by TGF-β, but not with LNCaP cells.25 DU-145 and LNCaP cells were demonstrated to contain fairly high levels of both EGF-R, TGF-α, and to a lesser extent EGF.25 In contrast, PC3 cells exhibited a much lower expression level of EGF-R and the ligands EGF or TGF-α.25 The androgens 5α-DHT and testosterone were shown to be able to stimulate the synthesis of TGF-α receptor mRNAs and EGF/TGF-α receptor levels in the androgen-sensitive cell line ALVA-101, indicating a possible TGF-α autocrine loop in these cells with EGF/TGF-α receptors.148

It has recently been demonstrated that stromal cell-conditioned media can strongly inhibit the proliferation of PC-3 and LNCaP cells.121 PC-3 cells display a very strong binding for TGF-β and also contain relatively high levels of endogenous TGF-β.25 Muir et al.173 have demonstrated TGF-β1 as an epithelial cell growth inhibitor that can be induced in hormonal therapies against prostate cancer in vivo. It has been hypothesized that the relapse of hormonally treated prostate cancer may be associated with the failure of the epithelial cells to respond to TGF-β1 secreted by the stroma.173 Since some prostate
cancer cells can express high levels of TGF-β, therefore they retain some sensitivity to the growth inhibitory effect of TGF-β, but have devised a way to protect themselves from growth inhibition by TGF-β in vivo. These type of mechanisms have also been demonstrated in breast cancer. For example, the hormone-dependent human breast cancer cell line MCF-7, when treated with antiestrogens at concentrations known to inhibit their growth, it secretes TGF-β that inhibits the growth of an ER-negative human breast cancer cell line in coculture. This inhibition was reversed with anti-TGF-β antibodies.

(iii.) c-myc and bcl-2

c-myc is a proto-oncogene involved in cell proliferation and is associated with mitogen-stimulated growth of cells. Its overexpression has been reported in breast cancer. Its constitutive expression has been implicated in the progression of breast cancer tumors to a hormone-independent state. In prostate, c-myc is regulated by androgen. Though its deregulation occurs in virtually all tumor cells, it is also a potent trigger for apoptosis. The anti-apoptotic oncogene bcl-2 suppresses c-myc induced apoptosis. bcl-2 or c-myc levels have been reported to increase in androgen-independent carcinomas.

myc-induced apoptosis has been shown to occur only in cells deprived of growth factors or in cells arrested with cytostatic drugs. Moreover, induction of apoptosis by c-myc requires association with c-myc’s heterologous partner, max. All of this strongly implies that c-myc drives apoptosis through a transcriptional mechanism: presumably by modulation of target genes.

(iv.) Insulin-like Growth Factors

Insulin-like growth factors (IGFs) are important and powerful growth stimulators of PEC in culture, that can bind with high affinity and specificity to IGF receptors and IGF-binding proteins (IGFBPs), and appear to have a major role in intercellular communication within the prostate. In PEC there is an abundance of type 1 IGF receptors but no evidence of type 2 IGF receptors. The growth of PEC was stimulated by IGF-I, while IGF-II and insulin were 1 and 3 orders of magnitude, respectively,
less effective in stimulating PEC growth. It is thus speculated that the actions of IGFs in PEC are mediated through the type I IGF-receptor. Furthermore, PEC may produce IGFBPs (IGFBP-2 and a 24 kDa IGFBP) which may also participate in the regulation of IGF action in these cells.

DU-145 cells have been shown to possess type I IGF receptors. When IGF-I or IGF-II was added to serum free medium, there was an increase in the rate of thymidine uptake by DU-145 cells. DU-145 cells also secrete IGBP-1 into their conditioned medium, and this secretion was suppressed when the EGF autocrine loop was interrupted. Removal of IGFBPs from their conditioned medium resulted in no secretion of either IGF-I or IGF-II by DU-145 cells.

(v.) c-fos/c-jun.

These two oncogenes are the primary targets in the signal transduction pathway from growth factor receptor activation to cellular proliferation. Their expression is rapidly down-regulated and their gene products bind DNA only after formation of heterodimers with AP-1. The dimeric transcription factor AP-1 interacts with TPA response elements (TRE). Increases in TRE-dependent transcription activity results in cellular growth and transformation.

Prostatic cell death may be accompanied by a sequential pattern of gene activity (c-fos→c-myc→70 kDa heat shock protein) which has been termed "reactive cascade". This cascade of gene activity has been speculated to reflect the molecular events that occur during stimulation of cultured cells to proliferate, such that both cell death and proliferation may share common signal mechanisms.

(vi.) Retinoic Acid.

Retinoic acid (RA) has been shown to inhibit the growth of ER-positive breast cancer cell lines but not ER-negative breast cancer cells. It has been shown that ER-negative breast cancer cell lines transfected with ER cDNA respond to RA with decreased growth. This has been attributed to increased RA receptor (RARα) mRNA in these cells. Furthermore, RA resistance of estradiol-independent breast cancer cells has been attributed to coincide with diminished RAR function. DHT and all-trans-retinoic acid have also been shown to down-regulate AR mRNA levels in T-47D (ER+ and
PR+) cells 6 hours after treatment, whereas estradiol had no effect. RA however, increased the level of AR mRNA in MDA-MB-453 (ER-, PR-) cells.

RA has also been shown to inhibit the growth of normal and prostate cancer cells in vitro and in vivo. Recent studies have demonstrated that RA can repress the growth of human prostatic epithelial cells. In LNCaP cells, androgen-binding activity was reduced by 30-40% in cells treated with 10^-5 M RA plus 6 nM DHT, as compared to cells with DHT alone. Retinoic acid has also been shown to have a biphasic effect on LNCaP cell growth. Fong et al. have shown that in the absence of DHT, 0.01 μM RA stimulated while 0.1 μM RA inhibited LNCaP growth; 10 μM RA but not lower doses reduced LNCaP cell proliferation in the presence of 0.1 nM DHT; 0.1 μM RA however, reduced LNCaP cell growth in the presence of 10 nM DHT. Therefore, suppression of proliferation and function of prostatic cells by RA may be via modulatory effects on the AR. It was also shown that 10 μM RA reduced LNCaP growth response to either EGF or TGF-α and potentiated the growth inhibitory effect of TGF-β.
1-7. THESIS HYPOTHESIS

A lack of understanding of the mechanisms involved in the evolution of prostate tumors from an androgen-dependent state to an androgen-independent state appears to preclude the development of more efficacious treatments for prostate cancer. The loss or absence of androgen sensitivity in advanced prostate cancer cells may be associated with the expression of a different set of genes enabling the cell to take advantage of alternative mitogenic factors or pathways available to them.

Results of AR transfections with non-episomal vectors have yielded somewhat contradictory findings. Whereas Yuan et al. reported a slowed growth with transfection of pSG5-AR, these cells apparently suffered massive changes in their chromosomal make-up. Further, studies by Hansen and Brass reported that transfection with the same constituent yielded cells that were dependent upon androgen for survival. Lastly, Marcelli et al. indicate that expression of a constitutively (albeit mutated) receptor slows growth. Unfortunately, most of these transfection studies, including those in breast cancer cells involve non-episomal vectors and co-transfection strategies. Further, the criticism leveled at the work with breast cancer cells stems largely from the use of vectors that overexpress ER. No attempt has been made to establish clonal cell lines with different levels of expression of the transfected receptor. This is critical in comparing responses to hormone treatment, at different levels of receptor expression, with hormone receptor-positive and hormone-sensitive control cell lines.

The main objective of this thesis was to develop several clonal lines of PC-3 cells transfected with an episomal vector (pCEP4) containing a full-length human AR cDNA. It was hoped that a critical range could be attained that would bracket that of an AR-positive and androgen-sensitive cell line, LNCaP. Our hypothesis is that expression of AR in PC-3 cells causes growth inhibition through cellular differentiation induced by DHT treatment. Further, we expect the manifestation of this effect to be directly related to the level of AR expressed. The vector system chosen to achieve this is unique in that it replicates independently of genomic DNA by utilization of host cellular factors and the protein EBNA-1 which is coded for by the vector itself. This, in theory, allows for the establishment of
normal cell lines without any alterations to the host cell’s genome that can often occur from the introduction of a non-episomal vector. The vector itself also carries the hygromycin B gene for stable selection of transfectants with the aminocyclitol antibiotic Hygromycin B,\textsuperscript{47,62,80,81,157} without the need of a second vector to be co-transfected.
CHAPTER II

Materials and Methods

2-1. Cell lines and cultures.

PC-3 (Kaighn, M.E et al., 1979; ATCC CRL-1435, human prostate adenocarcinoma metastasis to bone, at passage 21; batch date 04/01/1993 F-11154) and LNCaP (Horoszewicz, J.S. et al., 1983; ATCC CRL-1740 FGC clone, human lymph node prostate carcinoma, at passage 19; batch date 05/01/1993 F-11278) cell lines were obtained directly from the American Type Culture Collection (Rockville, MD). Cells were maintained in RPMI 1640 supplemented with 5% and 10% (for PC-3 and LNCaP cells, respectively) Fetal Bovine Serum (FBS, GIBCO-BRL #16140-022, Burlington, ON, Canada), 1% (vol/vol) penicillin-streptomycin (5,000 U/ml and 5,000 μg/ml, respectively, GIBCO-BRL #15070-014) and with 0.25% (vol/vol) fungizone (250 μg/ml amphotericin B and 250 μg/ml sodium desoxycholate, GIBCO-BRL #15295-017). All cell cultures were grown at 37°C in a humidified 5% CO2 atmosphere and subcultured at 1:3 (LNCaP) or 1:5 (PC-3) dilutions by trypsinization with 0.25% trypsin (GIBCO-BRL #15090-046) for 3-5 minutes (37°C).

2-2. Preparation of Charcoal-Stripped Fetal Bovine Serum.

FBS was stripped of steroids by charcoal adsorption. In brief, 32.5 g of charcoal (Carbon, Decolorizing, Alkaline; Fisher Scientific #C176-500) was mixed with 500 ml of FBS and stirred for 5 minutes at room temperature. The mixture was then placed at 4°C for 48 hours with continuous mixing. The charcoal was removed by centrifugation at 18,000 rpm (SS-34 Sorvall) for 1 hour at room temperature. Any residual charcoal remaining in the supernatant (charcoal stripped FBS or sFBS) was removed by filtering through Whatmann #20 filter paper. The sFBS was aliquoted and stored at -20°C until required.
2-3. **Preparation of RPMI 1640 Media for Cell Cultures.**

RPMI 1640 media (with L-glutamine, without phenol red, without sodium bicarbonate; heat-inactivated, and virus and myco-plasma tested) was purchased from GIBCO-BRL (#13200-076). In brief, 10.4 g was used per litre of media in double distilled water (ddH₂O), supplemented with either FBS or sFBS, fungizone and penicillin-streptomycin as described above, and 2.2 g/L of sodium bicarbonate (GIBCO-BRL, ACS grade #895-18101N). All pH of the media was adjusted to 7.45 with concentrated HCl. Media was filter-sterilized by passage through 0.2 μ filter units (Corning), prior to culturing with cells.

2-4. **Freezing and Thawing of Cells.**

Cell culture mono-layers were washed twice with 1X Dulbecco’s Phosphate Buffer Saline (PBS: 140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4), trypsinized in the presence of 10% nFBS RPMI media, and resuspended in 750 μl freezng solution [10% (v/v) glycerol in FBS] and then transferred into cryogenic vials. Sample vials were incubated at 4°C (10 minutes), then at -20°C (10 minutes), followed by incubation at -86°C for at least 10 minutes to a week, and then transferred over to a liquid nitrogen tank (-270°C) via dry ice (-70°C).

When cells were required, the cryogenic vials were taken from liquid nitrogen stores and transferred to a 37°C incubator via dry ice, for rapid thawing. Cells were then transferred and resuspended in 6 mL of propagation medium (e.g., 10% FBS RPMI 1640) and centrifuged for 5 minutes at 400 g. This step was repeated two more times to ensure complete removal of glycerol. The cells were finally plated into culture flasks or tissue culture dishes with freshly prewarmed medium for propagation.

2-5. **Preparation of Competent Bacteria.**

Bacteria were grown overnight on LB agar plates. Single colonies were selected and used to inoculate 1 mL of LB media [Luria-Bertani Medium: 1% (m/v) bacto-tryptone, 0.5% (m/v) bacto-yeast
extract, 0.17M NaCl, pH 7.0 (with approximately 2 ml of 5N NaOH)] followed by vortexing at moderate speed and then diluting to 100 mL with LB medium. The cultures were left to grow for several hours [i.e., 2.5 to 3.0 hours for DH5α E. Coli or 6.0 to 6.5 hours for TOP10 E. Coli (Invitrogen)] at 37° C in a shaker incubator (300 rpm's). The number of bacteria was analyzed at OD∞ so as to make sure that the reading was between 0.4 to 0.5, that is not to exceed 100,000,000 bacteria/mL. Twenty-five milliliters of this bacterial culture was then transferred to sterile, pre-chilled, 50 mL polypropylene tubes and incubated on ice for 10 minutes. This was followed by centrifugation at 2700 g for 10 minutes at 4° C. The supernatant was then decanted and tubes were left to stand inverted for 1 minute. The pellet was resuspended in 4 mL (or 10 ml per 50 ml tube of culture) of ice cold 0.1M CaCl₂, vortexed gently and incubated on ice for 10 minutes, followed by centrifugation as before. The supernatant was once again decanted, the cell pellet resuspended in 2 mL of ice-cold 0.1M CaCl₂, and vortexed gently.

**Long-term Storage of Competent Bacteria.** Competent bacteria in 2 mL of 0.1M CaCl₂ were mixed with 300 µl of 80% glycerol by gently swirling. Two-hundred microlitre aliquots were then dispensed into chilled sterile microfuge tubes and stored at -86° C until required.

2-6. **Transformation of Competent Bacteria.**

Two-hundred microlitres of competent bacteria were incubated with 50 ng of DNA (in no more than 10 µl) on ice for 30 minutes. The sample was then incubated in a 42° C water bath for 90 seconds, chilled on ice for 1 to 2 minutes, and then mixed with 800 µL of LB medium [0.02 g/ml bacto-tryptone, 0.005 g/ml bacto-yeast extract, 5 mg/ml NaCl, 2.5 mM KCl, pH 7.0 with 5N NaOH, 0.01 mM MgCl₂, and 20 mM Glucose]. This was followed by incubating in a 37° C water bath for 45 minutes. Then 100 uL was spread onto an LB-Agar [LB media with 15 mg/ml bacto-agar or agarose] plate with the appropriate antibiotic (e.g., 100 µg/ml ampicillin). The bacteria were left to absorb into the agar by inverting the plate, and then incubated at 37° C overnight.
Minipreps were carried out to isolate small amounts of DNA (up to 5 µg), for verification of inserts and for cloning purposes. Transformed competent bacteria were propagated in 5 mL of LB media [10 mg/ml bacto-tryptone, 5 mg/ml bacto-yeast extract, 0.17 M NaCl, pH 7.0 (with approximately 2 ml of 5N NaOH)] supplemented with 50 µl of 10 mg/mL (in methanol) Ampicillin (100 µg/ml final concentration) in 17 X 100 mm polystyrene tissue culture tubes. Individual colonies were lifted from agar plates with a sterile toothpick and allowed to grow overnight at 37°C in a shaker incubator. A 1.5 ml aliquot of bacterial culture was then centrifuged briefly (20 sec) to pellet the cells in a sterile microfuge tube. The supernatant was aspirated off and the bacteria were resuspended in 350 uL of Sucrose Solution [0.08 g/ml sucrose, 5% (v/v) Triton-X, 10 mM Tris-HCl, pH 8.0, 50 mM EDTA] with 25 µL of 10 mg/ml lysozyme (0.0167 mg/ml final concentration) and let to stand at room temperature for 5 minutes. The samples tubes were then placed in boiling water for precisely 40 seconds and centrifuged at 13,000 rpm (maximum speed in a microfuge) for 15 min. The resulting pellet, consisting primarily of chromosomal DNA was removed and discarded. An equal volume of isopropanol was then added to the supernatant, mixed and left to precipitate for 10 minutes at -20°C. The samples were centrifuged for 15 minutes at 4°C, the supernatant was discarded, and the pellet resuspended in 100 µL of sterile H₂O or TE buffer [10 mM Tris, 1 mM EDTA, pH 8.0]. The resulting DNA samples were then subjected to endonuclease digestion with specific restriction enzymes in verification of inserts or for cloning purposes as follows: 2 µL of 10X React 3 Buffer (GIBCO-BRL, 500 mM Tris-HCl, 100 mM MgCl₂, 1 M NaCl, pH 8), 16 µL of DNA sample, 2 µL restriction enzyme, and followed by 2 µL of RNase A. Enzyme digestion reactions were carried out at 37°C for 1 to 3 hours as required.

Running DNA Mini Gels. A 1% agarose gel was usually prepared to separate DNA fragments of 500 base pairs or greater, and a 2% gel for DNA fragments less than 500 base pairs. Agarose gels were prepared in either 0.5X TBE [0.45 M Tris-borate, 20 mM EDTA, pH 8.0] or 1X TAE [0.04 M Tris-acetate, 1 mM EDTA, pH 8.0]. After boiling the gels in a microwave oven, ethidium bromide was added.
to give a final concentration of 0.5 µg/ml of gel. The ethidium bromide stains nucleic acids and allows for its visualization under a UV light source.\textsuperscript{216} To prepare DNA samples for loading onto gels, 4 µl of 6X Dye Gel Loading Buffer [trace amounts of bromophenol blue, xylene cylenol in 1X TBE buffer with 30% (v/v) glycerol] was added to 20 µl of DNA sample. The gels were run at 120 V in a horizontal mini-gel apparatus for about 1 hour or until the dye front (contains 200 or less base pair DNA fragments\textsuperscript{216}) reached the end of the gel.

2-8. \textit{Large-Scale Preparation (Maxiprep) of Plasmid DNA.}

This procedure was carried out for preparing larger amounts of DNA (up to 500 µg), which was necessary during transfections. A single colony of \textit{E. coli} strain TOP10 (Invitrogen) transformed with either pCEP4 (Invitrogen) or pCEP4-hAR plasmid DNA was used to inoculated 30 ml of LB media in the presence of 100 µg/ml of ampicillin. Bacteria were then grown at 37° C in a shaker incubator (300 rpm’s) for 6 to 6.5 hours or until an OD reading of approximately 0.6 to 0.7 (log-phase growth) was obtained at 600 nm. Then 25 ml of log-phase bacteria was added to 500 ml LB media with 100 µg/ml ampicillin final concentration and grown in a shaker incubator (37° C at 300 rpm’s) for 2.5 to 3.5 hours. At this point 2.5 ml of 34 mg/ml of \textit{chloramphenicol} was added to stop bacterial cell division but allow for DNA replication to continue. Bacteria at this point were allowed to grow overnight (12 to 16 hours in a shaker incubator at 37° C at 300 rpm’s).

Sterile QIAGEN TIP-500 COLUMNS (Qiagen Plasmid Midi Kit: QIAGEN #12143) were used to purify plasmid DNA from harvested bacteria. Bacteria obtained from the above maxiprep were centrifuged at 6000 g (~6000 rpm's in a Sorvall GSA or GS3 rotor) at 4° C for 15 minutes. The bacterial pellet was resuspended in 10 ml of sterile Buffer P1 [resuspension buffer: 100 µg/ml RNase A, 50 mM Tris-HCl, 10 mM EDTA, pH 8.0, 4° C] and gently mixed with 10 ml of sterile Buffer P2 [lysis buffer: 200 mM NaOH, 1% (m/v) SDS, 25° C] by inverting 4-6 times, and incubating at room temperature for 5 minutes. This was followed by the addition of 10 ml of ice-cold sterile Buffer P3 [neutralization buffer:
3.0M Potassium acetate, pH 5.5, 4°C, and incubation on ice for 20 minutes in a 50 mL-Beckman Ultracentrifuge tube. Contents were mixed by inverting 4 to 6 times. This mixture was centrifuged at 4°C for 30 minutes at ~16,000 rpm’s in a Sorvall SS-34 rotor. An aliquot (125 µl) of supernatant (SAMPLE 1) was collected for analytical gel. The remaining supernatant was then loaded immediately onto a previously equilibrated (with 10 ml of sterile Buffer QBT [equilibration buffer: 750 mM NaCl, 50 mM MOPS, 15% (v/v) ethanol, pH 7.0, 0.15% (v/v) Triton X-100, 25°C] by gravity flow) QIAGEN TIP-500 column and allowed to enter the resin by gravity flow. A 125 µl (SAMPLE 2) was collected for analysis on gel. The column was then washed 2 times with 30 ml of sterile Buffer QC [wash buffer: 1.0 M NaCl, 50 mM MOPS (4-Morpholinepropanesulfonic acid), 15% (v/v) ethanol, pH 7.0]. The plasmid DNA was then eluted from the column with 15 ml sterile Buffer QF [elution buffer: 1.25 M NaCl, 50 mM Tris-HCl, 15% Ethanol, 25°], precipitated with 0.7 to 1.0 volumes of isopropanol previously equilibrated to room temperature, and centrifuged at 9,500 to 12,000 rpm’s (≥15,000 g) in a Sorvall HB-4 swinging bucket rotor at 4°C for 30 minutes. The supernatant was then carefully removed and the DNA washed with 5 to 15 ml ice-cold 70% ethanol (more volatile than isopropanol, removes any precipitated salt and makes DNA easier to dissolve) and centrifuged again. The 70% ethanol supernatant was carefully removed and the DNA pellet was allowed to dry for 10 minutes on the bench. The DNA was dissolved in a small volume (~ 1.0 mL) of TE buffer [10 mM Tris, 1 mM EDTA, pH 8.0] and OD$_{260}$ vs. OD$_{280}$ obtained (OD$_{260}$ = 1 corresponds to 50 µg/ml of dsDNA).

Analysis on Gel. SAMPLES 1 and 2 were precipitated with 0.7 to 1.0 volumes of isopropanol and microfuged for 15 minutes. The pellets were rinsed with 70% ethanol and air dried and resuspended in 50 µl of TE buffer. Two microliters was used for analysis on 1% agarose gel.


To isolate and recover DNA fragments, 0.5% Low-Melt Agarose (FMC Bioproducts #50101, Rockland, ME) was used in lieu of normal agarose. Samples were electrophoresed at 0-4°C. The
fragments were identified under a U.V. light source and excised from the gel with a scalpel or razor blade. Each gel fragment was placed in a 1.5 mL sterile eppendorf microfuge tube and incubated at 65°C for 2 min. to melt the agarose. Four volumes (40 μl) of sterile H₂O or TE buffer (pH 8.0) was then added and the mixture was incubated at 65°C for 5 min. This was followed by aggressive vortexing. The samples were stored at -20°C.

2-10. Construction of the expression plasmid pCEP4-hAR.

The full length human AR cDNA clone (3.72 kb) contained within the plasmid pGEM-3Z (2.74 kb) (Figure 2A) was kindly provided by S. Liao (PNAS 85: 7211, 1988). Endonuclease digestion with BamHI and NheI resulted in a 3.29 kb AR cDNA fragment containing both initiation and termination sites (Figure 2B). A third restriction enzyme, AartII, was also used to allow resolution and isolation of the 3.29 kb AR cDNA fragment on agarose gel. This reaction was carried out as follows: 4 μl 10X Buffer A (333 mM Tris acetate, 100 mM Mg-acetate, 666 mM K-acetate, 5 mM DTT, pH at 37°C 7.9; Boehringer Mannheim Biochemica), 20 μl of 30 ng/μl pGEM-3Z/hAR cDNA, 2 μl AatII (Pharmacia Biotech #27-0953, 20000 units/ml), 1 μl Bam HI (GIBCO-BRL #15201-023, 10 units/μl), 1 μl Nhe I (GIBCO-BRL #5444SA, 10 units/μl), and 3 μl RNase A (10 mg/ml), in a 1.5 ml sterile eppendorf tube at 37°C for 2 hours. Digestion of pCEP4 (Invitrogen #V044-50, 10.4 kb) at BamHI and NheI sites within the polycloning region resulted in a loss of 40 bases and linearization of the plasmid with ends compatible with the 3.29 kb AR cDNA fragment from above (Figure 2C). This reaction was carried out as follows: 2 μl 10X Buffer A (Boehringer Mannheim Biochemica), 14 μl 2.5 ng/μl pCEP4 cDNA, 1 μl Nhe I, 1 μl Bam HI, and 2 μl RNase A, in a 1.5 ml sterile eppendorf tube at 37°C for 2 hours.

Ligation of the AR cDNA, using T4 DNA Ligase (GIBCO-BRL #15224-017), into the polycloning site of pCEP4 produced a 13.69 kb plasmid, pCEP4-hAR (Figure 2D). The AR cDNA fragment (estimated at ~200 ng by band intensity) was isolated from the gel, as described above, and extracted/precipitated with one-tenth the volume of 3 M sodium acetate (pH 8) and 2 volumes of ice-cold
ethanol at -20° C for 1 hour, followed by centrifugation at 13,000 rpm for 15 minutes. The resultant pellet was resuspended in approximately 20 µl of sterile water. The ligation reaction was carried out as follows: 7 µl AR cDNA, 2 µl pCEP4 cDNA, 1.5 µl 10X ligation buffer (Boehringer Mannheim), 1.0 µl T4 DNA Ligase (1 unit/µl, GIBCO-BRL #15224-017), and 4.0 µl of sterile H2O in a 1.5 ml sterile eppendorf tube. The reaction mixture was left to incubate for 12-16 hours (i.e., overnight) at room temperature. Proper ligation was confirmed by restriction mapping (Figure 3).

2-11. Viability of PC-3 cells in Media Containing Hygromycin B.

Cells were plated in 24-well plates with each well containing an equal number of cells (i.e. 10,000 cells/well). The cells were propagated in 2.0 mL of media supplemented with 0, 1, 2, 5, 10, 25, 50, 100, 200, 400, 500, or 600 µg/mL of Hygromycin B. The cells were then allowed to propagate for a period of 10 days with cell counts taken every 24 or 48 hours using a hemacytometer.

2-12. Stable Transfection of PC-3 cells by the Calcium Phosphate-DNA Coprecipitation Method.

PC-3 cells were grown to about 70% confluence in 60 mm tissue culture plates (Corning #25010) with Minimum Esssential Medium containing 5% FBS. The calcium phosphate (CaPO4)-DNA coprecipitate was prepared by placing 220 µl of 0.1X TE buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 8.0; sterilized by filtration through a 0.22 micron syringe filter; storage at 4°C) containing 88 µg of either pCEP4-hAR or pCEP4 (for mock transfections) cDNA, and 250 µl 2X Hepes-Buffered Saline (HBS: 280 mM NaCl, 10 mM KCl, 1.5 mM Na2HPO4:2H2O, 12 mM dextrose, 50 mM HEPES, pH 7.05; sterilized by filtration through a 0.22 micron filter; storage at -20°C, 5 ml aliquots) in a sterile 5 ml (12 X 75 mm) plastic tube. Following this,
FIGURE 2:

- **A**
  - pGEM-3Z/hAR
  - (6.46 kb)

- **B**
  - hAR
  - (3.29 kb)

- **C**
  - pCEP4
  - (10.4 kb)

- **D**
  - pCEP4-hAR
  - (13.6 kb)
**FIGURE 2: Construction of pCEP4-hAR.** pCEP4 (C. Invitrogen) is an Epstein Barr Virus-based vector that is maintained extrachromosomally when transfected into primate cell lines. Replication is dependent on the EBV origin for plasmid replication (oriP) and the transacting nuclear antigen EBNA-1, allowing high copy episomal replication. AR cDNA contained within the plasmid pGEM-3Z/hAR [A. supplied by S. Liao, PNAS 85: 7211, 1988], was digested with BamHI and NotI, resulting in a 3.29 kb AR cDNA fragment containing both initiation and termination sites. A third restriction enzyme, AattII, was also used to cut the remaining 3.29 kb fragment (B) and allow resolution and isolation of the AR cDNA fragment. Digestion of pCEP4 at BamHI and NotI sites within the polycloning region resulted in loss of 40 base pairs and linearization of the plasmid with ends compatible with the 3.2 kb AR cDNA fragment. Ligation of the AR cDNA into the polycloning site of pCEP4 produced a 13.6 kb plasmid, pCEP4-hAR (D). The AR cDNA was inserted 3' to the constitutive CMV promoter and 5' to the SV40 polyadenylation sequence. This vector also encodes for production of a phosphatransferase which inactivates hygromycin B and confers resistance to this antibiotic.
FIGURE 3: Characterization of pCEP4-hAR by Restriction Enzyme Digestion. pCEP4-hAR was amplified in the TOP 10 strains of E. coli (Invitrogen), isolated by bacterial lysis, then subjected to digestion with BamHI (lanes 9-10), Nhel (lanes 11-12) or BamHI+Nhel (lanes 5-8). Digestions were separated by gel electrophoresis. Non-digested pCEP4-hAR (lanes 1-4) migrates slower than the top band of the DNA ladder (> 12 kb pairs). Digestion with either enzyme alone (lanes 9-12) results in a slightly faster migration than non-digested plasmid due to linearization. Digestion with both enzymes produces two fragments, corresponding to the pre-transfected plasmid pCEP4 (10.4 kb pairs) and the AR cDNA (3.2 kb pairs).
31 μl of 2M CaCl₂ (10.8 g CaCl₂·6H₂O + 20 mL ddH₂O; sterilized by filtration through a 0.22 micron filter; storage at -20°C, 1 ml aliquots) was slowly added with gentle mixing over a period of 30 seconds. Then the mixture was incubated for 20-30 minutes at room temperature to form a fine CaPO₄-DNA coprecipitate. Thereafter the precipitate was resuspended by pipetting the mixture up and down once. 0.5 ml of this suspension was then transferred to the medium above the cell monolayer and mixed by shaking the culture dish gently until the medium appeared yellow-orange and turbid. The cells were then incubated for 3 to 5 hours at 37°C in a 5% CO₂ incubator. This was followed by a 3.5 minute glycerol shock as follows: (i) medium was removed by aspiration; (ii) the monolayer was washed once with PBS; (iii) 1.5 ml 15% glycerol in 1X HBS was added and followed by incubation for 3.5 minutes at 37°C; (iv) the glycerol was gently aspirated off and the cell monolayer was washed twice with PBS; (v) fresh media was added (RPMI 1640 with 5% FBS) and the cell cultures were incubated for 48 hours before replating in media (RPMI 1640 with 5% FBS) containing hygromycin B.

At first, pCEP4-hAR or pCEP4 transfectants were selected by passaging of cells in RPMI supplemented with 5% FBS and progressively increasing concentrations of hygromycin B (5-10-25-50-100 μg/mL) over a period of 5-6 weeks. Ultimately, cells were maintained in media containing 100 μg/mL hygromycin B. Transfected cells were then examined for the expression of AR by Western blotting with reactivity to the PAR-1 rabbit anti-AR antibody and by ligand binding assays.


Two different methods for colony selection were used in these studies. The first method which involved cloning rings was used to isolate colony 2 [PC-3(AR)₂ cell line]. The remaining colonies [PC-3(AR)₃ - PC-3(AR)₁₃ cell lines] were selected via a dilution method using 96-well culture plates.

METHOD 1: COLONY ISOLATION VIA CLONING RINGS.

Heterogeneous populations of PC-3 cells transfected with the pCEP4-hAR plasmid (PC-3(AR) cells) were allowed to grow in 100 mm plates with 5% FBS RPMI 1640 containing 100 μg/ml
hygromycin B, until several colonies were formed. The culture plate containing the colonies was then washed twice with PBS. Sterile Cloning Rings (Fisher Scientific) with sterile adhesive spread evenly around their lower outer edges, were handled with sterile forceps and applied around the colonies to be harvested. Four to five drops of 0.25% trypsin was added into each ring with a sterile pipette tip. After 2 to 3 minutes, each colony was broken up by pipetting the trypsin up and down 2 to 3 times. The trypsinized cells were transferred to a single well (per colony) in a 24-well plate after washing twice with 10% FBS RPMI 1640 medium to remove residual trypsin. Some of the media from the 24-well plate was used to rinse the inside of the cloning ring to remove any residual cells and transfer back to the well. After 24 hours, each well was examined with a microscope to determine if the cells had attached. The media was replaced with fresh complete media (5% FBS RPMI 1640) in order to remove any remaining trypsin. Once the small cultures had grown to confluence they were transferred to a progressively larger culture vessel (i.e., 60 mm plates then 100 mm culture plates).

**METHOD 2: COLONY ISOLATION BY DILUTION IN 96-WELL PLATES.**

Heterogeneous populations of PC-3(AR) cells, were plated at a ratio of one cell per well in 96-well plates and allowed to propagate in 5% FBS RPMI 1640 medium containing 100 μg/ml hygromycin B, until well defined colonies were formed. Cells received fresh media every 4 to 5 days. After several weeks, several wells were examined for formation of a single colony per well. Some wells contained more than one colony and were discarded. Only wells containing a single colony of cells were considered for isolation.

Selected clones (PC-3(AR)3,4,5,6,7,8,9,10,11,12,13) were harvested from their wells with 0.25% trypsin in PBS, washed twice with 10% FBS RPMI 1640 media to remove any residual trypsin, and plated in 60 mm plates with 5% FBS RPMI 1640 containing 100 μg/ml hygromycin B. The cells were allowed to grow to confluence and replated into 100 mm plates, where they were maintained as homogeneous separate cell lines, PC-3(AR)3-PC-3(AR)13, with respect to the heterogeneous population of cells, PC-
3(AR). All cell clones were maintained in similar media, 5% FBS RPMI 1640 with 100 μg/ml hygromycin B.

2-14. **Reporter Gene Studies.**

Expression vectors for either Rous sarcoma virus (RSV) or Mouse mammary tumor virus (MMTV) promoter fused to the luciferase gene were transfected into PC-3(M), PC-3(AR), and LNCaP cells by the calcium phosphate method. After 24 hr in RPMI-1640 + 5% sFBS, the transfected cells were treated with DHT (1 nM) or hydroxyflutamide (10 μM) for 24 hr, then harvested in 0.25 ml buffer containing 50 mM Tris/MES (2-[N-morpholino]ethanesulfonic acid), pH 7.8, 1 mM dithiothreitol, and 0.1% Triton X-100. After vortexing briefly in 1.5 ml microfuge tubes, solubilized suspensions were centrifuged at 15,000 g for 3 min. The supernatant (0.2 ml) was added to a mixture of 10 mM MgCl₂, 2.5 mM luciferin and 6 nM ATP (final concentrations) and assayed for luciferase activity using a Berthold LB 9501/16 luminometer (Fisher). Luciferin¹³⁶ was used as the substrate for luciferase and to modulate luciferase activity.

2-15. **Western blot analyses**

Cells at confluence were washed twice with ice-cold PBS and lysed with 1% NP40 lysis buffer [50 mM Hepes (pH 7.25), 150 mM NaCl, 0.1 mM ZnCl₂, 2 mM EDTA, and 1% (v/v) NP40, pH 7.25] in the presence of 5 μl PMSF (phenylmethylsulphonyl fluoride) per ml of buffer. Cells were then agitated at 4°C for 15 minutes with vigorous shaking and transferred to a prechilled microfuge tube and microfuged for 15 minutes (4°C). The supernatant (whole cell extract) was collected and total protein content was determined using the BCA Protein Assay Reagent KIT (PIERCE #23225X), protocol for microtiter plate.

Immunoprecipitates were prepared using 25-35 μl of 20% Protein-A Sepharose CL-4B (in 1X Borate Buffer) per immune complex. Sepharose beads were washed 3X with 1% NP-40 Lysis Buffer and incubated with a polyclonal AR antibody, PAR-1,²⁰⁴ at 4°C on a rotary mixer for 1-16 hours and then washed 3X. Cell lysates at 1-3 mg/ml were incubated at 4°C for 1 hour with immune complex and...
then washed 3X with 1% NP-40 Lysis Buffer. Immunoprecipitates were suspended in 20 μl of Urea SDS-PAGE Buffer [62.5 mM Tris-Cl (pH 6.8), 2% SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 7M urea, and trace amounts of bromophenol blue]. Samples were then boiled for 5 minutes and stored (optional) at -20°C for no more than a week.

Fifteen microlitres of each immunoprecipitated sample was then loaded onto an 8% SDS-polyacrilamide gel. Following electrophoresis (at 150 V for 1-1.5 hours), proteins were transferred onto a Immobilon™ PVDF (MILLIPORE, Transfer Membrane for Protein/Western Blotting) nylon membrane at 20 V overnight at 4° C. The membrane was then incubated in a heat-sealable bag with TBST blocking solution [50 mM Tris-Cl (pH 7.4), 0.15 M NaCl, 0.2% (v/v) Tween 20, and 5% (m/v) powdered milk] for 1 hour, and then incubated sequentially with an affinity-purified polyclonal anti-AR antibody, PAR-1,204 for 1 hour and with 125I-labeled antirabbit immunoglobulin A (1 hour). Immunoreactive bands were visualized after autoradiography.

2-16. Androgen Receptor Ligand Binding Assay - Cytosol

Near confluent cultures in 100 X 20 mm plates (FALCON #3003) were incubated in RPMI-1640 supplemented with 5% sFBS overnight. The cells were washed twice and harvested in Dulbecco’s PBS, and centrifuged at 400 g for 5 minutes at 4°C. The cells were then suspended in 1.5 ml TEGDMo buffer (10 mM Tris-HCl, 1.5 mM EDTA, 10% (v/v) glycerol, 0.1 mM dithiothreitol, and 40 mM sodium molybdate, pH 7.4) and homogenized at 4° C using a Teflon pestle. The homogenate was centrifuged at 104,000 g (50,000 rpm’s in a TL-100 Beckman Ultracentrifuge) for 45 minutes at 4° C. Aliquots (75 μl) of the resulting supernatant (cytosol) were incubated overnight at 4° C with 50 μl of TEGDMo containing 0.1-12 nM of [3H]R1881 (sp. act. = 86 Ci/mmol; Dupont NEN, Boston, MA). Parallel incubations were conducted in the presence of 1 μM unlabeled R1881 to determine non-specific binding at each [3H]R1881 concentration. All incubates also contained 10 μM TA to suppress binding of [3H]R1881 to the progestin receptor.286 Bound [3H]R1881 was separated from unbound by gel filtration on 7 X 32 mm Sephadex LH-20 columns at 4° C.79 Alliquots of incubate (100 μl) were loaded onto the columns and washed into the column bed with 100 μl TEGDMo. Thirty minutes after sample
application, the macromolecular fraction containing the bound $[^3]$H]R1881 was eluted into scintillation vials with 400 µl TEGDMo. After overnight extraction into 5 ml Betacount (ICN) liquid scintillation fluid, radioactivity was quantified at 50% efficiency. A small aliquot (20 µl) of residual incubation mixture was taken at the end of the incubation period to determine the actual $[^3]$H]R1881 concentration. Cytosol protein content was determined by the dye-binding method of Bradford with bovine serum albumin used as standard. Specific binding was calculated as the difference between total binding (measured in the absence of unlabeled R1881) and non-specific binding (measured in the presence of unlabeled R1881). The resulting data were analyzed by the method of Scatchard using a computer-assisted nonlinear curve fitting method adapted to an IBM-PC microcomputer.

2-17. Androgen Receptor Ligand Binding Assay - Nuclear.

Near confluent cultures in 100 X 20 mm plates (FALCON #3003) were incubated in RPMI-1640 supplemented with 5% sFBS overnight and treated with 1 nM DHT or 0.01% ethanol vehicle for 20 minutes before harvesting. Cells were harvested as described above and homogenized in 1.5 ml N$_{11}$D buffer (1 mM KH$_2$PO$_4$, 0.32 M sucrose, 3 mM MgCl$_2$, 1 mM DTT, pH 7.4) at 4°C using a Teflon pestle. Homogenates were centrifuged at 1,000 g for 5 minutes and the supernatant was decanted and processed for cytosolic AR binding as above, except that buffer N$_{11}$D was used throughout. The cell nuclei were then purified by resuspending them in 0.4 ml N$_{11}$D buffer and adding 2.2 ml of buffer N$_{111}$D (1 mM KH$_2$PO$_4$, 2.39 M sucrose, 1 mM MgCl$_2$, 1 mM DTT, pH 6.8). The samples were mixed well, and centrifuged at 25,000 g (13,000 rpm's in a HB-4 swing bucket rotor) for 20 min at 4°C. The supernatant was discarded and the walls of the tube were dried. Nuclear bound receptors were salt-extracted by resuspending the pellets in 0.5 ml TEGDBAL buffer (TEGD buffer, 1 mM Bacitracin, 0.6 mM AEBSF, and 0.25 mM leupeptin). After 15 minutes, an equal volume of TEGDBALK$_{12}$ (TEGDBAL containing 1.2 M KCl) was then added and left for 15 to 30 minutes at 4°C with occassional mixing by vortexing. Solubilized receptor was isolated by centrifugation at 25,000 g for 10 min at 4°C. Alliquots (75 µl) of the resulting supernatant were incubated for 24 hr at 4°C with 50 µl TEGDBALK$_{12}$ containing 0.1-12 nM $[^3]$H]R1881 ± 10 µM unlabelled R1881 to determine non-specific binding at each $[^3]$H]R1881 concentration. All incubates contained 10 µM TA to suppress binding of $[^3]$H]R1881 to the progestin.
receptor. Bound [\(^{3}\text{H}\)]R1881 was separated from unbound by gel filtration on 7 X 32 mm Sephadex LH-20 columns at 4° C as described above. The resultant DNA pellet was digested in 1 ml of 0.3N KOH and DNA content was determined by the dye-binding method of Burton with calf thymus DNA used as standard. Specific binding was calculated as the difference between total binding (measured in the absence of unlabeled R1881) and non-specific binding (measured in the presence of unlabeled R1881).

2-18. Acid Phosphatase Activity.

AcP activity was measured as described by Suzuki et al. Cells (1 X 10⁵) were cultured in 24-well plates containing 1 ml RPMI-1640 supplemented with sFBS for 24 hr and then in the presence of 10 nM DHT or 0.01% ethanol vehicle for 72 hr. The cells were harvested by trypsinization, suspended in 500 ml 10 mM Tris-HCl buffer (pH 7.4), and disrupted by sonication. The samples were then centrifuged at 12,000 g for 10 min and 400 ml of the resultant supernatant were mixed with 100 ml 150 mM sodium citrate (pH 4.9) and 30 mM disodium p-nitrophenyl phosphate (dNP, SIGMA #N-4645). Following incubation at 37°C for 1 hr, 1.5 ml of 1 N NaOH were added to stop the reaction. Dephosphorylation of dNP was determined by measuring the absorbance at 405 nm. Protein content was measured in the supernatant by the method of Bradford. Results are expressed as the optical density value at 405 nm per mg protein.


Cell proliferation was determined by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma #M-2128, St. Louis, MO) reduction method of Romijn et al. Romijn et al. (1988) reported good correlations of the results obtained with the MTT-test, as compared with a thymidine incorporation assay or with direct DNA measurements. For the studies presented in this thesis, cells were plated in 200 ml of RPMI 1640 supplemented with sFBS in 96-well microtiter plates and treated as described in the Results section. At the time of assay, 30 ml of 5 mg/ml solution of MTT in RPMI 1640 were added to each well to be analyzed, under subdued lighting. Cells were
incubated for 3 to 4 hours with MTT at 37°C, after which the medium was carefully aspirated and 100 ml DMSO were added to each well. Plates were placed in a microplate shaker for 5 min and absorbance at 570 nM was determined with an ELIZA microplate reader (Molecular Devices). Wells containing medium alone served as blanks. Results are expressed as the mean ± S.E.M. of eight replicate wells.

2-20. **Cell Proliferation: Protocol for Counting Cells.**

Cells from culture dishes or flasks were resuspended in 1 mL of propagation media (e.g., 10% FBS RPMI). Fifty microlitres of this suspension were taken and added to a 12 X 75 mm glass culture tube containing either 30 µl of 0.4% Trypan Blue (GIBCO-BRL #15250-020) with 250 µl of 1X PBS or 60 µl of 0.2% Trypan Blue with 220 µl of 1X PBS, to make a final volume of 330 ul of cell suspension for counting cells. Using a Hemacytometer (VWR Scientific, .1 mm in depth) and a light microscope, cells were counted per field for 5 fields. The average number of cells per field was then obtained and the following formula was used to calculate the number of cells per ml from the original cell suspension:

\[
\text{AVERAGE # CELLS PER FIELD} \times 330 \mu l \times 10,000 \\
50 \mu l
\]

2-21. **Analysis for DNA Fragmentation - Gel Assay.**

The most striking biochemical event in apoptosis is the DNA cleavage between nucleosomes that produces fragments in multiples of approximately 185 bp. This phenomenon is most often analyzed by agarose gel electrophoresis which measures DNA fragmentation in nuclear extracts showing a typical "DNA ladder" configuration. Two different methods were used to examine the possibility of apoptosis, through DNA fragmentation, in transfected cells.

DNA FRAGMENTATION IN TOTAL GENOMIC DNA: METHOD 1.

One to five million cells (i.e., at ~80% confluence in 1 X 100 mm culture dishes) were harvested and resuspended in 20 to 30 µl of cold PBS in a 1.5 ml microtube tube. Lysis Buffer at 2X concentration [230 mM Tris, 100 mM EDTA, 16% sarcosine, pH 8.0] was added at a volume of 20 - 30 µl with 50
μg/ml Proteinase K and mixed by vortexing. This was followed by adding a drop of sterile paraffin oil to prevent from evaporation. The samples were incubated at 55°C for at least 3 hours or overnight. After adding DNase-free RNase A to a final concentration 50 μg/ml, the mixture was incubated in a 37°C water bath for another 30 minutes. The resulting DNA solution was then analyzed on a 1 - 2% agarose gel.

DIRECT ISOLATION OF FRAGMENTED DNA: METHOD 2.

Five hundred thousand to one million cells were harvested into a 1.5 ml microfuge tube. Because apoptotic cells often dissociate from the monolayer and float in the culture medium, cells were harvested in the existing culture medium. The cells were then centrifuged at 12,000 rpm for 5-10 seconds (sufficient to pellet the cells). The supernatant was discarded and cells were resuspended in 1.0 ml cold PBS. The cells were centrifuged once more and the supernatant was discarded. One hundred microliters of GT Solution [5 M guanidine thiocyanate, 0.5% sarkosyl, 25 mM sodium citrate, pH 7.0, 100 mM β-mercaptoethanol] was added and the mixture was vortexed aggressively at high speed until completely in solution. This was followed by adding 100 μl AAG Solution (7.5 M ammonium acetate, 0.8 mg/ml glycogen) and vortexed. A precipitate was formed in some samples after this step, therefore the samples were centrifuged to pellet the precipitate and the clear supernatant was transferred to a fresh tube. Ice cold 100% ethanol (300 μl) was added and the mixture was vortexed vigorously. The mixture was left to stand on ice for 30 minutes and centrifuged at 13,000 rpm for 30 minutes at 4°C. The resulting pellet was washed with ice cold 75% ethanol. The tube was then air dried by removing the ethanol and inverting the tube over a Kimwipe and the ethanol was allowed to drain completely. A sterile swab was also used (if required) to dry the edges of the tube. The DNA pellet was then solubilized in TE buffer (pH 8.0) containing 75 μg/ml DNase-free RNase A. Generally 60 μl of TE buffer was used, but if the sample did not go into solution a greater volume was used to get the sample into solution. It was necessary to pipette the solution up and down 20 to 30 times to get the DNA into solution, and the sides of the tube were washed as nearly 50% of the DNA can be located there. The
samples were then incubated at 37° C for 20 minutes to allow the RNase A to work completely and then analyzed on 1 to 2% large format agarose gel. Ethidium bromide was added to the gel solution at 0.25 µg/ml after it had cooled a bit.

2-22. **Cell Cycle Analysis (Flow Cytometry) by Propidium Iodide Staining.**

Flow cytometry (FACS Analysis) can be used to indicate the degree of DNA degradation occurring in a cell population. The DNA content was measured by staining with propidium iodide.

One to three million cells were harvested in their existing culture medium into a 15 ml conical Falcon tube and centrifuged to remove the supernatant. The cells were washed with cold PBS and fixed with 80% ethanol for 1 hour on ice. The cells were washed again in cold PBS, followed by washing in Propidium Iodide Staining Buffer [PIB: PBS, 0.12% Triton X-100, 0.12 mM EDTA]. Cells were then resuspend in PIB (~0.9 ml) containing 50 - 100 µg of DNase-free RNase A and incubated at 37°C for 30 to 45 min. Propidium Iodide (PI) was then added to the tube to give a final concentration of 50 µg/ml (stock PI solution = 1 mg/ml in PIB; aliquots were stored at -86°C in the dark). Cells were then stained in the dark for 1 to 24 hours (overnight) at room temperature. The cells were analyzed using FL-2 by gating out doublets using doublet discrimination (FL2 area vs FL2 width).
CHAPTER III

Results

3-1. *Viability of PC-3 cells in Media Containing Hygromycin B.*

In order to determine if non-transfected PC-3 cells were viable in medium containing hygromycin B, their growth was analyzed in the presence of various concentrations of this aminocyclitol antibiotic (Figure 4). At all concentrations tested (50 - 600 µg/ml in Figure 4A and 0 - 10 µg/ml in Figure 4B), PC-3 cell growth was effectively hindered (Two way ANOVA: \( F_{(30,84)} = 268.4 \) in Figure 4A, \( F_{(24,105)} = 685.4 \) in Figure 4B; \( P < 0.0001 \)).

3-2. **Expression of Androgen Receptor in Transfected PC-3 Cells.**

PC-3 cells transfected with the expression vector pCEP4-hAR that contained a constitutively active AR cDNA (PC-3(AR) cells) were selected in medium containing 100 µg/ml hygromycin B. PC-3 cells transfected with the expression vector pCEP4 (mock transfections) and containing no constitutively active AR cDNA (PC-3(M) cells) were also selected in medium containing 100 µg/ml hygromycin B. In the initial attempt to transflect androgen-independent PC-3 cells with the full-length human AR cDNA, a new cell line (PC-3(AR')) cells) was developed that expressed an apparent shorter isoform of the AR protein (see Appendix).

On repeating the transfection using the same AR cDNA sequence (from the same batch of isolated DNA) into the PC-3 parent cell line, PC-3(AR) cells, were produced that constitutively expressed an AR immunoreactive protein migrating at approximately 110 kDa (Figure 5). This protein migrated at the same rate as the AR from LNCaP cells (Figure 5) and was absent in the mock-transfected PC-3(M) cells (Figure 5) and in ordinary PC-3 cells (Figure 5).
FIGURE 4: Viability of PC-3 Cells Grown in the Presence of the Aminocyclitol Antibiotic Hygromycin B, the Selective Agent for Transfectants. Cells were propagated in 24-well culture plates with 5% FBS RPMI 1640 media and supplemented with various concentrations of hygromycin B. Cells were effectively hindered from growth by 24 hours (panel A), $p < 0.05$ (Student-Newman-Keuls Test) for all treatments except for * (not statistically different) with respect to cells not treated with hygromycin B. When a lower dose range was used, cells were significantly hindered from growth by 4 days (panel B) except at *.
FIGURE 5: Western Blot Analysis of Heterogeneous Population of AR-Transfected PC-3 Cells. Cell lysates were immunoprecipitated with 1 µg/ml PAR-I antibody. Immunoprecipitates were separated on a 8% SDS-PAGE, transferred to Immobilon PVDF membrane (Millipore), probed with 1 µg/ml PAR-I antibody (Figure 3) and visualized after probing with 100 nCi/ml 125I-Protein A. No immunoreactive species were observed in PC-3 cells transfected with pCEP4 (mock transfections). Transfection of the parent PC-3 cell line with the full-length AR cDNA resulted in a new cell line, PC-3(AR) cells that expressed an immunoreactive protein migrating with an apparent molecular weight (~110 kDa) similar to the native wild-type AR (110 kDa) found in LNCaP cells.

3-3. Variable Expression of Androgen Receptor in Clonally Selected pCEP4-hAR Transfectants.

The expression of a full-length AR in selected clonal cell lines of PC-3(AR) cells (PC-3(AR)2-PC-3(AR)13) was demonstrated by both a binding assay using the synthetic androgen [3H]R1881 as ligand (Table 2) and by a Western blot (Figure 6) analysis. The concentration of AR produced in the selected colonies was determined by saturation binding analysis performed on cytosol protein extracts using [3H]R1881 as ligand. A saturable, single class androgen binding site of high affinity (Kd = 0.27 to 0.54 nM) was detected in these clones (Table 2). The colonies exhibited varying concentrations of AR ranging from 273 to 1621 fmol/mg cytosol protein, which bracketed the level of receptor binding measured in LNCaP cells (935 fmol/mg cytosol protein). No [3H]R1881 binding was detected in cytosol extracts from mock-transfected PC-3(M), ordinary PC-3 cells, or clones PC-3(AR)3, PC-3(AR)4, PC-3(AR)7, PC-3(AR)9, and PC-3(AR)11. These findings were confirmed by western blot analysis using the polyclonal anti-human AR antibody PAR-I (Figure 6).
TABLE 2: Saturation Binding Analysis of AR Binding in Cytosol Extracts of Clonally Selected PC-3 Cells Transfected with pCEP4-hAR. Cells were grown in RPMI-1640 + sFBS, harvested, and cytosol extracts were incubated overnight at 4°C with a range of [3H]R1881 concentrations (0.1 - 12.0 nM) in the presence or absence of 1 mM unlabeled R1881. Data were analyzed by the method of Scatchard using a computer-assisted least squares curve fitting method (LIGAND). The error indicated for $B_{\text{max}}$ values represent the S.D. estimate provided by LIGAND. Note that receptor content as determined by binding assay appeared to be relatively stable as binding was similar in cells with a 15 passage difference (*).

<table>
<thead>
<tr>
<th>Clonal Line</th>
<th>$K_d$ (nM)</th>
<th>$B_{\text{max}}$ (fmol/mg cytosol protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNCaP</td>
<td>0.244</td>
<td>935.0 ± 26.7</td>
</tr>
<tr>
<td>PC-3(M)</td>
<td>-</td>
<td>N.D.</td>
</tr>
<tr>
<td>PC-3(AR)$_3$</td>
<td>-</td>
<td>N.D.</td>
</tr>
<tr>
<td>PC-3(AR)$_4$</td>
<td>-</td>
<td>N.D.</td>
</tr>
<tr>
<td>PC-3(AR)$_7$</td>
<td>-</td>
<td>N.D.</td>
</tr>
<tr>
<td>PC-3(AR)$_9$</td>
<td>-</td>
<td>N.D.</td>
</tr>
<tr>
<td>PC-3(AR)$_{13}$</td>
<td>0.545</td>
<td>273.0 ± 16.9</td>
</tr>
<tr>
<td>PC-3(AR)$_5$</td>
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<td>464.8 ± 31.7</td>
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<tr>
<td>PC-3(AR)$_2$</td>
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<td>491.4 ± 22.3</td>
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<tr>
<td>PC-3(AR)$_2^*$</td>
<td>0.267</td>
<td>331.3 ± 27.3</td>
</tr>
<tr>
<td>PC-3(AR)$_{12}$</td>
<td>0.461</td>
<td>666.1 ± 78.6</td>
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<tr>
<td>PC-3(AR)$_6$</td>
<td>0.528</td>
<td>886.1 ± 58.2</td>
</tr>
<tr>
<td>PC-3(AR)$_8$</td>
<td>0.392</td>
<td>1138.4 ± 53.4</td>
</tr>
<tr>
<td>PC-3(AR)$_{10}$</td>
<td>0.356</td>
<td>1621.0 ± 90.6</td>
</tr>
</tbody>
</table>

*These cells were harvested at 15 passages earlier than PC-3(AR)$_2$.
FIGURE 6: Western Blot Analysis of Clonally Selected pCEP4-hAR Transfectants. Cell lysates were immunoprecipitated with PAR-1 antibody. Immunoprecipitates were separated by SDS-PAGE, transferred to Immobilon PVDF membrane (Millipore), probed with 1μg/ml PAR-1 antibody then visualized after probing with 100 nCi/ml $^{125}$I-Protein A. Immunoblot analysis of transfected PC-3(AR) clones, mock-transfected PC-3(M), and untransfected PC-3 cells were compared with the positive control cell line LNCaP. No immunoreactive species was observed in PC-3 cells transfected with pCEP4 [mock transfections, PC-3(M)] while an immunoreactive protein similar to the native 110 kDa AR in LNCaP was expressed and observed in some PC-3(AR) clones. Each lane was loaded with 15 μl of 2 mg/ml cellular protein that was immunoprecipitated with 1.5 μg/ml of PAR-1 antibody. The numbers on the left indicate the positions and sizes of prestained mol. wt. Markers (GIBCO-BRL, Burlington, ON) run in the same gel.
3-4. **Effect of Triamcinolone Acetonide on Androgen Receptor Binding**

All incubates contained 10 μM triamcinolone acetonide (TA) in saturation binding assays, to suppress binding of \[^{3}H\]R1881 to progestin receptor.\(^{286}\) Although PC-3 cells do not express PR,\(^{19}\) TA was included in the results possibility that androgen sensitivity could affect PR expression. Furthermore, at the time of initiating these assays we did not know if they expressed PR or not. Although LNCaP cells have also been shown to lack expression of PR,\(^{19}\) it was also necessary to determine if the AR in LNCaP cells was capable of binding this glucocorticoid (TA) because of its single point mutation in the binding domain.\(^{262,263}\) It was speculated that this mutation may affect its steroid binding characteristics in the presence of TA. TA was capable of blocking out \[^{3}H\]R1881 binding sites in cytosol incubates from transfected PC-3 clonal cells by 50% at a concentration of 100 μM, but not at 10 μM (used in the binding assays) or less (Figure 7C). In contrast, TA at 10 μM decreased \[^{3}H\]R1881 binding in LNCaP cells by 50% (Figure 7C). Therefore TA was excluded from scatchard binding assays for LNCaP cells (TABLE 2).
FIGURE 7: Effect of Triamcinolone Acetonide on Androgen Receptor Binding in LNCaP Cells. Cells were treated with RPMI media in the presence of sFBS for 48 h prior to harvesting. LNCaP cytosol was prepared and analyzed for [³H]R1881 binding both in the absence of TA (panel A) and in the presence of 10 µM TA (panel B). In the presence of TA there was a decrease in AR binding affinity and a decrease in binding capacity, while there was an increase in binding affinity and an increase in binding capacity in the absence of TA. The AR in LNCaP cells exhibited a 10-fold increase in binding affinity for TA than the AR in PC-3(AR)₈ cells (panel C). This suggests that 10 µM TA, the concentration used to block binding of [³H]R1881 to progesterin receptor in binding assays, is capable of suppressing [³H]R1881 binding in LNCaP cell cytosol.

* P < 0.05 against that of LNCaP cells in the absence of TA (paired t-test, p = 0.0473, n = 2)
† P < 0.05 against that of LNCaP cells treated with 0, 0.1, 1, and 10 µM, respectively (t-test, p < 0.05, n = 4)
3-5. **Nuclear binding of Transfected Androgen Receptor.**

The ability of DHT treatment to increase cell nuclear binding of transfected AR was assessed in PC-3(AR)2 cells. Cells were grown in RPMI 1640 with 5% sFBS and were treated with 1 nM DHT or the ethanol vehicle 20 min before harvest. [3H]R1881 binding in extracts from purified cell nuclei was assessed by saturation binding analysis. DHT treatment resulted in depletion of receptor binding in the cytosol compartment and a marked increase in binding in the nuclear compartment (Figure 8). A significant amount of androgen binding was detected in nuclear extracts from vehicle-treated cells.

DHT treatment resulted in depletion of binding in the cytosol and a decrease of receptor binding from 186.77 fmol/mg protein to non-detectable levels (Figure 8A, B). Furthermore, DHT increased cell nuclear receptor levels from 1020.4 fmol/mg DNA to 4990.8 fmol/mg DNA (Figure 8C, D). Therefore the expressed AR appears viable in that it is capable of binding both ligand and cell nuclear components, presumably androgen response elements.
FIGURE 8: Nuclear Translocation of Androgen Receptor. PC-3(AR) cells (colony 2) were grown in media containing charcoal stripped FBS then treated for 20 minutes with 1 nM DHT. Cytosol and nuclear extracts were prepared and [³H]R1881 binding was analyzed. DHT treatment resulted in a decrease in receptor levels measured in the cytosolic fraction (panel A and B) while an increase in receptor levels was observed in the nuclear fraction (panels C and D), suggesting increased chromatin bound receptor in response to DHT exposure.
3-6. Androgen Induced Expression of MMTV-Luciferase.

To assess the ability of the transfected AR to regulate gene transcription through a known androgen response element (ARE), PC-3(AR)\textsubscript{2} cells were transiently transfected with the reporter gene construct MMTV-luciferase. The MMTV promoter is androgen sensitive has been shown to contain an androgen response element.\textsuperscript{59,120,209} Cells were treated with 0.01 - 10 nM DHT 24 hr before measuring luciferase activity. PC-3(M) cells transfected with MMTV-luciferase served as controls. Only low levels of luciferase activity were detected in these cells.

DHT treatment of transfected PC-3(AR)\textsubscript{2} cells resulted in a dose-dependent increase in luciferase activity (Figure 9). Even in the absence of DHT treatment, an approximate 3-fold increase in luciferase above that measured in PC-3(M) cells was measured. To test the specificity of the androgen response, luciferase activity in PC-3(AR)\textsubscript{2} cells transiently transfected with MMTV-luciferase (Figure 10A, B) were compared to cells transiently transfected with RSV-luciferase (Figure 10C, D), following treatment with 1.0 nM DHT and/or 10 \textmu M hydroxyflutamide (OH-FLUT). The RSV promoter is not androgen sensitive and lacks an ARE. PC-3(AR)\textsubscript{2} cells transfected with MMTV-luciferase exhibited a 11-fold increase in luciferase activity (Figure 10B) compared to PC-3(M) cells (Figure 10A) in the absence of DHT treatment. DHT induced a further 113-fold increase in activity in the PC-3(AR)\textsubscript{2} cells (Figure 10B), but was without effect in PC-3(M) cells... (Figure 10A). When given alone, the AR antagonist OH-FLUT did not alter luciferase activity (Figure 10B); however, the DHT-induced increase in luciferase activity in PC-3(AR)\textsubscript{2} cells was largely attenuated in cells treated concurrently with DHT and OH-FLUT (Figure 10B). High levels of luciferase activity were measured in cells transfected with RSV-luciferase. However, no effect of either DHT or OH-FLUT was observed in cells transfected with RSV-luciferase (Figure 10C, D).
FIGURE 9: DHT-Induced Expression of MMTV-Luciferase Reporter Construct. PC-3(AR) cells (colony 2) were grown in medium containing 5% sFBS, and then transiently transfected with MMTV-luciferase. Cells were treated with various concentrations of DHT, lysed, and luciferase activity was measured. Results are expressed as a fold activation above control (luciferase activity in pCEP4 mock transfected cells). In the absence of added DHT, a 3.68-fold increase in luciferase activity was observed. DHT further increased luciferase activity in a dose-dependent manner.
FIGURE 10: Hydroxyflutamide Blockade of DHT-Induced Expression of MMTV-Luciferase Reporter Construct and Promoter Specificity. PC-3(AR) cells (colony 2) growing in media containing sFBS, were transiently transfected with MMTV-Luciferase (panels A and B) or RSV-Luciferase (panels C and D). Cells were lysed after 24 hours treatment with ethanol vehicle or 1.0 nM DHT +/- 10 μM hydroxyflutamide (OH-FLUT). Supplemental OH-FLUT was given 12 hr before harvesting. Ethanol vehicle was given to all cultures when not scheduled for hormone/drug treatment. Luciferase activity is expressed as a fold-increase above that measured in control PC-3(M) cells. In the absence of added DHT, a 10-fold increase in luciferase activity was observed. Bars represent the mean ± s.e.m. of 4-7 determinations. Two-way ANOVA revealed a statistically significant DHT (F_{1,26} = 55.6, P<0.0001) and OH-FLUT (F_{1,26} = 23.9, p<0.0001) effect, and a significant interaction of DHT and OH-FLUT (F_{1,26} = 25.6, p<0.0001) in PC-3(AR)_{2} cells transfected with MMTV-luciferase. No statistically significant treatment comparisons were found in PC-3(M) cells, or in PC-3(AR)_{2} cells transfected with RSV-luciferase. Within each panel, treatment groups that are statistically indistinguishable, as established by Student-Newman-Keuls test, are denoted by a similar letter above the bars (p<0.05).
3-7. Acid Phosphatase Activity.

To determine if AR transfected cell express an acid phosphatase (AcP) that is androgen regulated, AcP activity was compared in PC-3(M) cells and PC-3(AR) cells. PC-3 and PC-3(M) cells showed similar AcP activity in the presence and absence of DHT (TABLE 3). Although both PC-3(AR)_{2} and PC-3(AR)_{10} showed almost identical AcP activity to that of PC-3 cells in the absence of DHT, DHT significantly decreased AcP activity by 36% and 38% in PC-3(AR)_{2} and PC-3(AR)_{10} cells, respectively (TABLE 3). However, as shown in TABLE 3, DHT did not statistically significantly increase AcP activity in LNCaP cells, although a higher value was attained for DHT-treated cells than for vehicle-treated cells.

No acid phosphatase activity was detected in the culture medium from PC-3, PC-3(M) or PC-3(AR) cells in the absence or presence of DHT.

TABLE 3: Acid Phosphatase Activity in Androgen Receptor-Transfected PC-3 Cells. Cells were grown in RPMI 1640 containing 5% sFBS (10% sFBS was used for LNCaP cells) and supplemented with either 0.01% ethanol vehicle or 10 nM DHT. After 96 hours cells were harvested and disrupted by sonication. Cell lysates were centrifuged and samples of supernatant were incubated at 37°C for 1 hour with 30 mM sodium citrate (pH 4.9) and 30 mM disodium p-nitrophenyl phosphate. The reaction was stopped in the presence of 0.75 N NaOH (final concentration) and acid phosphatase activity was measured spectrophotometrically at 405 nm. Results are represented as absorbance per mg protein.

<table>
<thead>
<tr>
<th>CELL LINES</th>
<th>ETHANOL VEHICLE</th>
<th>5α-DIHYDROTESTOSTERONE</th>
</tr>
</thead>
<tbody>
<tr>
<td>†PC-3</td>
<td>100 ± 9.36</td>
<td>96.70 ± 6.18</td>
</tr>
<tr>
<td>†PC-3(M)</td>
<td>82.58 ± 3.90</td>
<td>96.09 ± 6.44</td>
</tr>
<tr>
<td>†PC-3(AR)_{2}</td>
<td>99.57 ± 7.70</td>
<td>64.57 ± 5.87*</td>
</tr>
<tr>
<td>†PC-3(AR)_{10}</td>
<td>99.09 ± 7.63</td>
<td>62.15 ± 5.55*</td>
</tr>
<tr>
<td>‡LNCaP</td>
<td>100 ± 12.80</td>
<td>126.68 ± 11.36</td>
</tr>
</tbody>
</table>

†Data are shown as MEAN ± SEM of percentage to acid phosphatase activity of PC-3 cells in the absence of DHT (13.146 ± 0.967 per mg protein as p-nitrophenol measured at 405 nm absorption) based on 8 independent samples.
‡Data are shown as MEAN ± SEM of percentage to acid phosphatase activity of LNCaP cells in the absence of DHT (27.041 ± 1.708 per mg protein as p-nitrophenol measured at 405 nm absorption) based on 8 independent samples.
*P < 0.05 against that of PC-3 cells in the absence of DHT (One way ANOVA: F_{(9,72)} = 5.70, P < 0.0001; Student-Newman-Keuls Multiple Comparison Method).
3-8. Proliferation of PC-3(AR) Cells:

In order to compare the cell proliferation rates between mock- and AR-transfected PC-3 cells (Figure 11), the tetrazolium salt MTT was employed. Based on the enzymatic reduction of this reagent by mitochondrial enzymes present in metabolically active cells to a purple-colored formazan soluble in dimethylsulfoxide (DMSO), O.D. 570 nm values were colorimetrically read using an ELIZA reader. In the absence of androgen the growth of PC-3(AR)2 cells was comparable to that of PC-3(M) cells (Figure 11). However, the growth of PC-3(AR)10 was significantly slower by day 3 and 4, as compared to both PC-3(M) and PC-3(AR)2. This difference disappeared by day 7 (168 hours), as all three cell lines seemed to reach a mutual growth rate with respect to one another.

The effect of androgen treatment on cell proliferation was examined in three clonal lines of pCEP4-hAR transfected PC-3 cells, expressing low [PC-3(AR)13], moderate [PC-3(AR)2], and high [PC-3(AR)10] levels of AR. Cells were grown in RPMI-1640 medium containing 5% FBS and 0.1 nM to 1.0 nM DHT; cells grown in the absence of DHT were treated with the 0.01% ethanol vehicle. Cell proliferation was determined daily for 3-5 days using the MTT reduction assay method. DHT treatment of mock transfected, PC-3(M) cells did not influence their growth rate (Figure 12A). However, DHT treatment caused a marked decrease in the growth of all three AR transfected PC-3 cell lines (Figure 12). This reduction was observed at all DHT dose levels examined. Furthermore, androgen-induced growth inhibition in PC-3(AR) cell lines was shown to occur in a dose-dependent fashion.
FIGURE 11: Proliferation of PC-3(AR) Cells. Twenty-five hundred PC-3(M), PC-3(AR)$_2$ or PC-3(AR)$_{10}$ cell lines were plated in 96-well culture plates with 5% sFBS RPMI 1640 media and allowed to proliferate for a period up to 7 days. Bars with similar letters are statistically similar with respect to time (hours). Bars with different letters, with respect to time, are statistically significantly different (One way ANOVA: $F_{119} = 183.0$, $P < 0.001$; Student-Newman-Keuls Multiple Comparison Test, $p < 0.05$).
FIGURE 12: Androgen Induced Growth Inhibition of PC-3(AR) Cells as Measured by MTT Reduction Assay. PC-3(AR) cells expressing low (panel B, PC-3(AR) Lo, 5,000 cells plated initially), moderate (panel C, PC-3(AR) M, 2,500 cells plated initially) and high (panel D, PC-3(AR) H, 5,000 cells plated initially) levels of AR, were growth inhibited in the presence of various concentrations (10^-10 M to 10^-6 M) of DHT (Two way ANOVA: F(15,151) = 6.472 in panel B, F(10,126) = 9.517 in panel C, F(20,204) = 8.462 in panel D; P < 0.0001). There was no significant effect of treatment in mock transfected cells (panel A, 2,500 PC-3(M) cells plated initially; Two way ANOVA: F(4,5) = 1.6758, P = 0.271). All AR-transfected cells were significantly inhibited by at least 48 hours. Furthermore, this inhibition generally occurred in a dose-dependent manner with respect to increasing concentrations of DHT. All bars with similar letters over them indicate no significant differences (P > 0.05, Duncan's Multiple Range Test) while different letters indicate significant effect due to DHT (P < 0.05, Duncan's Multiple Range Test). Each cell line was analyzed individually. Cell medium was changed after 3 days in all cultures. Note that cells in panels A and C also received 0.1% ethanol vehicle, and cells in panels B and D received 0.01% ethanol vehicle, as a final concentration.

To determine if AR transfected cells can recover from androgen-induced growth inhibition, PC-3(AR)_{13} (Figure 13A), PC-3(AR)_{2} (Figure 13B), and PC-3(AR)_{10} (Figure 13C) cell lines were grown in the presence of 1.0 nM DHT for three days. On the third day, the media was removed and replaced with RPMI 1640 + sFBS (i.e., no DHT). Control PC-3(AR) cultures of each colony received only the 0.01% ethanol vehicle. Cell proliferation was assessed daily by MTT reduction until four days after DHT withdrawal. In the continued absence of DHT treatment (vehicle treated control cells), PC-3(AR) cell lines proliferated at a steady rate for the duration of the experiment (Figure 13). In the continued presence of DHT treatment, PC-3(AR) cell lines were attenuated from proliferating at a steady rate for the duration of the experiment (Figure 13) with cell growth reaching approximately 50% of vehicle treated control cells. Following the withdrawal of DHT at 72 hr, cell proliferation as assessed by MTT reduction, increased at 120 hr and this trend continued slowly for the next 3 days until DHT was replaced at day 7 (168 hours). However, the DHT withdrawal between 72 hours and 168 hours did not allow the androgen induced inhibited cells to recover completely, as they were still proliferating slower than the control cells in the absence of DHT. This was clearly observed in PC-3(AR)_{2} and in PC-3(AR)_{10} cell lines but not in the low AR expressing cell line, PC-3(AR)_{13} (Figure 13). After replacement of DHT at 168 hours, all three cell lines were again growth inhibited for the next 4 days in the presence of DHT.

In comparison, LNCaP cells were growth stimulated in the presence of continuous DHT treatment and proliferated at an increasing steady rate for the duration of the experiment (Figure 14). Their growth rate was attenuated in the continuous absence of DHT treatment and proliferated at a slower steady rate. DHT withdrawal after 72 hours of treatment resulted in a decreased growth rate in comparison to LNCaP cells continuously treated with DHT for the duration of the experiment. Furthermore, this proliferation was not hindered to the same level as with the continuous absence of DHT treated LNCaP cells.
FIGURE 13:
FIGURE 13: Effect of Androgen Withdrawal on the Growth of PC-3(AR) Cells I. Twenty-five hundred cells were initially seeded in 96-well plates and grown in RPMI 1640 with 5% sFBS, and supplemented with either 0.01% ethanol vehicle (for 0 nM DHT treatment, n = 8 wells) or 1 nM DHT (n = 24) for 72 hours. At 72 hours the medium was aspirated off, cells washed once with RPMI 1640 containing 5% sFBS (to remove any residual DHT), and all media were freshly replaced. Cells treated with 1 nM DHT for the initial 72 hour period were split into two groups: one group received a similar treatment of 1 nM DHT (n = 8) and the other group received media containing 0.01% ethanol vehicle (1-0 nM DHT group, n = 16). Cells were grown for a further 96 hours and medium changed once again at this point. A group of cells from the 1-0 nM DHT group received medium containing 1 nM DHT (1-0-1 nM DHT group, n = 8). All cells were then grown for a final 96 hours. MTT Reduction Assays were carried out every 24 hours to determine amount of reduced dye at O.D.070. Data are shown as MEAN ± SEM of reduced MTT from 3 different clones of viable PC-3(AR) cells: cells expressing a low level of AR (PC-3(AR)$_{13}$ in panel A), a moderate level of AR (PC-3(AR)$_{22}$, panel B), and a high level of AR (PC-3(AR)$_{10}$, panel C). All data were analyzed using One way ANOVA ($F_{31} = 3.4903$ in panel A, $F_{31} = 0.1589$ in panel B, $F_{31} = 4.0047$ in panel C) followed by Duncan’s Multiple Range Test:

* P < 0.05 ( ● 1 nM DHT group against that of PC-3(AR) cells in the absence of DHT, ○○○); † P < 0.05 ( ○ 1-0 nM DHT group against that of PC-3(AR) cells in the absence of DHT, •••); ‡ P < 0.05 ( • 1-0-1 nM DHT group against that of PC-3(AR) cells in the absence of DHT, ○○○); § P < 0.05 ( □ 1-0 nM DHT group against that of PC-3(AR) cells in the presence of DHT, ●●●); ¶ P < 0.05 ( © 1-0-1 nM DHT group against that of PC-3(AR) cells in the presence of DHT, ●●●); £ P < 0.05 ( ℃ 1-0-1 nM DHT group against that of ■ 1-0 nM DHT group of cells).
FIGURE 14: Effect of Androgen Withdrawal on the Growth of LNCaP Cells. Twenty-five hundred cells were initially seed in 96-well plates and grown in RPMI 1640 with 10% sFBS and supplemented with either 0.01% ethanol vehicle (for 0 nM DHT treatment, n = 8 wells) or 1 nM DHT (n = 16) for 72 hours. Thereafter media was aspirated off, cells were washed once with RPMI 1640 containing 10% sFBS to remove any residual DHT, and media was freshly replaced. Cells treated with 1 nM DHT for this period were split into two groups: one group received a similar treatment of 1 nM DHT (n = 8) and the other group received media containing 0.01% ethanol vehicle (1-0 nM DHT, n = 8). Cells were then allowed to proliferate for a final 96 hours. MTT reduction assays were carried out every 24 hours to determine the amount of reduced dye at O.D. 570. Data are shown as MEAN ± SEM of reduced MTT from viable cells. All data were analyzed using One way ANOVA followed by Duncan’s Multiple Range Test:

* P < 0.05 ( ■ 1 nM DHT group against that in the absence of DHT, ○ 0 nM DHT).
† P < 0.05 ( ○ 1-0 nM DHT group against that in the absence of DHT, ○ 0 nM DHT).
‡ P < 0.05 ( ○ 1-0 nM DHT group against that in the presence of DHT, ■ 1 nM DHT).
3-10. Androgen Induced Cell Death of PC-3(AR) Cells.

To further examine this paradoxical growth inhibition in the presence of DHT, the effect of 1.0 nM DHT on growth of PC-3(AR)\textsubscript{13} and PC-3(AR)\textsubscript{2} cells, which express low and moderate levels of AR respectively, were monitored for 15 days in culture (Figure 15). Medium was replaced every 3 days in all cultures with DHT removed after 3, 6 or 9 days and replaced with steroid-free medium. By 15 days of androgen treatment, both cell lines experienced growth reduction in the continued presence of DHT; PC-3(AR)\textsubscript{2} cells progressed to this state more rapidly than PC-3(AR)\textsubscript{13} cells. Moreover, there were no PC-3(AR)\textsubscript{2} cells remaining after 15 days of continuous treatment. PC-3(AR)\textsubscript{2} cells were rescued from this apparent cell death when the DHT was withdrawn after 3 days of DHT exposure (Figures 13 and 15), but not after 6 or 9 days (Figure 15). After 6 days of DHT treatment, these cells appeared destined to die. PC-3(AR)\textsubscript{13} cells, in comparison, could be rescued after 6 days and perhaps after 9 days of exposure.

DNA cell cycle analysis of cells treated with DHT, ethanol-fixed, and stained with propidium iodide, demonstrated an increase in the proportion of cells at G\textsubscript{1} and a decrease in cells at S and G\textsubscript{2}M. Upon DHT exposure, a greater proportion of cells were found at G\textsubscript{1} than vehicle-treated control cells (Figure 16). This was accompanied by a decrease in the proportion of cells in the S phase and G\textsubscript{2}M. By 6 days of DHT exposure, the proportion of cells in G\textsubscript{1} had returned to control levels with a concomitant increase in the hypodiploid state (designated A\textsubscript{0}). Removal of DHT after 3 days of exposure resulted in a marked increase in the proportion of cells in the S phase and G\textsubscript{2}M phase as determined 3 days later (day 6). The removal of DHT at this time also prevented the increase in cells at A\textsubscript{0}. Removal of DHT after 6 days of treatment did not shift the cell cycle profiles appreciably from that observed with continued DHT exposure. Despite the fact that 200,000 cells were originally plated, only 3,000 cells could be recovered and sampled after 9 days of DHT followed by 3 days of vehicle (a minimum of 10,000 cells
FIGURE 15: Effect of Androgen Withdrawal on the Growth of PC-3(AR) Cells II. Effect of 1.0 nM DHT on the growth of low AR expressing PC-3(AR)$_{13}$ and moderate AR expressing PC-3(AR)$_{2}$ in RPMI 1640 medium containing stripped fetal bovine serum. DHT was removed after 3, 6, or 9 days and replaced with steroid-free medium (RPMI 1640 + stripped fetal bovine serum). Vehicle control cells received 0.1% ethanol vehicle and DHT cells were maintained in 1.0 nM DHT for the duration of the experiment. Cell medium was changed every 3 days in all cultures. The dotted lines indicate initial number of cells.
FIGURE 16: FACS Analysis. Summary of DNA cell cycle analysis performed on PC-3(AR)2 cells treated with 1 nM DHT for up to 9 days, or treated with DHT for 3, 6, or 9 followed by 3 days in the steroid-free medium. Control PC-3(AR)2 cells were treated with the ethanol vehicle. Cell medium was changed every 3 days. Note that $A_0$ is the quantifiable cell cycle region below $G_0/G_1$ and has been previously shown to represent cells undergoing apoptosis-associated DNA degradation\textsuperscript{254}.
should be sampled for cell cycle analysis), which together with the increase in cells found in $A_0$ suggest that massive cell death occurs in these cells after prolonged exposure to DHT.


There was no dramatic change in the morphology of PC-3(AR) cells as compared with PC-3(M) cells (Figure 17) due to the transfection process itself. Both cell lines were epithelial-like in appearance (Figure 17A, C, and E). Following DHT treatment, the morphology of PC-3(AR) cell lines was dramatically altered while that of PC-3(M) cells remained unchanged. Treated PC-3(AR) cells exhibited a marked increase in size consistent with swelling (Figure 17D, and E). Furthermore, some cells appear to be undergoing what seems to be consistent with or resembling membrane blebbing (Figure 17D) with vacuole formations.


Androgen withdrawal has been shown to result in decreased proliferation and programmed cell death or apoptosis in androgen-sensitive prostate cancer cells. DNA fragmentation has long been recognized as a hallmark for apoptosis caused by the activation of a calcium-dependent nuclear endonuclease. Because PC-3(AR) cells respond in an opposite manner to DHT (i.e., with a decrease in cell proliferation or apparent cell death), the possibility that DHT exposure triggers apoptosis in these cells was examined.

PC-3(AR)$_2$, PC-3(AR)$_{10}$, and PC-3(M) cells were treated with RPMI 1640 medium supplemented with 5% stripped FBS and 1 nM DHT for 24, 48, and 72 h periods. PC-3 cells treated with thapsigargin were used as a control. Using the two different methods described in Chapter II (Materials and Methods, section 2-21) to detect fragmented DNA on agarose gels, neither cell line exhibited signs of apoptosis with any of the treatments assigned, including thapsigargin-treated PC-3 control cells.
FIGURE 17:
FIGURE 17: Androgen-Induced Morphological Changes in PC-3(AR) Cells. PC-3(AR)₂ cells (panels C and D), PC-3(AR)₁₀ cells (panels E and F), and PC-3(M) cells (panels A and B) were grown for 3 days in RPMI 1640 containing 5% stripped FBS in the presence (panels B, D, and F) or absence (panels A, C, and E) of 10 nM DHT. mb, membrane blebbing; v, vacuole formations; n, nucleus; bar = 10 μm.
CHAPTER IV

Discussion

In this thesis, transfection of the pCEP4-hAR expression vector system into the androgen-independent cell line PC-3 resulted in the development of a new cell line, PC-3(AR). These cells, unlike the parental cells were highly resistant to the aminocyclitol antibiotic hygromycin B (100 μg/ml), and cells remained viable in its presence. This resistance was comparable to PC-3(M) cells, transfected with the pCEP4 expression vector lacking the AR cDNA insert. Because hygromycin B was highly toxic to normal PC-3 cells (Figure 4), this initial finding was used as a preliminary confirmation for the presence of the constitutive expression vectors, pCEP4 and pCEP4-hAR, in their respective cell lines.

The transfected AR was observed in both heterogeneous and selected clonal populations of PC-3(AR) cells, as evidenced by western blot analysis (Figures 5 and 6, respectively). Its molecular weight was comparable to the AR expressed in LNCaP cells. However, not all of the selected PC-3(AR) clonal lines expressed the AR as was demonstrated by both binding assays (TABLE 3) and the western blot in Figure 6. Furthermore, these clones were still resistant to hygromycin B in their media which indicated that they were at least transfected with the plasmid pCEP4-hAR that gave them this resistance, but for unknown reasons failed to express AR protein. Upon treatment with DHT, there was no significant effect on the proliferation of these cells. The mechanism underlying the lack of AR protein expression in these cells is currently unknown. Further analyses of these cells are required to determine if the vector contained in these cells is actually the pCEP4-hAR plasmid and if the proper AR cDNA sequence is in place. Nevertheless, 7 out of 12 clonal lines selected expressed the full-length AR.

The AR transfected into PC-3 cells was shown to be a viable receptor by binding assays (Figure 8) of nuclear and cytosolic cell extract. DHT treatment resulted in increased levels of nuclear bound receptor within the nuclear compartment (Figure 8). This shows the receptor is capable of binding androgen as well as nuclear components, presumably to AREs, i.e. it is a viable receptor. A significant amount of nuclear bound AR was observed in the absence of DHT treatment. This level of binding could
reflect residual DHT in the medium, however, a vigorous stripping method was employed to remove such DHT but the level could reflect residual bound receptor as the cells were transferred to sFBS medium only 48 hours before assay. Alternatively, the nuclear bound receptors could represent receptors that were trapped in the cell nuclei during cellular compartmentalization. Another possibility is that this level of nuclear binding represents ligand-independent activation of the transfected AR. In the absence of DHT, the level of receptors in the cytosol was significantly lower than that reported in TABLE 3. This discrepancy may be due to the different buffer systems used. In this particular experiment, our primary objective was to examine the ability of DHT to result in increased nuclear binding; therefore, the buffers were chosen for nuclear isolation rather than the preservation of cytosol receptors and hence sodium molybdate was absent in the N11D buffer.

The transfected AR appears fully functional, as evidenced by DHT-induced expression of an MMTV-luciferase reporter construct (Figure 9) and by inhibition of this expression in the presence of the antiandrogen hydroxyflutamide (OH-FLUT, Figure 10). A 4 to 10-fold increase in luciferase activity was observed in the absence of DHT which was not inhibited by OH-FLUT, suggesting i) the possibility of ligand-independent activation of the AR, or ii) a low level activation of MMTV through an additional mechanism (e.g. glucocorticoid receptor). Recently it has been shown that nuclear receptors possess evolutionary conserved motifs for constitutive transcriptional activation of genes through autonomous activation function (AFs) that are capable of modulating ligand-independent activation present in the N-terminus (AF-1 region) and ligand-dependent activation in the C-terminus (AF-2 region).5,178 Furthermore, the AF-2 region has been identified as being able to mediate both transactivation as well as transcriptional interference ("squelching") with other transcription factors.5 For example, the human ER has been shown to undergo ligand- and ligand-independent activation.235 Therefore, the AR in transfected PC-3 cells may have the ability to activate AR-dependent transcription of a target gene in a ligand-independent manner.
The DHT effect on luciferase expression is promoter specific. DHT increased luciferase activity only with presence of the MMTV promoter which contains a ARE element. No luciferase activity was induced by DHT from the reporter gene construct RSV-luciferase, which lacks AREs. These results demonstrate that, the effects of DHT were not due to changes in luciferase stability and can be assumed to be due to action at the promoter level. However, DHT-induced luciferase activity was not completely blocked by the presence of excess OH-FLUT (10 μM). This may be due to rapid metabolism of OH-FLUT. During these experiments, OH-FLUT had to be replenished every 12 hours (i.e., given twice daily) in order to maintain a blockade of androgen activity. Nevertheless, hydroxyflutamide acted as a true antiandrogen since it lacked agonist activity and was an inhibitor of androgen-induced transcriptional activation. Altogether, these studies demonstrate that the AR transfected into PC-3 cells, is viable and functional, and capable of specifically targeting hormone response elements in the genome.

In this thesis, it was identified that conferring androgen sensitivity to an androgen insensitive human prostate cancer cell line, by the introduction of a full length human AR cDNA, resulted in a profound stunting of 50-60% cell proliferation after 72 hours of incubation with exogenous androgen. Furthermore, continuous treatment with DHT beyond this time point resulted in an apparent time-dependent androgen induced cell arrest (Figure 12 and 13) or cell death (Figure 15). In PC-3(AR)2 cells, expressing moderate levels of functional AR, a critical time point was reached between 3 and 6 days of DHT exposure where DHT seemed to trigger a response that resulted in the cells apparent death (Figure 15), perhaps through a process that includes cellular differentiation. It is likely that this also occurs in PC-3(AR)3 cells that express low levels of AR, but perhaps with a longer time course of DHT exposure. These findings were unlike steroid-sensitive prostate cancer cells, such as LNCaP cells that typically respond to steroid hormone treatment with increased cell proliferation and survival.36,37

The inability of DHT removal to reverse this effect in the PC-3(AR)2 clone may be indicative of DHT-induced terminal differentiation, culminating in cell death. Removal of DHT after 6 days of treatment resulted in a decreased proportion of cells in G1 phase and this was accompanied by an
increase in a subG₁ population. Removal of the DHT after 3 days of treatment prevented this increase in the subG₁ population and instead resulted in a large shift in the proportion of cells from G₁ to S and G₂M. These effects were not observed when DHT was removed after 6 days of exposure as an increasing number of cells were approaching an apparent cell death state. These data are somewhat consistent with those obtained by Novichenko et al.¹⁸⁵ Within hours of exposure to TPA, JCA-1 cell proliferation decreased and reached approximately 80% reduction after 3 days with a blocking of cells entering into the replicating S and G₂M phases from G₁ phase.¹⁸⁵ These TPA-induced cells had a reduced growth rate but remained viable, much in the same way as did PC-3(AR) cells with respect to 3 days of DHT treatment. Interestingly, the growth-modulating activity of TPA was also observed with LNCaP cells.¹⁸⁵ Agarose gel electrophoresis of the chromosomal DNA from TPA treated cells exhibited a non-fragmented pattern,¹⁸⁵ as was observed in our DHT treated PC-3(AR) cells. Furthermore, in parallel to growth reduction, DHT-treated PC-3(AR) cells became larger in size presumably by swelling (Figure 17D and F), which is indicative of necrotic death, as did the TPA induced JCA-1 cells.¹⁸⁵ However, these two findings do not exclude the possibility of DHT-induced apoptosis of PC-3(AR) cells. There is insufficient evidence at the present to conclude that such an event might be occurring in these cells. Although the cell cycle analysis data leads one to speculate about apoptosis, analysis of DNA fragmentation on agarose gels was inconclusive. Nevertheless, these results indicate an induced growth reduction accompanied by a time-dependent cellular differentiation of prostatic cancer cells: JCA-1 cells by TPA¹⁸⁵ and PC-3(AR) cells by DHT (this thesis). The androgen-induced changes in morphology, growth, and cell cycle phases may suggest that establishing androgen responsiveness in PC-3 cells results in a decreased malignant state of this cell line.

Recently it was demonstrated that 0.1-100 nM dexamethasone and 0.5-50 ng/ml TGF-β₁ inhibited PC-3 cell proliferation and arrested cell progression at the G₁/G₀ phase of the cell cycle.²⁰⁹ This was shown to be mediated by the presence of glucocorticoid receptor (GR) in PC-3 cells that mediated the expression of TGF-β₁ in a ligand-dependent manner. This dexamethasone inhibition of PC-
3 cells was blocked by polyclonal anti-TGF-β1 antibodies. In our lab, we also showed, through ligand-binding assays, that the GR in PC-3 cells is down-regulated by the presence of the AR (Dr. Lawrence E. Heister, unpublished data). In clones exhibiting higher levels of AR (e.g., PC-3(AR)10) lower levels of GR were measured than in cells expressing lower levels of AR.

In the absence of DHT treatment, the growth of PC-3(M) and PC-3(AR)2 cells (Figure 11) is comparable. PC-3(AR)10 cells, in contrast, exhibited a slightly slower growth response by 72 and 96 hours of incubation, but eventually approached a similar growth rate to PC-3(M) and PC-3(AR)2 cells by 168 hours. These studies were carried out in RPMI 1640 media containing 5% sFBS and may reflect an early inhibitory response in PC-3(AR)10 cells due to minute levels of androgen remaining in charcoal-stripped FBS. Because the PC-3(AR)10 clonal line exhibits a higher level of AR content than normal, these cells may be more sensitive to lower levels of androgen than PC-3(AR)2 cells. These cells, perhaps with time, can escape this minor inhibitory response to residual androgens and approach a more normal proliferatory response. On the other hand this study may be reflecting some of the problems associated with the MTT assay. It has been noted that the MTT reduction rate can be affected by metabolic and other factors (culture age, cell line specificity, pH, and cellular and medium glucose levels) that may influence the quantitation of cell viability. Thus in the PC-3(AR)10 clone a high number of plasmid copies (reflective of high AR content) may be altering its cellular metabolism of MTT, resulting in differed growth pattern. However this may not be problem with these studies as the overall proliferation rate of each cell line can still be measured and compared; differences in growth was observed only with DHT-treatment of AR-transfected cells but not in mock cells.

The results demonstrated in this thesis and by others are in conflict with those reported by Hansen et al., showing that stable transfection of the human AR into PC-3 cells resulted in a reduced growth rate in androgen-free media (i.e., 5% or 10% charcoal-stripped serum (CSM) media or ITS Plus, a serum-free media additive, without androgen). This finding however, can be criticized on the basis of how these cells were analyzed. Cells were grown in media with DHT (10 nM) for 9 days (with
media changed every two days) at which point the media was changed to CSM or ITS Plus without DHT.

After 3 days, cell numbers were significantly lower for not only transfected cell lines, but also for LNCaP cells. However, this does not agree with previous studies showing LNCaP cells to be androgen-sensitive but not necessarily androgen-dependent. Furthermore, the significance of the transfected PC-3 cells reported by Hansen et al.90 cannot be ascertained at this time, from the data presented in their paper. The level of AR being expressed in these cells has not been quantified. Further, no attempt has been made to fully characterize the expression of the transfected AR in these cells. Therefore, the level of AR being expressed in these cells is still questionable. It is theoretically possible however, to select clones of cells expressing even lower levels of AR than has been reported in this thesis, that could respond to androgen in a positive manner, that is representing a return to an androgen-sensitive phenotype.

Kushner et al.125 have reported that in ER-transfected hamster ovarian cells that overexpress the ER, estrogen treatment induces cell lysis, presumably by inducing apoptosis. This has also been observed in ER-negative breast cancer cell lines transfected with the ER receptor gene.107,109,285 Levenson and Jordan135 attribute this phenomenon to transcription factors that interact with overexpressed ER to cause the negative effects of estrogen indirectly ("squelching") or to altered expression of target genes that cause growth inhibition. By coupling with other transcription factors, high expression of ERs may lessen the expression of growth promoting genes. If this were true, then one would hypothesize that different effects would be observed at low levels of receptor expression.

The data obtained in this thesis indicate that a different mechanism was responsible for the decreased proliferation of PC-3(AR) cells. The AR gene transfected into PC-3 cells was not overexpressed despite being controlled by a strong promoter, CMV. Ligand binding assays indicated that the amount of receptor produced in several different clones of these cells, bracketed that produced by androgen-responsive LNCaP226 and ALVA-41182 cells. However, these levels were higher than that reported for PC-82 human prostate androgen-dependent cells (120 fmol/mg protein)14 and higher than that reported for human ventral prostate tissue (26 fmol/mg protein)206 albeit this tissue contains many
non target cells. The different levels of AR expression in PC-3(AR) cell lines may be a consequence resulting from different number of plasmid copies obtained by different transfectants. However, the low level of AR expression in PC-3(AR) cells cannot be attributed to selection against transfectant cell lines expressing high levels of AR by growth inhibition even in the absence of exogenous androgen. This is reflected in the PC-3(AR)\textsubscript{10} clone, which expresses almost twice as much AR levels as found in LNCaP or ALVA-41 cells, and still survives in media in the absence of exogenous androgen.

Several of the hypotheses offered to explain the similar action of estradiol in ER-negative human breast cancer cell lines transfected with ER cDNA\textsuperscript{107,109,135} are based on the work by Kushner \textit{et al.,}\textsuperscript{125} but cannot be supported by this thesis. It has long been speculated that transfection has caused genetic rearrangements resulting in the expression of cytotoxic or growth inhibitory genes. However, given the wide range of cell lines examined it is difficult to conceive that these rearrangements are encountered so consistently. The present study involved an episomal expression vector that is thought not to incorporate into the genome. Thus in PC-3(AR) cells, genetic rearrangements should not have occurred. However, in practice, the possibility of incorporation cannot be completely excluded.

Kushner \textit{et al.}\textsuperscript{125} have also hypothesized that one possible explanation for the toxic effects of estrogen is that the ER is present in such abundance that it activates (or possibly inhibits) endogenous gene expression. This was observed with reporter genes lacking an estrogen response element (ERE) transfected into high ER expressing cell lines. Reporter gene expression was inhibited in cells expressing high levels of ER but not when transfected into cell lines with lower receptor levels.\textsuperscript{271} In the present studies however, there was no activation or inhibition, by DHT, of the luciferase reporter genes including that of RSV-luciferase, transiently transfected into the PC-3(AR)\textsubscript{2}. These data, therefore, indicate that a general effect upon gene expression is not occurring.

In ER-negative breast cancer cells transfected with the ER, overexpression of activated ER has been theorized to be involved in the cross-coupling of components involved in transcriptional activation of a wide scale of genes (referred to as a “squelching” effect).\textsuperscript{132,149,220} Expression of high levels of
receptor might shift this response such that inhibitory effects might be seen at lower dose levels.109,125 In the prostate carcinoma cell line LNCaP, a biphasic effect on proliferation has been reported only in the presence of retinoic acid.70 However, in terms of the AR transfections in PC-3 cells of this thesis, one clonal cell line was obtained that expressed high levels of functional receptor, PC-3(AR)10. In this clonal line, no biphasic effect was observed with the range DHT treatment used (0.1 nM to 1000 nM). All concentrations were inhibitory towards this cell line. However, no effects were observed at lower concentrations (10^-12 - 10^-15 nM) (Dr. Lawrence E. Heisler, unpublished data).

Another possibility for steroid-induced toxicity reported in breast cancer studies, is that estrogen-bound receptor binds widely to DNA and may physically interfere with some fundamental process, such as DNA replication. This is not likely the case in the present studies as growth inhibition with DHT was observed in cells expressing AR at levels below that measured in LNCaP and ALVA-41 cells.

Notwithstanding, the data obtained in this thesis are consistent with those obtained in similar studies on estrogen-regulated growth of estrogen-insensitive breast cancer cells. Jiang and Jordan137,109 recently introduced the human ER gene and selected mutations into estrogen-receptor negative breast cancer cells and noted the effect of estrogen on cell growth. As with the transfected cells of this thesis, exposure to steroid resulted in a marked decrease in proliferation. The extension of these findings to human prostate cancer cells raises the possibility that this may be a general property of steroid-insensitive neoplastic cells found in steroid target organs. Furthermore, the inhibitory effect of DHT on PC-3(AR) cells may be extended to breast cancer cells as well. Birrell et al.10 have recently shown that two AR-positive cell lines, T47-D and ZR-75-1 were growth inhibited in the presence of 1 nM DHT, while the proliferation of the AR-positive cell lines MCF-7 and MDA-MB-453 was increased. However, in this latter study it was concluded that androgen action in breast cancer cell lines may not be solely mediated by binding of androgen to the AR, and that metabolites of DHT with estrogenic activity, or androgen binding to receptors other than the AR may explain the divergent responses to androgens in different AR+ breast cancer cell lines.
A potential and critically important problem is the possibility that the androgen-induced inhibition observed in PC-3(AR) cells may be reflective of the episomal vector system used. pCEP4 is theorized to replicate independently of genomic DNA by utilizing host cellular factors and the protein EBNA-1 that is encoded for by the vector itself. Under certain conditions, the receptor could replicate out of synch with respect to the host cell’s genome, leading to overexpression of the inserted cDNA. Thus, DHT-induced inhibition of cell growth might not halt replication of the vector construct, leading to overexpression of the AR. Cell death could ensure essentially as described by Kushner with respect to ER overexpression in breast cancer cells or perhaps due to increased production of EBNA-1 protein, which can be toxic to cells when expressed in great abundance. Alternatively, as cells are growth inhibited by DHT, they may eject the episomal vector and with it, resistance to hygromycin. Cell death then may occur as a result of hygromycin toxicity as the growth studies were performed in the presence of hygromycin. These possibilities can be easily tested and these experiments are in progress. AR binding assays performed in cells exposed to DHT-induced growth expression will reveal if AR levels become overexpressed or if the cell ejects the vector (decrease in AR levels). Growth studies performed in the absence of hygromycin are also being conducted.

The progression of androgen-independent cells in patients undergoing androgen ablation therapy is unknown. These cells may have lost their sensitivity to androgen or were already androgen-insensitive from the start of a primary tumor and progressed only after the withdrawal of androgen or treatment with antihormonal therapy, to a malignant metastatic stage. Then of course the question remains as to why these cells proliferate in the absence of androgen. One possible alternative mechanism of proliferation is the EGF/TGF-α autocrine or paracrine loop to the epidermal growth factor receptor signaling pathway. As mentioned in the Introduction, it has been reported that PC-3 cells transfected with a vector containing the cDNA coding for the full length human PAcP, exhibited a diminished cellular growth rate. PC-3 cells are normally known not to express endogenous PAcP. In this thesis PAcP expression was examined in PC-3(AR) cells. It was hypothesized that perhaps androgen treatment may
upregulate PAcP expression, leading to dephosphorylation of the EGF-R, thereby causing slowed growth. DHT failed to increase PAcP activity in PC-3 and PC-3(M) cells whereas a 27% increase was detected in LNCaP cells. In androgen treated PC-3(A') cells there was a decrease in AcP activity as compared to vehicle treated cells. A possible modification to these experiments is that the conventional inhibitor of PAcP, L-(-)-tartrate, should have been used to distinguish PAcP from AcP. The L-(-)-tartrate-sensitive acid phosphatase (AcP) activity is normally taken to represent PAcP activity. From this, no definite conclusions regarding PAcP itself can be reached at this time until the proper control experiments are included. However, it is possible that in PC-3(AR) cells, decreased AcP activity may play a role in observed growth inhibition of these cells.

Further comparisons of these cells, PC-3(AR) cells, and androgen-dependent cell lines (LNCaP, ALVA-41, ALVA-101) are required to identify factors contributing to the differences in androgen-dependent and androgen-independent cell growth. The EGF-R remains an important factor to look at and expression of EGF-R in these cells should be compared to the parental cell line. Furthermore, although PC-3(AR) cells apparently undergoing cell death were recognized in a region of the cell cycle phase previously shown for cells undergoing apoptosis-associated DNA degradation (Figure 16), a second method is required to confirm androgen-induced apoptosis in these cells. One approach is to look at expression of members of the bcl-2 family such as Bcl-2, Mcl-2 and Bax. These homologs of bcl-2 play a key role in regulating physiologic cell death (apoptosis). For example, bcl-2 is a protooncogene whose protein product plays an important role in inhibiting apoptosis; the bax gene encodes a protein product involved in the induction of apoptosis. When Bax is overexpressed it can counteract the death repressing activity of Bcl-2 by forming homodimers. If Bcl-2 is dominantly expressed over that of Bax, it can heterodimerize with Bax and repress its apoptotic death activity. Therefore if DHT induced cell death of PC-3(AR) cells is a stimulus for an apoptotic pathway, then evaluating the ratio of Bcl-2 to Bax should be of critically importance in its identification.
It is possible that PC-3(AR) cells may reflect one of the population of cell types present in a heterogeneous prostate tumor. If so, androgen ablation therapy may inadvertently be causing these cells to proliferate, resulting in tumor progression along with androgen-insensitive cells and AR mutation-containing cells that do not require androgen for their growth. This idea has been previously speculated by Yuan et al.\textsuperscript{284} and leads to the conclusion that new treatment strategies are required for patients suffering from this disease. Furthermore, it is hopeful that different strategies developed for either prostate cancer or breast cancer treatment can be applicable to one another with respect to sex steroid hormones.

Recently, Westin et al.\textsuperscript{272} demonstrated that castration followed by a period of estradiol treatment was more effective in inducing epithelial cell apoptosis and a reduction in the total tumor volume in the androgen-sensitive rat prostatic adenocarcinoma (Dunning R3327PAP tumor model) subcutaneously inoculated into male rats. With the potential advent of PC-3(AR) cells, a treatment similar to this can be adopted for use in \textit{in vivo} models. For example, PC-3(AR) cells can be transplanted into castrated male “nude” mice for a period of time, and then followed by exposure to androgens to determine if there is an inhibition in tumor size. Similarly, these findings can be followed by a gene therapy model design in which human AR cDNA can be introduced into transplanted PC-3 cells in “nude” mice.

Kleinerman et al.\textsuperscript{116} have developed a potential prostate cancer gene therapy model using an adenoviral infection system to which PC-3 cells are very sensitive. They used this system to infect PC-3 cells with a tumor suppressor cell adhesion molecule (C-CAM1) and found that in a single dose of adenovirally infected cells they were able to repress the growth of PC-3 cells transplanted into “nude” mice. It is potentially possible that androgen induced PC-3(AR) cells are growth inhibited in a similar manner. That is, C-CAM1 or other cell adhesion molecules could be differently expressed in these cells following androgen treatment.
Another possible mechanism could involve the expression of activity of the enzyme telomerase. Telomeres are essential for chromosome stability and replication: maintaining a balance between shortening and lengthening is essential for cell viability. The synthesis of DNA and maintenance of these structures at chromosome ends are due to telomerase activity, and may be necessary for indefinite proliferation of human cells. Telomerase appears to be highly repressed in normal human somatic cells but reactivated in cancer, leading to a group of immortal cells that ultimately result in uncontrolled tumor growth. Telomerase appears to be reactivated in essentially all human cancers. Counter et al. have detected this enzyme in a variety of immortal human cells transformed by different viruses, including the Epstein-Barr Virus, that indicated the activation of telomerase activity may be a common step in immortalization of cells as they transform to a malignant state. In our lab it has been shown that PC-3 cells express very high levels of telomerase. It would be of great interest to determine if PC-3(AR) cells express lower levels of this enzyme activity when they are treated with androgen. It is possible that conferring androgen sensitivity in these cells, and exposing them to androgens, causes them to redifferentiate back to a normal somatic state.

In summary, the PC-3(AR) cell lines established and characterized as part of this thesis should serve as useful models to assess androgen action in prostate cancer cell lines. Given the paradoxical growth response to DHT stimulation and possibly DHT-induced death, work in these cell lines may establish a viable approach to the treatment of prostate cancer. Perhaps introduction of AR cDNA, through gene therapy mechanisms, into androgen insensitive cells followed by treatment with DHT or removal of androgen ablation drugs may result in slowed progression or even regression of the androgen-insensitive tumor. The data presented at least question the wisdom of the currently accepted treatment of Prostate Cancer -- that is complete androgen ablation therapy, and suggests that cyclical androgen withdrawal may be a preferable treatment approach.
APPENDIX

Initial transfections of PC-3 cells resulted in the development of a new cell line, PC-3(AR') cells that were also growth inhibited in the presence of various concentrations of the androgen 5α-dihydrotestosterone in their media (Figure 20A). These cells expressed an immunoreactive protein (detected using the PAR-1 Antibody201) migrating at 69 kDa (Figure 18). This low molecular weight isoform was later analyzed by immunoprecipitation of cell lysate with the polyclonal antibody PG-21 to confirm if this was a N-terminal truncated form of the AR (Figure 19), that is the 87 kDa AR that was previously detected in genital skin fibroblasts by Wilson and McPhaul.275

This lower molecular weight form of the AR was stably expressed in PC-3(AR') cells, and has been partially characterized. Using Saturation binding assays, no binding activity was detected in these cells with [3H]R1881 as the ligand. Furthermore, there was no androgen induced activation of the MMTV-luciferase reporter gene construct transiently transfected in PC-3(AR') cells. However, in a second growth curve study (performed “blind” by another observer), these cells were significantly growth inhibited by 96 hours in the presence of 10 nM DHT (Figure 20B).
FIGURE 18: Western Blot Analysis of the Androgen Receptor Expressed in PC-3(AR') cells. Cell lysates were immunoprecipitated with the 1 μg/ml PAR-1 antibody and separated on an 8% SDS-PAGE gel, transferred to Immobilon PVDF membrane (Millipore), probed with 1 μg/ml PAR-1 antibody and then visualized after probing with 100 nCi/ml 125I-Protein A. In the initial transfection of the full length androgen receptor cDNA in the parent PC-3 cell line resulted in the development of a new cell line, PC-3(AR'), that expressed an immunoreactive protein migrating with an apparent molecular weight of 69 kDa. Note that a similar band of approximately the same size seems to be present in another androgen-independent prostate cancer cell line, DU-145.

FIGURE 19: Western Blot Analysis Using PG-21 Antibody. Cell lysates from PC-3(AR') cells were immunoprecipitated with the PAR-1 antibody (1 μg/ml), separated by SDS-PAGE and transferred to Immobilon PVDF membrane (Millipore). The membrane was then probed with 2 μg/ml PG-21 [gift of Dr. Gail Prins, Chicago, IL202], an antibody directed against the first 21 amino acids of the N-terminal domain of the androgen receptor. The presence of an immunoreactive species in the PC-(AR') immunoprecipitate suggests that this low molecular weight variant is not due to an N-terminal truncation of the receptor.
FIGURE 20: Apparent Androgen-Induced Growth Inhibition of PC-3(AR') Cells. Cells were grown in RPMI media supplemented with 5% sFBS and various concentrations of the androgen 5α-DHT. Initially, straight cell counts using a hemacytometer by this author resulted in the observation of an androgen induced and dose-dependent inhibition of PC-3(AR') cells by 72 hours (panel A, 10000 cells plated initially). Later, in a “blind” test experiment by another observer to verify the results observed in panel A, resulted in the observation of cellular growth inhibition by 96 hours in the presence of 10 nM DHT (panel B, 20000 cells plated initially). Note that there exists no statistical significant interaction between time and treatment periods (Two way ANOVA: $F_{(10,36)} = 2.02, P = 0.060$ in panel A; $F_{(2,17)} = 1.78, P = 0.199$ in panel B).

* $P < 0.05$ against that in the absence of DHT for the same time point (Student-Newman-Keuls Test)
The precise nature of this form of the AR is currently unknown. However, it has been speculated that it may be an artefact of the initial transfection process in PC-3 cells. The AR cDNA sequence could have somehow become mutated in its introduction into the parent cell line allowing for the possibility of introducing a premature stop codon in the AR cDNA resulting in the observed effects. The fact that PG-21 is capable of detecting this protein coupled with the lack of androgen binding and MMTV luciferase activity (i.e., MMTV activation) indicates that this mutated AR lacks at least a significant portion of the ligand binding domain. Calculations to determine the exact size of this receptor, based on its apparent 69 kDa migration on 8% SDS-PAGE, indicate that it is a protein of approximately 621 amino acids or 1863 base pairs (i.e., 1 kb = 333 a.a. = 37 kDa molecular weight\(^1\)). It's known that the full-length AR consists of 917 amino acid residues with residues 1-555 coding for the transcriptional binding domain (N-terminus), 556-623 for the DNA binding domain, 624-665 for the hinge region, and 666-917 for the C-terminus hormone binding domain.\(^70,2^{58}\) Therefore an AR protein of 621 amino acids in length and having an intact N-terminus would indicate that it is missing approximately 296 amino acids or 888 base pairs from the C-terminus end. Thus the entire ligand binding domain and hinge region of the AR cDNA sequence as well as the last 2 amino acids of the DNA binding domain are not expressed (Figure 21). This would indicate that PC-3(AR') cells are constitutively expressing a AR protein that is still capable of binding DNA components.

This might be agreeable with the finding by Lobaccaro et al.\(^1^{50}\) that a 13 base pair deletion within exon 4 of the AR resulted in a frameshift in the open reading frame, yielding a premature stop codon at position 783 (of the ligand-binding domain) instead of 919, and encoding a 94 kDa protein. This mutated AR was incapable of binding androgens but was still able to bind target DNA without being able to transactivate a reporter gene. In PC-3(AR') cells, perhaps a X-base pair deletion in exon 1 or 2 of the transfected AR may also have resulted in a frameshift in the open reading frame leading to a premature stop codon in the DNA-binding domain, and the production of an apparently 69 kDa mutated AR protein. In fact, a single base-pair deletion could lead to the introduction of a stop codon near the
end of the DNA-binding domain (see Figure 21) that will result in the production of a 621 a.a. protein (see Figure 22). In future studies, single-strand conformation polymorphism analysis\textsuperscript{150} and sequencing of the AR cDNA from PC-3(AR') cells should be able to confirm this hypothesis.

Another possibility for production of the mutated AR in PC-3(AR') cells is a nonsense point mutation in the DNA-binding domain of the transfected AR cDNA. This would result in the formation of a premature terminal signal codon (TGA) causing the translation of the cDNA to cease.

Interestingly however, Marcelli et al.\textsuperscript{158} transfected into PC-3 cells a C-terminal truncated AR cDNA sequence, lacking 792 3'-terminal nucleotides. Although no androgen binding was detected in these cells (PC3-ARCA), they did express a transcriptionally active protein that constitutively induced transcription of the MMTV-CAT reporter gene construct.\textsuperscript{162} This stimulation was observed in the absence as well as in the presence (48 hours) of 2 nM DHT, when added to medium containing 5% charcoal-stripped serum.

If the 69 kDa band observed in PC-3(AR') cells lacks the AR binding region, the results obtained in our growth studies appear inconsistent. One possibility is that the DHT effect on growth are not exerted through the AR. This is unlikely as DHT is without effect in mock-transfected PC-3 cells. Moreover reporter genes were not affected by DHT treatment in mock-transfected cells. At present we attribute these findings as artefactual. Future studies may eventually provide sufficient insight to render these results interpretable.
FIGURE 21:

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FIGURE 21: Androgen Receptor cDNA Sequence. This cDNA sequence was adopted from Chang et al.\textsuperscript{35} The cDNA sequence coding for the N-terminal transactivating domain is given in normal font followed by the sequence for the DNA-binding domain (underlined), hinge region (CAPITALIZED font), and the C-terminal ligand binding domain (italicized font). Together, these four regions code for the full length (917 amino acids) androgen receptor protein. \texttt{arg} is the premature start codon leading to the production of the 87 kDa androgen receptor isoform. \texttt{gggatgact} could possibly represent the site for the premature truncation of the full-length AR and leading to a 621 amino acid AR protein being expressed in PC-3(AR') cells. A premature stop codon (\texttt{tga}) could be introduced at this site by a single base pair deletion of exon 1 or 2 resulting in a frameshift and causing the production of this mutated AR.
FIGURE 22:

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FIGURE 22: Androgen Receptor Amino Acid Sequence. In bold font is the amino acid sequence for the N-terminal (transactivating) domain of the AR, with the DNA binding domain sequence underlined, hinge region CAPITALIZED and the C-terminal ligand binding domain is italicized. It is precluded that the hinge region and ligand binding domains are the truncated segments from the full-length AR that lead to the production of a AR isoform (estimated to be 621 amino acids in length) being expressed in PC-3(AR') cells. If this is true then gly^{621} could represent the final residue in this isoform. (amino acid sequence adopted from Chang et al. \textsuperscript{33}).
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