ANDROGEN RECEPTOR EXPRESSION IN HUMAN PROSTATE CANCER CELL LINES

by

Vassiliki Samara

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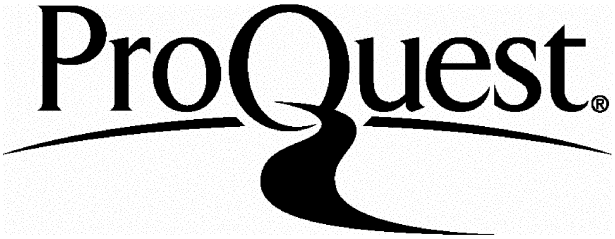

ABSTRACT

Androgens are essential for the growth and differentiation of the prostate. Androgen signalling is mediated by the androgen receptor (AR), a ligand-dependent transcription factor. Androgen ablation is the standard treatment for prostate cancer but nearly all patients relapse with androgen-independent disease. Progression of prostate cancer is often associated with changes in the AR-signalling pathway.

The project aimed to investigate molecular mechanisms underlying the regulation of AR gene expression in hormone-relapsed prostate cancer. An inducible and a constitutive gene expression system were used to overexpress AR in human prostate cancer cell lines.

The TetOff™ inducible gene expression system, which offers the advantage of quantitatively regulating AR gene expression in response to varying concentrations of tetracycline, was used. A highly tTA-expressing stable cell line (DUTetOff) was established in the AR-negative DU145 cell line, and a functional AR expression vector (pTRE-AR) was constructed. Transient assays with pTRE-AR in DUTetOff, DU145, PC-3, DUSF and COS-1 cells indicated that, while AR mRNA was expressed in all cells tested, the AR transcript was not translated in DUTetOff and DU145 cells.

To develop a constitutive AR gene expression system, the full-length human AR cDNA was introduced into a DU145-derived serum-free subline (termed DUSF). Stable clones were screened for AR expression by immunocytochemistry, Western analysis and RT-PCR. Up-regulation of AR mRNA and protein was detected in DUSF transfectants following androgen treatment. Endogenous PSA mRNA expression was observed in untransfected DUSF cells, while androgen treatment of the transfectants implied an AR- and androgen-independent mechanism for PSA regulation.

The work described in this thesis indicates that overexpression of AR in AR-negative cells permits androgen-mediated AR gene expression, and implies an alternative mechanism for PSA activation. The stable AR-expressing DUSF cells provide a useful model system for investigating androgen-independent regulatory elements involved in PSA gene regulation and, elucidating the mechanisms involved in prostate cancer progression.

To my parents,

Γιωργο και Κατινα

for all they have done for me over the years

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In the lab....

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The most exciting phrase to hear in science,
the one that heralds new discoveries,
is not "Eureka" but "That's funny..."

Isaac Asimov

LIST OF ABBREVIATIONS

aa	- amino acid(s)
Ab	- antibody
ABC	- avidin-biotin complex
ACTH	- adrenocorticotrophic hormone
AIS	- androgen insensitivity syndrome
Amp	- ampicillin
AMPS	- ammonium persulfate
AR	- androgen receptor
ARE	- androgen response element
bp	- base pairs
BPH	- benign prostatic hyperplasia
BSA	- bovine serum albumin
°C	- degrees Celsius
cAMP	- adenosine 3′, 5′-cyclic-monophosphate
cDNA	- complementary (to RNA) deoxyribonucleic acid
CIAP	- calf intestinal alkaline phosphatase
CMV	- cytomegalovirus
COX-1	- cyclooxygenase-1
CRTH	- corticotrophin-releasing hormone
DAB	- 3,3′-diaminobenzidine
DBD	- DNA-binding domain
DCC-FBS	- dextran-coated charcoal-treated fetal bovine serum
DEPC	- diethylpyrocarbonate
DHEA	- dehydroepiandrosterone
DHEAS	- dehydroepiandrosterone sulfate
DHT	- 5α-dihydrotestosterone
DMEM	- Dulbecco's modified Eagle medium
DMSO	- dimethylsulphoxide
DNA	- deoxyribonucleic acid
Dox	- doxycycline
DRE	- digital rectal examination
dsDNA	- double-stranded DNA
DTT	- dithiothreitol
DUSF	- DU145 serum-free
ECL	- enhanced chemiluminescence
E.coli	- Escherichia coli bacterium
EDTA	- ethylenediaminetetraacetic acid
EGF	- epidermal growth factor
ER	- estrogen receptor
EtBr	- ethidium bromide
FBS	- fetal bovine serum
FGF	- fibroblast growth factor
GR	- glucocorticoid receptor
HRP	- horseradish peroxidase
hsp	- heat shock protein
ICC	- immunocytochemistry

IGF-I	- insulin-like growth factor I
IL-6	- interleukin-6
kb	- kilobase pairs
kDa	- kilodalton
KGF	- keratinocyte growth factor
LA	- Luria agar
LB	- Luria broth
LBD	- ligand-binding domain
LH	- luteinising hormone
LHRH	- luteinising hormone-releasing hormone
MAB	- maximal androgen blockade
Mb	- megabase pairs
mg	- milligrams
µg	- micrograms
ml	- millilitres
µl	- microlitres
mM	- millimolar
µM	- micromolar
MRI	- magnetic resonance imaging
mRNA	- messenger ribonucleic acid
MW	- molecular weight
neo	- neomycin
ng	- nanograms
NLS	- nuclear localisation signal
nm	- nanometres
NTD	- amino-terminal domain
OD	- optical density
PAGE	- polyacrylamide gel electrophoresis
PAP	- prostatic acid phosphatase
PBS	- phosphate buffer saline
PCR	- polymerase chain reaction
PIN	- prostatic intraepithelial neoplasia
PKA	- protein kinase A
PMSF	- phenylmethanesulphonyl fluoride
PR	- progesterone receptor
PSA	- prostate-specific antigen
PSMA	- prostate-specific membrane antigen
RNA	- ribonucleic acid
rpm	- revolutions per minute
RPMI	- Roswell Park Memorial Institute medium
RT	- room temperature
SBMA	- X-linked spinal and bulbar muscular dystrophy
SDS	- sodium dodecyl sulfate
SEM	- standard error of the mean
ssDNA	- single-stranded DNA
SV40	- simian virus 40
TAE	- tris acetate EDTA buffer
TBE	- tris borate EDTA buffer

Tc	- tetracycline
TE	- tris EDTA
TEMED	- N, N, N', N'-tetramethylethylene diamine
TNM	- tumour node metastasis
TRE	- tetracycline-responsive element
Tris	- tris - (hydroxymethyl) - aminoethane
Triton X-100	- octyl phenoxy polyethoxyethanol
TRUS	- trans-rectal ultrasound
Tween 20	- polyoxyethylene-sorbitan monolaurate
UTR	- untranslated region
UV	- ultra-violet radiation
V	- volts
VDR	- vitamin D receptor
v/v	- volume per unit volume
w/v	- weight per unit volume

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Chapter 1

Introduction

PART I *PROSTATE GLAND*

1.1 The anatomy and development of the prostate gland

1.1.1 Anatomy

The prostate gland is about the size and shape of a walnut and surrounds the male urethra at its origin from the bladder (Figure 1.1). It is composed of numerous tubuloalveolar glands which vary in size. The glands produce and store a secretion that drains into the prostatic urethra. The prostatic secretion together with sperm forms the semen. The secretion is a thin, milky, slightly alkaline liquid that aids fertilisation, most probably by protecting the sperm in the acidic vaginal environment (Smith and Gillatt, 1997).

According to McNeal's (1981) anatomical model, the prostate is divided into a fibromuscular and four glandular zones (Figure 1.2). The fibromuscular zone, called the **anterior fibromuscular stroma**, represents one third of the mass of the gland. It is mainly nonglandular consisting of smooth muscle cells, and is the anchoring point of the urethral sphincter that controls urination.

The four glandular zones are:

- The **central zone** is a cone-shaped structure surrounding the ejaculatory ducts and comprising 25% of the total glandular tissue. It consists of large glands embedded in a dense stroma.
- The **peripheral zone** surrounds the central zone and consists of smaller glands embedded in a loose stroma. It is the largest zone, comprising 70% of the total glandular tissue, and is the area where most prostate adenocarcinomas arise.
- The **transition zone** represents only 5% of the glandular tissue and consists of two small paraurethral lobes, located mid-level of the prostate. The glands in this zone are identical to the glands in the peripheral zone, but are less numerous and surrounded by a more dense stroma.
- The **periurethral gland region** is the smallest region and represents less than 1% of the glandular tissue.

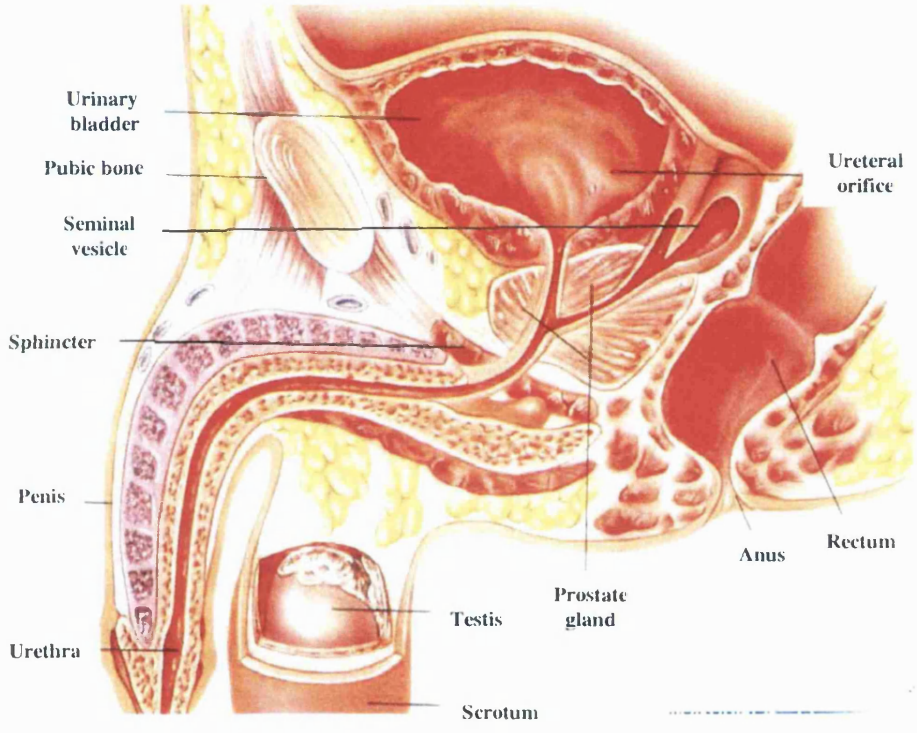


Figure 1.1 **The male reproductive system.**

The prostate gland is part of the male reproductive system and is located below the bladder, behind the pubic bone, and in front of the rectum.

Adapted from http://www.cancer-prostate.com (compiled by Jhaveri F, 1999).

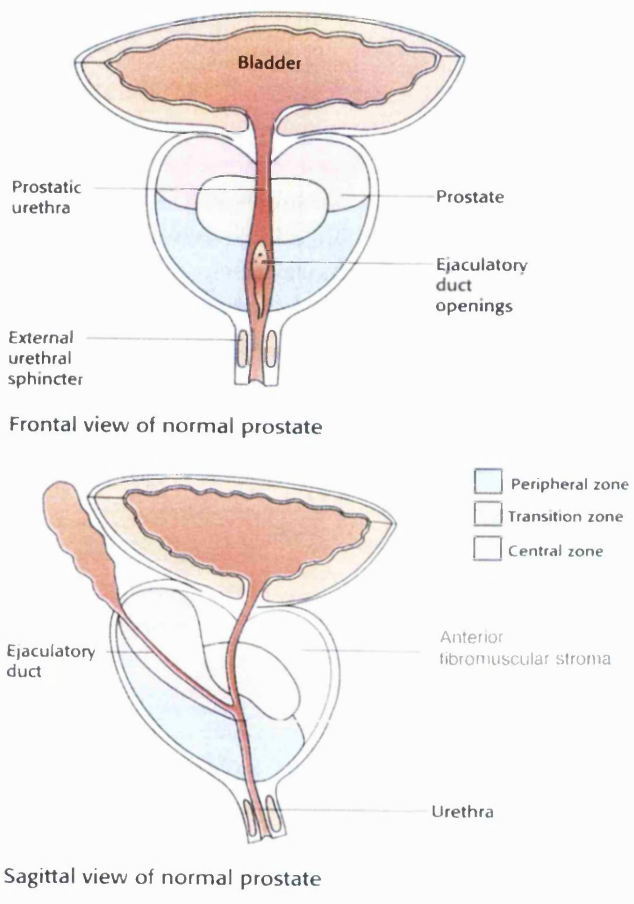


Figure 1.2 **Anatomical model of the prostate gland.**
Schematic representation of the zonal subdivision of the prostate in NcNeal's (1981) model. The position of the glandular and fibromuscular zones is shown (adapted from Kirby and McConnell, 1999).

1.1.2 Prostate Development

The prostate is a secondary sex gland affected by hormonal stimulation. Prostatic development begins in the third month of fetal life (Shapiro, 1990). There is little change in the gland during the remainder of fetal life and childhood. During puberty, when androgen levels increase, prostatic growth becomes rapid and secretory activity starts. In adult life, androgens maintain the structure and the secretory activity of the organ.

1.2 Histology

A fibroelastic capsule containing a number of veins surrounds the whole prostate. The glands are embedded within an abundant and dense fibroelastic stroma containing numerous smooth muscle fibers (Chang et al., 1989). Epithelial cells make up the glandular portion of the prostate while stromal cells make up the surrounding muscle and connective tissues (McNeal, 1988; Coffey, 1993).

1.3 Role of androgens in the biology of the human prostate gland

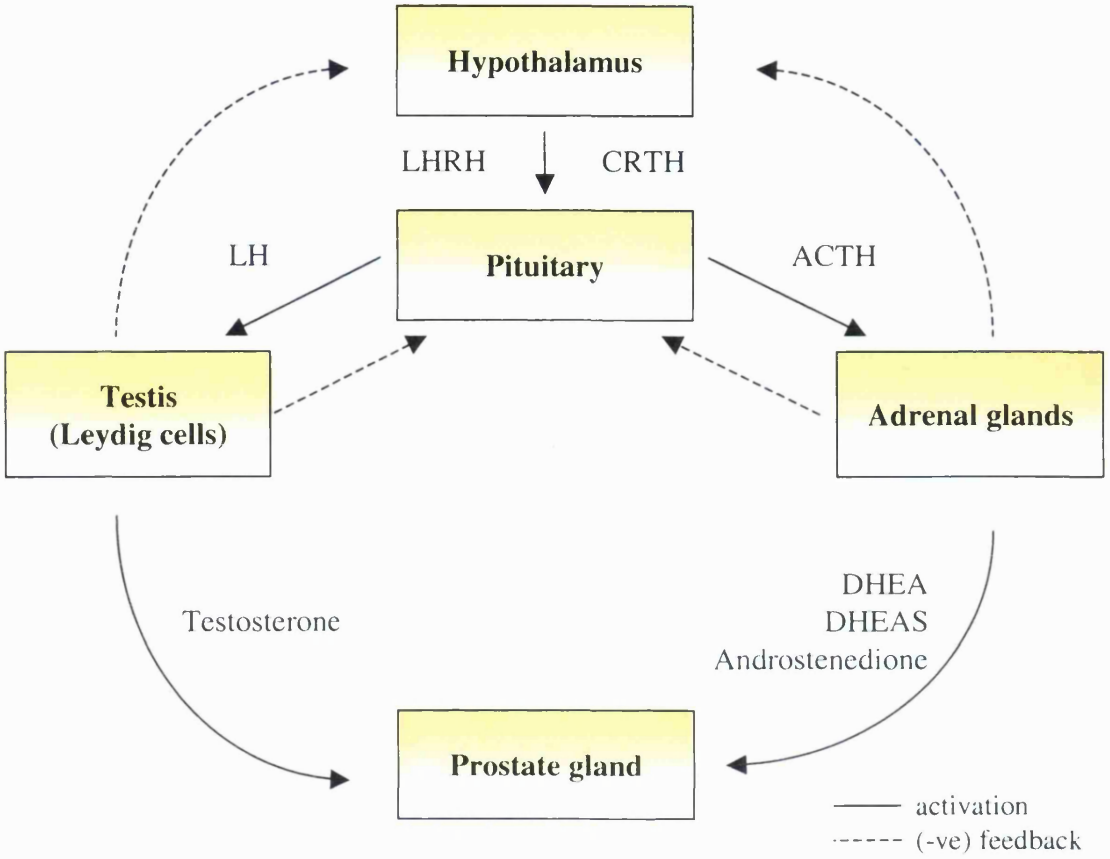
Androgens (testosterone and 5α-dihydrotestosterone [DHT]) control the development and function of the prostate gland, mediate male sexual differentiation (i.e. masculinisation of the urogenital tract in a 46,XY embryo), and play the major role in development of secondary male sexual characteristics at puberty (Wilson et al., 1981; Coffey, 1992; Griffin, 1992). Androgens are also known to directly stimulate the growth of prostate cancer cells and thus are believed to play an important role in prostate carcinogenesis (Kyprianou et al., 1990; Henderson et al., 1991; Kirschenbaum et al., 1993).

Testosterone is the primary androgen secreted by the testis. 5α-dihydrotestosterone (DHT) is the active form of testosterone and is the predominant androgen in the prostate (George, 1997). Testosterone diffuses passively into target tissues where it is converted to DHT by the 5α-reductase enzyme. There are two 5α-reductase isoforms: Type-1 enzyme is ubiquitously expressed, whereas Type-2 isoform is expressed

predominantly in tissues of the male urogenital tract (Normington and Russell, 1992; Berman and Russell, 1993; Russell et al., 1994; Berman et al., 1995).
The adrenal glands are the second site of androgen synthesis and produce, in smaller quantities, the inactive precursors of testosterone androstenedione, dehydro-epiandrosterone (DHEA) and its sulfate DHEAS (Harper et al., 1974).

Testosterone levels in the body are controlled by the release of the luteinising hormone-releasing hormone (LHRH) from the hypothalamus in the brain. LHRH stimulates the release of luteinising hormone (LH) from the pituitary gland, which in turn stimulates the testicular Leydig cells to produce testosterone. Adrenal androgen levels are also controlled by the hypothalamus through the release of the corticotrophin-releasing hormone (CRTH). CRTH stimulates the release of adrenocorticotrophic hormone (ACTH) from the pituitary causing the adrenal glands to produce the adrenal androgens (androstenedione, DHEA and DHEAS). A negative feedback loop upon the hypothalamic-pituitary system controls androgen production in the body (McConnell, 1991) (Figure 1.3).

Testosterone and DHT mediate different androgen effects. Testosterone is responsible for the regulation of spermatogenesis and the virilisation of the Wolffian duct during embryogenesis, while DHT induces virilisation of the urogenital tract and external genitalia during embryogenesis and is responsible for the maturation events during male puberty (Wilson et al., 1981). Even though testosterone and DHT mediate different androgen effects, both their actions are mediated by one androgen receptor (AR) (Figure 1.4). DHT binds to AR with much higher affinity (Wilbert et al., 1983; George and Noble, 1984) and stabilises the receptor (Zhou et al., 1995). Upon binding of the androgen, the hormone-receptor complex interacts with specific DNA sequences located in the flanking regions of target genes, thereby regulating gene expression.



LHRH: Luteinising hormone-releasing hormone
LH: Luteinising hormone
CRTH: Corticotrophin-releasing hormone
ACTH: Adrenocorticotrophic hormone
DHEA: Dehydroepiandrosterone
DHEAS: Dehydroepiandrosterone sulfate

Figure 1.3 **Schematic diagram of the normal hypothalamus-pituitary-testis system.** Androgen regulation starts in the hypothalamus which synthesizes and releases, in pulsatile manner, the LHRH and CRTH hormones. LHRH and CRTH act directly on the anterior pituitary gland that synthesises and secretes LH and ACTH, respectively. LH stimulates the production of testosterone from the testis, while ACTH stimulates the secretion of adrenal androgens from the adrenal glands. Endogenous circulating levels of androgens feed back to the hypothalamus and the pituitary to shut off the production. Broken lines indicate negative feedback (adjusted from Galbraith and Duchesne, 1997).

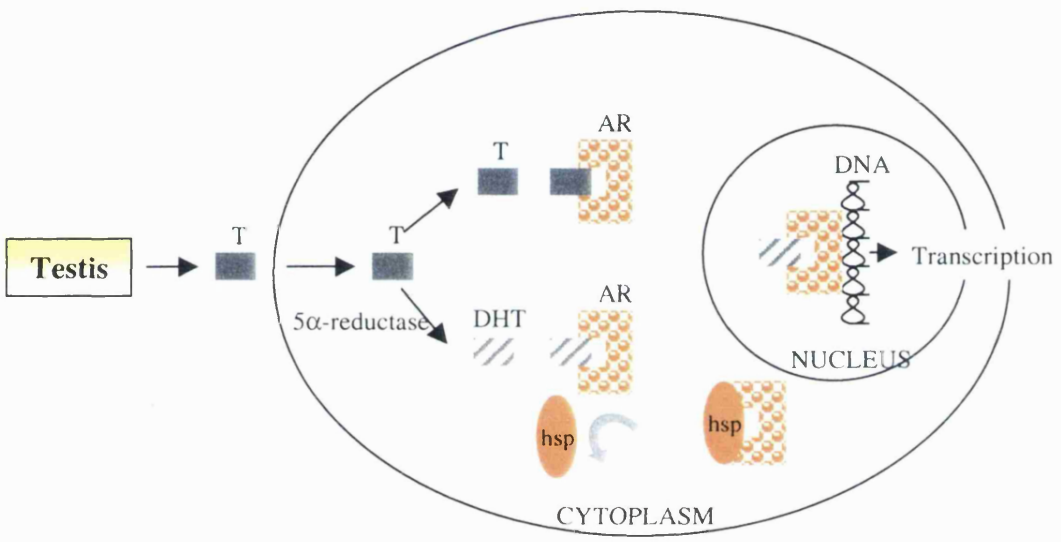


Figure 1.4 **Simplified diagram of androgen action.**

Testosterone (T) secreted by the testis diffuses passively into the cell and binds directly or indirectly (after conversion to dihydrotestosterone, DHT) to the androgen receptor (AR). The unliganded AR is located in the cytoplasm and is associated with heat-shock proteins (hsp). Upon ligand-binding (T or DHT), the AR translocates to the nucleus where it binds DNA and regulates the transcription of androgen-regulated genes (adjusted from Griffin, 1992).

1.4 Prostate Pathology

1.4.1 Benign prostatic hyperplasia (BPH)

Benign Prostatic Hyperplasia (BPH) is a common condition resulting from enlargement of the prostate gland and its symptoms increase dramatically with age (Berry et al., 1984; Carter and Coffey, 1990). BPH is a stromal cell hyperplasia usually originating in the transition zone of the prostate. BPH often results in obstruction of urinary outflow, haematuria, and frequent micturation (Figure 1.5b).

1.4.2 Prostatitis

Apart from enlargement of the prostate (BPH), urinary problems can also be caused by prostatitis. Prostatitis is the inflammation or infection of the prostate and tends to occur in relatively young men, between the ages of 20 and 50 (Smith and Gillatt, 1997).

1.4.3 Prostatic adenocarcinoma

Adenocarcinoma of the prostate originates from the prostatic glandular epithelium. The symptoms of prostate cancer are initially similar to those of BPH (Figure 1.5c). Tumours arise principally in the peripheral zone of the prostate and may be palpable by rectal examination. Early-stage tumours can be discovered incidentally in the transition zone during BPH surgery. Tumours usually grow peripherally through the capsule of the gland and often invade the seminal vesicles and the neck of the bladder. Their metastatic spread is both lymphatic and haematogenous. Lymphatic spread affects the obturator and iliac nodes first (Fowler and Whitmore, 1981). Haematogenous spread is mainly to the bones and less to the lungs and liver (Jacobs, 1983; Saitoh et al., 1984). Spinal spread can extend to the epidural space and cause compression of the spinal cord and weakness in the legs, leading to paraplegia.

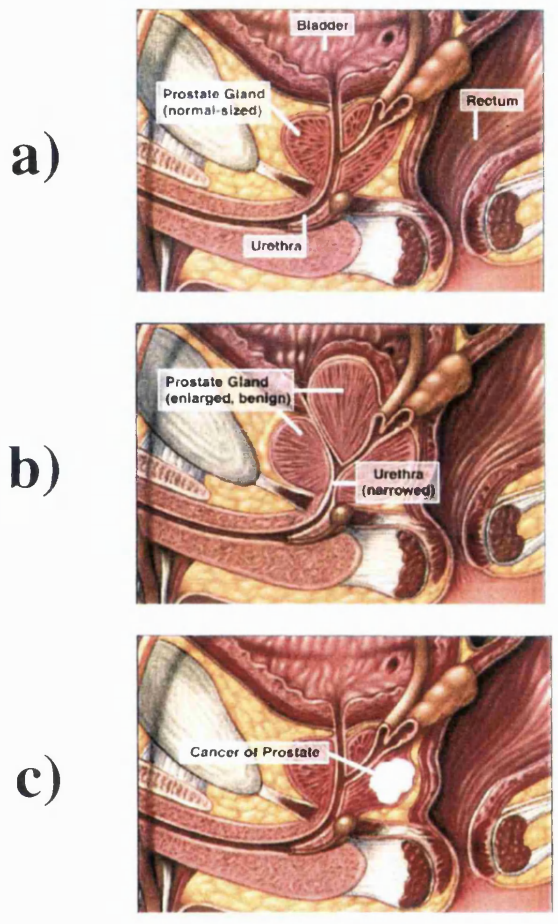


Figure 1.5 **Prostate gland abnormalities.**

Schematic diagrams indicating common abnormalities of the prostate gland.

a) Normal prostate

b) Benign prostatic hyperplasia (BPH)

c) Prostate cancer

Adapted from http://www.mayo.edu (Mayo Foundation for Medical Research, 1997).

The etiology of prostate cancer is unknown, but there is compelling evidence to support the hypothesis of a hormonal etiology involving androgen action (Ross et al., 1983; Nomura and Kolonel, 1991; Wilding, 1992).

PART II *PROSTATE CANCER*

1.5 Incidence

Prostate cancer is the most frequently diagnosed malignancy in men in the United States and Europe, and its incidence is rising. There are about 135,000 cases of prostate cancer diagnosed each year in men in the EU and about 55,000 deaths from the disease (http://www-dep.iarc.fr, 2000; Cussenot and Valeri, 2001). Improvements in life expectancy, registration efficiency and detection of early and latent disease may account for the increase in incidence. Incidence increases with age as prostate cancer is an age-related disease, varying from one per 100,000 at the age of 45 years to one per 1000 at the age of 65 years and 5 per 1000 at the age of 75 years. In the UK, the median age at presentation is 67 years (Henry and O'Mahony, 1999).

1.6 Risk factors

There are differences in prostate cancer incidence, biological behaviour and mortality (Nomura and Kolonel, 1991; Shibata and Whittemore, 1997; Pettaway, 1999). These might be explained by the interaction among diverse environmental factors and genetic mechanisms. Studies on the epidemiology of prostate cancer have reported several factors that can affect prostate cancer risk.

a) Ethnic factors

Variation in prostate cancer risk has been observed amongst different ethnic groups. African-American men have higher clinical incidence of prostate cancer than any other population in the world, while Asian men have the lowest prostate cancer incidence (Levine and Wilchinsky, 1979; Ross et al., 1986; Shibata and Whittemore, 1997).

b) Genetic factors

First-degree relatives of men with prostate cancer have a two- to three-fold risk for prostate cancer; for two first-degree relatives, prostate cancer risk is increased five-fold (Steinberg et al., 1990; Hayes et al., 1995; Monroe et al., 1995; Sun et al., 1995; Whittemore et al., 1995). Several predisposition loci for familial prostate cancer have been suggested, including HPC1 (hereditary prostate cancer 1), HPC20 and HPCX genes at chromosome 1, 20 and X, respectively (for a review see Cussenot and Valeri, 2001). However, the familial form of inheritance is estimated to account for only 9% of all prostate cancers; the majority of cases are sporadic (Carter et al., 1993).

The prevalence of certain alleles of specific genes related to the metabolism and function of male sex hormones has also been explored. The thymine-adenine (TA) dinucleotides repeat in the 3′ untranslated region (UTR) of the type II 5α-reductase gene was shown to be polymorphic. Three alleles have been considered, but the 18-repeats allele (TA_{18}) is common in black-American men, the ethnic group with the highest incidence worldwide (Ross et al., 1992; Davis and Russell, 1993; Reichardt et al., 1995). Another polymorphism of the cytosine-adenine-guanine (CAG) trinucleotide repeat in exon 1 of the androgen receptor gene has been shown to correlate with prostate cancer risk. A negative relationship between repeat length and transactivation activity of the gene has been reported (Chamberlain et al., 1994; Schoenberg et al., 1994; Giovannucci et al., 1997; Stanford et al., 1997). Mutated alleles of the breast cancer, BRCA1 and BRCA2 genes have also been implicated in increased prostate cancer risk (Ford et al., 1994; Langston et al., 1996). In addition, the length of a polyA tract in the 3′ UTR of the vitamin D receptor (VDR) gene has been associated with prostate cancer risk. Longer alleles show decreased VDR expression and subsequent decreased transcription of VDR-regulated genes, such as the metastasis-related gene fibronectin, thereby increasing the risk (Ingles et al., 1997).

c) Hormonal factors

African-American men with the highest risk of prostate cancer in the world, have 10-15% higher concentrations of circulating testosterone compared to Asian men.

Increased amounts of circulating androgens might be implicated in the high incidence of prostate cancer (Henderson et al., 1991; Ross et al., 1992; Pettaway, 1999).

d) Dietary factors

High intake of fresh fruits and vegetables, soyabean products, cereals, and vitamins C and D may exert a protective role against prostate cancer (Messina and Bennink, 1998; Ekman, 1999). Consumption of red meat and a diet high in fat (mostly saturated fat, monounsaturated fat and α-linoleic acid) have been correlated with increased risk of prostate cancer (Giovannucci et al., 1993; Pienta and Esper, 1993).

e) Infective agents

Two viruses have been suggested as possible etiologic agents: herpes virus type 2, and cytomegalovirus (CMV). Higher antibody titrations for these viruses are reported among prostate cancer cases (Steele et al., 1971; Schuman et al., 1977).

f) Social factors

Studies of prostatic cancer patients suggest that men with great sexual drive, many sexual partners, earlier marriage and great sexual activity within marriage might have increased risk of prostate cancer (Steele et al., 1971; Greenwald et al., 1974; Mishina et al., 1985).

In summary, epidemiological studies of prostate cancer have revealed the potential of the above factors in increasing the risk for the disease, but there is no evidence that these are directly implicated. Prostate carcinogenesis cannot be explained by a single-gene model but rather follows a multistage pathway, where more than one genetic alteration together with various environmental and dietary factors are involved.

1.7 Methods for prostate cancer detection

1.7.1 Digital rectal examination (DRE)

Digital rectal examination (DRE) remains a widely used method for the screening and diagnosis of prostate cancer. Recent studies have suggested that the sensitivity of DRE is about 30% and its specificity is about 40% for organ-confined disease (Mettlin et al., 1993).

1.7.2 Transrectal Ultrasonography (TRUS)

Transrectal ultrasonography is reported to be more sensitive than DRE (Palken et al., 1991). It is an imaging technique which can detect hypoechoic lesions (consistent with prostate cancer) as small as 5mm in diameter. This method is most promising for early detection of prostate cancer, although it has a significant false negative rate (Rabrani et al., 1998). It is offered to most men with serum PSA levels of 4-10ng/ml (Dearnaley et al., 1999).

1.7.3 Magnetic resonance imaging (MRI)

Magnetic resonance imaging (MRI) uses magnetism instead of X-rays to build up cross-sectional images of the body. MRI allows a high degree of resolution and sensitivity for prostate cancer and can prevent unnecessary biopsies (Schnall et al., 1989).

1.7.4 Diagnostic and prognostic markers

Ideally, tumour markers should not only be specific, sensitive and reproducible, but they should also be organ- and cancer-specific. Unfortunately, the markers currently used for diagnosis and prognosis of prostate cancer have limited specificity and sensitivity and cannot accurately detect very early localised prostate tumours or predict their aggressiveness. The conventional protein markers (PAP, PSMA, PSA)

together with some of the most frequent chromosomal alterations and PIN (Prostatic Intraepithelial Neoplasia) are summarised below (for a detailed review see Gao et al., 1997):

a) Prostatic acid phosphatase (PAP)

Prostatic acid phosphatase (PAP) was originally described in the late 1930s (Gutman et al., 1936), but it was not until the 1970s that was it used for immunochemical assays. Human PAP is a glycoprotein secreted under androgen control into the seminal plasma (Ostrowski and Kuciel, 1994). Elevated serum PAP levels have been reported in patients with prostate cancer (Oesterling et al., 1987; Sakai et al., 1993). However, PAP is only a small portion (10-25%) of the total acid phosphatase in the serum of the normal adult male. Hence, cross-reactivity with serum acid phosphatases of non-prostatic origins together with variation in the PAP levels depending on different assaying methods complicates its use as a marker in the staging of prostate cancer.

b) Prostate-specific membrane antigen (PSMA)

Prostate-specific membrane antigen (PSMA) is a transmembrane glycoprotein originally identified by Horoszewicz et al. (1987). PSMA expression is elevated in the serum of prostate cancer patients compared to those with normal prostate or BPH (Rochon et al., 1994; Murphy et al., 1995a; Murphy et al., 1995b; Silver et al., 1997). PSMA has been found to positively correlate with pathological grade but not with clinical stage i.e. PSMA expression is high in poorly differentiated prostate cancer but expression does not correlate with nodal status, extracapsular penetration, or seminal vesicle status (Wright et al., 1995). However, PSMA expression is not only restricted to prostatic tissues; it has also been detected in the brain, salivary glands and the small intestine (Israeli et al., 1993).

c) Prostate specific antigen (PSA)

Prostate specific antigen (PSA) is currently the most useful prostate marker in practice for screening, diagnosis and monitoring of prostate cancer (Catalona et al., 1991; Brawer et al., 1992; Labrie et al., 1992).

Prostate-specific antigen (PSA) is the most studied androgen-responsive gene. Its product was first described in 1971 and purified in 1979 in the seminal plasma of the prostate (Wang et al., 1979). It is a member of the kallikrein-like serine family of proteases and is synthesised by the luminal epithelial cells of the prostate gland. PSA has a trypsin-like activity, liquifying the seminal coagulum by proteolysis (Lilja, 1985).

The human PSA gene (hPSA) is a single copy gene, comprised of five exons located on chromosome 19q13.2-13.4 (Riegman et al., 1989; Riegman et al., 1992). It encodes a single-chain, 240 amino acids long, glycoprotein with a molecular weight of ~34kDa (Watt et al., 1986).

PSA is present in the serum of men with both benign and malignant prostatic disease. A direct relationship between serum PSA and tumour volume has been reported (Kleer et al., 1993; Kabalin et al., 1995). As a general rule, as prostate cancer progresses, PSA levels rise. A PSA value of 4ng/ml was selected as the concentration to differentiate normal from elevated PSA levels (Catalona et al., 1991).

Initially, PSA was thought to be exclusively secreted by prostate cells. However, PSA expression has been demonstrated in urine, periurethral glands, perianal gland, saliva, amniotic fluid, milk of lactating women, and serum of normal women (Frazier et al., 1992; Iwakiri et al., 1993; Yu and Diamandis, 1995a; Yu and Diamandis, 1995b; Breul et al., 1997). In addition, PSA protein and/or mRNA have been detected in tumours of the skin, salivary glands, ovary, and breast (Papotti et al., 1989; van Krieken, 1993; Diamandis et al., 1994; Yu et al., 1995).

Despite the fact that PSA is not disease-specific and cannot accurately predict the exact pathological stage, it has been proved the marker available to date with the highest sensitivity and specificity (Ploch and Brawer, 1994). Recent studies examining prostate cancer mortality rates in the USA have documented a decrease in mortality since the introduction of PSA screening (Labrie, 2000b; Mettlin, 2000; Tarone et al., 2000).

d) Chromosomal alterations

The chromosomal aberrations frequently occurring in prostate cancer seem to be related to later phases of disease progression (for a review see Nupponen and Visakorpi, 1999). The most common alterations in the locally recurrent hormone refractory prostate cancers are losses of loci on chromosomes 8p, 13q, 1p, 22, 19, 10q, 17p and 16q, and gains of loci on chromosomes 8q, 7q, Xq, and 18q. Inactivation of tumour suppressor genes and activation of oncogenes located at these loci seem to be associated with progression of the disease.

Deletions of parts of 16q are of interest as this is the location of the E-cadherin gene. Loss of E-cadherin function is likely to promote tumour invasion and metastasis, as E-cadherin is involved in cell-cell adhesion (Umbas et al., 1992; Kemler, 1993). E-cadherin expression is decreased or absent in a variety of poorly differentiated carcinomas including that of the prostate (Shiozaki et al., 1991; Umbas et al., 1992). Allelic loss of the E-cadherin gene locus and aberrant DNA hypermethylation have been implicated as mechanisms by which the expression of E-cadherin is silenced (Graff et al., 1995; Yoshiura et al., 1995).

Losses of chromosome 17p where the tumour suppressor gene p53 is located have been described in a variety of human cancers. Aberrant function of p53 leads to de-regulation of the cell cycle checkpoint and replication, defective or inefficient DNA repair, selective growth advantage and ultimately tumour formation and progression (for a review see Gao et al., 1995). Transfection of wild type p53 cDNA into human prostate cancer cell lines suppresses their tumourigenicity (Isaacs et al., 1991). p53 abnormalities (allelic deletion, low expression, mutation) correlate with tumour grade and stage and are considered to be a late event in the development of prostate cancer (Bookstein et al., 1993; Navone et al., 1993; Chen et al., 1994; Massenkeil et al., 1994).

Gain of chromosome 8q where the oncogene c-myc is located is also of great interest, as c-myc is commonly amplified in prostate cancer (Jenkins et al., 1997; Nupponen et al., 1998).

e) Prostatic intraepithelial neoplasia (PIN)

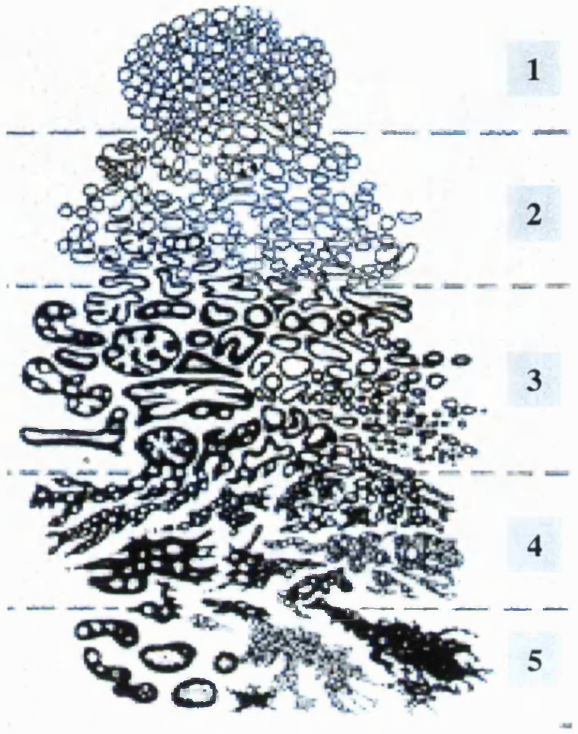
PIN is associated with progressive phenotypic and genotypic abnormalities. A PIN lesion consists of atypical cells with high nuclear to cytoplasmic ratio. High-grade PIN is considered the most likely pre-invasive stage of adenocarcinoma (Zlotta et al., 1996). Its identification warrants repeat biopsy for concurrent or subsequent invasive carcinoma (Weinstein and Epstein, 1993). PIN does not significantly elevate the levels of PSA and can only be detected by biopsy not ultrasonography.

Of the diagnostic methods described above, a combination of the results of serum PSA, transrectal ultrasound and digital rectal examination is often preferred to each test on its own for the diagnosis of prostate cancer. No detection marker can predict the future clinical behaviour of the tumour.

1.8 Grading and Staging

As prostatic carcinoma shows a variable degree of differentiation, a uniform histological grading is necessary. The system developed by Gleason (1977) is widely used and appears to correlate with tumour size, metastases to pelvic lymph nodes, and the level of PSA (McNeal, 1988). The Gleason system takes into account the degree of glandular differentiation of epithelial cells. The grades range from well-differentiated tumours with well preserved glandular differentiation (low grade) to undifferentiated tumours with minimal or no glandular differentiation (high grade). A Gleason sum (2-10) is the sum of primary and secondary morphological patterns in heterogeneous tumours (Figure 1.6).

Staging of prostate cancer dictates therapy and distinguishes between clinically localised, locally advanced, and metastatic disease. The most commonly used staging system is the Tumour-Node-Metastasis (TMN) classification system (Catalona and Whitmore, 1989; Armstrong, 1998) (Table 1.1).



Grade	Description
1	Very well differentiated adenocarcinoma. Tumours arranged in very well defined clumps and composed of regular round or oval cells separated by a very fine stroma.
2	A well differentiated adenocarcinoma. Tumours arranged in less defined clumps and composed of less uniform regular round or oval cells separated by a slightly more abundant stroma.
3	A moderately differentiated adenocarcinoma. Tumours arranged in poorly defined clumps and composed of polymorphic differentiated glands separated by abundant stroma.
4	A poorly differentiated adenocarcinoma. Tumours composed of poorly defined glands.
5	An undifferentiated adenocarcinoma with minimal gland formation. Tumours composed of bands or sheets of isolated cells with marked infiltration of the stroma.
2-10	sum of primary and secondary morphological patterns in heterologous tumours

Figure 1.6 **Gleason grading system.**

Five distinct glandular patterns are identified and their characteristics are described. For more than one pattern present, the sum of the two predominant ones yields the final grade. (Modified from Gittes, 1991).

Table 1.1 tumour-Node-Metastasis staging system (TNM)

Stage		TNM		Description
A	Incidental		Tx	cannot be assessed
			T0	no evidence
		T1		clinically unapparent tumour not palpable or visible by imaging
			T1a	incidental histological finding in <5% of resected tissue
			T1b	incidental histological finding in >5% of resected tissue
			T1c	identified by needle biopsy not palpable or visible by imaging
B	Palpable, organ-confined	T2		tumour confined within the prostate
			T2a	involves half a lobe or less
			T2b	involves more than half a lobe but not both lobes
			T2c	involves both lobes
C	Locally invasive	T3		tumour extends through the prostate capsule
			T3a	Unilateral extracapsule extension
			T3b	Bilateral extracapsule extension
			T3c	seminal vesicles invasion
D	Metastasis	T4		tumour invades adjacent structures
			T4a	invasion of bladder neck, external sphincter or rectum
			T4b	invasion of elevator muscles and/or is fixed to pelvic wall
N	Regional lymph nodes	Nx		cannot be assessed
		N0		no regional lymph node metastasis
		N1		metastasis in a single lymph node, <2cm in dimension
		N2		metastasis in a single lymph node, 2-5cm in dimension, or multiple nodes <5cm in dimension
		N3		metastasis in a regional lymph node, >5cm in dimension
M	Distant metastases		Mx	cannot be assessed
			M0	no distant metastasis
		M1		distant metastasis
			M1a	non-regional lymph nodes
			M1b	bone
			M1c	other sites

Adapted from Catalona and Whitmore, 1989

1.9 Treatment

The dependence of prostate cancer growth on androgens led Huggins and Hodges (1941) to develop castration as a therapy for human prostate carcinoma. Chemical or surgical castration are standard treatment used for prostatic carcinoma. Androgen ablation (chemical or surgical castration) at the cellular level induces apoptosis (programmed cell death), causing shrinkage of the prostate and primary tumours in approximately 80% of the patients (Lepor et al., 1982).

A wide spectrum of treatment options currently exists ranging from watchful waiting to therapy including orchidectomy, prostatectomy, radiotherapy, and administration of LHRH agonists, anti-androgens, or estrogens. Curative therapy (prostatectomy or radiation therapy) is possible only for localised disease. Controversy surrounds the choice of the appropriate treatment for advanced-stage disease, as none of them appears to be curative. Nearly all patients with advanced prostate cancer relapse with androgen-independent tumours that are no longer controllable by hormonal therapy. The progression of human prostate cancer from androgen-dependent to androgen-independent is a major clinical problem and, once this progression has occurred, further treatment options are severely limited.

1.9.1 Watchful waiting (conservative management)

Autopsy from one third of men over the age of 75 showed microscopic foci of well-differentiated adenocarcinoma in sections of prostate glands considered to be normal ("latent" or "incidental" tumours) (Catalona, 1994). Advances in screening for prostate cancer have increased the diagnosis of these "latent" or "incidental" tumours, which would otherwise have remained undetected. Only about 10% of the "latent" tumours will develop into clinically significant tumours during the patient's life (Gittes, 1991). Watchful waiting is considered a reasonable option for men with grade 1 or 2 clinically localised prostate cancer, especially if their life expectancy is 10 years or less (Chodak et al., 1994). Despite the fact that no adequate large-scale trials comparing treatment with watchful waiting have been completed, watchful waiting is not advised for a patient under 60 years old.

1.9.2 Radical prostatectomy

Organ-confined prostate cancer can be eradicated by radical prostatectomy, which involves removal of the whole prostate gland, its capsule and the seminal vesicles. However, impotence and incontinence are two frequent complications resulting from the operation.

1.9.3 Radiation therapy

Radiotherapy can be curative or palliative and is an alternative to radical surgery. However, side effects and complications can arise from radiation to the organs and tissues surrounding the prostate.

Interstitial radiotherapy (brachytherapy) is another way of focusing maximum radiation on the prostate. In brachytherapy, radioactive particles are implanted, temporarily or permanently, into the prostate with the help of transrectal ultrasound. Patients with small tumour volumes and low Gleason grade seem to benefit (Kuyu et al., 1999). Despite the fact that brachytherapy has a low complication rate and is a simple procedure with low invasiveness, there is no evidence that it is as effective as radical prostatectomy (Wirth and Hakenberg, 1999).

1.9.4 Surgical castration (orchidectomy)

Bilateral subcapsular orchidectomy removes about 90% of circulating androgens (the remaining 5-10% being secreted by the adrenal glands). This treatment option is not popular as it leads to irreversible side effects including impotence, loss of libido, tiredness, feminisation and hot flushes.

1.9.5 Chemical castration (hormonal therapy)

Hormonal therapy is traditionally administered continuously, using drugs that produce castrated levels of testosterone. Chemical castration results in the death (by apoptosis) of androgen-sensitive cells. PSA monitoring has created a dramatic shift in the

population of patients in whom hormonal therapy is initiated. Hormonal therapy includes LH-RH analogues, estrogens, and anti-androgens which, although they extend survival, are not curative and have an impact on the patient's quality of life.

a) LHRH analogues

LHRH analogues prevent the production of androgens by blocking the production of LH hormone from the pituitary gland. The initial stimulation of the pituitary regulatory system prior to its desensitisation temporarily increases the androgen levels, thereby resulting in a rapid rise in serum testosterone levels and a tumour flare.

b) Estrogens

Estrogens produce their effects by suppressing the secretion of LHRH from the hypothalamus, thereby inhibiting the release of LH from the pituitary and subsequently blocking secretion of testosterone by the testis. High risk of cardiovascular side effects and thromboembolic phenomena complicates their use.

c) Anti-androgens

Anti-androgens compete with androgens for binding to the androgen receptor, and when bound they have antagonistic action. They are classified as steroidal and non-steroidal (pure anti-androgens). The biological effects of the steroidal versus nonsteroidal agents are distinguished by differences in their effect on serum testosterone levels, and by their activity on receptors other than the AR. The major side effects from anti-androgen administration include gynecomastia, depression, impotence and loss of libido.

Steroidal anti-androgens (e.g. cyproterone acetate) inhibit LH secretion from the pituitary. However, at higher concentrations estrogenic and progestogenic steroids can induce the same effects on the AR as physiological doses of androgens (Kemppainen et al., 1992).

Non-steroidal anti-androgens (i.e. flutamide and its derivatives, hydroxyflutamide, bicalutamide [Casodex], and Nilutamide) block binding of androgens to the AR and

oppose the negative feedback of testosterone on the pituitary, thereby increasing serum testosterone levels.

Surprisingly, withdrawal of anti-androgens (flutamide) in a subset of prostate cancer patients with therapy-resistant disease is found to be clinically beneficial (Scher and Kelly, 1993; Scher and Kolvenbag, 1997). This is known as anti-androgen (or flutamide) withdrawal syndrome. Currently, it is not possible to identify the tumours that will respond to anti-androgen withdrawal.
A recent study has suggested that the non-steroidal androgen bicalutamide (Casodex) also acquires agonistic properties during long-term androgen ablation (Culig et al., 1999).

Intermittent androgen ablation is another therapeutic option for advanced prostate cancer which results in improved quality of life in the off-treatment interval (Akakura et al., 1993). The rational for this therapy is that re-exposure of prostate cancer cells to androgen may re-induce an androgen-sensitive phenotype, or that the clonal selection of androgen-independent cells is delayed and therefore survival is improved (Klotz et al., 1986; Bruchovsky et al., 1990). Uncertainty remains with respect to the long-term effect of intermittent androgen ablation therapy on patient survival.

d) Total androgen blockade (MAB)

Maximal or total androgen blockade (MAB) is a treatment for advanced prostate cancer where the action of both the testicular and adrenal androgens is blocked. MAB combines chemical or surgical castration (i.e. orchidectomy or the use of LHRH analogues) with anti-androgens. MAB has been shown to produce a small improvement in the response rates of some patients with metastatic prostate cancer compared to that of surgical castration alone (Denis, 1996). MAB produces a modest (3%) overall and cancer-specific survival at 5 years but is associated with increased adverse events and reduced quality of life (Klotz, 2000). However, the survival benefits from MAB and the timing of therapy remain controversial which make it difficult to consider MAB as a standard treatment for advanced prostate cancer (Kuyu et al., 1999).

1.9.6 Cytotoxic chemotherapy

The role of chemotherapy in the management of androgen-independent, hormone-refractory disease (progressing after adrenal suppression) remains controversial, as limited experimental data is available. Treatment with standard cytotoxic agents in hormone-insensitive patients is directed to improve the limited survival and quality of life of patients.

In summary, medical advances permit earlier diagnosis and more accurate staging of prostate cancer. However, curative therapy (radical prostatectomy or radiation therapy) is possible only for localised disease. In advanced disease, androgen ablation therapy remains the only treatment with many controversial and unresolved consequences. At present, there is no consensus as to the best treatment course for advanced prostate cancer. Factors that ought to be taken into consideration include the normal life expectancy of the patient, the stage of the disease and the side effects of the treatment.

1.10 Models of prostate cancer

A number of models have been developed over the years in order to study prostate cancer *in vitro* (Royai et al., 1996). Among these, the ones widely used are: the Dunning rat model (Dunning, 1963), the CWR-22 xenograft model (Wainstein et al., 1994), the Transgenic Adenocarcinoma of Mouse Prostate, TRAM (Greenberg et al., 1995), and cultures of established cell lines (for a review see Mitchell et al., 2000). Compared to other tissues, such as breast cancer for example, where 100 to 150 different cell lines are available (Royai et al., 1996), few prostate cancer cell lines have been developed. Furthermore, most do not exhibit features commonly seen in human prostate cancer. The majority of them are androgen-insensitive and fail to express the androgen receptor (AR) and prostate-specific antigen (PSA). The most widely used established human prostate cancer cells lines are LNCaP, DU145 and PC-3, all derived from metastatic lesions.

1.10.1 The LNCaP prostate cancer cell line

LNCaP (**L**ymph **N**ode **Ca**rcinoma of the **P**rostate) is an epithelial cell line which was established from a metastatic lesion in the left lymph node of a 50 year old prostate cancer patient (Horoszewicz et al., 1980). Karyotypic analysis showed that the cells are aneuploid with a near tetraploid chromosome number.

The cells have a spindle-like shape with features characteristic of neoplastic and epithelial cells. LNCaP cells show anchorage-independent proliferation in soft agar and produce poorly differentiated adenocarcinomas in nude mice (Rembrink et al., 1997).

LNCaP is the most frequently used cell line as it is the only cell line which, like normal prostate and prostate cancer epithelial cells, expresses AR and PSA, and is hormone-responsive to androgens (Tilley et al., 1990; Pousette et al., 1997). However, a mutation in the steroid-binding domain (^{868}Thr→Ala) of the AR of LNCaP cells is responsible for aberrant response of the cells to estrogen, progesterone and anti-androgens (Trapman et al., 1990; Veldscholte et al., 1990a; Veldscholte et al., 1990b; Veldscholte et al., 1994).

1.10.2 The DU145 prostate cancer cell line

DU145 is an epithelial cell line which was established from a metastatic lesion in the brain of a 69 year old prostate cancer patient (Stone et al., 1978). Both primary and metastatic lesions have a similar histological appearance. However, the prostatic tissue was described as a poorly differentiated adenocarcinoma, while the brain metastasis was described as a moderately differentiated adenocarcinoma. Karyotypic analysis showed that the cell line is near triploid with extensive chromosomal rearrangements.

The cells are small and polygonal, and the shape of their nuclei varies from round to highly irregular. Cell processes of adjacent cells overlap extensively, forming double or triple layers locally. DU145 cells form colonies in soft agar, characteristic of tumourigenicity.

The DU145 cell line is not hormone-sensitive. These cells do not express the androgen receptor (AR) or prostate-specific antigen (PSA), even though both wild type genes are present (Tilley et al., 1990; Culig et al., 1993b; Royai et al., 1996).

1.10.3 The PC-3 prostate cancer cell line

PC-3 is an epithelial cell line which was established from a metastatic lesion in the bone of a 62 year old prostate cancer patient (Kaighn et al., 1979). The bone metastasis was described as a poorly differentiated adenocarcinoma. Karyotypic analysis showed that the cells are aneuploid with a near triploid chromosome number. The cells are spherical with features characteristic of neoplastic and epithelial cells. Anchorage-independent growth in soft agar and tumourigenicity in nude mice was also described (Rembrink et al., 1997).

Growth of the PC-3 cell line is not responsive to androgens, glucocorticoids, or growth factors (epidermal growth factor, fibroblast growth factor). PC-3 cells do not express either the androgen receptor (AR) or prostate-specific antigen (PSA), even though both wild type genes are present (Kaighn et al., 1979; Tilley et al., 1990; Trapman et al., 1990; Culig et al., 1993b; Marcelli et al., 1995).

PART III *ANDROGEN RECEPTOR*

1.11 The nuclear receptor superfamily

The superfamily of nuclear receptors (for a review see Parker, 1993; Kumar and Tindall, 1998) comprises receptors for steroids (androgen, estrogen, progesterone, glucocorticoid and mineralocorticoid), retinoids, thyroid hormone, vitamin D, and fatty acids (Laudet et al., 1992; Mangelsdorf et al., 1995; Laudet, 1997). In addition, the superfamily contains a large group of orphan receptors for which a ligand has either not yet been identified or may not exist (Enmark and Gustafsson, 1996). Steroid receptors are able to bind to specific sequences (hormone-responsive elements, HREs) in the control region of the target genes and thus regulate transcription of these genes (Evans, 1988; Beato, 1989).

1.12 Structure and function of the Androgen Receptor

All members of the steroid-thyroid-retinoid receptor superfamily, including AR, share basic structural and functional homology (Giguere et al., 1986; Gronemeyer et al., 1987; Kumar et al., 1987; Lubahn et al., 1988a; Dobson et al., 1989). AR is most closely related to the glucocorticoid and the progesterone receptor (Tenbaum and Baniahmad, 1997). The androgen receptor is a ligand-activated nuclear transcription factor that mediates androgen action in the normal prostate gland and other target organs (for a review see Culig et al., 2000; Gnanapragasam et al., 2000b).

The androgen receptor was first described by Fang et al. (1969) and the human AR gene was cloned in the late 1980s (Chang et al., 1988a; Lubahn et al., 1988b; Tilley et al., 1989). AR is a single copy gene (>90kb in length) located on chromosome Xq11-12 (Brown et al., 1989; Kuiper et al., 1989). It encodes a 910-919 amino acids protein with a molecular weight of 110-114kDa. The AR gene is comprised of 8 exons (Lubahn et al., 1989) with exon/intron boundaries conserved among the progesterone, estrogen and androgen receptor genes (Huckaby et al., 1987; Ponglikitmongkol et al., 1988; Kuiper et al., 1989). The eight exons encode three distinct functional domains: the amino-terminal, the DNA-binding, and the ligand-binding domains (Lubahn et al., 1988b) (Figure 1.7).

1.12.1 The amino-terminal domain (NTD)

The amino-terminal domain (NTD), constitutes 60% of the AR coding sequence and is the least conserved of the three major domains between members of the family of nuclear receptors (Strahle et al., 1988).

NTD is encoded by exon 1 and is variable in length due to the presence of three homopolymeric repeated regions: a polyglutamine (Gln), a polyglycine (Gly) and a polyproline (Pro). The multiple alleles of the normal AR gene differ in the glutamine (CAG) and glycine (GGC) repeat length. The CAG trinucleotide repeat normally ranges from 8-31 repeats (Edwards et al., 1992).

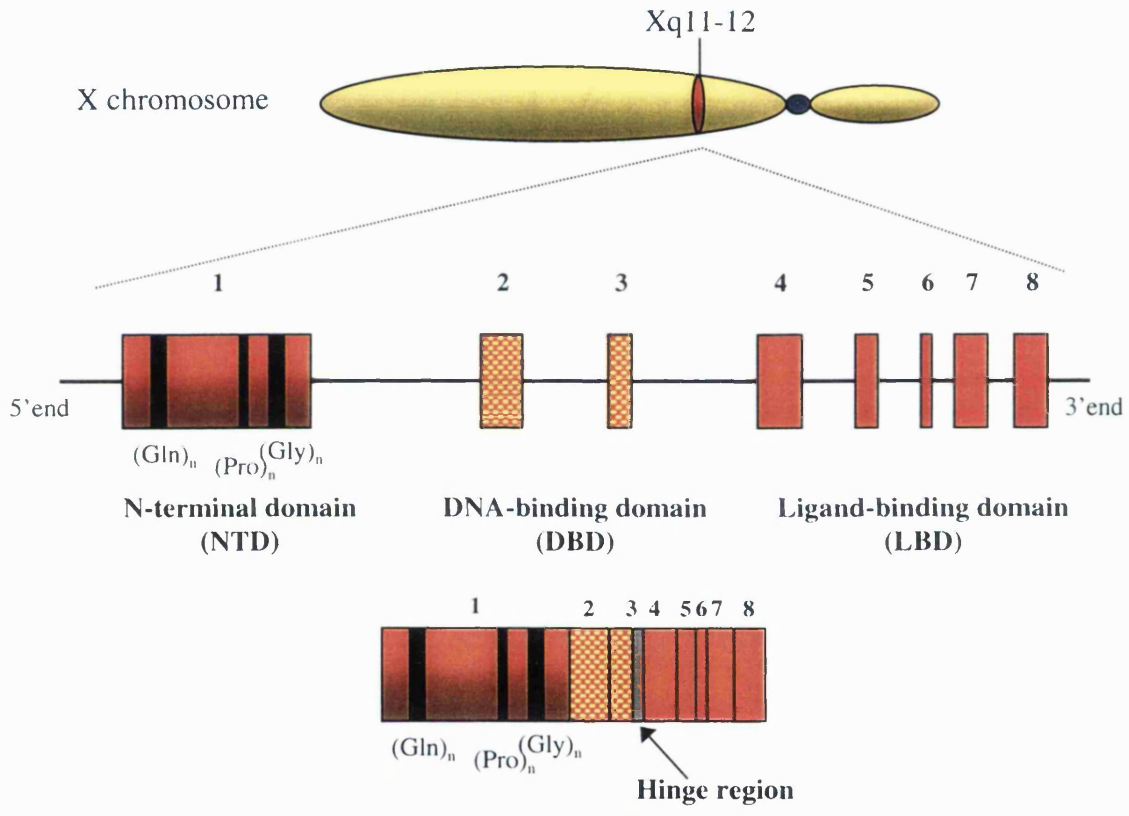


Figure 1.7 **Schematic diagram of the domains of the human AR gene.**

The androgen receptor gene is encoded by eight exons (boxes). Within the transactivation (N-ternimal) domain, encoded by exon 1, there are several stretches of polyamino acid repeats (black boxes). The DNA-binding domain is encoded by exon 2 and 3, and the ligand-binding domain is encoded by exons 4 to 8. Within the hinge region (junction of exon 3 and exon 4), the nuclear localisation signal (NLS) is located. The numbers above the boxes indicate the exon number. Exons and intervening introns are shown in relative scale.

In functional studies, progressive expansion of the repeat length in the human AR has been associated with a linear decrease in transactivation function (Chamberlain et al., 1994). This supports the hypothesis that there is an optimal repeat length; shorter repeats may increase the level of receptor transactivation, thereby increasing the risk of prostate cancer (Irvine et al., 1995; MacLean et al., 1995; Hardy et al., 1996; Hakimi et al., 1997; Ingles et al., 1997). The polyglycine $(GGC)_n$ stretch normally ranges from 10-31 repeats (Lumbroso et al., 1997) and its decrease in size has also been linked with an increased risk of developing prostate cancer (Irvine et al., 1995; Hardy et al., 1996; Giovannucci et al., 1997). The average size of the polyproline stretch is 8-9 repeats (Hakimi et al., 1996; Giovannucci et al., 1997) but its role remains unknown.

The amino-terminal domain is involved in transcriptional activation (transactivation), via interactions with the basal transcriptional machinery or other accessory factors (coactivators, corepressors). Hence, it contains sequence information that optimises the transactivation capability of the AR (Jenster et al., 1995). Deletion of the NTD renders the AR transcriptionally inactive, despite its ability to bind androgen with high affinity (Simental et al., 1991; Jenster et al., 1995).
Two transactivation regions, designated as AF-1 and AF-5 (activating function-1 and -5, respectively) are located in the N-terminal domain of AR (Jenster et al., 1995; Chamberlain et al., 1996; Ikonen et al., 1997). Removal of either one of these domains results in 50% decrease in AR activity (Chamberlain et al., 1996). The activity of AF-1 is ligand-dependent, while AF-5 operates in a ligand-independent manner (Brinkmann et al., 1999).

1.12.2 The DNA-binding domain (DBD)

The central, DNA-binding domain of AR is the most highly conserved region in the nuclear receptor superfamily, supporting the functional importance of this region (Chang et al., 1988b; Lubahn et al., 1988a). The AR DBD shares 80% homology with the same domain of the glucocorticoid (GR) and progesterone (PR) receptors (Giguere et al., 1986; Gronemeyer et al., 1987; Kumar et al., 1987; Lubahn et al.,

1988a; Dobson et al., 1989). It is a cysteine-rich domain, and is encoded by exons 2 and 3. DBD recognises and interacts with specific DNA sequences (androgen-responsive elements, ARE) which are 15-base pair (bp) long sequences located in the flanking regulatory regions of androgen-responsive genes (Roche et al., 1992; Ho et al., 1993; Kuil et al., 1995). Specifically, the consensus DNA-binding site for the AR is comprised of two imperfect 6-bp indirect repeats separated by a 3-bp spacer, 5'-GGA/TACANNNTGTTCT-3' (Roche et al., 1992).

There are two structurally and functionally distinct zinc finger motifs present in this domain which are both necessary for transcriptional activation (Quigley et al., 1992a). Each zinc atom is tetrahedrically co-ordinated with four cysteines required for proper folding and sequence-specific DNA binding (Freedman et al., 1988). Nucleotides at the first zinc finger, referred to as the P box, are responsible for recognition of the specific target DNA sequence (Luisi et al., 1991; Quigley et al., 1992a). Nucleotides at the second zinc finger, referred to as the D box (Freedman, 1992), are responsible for orienting the receptor to bind the target DNA sequence. The D box also recognises the spacing between ARE half-sites, induces androgen-dependent dimerization of two receptor molecules during their association to DNA, stabilises the DNA-protein complex and finally directly activates transcription (Dahlman-Wright et al., 1991; Freedman, 1992; Wong et al., 1993; Warriar et al., 1994).

A ligand-independent nuclear localisation signal (NLS) has been identified in the hinge region (junction of exon 3 and 4) of the AR (Jenster et al., 1993). Mutations in the hinge region cause an almost complete cytoplasmic localisation of the receptor and absence of its transactivation capacity (Zhou et al., 1994). However, deletion of the hinge region still permits nuclear localisation in the presence of androgen, suggesting the presence of a second ligand-dependent NLS (Jenster et al., 1991). The second NLS has been identified and localised in the ligand-binding domain of the receptor (Jenster et al., 1993; Zhou et al., 1994).

1.12.3 The ligand-binding domain (LBD)

The ligand-binding domain shares a 50-55% homology with similar domains in the GR and PR receptors (Hollenberg et al., 1985; Trapman et al., 1988). LBD is a

hydrophobic domain encoded by exons 4 to 8. It is responsible for ligand binding and interacts with heat-shock proteins (hsp90, hsp70 and hsp56) in the absence of androgen (Marivoet et al., 1992; Nemoto et al., 1992; Veldscholte et al., 1992a; Stenoien et al., 1999). Androgen binding releases the receptor from the inhibitory complexes, causing a conformational change and allowing the DBD to bind to the ARE of a specific gene, thereby regulating its transcription (Jenster et al., 1991; Simental et al., 1991; Veldscholte et al., 1992b). Point mutations in the LBD of AR result in transcriptionally inactive receptors (Quigley et al., 1995), whereas extensive deletions of the LBD yield molecules with varying degrees of constitutive transcriptional activity, suggesting that in the absence of the ligand the LBD plays an inhibitory role (Jenster et al., 1991; Simental et al., 1991).

One ligand-dependent transactivation region (designated as AF-2) and a ligand-dependent dimerisation region have been identified in the LBD of AR (Jenster et al., 1991; Simental et al., 1991; Danielian et al., 1992; Wong et al., 1993)

Intramolecular interaction between the N-terminal transactivation and the C-terminal ligand-binding domain stabilises AR (Langley et al., 1995; Zhou et al., 1995), and modulates receptor dimerisation and DNA binding in the presence of androgen (Jenster et al., 1991; Simental et al., 1991; Nemoto et al., 1994).
Androgen-dependent intermolecular interaction between the NTD and LBD domains has been reported, suggesting an antiparallel arrangement of the two AR monomers (Wong et al., 1993; Langley et al., 1995; Langley et al., 1998). According to this model, AR binds to the ARE as a homodimer with each monomer recognising one half-site of the palindromic response element.

In summary, the broadly defined N-terminal, DNA-binding and ligand-binding domains are further divided into smaller sub-regions. These sub-regions are involved in different functions and interactions between different parts of the receptor molecule and are required for normal receptor action.

1.13 Expression and localisation of the androgen receptor

Similar to the expression of most other members of the steroid-thyroid-retinoid receptor superfamily, expression of AR is regulated in a tissue-, cell- and age-dependent manner.

Immmunohistochemical assays show AR to be predominantly expressed in the male reproductive tissues. Specifically, ARs are found in androgen-sensitive organs such as the prostate, seminal vesicles, hair follicles, sebaceous and preputial glands (Liao, 1975). In addition, high levels of AR are found in the kidney, liver, adrenal cortex, pituitary gland, endometrium, uterus and ovary (Takeda et al., 1990; de Winter et al., 1991; Kimura et al., 1993; Janssen et al., 1994; Wilson and McPhaul, 1996).

In the early developing prostate, AR is mainly expressed in the mesenchymal cells. In later stages of development, AR expression can be visualised in the epithelial cells. In the mature human prostate, the AR is present in the luminal epithelial and absent in the basal cell layer (de Winter et al., 1991). The level of AR expression in the stromal cells (fibroblasts and smooth muscle cells) seems to be variable (van Laar et al., 1989; de Winter et al., 1990; Husmann et al., 1990; de Winter et al., 1991).
A number of immunohistochemical investigations have also shown the expression of AR protein in both primary and hormone-refractory prostate cancers (de Winter et al., 1990; Tilley et al., 1990; Sadi et al., 1991; van der Kwast et al., 1991; de Winter et al., 1994; Hobisch et al., 1995; Taplin et al., 1995; Hobisch et al., 1996).

There has been much debate as to whether the AR protein is nuclear or cytoplasmic, and whether hormone-dependent nuclear localisation occurs. Nuclear localisation of AR in the absence of hormone has been reported in normal and castrated rats and in prostate cancer patients under hormonal ablation therapy (Lubahn et al., 1988a; Husmann et al., 1990; Sar et al., 1990; van der Kwast et al., 1991). In contrast, other studies have reported a nuclear AR expression only in the presence of androgen, while in the absence of hormone AR was predominantly localised in the cytoplasm with some nuclear staining (Tan et al., 1988; Jenster et al., 1991; Simental et al., 1991;

Kemppainen et al., 1992; Simental et al., 1992; Stenoien et al., 1999). The contradictory results obtained for AR localisation imply tissue-specific effects (Jenster et al., 1993) or differences in the experimental procedures, including fixation techniques (Jacobson et al., 1995).

Further evidence for the cytoplasmic localisation of AR in the absence of a ligand was provided by a recent study reporting that binding of filamin, an actin-binding protein to the HBD of AR was necessary for AR nuclear translocation (Ozanne et al., 2000).

1.14 Phosphorylation of the androgen receptor

The androgen receptor is a phosphoprotein (like ER, PR and GR) and modulation of its phosphorylation status influences ligand binding and consequently transcriptional activation of androgen-responsive genes (van Laar et al., 1991). AR is synthesised as a single 110kDa protein, which becomes rapidly phosphorylated (in the absence of hormone) to a 112kDa protein (van Laar et al., 1991; Kemppainen et al., 1992; Kuiper et al., 1992). Both isoforms can bind hormone and undergo hormone-dependent transformation into a nuclear binding form (van Laar et al., 1990; van Laar et al., 1991; Kuiper et al., 1992; Kuiper and Brinkmann, 1995). There is an almost two-fold increase in receptor phosphorylation within 30 min after hormone addition (van Laar et al., 1991). Phosphorylation in the absence of hormone is important for the acquisition of the hormone-binding capacity (Housley et al., 1982); while phosphorylation in the presence of hormone is involved in the transformation process and the regulation of gene transcription (Sheridan et al., 1988; Hoeck et al., 1989).

Phosphorylation mainly occurs in the NTD suggesting a role for phosphorylation in transcriptional activation (Kuiper et al., 1993; Sadar et al., 1999)

The 110kDa AR protein together with the two AR isoforms produced through hormone-independent and hormone-dependent post-translational phosphorylation (i.e. 112, and 114kDa, respectively), migrate as a triplet on a SDS-PAGE gel (Jenster et al., 1994; Bruggenwirth et al., 1997). All three isoforms (i.e. 110, 112, and 114kDa)

also exist in several androgen-responsive cell lines in the presence of androgens and migrate as a triplet (Brinkmann et al., 1999).

1.15 Androgen receptor abnormalities

The androgen receptor plays an important role in development, growth and behaviour. Therefore, any defect in the androgen receptor can result in abnormalities in male sex differentiation and development. There are two pathological conditions associated with abnormal AR structure and function: a) the Androgen Insensitivity Syndrome (AIS) and b) Spinal and Bulbar Muscular Atrophy (SBMA).

1.15.1 Androgen Insensitivity Syndrome (AIS)

Androgen Insensitivity Syndrome (AIS) is a form of male pseudo-hermaphroditism that occurs in males with a normal 46,XY karyotype. AIS has an X-linked recessive mode of inheritance (French et al., 1990). The syndrome affects between 1 in 20,000 and 1 in 60,000 newborn XY infants (Jagiello and Atwell, 1962). AIS results from a defective cellular response to androgen caused by an absent or dysfunctional AR and the phenotype is highly variable among affected individuals (Griffin, 1992; Patterson et al., 1994b). AIS is clinically classified into two forms: complete androgen insensitivity syndrome (CAIS or testicular feminisation syndrome, Tfm) and partial androgen insensitivity syndrome (PAIS or Reifenstein syndrome).

The complete androgen insensitivity syndrome (**CAIS**) is the most severe form. It results in complete lack of virilisation and hence a normal female appearance at birth. Males with CAIS have a normal female phenotype with a blind-ending vagina at birth, testis present internally instead of ovaries, breast development at puberty and no body hair.

The partial androgen insensitivity syndrome (**PAIS**) presents at birth when the infant is born with ambiguous or abnormal genitalia, which may be predominantly male or female in appearance. Genital abnormalities of males with PAIS vary, ranging from

primarily female with some virilisation such as clitomegaly or labial fusion to primarily male with undervirilisation such as hypospadias or micropenis.

The clinical heterogeneity of AIS is matched by a diversity of abnormalities of the AR receptor, varying from the absence of detectable androgen binding to alterations in the stability of the AR protein. More severe defects are caused by major alterations in the AR gene structure. However, the majority appears to be due to small defects such as single nucleotide changes (i.e. point mutations causing substitution of amino acids). The great majority of mutations are found in the hormone-binding domain of AR (Brown et al., 1988; Lubahn et al., 1989; Brown et al., 1990; Marcelli et al., 1990b; Sai et al., 1990; Marcelli et al., 1991; McPhaul et al., 1991a; McPhaul et al., 1991b), while only 20% of the mutations occur within the DNA-binding domain (Patterson et al., 1994a). Deletions (Brown et al., 1988; Trifiro et al., 1991; Quigley et al., 1992a; Quigley et al., 1992b), aberrant splicing (Ris-Stalpers et al., 1990; Brinkmann et al., 1991; Yong et al., 1994), and nonsense mutations (Marcelli et al., 1990a; Marcelli et al., 1990b; Sai et al., 1990; Trifiro et al., 1991) occur less frequently. The information regarding the nature and the location of the mutation, the androgen-binding affinity of the receptor, the clinical phenotype, and the family history of the patient are available from the AR mutation database (http://www.mcgill.ca/androgendb/; Gottlieb et al., 1998; Gottlieb et al., 1999).

1.15.2 X-linked Spinal and Bulbar Muscular Dystrophy (SBMA or Kennedy's syndrome)

Spinal and bulbar muscular atrophy (SBMA or Kennedy's syndrome) affects adult males and is a rare X-linked hereditary disease characterised by a progressive degeneration of motor neurons that leads to muscle weakness and atrophy (Kennedy et al., 1968; Sobue et al., 1989). The onset of the disease usually occurs between the third and the fourth decade in affected males (Kennedy et al., 1968). Males with SBMA may also have gynecomastia, reduced fertility and show partial androgen insensitivity, suggesting the presence of a dysfunctional androgen receptor (Brooks and Fischbeck, 1995).

Expansion of the polyglutamine (CAG) stretch in the N-terminal domain of the AR (>40 residues; normal: 8-31) is associated with reduced androgen-responsive gene transcription and reduced affinity for androgens (La Spada et al., 1991). This altered CAG repeat length does not interfere with the role of AR in masculinisation but does interfere with normal AR function in motor neurons at adulthood (La Spada et al., 1991). Androgen receptors are present in the normal brain and spinal cord (Matsuura et al., 1993), but their role there is unknown.

1.16 Androgen receptor abnormalities in prostate cancer

Progression of androgen-dependent prostate cancer to androgen-independent disease has been studied extensively and is suggested to be associated with changes in the structure and function of AR as well as with changes in the androgen-signalling pathway. However, the molecular events leading to endocrine therapy failure are not fully understood.

Changes of the AR gene that have been implicated in the development and progression of prostate cancer include: a) mutations, b) gene amplification, c) activation in a ligand-independent manner by growth factors and cytokines d) amplification of coactivators.

1.16.1 Androgen receptor gene mutations

AR mutations have been found in clinical prostate cancer, both prior to hormonal therapy (untreated) and in hormone-refractory disease. However, the frequency of AR mutations at different stages of the disease is poorly documented and contradictory results have been published.

The vast majority of the studies suggest that mutations are rare in both untreated early- and late-stage as well as in hormone-refractory recurrent prostate cancers (Newmark et al., 1992; Culig et al., 1993a; Culig et al., 1993b; Suzuki et al., 1993; de Winter et al., 1994; Schoenberg et al., 1994; Visakorpi et al., 1995; Evans et al., 1996; Hakimi et al., 1996; Koivisto et al., 1997; Wallen et al., 1999). These reports support

the hypothesis that the absence of AR mutations in the majority of early-stage tumours suggests that the role of androgen in the development of clinical prostate cancer is mediated predominantly by a normal AR gene.

However, there are three papers which report mutations at high frequency in both untreated and hormone-refractory prostate cancers and suggest a potential role of AR mutations in contributing to the hormone-insensitivity (Gaddipati et al., 1994; Taplin et al., 1995; Tilley et al., 1996). The presence of mutations in the AR in early disease suggests a cell-selective growth advantage in reduced levels of androgens, while mutations present in late-stage disease might allow the development of hormone resistance.

Most mutations have been located in the AR region encoding the ligand-binding domain of the receptor (Newmark et al., 1992; Culig et al., 1993a; Suzuki et al., 1993; Taplin et al., 1995). These mutations result in a promiscuous receptor with a broad hormone-binding and transactivation spectrum. Mutated receptors with an unusually high affinity for estrogens, progestagens or anti-androgens have been described (Veldscholte et al., 1990a; Veldscholte et al., 1990b; Culig et al., 1993a; Suzuki et al., 1993; Gaddipati et al., 1994; Taplin et al., 1995). These receptors might provide a selective advantage for the cancer cells carrying them and may contribute in the progression of the tumour (Koivisto et al., 1998).

It is unclear whether androgen ablation itself favours the growth or persistence of cells that express mutant AR genes, or whether the increased frequency of cells with mutant AR genes in metastatic lesions is a consequence of the natural course of the disease.

1.16.2 Androgen receptor gene amplification

Androgen receptor gene amplification has been reported in 30% of samples from patients with locally recurrent disease after endocrine therapy failure and also from patients prior to androgen ablation therapy (Visakorpi et al., 1995; Koivisto et al., 1997; Koivisto and Helin, 1999; Wallen et al., 1999; Palmberg et al., 2000; Ware et

al., 2000). Amplification is not present in the primary tumours available from patients with recurrent disease or in recurrent tumours from patients who had not received endocrine therapy, suggesting that it is a result of androgen deprivation therapy. The increased AR mRNA levels observed in tumours with AR gene amplification are suspected to increase AR protein levels, thereby enhancing the ability of cells to grow in reduced levels of androgens, and contributing to therapy resistance.

1.16.3 Androgen receptor activation by growth factors and cytokines

There is evidence that AR can be activated in the absence of its cognate ligand. Such activation is termed ligand-independent activation and has been suggested to occur in androgen-deprived cells and androgen-independent prostate cancer cells leading to disease progression. Specifically, AR has been shown to be activated in the absence of androgen by various growth factors, protein kinase A (PKA) activators and compounds that elevate second messenger cyclic adenosine monophosphate (cAMP) levels (Culig et al., 1994; Nazareth and Weigel, 1996; Sadar, 1999). However, the mechanism of such ligand-independent AR activation has not been defined.

Prostatic cells, both stromal and epithelial, express a number of growth factors and growth factors receptors that are part of signal transduction pathways and transmit biochemical messages from the cell surface to the nucleus. Signal interruption or aberrant signalling might result in altered cell proliferation, and promote invasion and metastasis.

In transfection experiments where cells were exposed to growth factors such as the insulin-like growth factor-I (IGF-I), the keratinocyte growth factor (KGF), and the epidermal growth factor (EGF), aberrant AR activation was observed in the absence of androgens. The pure anti-androgen Casodex completely inhibited AR activation, indicating that the effects were AR-mediated (Culig et al., 1994; Ikonen et al., 1994; Brass et al., 1995; Culig et al., 1995; Nazareth and Weigel, 1996; Culig et al., 1997; Ye et al., 1999). As growth factors cause tyrosine phosphorylation of various effector molecules upon binding to their receptors, it is thought that such phosphorylation

events signal AR to enhance its transactivation property (Reinikainen et al., 1996). However, it is still unclear how signalling of kinases results in activation of AR.

Growth factors have been implicated in prostate carcinogenesis by supporting the proliferation of transformed cells and enhancing cell motility and invasion. IGF-I and EGF are potent mitogens of prostate epithelial cells (Chaproniere and McKeehan, 1986; Cohen et al., 1991). High levels of IGF-I are associated with increased risk for prostate cancer (Chan et al., 1998). Increased levels of IGF-I have been found in bone cells and it is suggested that they are involved in metastatic spread to the bone by supporting the growth of prostate cancer cells (Centrelia et al., 1990). There is also evidence that IGF-II increases AR expression in LNCaP cells (Gnanapragasam et al., 2000a). The human prostatic secretion contains high EGF levels which are regulated by serum androgen concentrations (Gregory et al., 1986; Hiramatsu et al., 1988; Jacobs et al., 1988). EGF receptor and its ligands, EGF and TGF-α stimulate prostate epithelial and stromal cell growth *in vitro* (Chaproniere and McKeehan, 1986). The levels of the EGF receptor in prostate cancer are negatively related to tumour grade (Maddy et al., 1989). KGF (FGF7) is a member of the fibroblast growth factor (FGF) family and is involved in modulation of prostate cell proliferation by mediating signal transmission between the stromal and epithelial compartments of the prostate (Yan et al., 1992; Ropiquet et al., 1999). KGF is overexpressed in androgen-independent prostate cancer (Leung et al., 1997).

Activation of AR in a ligand-independent manner through a protein kinase A (PKA) signalling pathway or elevation of intracellular levels of cAMP has also been reported (Ikonen et al., 1994; Nazareth and Weigel, 1996; Sadar et al., 1999). Activators of PKA (forskolin), can activate AR either directly or indirectly by increasing intracellular cAMP (Ikonen et al., 1994; Nazareth and Weigel, 1996; Culig et al., 1997). LHRH peptide is a cellular regulator that uses cAMP as a second messenger system and increases intracellular cAMP levels. LHRH is used in prostate cancer endocrine therapy and was reported to cause weak activation of AR in the absence of androgens, while there was a synergism between LHRH and androgen for enhanced AR activation (Culig et al., 1997).

Interleukin-6 (IL-6) was shown to stimulate AR-mediated reporter gene transcription in the absence of androgens in AR-transfected DU145 cells (Hobisch et al., 1998). AR activation was completely inhibited by the pure AR antagonist Casodex, indicating that the effects were AR-mediated. IL-6 is a pleiotropic cytokine, which regulates antigen-specific immune responses and inflammatory reaction (Kishimoto et al., 1992). It is implicated in the growth and differentiation of several tumours and levels are frequently elevated in the sera of prostate cancer patients (Seymour et al., 1995; Twillie et al., 1995; Seymour and Kurzrock, 1996; Drachenberg et al., 1999). IL-6 receptors are expressed in prostate cancer cell lines, in BPH and prostate cancer specimens (Siegall et al., 1990; Siegsmund et al., 1994; Borsellino et al., 1995).

Mitogen-activated protein kinase kinase kinase 1 (MEKK1) was also shown to stimulate AR transcriptional activity in the absence of androgens in AR-transfected DU145 cells (Abreu-Matrin et al., 1999). MEKK1 plays an important role in signaling involving the stress response (Derijard et al., 1994; Chen et al., 1996) and NFkB activation (Lee et al., 1997). Constitutive activation of MEKK1 induces apoptosis in AR-positive but not in AR-negative prostate cancer cells. Reconstitution of the AR signalling pathway in AR negative prostate cancer cells restores MEKK1-induced apoptosis (Abreu-Matrin et al., 1999).

1.16.4 Amplification of androgen receptor coactivators

Studies within the past six years have led to the identification of a number of intermediary proteins that interact with nuclear hormone receptors and play essential roles in mediating their transcriptional effects.

Several putative cofactors (coactivators or corepressors) for steroid receptors have been identified. It is suggested that these cofactors play essential roles in the regulation of target gene transcription by interacting with general transcription factors and remodelling of the chromatin (Chen et al., 1997; Jenster et al., 1997).

Cellular and promoter specificity of AR transactivation is determined to a large extent by the occurrence and relative abundance of other transcription factors and receptor cofactors in different tissues. Cofactors can either enhance (coactivators) or repress (corepressors) receptor activity (Horwitz et al., 1996; Heery et al., 1997) (Table 1.2).

Table 1.2 Androgen receptor interacting proteins

Name		Function	Interacts with	Reference
ANPK	AR-interacting nuclear protein kinase	coactivator	DBD-hinge	(Moilanen et al., 1998a)
ARA160	AR-associated protein 160	coactivator	NTD, DBD-LBD	(Hsiao and Chang, 1999)
ARA24	AR-associated protein 24	coactivator	NTD	(Hsiao et al., 1999)
ARA54	AR-associated protein 54	coactivator	LBD	(Kang et al., 1999)
ARA55	AR-associated protein 55	coactivator	LBD	(Fujimoto et al., 1999)
ARA70	AR-associated protein 70	coactivator	LBD	(Yeh and Chang, 1996)
ARIP3	AR-interacting protein 3	coregulator	DBD	(Moilanen et al., 1999)
ARIP4	AR-interacting protein 4	coactivator	DBD-hinge	(Janne et al., 2000)
BRCA1	Breast cancer susceptibility gene 1	coactivator	NTD	(Park et al., 2000)
c-jun		coregulator	DBD-hinge	(Bubulya et al., 1996)
calreticulin		corepressor	DBD	(Dedhar et al., 1994)
CBP	cAMP response element-binding protein	coactivator	NTD, LBD (rAR)	(Aarnisalo et al., 1998)
Cyclin D1		corepressor	DBD-LBD	(Knudsen et al., 1999)
Cyclin E		coactivator		(Yamamoto et al., 2000)
ERM	Ets family member	corepressor	NTD, DBD, HBD	(Schneikert et al., 1996)
GRIP-1	GR-interacting protein 1	coactivator	NTD, LBD	(Hong et al., 1997)
PDEF	prostate-derived Ets factor	coactivator	DBD	(Oettgen et al., 2000)
RAF	receptor accessory factor	coactivator	NTD-DBD	(Kupfer et al., 1994)
RB	retinoblastoma	coactivator	NTD-DBD	(Yeh et al., 1998)
RIP140	receptor interacting protein	coactivator	LBD	(Ikonen et al., 1997)
SNURF	small nuclear RING finger protein	coactivator	DBD-hinge	(Moilanen et al., 1998b)
SRC-1	steroid receptor coactivator 1	coactivator	NTD, LBD	(Alen et al., 1999)
TFIIH	transcription factor IIH	coactivator	NTD	(Lee et al., 2000)
TIF2	transcriptional intermediary factor 2	coactivator	LDB	(Voegel et al., 1998)
Tip60	Tat-interacting protein 60	coactivator	LBD	(Brady et al., 1999)
TR4	testicular orphan receptor	corepressor	NTD, DBD, LBD	(Lee et al., 1999)
TRAM-1	thyroid hormone receptor activator molecule 1	coactivator	NTD, DBD-LBD	(Tan et al., 2000)
Ubc9	ubiquitin-conjugating enzyme homologue	coactivator	DBD-hinge	(Poukka et al., 1999)

Adapted from http://www.mcgill.ca/androgendb/ (compiled by Beitel LK, 2000)

Cofactors do not bind directly to the basal transcriptional apparatus, and it is unlikely they function as bridging molecules for the AR and the basal transcriptional initiation factors.

Miyamoto et al. (1998) reported that in transient transfection experiments, anti-androgens could promote interaction between AR and its coactivator ARA70, enhancing its AR transcriptional activity. Anzick et al. (1997) reported that AIB1 (amplified in breast cancer), an ER coactivator, is amplified and overexpressed in several ER-positive breast and ovarian cancer cell lines, as well as in primary breast cancer specimens.This implies that altered expression of AIB1 may contribute to the development of hormone-dependent cancers.

Because cofactors mediate receptor activity, an increased coactivator expression will result in an increased receptor transactivation. However, their relevance to prostate tumour progression has yet to be established.

In summary, the key mechanisms thought to be involved in the failure of endocrine therapy in advanced prostate cancer include changes in the AR signalling pathway. These changes can cause either selective clonal growth of cells never responsive to hormonal therapy or adaptation to an androgen-depleted environment (hyper-sensitivity). Hypersensitivity means that proliferation and/or expression of prostate-specific genes may be stimulated by extremely low doses of androgens, by other steroids (estrogens, progestins), AR antagonists, growth factors or substances that increase intracellular kinase activity.

1.17 Thesis Aims

The development and progression of prostate cancer is a multistep process involving oncogenes, tumour supressor genes, growth factors and the AR gene. Any alterations causing disruption in the normal regulation of the pathway can result in uncontrolled cellular proliferation.

The androgen-signalling pathway is one of the possible sites of intervention in prostate cancer prevention efforts and has therefore been extensively studied. However, lack of functionally relevant model systems of advanced prostate cancer has limited prostate cancer research (Dijkman and Debruyne, 1996). In the majority of the model systems, lack of significant AR gene expression underlies their androgen-independence.

The purpose of the work described in this thesis was to investigate the role of the AR in the development and progression of prostate cancer, and in particular, the regulation of AR gene expression in hormone-relapsed prostate cancer. Specifically, the aims were to:

a) Establish a new *in vitro* model for metastatic prostate cancer which more closely resembles the situation in the androgen-ablated patients than previous models.
b) Utilise the *in vitro* model in order to study, in a comparative fashion, the percentage of AR positivity and testosterone sensitivity, and investigate the effect of AR expression on the regulation of the androgen-induced signalling pathway.

The implication from all AR studies is that the AR (normal or mutated) has a critical biological role in a subgroup of patients with advanced prostate cancer. Therefore, identification of the molecular basis of AR activation and expression in androgen-independent disease will contribute in distinguishing tumours with a high risk of progression from those with a low risk and subsequently, in developing a more effective therapy for advanced prostate cancer.

Chapter 2

Materials and Methods

All experiments were carried out according to the university's safety rules; chemicals and reagents were handled according to COSHH (Control Of Substances Hazardous to Health) regulations. All bacterial work was carried out under aseptic conditions and tissue culture work was performed in a class II tissue culture cabinet.

MATERIALS

2.1 Standard materials

All chemicals and solvents were of analytical or molecular biology grade and were purchased from either Sigma-Aldrich Co. Ltd., UK or British Drug Houses (BDH) Ltd., UK, unless otherwise stated. All restriction endonucleases and their buffers were purchased from GibcoBRL (Life Technologies Ltd., UK).

2.2 Cell culture reagents

All materials used in routine cell culture were of cell culture grade and were purchased from GibcoBRL (Life Technologies Ltd., UK). Fetal bovine serum (FBS) was purchased from Sigma-Aldrich Co. Ltd., UK. Mibolerone was purchased from NEN (Life Science Products Inc., USA). All plasticware for cell culture use was manufactured by Nunc Ltd., UK.

2.3 Buffers

All solutions were prepared using distilled and deionised water and were stored at room temperature (RT) unless otherwise stated in the text.

2.3.1 Buffers for DNA extraction and conventional gel electrophoresis

Chloroform:	As used in organic/aqueous extractions: 24:1 (v/v) chloroform : isoamyl alcohol
Phenol:	Phenol liquified, under 100mM Tris-Cl buffer (pH 8.0)

Phenol/chloroform: 1:1 mixture of the above with 24:1 (v/v) chloroform : isoamyl alcohol

Dish I (Resuspension buffer): 50mM glucose, 10mM Na_2EDTA (pH 8.0), 25mM Tris-Cl

Dish II (Lysis buffer): 0.2M NaOH, 1% (w/v) SDS

Dish III (Neutralising buffer): 3M KOAc

Lysis buffer: 0.33M sucrose, 10mM Tris-Cl (pH 7.5), 3mM $MgCl_2$, 1% (v/v) Triton X-100

Incubation buffer: 75mM NaCl, 25mM Na_2EDTA (pH 8.0), 0.5% (w/v) SDS, 200µg/ml Proteinase K (Sigma)

TE: 10mM Tris-Cl, 1mM Na_2EDTA (pH 8.0)

50 x TAE: 2M Tris-acetate, 0.05M Na_2EDTA

10 x TBE: 0.90M Tris-borate, 0.02M Na_2EDTA (pH 8.3)

Loading buffer for DNA (5x): 5 x TBE, 15% (w/v) Ficoll-400, 0.05% (w/v) bromophenol blue

Loading buffer for DNA (5x): 5 x TBE, 15% (w/v) Ficoll-400, 0.05% (w/v) xylene cyanol

2.3.2 Buffers for RNA extraction and conventional gel electrophoresis

GTC Extraction buffer: 4M guanidinium isothiocyanate, 25mM sodium citrate (pH 7.0), 0.5% (w/v) Sarkosyl, 1% (v/v) antifoam A, 0.104M 2-mercaptoethanol in DEPC-treated water (0.1% v/v) to minimise contamination from RNAases

Loading buffer for RNA (2x): 95% (v/v) formamide, 18mM Na_2EDTA, 0.025% (w/v) bromophenol blue, 0.025% (w/v) xylene cyanol, 0.025% (w/v) SDS

2.3.3 Buffers for Western blotting

Extraction buffer: 50mM Tris (pH 7.5), 1mM Na_2EDTA, 250mM NaCl, 0.1% (v/v) Triton X-100, 2µg/ml

Leupeptine, 1μg/ml Pepstatine, 97μg/ml PMSF, 2μg/ml Aprotonine

Electrode running buffer (5x): 25mM Tris (pH 8.3), 192mM Glycine, 0.1% (w/v) SDS

Loading buffer (2x): 125mM Tris (pH 6.8), 6% (w/v) SDS, 20% (v/v) glycerol, 0.02% (w/v) bromophenol blue, 10% (v/v) 2-mercaptoethanol

Transfer buffer (pH 8.1 to 8.4): 25mM Tris, 192mM Glycine, 20% (v/v) methanol

2.3.4 Buffers for transfection

2 x HBS (**H**EPES-**B**uffered **S**aline): 280mM NaCl, 10mM KCl, 1.5mM $Na_2HPO_4 \cdot 2H_2O$, 12mM Dextrose, 50mM HEPES (pH adjusted to 7.05 with 0.5M NaOH)

2.4 Cell lines

The established cell lines used, their characteristics and their culture conditions are shown in Table 2.1 and Table 2.2, respectively.

Table 2.1 Cell lines and their characteristics

Cell line	First described	Cell type	Origin	AR gene	AR protein	PSA gene	PSA protein
DU145	Stone et al., 1978	human prostate epithelial	brain metastasis	wild type (wt)	absent	wt	absent
DUSF	*	human prostate epithelial	**DU**145 in **S**erum- **F**ree medium	wt	absent	wt	present **
PC-3	Kaighn et al., 1979	human prostate epithelial	bone metastasis	wt	absent	wt	absent
LNCaP	Horoszewicz et al., 1980	human prostate epithelial	lymph node metastasis	mutated ^{868}Thr→Ala	present	wt	present
COS-1	Gluzman, 1981	monkey kidney fibroblasts	SV40-transformed CV-1 cells	absent	absent	absent	absent

* Developed by J.R. Masters, Institute of Urology, UCL

** As shown from the work described in this thesis.

Table 2.2 Cell lines and their culture conditions

Cell line	Source	Culture medium
DU145	Stone et al.	RPMI 1640, 8% (v/v) FBS, 2mM L-glutamine
DUSF	Institute of Urology, UCL	OPTIMEM
PC-3	Kaighn et al.	RPMI 1640, 8% (v/v) FBS, 2mM L-glutamine
LNCaP	Horoszewicz et al.	RPMI 1640, 8% (v/v) FBS, 2mM L-glutamine
COS-1	Imperial Cancer Research Fund (ICRF)	RPMI 1640, 8% (v/v) FBS, 2mM L-glutamine

2.5 Antibodies

Titration experiments were performed to establish the hybridisation conditions of the antibodies used for the Western analyses. The hybridisation conditions of the antibodies used for immunocytochemical analysis were determined previously in the laboratory (Fry et al., 2000). The antibodies used and their hybridisation conditions are shown in the table below:

Table 2.3 Antibodies and their hybridisation conditions

Antibodies	Clone	Species	Source	Hybridisation conditions
Anti-human AR monoclonal	F39.4.1	mouse	Biogenex	(WESTERN) 1:1000 in 1x PBS/0.1% (v/v) Tween, 3% (w/v) milk
				(ICC) 1:100 in 1x PBS
Anti-human AR monoclonal	G122-434	mouse	PharMingen	(WESTERN) 1:1000 in 1x PBS/0.1% (v/v) Tween, 3% (w/v) milk
Anti-mouse peroxidase-conjugated		rabbit	Dako	(WESTERN) 1:5000 in 1x PBS/0.1% (v/v) Tween, 3% (w/v) milk
Anti-mouse biotinylated		horse	Vector Labs	(ICC) 1:200 in 1x PBS, 1.5% (v/v) normal serum

2.6 Plasmids

The following plasmid constructs were used:

- pTetOff
- pTRE-Luc
- pTRE
- pcDNA-AR
- pSVARo
- pCMVhAR

2.6.1 pTetOff plasmid

This is a commercially available mammalian expression vector provided as part of the TetOff™ gene expression system (CLONTECH Laboratories, Inc., USA). It expresses the tTA regulator protein under the control of the P_{hCMV} (human cytomegalovirus) promoter and was originally described as pUHD15-1neo (Gossen and Bujard, 1992; Resnitzky et al., 1994) (Figure 3.2b).

2.6.2 pTRE-Luc plasmid

This is a commercially available mammalian expression vector provided as part of the TetOff™ gene expression system (CLONTECH Laboratories, Inc., USA). It carries the tet-responsive element (TRE) which is activated by the tTA protein, thereby switching on transcription of the luciferase gene. The luciferase gene is located downstream of the minimal immediate early promoter of cytomegalovirus (P_{minCMV}). This promoter is dependent upon activation from tTA and is otherwise virtually silent. It was originally described as pUHC13-3 (Gossen and Bujard, 1992) (Figure 3.2b).

2.6.3 pTRE plasmid

This is a commercially available mammalian expression vector provided as part of the TetOff™ gene expression system (CLONTECH Laboratories, Inc., USA). It carries the tet-responsive element (TRE) which is activated by the tTA protein, thereby switching on transcription of the gene of interest. The gene of interest is located downstream of the minimal immediate early promoter of cytomegalovirus (P_{minCMV}). This promoter is dependent upon activation from tTA and is otherwise virtually silent. It was originally described as pUHD10-3 (Gossen and Bujard, 1992).
This plasmid was used to construct the pTRE-AR vector where AR cDNA from pcDNA-AR was subcloned into pTRE (Figure 3.5).

pTetOff, pTRE-Luc and pTRE plasmids were a kind gift from Dr. P. Corish, University of Oxford, UK.

2.6.4 pcDNA-AR plasmid

This mammalian expression vector was kindly provided by Dr. C.N. Robson, University of Newcastle, UK. It contains a wild type, full-length human AR cDNA subcloned into the *Xba*I site of the pcDNA3 plasmid (Invitrogen, Netherlands). Specifically, the AR insert represents the complete coding sequence with a *Xba*I PCR-generated site immediately adjacent to the ATG starting codon. At the 3′ end, there is ~0.4kb of 3′ untranslated region before the *Bam*HI restriction site from the pGEM-3Z plasmid (Chang et al., 1988a) (Figure 4.1).
The AR cDNA from pcDNA-AR was used to construct pTRE-AR expression vector (Figure 3.5).

2.6.5 pSVARo plasmid

This mammalian expression vector was kindly provided by Dr. J. Trapman, Erasmus University Rotterdam, The Netherlands. It contains a wild type, full-length human AR cDNA subcloned into the *Sal*I site of the pBR328A plasmid (Van Heuvel et al., 1986; Trapman et al., 1988; Brinkmann et al., 1989) (Figure 5.1).

2.6.6 pCMVhAR plasmid

This mammalian expression vector was kindly provided by Dr. R. Buettner, University Hospital, Regensburg, Germany. The full-length coding sequence of a wild type human AR is contained within the *Bgl*II-*Bsp*HI (blunt) cDNA insert subcloned into the pCMV5 plasmid (Anderson et al., 1989) (Figure 5.2). It was originally described as p5HBhAR-A (Brown et al., 1990).

METHODS

MOLECULAR BIOLOGY TECHNIQUES

2.7 Preparation of DNA from cultured cells

Cells grown to approximately 80% confluency after removal of culture media were washed with 0.25% (w/v) trypsin in 0.5mM versene to detach the monolayer of cells and were counted (see section 2.18 and 2.19). Approximately one to two million cells were lysed at 4°C for at least 2 hours using 5ml of lysis buffer (see section 2.3.1). The cells were then centrifuged at 3,000rpm in a Sorvall Super T21 centrifuge (Du Pont Ltd., UK) at 4°C for 10 min. The pellet was resuspended in 1.5ml incubation buffer (see section 2.3.1) and incubated with gentle agitation for 2 hours at 37°C. Two organic extractions were performed by adding equal volumes of phenol:chloroform: isoamyl alcohol (25:24:1), mixing by vortexing and spinning at 3,000rpm for 10 min. The aqueous upper (DNA-containing) phase was carefully removed and 0.1 volumes of 3M sodium acetate (pH 5.2) followed by two volumes of 100% ethanol were added. DNA was allowed to precipitate for half an hour at -70°C (or at -20°C for at least 2 hours) and recovered by centrifugation in an Eppendorf centrifuge (13,000rpm) for 10 min at RT. The supernatant was discarded and the pellet washed in 70% (v/v) ethanol, centrifuged again and air-dried for approximately 20 min at RT before dissolving in water.

2.8 Isolation and purification of plasmid DNA

2.8.1 Small scale (mini prep)- Alkaline lysis method

A single colony of transformed *E.coli* cells was inoculated into 10-20ml of Luria Broth (LB), containing the appropriate antibiotic. The culture was incubated for 12-16 hours at 37°C with vigorous agitation. Cells were pelleted by centrifugation in a Sorvall Super T21 centrifuge (Du Pont Ltd.) at 3,000rpm for 10 min. The pellet was resuspended in 300µl of Dish I containing lysozyme at 5mg/ml (see section 2.3.1), transferred into a 1.5ml eppendorf tube and incubated for 5 min at RT. Then 600µl of

freshly made Dish II (see section 2.3.1) were added, mixed gently by inverting and the mixture placed on ice for 5 min. Finally, 450µl of chilled Dish III (see section 2.3.1) were added, mixed by vortexing and left on ice for 20 min. Cell debris and bacterial DNA were pelleted by centrifugation (13,000rpm) at 4°C for 15 min and the supernatant (plasmid DNA) was transferred into a clean 1.5ml eppendorf tube. One organic extraction was carried out by adding 600µl of phenol:chloroform:isoamyl alcohol (25:24:1), mixing by vortexing and spinning at 13,000rpm for 2 min. The aqueous upper (DNA-containing) phase was carefully removed and two volumes of ice-cold 100% ethanol were added. DNA was allowed to precipitate at -20°C for 15-30 min and recovered by centrifugation at 13,000rpm for 15 min at RT. The supernatant was discarded and the pellet was washed and dissolved in water, as described in section 2.7.

2.8.2 Large scale (maxi prep)

A single colony of transformed *E.coli* cells was inoculated into 5-10ml of LB, containing the appropriate antibiotic. The culture was incubated at 37°C with vigorous agitation for 6-8 hours until turbid. It was then transferred into 250ml of LB, containing the appropriate antibiotic and left shaking (250rpm) for 12-16 hours in the 37°C incubator. Cells were pelleted by centrifugation (6,000rpm in a Sorvall centrifuge) at 4°C for 10 min. The pellet was completely resuspended in 10ml Dish I (see section 2.3.1) by vortexing until no cell clumps were visible and placed on ice. Twenty ml of freshly made Dish II (see section 2.3.1) were then added dropwise and the mixture was incubated on ice for no more than 10 minutes. Finally, 10ml of ice-cold Dish III (see section 2.3.1) were added, mixed by swirling and left on ice for at least 30 min. After centrifugation (10,000rpm at 4°C for 10 min) the supernatant was filtered through an absorbent cotton gauze (BP type 13 light by Smith & Nephew Textiles Ltd., UK) into a clean tube. DNA was precipitated by adding two volumes of 100% ethanol and recovered by centrifugation at 10,000rpm for 10 min at RT. The supernatant was discarded and the pellet washed in 70% (v/v) ethanol, centrifuged again and air-dried for approximately 20 min at 37°C before resuspending in 4ml TE buffer.

To purify the plasmid DNA, ethidium bromide-caesium chloride (EtBr-CsCl) density gradient centrifugation was then performed. A density gradient is produced by centrifugation of a CsCl solution at a very high speed. Macromolecules present in the CsCl solution form bands at distinct points in the gradient depending on their buoyant density (Figure 2.1a). Density gradient centrifugation in the presence of EtBr is used to separate supercoiled DNA from non-supercoiled molecules. EtBr binds DNA causing partial unwinding of the helix which results in a decrease about 0.125g/cm^3 in buoyant density for linear DNA. Supercoiled DNA, having no free ends, can bind only a limited amount of EtBr causing a decrease in buoyant density of about 0.085g/cm^3. When the cell lysate is subjected to the above procedure, plasmid DNA is separated from linear bacterial DNA (Figure 2.1b).

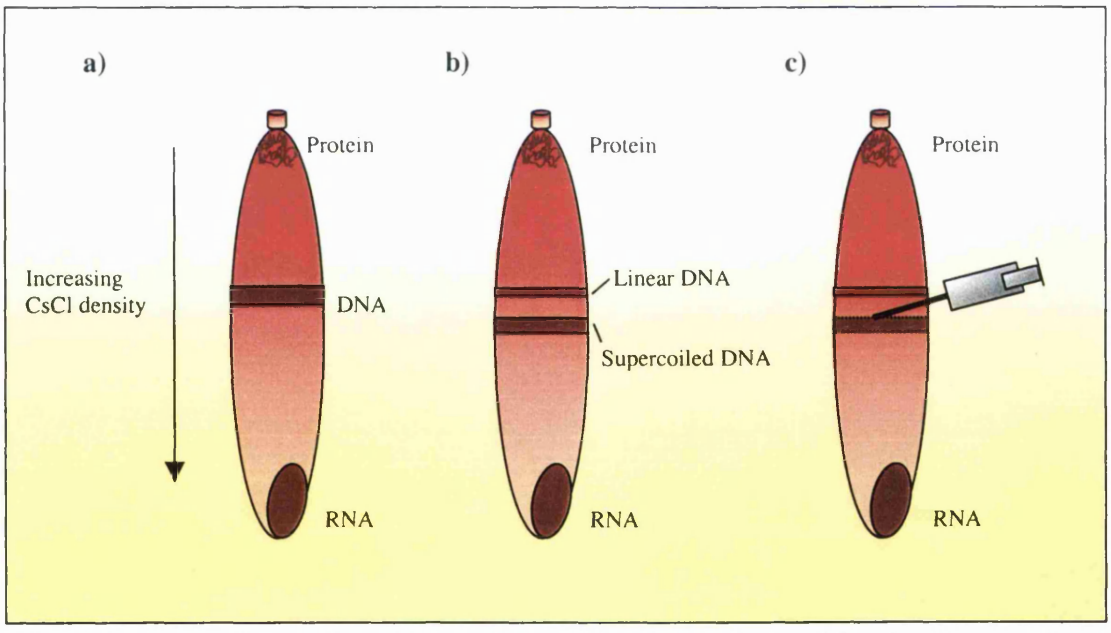


Figure 2.1 **Ethidium bromide-caesium chloride (EtBr-CsCl) density gradient centrifugation.** a) Separation of macromolecules in a density gradient, b) Density gradient in the presence of EtBr separates supercoiled (plasmid) DNA from linear (bacterial) DNA, and c) Removal of supercoiled (plasmid) DNA.

Subsequently, 4.8g of CsCl were added to the 4ml DNA suspension, the tube was gently inverted to dissolve the CsCl before adding 500µg/ml EtBr. The mixture was then centrifuged (3,500rpm in a Denley BS400 centrifuge) for 10 min, and the clear red solution was transferred into a Quick-Seal tube (Beckman Ltd., UK). The tubes

were balanced and spun overnight (12-16 hours) at RT in a Beckman L-80 ultra-centrifuge (NVT 90 rotor) at 70,000rpm.

Next day, the plasmid DNA band was removed by puncturing the side of the Quick-Seal tube and withdrawing it into a syringe (Figure 2.1c). The EtBr bound to plasmid DNA was extracted at least three times with equal volumes of isoamyl alcohol saturated with water. The mixture was spun each time at 3,000rpm for 3 min (Denley BS400 centrifuge) and the aqueous lower (DNA-containing) phase was removed. Two volumes of water were then added to reduce CsCl concentration and the DNA was precipitated with two volumes of ice-cold 100% ethanol at –20°C for at least 15 min.

The DNA pellet was recovered by centrifugation in a Sorvall centrifuge (10,000rpm) for 15 min at 4°C, resuspended in 1ml of water and transferred into an 1.5ml eppendorf. Three organic extractions, as described in section 2.7, were performed: two with phenol:chloroform:isoamyl alcohol (25:24:1) and one with chloroform :isoamyl alcohol (24:1). Each time, the aqueous upper (DNA-containing) phase was carefully removed into a clean 1.5ml eppendorf. DNA was precipitated, washed and dissolved in water (see section 2.7).

2.9 Measurement of DNA concentration

DNA concentrations were measured by UV absorbance spectrophotometry using a DU650 series spectrophotometer (Beckman Ltd., UK). Genomic or plasmid DNA was diluted (1/100) in TE buffer and 500µl were added to a microcuvette. The spectrophotometer was blanked with TE buffer, the density of the sample was measured at 260nm and at 280nm and the DNA concentration was calculated as follows:

$$OD_{260} \times 50\mu g/ml \times \text{dilution factor}$$

One OD unit at 260nm is defined as 50µg/ml for double stranded DNA. The 260nm/280nm ratio represents the purity of the sample. In high quality DNA, free of contaminants such as protein or phenol, the 260nm/280nm ratio ranges from 1.8-2.0. Concentration and quality of DNA were also verified by conventional gel electrophoresis (see section 2.11).

2.10 Restriction enzyme digestion

Restriction digest analysis was performed after plasmid DNA preparation to verify the integrity of the plasmid. A series of digests was carried out and the number and sizes of the fragments produced by each digest were compared to the plasmid's known restriction map. Restriction digests were also carried out before subcloning to linearise the vector and excise the DNA fragment to be cloned.

Restriction digests were done either in buffers supplied or recommended by the manufacturers, although compromises were sometimes made in double digests. Digests of genomic DNAs were carried out for 12-16 hours, using a three-fold excess of enzyme; plasmid digestions were performed for 2-16 hours, with an excess of enzyme between 2 and 10-fold, at the recommended temperature, usually 37°C. One unit of enzyme is generally defined as the amount required to digest 1μg of DNA in one hour at the appropriate temperature.

2.10.1 Plasmid digest precipitation

Plasmid digests carried out for subcloning purposes were performed in a large volume and were precipitated before agarose electrophoresis by the following procedure: sodium chloride was added to a final concentration of 200mM, followed by 2 volumes of ice-cold 100% ethanol. DNA was allowed to precipitate on ice for 15 min, and was recovered by spinning in an Eppendorf centrifuge (13,000rpm) for 5 min at RT. The pellet was washed in a large excess of 70% (v/v) ethanol, spun again, and air-dried before dissolving in water.

Removal of salt and other impurities (i.e. enzymes) by precipitation increased the quality of DNA and the sharpness of bands which were analysed on an agarose gel (see section 2.11). The DNA restriction fragments of interest were isolated from the gel and purified (see section 2.12) before subcloning (see section 2.13).

2.11 Conventional agarose gel electrophoresis

Small gels were run rapidly in a horizontal apparatus (Anachem Ltd., UK), using 1x TAE or 1x TBE buffer and 1-3% (w/v) agarose. The desired amount of agarose was dissolved in buffer for 3-4 min at full power in a conventional microwave oven. The gel was then cooled to 50°C, supplemented with 1μg/ml ethidium bromide and poured onto a clean plastic gel plate to set. Small fragments were analysed on higher percentage gels. An appropriate volume of 5x DNA loading buffer (see section 2.3.1) was added to each sample prior to loading. Gels were viewed on a UV transilluminator, images captured using Gel Doc system (Quantity One software, Bio-Rad Ltd., UK) and printed on thermal paper (Mitsubishi P91 thermal printer).

For most gels, λ phage DNA digested with *Hin*dIII was used as a size marker. For low molecular weight molecules, φX174 DNA digested with *Hae*III, a 1kb ladder and the 50bp marker were used (all markers from GibcoBRL).

The size of these fragments (in bp) are as follows:

λ/*Hin*dIII:	23130; 9416; 6557; 4361; 2322; 2027; 564; 125
φX/*Hae*III:	1353; 1078; 872; 603; 310; 281; 271; 234; 194; 118; 72
1kb ladder:	12216; 11198; 10180; 9162; 8144; 7126; 6108; 5090; 4072; 3054; 2036; 1636; 1018; 517; 506; 396; 344; 298; 220; 201; 154; 134; 75
50bp marker:	800; 750; 700; 650; 600; 550; 500; 450; 400; 350; 300; 250; 200; 150; 100; 50

Such gels were used to estimate concentration and quality of plasmid DNAs or total RNA; to check whether DNA was adequately digested; to determine the size of restriction fragments; to visualise PCR products.

For the isolation of specific restriction fragments of plasmid DNAs to be purified and used for subcloning, LMP (low melting point) agarose gels (Sigma) were run, using 1x TAE as buffer.

For a quick estimation of concentration and purity of total RNA, 1% (w/v) agarose gels were run using 1x TBE as buffer. The appropriate volume of 2x RNA loading

buffer (see section 2.3.2) was added to each sample. Prior to loading, samples were denatured for 3 min at 94°C.

2.12 Purification of DNA restriction fragments from agarose gels

DNA fragments used for subcloning purposes were purified using the silica-based QIAEX II Agarose Gel Extraction Kit (QIAGEN Ltd., UK). The sample was digested and one-tenth of the digestion mixture was checked on a test gel for its concentration and completeness of the digest. The remainder of the sample was then resolved on a 1% (w/v) LMP agarose (Sigma), 1x TAE gel. Using a clean scalpel blade, the fragment of interest was cut out from the gel as quickly as possible to minimise UV-mediated DNA damage. DNA was extracted and purified according to manufacturer's instructions. Briefly, the high-salt QX1 buffer solubilised agarose at 50°C for 10 min and allowed binding of DNA to QIAEX II silica-gel particles. DNA molecules bound to the QIAEX II particles were pelleted by centrifugation, washed with QX1 buffer to remove residual agarose, followed by two washes with the ethanol-containing buffer PE to remove residual salts. DNA was eluted from the QIAEX II particles with water by vortexing and heating to 50°C for 5 min. QIAEX II particles were pelleted by centrifugation and the supernatant, containing purified DNA, was transferred into a clean tube.

2.13 Subcloning procedures

The pTRE vector was used for sub-cloning. Subcloning was performed using purified Androgen Receptor (AR) cDNA from the pcDNA-AR plasmid, and dephosphorylated purified vector (pTRE). Ligation mixes were transformed into *E.coli* DH5α™ competent cells (GibcoBRL) and plated on media containing ampicillin (50μg/ml) for selection.

2.13.1 Dephosphorylation of pTRE plasmid

Digestion of the pTRE vector with *Xba*I resulted in protruding single-stranded ends which could religate the linearised vector and increase the number of bacterial colonies that contain plasmids with no inserts (non-recombinant colonies). In order to prevent re-circularisation, Calf Intestinal Alkaline Phosphatase, CIAP (Promega Ltd., UK) was used to remove the phosphate groups from both the 5′-termini of the linearised vector and prevent recircularisation.

pTRE plasmid was digested for 12-16 hours with *Xba*I and then CIAP (0.01units/pmol ends) was directly added to the linearised plasmid and incubated at 37°C for 30 min, according to manufacturer's instructions. pmol ends of linear dsDNA were calculated as follows (Promega protocols and applications guide, 3rd edition, 1996):

$$\frac{\mu g\ DNA}{DNA\ size\ in\ kb} \times 3.04$$

A second aliquot of CIAP was then added and the mixture was incubated at 37°C for a further 30 min. The reaction was stopped with 300µl stop buffer (10mM Tris-Cl pH 7.5, 1mM Na_2EDTA, 200mM NaCl, 0.5% w/v SDS), and the dephosphorylated linear plasmid was precipitated (as described in section 2.10.1) to remove salt and CIAP enzyme. The completeness of the digest was verified by agarose gel electrophoresis (as described in section 2.11) and the dephosphorylated linear plasmid was isolated from the gel and purified (as described in section 2.12).

2.13.2 Ligation

The last step in the construction of a recombinant molecule is the ligation of the dephosphorylated purified vector to the purified DNA insert. T4 DNA ligase (Promega Ltd., UK) was used to catalyse the reaction, according to manufacturer's instructions. A 3:1 molar ratio of insert:vector DNA was used for the ligation. Molar ratio was converted to mass ratio as follows:

$$\frac{ng\ of\ vector \times kb\ size\ of\ insert}{kb\ size\ of\ vector} \times molar\ ratio\ of\ \frac{insert}{vector} = ng\ of\ insert$$

A ten microlitre ligation reaction was carried out using a three-fold excess of enzyme at RT for 3 hours. Half of the ligation mix was used immediately for transformation while the remainder was kept at 4°C. As negative control, a vector-only ligation reaction was performed. After the dephosphorylation step (see section 2.13.1), the vector-only ligation reaction was expected to generate no or a small number of colonies, depending on the efficiency of the CIAP enzyme.

2.13.3 Bacterial transformation

Bacterial DH5α™ competent cells (GibcoBRL) were removed from the -70°C freezer and thawed on ice. Half of the DNA ligation mix was added to 50µl competent cells and mixed by gently tapping the tube. The cells were incubated on ice for 30 min and were then heat-shocked at 37°C for 2 min, before placing on ice for a further 2 min. For cells to recover and allow expression of the antibiotic-resistance gene (*amp*^{r}), 300µl of Luria Broth, LB (10g/lt tryptone, 5g/lt yeast extract, 10g/lt NaCl) medium were added and the cells were incubated at 37°C shaking (250rpm) for one hour. After expression, the reaction was plated on Luria Agar, LA (15g/lt agar, 10g/lt tryptone, 5g/lt yeast extract, 10g/lt NaCl) plates containing ampicillin (50µg/ml) and incubated overnight (12-16 hours) at 37°C. Next day, individual colonies were picked using sterile toothpicks and plasmid DNA was isolated, as described in section 2.8.

2.14 Western analysis

During Western analysis, the translation product (i.e. protein) of the gene of interest is detected. Initially, the cells were lysed, proteins were isolated and separated on a polyacrylamide gel. They were then transferred onto a membrane, and hybridised to a monoclonal antibody against the protein of interest. Gene expression (i.e. production of protein of interest) was detected by chemiluminescence.

2.14.1 Preparation of protein from cultured cells

Exponentially growing cells (70-80% confluency) were washed with 0.25% (w/v) trypsin in 0.5mM versene to detach the monolayer of cells, and were counted (see section 2.18 and 2.19). Five million cells were centrifuged at 1000rpm (Denley BS400 centrifuge) for 5 min. The supernatant was discarded and the cell pellet was washed twice with 1x PBS. Thirty microlitres of freshly made extraction buffer (see section 2.3.3) were then added and the lysate was placed on ice for 30 min. The mixture was centrifuged at 13,000rpm for 20 min at 4°C and the supernatant, containing the proteins, was recovered and stored at -70°C.

2.14.2 Measurement of protein concentration

Protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad Ltd., UK). This is a dye-binding assay where the Coomassie blue dye of the Reagent Concentrate changes colour depending on the amount of protein present in the sample. BSA (2mg/ml) was used as a standard. Four dilutions of the protein standard were prepared, Reagent Concentrate (1x) was added and using polystyrene cuvettes (BDH Ltd., UK) the DU650 spectophotometer (Beckman Ltd., UK) was blanked with 1x PBS buffer. The absorbance at 595nm was measured and a standard curve was plotted. Four dilutions of each protein sample were prepared, Reagent Concentrate (1x) was added and the absorbance was read from the standard curve. The protein concentration (mg/ml) of each sample was calculated as follows:

$$OD_{595} \times \text{dilution factor}$$

2.14.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

All gels were run using Bio-Rad Mini Protean II apparatus and all gel plates were assembled according to manufacturer's instructions. A 7.5% (v/v) polyacrylamide resolving gel containing 375mM Tris pH 8.8, 0.1% (w/v) SDS, 1% (v/v) glycerol, 2mM Na_2EDTA, 7.5% (v/v) acrylamide:bisacrylamide 37.5:1 (Protogel ULTRA

PURE solution by Flowgen Ltd., UK), 0.1% (w/v) AMPS, 0.1% (v/v) TEMED was poured up to three quarters of the gel mould. The resolving gel was allowed to set for 1 hour and was then overlaid with a 4% (v/v) stacking gel containing 125mM Tris pH 6.8, 0.1% (w/v) SDS, 2mM Na_2EDTA, 4% (v/v) acrylamide:bisacrylamide 37.5:1 (Protogel ULTRA PURE solution by Flowgen Ltd., UK), 0.1% (w/v) AMPS, 0.25% (v/v) TEMED. The second gel was allowed to polymerise also for 1 hour. An appropriate volume of 2x protein loading buffer (see section 2.3.3) was added to each sample prior to denaturation (10 min at 100°C). Forty micrograms of protein were loaded into each well. The gel tank was filled with Electrode Running Buffer (1x) (see section 2.3.3) and the electrophoresis was carried out at 192 Volts for approximately 1 hour and 15 min.

For all gels, kaleidoscope prestained standard (Bio-Rad Ltd., UK) was used as a size marker. The sizes of the fragments (in kDa) are as follows:

192; 127; 73; 43; 32.3; 17; 6.6

2.14.4 Transfer of proteins to a membrane

The resolving gel was electroblotted onto an Immobilon-P transfer membrane (Millipore Ltd., UK) according to manufacturer's instructions. Briefly, all the components (membrane, Whatman 3MM filter papers, pads) were presoaked in transfer buffer (see section 2.3.3). The membrane was placed on top of the gel and both were sandwiched between two 3MM filter papers on each side. A foam pad covered each side of the sandwich which was placed in a transfer cassette holder, and then into the tank of the blotting apparatus. The blotting apparatus was assembled in such a way that the gel was facing the cathode (-). Application of an electric current caused migration of the negatively-charged proteins towards the anode (+) carrying them from the gel onto the membrane (Figure 2.2). Proteins were blotted at 30V overnight (or 100V for 1 hour) at 4°C.

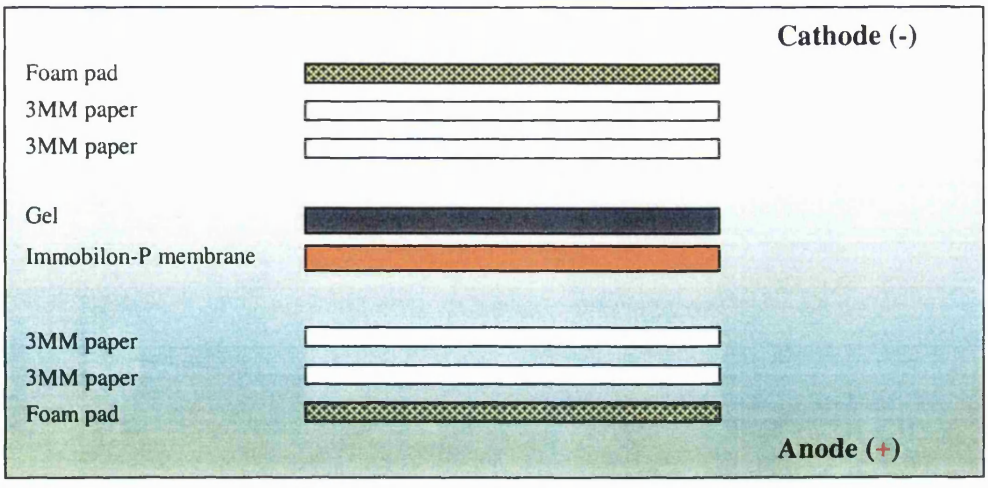


Figure 2.2 **Transfer stack for protein electroblotting.**

2.14.5 Hybridisation, washing, immunodetection and autoradiography

All membranes were hybridised by gentle agitation on a Coulter Mixer (Coulter Electronics Ltd., UK) at RT in 50ml Falcon tubes. Prior to hybridisation, the membrane was blocked in 1x PBS containing 10% (w/v) milk powder (Marvel) for 3 hours. Incubations with the primary and secondary antibody and washes between the incubations were carried out in 1x PBS/0.1% (v/v) Tween, 3% (w/v) milk solution. Hybridisation with the AR primary antibody (1/1000) was allowed for 2 hours. Unbound antibody was removed by washing (4 x 5 min). The membrane was then incubated with a peroxidase-conjugated secondary antibody (1/5000) for 45 min. Excess secondary antibody was removed by washing (4 x 5 min) in 1 x PBS/0.1% (v/v) Tween solution.

Enhanced Chemiluminescence (ECL) detection system (Amersham-Pharmacia Biotech., UK) was used to detect the presence of the protein of interest. This is a non-radioactive method where the light emitted during the oxidation of luminol by the peroxidase-conjugated secondary antibody is captured on an autoradiogram.

Subsequently, after the last wash, the membrane was incubated without agitation for approximately 1 min with equal volumes of detection solution 1 and detection solution 2, according to manufacturer's instructions. The excess detection reagent was drained off using 3MM filter paper, the membrane was wrapped in SaranWrap (Dow

Chemical Co.), placed in a Kodak cassette, and exposed to autoradiography film (Hyperfilm ECL) at RT for 20 min.

To check for equal protein loading:

i. The gel was stained after electroblotting in 0.1% (w/v) Coomassie Blue in 40% (v/v) methanol, 10% (v/v) acetic acid for 30 min at RT, and destained in 40% (v/v) methanol, 10% (v/v) acetic acid until the protein bands were visible and the background reduced.

ii. The membranes were stained briefly in Ponceau S and destained in water until the protein bands were visible and the background reduced. Membranes were then wrapped in SaranWrap, covered with aluminum foil and stored at 4°C.

2.15 Preparation of total RNA from cultured cells

All RNA work was carried out in a tissue culture cabinet and care was taken to minimise the contamination with RNAases liberated during cell lysis, or present in general laboratory glassware and plasticware. To achieve this, processing of RNA was carried out as quickly as possible and disposable plasticware was used. Glassware and water were incubated overnight (12-16 hours) at 37°C with DEPC (0.1% v/v in water), which is a strong inhibitor of RNAases. Next day, DEPC traces were removed by autoclaving. Gloves were worn during the RNA isolation and analysis to eliminate contamination with RNAases through dust and skin.

Total RNA was isolated using the following protocols:

- Single-step RNA isolation method
- RNeasy® Mini kit (QIAGEN Ltd., UK)

2.15.1 Single-step RNA isolation method

Total RNA from cultured cells was isolated by a guanidinium thiocyanate-phenol-chloroform extraction in a single step as described by Chomczynski and Sacchi (1987). Specifically, cells grown to 80% confluency after removal of culture media were washed once with 1x PBS to ensure removal of media. A sufficient amount of GTC buffer (see section 2.3.2) to cover the cell monolayer was added and the dish

was gently agitated for approximately 5 min until all cells were detached. The lysate was removed from the dish and transferred into a 15ml Sarstedt tube.

To avoid DNA contamination of the RNA sample, RNA precipitation and deproteinisation were carried out under acidic conditions (pH 4.0). In a single step, RNA was precipitated and deproteinised by the addition of 0.1 volumes of 2M sodium acetate (pH 4.0) followed by an equal volume of phenol (pH 4.0) and 0.2 volumes chloroform. The mixture was vortexed and placed on ice for 20 min. RNA was recovered by centrifugation (10,000rpm) in a Sorvall Super T21 centrifuge (Du Pont Ltd.) at 4°C for 20 min. The aqueous upper (RNA-containing) phase was carefully removed into a clean Sarstedt tube. An equal volume of isopropanol was added and the RNA was allowed to precipitate for half an hour at 4°C (or at -20°C for 12-16 hours) before recovering by centrifugation (10,000rpm at 4°C for 20 min). The RNA pellet was allowed to air-dry before dissolving in DEPC-treated water, containing RNAase inhibitor RNASEOUT™ (GibcoBRL) at 1unit/μl. RNA was then aliquoted into clean 1.5ml eppendorfs and stored at -70°C.

2.15.2 RNeasy® Mini kit (QIAGEN Ltd., UK)

Total RNA was also prepared using the silica-gel-based RNeasy® Mini kit (QIAGEN Ltd., UK) according to manufacturer's instructions. Briefly, cells grown to 80% confluency were initially lysed with RLT buffer (a guanidinine isothiocyanate (GITC)-containing buffer) and the cell lysate was homogenised using a QIAshredder spin column (QIAGEN). An equal volume of 70% (v/v) ethanol was then added to the homogenised lysate to ensure selective binding of RNA onto the silica-based RNeasy spin column, and the sample was loaded onto the RNeasy column. A wash with RW1 buffer followed by two washes with RPE buffer removed sheared genomic DNA, and protein contaminants. RNA was eluted from the RNeasy spin column with DEPC-treated water and recovered by centrifugation. The RNAase inhibitor RNASEOUT™ (GibcoBRL) was then added to the sample at 1unit/μl and RNA was stored at -70°C.

2.15.3 Removal of DNA contaminant from RNA

Even traces of DNA contaminant can become problematic during the PCR steps, resulting in false positive signals. Therefore, total RNA was purified prior to RT-PCR. The following protocols were used:

- MessageClean™ kit (GenHunter Corp., USA)
- RNase-Free DNase set (QIAGEN Ltd., UK)

a) MessageClean™ kit (GenHunter Corp., USA)

RNA samples were digested with DNase I of the MessageClean™ kit to remove all traces of DNA, according to manufacturer's instructions. Briefly, 25μg of total RNA were incubated for 30 min at 37°C with 10units of RNase-free DNase I, in the buffer supplied. The protein contaminants were removed by adding an equal volume of phenol:chloroform (3:1) (pH 7.0), mixing by vortexing and spinning at 13,000rpm for 10 min at 4°C. The upper (RNA-containing) phase was carefully removed and RNA was allowed to precipitate overnight (12-16 hours) at -70°C in the presence of 3M sodium acetate (pH 5.5) and 100% ethanol. Next day, RNA was pelleted by centrifugation (13,000rpm for 30 min at 4°C), washed in 70% (v/v) ethanol, centrifuged again and air-dried before dissolving in DEPC-water containing the RNASEOUT™ inhibitor at 1unit/μl.

b) RNase-Free DNase set (QIAGEN Ltd., UK)

The RNase-Free DNase set is used with the RNeasy® Mini kit and provides on-column digestion of DNA contaminants during RNA purification. Lysis and homogenisation of the RNA sample was carried out as described in the RNeasy® protocol (see section 2.15.2). A DNase treatment step (15 min at RT with 30 units of DNase I) was performed while the RNA was bound onto the silica-gel column. The DNase was removed by washing with RW1 and RPE buffers and RNA was eluted from the RNeasy spin column as described in section 2.15.2.

2.16 Measurement of RNA concentration

RNA concentrations were measured by UV absorbance spectrophotometry using a DU650 series spectrophotometer (Beckman Ltd., UK). Total RNA was diluted (1/100) in water and 500µl were added to a microcuvette. The spectrophotometer was blanked with water, the density of the sample was measured at 260nm and at 280nm and the RNA concentration was calculated as follows:

$$OD_{260} \times 40\mu g/ml \times \text{dilution factor}$$

One OD unit at 260nm corresponds to 40µg/ml of RNA. The 260nm/280nm ratio reflects the purity of the sample and is influenced by pH. As water is unbuffered, the same RNA sample may show different 260nm/280nm ratio in different types of water, ranging from 1.5-1.9. Therefore, concentration and quality of RNA were also verified by conventional gel electrophoresis (see section 2.11).

2.17 Reverse Transcription- Polymerase Chain Reaction (RT-PCR)

RT-PCR investigates gene expression at the mRNA level. The key to this technique is the enzyme Reverse Transcriptase which synthesises a complementary DNA (cDNA) strand from a RNA strand. cDNA is then used as a template for PCR analysis, where a chosen region of the DNA molecule is selectively amplified.

2.17.1 Reverse Transcription (RT)

Total RNA (2.5µg) was reverse transcribed into cDNA in a 20µl reaction containing 500µg/ml OligodT primer (GibcoBRL), 0.5mM dNTPs (GibcoBRL), 10mM DTT (GibcoBRL) and 200units of reverse transcriptase SUPERSCRIPT™ II (GibcoBRL) in the buffer supplied, according to the manufacturer's instructions. Briefly, total RNA was denatured at 70°C for 10 min before the reaction mixture was added. The reaction was then incubated at 42°C for 50 min to allow synthesis of cDNA. The enzyme was inactivated by heating at 70°C for 15 min. The cDNA synthesized was used as a template for PCR amplification.

2.17.2 Purification of cDNA

Prior to PCR analysis, the reverse transcribed cDNA was purified using the guanidine thiocyanate-based High Pure PCR Product Purification Kit (Roche Diagnostics, Germany), according to manufacturer's instructions. Briefly, DNA (minimum length of 100bp) bound selectively to glass fibers pre-packed in the High Pure filter tube in the presence of a guanidine thiocyanate-containing buffer. Removal of primers, unincorporated nucleotides, salts and other impurities (i.e. reverse transcriptase or thermostable polymerase) was achieved by two wash-and-spin steps (13,000rpm for 30 sec) using a low salt solution (20mM NaCl, 2mM Tris-HCl, pH 7.5). Purified DNA was eluted from the glass fibers with water.

Amplified DNA products, prior to nested PCR analysis were also purified using the method described above. The High Pure PCR Product Purification method increased the quality of cDNA (or amplified DNA), and reduced the number of non-specific PCR products.

2.17.3 Polymerase Chain Reaction (PCR)

All PCR tips and tubes were autoclaved before use to ensure that they were not contaminated with DNA. Moreover, all PCR procedures were carried out in a class II cabinet and gloves were worn at all times.

a) Preparation of primers

Primers were synthesised by MWG Biotech Ltd., UK or GibcoBRL (Life Technologies Ltd., UK) and supplied as lyophilised stock. For each primer an equal volume of water (in µl) as the amount of nmoles supplied was added to provide a stock of 100µM.

b) PCR assays

PCR was carried out in a Perkin Elmer-GeneAmp 2400 thermal cycler (Perkin Elmer Ltd., UK). Ten microlitre reactions contained 1µM of each primer, 0.5mM dNTPs, 3.75mM $MgCl_2$ and 1.25 units of AmpliTaq® DNA polymerase (Perkin Elmer Ltd.,

UK) or Red Hot® DNA polymerase (Advanced Biotechnologies Ltd., UK) in the buffer supplied. Amplification generally used 100-250ng DNA or one-tenth of the reverse transcribed cDNA as template and varying cycling conditions, depending on the class of template.

Sequences of the individual primer pairs and the thermal cycling conditions used are summarised in Table 2.4.

Table 2.4 Primers and their thermal cycling conditions

Name	Sequence 5′→3′	Thermal cycling conditions	No of cycles
ARAs (exon 1)	*1925CGAAATGGGCCCCTGGATGGATAG	30 sec at 94°C 30 sec at 65°C 40 sec at 72°C	30-40
ARDa (exon 4)	*2448AGTCGGGCTGGTTGTTGTCGTGTC		
AR exon 6	*2720AATGAGGCACCTCTCTCAAGAGT	30 sec at 94°C 30 sec at 53°C 40 sec at 72°C	40
Anchor 1	CTAATACGACTCACTATAGGGCT		
AR exon 7/8	*2962AGCCTATTGCGAGAGAGCTGCAT	30 sec at 94°C 30 sec at 56°C 40 sec at 72°C	30
Anchor nested 2	ACTCACTATAGGGCTCGAGCGGC		
AR exon 7s	*2872AACTCGATCGTATCATTGCATGC	30 sec at 94°C 30 sec at 60°C 40 sec at 72°C	40
AR exon 8a	*3069CTTGCACAGAGATGATCTCTGC		
PSAs	*183ACTGCATCAGGAACAAAAGCGTGA	30 sec at 94°C 30 sec at 65°C 40 sec at 72°C	20-35
PSAa	*544CGCACACACGTCATTGGAAATAAC		
Actin s	*2275GCCGAGCGGGAAATCGTGCGTG	5 min at 94°C 30 sec at 94°C 1 min at 65°C 2 min at 72°C	35
Actin a	*2987CGGTGGACGATGGAGGGGCCG	5 min at 72°C	
COX-1s	*1028AAATACCCATTGCCCCAGACCC	5 min at 94°C 30 sec at 94°C 1 min at 60°C 2 min at 72°C	35
COX-1a	*1740CTGTCCTCTCTCGCTGCTGCC	5 min at 72°C	

Anchor-oligodT_{21} CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGTn(n=21)

* The number indicates the 5′ nucleotide position on the cDNA of AR (Lubahn et al., 1988b), PSA (Lundwall and Lilja, 1987), actin (Nakajima-Iijima et al., 1985) and COX-1 (Hla, 1996). Non-designated primers are located in the anchor sequence of the anchor-oligodT_{21} primer. The nucleotide sequences of AR, PSA, actin and COX-1 are available from GenBank under accession numbers J03180, X05332, M10277, and U63846 respectively.

s: sense primer and a: antisense primer.

CELL CULTURE TECHNIQUES

2.18 Routine cell culture and maintenance of cell lines

Cell lines (Table 2.2) were routinely grown to approximately 80% confluency at 37°C in a humidified incubator in an atmosphere of 5% CO_2 before being passaged. Briefly, after removal of culture media the cells were washed once with 1x PBS to ensure removal of serum. They were then washed with 0.25% (w/v) trypsin in 0.5mM versene, and incubated at 37°C for 3 min to detach the monolayer of cells. The detached cells were resuspended in the appropriate volume of fresh medium to subculture at the required density.

2.19 Preparation of single-cell suspension

Exponentially growing cells (approximately 80% confluency) were detached from the plastic as described in section 2.18. The detached cells were resuspended in 5ml medium and the suspension was passed several times through a 10ml plastic pipette until no cell clumps were visible. A 100µl aliquot of the cell suspension was mixed with an equal volume of 0.1% (w/v) trypan blue solution. Viable cells were counted using an Improved Neubauer haemocytometer (Weber Scientific Ltd., UK). The number of cells per ml was calculated as follows:

$$\text{average num of cells} \times \text{dilution factor} \times 10^4$$

For the final number of cells required for each experiment, an appropriate cell dilution was prepared.

2.20 Preparation of steroid-depleted fetal bovine serum

To study androgen-induced responses of cells, fetal bovine serum (FBS) was stripped of its steroids using dextran-coated charcoal (DCC), as described by Leake et al. (1987). Specifically, 500ml of 10mM HEPES buffer containing 1.5mM $MgCl_2$ and 0.25M sucrose were initially prepared and the pH adjusted to 7.4 at 4°C. Then 0.25% (w/v) Norit A charcoal (Sigma) and 0.0025% (w/v) dextran T70 (Amersham-

Pharmacia Biotech., UK) were added and the suspension was incubated with stirring overnight (12-16 hours) at 4°C. Next day, the DCC suspension was centrifuged at 2200rpm in a Sorvall centrifuge for 10 min, the supernatant was discarded and an equal volume of heat-inactivated FBS (30 min at 56°C) was added to the charcoal pellet. The mix was incubated with stirring for 12-16 hours at 4°C. Charcoal was removed from serum by centrifugation (2200rpm for 20 min). The resulting dextran-coated charcoal-treated serum (DCC-FBS) was filter-sterilised through a 0.2μm filter and stored in 25ml aliquots at –20°C.

2.21 Transfection of cell lines

There are a number of transfection techniques available offering a variety of transfection efficiencies depending mostly on a) the cell line b) quality (purity) and quantity of the expression vector preparation. For the work described in this thesis, the calcium phosphate-mediated transfection protocol was used.

2.21.1 Optimisation of geneticin concentration and plating density

The plasmids used for the transfection experiments carried the neomycin-resistant gene (*neo*^{r}). The *neo*^{r} gene is frequently used as a selectable marker in mammalian cells and confers resistance to the antibiotic Geneticin® (G-418 Sulphate, GibcoBRL). Selection of stable transfectants is achieved by growing the transfected cells in otherwise cytotoxic concentrations of Geneticin®-containing medium. Cell lines vary widely in their sensitivity to Geneticin® and there is also lot-to-lot variation in the antibiotic's toxicity. Therefore, a titration of the Geneticin® concentration is necessary. The minimum cytotoxic concentration of Geneticin® was determined by exposing 10^6 cells grown in triplicate in 10cm dishes to varying concentrations of the antibiotic (range 0.2- 1.2mg/ml) for 15 days. Selective medium was replaced every four days.

It is also important to estimate the number of cells plated per dish that would reach 80% confluency before massive cell death after addition of the selective medium.

Selection of stable transfectants can be complicated if plating cell density is too high or too low. High cell density allows cells to reach confluency before selection takes place, thereby increasing the chance of losing the selection and obtaining non-resistant clones. Low cell density makes it difficult to obtain enough resistant clones. Optimal plating density for each cell line was determined by plating cells at several densities in 10cm dishes (0.3×10^6, 0.5×10^6, 1×10^6, 5×10^6 cells/10cm dish).

2.21.2 Calcium phosphate-mediated transfection

Calcium phosphate-mediated transfection is a process where DNA is mixed with $CaCl_2$ and a phosphate buffer to form a fine precipitate which is then added to the cell monolayer. DNA precipitates on the cell surface, binds to the plasma membrane and is taken up by endocytosis. This is one of the oldest techniques used to introduce DNA into cells in culture and was first described by Graham and van der Eb (1973). Briefly, exponentially growing cells were seeded at confluency no more than 40-60%. Next day, media was changed before addition of the transfection mixture. The transfection mixture (200µl/well for a 6-well dish or 1ml/10cm dish) was prepared as follows: 5-20µg expression vector (plasmid DNA), were mixed with 0.125M $CaCl_2$ and water before adding dropwise 1x HBS buffer (see section 2.3.4). Precipitate was allowed to form at RT for 20 min and was then added to the cells which were incubated at 37°C in 5% CO_2 for 12-14 hours. Next day, the precipitate-containing medium was removed and fresh medium was added to the cells.

a) Transient transfection

After transfection, cells were incubated for 48 hours to allow expression before harvesting to investigate the expression of genes of interest.

b) Stable transfection

After transfection, cells were incubated for 48 hours to allow expression before applying antibiotic selection. The cells were allowed to grow for 3-5 weeks, until individual colonies of transfectants were formed. The medium was changed regularly to remove dead cells and antibiotic selection was maintained continuously.

2.22 Ring cloning

The transfectants (antibiotic-resistant colonies) were marked using a permanent marker (Lumocolor, Staedtler) under the dish. Cloning rings (8mm, Sigma) were placed around each marked colony using forceps. Each ring was slightly lubricated with high-vacuum grease (Dow Corning) at its base to facilitate attachment of the ring onto the dish. Each colony was gently washed with 1x PBS and was detached from the plastic with 0.25% (w/v) trypsin in 0.5mM versene. The detached cells from an individual colony were transferred into a T25 flask containing the appropriate medium (see Table 2.2) and allowed to grow until confluency before frozen stocks were prepared.

2.23 Preparation of frozen stocks

Frozen stocks were made from low passage exponentially growing cells. Briefly, cells growing in T75 flasks after removal of culture media were washed with 0.25% (w/v) trypsin in 0.5mM versene to detach the monolayer of cells. The detached cells were resuspended in the appropriate medium (see Table 2.2) and centrifuged at 1,000rpm for 5 min (Denley BS400 centrifuge). The cell pellet was resuspended in freezing medium (20% v/v FBS, 10% v/v DMSO, 70% v/v culture medium) and was aliquoted into cryovials pre-labelled with cell line name, passage number, and date of freezing. Using a special neck plug (Union Carbide) on a liquid nitrogen container, cells were frozen at -1°C/min for 3-4 hours. The vials were then quickly attached to canes and protected in cardboard tubes before immersing into the liquid nitrogen tank (-196°C).

2.24 Reporter assays

Reporter assays are used to study gene expression. In such assays, the gene of interest is replaced by a test (reporter) gene which is under exactly the same control sequences as the gene of interest. The reporter gene must have a phenotype easy to detect and assay quantitatively.

The gene encoding firefly luciferase is widely used as a reporter gene, since the assay measuring enzyme activity has the advantages of speed, sensitivity, and simplicity. Emission of light is generated when the luciferin substrate and luciferase enzyme are mixed together. Light emission is measured by a luminometer.

2.24.1 Preparation of cell lysates

Forty-eight hours after transfection, medium was removed from the cells and they were washed twice with 1x PBS. A sufficient amount of 1x Cell Culture Lysis Reagent (Promega Ltd., UK) to cover the cells was added. Cells were left at RT for 5-10 min to dislodge and were then scraped off and the cell lysate transferred into an 1.5ml eppendorf.

2.24.2 Luciferase assay

Twenty µl of cell lysate were added to 100µl luciferase Assay Reagent (Promega Ltd., UK) and the luminescence was immediately measured using an 1250 LKB Wallac luminometer (LKB Wallac, Finland). The measurement within the first 10 sec was the reading recorded.

2.25 Immunocytochemistry (ICC)

Immunocytochemistry was performed using the immunoperoxidase Vectastain *Elite* ABC system which is based on the avidin-biotin peroxidase method (Vector laboratories Inc., USA). Briefly, exponentially-growing cells in 5mm dishes (at a confluency no more than 40%) were fixed for 10 min in 1:1 (v/v) methanol:acetone at –20°C. The fixative was removed and cells were washed (3 x 5 min) in 1x PBS. Endogenous peroxidase was saturated for 30 min with 0.3% (v/v) H_2O_2 in 70% (v/v) methanol at RT. The cells were then washed (3 x 5 min) in 1x PBS. To minimise non-specific binding of reagents in subsequent steps, cells were incubated at RT with 1.5% (v/v) normal blocking serum in PBS for 20 min. Blocking solution was then removed and the cells were incubated at RT with the AR primary antibody (clone F39.4.1,

Biogenex) diluted 1:100 in PBS for an hour. The excess primary antibody was removed by washing (3 x 5 min) in 1x PBS. For the negative controls, the primary antibody incubation step was omitted. Cells were then incubated at RT with the biotinylated secondary antibody diluted 1:200 in PBS containing 1.5% (v/v) serum for 30 min. The ABC reagent (**A**vidin-**B**iotinylated horseradish peroxidase **C**omplex) prepared according to manufacturer's instructions was then applied to the cells for 30 min at RT. After rinsing (3 x 5 min) the cells in 1x PBS, antibody staining was detected with 3,3′-diaminobenzidine (DAB) substrate for peroxidase (Vector laboratories). A reddish brown stain produced by DAB was developed within 2-10 min. The chromogenic reaction was terminated by rinsing in water, cells were counterstained with Mayer's haematoxylin (Sigma) for 30 sec, dehydrated and mounted with Gelvatol (Monsanto Chemicals).

Chapter 3

Overexpression of the androgen receptor (AR) gene in AR-negative cells using an inducible gene expression system

3.1 Introduction

The complex nature of eukaryotic cells limits the analysis of the expression of individual genes. Inability to experimentally regulate individual gene expression in these cells further complicates the analysis of gene function. Therefore, expression systems that allow stringent control of individual gene activity provide a means for regulating and studying the effects of gene expression on growth and differentiation. Such systems permit transcription to occur not only in a threshold, but rather in a graded manner. This implies that gene expression can be modulated not only in an "on/off" state but also limited expression at a defined level is possible. Hence, such systems provide a tool for research that greatly facilitates control and analysis of gene activity.

The Tetracycline-inducible gene expression system (TetOff™-system), developed by Gossen and Bujard (1992), is a regulatory system that fulfils the above characteristics and was used for the work described in this chapter. The TetOff™ system is advantageous for studies of gene expression as it allows controlling of the timing and levels of gene expression. Cloned genes are quantitatively regulated in response to varying concentrations of tetracycline or tetracycline derivatives (doxycycline). In the TetOff™ system, gene expression is turned off in a dose-dependent manner as tetracycline is added to the culture medium.

To develop the Tet-inducible system, two plasmids need to be introduced into the cells: the regulatory plasmid (pTetOff) which encodes the transcriptional activator (tTA), and the response plasmid (pTRE) which carries the tet-responsive element (TRE). In the absence of tetracycline, tTA binds to TRE and activates transcription of the gene of interest which is under the control of TRE (Figure 3.1).

There are two elements that provide the basis for the TetOff™ system – the *tet*R protein and the *tet*O regulatory sequence. Both regulatory elements are from the Tn*10*-specified tetracycline-resistance operon of *E.coli*.

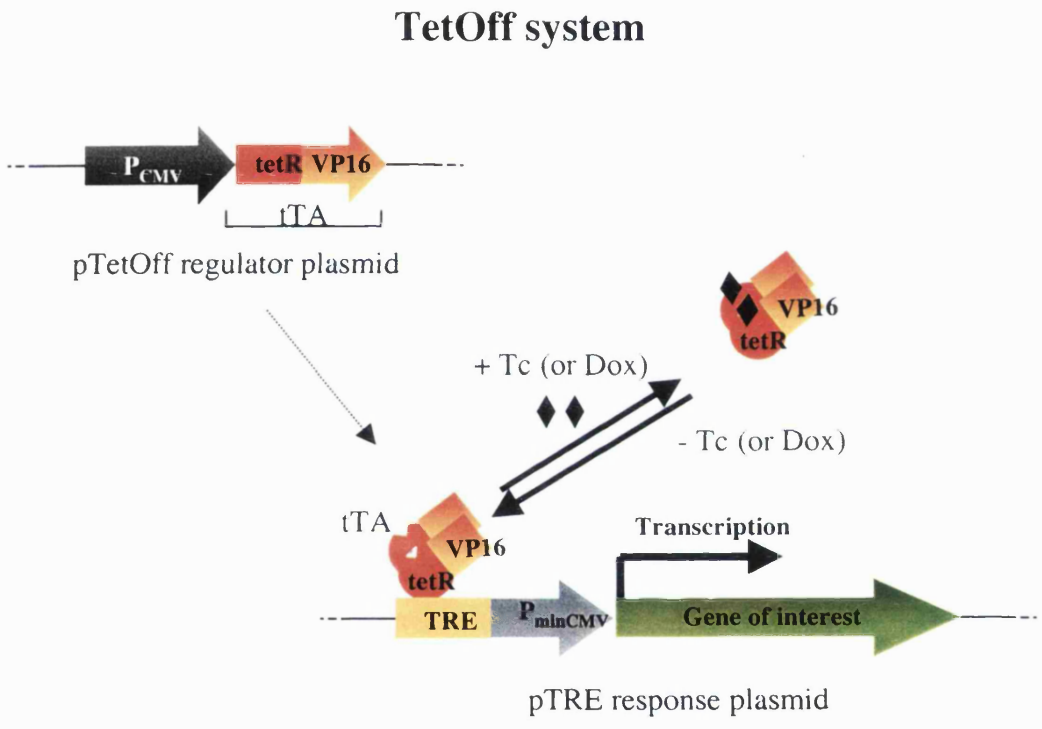


Figure 3.1 **The TetOff™ inducible gene expression system.**

The repressor protein *tet*R when fused to VP16 activation domain of herpes simplex virus is converted into a eukaryotic transactivator (tTA) encoded by the regulator plasmid pTetOff. The tet-responsive element (TRE) is encoded by the response plasmid (pTRE). In the absence of tetracycline (Tc) or doxycycline (Dox), a Tc derivative, tTA binds the tet-responsive element (TRE) and activates transcription of the gene of interest (adapted from CLONTECH user manual).

See text for a detailed description of mode of action.

The *tet*R protein is a repressor that negatively regulates transcription of resistance-mediating genes. Specifically, binding of *tet*R to the tet operator sequences (*tet*O) in the absence of tetracycline blocks transcription (Hillen and Wissmann, 1989). The repressor protein *tet*R is converted into a eukaryotic transactivator when fused with the C-terminal domain of virion protein 16 (VP16 protein) of herpes simplex virus (HSV). The C-terminal domain of VP16 protein functions as an activating domain in mammalian cells (Triezenberg et al., 1988). The hybrid protein called tetracycline-responsive transcriptional activator (tTA) is under the control of P_{hCMV} (human cytomegalovirus) promoter and is encoded by the regulator plasmid (pTetOff plasmid).

The *tet*O regulatory sequence is an inverted repeat to which *tet*R binds as a dimer. Seven copies of the *tet*O sequence form the tet-responsive element (TRE) of the response plasmid (pTRE).

The gene of interest is under the control of TRE which is located upstream of the minimal immediate early promoter of cytomegalovirus (P_{minCMV}). This promoter is dependent upon activation from tTA and is otherwise virtually silent. Consequently, there is no expression of the gene of interest in the absence of binding of tTA to TRE.

Compared to other mammalian regulatory systems (induction by heavy metals, hormones, heat shock, for a review see Gossen et al., 1993; Gossen et al., 1994), the Tet™ system appears to have several advantages:

1. The Tet™ system is based on prokaryotic regulatory mechanisms (*tet* operon of *E.coli*). Utilisation of regulatory elements from organisms that are evolutionary distant (i.e. *E.coli* and mammalian cells), increases the specificity of the induction as their regulatory DNA sequences are not similar and thus do not interfere with their physiology. On the contrary, most of the other mammalian systems are based on eukaryotic regulatory elements. Hence, pleiotropic effects caused by the inducing stimulus affecting genes other than the gene of interest, result in non-specific induction. Moreover, in the systems which are based on eukaryotic regulatory elements there is no tight control of gene expression in the inactivated state, resulting in "leakiness" and greatly limiting the analysis of proteins with cytotoxic properties.

2. The Tet™ system has a higher level of expression in the activated state compared to strong, constitutive mammalian promoters (Yin et al., 1996). Also the wide range of inducibility offers monitoring of varying levels of gene expression under different conditions.
3. Activation of a silent promoter (P_{minCMV}) in the absence of the inducing stimulus (tetracycline) is another essential feature. Increasing concentrations of tetracycline result in a dose-dependent response, while the gene activity can be controlled in a reversible and temporarily defined manner.
4. The doses of tetracycline or doxycycline required for regulation of gene expression are below cytotoxic levels and have no significant effect on cell proliferation even with continuous treatment (Mayford et al., 1996; Bohl et al., 1997).

In conclusion, the Tet™ system combines the advantages of tight regulation and specificity, with high levels of induction.

3.2 Aims of the chapter

The objective of the work described in this chapter was to establish an inducible gene expression system in order to study the effects of androgen exposure on cells expressing a range of androgen receptor concentrations.

Specifically, the aims were to:

- Select an AR-negative human prostate cancer cell line compatible with the TetOff™ system.
- Stably transfect tTA activator (encoded by pTetOff regulator plasmid) into the host cell line.
- Select the stable clone with the highest expression levels of tTA activator.
- Subclone the wild type, full-length human AR cDNA into the pTRE response plasmid.
- Transiently assay pTRE-AR for AR expression.
- Stably transfect pTRE-AR into the tTA-expressing cells and generate double-stable clones.

- Select the double-stable clone with the highest AR expression levels.

3.3 Compatibility of DU145 and PC-3 cell lines with the system

It has previously been reported that the efficacy of the system is influenced by cell type (Ackland-Berglund and Leib, 1995; Gossen and Bujard, 1995; Gossen et al., 1995; Yin et al., 1996). Therefore, a transient expression assay was performed before the development of the stable TetOff cell line in order to assess the compatibility of the system with the potential host cell lines. Two androgen receptor-negative human prostate cancer cell lines (DU145 and PC-3) were transiently tested by co-transfection with pTetOff regulator plasmid and pTRE-Luc response plasmid. The level of expression of the luciferase reporter gene (encoded by pTRE-Luc) reflects the inducibility of the system with the potential host cell lines.

Prior to the transient transfection experiment, sufficient amounts of the pTetOff and pTRE-Luc plasmids were prepared. One microgram of each plasmid was used to transform bacterial cells, as described in section 2.13.3. It is important that the DNA used for transfections is free of impurities. Therefore, plasmid DNA isolated from individual bacterial colonies by the maxi prep method was further purified by ethidium bromide-caesium chloride density gradient centrifugation (see section 2.8.2). Restriction enzyme analysis (see section 2.10) was carried out to estimate the quality and integrity of plasmid DNA. The restriction enzyme analysis for pTetOff and pTRE-Luc plasmids yielded the expected fragment sizes, confirming the integrity of the plasmids (Figure 3.2a). A restriction map (partial) for pTetOff and pTRE-Luc plasmids is also shown (Figure 3.2b).

For each cell line, 5 x 10^5 cells were seeded in triplicate in 10cm dishes and co-transfected with 10μg pTetOff and 10μg pTRE-Luc plasmids using the calcium phosphate transfection method (see section 2.21.2). The cells were transfected in the absence and presence of 2μg/ml tetracycline (Tc) or 5ng/ml doxycycline (Dox), a Tc derivative, as recommended by the manufacturer. The expression of the pTRE-Luc response plasmid was measured 48 hours after transfection by the luciferase assay (see section 2.24).

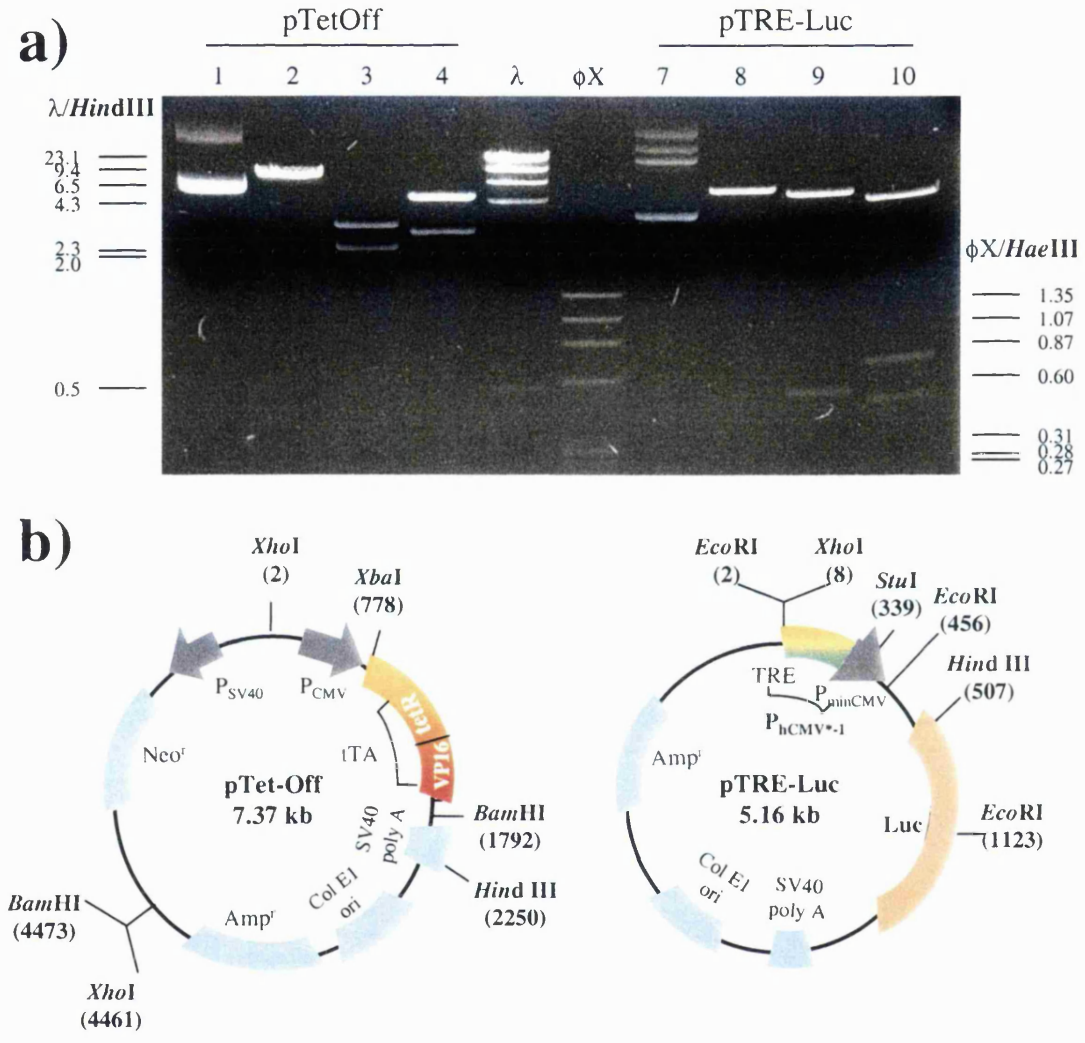


Figure 3.2 **Restriction enzyme analysis of pTetOff and pTRE-Luc plasmids.**

a) EtBr-stained gel showing restriction enzyme digestions of pTetOff and pTRE-Luc plasmids. Lanes 1 and 7 show the uncut pTetOff and pTRE-Luc plasmids respectively. Lanes 2 and 8 show the linearised pTetOff (*Xba*I digest, 7.37kb) and pTRE-Luc (*Stu*I digest, 5.16kb) plasmids respectively. Lanes 3 and 9 show the *Xho*I/*Hin*dIII digest of pTetOff (2.91kb, and the unresolved 2.25/2.21kb doublet) and pTRE-Luc (4.66 and 0.50kb) plasmids respectively, as recommended by the manufacturers (CLONTECH). Lanes 4 and 10 show the *Bam*HI digest of pTetOff (4.68 and 2.68kb) and the *Eco*RI digest of pTRE-Luc (4.0, 0.667 and 0.454kb) plasmids respectively, where the integrity of the plasmids was further confirmed. λ: *Hin*dIII fragments (in kb) of λ phage DNA. φX: *Hae*III fragments (in kb) of phage φX174 DNA.

b) Restriction map (partial) of pTetOff and pTRE-Luc plasmids

Researchers have reported higher induction levels by using an excess of the regulator over the response plasmid while maintaining the same total amount of DNA (Gossen and Bujard, 1995; Corish P., personal communication). Hence, the transient assay was also performed using 18μg of pTetOff plasmid and 2μg of pTRE-Luc. The relative amounts of the two plasmids were arbitrarily chosen while maintaining the same total amount of DNA.

As a negative control, cells were transfected with the pTRE-Luc plasmid alone. The luciferase (Luc) reporter gene, being under the control of the virtually silent P_{minCMV} promoter, was expected to show no induction in the absence of binding of tTA transactivator (encoded by the pTetOff regulator plasmid) to TRE.

The luciferase activity in the absence of tetracycline divided by that in 2μg/ml tetracycline was plotted as fold increase for each cell line (Figure 3.3).

The transient transfection assays indicated that both prostate cancer cell lines could be used as potential hosts for the TetOff™ system. There was a higher induction in DU145 than in PC-3 cells. Therefore, DU145 cells were chosen as the host cell line.

Both antibiotics successfully regulated luciferase gene expression but there was higher background expression when Dox was used. Therefore, Tc was chosen as the inducing stimulus.

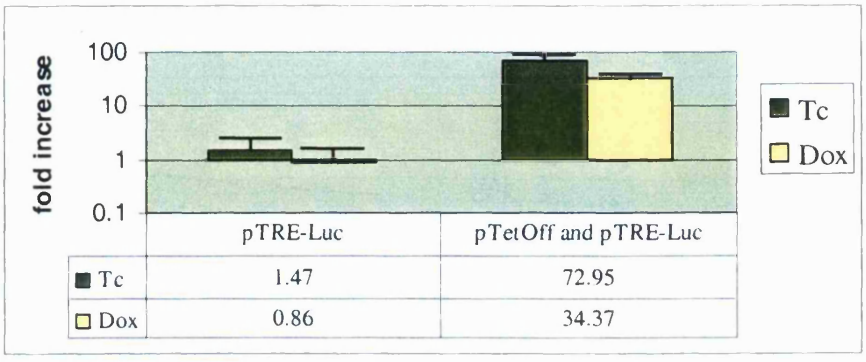
Induction levels were higher when a large excess of pTetOff (18μg) over pTRE-Luc (2μg) was used, compared to induction levels using equal amounts of both plasmids (10μg). Consequently, the former was chosen as the preferred plasmid ratio in further similar experiments.

In the negative control transfections with pTRE-Luc alone, no induction was expected. However, some background luciferase activity was detected.

3.4 Optimisation of geneticin concentration and plating density for DU145 cells

Before the development of the stable TetOff cell line, titration of geneticin concentration was performed and the optimal plating density for DU145 cells determined (see 2.21.1).

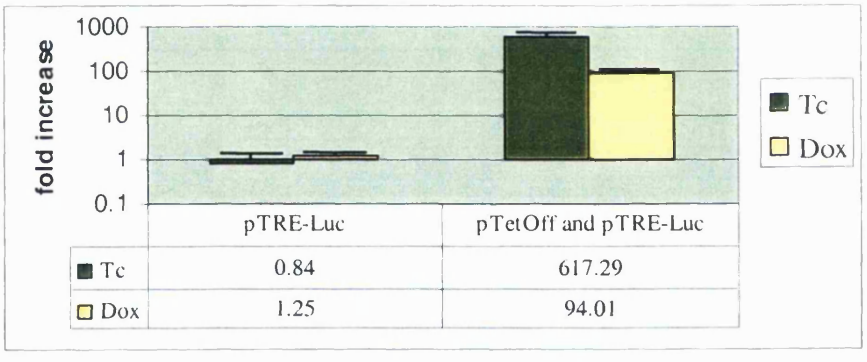
a)



	pTRE-Luc	pTetOff and pTRE-Luc
Tc	1.47	72.95
Dox	0.86	34.37

PC-3 cells

b)



	pTRE-Luc	pTetOff and pTRE-Luc
Tc	0.84	617.29
Dox	1.25	94.01

DU145 cells

Figure 3.3 **Transient luciferase expression assays in potential host cell lines: PC-3 (a) and DU145 (b).**

5 x 10^5 cells were seeded in triplicate in 10cm dishes and co-transfected with 18μg pTetOff and 2μg pTRE-Luc plasmids. The expression of pTRE-Luc plasmid was measured 48hrs after transfection by the luciferase assay. Cells were transfected in the absence and presence of 2μg/ml tetracycline (Tc) or 5ng/ml of doxycycline (Dox), a Tc derivative. A higher induction was apparent in DU145 than in PC-3 cells. The luciferase activity in the absence of Tc divided by that in 2μg/ml Tc is plotted as fold increase (■). The luciferase activity in the absence of Dox divided by that in 5ng/ml Dox is plotted as fold increase (□). Error bars are SEM for two independent experiments.

The regulator plasmid (pTetOff), used for the development of the stable TetOff cell line, carries the neomycin-resistant gene (*neo*^{r}) which confers resistance to the antibiotic Geneticin® (G-418 Sulphate) and allows for the selection of the transfected cells.

A minimum concentration of 1mg/ml Geneticin® was found to kill all cells within 15 days. This dosage was chosen as the optimal concentration for the development of the stable TetOff cell line.
One million cells/10cm dish was the number of cells that reached maximum of 80% confluency during selection. This number of cells was chosen as the optimal plating density for the development of the stable TetOff cell line.

3.5 Transfection of DU145 cells and selection of stable cell lines

For the establishment of the TetOff™ expression system two consecutive stable transfections rather than a simultaneous transfection are preferred (Yin et al., 1996). Simultaneous transfection can induce high basal expression levels of the gene of interest as the result of plasmid cointegration. In addition, it is likely that differences in the expression levels of the gene of interest are a result of clone-to-clone variation in the tTA expression and not Tc regulation. Consecutive transfections, on the other hand, can generate a double-stable clone expressing high levels of the tTA activator with low basal expression levels of the gene of interest.

Consequently, the pTetOff regulator plasmid was introduced into DU145 prostate cells by the calcium phosphate transfection method (see section 2.21.2). One million cells were seeded per 10cm dish. Cells were transfected with 20μg pTetOff plasmid and 48 hours after transfection selective medium containing Geneticin® at 1mg/ml was added. Ten replicates in 10cm dishes were set and non-transfected cells were grown in duplicate in the absence and presence of 1mg/ml Geneticin® as controls. The selective medium was replaced every four days. Cells were allowed to grow for three weeks. Thirty-two geneticin-resistant colonies were ring cloned (see section 2.22) and expanded for further analysis.

3.6 Transient luciferase expression assays in stable cell lines

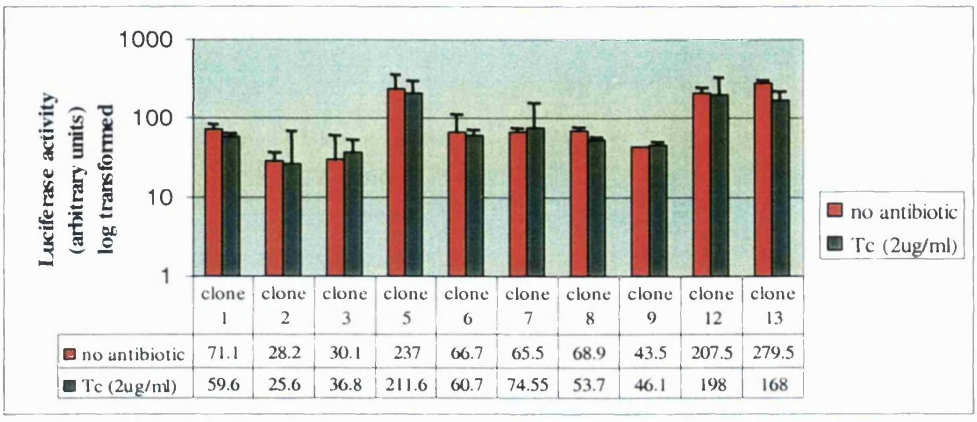
The expression levels of tTA transactivator encoded by the pTetOff plasmid depend on the site of plasmid integration. The objective of the first stable transfection was to generate a cell line with high tTA expression levels. Therefore, the geneticin-resistant colonies isolated were assayed for the transient expression of the luciferase gene in pTRE-Luc plasmid. The transient transfection experiment provided a functional assay for the tTA regulatory protein. The aim was to identify a stable clone that had high expression levels of luciferase in the "on" state and low basal expression in the "off" state.

For each stable clone, 3 x 10^5 cells were seeded in duplicate in 6-well dishes and transfected with 5μg pTRE-Luc plasmid using the calcium phosphate transfection method (see section 2.21.2). The cells were transfected in the absence and presence of 2μg/ml tetracycline (Tc). The expression of the pTRE-Luc response plasmid was measured 48 hours after transfection using the luciferase assay (see section 2.24). Twenty-seven (27/32) clones were tested, five being lost to bacterial contamination during expansion. All the stable clones assayed showed high luciferase induction with high background, except for clone 16 (Figure 3.4). Interestingly, clone 16 which showed the same high background levels as the other clones in the "off" state, induced the highest maximal luciferase activity in the "on" state. Therefore, clone 16 was designated as the stable cell line (DUTetOff cell line) to be used for the establishment of the double-stable TetOff cell line, and frozen stocks were prepared (see section 2.23).

3.7 Construction of pTRE-AR expression plasmid

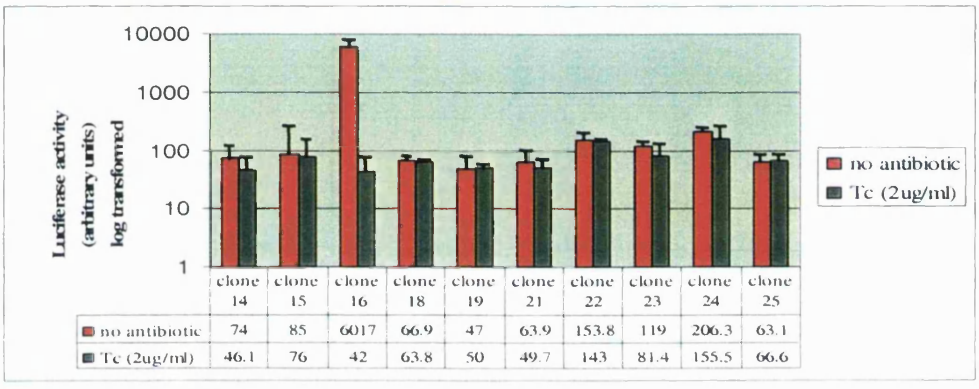
The next step in the development of the TetOff™ inducible system was the subcloning of the complete human androgen receptor (AR) cDNA into the pTRE response plasmid to construct the AR-expressing vector pTRE-AR.

a)



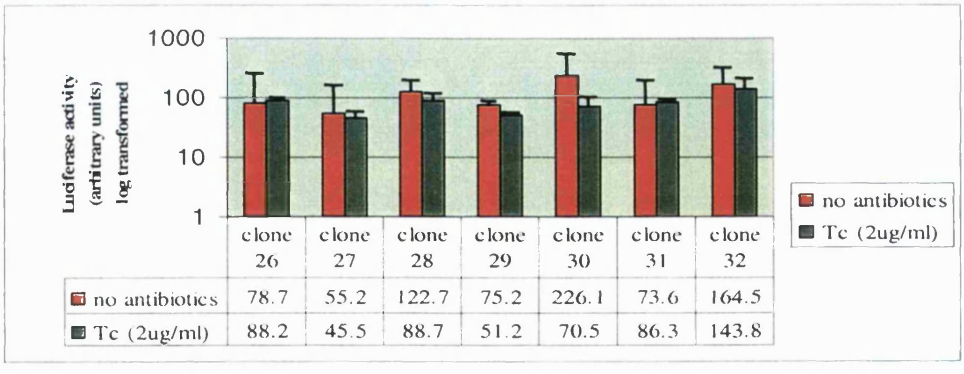
	clone 1	clone 2	clone 3	clone 5	clone 6	clone 7	clone 8	clone 9	clone 12	clone 13
no antibiotic	71.1	28.2	30.1	237	66.7	65.5	68.9	43.5	207.5	279.5
Tc (2ug/ml)	59.6	25.6	36.8	211.6	60.7	74.55	53.7	46.1	198	168

b)



	clone 14	clone 15	clone 16	clone 18	clone 19	clone 21	clone 22	clone 23	clone 24	clone 25
no antibiotic	74	85	6017	66.9	47	63.9	153.8	119	206.3	63.1
Tc (2ug/ml)	46.1	76	42	63.8	50	49.7	143	81.4	155.5	66.6

c)



	clone 26	clone 27	clone 28	clone 29	clone 30	clone 31	clone 32
no antibiotics	78.7	55.2	122.7	75.2	226.1	73.6	164.5
Tc (2ug/ml)	88.2	45.5	88.7	51.2	70.5	86.3	143.8

Figure 3.4 **Transient luciferase expression assays of stable clones.**

3 x 10^5 cells from each stable clone were seeded in duplicate in 6-well dishes and transfected with 5µg pTRE-Luc plasmid. Twenty-seven stable clones were assayed [a), b) and c)]. The expression of pTRE-Luc plasmid was measured 48hrs after transfection by the luciferase assay. Cells were transfected in the absence (■) and presence (■) of 2µg/ml tetracycline (Tc). Only in Clone 16 (b) consistently higher levels of luciferase were induced in the absence of Tc. The mean of luminometer readings from duplicate samples were plotted as the luciferase activity. Error bars are SEM for two independent experiments.

The wild type, full-length human AR cDNA contained within the pcDNA-AR plasmid (see section 2.6.4) was used for the subcloning.

To generate pTRE-AR, both pTRE and pcDNA-AR plasmids were digested with the restriction enzyme *Xba*I. This digest linearised pTRE (3.146kb) and excised the AR cDNA (3.1kb) from pcDNA-AR (Figure 3.5). The linearised pTRE plasmid was then dephosphorylated (see section 2.13.1) to prevent re-circularisation. Before ligation, both the insert (AR cDNA) and the dephosphorylated vector (pTRE) were resolved on an agarose gel, isolated and purified as described in section 2.12. Finally, the dephosphorylated purified pTRE was ligated to the purified AR cDNA (see section 2.13.2) and the ligated product was used to transform bacterial cells (see section 2.13.3). DNA from six recombinant clones was isolated by the mini prep method (see section 2.8.1).

Extensive restriction enzyme analysis confirmed the ligation of AR cDNA to pTRE, and demonstrated the integrity of the newly generated pTRE-AR plasmid and the correct orientation of the AR cDNA into pTRE-AR.

The restriction analysis of three recombinant clones (Clone IV, V, VI) with three indicative enzymes (*Eco*RI, *Hinc*II, *Sma*I) is shown in Figure 3.6. These enzymes yielded restriction fragments whose sizes distinguished between the correct (Clone V) and incorrect (Clone IV, and Clone VI) insertion of AR cDNA.

From the restriction analysis with *Hinc*II, it was apparent that the expected fragment (shown in bold in the table of Figure 3.6a) gave rise to two fragments. This implied the presence of an extra *Hinc*II site in the pTRE-AR plasmid, probably generated through mutation. It was important to verify the location of this extra *Hinc*II site on pTRE-AR to ensure that it did not interfere with the expression of AR. Further restriction analysis was, therefore performed on pTRE and on AR cDNA (Figure 3.7). The extra *Hinc*II site was localised (around position 2600-2650) on the pTRE vector. This site is not located within the vector regulatory sequences, suggesting that is unlikely that the extra *Hinc*II site will interfere with pTRE (or subsequently pTRE-AR) expression.

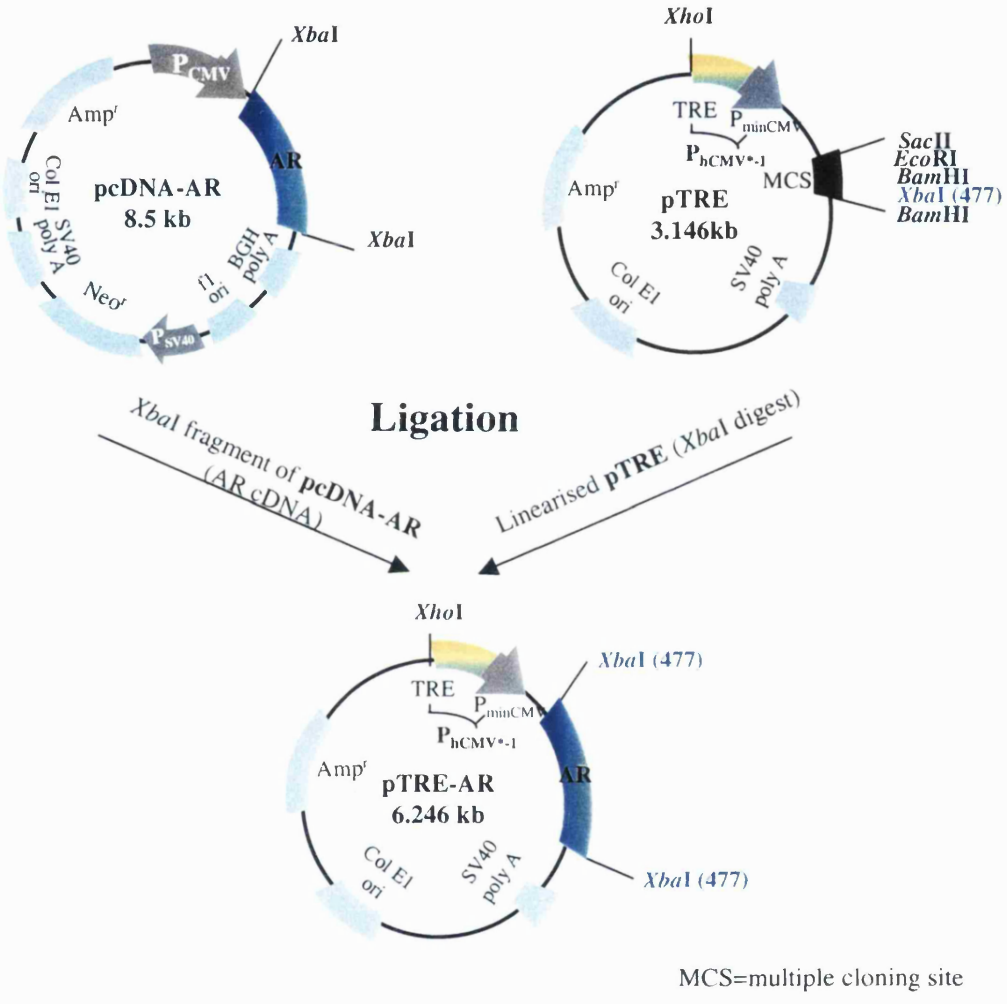
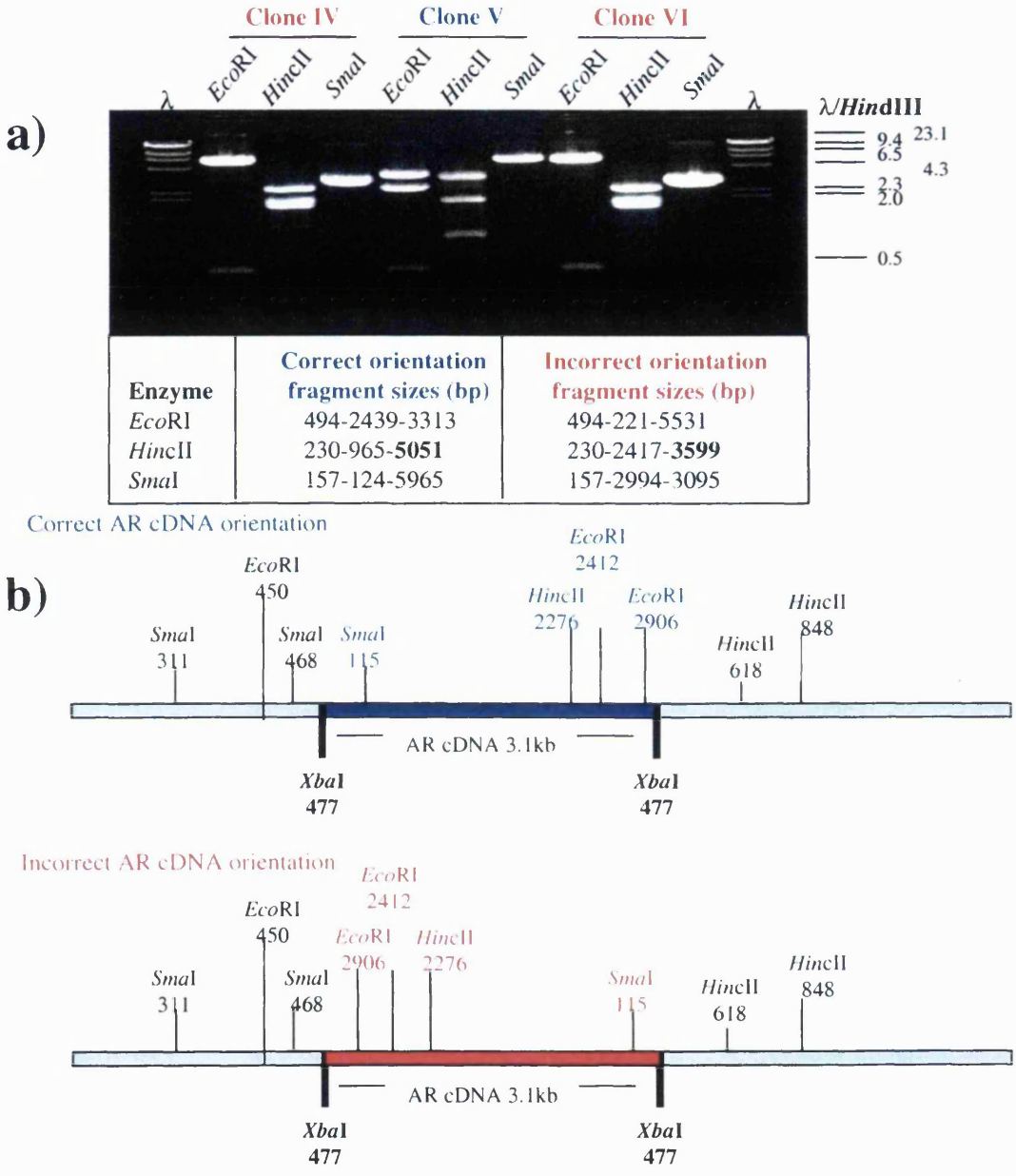


Figure 3.5 **Construction of pTRE-AR expression plasmid.**

Schematic representation of restriction enzyme digestion and ligation steps in the preparation of pTRE-AR, the AR encoding expression vector. *Xba*I digest excised AR cDNA (3.1kb) from pcDNA-AR plasmid and linearised pTRE plasmid (3.146kb). AR cDNA and the linearised pTRE were ligated to generate pTRE-AR vector.



Enzyme	Correct orientation fragment sizes (bp)	Incorrect orientation fragment sizes (bp)
*Eco*RI	494-2439-3313	494-221-5531
*Hinc*II	230-965-**5051**	230-2417-**3599**
*Sma*I	157-124-5965	157-2994-3095

Figure 3.6 **Confirmation of correct orientation of AR cDNA in pTRE-AR.**

a) EtBr-stained gel showing the restriction enzyme analysis of three recombinant clones (Clone IV, V, and VI) with *Eco*RI, *Hinc*II and *Sma*I enzymes. The patterns for clones IV and VI (incorrect AR cDNA orientation) are identical, whilst different from Clone V (correct AR cDNA orientation). The table shows the expected fragment sizes (correct and incorrect orientation of AR cDNA) from the restriction enzyme analysis of pTRE-AR. The fragment sizes in bold (*Hinc*II digest) do not correspond to the pattern on the gel (see Figure 3.7 for details). In lanes 3, 4, 9, and 10 unresolved doublets are apparent. λ: *Hind*III fragments (in kb) of λ phage DNA.

b) Cartoons of linearised pTRE-AR (6.246kb). The restriction sites of the three enzymes (*Eco*RI, *Hinc*II and *Sma*I) are shown on the plasmid and AR cDNA.

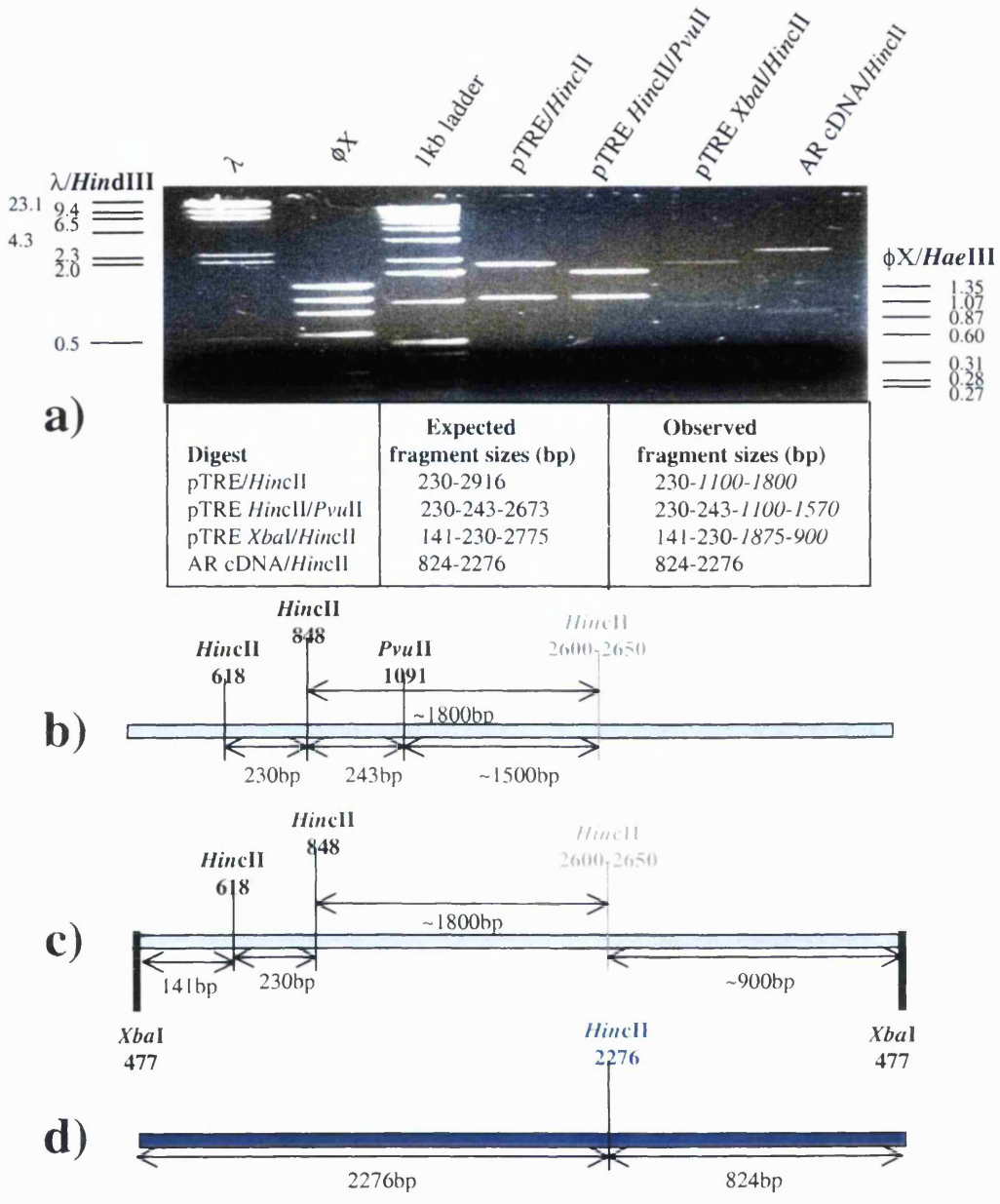


Figure 3.7 **Verification of location of the extra *Hin*cII site in pTRE-AR plasmid.**

a) EtBr-stained gel showing the restriction enzyme analysis of pTRE plasmid and AR cDNA. The table shows the restriction fragment sizes (expected and observed) of pTRE and AR cDNA. The observed fragment sizes in italic are the approximate sizes (in bp) as read from the gel using DNA markers as reference. There was no difference between the expected and the observed restriction fragments of AR cDNA suggesting that the extra *Hin*cII site is located on pTRE. λ: *Hin*dIII fragments (in kb) of λ phage DNA. φX: *Hae*III fragments (in kb) of phage φX174 DNA.

b) and c) Cartoons of linearised pTRE (3.146kb). The restriction sites of *Pvu*II, *Xba*I, and *Hin*cII (the extra site is shown in grey) are shown.

d) Cartoon of AR cDNA (3.1kb). The *Hin*cII site is shown.

The extensive restriction analysis of Clone V confirmed the presence, integrity and correct orientation of the AR cDNA. This clone was designated as pTRE-AR, the AR-encoding expression vector to be used for the establishment of the double-stable TetOff cell line.

3.8 Transient expression assays with pTRE-AR

Prior to the development of the double-stable TetOff cell line overexpressing AR, the newly established pTRE-AR expression plasmid was tested transiently in DUTetOff cells for its functionality. Transient assays give a quick indication of whether or not the plasmid will function properly in a particular cell line.

3 x 10^5 DUTetOff cells were seeded in duplicate in 6-well dishes and transfected with 5μg pTRE-AR plasmid using the calcium phosphate transfection method (see section 2.21.2). The cells were transfected in the absence and presence of 2μg/ml tetracycline (Tc). Cells were assayed for AR protein expression 48 hours after transfection by Western analysis (see section 2.14). As a negative control, cells were transfected with pTRE plasmid lacking the AR-insert. AR protein expression was expected in the cells transiently transfected with pTRE-AR grown in the absence of Tc. However, no AR protein expression was seen (Figure 3.8a).

Over time Tc-responsiveness can be lost i.e. Tc-regulated plasmids cease to be expressed, a characteristic dependent on cell type (Hofmann et al., 1996; Corish P, personal communication; Sharrad RM, unpublished observations). Therefore, the functionality of pTRE-AR was tested transiently by co-transfecting pTetOff and pTRE-AR in DU145 cells.

5 x 10^5 cells were seeded in duplicate in 10cm dishes and co-transfected with 18μg pTetOff and 2μg pTRE-AR plasmids using the calcium phosphate transfection method (see section 2.21.2). The cells were transfected in the absence and presence of 2μg/ml tetracycline (Tc). The expression of the pTRE-AR response plasmid was assayed 48 hours after transfection by Western analysis (see section 2.14).

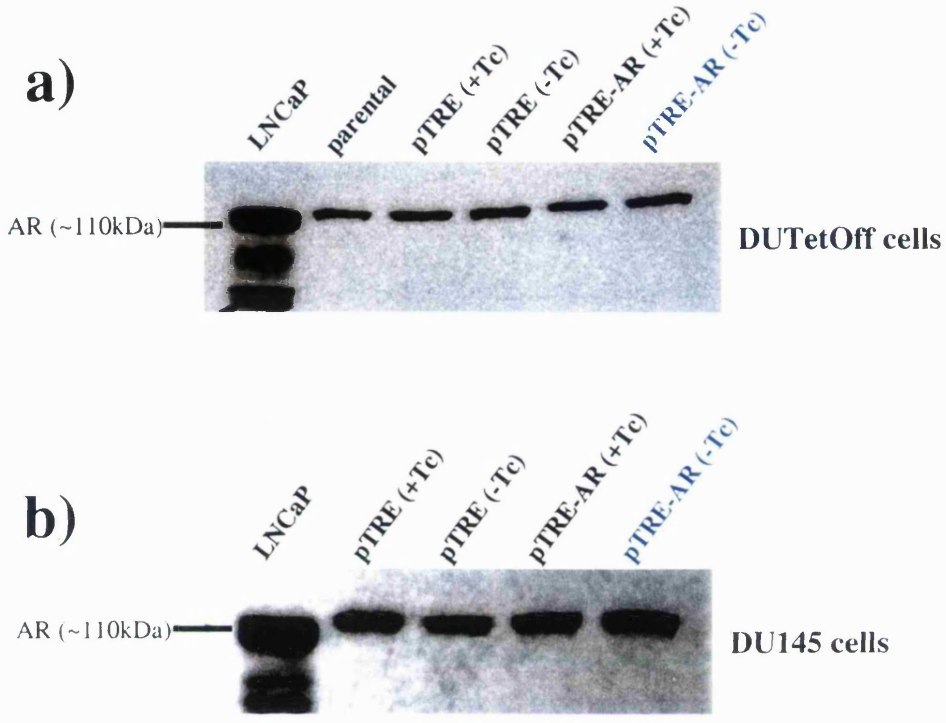


Figure 3.8 **Western analysis of DUTetOff and DU145 cells transiently transfected with the newly established pTRE-AR plasmid**.

Cells were transfected in the absence and presence of 2μg/ml tetracycline (Tc) and assayed 48hrs after transfection for AR protein expression. Transfections with pTRE lacking the AR-insert (+/- Tc) were used as negative control together with lysates from non-transfected (parental) cells. Loading of protein from LNCaP cells indicated the size of the expected AR-specific band (~110kDa). AR protein expression was expected in the cells transfected with pTRE-AR grown in the absence of tetracycline (-Tc). Equal loading was confirmed by Ponceau S staining (data not shown). No AR protein was detected. The presence of a non-specific band of slightly higher molecular weight than AR is apparent in all samples tested (see section 3.9 for details).

a) Western blot of DUTetOff stable cells transiently transfected with pTRE-AR.

b) Western blot of DU145 cells transiently co-transfected with pTetOff and pTRE-AR.

As negative control, cells were transfected with pTRE plasmid lacking the AR-insert. AR protein expression was expected in the transiently transfected cells with pTRE-AR grown in the absence of Tc. However, no AR protein expression was seen (Figure 3.8b).

To ensure that the pTetOff plasmid was still present in the DUTetOff cell line, a transient luciferase expression assay (as described in section 3.6) with pTRE-Luc plasmid was performed. As expected, there were high levels of luciferase expression in the absence of Tc, with low background expression in the presence of 2μg/ml Tc. This confirmed the presence of the pTetOff plasmid in DUTetOff cells and indicated that DUTetOff cells maintained the same levels of induction after 2 months of continuous culture.

At this point, the monkey kidney fibroblastic cell line COS-1 was utilised. This is a highly inducible cell line commonly used in transfection experiments. The pTRE-AR plasmid was transiently assayed for its functionality in COS-1 cells by co-transfecting pTetOff and pTRE-AR (as described in co-transfection of pTetOff and pTRE-AR in DU145 cells).

AR protein expression was apparent, as expected, in the cells transiently transfected with pTRE-AR, grown in the absence of Tc (Figure 3.9). This confirmed the functionality of the newly established pTRE-AR expression plasmid.

To ensure that the lack of AR expression seen in DUTetOff and DU145 cells was not due to a prostate cell line-specific problem, a transient co-transfection experiment with pTetOff and pTRE-AR plasmids was carried out in PC-3 and DUSF prostate cancer cells (as described in co-transfection of pTetOff and pTRE-AR in DU145 cells). AR protein expression was apparent, as expected, in the cells transiently transfected with pTRE-AR grown in the absence of Tc (Figure 3.10). This also confirmed the functionality of the newly established pTRE-AR expression plasmid and implied a cell line-specific problem with DU145 cells.

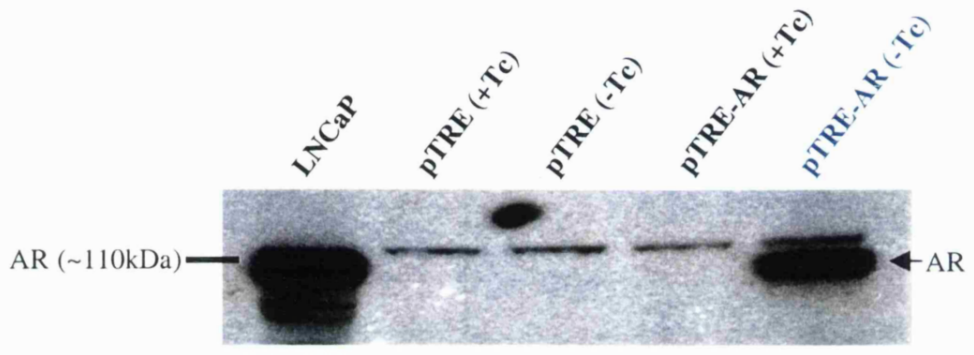


Figure 3.9 **Western analysis of COS-1 cells transiently transfected with the newly established pTRE-AR plasmid.**

Cells were co-transfected with pTetOff and pTRE-AR in the absence and presence of 2μg/ml tetracycline (Tc) and assayed 48hrs after transfection for AR protein expression. Transfections with pTRE lacking the AR-insert (+/- Tc) were used as negative control. Loading of protein from LNCaP cells indicated the size of the expected AR-specific band (~110kDa). AR protein expression was detected, as expected, in cells transfected with pTRE-AR grown in the absence of tetracycline (-Tc). Equal loading was confirmed by Ponceau S staining (data not shown). The presence of a non-specific band of slightly higher molecular weight than AR is apparent in all samples tested (see section 3.9 for details).

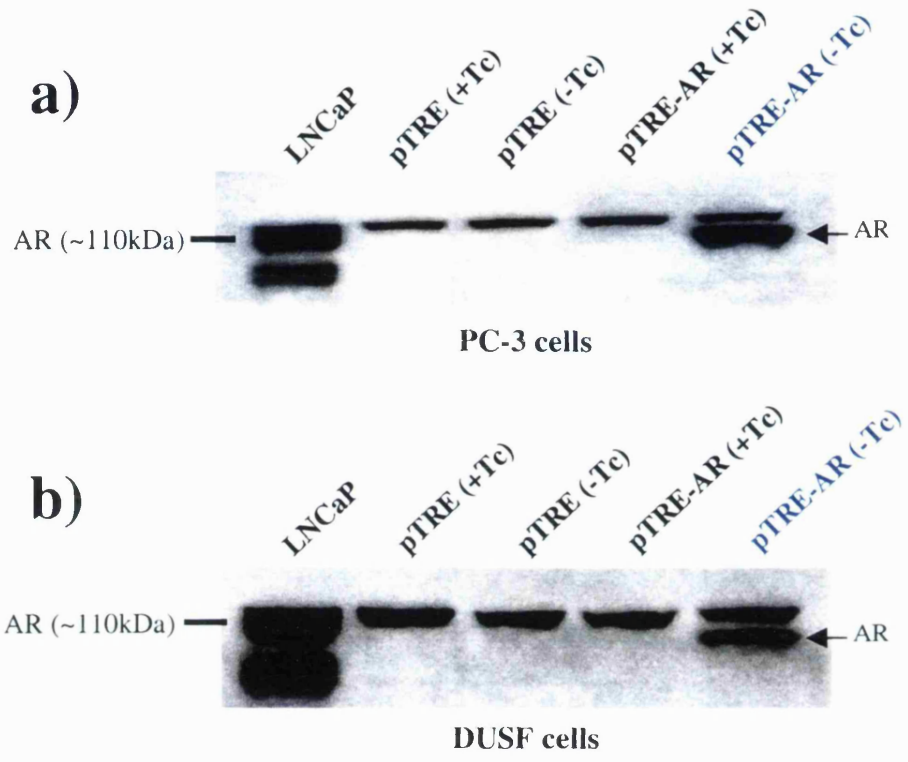


Figure 3.10 **Western analysis of PC-3 and DUSF cells transiently transfected with the newly established pTRE-AR plasmid.**

Cells were transfected in the absence and presence of 2μg/ml tetracycline (Tc) and assayed 48hrs after transfection for AR protein expression. Transfections with pTRE lacking the AR-insert (+/- Tc) were used as negative control. Loading of protein from LNCaP cells indicated the size of the expected AR-specific band (~110kDa). AR protein expression was detected, as expected, in the cells transfected with pTRE-AR grown in the absence of tetracycline (-Tc). Equal loading was confirmed by Ponceau S staining (data not shown). The presence of a non-specific band of slightly higher molecular weight than AR is apparent in all samples tested (see section 3.9 for details).

a) Western blot of PC-3 cells transiently co-transfected with pTetOff and pTRE-AR.

b) Western blot of DUSF cells transiently co-transfected with pTetOff and pTRE-AR.

3.9 Immunodetection of AR expression

From the Western analyses shown in Figure 3.8, 3.9 and 3.10, a band of slightly higher molecular weight than the AR-specific band was apparent in all the samples tested. Suprisingly, this band was present even in the cell extracts from parental AR-negative cells (Figure 3.8a), and also in COS-1 cells (Figure 3.9) which do not express the androgen receptor gene (Brinkmann et al., 1989). From the autoradiographs it was apparent that the size of this band was just a few kDa bigger than the 112kDa AR-specific band (corresponding to a phosphorylated AR isoform) (Jenster et al., 1991; Kuiper et al., 1991). Both the slower migrating band and the AR-specific band, when present, migrated as a closely spaced doublet on SDS-polyacrylamide gels and were inseparable unless the gel was run for longer. However, in LNCaP cells due to the intensity of the AR signal, this slower migrating band was rarely noticeable.

3.9.1 Evidence for AR expression using F39.4.1 monoclonal antibody

The mouse monoclonal antibody, clone F39.4.1 (Biogenex, UK) used for AR immunodetection was generated against a fragment of the N-terminal domain of the androgen receptor (hAR residues 301-320) and does not cross-react with other steroid hormone receptors (Zegers et al., 1991). The F39.4.1 antibody has already been successfully used for immunocytochemistry (Masai et al., 1990; de Winter et al., 1991; van der Kwast et al., 1991; Chodak et al., 1992; Fry et al., 2000) and Western analysis (Jenster et al., 1991; van Laar et al., 1991; Kuiper et al., 1992; Kuiper et al., 1993). However, these researchers followed a different protocol for Western analysis whereby the AR protein was initially immunoprecipitated from whole cell lysates with the F39.4.1 monoclonal antibody, and subsequently immunodetected on a Western blot with a polyclonal antibody. The detected wild type hAR migrated as a 110 to 112kDa doublet on SDS-PAGE, reflecting different degrees of phosphorylation, while no additional bands of higher molecular weight than AR were

apparent (van Laar et al., 1990; Jenster et al., 1991; Kuiper et al., 1992; Kuiper et al., 1993).

To verify whether the prominent band was an artifact caused by the secondary antibody or the ECL detection system, cell extracts from LNCaP and COS-1 cells were analysed. Lysates from both cell lines were subjected to SDS-polyacrylamide electrophoresis in groups of two. Four hybridisations were performed omitting each time an incubation step. As expected, no band was detected in the hybridisations with the primary antibody alone, the secondary antibody alone and the ECL detection system alone (Figure 3.11). These findings implied that neither the secondary antibody nor the ECL detection system were generating the additional band.

3.9.2 Comparison of AR monoclonal antibodies (clone F39.4.1 vs clone G122-434)

According to Brinkmann A.O. (personal communication) a direct Western blot, (without the immunoprecipitation step) with the F39.4.1 monoclonal antibody generates some additional bands that are different from the AR isoforms. Due to this, the immunoprecipitation step is included routinely to increase specificity.

In order to substantiate this, another mouse monoclonal antibody, clone G122-434 (PharMingen) was used to detect AR protein expression in cells transiently transfected with an AR expression plasmid. G122-434 antibody was generated against residues 33-485 of the N-terminal domain of the human androgen receptor (hAR) and does not recognise estrogen and progesterone receptors (Chang et al., 1992).
Transiently transfected cells from PC-3 and COS-1 cell lines were assayed for AR protein expression 48 hours after transfection by Western analysis (see section 2.14). Cell lysates from non-transfected (parental) cells were included as negative control. AR protein expression was expected in the transiently transfected cells with the AR expression vector and in the LNCaP cells that were included as positive control.

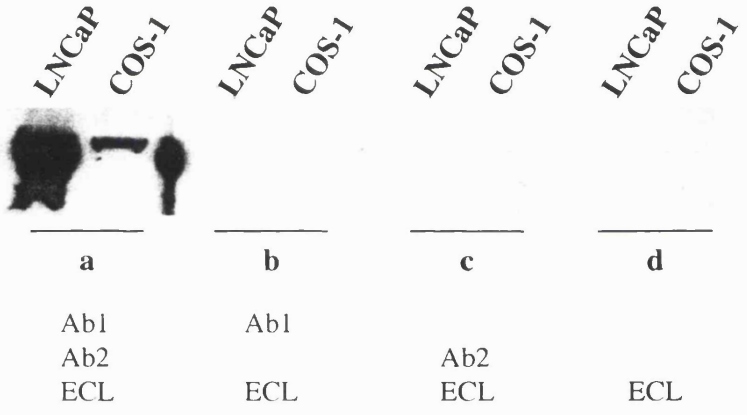


Figure 3.11 **Specificity control experiment for the secondary antibody and ECL detection system.**

Cell extracts in groups of two from LNCaP and COS-1 cells were assayed for AR protein expression by Western analysis. Four hybridisations were performed omitting a step each time: a) as described in 2.14) b) primary antibody and ECL c) secondary antibody and ECL and d) only ECL detection system. Loading of protein from LNCaP cells indicated the size of the expected AR-specific band (~110kDa). Loading of protein from COS-1 indicated the non-specific band. Equal loading was confirmed by Ponceau S staining (data not shown). Neither the secondary antibody nor the ECL detection system on their own generated the non-specific band.

The results of the Western analysis using the two different monoclonal antibodies are shown in Figure 3.12. From this experiment, it is clear and convincing that the prominent band is an artifact caused by the F39.4.1 monoclonal antibody.

3.10 Conclusions

The aim of the work described in this chapter was to develop an inducible gene expression system (TetOff™ system) where androgen-mediated changes in cells overexpressing a range of AR levels could be studied.

Two androgen receptor-negative prostate cancer cell lines (DU145 and PC-3) were tested as potential hosts for the TetOff™ system. Transient luciferase assays indicated that both cell lines were compatible with the system, but DU145 cells showed a higher luciferase induction in the absence of Tc and were therefore used as the host cell line. For the development of a highly inducible tTA-expressing cell line, pTetOff plasmid was stably introduced into DU145 cells and clones expressing the tTA transactivator were screened by transient luciferase assays. One out of 27 clones (less than 5%) showed high transcriptional activity and this clone was designated as DUTetOff cell line.

These results are consistent with previous studies where the efficiency of the system has been shown to be influenced by cell type, and only a small number (less that 10%) of the isolated stable clones has been shown to express high levels of the tTA transactivator (Ackland-Berglund and Leib, 1995; Gossen and Bujard, 1995; Gossen et al., 1995; Howe et al., 1995; Yin et al., 1996). The residual luciferase activity observed in the presence of Tc in the transient assays is also in agreement with published reports (Gossen and Bujard, 1995; Yin et al., 1996). Tandem integration of pTetOff and pTRE-Luc plasmids can raise the basal levels of gene expression (Yin et al., 1996). Only chromosomal integration of the minimal promoter (P_{minCMV}), results in low basal activity because then the copy number per cell is low and basal promoter activities are repressed after chromatin assembly (Gossen and Bujard, 1995).

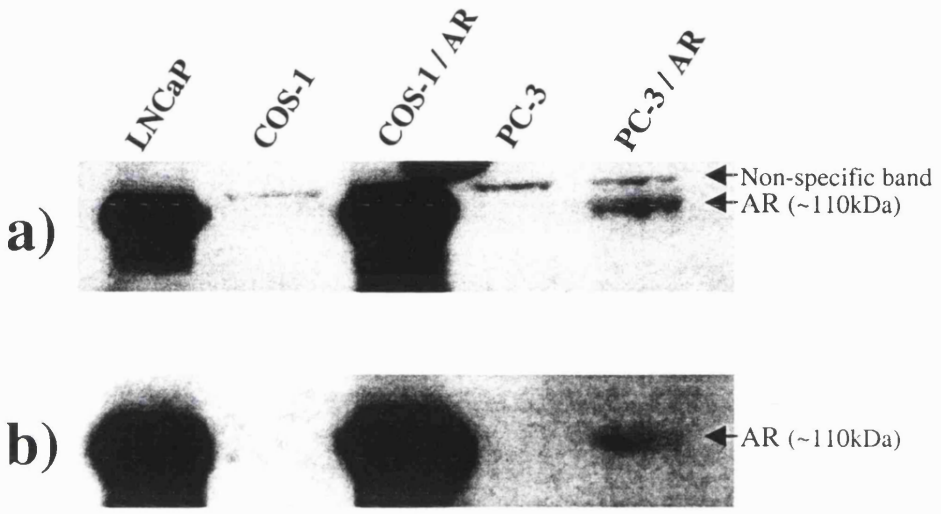


Figure 3.12 **Comparison of AR mouse monoclonal antibodies (clone F39.4.1 vs clone G122-434).**

Cells from COS-1 and PC-3 cell lines were transiently transfected with an AR expression plasmid (pTRE-AR) and analysed 48hrs after transfection for AR protein expression. Lysates from non-transfected (parental) cells were included as negative control. Loading of protein from LNCaP cells indicated the size of the expected AR-specific band (~110kDa). Western analysis with two AR monoclonal antibodies: a) Clone F39.4.1 (Biogenex) and b) Clone G122-434 (PharMingen) was performed as described in 2.5 and 2.14. Equal loading was confirmed by Ponceau S staining (data not shown). The band of higher molecular weight than the AR-specific band is non-specific and is generated by the F39.4.1 monoclonal antibody.

In addition to the DUTetOff cell line, a functional AR expression vector was constructed (pTRE-AR). However, transient assays of DUTetOff cells with pTRE-AR suggested a potential cell line-specific problem; it was therefore decided not to proceed with the double-stable experiments. The cell line-specific expression problem is investigated further in Chapter 5.

This chapter also describes the specificity of the monoclonal antibody used for Western analysis. From the Western blots, a prominent band of higher molecular weight than the AR-specific band was evident in all samples tested. Experiments with another AR monoclonal antibody (clone G122-434) proved that the additional band was not the androgen receptor or an AR isoform, but rather a non-specific band generated by the F39.4.1 monoclonal antibody. Because the non-specific band seemed to reflect the loading, the F39.4.1 antibody was used for the experiments described in the following chapters.

Chapter 4

Overexpression of the androgen receptor (AR) gene in AR-negative cells using a constitutive gene expression system

4.1 Introduction

The constitutive gene expression system is the traditional approach to the study of genes. To develop such a system, a plasmid carrying the gene of interest under the control of a constitutive promoter needs to be stably introduced into the host cells in a single transfection step. The constitutively expressing cells are isolated using a drug selectable marker.

As the expression pattern of a cloned gene is influenced by the sites of vector integration and the copy number of the integrated gene, differences in the levels of expression are expected (Doerfler et al., 1995; Doerfler, 1996; Knoblauch et al., 1996; Doerfler et al., 1997; Choi et al., 2000). Therefore, a number of stable clones need to be isolated for the analysis of cells expressing different amounts of the protein of interest.

For the work described in this chapter, a CMV promoter/enhancer-driven system encoding a wild type, human androgen receptor was used.

4.2 Aims of the chapter

The objective of the work described in this chapter forms a continuum in the development of an *in vitro* prostate cancer model where the effect of androgen exposure on cells expressing a range of androgen receptor concentrations could be studied.

Specifically, the aims were to:

- Stably transfect the wild type, full-length human AR cDNA into three AR-negative human prostate cancer cell lines (DU145, PC-3 and DUSF).
- Select, expand and characterise the cells expressing different AR levels.
- Assess the effects of androgen exposure on AR mRNA and protein levels.
- Assess the effects of androgen exposure on cell proliferation and cloning efficiency.

4.3 Optimisation of geneticin concentration and plating density

Before the generation of stable prostatic cell lines expressing different levels of the human androgen receptor, the optimal geneticin concentration and plating density for the host cell lines (DU145, PC-3 and DUSF) were determined as described in 2.21.1. The expression plasmid (pcDNA-AR), used for the transfection experiments, carried the neomycin-resistance gene (*neo*^{r}) which confers resistance to the antibiotic Geneticin® (G-418 Sulphate) and allows for selection of the transfected cells.

For all three cell lines, 1mg/ml Geneticin® was chosen as the optimal dosage for selection, while 1 x 10^6 cells/10cm dish was chosen as the optimal plating density.

4.4 Transfection of cells and selection of stable clones

In a single transfection step, the expression plasmid pcDNA-AR was stably introduced into three androgen receptor-negative human prostate cancer cell lines (DU145, PC-3 and DUSF). pcDNA-AR is a vector with a constitutive mammalian promoter (P_{CMV}) which drives the expression of a wild type, full-length human androgen receptor (AR) gene.

Before the transfection experiments, a sufficient amount of plasmid DNA (pcDNA-AR) was prepared and purified as described in section 2.8.2. Restriction enzyme analysis (see section 2.10) confirmed the quality and integrity of the plasmid (Figure 4.1a). A restriction map (partial) for pcDNA-AR plasmid is also shown (Figure 4.1b).

All three cell lines (DU145, PC-3 and DUSF) were transfected with pcDNA-AR plasmid by the calcium phosphate transfection method (see section 2.21.2). One million cells per 10cm dish were transfected with 10μg plasmid DNA. Mock-transfected cells were generated by parallel transfection with pcDNA vector lacking the androgen receptor cDNA insert. Selective medium (1mg/ml Geneticin®) was added 48hrs after transfection to both mock- and androgen receptor-transfected cells and was replaced every four days.

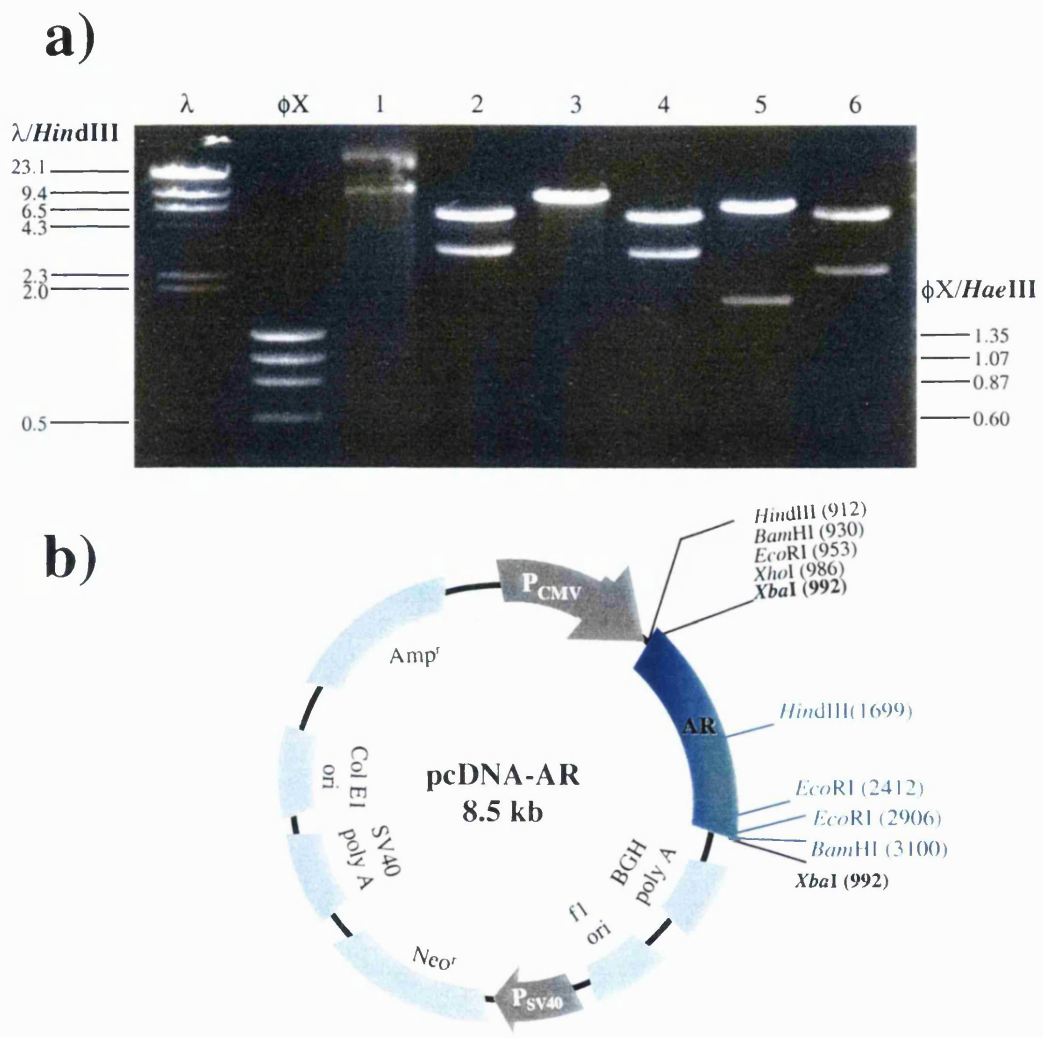


Figure 4.1 **Restriction enzyme analysis of pcDNA-AR plasmid.**

a) EtBr-stained gel showing restriction enzyme digestions of pcDNA-AR plasmid. Lane 1 shows the uncut pcDNA-AR plasmid. Lanes 2 and 4 show the *Bam*HI and *Xba*I digest respectively where the AR cDNA (3.1kb) is excised from the pcDNA vector backbone (5.4kb). Lane 3 shows the linearised pcDNA-AR plasmid (*Xho*I digest, 8.5kb). Lanes 5 and 6 show the *Hin*dIII digest (6.72 and 1.78kb) and the *Eco*RI digest (5.55, 2.45 and 0.49kb) respectively where the integrity of the plasmid was further confirmed. λ: *Hin*dIII fragments (in kb) of λ phage DNA. φX: *Hae*III fragments (in kb) of phage φX174 DNA.

b) Restriction map (partial) of pcDNA-AR plasmid. The restriction sites on AR cDNA are shown in blue.

As a control, non-transfected cells were grown in the absence and presence of Geneticin®. Cells were incubated until geneticin-resistant colonies appeared. Six AR-transfected clones from PC-3 and DUSF transfectants and five AR-transfected clones from DU145 transfectants were isolated and ring cloned (see section 2.22). In addition, from each transfected cell line, one mock-transfected clone was isolated and ring cloned. Both mock- and androgen receptor-transfected clones were then expanded to be tested for AR expression and frozen stocks were prepared (see section 2.23).

4.5 Western analysis of stable clones

The stable AR-transfected clones isolated were expected to express AR, as the gene was under the control of a constitutive promoter. Moreover, as discussed in section 4.1, the AR expression levels between the clones were expected to vary depending on differences in the copy number and insertion site of plasmid DNA into the genome.

The stable AR-transfected clones from the three cell lines (DU145, PC-3 and DUSF) were assayed for AR protein expression by Western analysis (see section 2.14). Mock-transfected clones together with cell lysates from parental, non-transfected cells were also assayed. AR protein expression was expected in the AR-transfected cells and in the LNCaP cells that were included as positive control. As indicated by the Western analysis, there was no difference between the non-transfected (parental), mock-transfected and AR-transfected cells (Figure 4.2). An AR immunoreactive band was present only in LNCaP cells. The absence of AR expression in both non-transfected and mock-transfected cells was expected. However, the absence of AR expression in AR-transfected cells from all three cell lines was surprising.

4.6 Lack of AR expression in the AR-transfected cells

Several reasons for the lack of AR expression in the AR-transfected cells were considered.

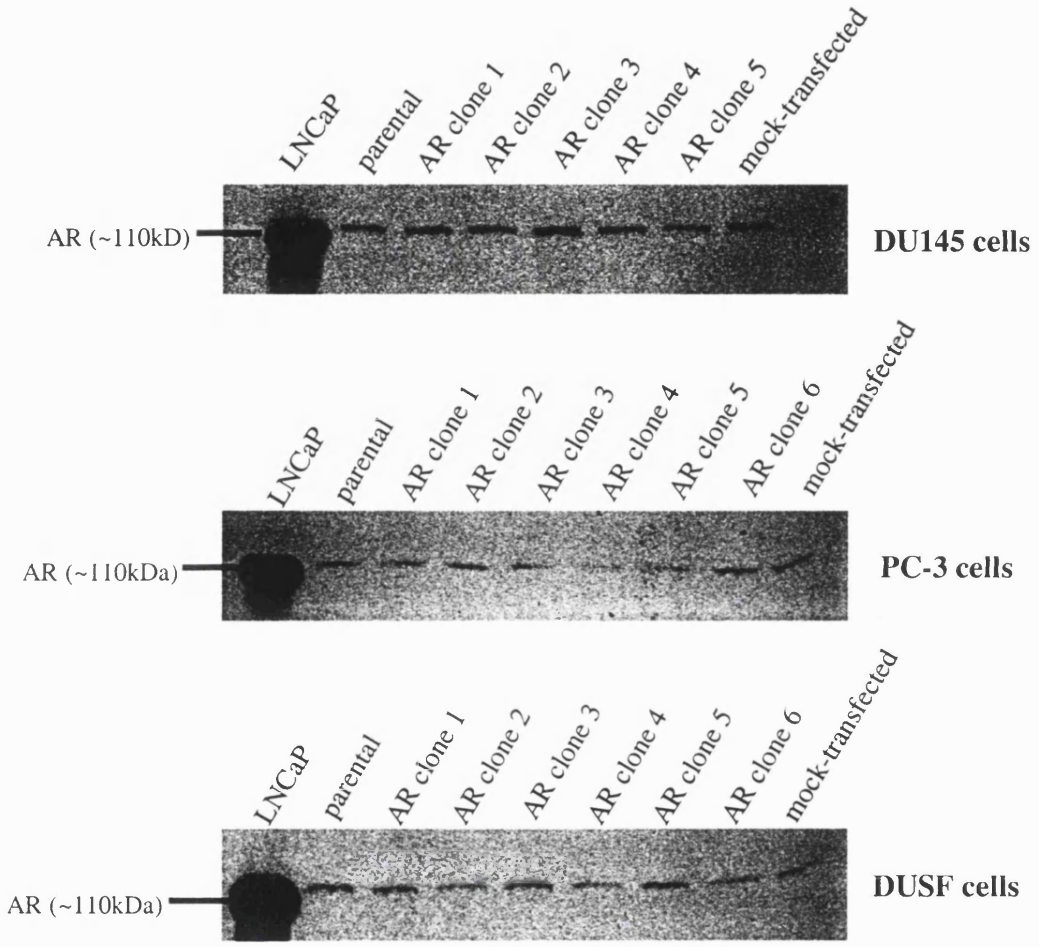


Figure 4.2 **Western analysis of DU145, PC-3 and DUSF cells stably transfected with the constitutive pcDNA-AR plasmid.**

AR clones from the three cell lines (DU145, PC-3 and DUSF) stably transfected with pcDNA-AR plasmid were assayed for AR protein expression. Lysates from mock-transfected and non-transfected (parental) cells were included as negative control. Loading of protein from LNCaP cells indicated the size of the expected AR-specific band (~110kDa). No AR protein was detected in the AR clones. Equal loading was confirmed by Ponceau S staining (data not shown). The presence of a non-specific band of slightly higher molecular weight than AR is apparent in all samples tested (see section 3.9 for details).

1. The possibility of plasmid integration into a 'silent' stretch of the genome could explain the absence of AR expression in some clones (Doerfler et al., 1995). However, this possibility seemed highly unlikely as all AR-transfectants from all three cell lines lacked AR expression.

2. As mentioned in section 4.4, six AR-transfected clones from PC-3 and DUSF transfectants and five AR-transfected clones from DU145 transfectants were selected to be analysed. Chaudhary KS (1999) has reported that only a small number (5/23) of the AR-transfected DU145 clones he generated express AR protein when immunocytochemically screened. Moreover, only 2/5 of the AR clones that stain AR positive contain >90% AR-expressing cells, the rest (3/5) have <10% AR-expressing cells. Hence, it is likely that more AR-transfected clones needed to be isolated to obtain a number of stable clones expressing different levels of the AR gene.

3. Alternatively, the lack of AR expression in the AR-transfected clones could be a result of selection against transfectants expressing high levels of AR by growth inhibition, even in the absence of exogenous androgen (Gill and Ptashne, 1988; Fiering et al., 1990; Sharrad RM, personal communication).

4. Another possible explanation, could include transcriptional blockage resulting from methylation of the plasmid (Sutter et al., 1978; Orend et al., 1995; Doerfler et al., 1997) (see Chapter 5).

5. Finally, a non-functional expression plasmid could explain the lack of AR expression in the AR-transfected clones. pcDNA-AR plasmid has previously been sequenced and successfully used in transfection experiments (Robson CN, personal communication). Restriction enzyme analysis of pcDNA-AR (section 4.4), yielded the expected fragment sizes confirming the integrity of the plasmid. Moreover, successful transient assays of DUSF, PC-3 and COS-1 cells with pTRE-AR (carrying the AR cDNA insert from pcDNA-AR) were described in Chapter 3 (section 3.8).

4.7 Transient expression assays with pcDNA-AR

In order to confirm functionality of pcDNA-AR, transient transfection assays were carried out.

3 x 10^5 cells from the three cell lines (DU145, PC-3 and DUSF) were seeded in duplicate in 6-well dishes and transfected with 5μg pcDNA-AR plasmid using the calcium phosphate transfection method (see section 2.21.2). Cells were assayed for AR protein expression 48 hours after transfection by Western analysis (see section 2.14). As negative control, cells were transiently transfected with pcDNA vector lacking the androgen receptor cDNA insert (mock-transfected). Cell lysates from non-transfected (parental) cells were also included as negative control. AR protein expression was expected in the transiently transfected cells with pcDNA-AR and in the LNCaP cells that were included as positive control. No AR protein expression was seen in any of the three cell lines (Figure 4.3).

To ensure that the lack of AR expression seen in DU145, PC-3 and DUSF cells was not due to a prostate cell line-specific problem, a transient transfection assay with pcDNA-AR (as described above) was performed in COS-1 monkey kidney fibroblasts. AR protein expression was expected in the transiently transfected COS-1 cells with pcDNA-AR. No AR protein expression was detected (Figure 4.4). This implied a functional problem with pcDNA-AR plasmid.

At this point, it was important to verify whether there was an expression problem with the plasmid when originally received or whether there was an inactivating mutation introduced into it while being propagated. Therefore, 1μg of the original pcDNA-AR was used to transform bacterial cells, as described in section 2.13.3. Six individual bacterial colonies were selected and plasmid DNA from the colonies was isolated and purified, as described in section 2.8.2. Restriction enzyme analysis (see section 2.10) confirmed the integrity of plasmid DNA from the six maxi preps (pcDNA-AR 1→6).
3 x 10^5 cells from COS-1 cell line were seeded in duplicate in 6-well dishes and transfected with 5μg from each plasmid maxi prep (pcDNA-AR 1→6) using the calcium phosphate transfection method (see section 2.21.2). AR protein expression was measured 48 hours after transfection by Western analysis (see section 2.14). Cell lysates from mock-transfected and non-transfected (parental) cells were included as negative control. AR protein expression was apparent, as expected, in the transiently transfected cells with all six (pcDNA-AR 1→6) plasmid preps (Figure 4.5).

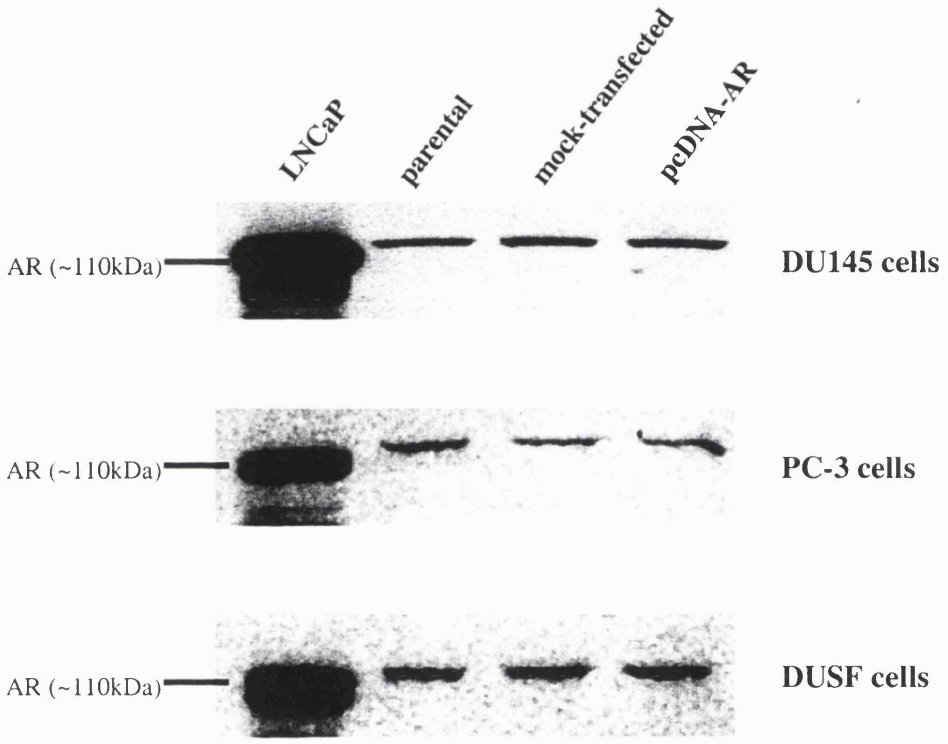
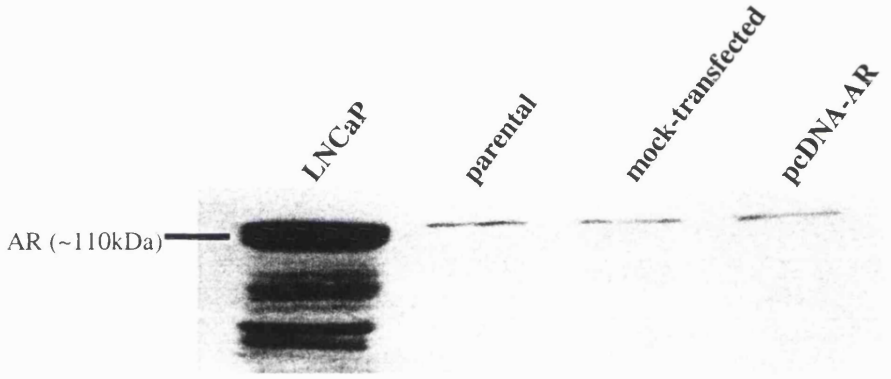


Figure 4.3 **Western analysis of DU145, PC-3 and DUSF cells transiently transfected with the constitutive pcDNA-AR plasmid.**

Cells from the three cell lines (DU145, PC-3 and DUSF) were transiently transfected with pcDNA-AR plasmid and assayed 48hrs after transfection for AR protein expression. Lysates from mock-transfected and non-transfected (parental) cells were included as negative control. Loading of protein from LNCaP cells indicated the size of the expected AR-specific band (~110kDa). AR protein expression was expected in the cells transfected with pcDNA-AR. Equal loading was confirmed by Ponceau S staining (data not shown). No AR protein was detected. The presence of a non-specific band of slightly higher molecular weight than AR is apparent in all samples tested (see section 3.9 for details).



COS-1 cells

Figure 4.4 **Western analysis of COS-1 cells transiently transfected with the constitutive pcDNA-AR plasmid.**

COS-1 cells were transiently transfected with pcDNA-AR plasmid and assayed 48hrs after transfection for AR protein expression. Lysates from mock-transfected and non-transfected (parental) cells were included as negative control. Loading of protein from LNCaP cells indicated the size of the expected AR-specific band (~110kDa). AR protein expression was expected in the cells transfected with pcDNA-AR. Equal loading was confirmed by Ponceau S staining (data not shown). No AR protein was detected. The presence of a non-specific band of slightly higher molecular weight than AR is apparent in all samples tested (see section 3.9 for details).

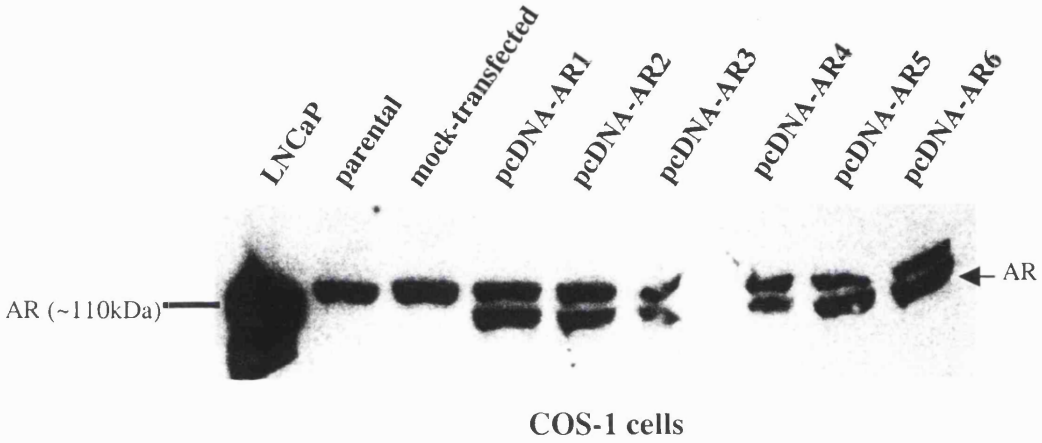


Figure 4.5 **Western analysis of COS-1 cells transiently transfected with the constitutive pcDNA-AR 1→ 6 plasmids.**

COS-1 cells were transiently transfected with pcDNA-AR 1→6 plasmid preps and assayed 48hrs after transfection for AR protein expression. Lysates from mock-transfected and non-transfected (parental) cells were included as negative control. Loading of protein from LNCaP cells indicated the size of the expected AR-specific band (~110kDa). AR protein expression was detected, as expected, in the cells transfected with pcDNA-AR 1→6. Equal loading was confirmed by Ponceau S staining (data not shown). The presence of a non-specific band of slightly higher molecular weight than AR is apparent in all samples tested (see section 3.9 for details).

This indicated that the original plasmid was functional and implied that the plasmid preparation used for the stable transfections carried an inactivating mutation resulting in lack of AR expression.

4.8 Conclusions

The work described in this chapter was designed to develop an experimental model where overexpression of the AR gene could be studied. Specifically, the aim was to assay the effect of androgen treatment on stable cell lines constitutively expressing different levels of the human androgen receptor.

Three AR-negative prostate cancer cell lines (DU145, PC-3 and DUSF) were stably transfected with a constitutive expression vector (pcDNA-AR). Clones stably expressing the plasmid were generated from all three cell lines and assayed for AR protein expression by Western analysis. The results demonstrated that none of the stable AR clones developed was overexpressing AR. Transient transfection assays of DU145, PC-3, DUSF and COS-1 cells with different preparations of pcDNA-AR plasmid indicated that the reason for the lack of AR expression in the stable AR clones was the non-functional plasmid preparation used.

Chapter 5

Characterisation of cell lines transiently transfected with AR expression vectors

5.1 Introduction

The results of Chapter 3 offer the hypothesis that DU145 cells suppress AR expression from the inducible pTRE-AR vector. Therefore, a series of experiments was designed to study the regulation of AR expression in DU145 cells.

For the experiments, three constitutive expression vectors (pcDNA-AR, pSVARo, pCMVhAR), all carrying a wild type, human AR cDNA were employed. The constitutive pcDNA-AR vector was used previously in Chapter 4. The constitutive pSVARo vector has been employed in a number of AR studies mostly with COS-1 cells (Trapman et al., 1988; Brinkmann et al., 1989; Veldscholte et al., 1990a; Veldscholte et al., 1990b; Jenster et al., 1991; Bevan et al., 1996; Koivisto et al., 1997). The constitutive pCMVhAR vector has also been used in a number of AR studies mostly with COS-1 and CV-1 cells (Brown et al., 1990; De Bellis et al., 1992; Quigley et al., 1992a; Choong et al., 1996a; Choong et al., 1996b; Lobaccaro et al., 1996; Langley et al., 1998).

5.2 Aims of the chapter

The objective of the work described in this chapter was to investigate the hypothesis that a mechanism operating either at the transcriptional or at the translational level suppresses AR expression from the pTRE-AR plasmid in DU145 cells.

Specifically, the aims were to:

- Perform transient assays with three constitutive AR expression vectors.
- Measure AR protein expression of transfectants by Western analysis.
- Measure AR mRNA expression of transfectants by RT-PCR.

5.3 Transient expression assays

Transient transfection assays with the three constitutive AR constructs were performed in three AR-negative prostate cancer cell lines (DU145, PC-3 and DUSF) and in COS-1 cells.

Before the transfection experiments, sufficient amounts of pSVARo and pCMVhAR plasmids were prepared and purified as described in section 2.8.2. The quality and integrity of the plasmids was confirmed by extensive restriction enzyme analysis (see section 2.10) (Figure 5.1a and Figure 5.2a, respectively). A restriction map (partial) for pSVARo and pCMVhAR plasmids is also shown (Figure 5.1b and Figure 5.2b, respectively).

For each cell line (DU145, PC-3, DUSF and COS-1), 3 x 10^5 cells were seeded in duplicate in 6-well dishes and transfected with 5μg of each of the three AR expression vectors using the calcium phosphate transfection method (see section 2.21.2). Mock-transfected cells (i.e. water replaced plasmid DNA in the transfection mix) were included as negative control.

5.4 Western analysis of transfectants

Cells from the four cell lines (DU145, PC-3, DUSF, and COS-1) were assayed for AR protein expression 48 hours after transfection by Western analysis (see section 2.14). AR protein expression was expected in the transiently transfected cells with the three AR expression vectors and in the LNCaP cells that were included as positive control. As indicated by the Western analysis, an AR immunoreactive band was present in LNCaP cells, while in both non-transfected (parental) and mock-transfected cells AR expression was absent, as expected.

There was no AR expression in all three prostate cancer cell lines (DU145, PC-3 and DUSF) transfected with pcDNA-AR vector (Figure 5.3). pcDNA-AR has previously been shown to successfully express AR in COS-1 cells (see section 4.7, Figure 4.5).

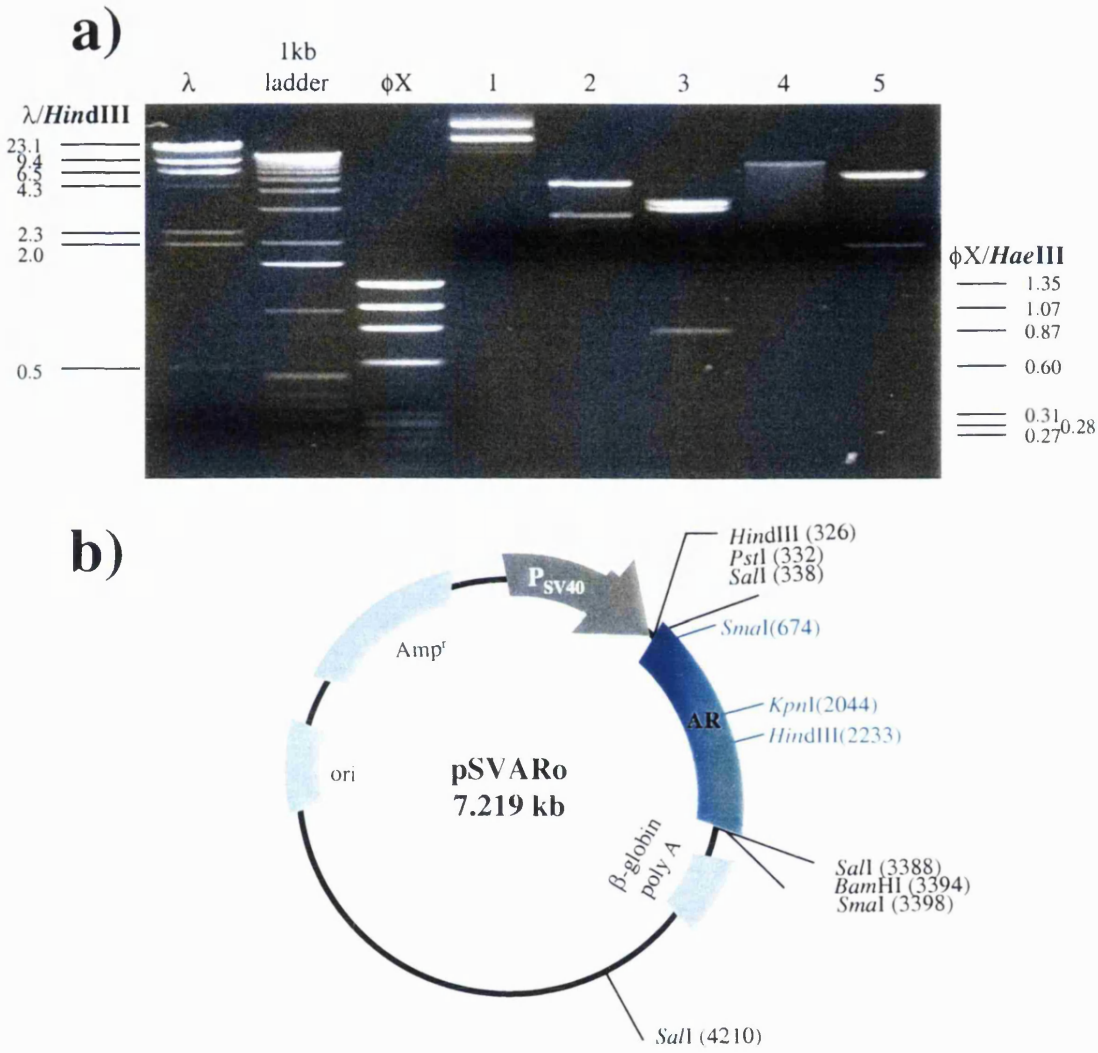


Figure 5.1 **Restriction enzyme analysis of pSVARo plasmid.**

a) EtBr-stained gel showing restriction enzyme digestions of pSVARo plasmid. Lane 1 shows the uncut pSVARo plasmid. Lane 2 shows the *Sma*I digest (2.72 and 4.50kb) where most of the AR cDNA (3.03kb) is excised from the vector backbone 4.18kb). Lane 3 shows the *Sal*I digest (3.34, 3.05 and 0.82kb) where the AR cDNA (3.03kb) is excised from the vector backbone. Lane 4 shows the linearised pSVARo plasmid (*Kpn*I digest, 7.219kb). Lane 5 shows the *Hin*dIII digest (5.31 and 1.90kb) where the integrity of the plasmid was further confirmed. λ: *Hin*dIII fragments (in kb) of λ phage DNA. φX: *Hae*III fragments (in kb) of phage φX174 DNA.

b) Restriction map (partial) of pSVARo plasmid. The restriction sites on AR cDNA are shown in blue.

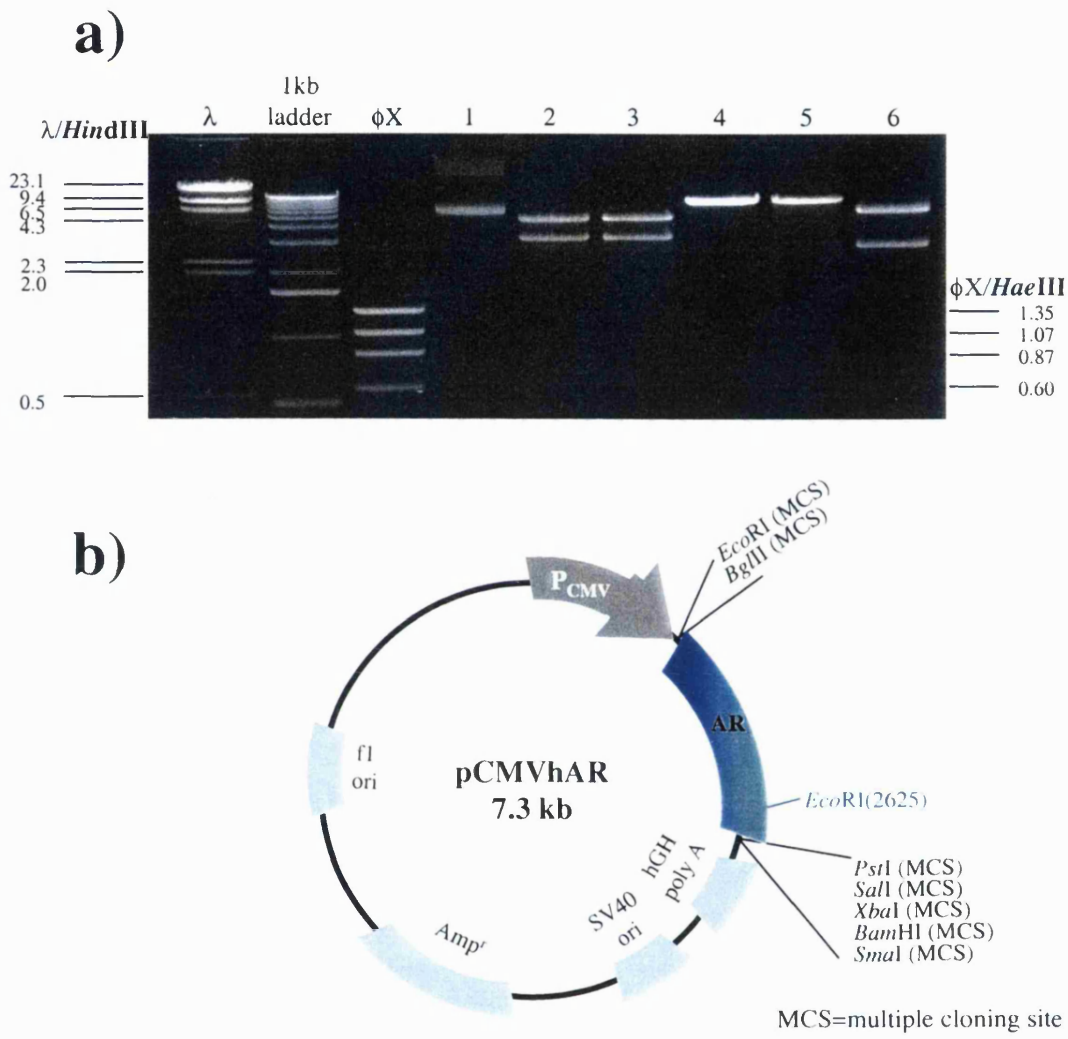


Figure 5.2 **Restriction enzyme analysis of pCMVhAR plasmid.**

a) EtBr-stained gel showing restriction enzyme digestions of pCMVhAR plasmid. Lane 1 shows the uncut pCMVhAR plasmid. Lanes 2 and 3 show the *Bgl*II/ *Xba*I and the *Bgl*II/ *Sal*I digest respectively where the AR cDNA (3.1kb) is excised from the pCMV5 vector backbone (4.2kb). Lanes 4 and 5 show the linearised pCMVhAR plasmid (*Bgl*II and *Xba*I digest, respectively). Lane 6 shows the *Eco*RI digest (2.62 and 4.69kb) where the integrity of the plasmid was further confirmed. λ: *Hin*dIII fragments (in kb) of λ phage DNA. φX: *Hae*III fragments (in kb) of phage φX174 DNA.

b) Restriction map (partial) of pCMVhAR plasmid. The restriction sites on AR cDNA are shown in blue.

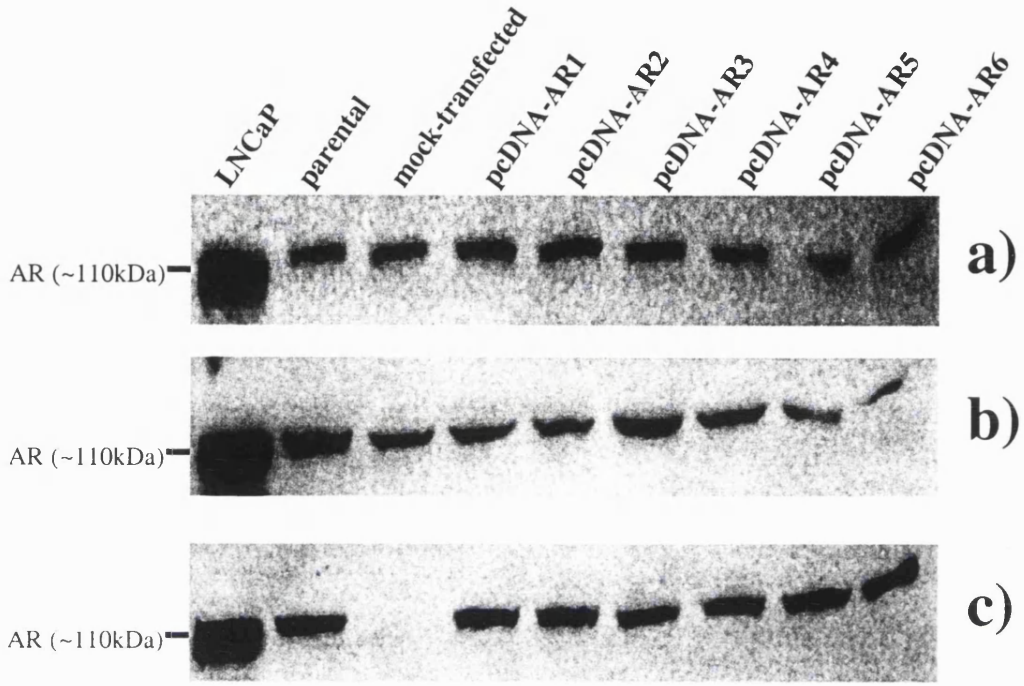


Figure 5.3 **Western analysis of DU145, PC-3 and DUSF cells transiently transfected with the constitutive pcDNA-AR plasmid.**

Cells from the three cell lines (DU145, PC-3 and DUSF) were transiently transfected with pcDNA-AR 1→6 plasmids (see section 4.7) and assayed 48hrs after transfection for AR protein expression. Lysates from mock-transfected and non-transfected (parental) cells were included as negative control. Loading of protein from LNCaP cells indicated the size of the expected AR-specific band (~110kDa). AR protein expression was expected in the cells transfected with pcDNA-AR. Equal loading was confirmed by Ponceau S staining (data not shown). No AR protein was detected. The presence of a non-specific band of slightly higher molecular weight than AR is apparent in all samples tested (see section 3.9 for details). In lane 3 (mock-transfected) of DUSF cells (c), the absence of the non-specific band cannot be explained, because staining with Ponceau S confirmed the presence of protein.

a) DU145 cells, b) PC-3 cells and c) DUSF cells.

There was no AR expression in the prostate cancer cell lines (DU145, PC-3 and DUSF) transfected with pSVARo vector. However, pSVARo successfully expressed AR in COS-1 cells (Figure 5.4).
AR expression was detected in all four cell lines (DU145, PC-3, DUSF and COS-1) transfected with pCMVhAR vector (Figure 5.4).

The experiments described in this section indicated that only the constitutive pCMVhAR plasmid expressed AR protein in DU145 cells. The other two constitutive AR expression vectors (pcDNA-AR and pSVARo) did not produce AR protein in any of the prostate cancer cells (DU145, DUSF, PC-3), despite the fact that both plasmids induced AR protein expression in COS-1 cells.

The results from the Western analysis of transfectants with the constitutive AR expression vectors are summarised in Table 5.1. The results from the transient transfection experiments with the inducible vector pTRE-AR, described in Chapter 3, are also included.

Table 5.1 AR protein expression of cells transfected with four AR vectors

Cell line	Plasmids			
	pcDNA-AR	pSVARo	pCMVhAR	pTRE-AR
DU145	-	-	+	-
DUSF	-	-	+	+
PC-3	-	-	+	+
COS-1	+	+	+	+

5.5 Transfection efficiency of DU145 prostate cells

To ensure that the lack of AR expression seen in DU145 was not due to a transfection efficiency problem, DU145 and COS-1 cells were transiently transfected with increasing amounts of pCMVhAR vector, the only vector expressing AR protein in DU145 cells. COS-1 cells were included in the experiment as positive control.

Hence, 3 x 10^5 cells from the two cell lines (DU145 and COS-1) were seeded in duplicate in 6-well dishes and transfected with increasing amounts (range 1-40μg) of

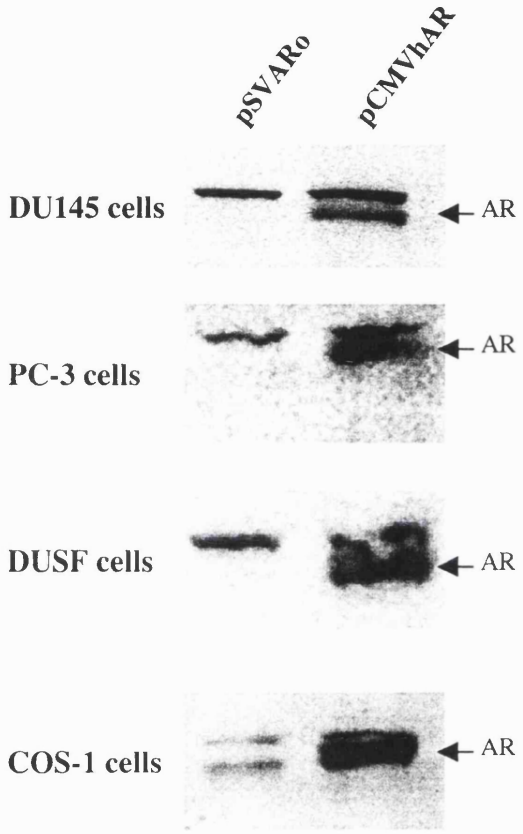


Figure 5.4 **Western analysis of DU145, PC-3, DUSF and COS-1 cells transiently transfected with the constitutive pSVARo and pCMVhAR plasmids.**

DU145, PC-3, DUSF and COS-1 cells were transiently transfected with pSVARo and pCMVhAR plasmids and assayed 48hrs after transfection for AR protein expression. AR protein expression was apparent, as expected, in all cells transfected with pCMVhAR plasmid. However, AR protein expression in cells transfected with pSVARo plasmid was detected only COS-1 cells. The arrows indicate the AR-specific band (~110kDa). Equal loading was confirmed by Ponceau S staining (data not shown). The presence of a non-specific band of slightly higher molecular weight than AR is apparent in all samples tested (see section 3.9 for details).

pCMVhAR plasmid using the calcium phosphate transfection method (see section 2.21.2). Mock-transfected cells (i.e. water replaced plasmid DNA in the transfection mix) were included as negative control. The transfectants were assayed for AR protein expression 48 hours after transfection by Western analysis (see section 2.14).

A dose-response pattern of AR protein expression was apparent, as expected, in COS-1 cells. AR expression as high as the AR expression levels in LNCaP cells was detected in COS-1 cells transfected with 5μg plasmid DNA. No AR protein expression was detected in cells transfected with more than 10μg pCMVhAR plasmid, suggesting saturation of the system at concentrations of transfected DNA higher than 10μg (Figure 5.5).
AR protein expression in DU145 cells also followed a dose response pattern, but did not reach the AR protein levels detected in LNCaP cells. The system became saturated at a lower concentration of transfected DNA than COS-1 cells. No AR protein expression was detected in cells transfected with more than 5μg pCMVhAR plasmid (Figure 5.6).

The experiments described in this section indicated that, as expected, increasing amounts of plasmid DNA induced increasing amounts of AR protein production. Moreover, the results confirmed that the amount (5μg) of plasmid DNA used in the transient transfection experiments was suitable and in accordance with previously published studies (Brown et al., 1990; De Bellis et al., 1992; Quigley et al., 1992a; Choong et al., 1996a; Langley et al., 1998). These findings implied that it was unlikely that a transfection efficiency problem resulted in the lack of AR protein expression seen in DU145 cells.

5.6 RT-PCR analysis of transfectants

Transient transfection experiments with the four AR expression vectors (pcDNA-AR, pSVARo, pCMVhAR and pTRE-AR) were performed and AR gene expression at the mRNA level was investigated.

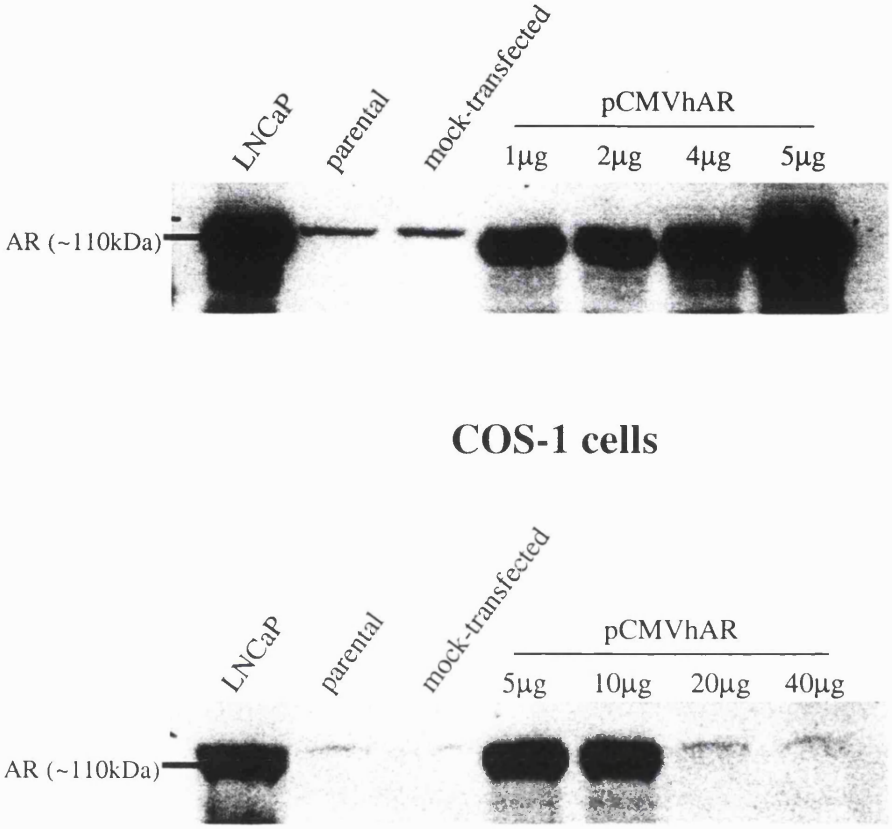


Figure 5.5 **Western analysis of COS-1 cells transiently transfected with increasing amounts of pCMVhAR plasmid.**

COS-1 cells were transiently transfected with increasing amounts of pCMVhAR plasmid (range 1-40μg) and assayed 48hrs after transfection for AR protein expression. Lysates from mock-transfected and non-transfected (parental) cells were included as negative control. Loading of protein from LNCaP cells indicated the size of the expected AR-specific band (~110kDa). A dose-response pattern of AR protein expression was detected, as expected, in cells transfected with increasing amounts of plasmid DNA. Plasmid concentrations above 10μg did not induce any AR expression, probably due to saturation of the system. Equal loading was confirmed by Ponceau S staining (data not shown). The presence of a non-specific band of slightly higher molecular weight than AR is apparent in all samples tested (see section 3.9 for details).

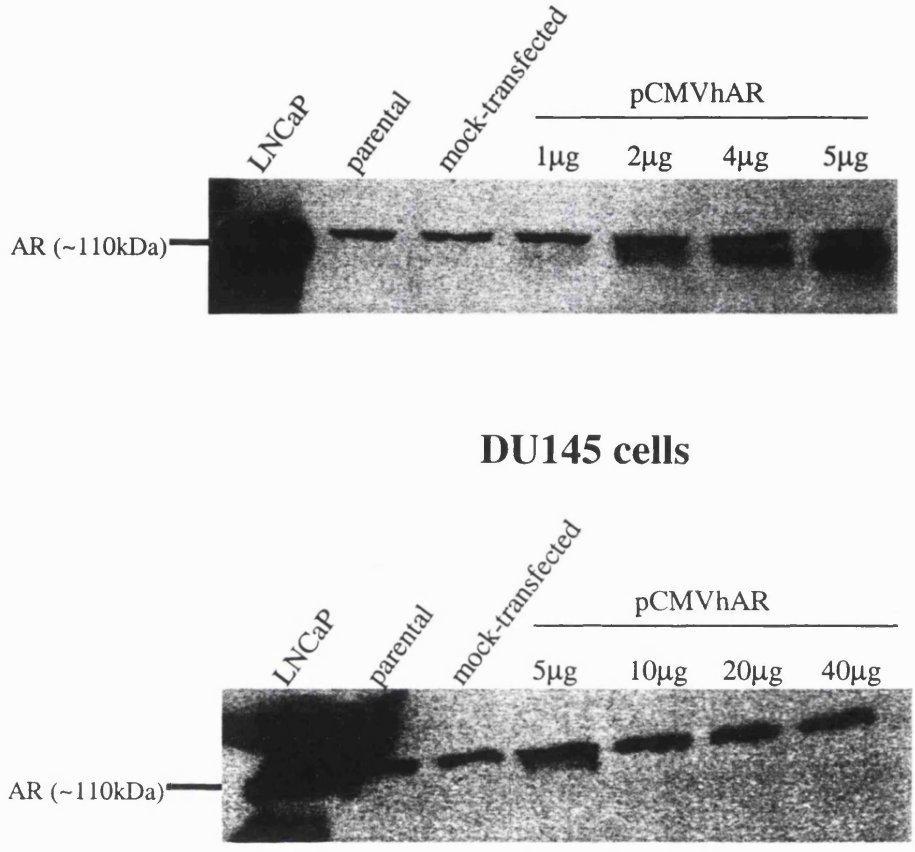


Figure 5.6 **Western analysis of DU145 cells transiently transfected with increasing amounts of pCMVhAR plasmid.**

DU145 cells were transiently transfected with increasing amounts of pCMVhAR plasmid (range 1-40μg) and assayed 48hrs after transfection for AR protein expression. Lysates from mock-transfected and non-transfected (parental) cells were included as negative control. Loading of protein from LNCaP cells indicated the size of the expected AR-specific band (~110kDa). A dose-response pattern of AR protein expression was detected, as expected, in cells transfected with increasing amounts of plasmid DNA. Plasmid concentrations above 5μg did not induce any AR expression. The variation in the intensity of the signal from the transfection with 5μg pCMVhAR between the two blots is the result of variation in the transfection technique.

Specifically, 3 x 10^5 cells from the four cell lines (DU145, PC-3, DUSF and COS-1) were seeded in duplicate in 6-well dishes and transfected with 5μg of each of the three constitutive AR expression vectors (pcDNA-AR, pSVARo and pCMVhAR) using the calcium phosphate transfection method (see section 2.21.2). Mock-transfected cells (i.e. water replaced plasmid DNA in the transfection mix) were included as negative control.

For the transfection experiments with the inducible pTRE-AR expression plasmid, 5 x 10^5 cells were seeded in duplicate in 10cm dishes and co-transfected with 18μg pTetOff and 2μg pTRE-AR plasmids using the calcium phosphate transfection method (see section 3.8). The cells were transfected in the absence and presence of 2μg/ml tetracycline (Tc). As negative control, cells were transfected with pTRE plasmid lacking the AR-insert ($^+$/- 2μg/ml Tc).

Cells were assayed for AR mRNA expression by RT-PCR analysis (see section 2.17). Specifically, 48 hours after transfection total RNA was isolated (see section 2.15) and its concentration and quality were verified (see section 2.11 and 2.16). Total RNA (2.5μg) was then reverse transcribed into cDNA (see section 2.17.1) which was purified (see section 2.17.2) to remove contaminants from the RT reaction (i.e. reverse transcriptase, unincorporated nucleotides, salts) prior to PCR analysis.

To ensure a DNA-free total RNA preparation, two protocols specific for removing DNA contaminants from RNA were followed (MessageClean® kit by GenHunter Corp., and RNase-Free DNase Set by QIAGEN Ltd., for both see section 2.15.3). Moreover, PCR primers (ARAs and ARDa) hybridising to different exons (Exon 1 and Exon 4) of the AR gene were used to exclude amplification of contaminating genomic DNA i.e. only the PCR product from AR mRNA (524bp) would be visible on an EtBr-stained agarose gel, because the expected PCR product from genomic DNA (>65kb) would be too big to amplify.

A control experiment in which no reverse transcriptase was added prior to PCR was also included to test for plasmid DNA contamination. i.e. a mock reverse transcription reaction of DNA-free RNA yields RNA, a template not ampified by PCR. Hence, the

presence of an amplified fragment of the expected size from a mock RT-reaction would subsequently imply contamination from plasmid DNA.

None of the protocols (MessageClean® kit and RNase-Free DNase Set) proved successful in completely removing plasmid DNA from the RNA samples. RT-PCR being a very sensitive technique was able to amplify even traces of DNA contaminants, resulting in false positive signals (Figure 5.7). This made it impossible to distinguish between AR expression from the plasmid and plasmid AR cDNA itself.

To overcome the problem, nested anchored RT-PCR analysis (Sharrad RM, personal communication) was performed (Figure 5.8). The key to this approach is reverse transcription of total RNA using an anchor-oligodT primer consisting of 39 bases (anchor) followed by 21 T (oligodT) residues. The anchor sequence shares no homology with the AR sequence and provides a region for designing non-specific primers. Hence, a forward primer in an exon of AR cDNA ensures annealing to AR sequences, while an anchor-specific reverse primer ensures annealing to the mRNA template. A second-stage PCR (nested PCR) amplifies the inner portion of the AR-specific product from the first PCR. Consequently, nested anchored RT-PCR analysis allows amplification only of the AR transcripts, thereby enabling monitoring of changes in AR gene expression.

For nested anchored RT-PCR analysis, two sets of primers (an external and an internal pair) were used to amplify the purified cDNA. The external set of primers, AR exon 6 and Anchor 1, corresponded to residues in exon 6 of AR and residues in the anchor of the OligodT primer, respectively. The forward primer of the internal set, AR exon 7/8, was an intron 7-spanning primer, while the reverse primer, Anchor nested 2, corresponded to residues in the anchor of the OligodT primer. Amplified DNA from the first PCR (with the external primers) was purified (see section 2.17.2) prior to nested PCR (with the internal primers) to increase specificity and reduce the number of non-specific PCR products.

Actin mRNA was amplified as a control for relative amounts and integrity of the mRNA. The reaction mixture containing all components except for the template RNA was used as negative control for the RT-reaction [(-ve) RT control].

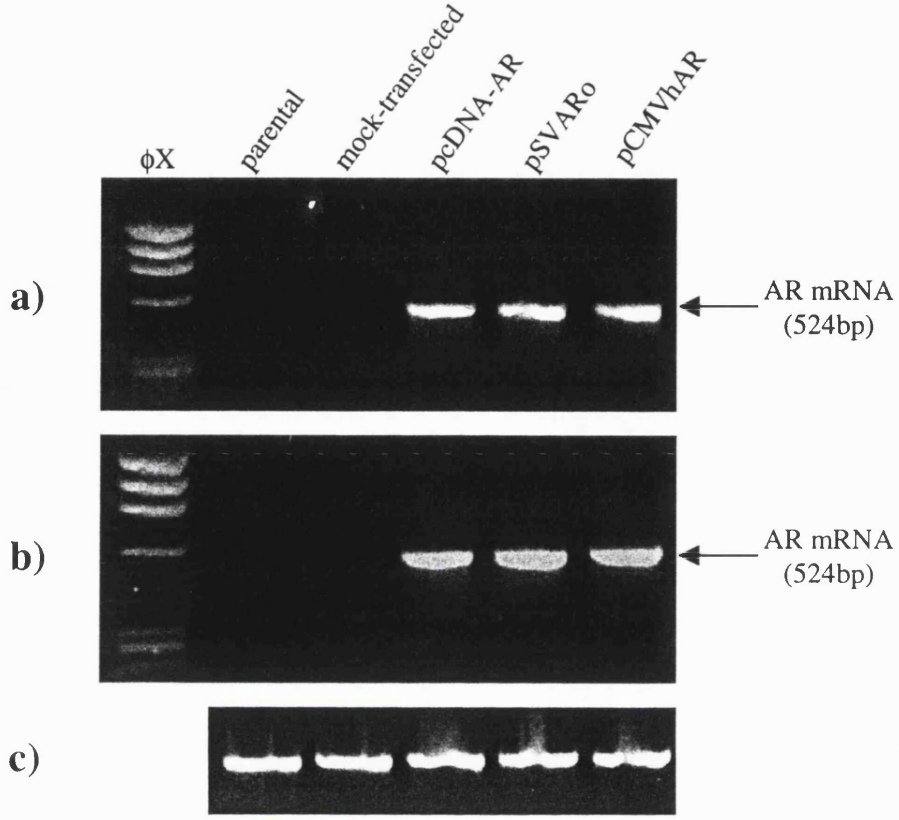


Figure 5.7 **RT-PCR analysis of DNase-treated total RNA.**

PC-3 cells were transfected with 5μg of each of the constitutive AR expression vectors (pcDNA-AR, pSVARo and pCMVhAR). Total RNA, isolated 48 hours after transfection, was DNase-treated (MessageClean® kit, GeneHunter Corp.) prior to RT-PCR analysis. Total RNA from mock-transfected and non-transfected (parental) cells was included as negative control. Reverse transcribed cDNA was amplified using ARAs and ARDa primers, located in exon 1 and exon 4 of AR, respectively. A mock-reverse transcription reaction (-RT) was included as negative control to test for the presence of DNA contaminants. A PCR product (524bp) was apparent, as expected, in PC-3 cells transfected with the three constitutive AR plasmids. The presence of the 524bp PCR product in the mock-reverse transcribed samples (b), suggested contamination from plasmid DNA. Equal loading and integrity of the RNA was confirmed by amplification of actin mRNA (c). φX: *Hae*III fragments (in kb) of phage φX174 DNA.

a) EtBr-stained gel showing RT-PCR analysis with ARAs and ARDa primers.

b) EtBr-stained gel showing RT-PCR analysis with ARAs and ARDa primers (mock-RT).

c) EtBr-stained gel showing RT-PCR analysis with actin primers.

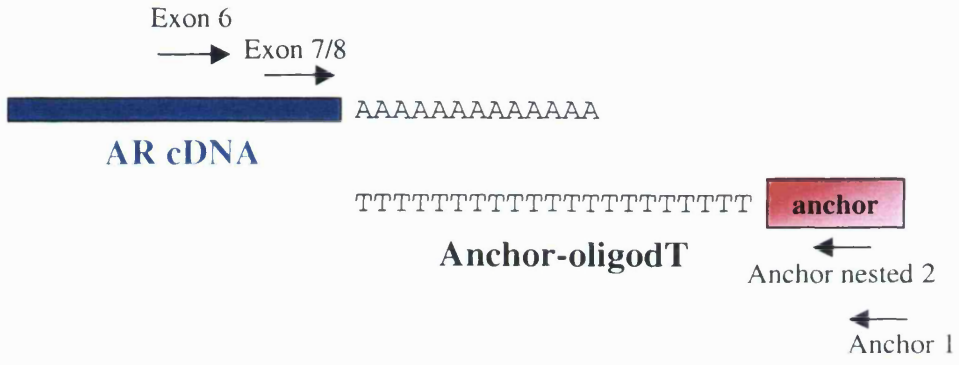


Figure 5.8 **Nested anchored RT-PCR.**

Reverse transcribed cDNA was amplified using nested anchored RT-PCR which allowed amplification only of the AR transcripts and excluded priming of plasmid DNA contaminants. The key to this analysis was reverse transcription of total RNA with an anchor-oligodT primer consisting of 39 bases (anchor) followed by 21 T residues. The anchor shared no homology with the AR sequence. Initially, AR cDNA was amplified using an external set of primers: an AR-specific primer (AR Exon 6) and an anchor-specific primer (Anchor 1). Then, the first-PCR product was further amplified (nested PCR) to increase specificity using an internal set of primers: an AR-specific, intron 7-spanning primer (AR Exon7/8) and an anchor-specific primer (Anchor nested 2).

The reaction mixture containing all components except for the template DNA was used as negative control for the PCR-reaction [(-ve) PCR control]. The primers sequences and their thermal cycling conditions are shown in section 2.17.3, Table 2.4.

The AR expression vectors used for the transfection experiments all contain the complete protein coding part of the human AR cDNA, but differ in the length of 5′ and 3′ UTRs (untranslated regions) of AR (Figure 5.9). Consequently, a difference in the size of PCR products from cells transfected with the different AR expression vectors was expected. The exact size of the expected PCR products from the different transfectants was difficult to estimate because the exact length of the polyA tail of the AR transcripts, as well as the exact location where the anchor-oligodT primer annealed, were not known. However, the minimum expected size of the nested PCR products (PCR_{min}) could be estimated; any smaller PCR product than PCR_{min} was considered non-specific. The minimum expected size of the nested PCR products (in bp) was calculated as follows:

PCR_{min}= [end of the 3′UTR – start of $P_{AR\ Exon\ 7/8}$] + 21 T residues + size of $P_{anchor\ nested\ 2}$

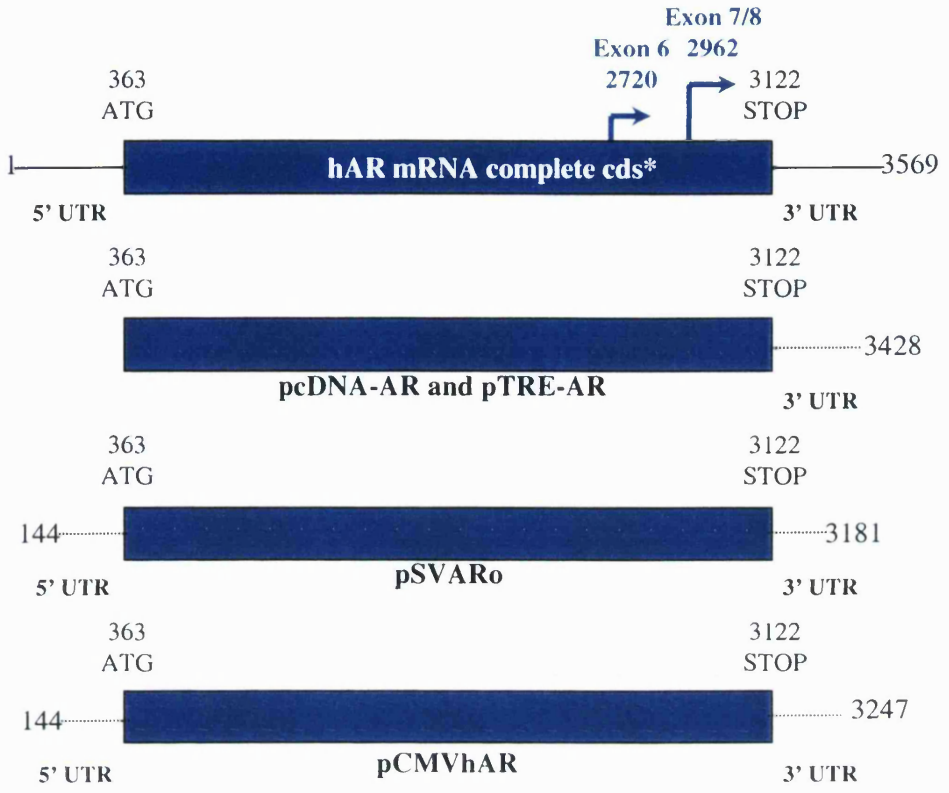
The length of the sequence between AR Exon 7/8 primer and the end of the 3′UTR depended on the AR construct (Figure 5.9). Hence, for example, for pCMVhAR vector the minimum expected size of the nested PCR product would be:

$$PCR_{min}= [3247-2962] + 21+ 31= 338bp$$

The nested PCR product from LNCaP cells was used as positive control and was expected to be few bp bigger than the PCR product from cells transfected with the constitutive pcDNA-AR and the inducible pTRE-AR plasmids.

The same size nested PCR product was expected from transfectants with pcDNA-AR and pTRE-AR plasmids because AR cDNA from pcDNA-AR was used to construct pTRE-AR (see section 3.7). The smallest PCR product was expected from cells transfected with the constitutive pSVARo plasmid because this plasmid contains the shortest 3′untranslated region.

As indicated by the RT-PCR analysis of transfectants, AR mRNA was present in LNCaP cells that were included as positive control, while in both non-transfected (parental) and mock-transfected cells AR expression was absent, as expected.



Plasmids	Nested PCR product (in bp) PCR_{min}
pcDNA-AR and pTRE-AR	519
pSVARo	272
pCMVhAR	338
LNCaP	660

Figure 5.9 **Comparison of hAR cDNA sequences contained within the AR expression vectors.**

The schematic drawing compares hAR cDNA sequences contained within the four AR expression vectors. All AR plasmids used for the transfection experiments contain the complete coding sequence (cds) of the human AR cDNA ($ATG_{363} \rightarrow STOP_{3122}$). However, they differ in their 5´ and 3´ untranslated regions (UTR). pcDNA-AR and pTRE-AR contain identical sequences of AR cDNA (see section 3.7). Due to differences in the 3´ UTR sequences contained within each plasmid, the amplified fragments from nested anchored RT-PCR analysis are expected to differ in size. The minimum expected size PCR product (PCR_{min}) from each plasmid is shown in the table. See text for a detailed description. The position of the two forward AR-specific primers (Exon 6 and Exon 7/8) used for nested anchored RT-PCR analysis are shown in blue.

*The nucleotide sequence of the hAR is available from GenBank under accession number J03180. Numbering from Lubahn et al. (1988a).

A PCR product of the expected size was present in transfectants from all four cell lines, indicating that AR mRNA was expressed from all four AR expression plasmids.

RT-PCR analysis of transfectants with the constitutive pcDNA-AR and the inducible pTRE-AR yielded the same size nested PCR product, as expected.
RT-PCR analysis of transfectants with the constitutive pSVARo plasmid yielded three bands. All three were likely to be AR-specific (their size was bigger than the minimum expected size), produced from annealing of the anchor-specific primer to multiple sites on the polyA tail of the AR transcript.
RT-PCR analysis of transfectants with the constitutive pCMVhAR plasmid yielded a single strong PCR band, approximately 200bp smaller than the band from pcDNA-AR and pTRE-AR, as expected.
RT-PCR analysis of transfectants with the inducible pTRE-AR yielded the expected size band both in the presence and absence of Tc, implying that the TetOff system is not completely switched off in the presence of Tc. However, a difference in the signal intensity between (+) and (–) Tc was apparent and reproducible in all cell lines tested. This implied that despite the background expression ('leakiness') in the presence of Tc, the system was inducing, as expected, an increase in the AR mRNA expression in the absence of Tc. No band was detected from the RT-PCR analysis of transfectants with pTRE plasmid lacking the AR-insert ($^{+}$/- 2μg/ml Tc).

The nested PCR result from COS-1 and DU145 transfectants is shown in Figure 5.10 and Figure 5.11, respectively. PC-3 and DUSF cells showed the same pattern of AR mRNA expression as DU145 and COS-1 cells. No discrete band was visible on an EtBr-stained agarose gel from amplified DNA with the external set of primers (AR exon 6 and Anchor 1).

The experiments described in this section indicated that all four AR expression plasmids (constitutive and inducible) expressed AR mRNA in DU145 cells. These results suggested that the lack of AR protein expression (see Table 5.1) was not due to a transcriptional blockage mechanism.

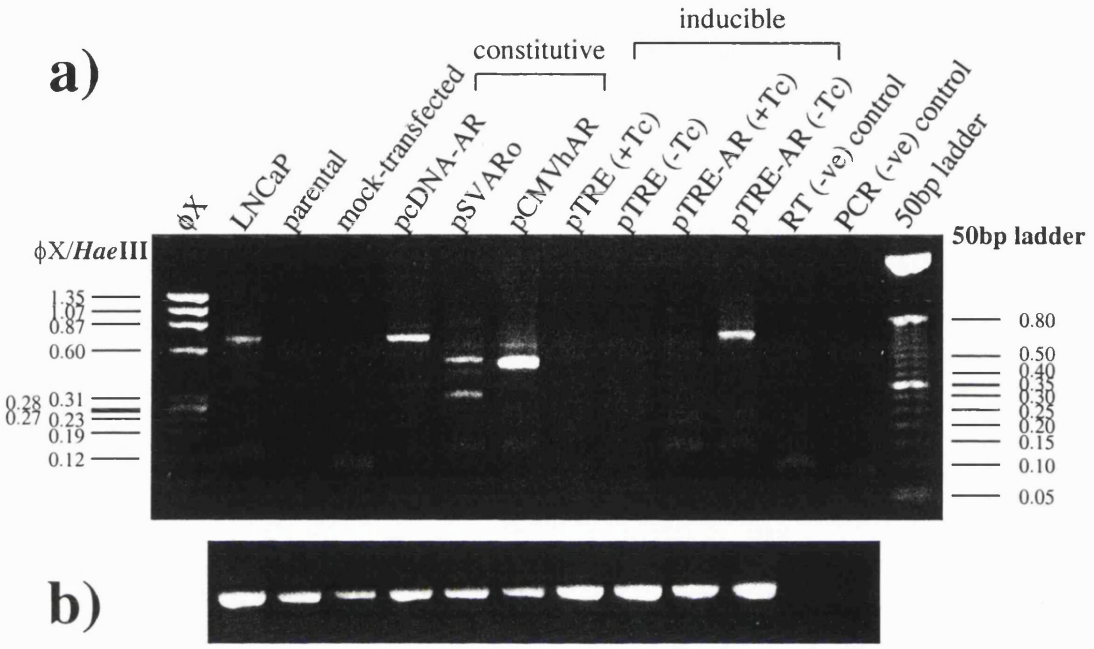


Figure 5.10 **Nested anchored RT-PCR analysis of COS-1 transfectants.**

COS-1 cells were transiently transfected with the four AR expression vectors (pcDNA-AR, pSVARo, pCMVhAR, and pTRE-AR). Total RNA, isolated 48 hours after transfection, was reverse transcribed into cDNA which was then amplified using nested anchored RT-PCR. Total RNA from mock-transfected and non-transfected (parental) cells was included as negative control. Initially, cDNA was amplified using an external pair of primers (AR Exon 6, an AR-specific primer and Anchor 1, an anchor-specific primer). Then, the first-PCR product was further amplified to increase specificity using an internal set of primers (AR Exon7/8, an AR-specific, intron 7-spanning primer and Anchor nested 2, an anchor-specific primer). A nested PCR product was apparent, as expected, in the LNCaP cells that were included as a positive control and in COS-1 cells transfected with all four AR plasmids (see text for a detailed description). The reaction mixture containing all components except for the template RNA (DNA) was used as negative control for the RT (PCR) reaction. Equal loading and integrity of the RNA was confirmed by amplification of actin mRNA. φX: *Hae*III fragments (in kb) of phage φX174 DNA.

a) EtBr-stained gel showing the nested PCR product with AR Exon7/8 and Anchor nested 2 primers.

b) EtBr-stained gel showing RT-PCR analysis with actin primers.

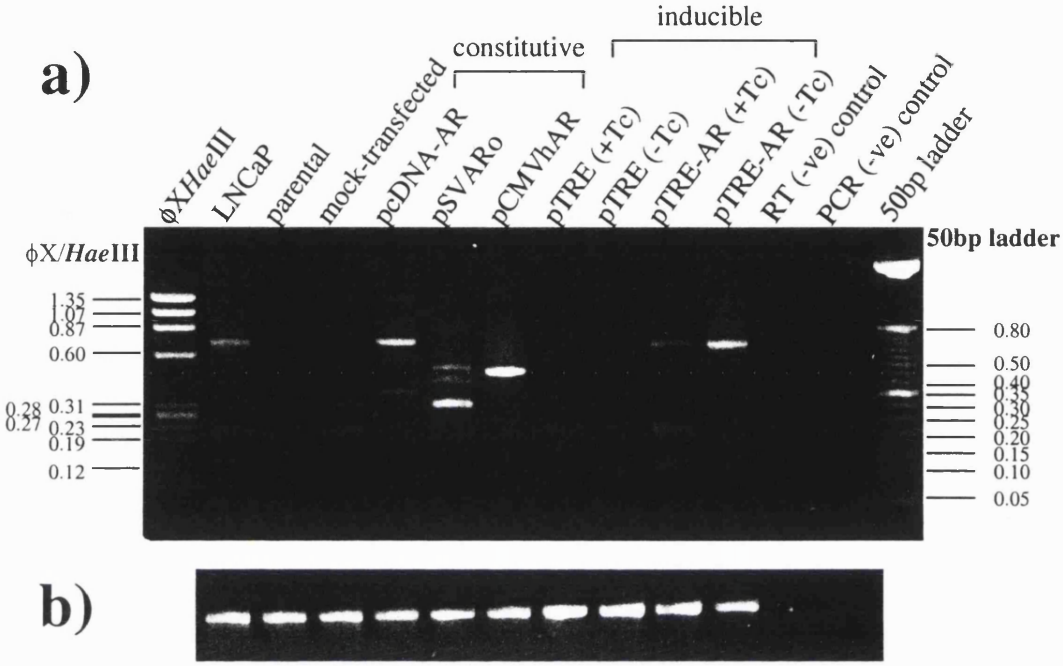


Figure 5.11 **Nested anchored RT-PCR analysis of DU145 transfectants.**

DU145 cells were transiently transfected with the four AR expression vectors (pcDNA-AR, pSVARo, pCMVhAR, and pTRE-AR). Total RNA, isolated 48 hours after transfection, was reverse transcribed into cDNA which was then amplified using nested anchored RT-PCR. Total RNA from mock-transfected and non-transfected (parental) cells was included as negative control. Initially, cDNA was amplified using an external pair of primers (AR Exon 6, an AR-specific primer and Anchor 1, an anchor-specific primer). Then, the first-PCR product was further amplified to increase specificity using an internal set of primers (AR Exon7/8, an AR-specific, intron 7-spanning primer and Anchor nested 2, an anchor-specific primer). A nested PCR product was apparent, as expected, in the LNCaP cells that were included as a positive control and in DU145 cells transfected with all four AR plasmids (see text for a detailed description). The reaction mixture containing all components except for the template RNA (DNA) was used as negative control for the RT (PCR) reaction. Equal loading and integrity of the RNA was confirmed by amplification of actin mRNA. φX: *Hae*III fragments (in kb) of phage φX174 DNA.

a) EtBr-stained gel showing the nested PCR product with AR Exon7/8 and Anchor nested 2 primers.

b) EtBr-stained gel showing RT-PCR analysis with actin primers.

The results from the RT-PCR analysis of transfectants with all four (constitutive and inducible) AR expression vectors are summarised in Table 5.2.

Table 5.2 AR mRNA expression of cells transfected with four AR vectors

Cell line	Plasmids			
	pcDNA-AR	pSVARo	pCMVhAR	pTRE-AR
DU145	+	+	+	+
DUSF	+	+	+	+
PC-3	+	+	+	+
COS-1	+	+	+	+

5.7 Conclusions

The work described in this chapter was designed to investigate the lack of AR expression from the inducible pTRE-AR vector in DU145 cells. RT-PCR and Western analysis were used to study the potential suppression of AR expression at the transcriptional and translational levels, respectively.

Four AR expression vectors (pcDNA-AR, pSVARo, pCMVhAR and pTRE-AR) were used to transiently transfect three AR-negative prostate cancer cell lines (DU145, PC-3 and DUSF) and COS-1 cell line. PC-3 and DUSF cells were included in the study to investigate whether the lack of AR expression was specific to DU145 cells, while COS-1 cells were used as positive control.

All four AR vectors were expected to induce AR mRNA and protein expression in the transfectants because expression of AR cDNA was under the control of the constitutive CMV and/or SV40 promoters, or under the tetracycline-inducible system (Table 5.3).

Specifically, in pSVARo plasmid the AR gene is under the control of the SV40 promoter/enhancer, while in pCMVhAR plasmid the stronger CMV promoter /enhancer drives the expression of the AR gene, and can synergise with the SV40 promoter/enhancer for an increased expression (Anderson et al., 1989; Russell DW personal communication). The inducible pTRE-AR plasmid is activated by tTA

protein and drives transcription of the AR gene from the activated P_{minCMV} promoter which is slightly stronger than the CMV promoter/enhancer (Yin et al., 1996).

Table 5.3 Characteristics of AR expression vectors

Plasmids	Type	Promoter	AR sequence*
pcDNA-AR	constitutive	CMV	$363_{(ATG)}$-3428
pSVARo	constitutive	SV40	144-3181
pCMVhAR	constitutive	CMV synergising with SV40	144-3247
pTRE-AR	inducible	TRE-P_{minCMV}	$363_{(ATG)}$- 3428

(*Numbering from Lubahn et al., 1988a)

Transfectants were assayed for AR protein expression (Western analysis), and for AR mRNA expression (RT-PCR). The results from the Western analysis demonstrated that only the constitutive pCMVhAR plasmid expressed AR protein in all cell lines tested. The constitutive pcDNA-AR and pSVARo expressed AR protein in COS-1 cells, but not in the prostate cancer cells (DU145, PC-3 and DUSF). Transient transfection assays in DU145 and COS-1 cells with increasing amounts of pCMVhAR plasmid excluded a potential transfection efficiency problem in DU145 cells. The results from the RT-PCR analysis demonstated that all plasmids expressed AR mRNA in all cell lines tested. According to these findings, it is likely that a post-transcriptional mechanism suppressed AR protein expression in DU145 cells.

One possible explanation for the absence of AR protein expression seen in the prostate cancer cells (see Table 5.1) could be the lack of sensitivity of the Western analysis. It is likely that RT-PCR being more sensitive, enabled detection of even low gene expression levels. However, the RT-PCR analysis used for the experiments did not discriminate between high and low levels of AR mRNA expression. The differences in the AR mRNA levels produced by the different plasmids can be tested by quantitative RT-PCR (Q-RT-PCR). Quantification of the AR mRNAs is expected to reflect the activity of the respective plasmid.

Another reason for the absence of AR protein in the transfectants despite AR mRNA expression is the fact that the presence of a transcript does not always imply translation into the protein product. Nozawa et al. (2000) have shown that an Ets-

related transcription factor (termed hPSE) is transcribed in normal prostate epithelial cells and in PC-3 and LNCaP cell lines. However, the hPSE transcript is translated only in normal epithelial cells and not in malignant cells.

Several factors such as mRNA secondary structure, and mRNA stability have been implicated in affecting translation speed and efficiency (Kozak, 1989; Stansfield et al., 1995; Pain, 1996; Day and Tuite, 1998).

As discussed in section 5.6, all plasmids contain the complete coding sequence of the hAR cDNA, but differ in the length of 5′ and 3′ UTR AR sequences contained within each plasmid (Figure 5.9 and Table 5.3). Therefore, it is possible that due to these differences, some of the transcripts from the different plasmid constructs could fold their mRNA into a secondary structure that would make it inaccessible to the ribosome, thereby blocking translation.

Decreased translational efficiency due to mRNA instability in the absence of androgens has also been reported (Krongrad et al., 1991; Wolf et al., 1993; Dai and Burnstein, 1996; Mora et al., 1996; Mora and Mahesh, 1999; Yeap et al., 1999). Androgens regulate AR expression post-transcriptionally by stabilising the AR mRNA (Mora and Mahesh, 1999). Therefore, degradation of AR mRNA from the transfectants grown in the absence of exogenous androgens, could be a possible explanation for the lack of AR protein observed. To test this, AR mRNA levels from cells grown in the absence and presence of androgens should be compared.

It is becoming clear that 3′ UTRs of some mRNAs can play important roles in translation of these mRNAs (Gallie and Walbot, 1990; Leathers et al., 1993; Ostareck-Lederer et al., 1994). It is likely that such translation regulatory sequences are present in 3′ UTR of the androgen receptor. Subsequently, it is possible that prostate cells would differentially regulate the translation of AR transcripts that differ at their 3′ UTR. In COS-1 cells, such potential post-transcriptional regulation is unlikely to exist as prostate- or AR-specific translation regulatory proteins are not present.

In summary, the set of experiments described in this chapter implied that AR mRNA instability and subsequent degradation in the absence of androgens resulted in lack of

AR protein expresssion. This, together with differences in the promoter strength of the four AR vectors could explain the results of the Western and RT-PCR analysis.

A possible scenario: all plasmids express AR mRNA but probably different levels due to differences in promoter strength. The pCMVhAR and pTRE-AR plasmids carrying stronger promoter/enhancer elements than pcDNA-AR and pSVARo plasmids are inducing AR protein expression. Lack of AR protein expression from the plasmids with the weaker promoters could be due to a) lack of Western sensitivity, or b) mRNA degradation in the absence of androgens.

Chapter 6

Characterisation of DUSF androgen-independent prostate cancer cells stably expressing the androgen receptor

6.1 Introduction

As described in Chapter 5, only the constitutive pCMVhAR expression vector, carrying the strongest promoter sequences (the strong CMV promoter/enhancer synergising with the SV40 promoter/enhancer), expressed AR protein in all prostate cancer cells tested (DU145, PC-3 and DUSF). This plasmid was therefore, chosen to stably transfect DUSF androgen-independent prostate cancer cells. DUSF cells offer the advantage of studying androgen effects on cells growing in a fully defined serum-free medium eliminating the complications arising from the use of charcoal-treated serum. Charcoal stripping of the serum depletes a large range of endogenous steroid hormones and related molecules. Re-addition of androgens does not completely reverse the effect.

6.2 Aims of the chapter

The objective of the work described in this chapter forms a continuum in the establishment of a constitutive gene expression system where the effect of androgens on prostate cells expressing a range of androgen receptor concentrations could be analysed.

Specifically, the aims were to:

- Stably transfect the wild type, full-length human AR cDNA into the DUSF AR-negative human prostate cancer cell line.
- Select, expand and characterise the AR transfectants.
- Assess the effect of androgens on AR mRNA.
- Assess the effect of androgens on AR protein.
- Assess the effect of androgens on PSA mRNA.

6.3 Transfection of DUSF cells and selection of stable clones

The constitutive pCMVhAR expression plasmid does not carry a selectable marker for mammalian cells (see Figure 5.2). Therefore, pcDNA3 plasmid (the backbone of

pcDNA-AR, see Figure 4.1) carrying the neomycin-resistance gene (*neo*^{r}) was used to co-transfect DUSF cells in a single transfection step.

One million cells per T25 flask were co-transfected with pCMVhAR and pcDNA3 plasmids at a molar ratio of 20:1 [20 and 1μg, respectively (Brown et al., 1990)] by the calcium phosphate transfection method (see section 2.21.2). Mock-transfected cells were generated by parallel transfection with the neomycin-encoding pcDNA3 plasmid (10μg). Selective medium (1mg/ml Geneticin®) was added 48hrs after transfection to both mock- and AR-transfected cells and was replaced every four days. As a control, non-transfected cells were grown in the absence and presence of Geneticin®. The cells were incubated until geneticin-resistant colonies appeared. Thirty-four AR-transfectants were ring cloned (see section 2.22) and expanded for further analysis.

6.4 Immunocytochemical analysis of stable clones

Initially, the thirty-four stable transfectants were screened for AR expression by immunocytochemical (ICC) staining (see section 2.25). The cells were fixed and stained for AR using the mouse monoclonal anti-AR antibody (F39.4.1) that recognises residues 301-320 of the N-terminal domain of the androgen receptor (Zegers et al., 1991). The ICC analysis detected eight transfectants expressing AR. The staining was mostly localised to the nucleus of cells, with some cytoplasmic reactivity (Figure 6.1).

6.5 Morphology

The morphology of the stably-transfected DUSF cells was similar to that of the non-transfected (parental) and mock-transfected DUSF cells (Figure 6.2).

6.6 PCR analysis of stable clones

PCR analysis was used in order to verify the presence of the pCMVhAR expression vector in the transfected cells. Specifically, DNA from an AR-transfectant and from a

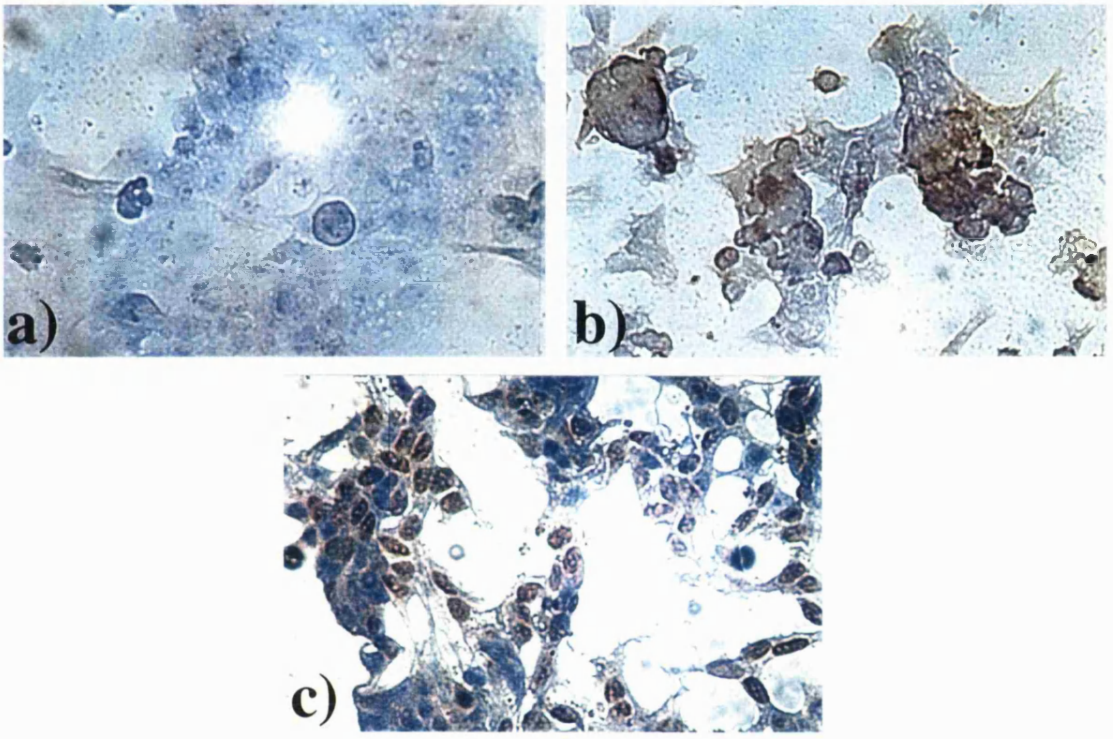


Figure 6.1 **Immunocytochemical staining of DUSF cells stably transfected with the constitutive pCMVhAR plasmid.**

Cells grown in OPTIMEM medium were fixed and stained with the mouse anti-AR monoclonal antibody (F39.4.1). Immunoreactive AR was detected in the nucleus of AR-transfected cells (b), while some cytoplasmic reactivity was also present. No immunostaining was detected in mock-transfected cells (a), as expected. LNCaP cells (c) were included as a positive control. Nuclear counterstaining with Mayer's haematoxylin. Original magnification x 200.

a) mock-transfectant, b) AR-transfectant, c) LNCaP cells.

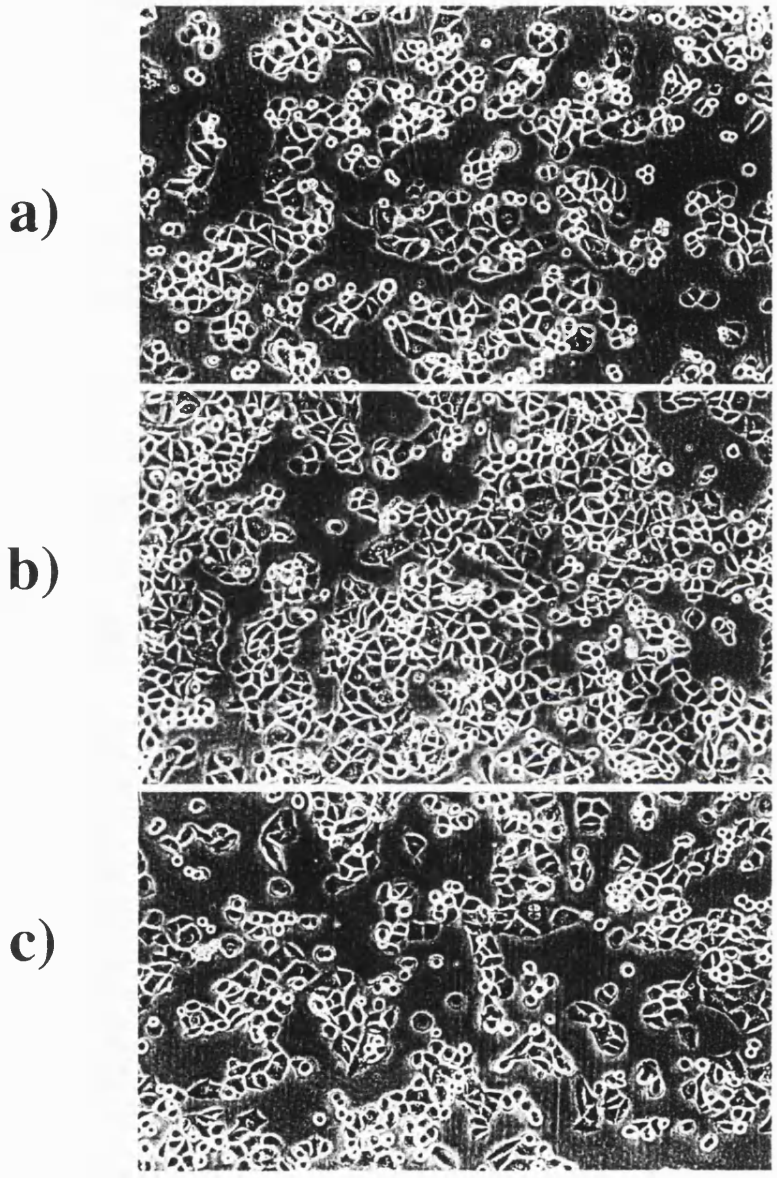


Figure 6.2 **Phase contrast microscopy of DUSF cells stably transfected with the constitutive pCMVhAR plasmid.**

AR-transfected cells exhibited similar morphological characteristics as compared with the mock-transfected and parental DUSF cells. There were no differences in the size and shape of transfectants. Cells were grown in OPTIMEM medium. Original magnification x 200.

a) AR-transfectant, b) mock-transfectant, c) parental.

mock-transfectant were analysed using primers spanning intron 7 (AR Exon 7s and AR Exon 8a). DNA from DUSF parental, non-transfected cells was included as a control. The expected size PCR product (198bp) from the AR cDNA encoded by pCMVhAR plasmid was apparent, as expected, only in the AR-transfectant. The expected size PCR product (898bp: intron 7 is 700bp) from the endogenous AR gene was apparent, as expected, in all samples (parental, AR- and mock-transfectant) (Figure 6.3).

6.7 Western analysis of stable clones

Depending on the site of expression vector integration, and on the number of copies of the exogenous DNA integrated, variation in expression levels between different transfected clones was expected.

Five (5/8) randomly chosen DUSF stable transfectants which stained positive for AR after ICC analysis were assayed for AR protein expression by Western analysis (see section 2.14). Cell lysates were analysed using the mouse monoclonal anti-AR antibody (G122-434) that recognises residues 33-485 of the N-terminal domain of the androgen receptor (Chang et al., 1992). An AR immunoreactive band was present in all AR-transfectants analysed (very low AR levels in clone 20). Moreover, variable expression of the androgen receptor was apparent as indicated by differences in the intensity of the protein band (Figure 6.4). Two AR transfectants, clone 44 and clone 17, expressing relatively high and low AR protein respectively, were chosen for further analysis.

A Western blot of the clones chosen for further analysis is shown in Figure 6.5. As an additional control for the variable AR expression levels, cell lysates were assayed using the mouse monoclonal anti-AR antibody (F39.4.1). This antibody recognises residues 301-320 of the N-terminal domain of the androgen receptor (Zegers et al., 1991). AR protein expression in DUSF transfectants did not reach the AR protein levels detected in LNCaP cells, that were included as positive control. Mock-transfected and non-transfected (parental) cells were included as negative controls.

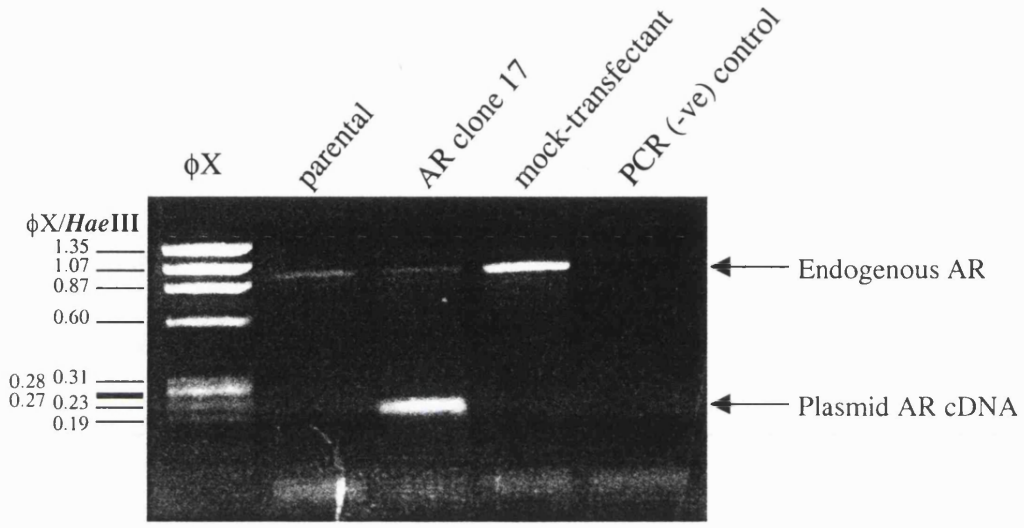


Figure 6.3 **PCR analysis of stable clones.**

EtBr-stained gel showing the PCR analysis with the intron 7 spanning primers: AR Exon 7s and AR Exon 8a. A PCR product (898bp) from the endogenous AR gene was apparent, as expected, in all samples tested (parental, AR clone 17, mock-transfectant). A PCR product (198bp) from the AR cDNA encoded by pCMVhAR plasmid was apparent, as expected, only in the AR clone. The reaction mixture containing all components except for the template DNA was used as negative control for the PCR reaction. φX: *Hae*III fragments (in kb) of phage φX174 DNA.

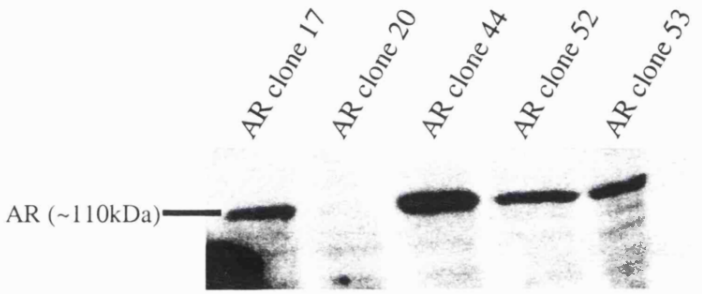


Figure 6.4 **Western analysis of AR-transfected DUSF cells.**

DUSF cells stably transfected with the constitutive pCMVhAR plasmid were assayed for AR protein expression by Western analysis. Cell lysates were analysed using the mouse anti-AR monoclonal antibody (G122-434). An AR immunoreactive band of the expected size (~110kDa) was apparent in all clones tested (very low AR levels detected in clone 20). Variable AR expression levels between the clones were also detected, as indicated by the differences in the intensity of the protein band. Equal loading was confirmed by Ponceau S staining (data not shown).

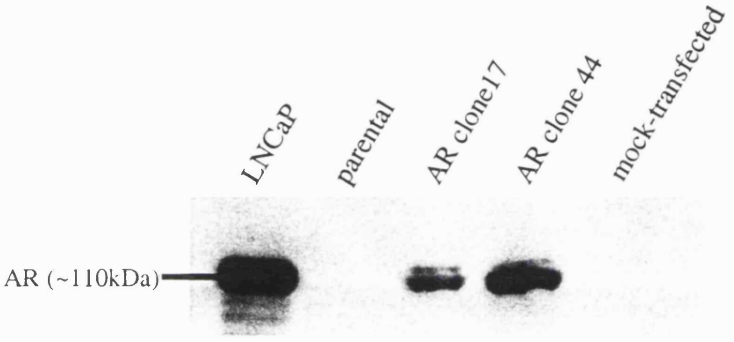


Figure 6.5 **Western analysis of DUSF cells stably transfected with the constitutive pCMVhAR plasmid.**

DUSF cells stably transfected with the constitutive pCMVhAR plasmid were assayed for AR protein expression by Western analysis. Cell lysates were analysed using the mouse anti-AR monoclonal antibody (F39.4.1). Loading of protein from LNCaP cells indicated the size of the expected AR-specific band (~110kDa). Lysates from mock-transfected and non-transfected (parental) cells were included as negative controls. An AR immunoreactive band was apparent, as expected in the two AR clones. Variable AR expression levels between the AR clones were also detected, as indicated by the differences in the intensity of the protein band. Equal loading was confirmed by Ponceau S staining (data not shown). The presence of a non-specific band of slightly higher molecular weight than AR is apparent in the AR clones tested (see section 3.9 for details).

6.8 RT-PCR analysis of stable clones

The two AR-transfectants (clone 17 and clone 44) expressing different levels of AR protein and one mock-transfectant were further characterised by RT-PCR analysis. Specifically, total RNA was isolated from exponentially growing cells (see section 2.15) and reverse transcribed into cDNA (see section 2.17.1). cDNA was purified (see section 2.17.2) to remove contaminants from the RT reaction (i.e. reverse transcriptase, unincorporated nucleotides, salts) and subjected to PCR analysis. Primers (ARAs and ARDa) hybridising to different exons (Exon 1 and Exon 4) of the AR gene were used to exclude amplification of contaminating genomic DNA. In addition, a mock reverse transcription reaction (-RT) was performed to exclude amplification of contaminating plasmid DNA.
Actin mRNA was amplified as a control for relative amounts and integrity of the mRNA. The reaction mixture containing all components except for the template DNA was used as negative control for the PCR-reaction [(-ve) PCR control]. The primers sequences and their thermal cycling conditions are shown in section 2.17.3, Table 2.4.

As indicated by the RT-PCR analysis of transfectants, the expected size PCR product (524bp) was present in LNCaP cells that were included as positive control, and in both AR-transfectants. AR transcripts were absent in non-transfected (parental) and mock-transfected cells, as expected. Despite the fact that the two AR-transfectants expressed different levels of AR protein, there was no difference in their level of AR mRNA expression, as indicated by the evaluation of a range of PCR cycles (Figure 6.6). No band was visible on an EtBr-stained agarose gel from a PCR using a mock reverse transcription reaction (-RT) as template. This confirmed the integrity of the PCR results and indicated that total RNA was free from plasmid DNA contaminants. Chromosomal integration of the transfected DNA decreases DNA contamination risk during total RNA preparation.

6.9 Androgen effects on AR mRNA expression

To assess restoration of androgen-sensitivity in DUSF cells stably transfected with a constitutively active AR cDNA, the expression of androgen receptor in the presence

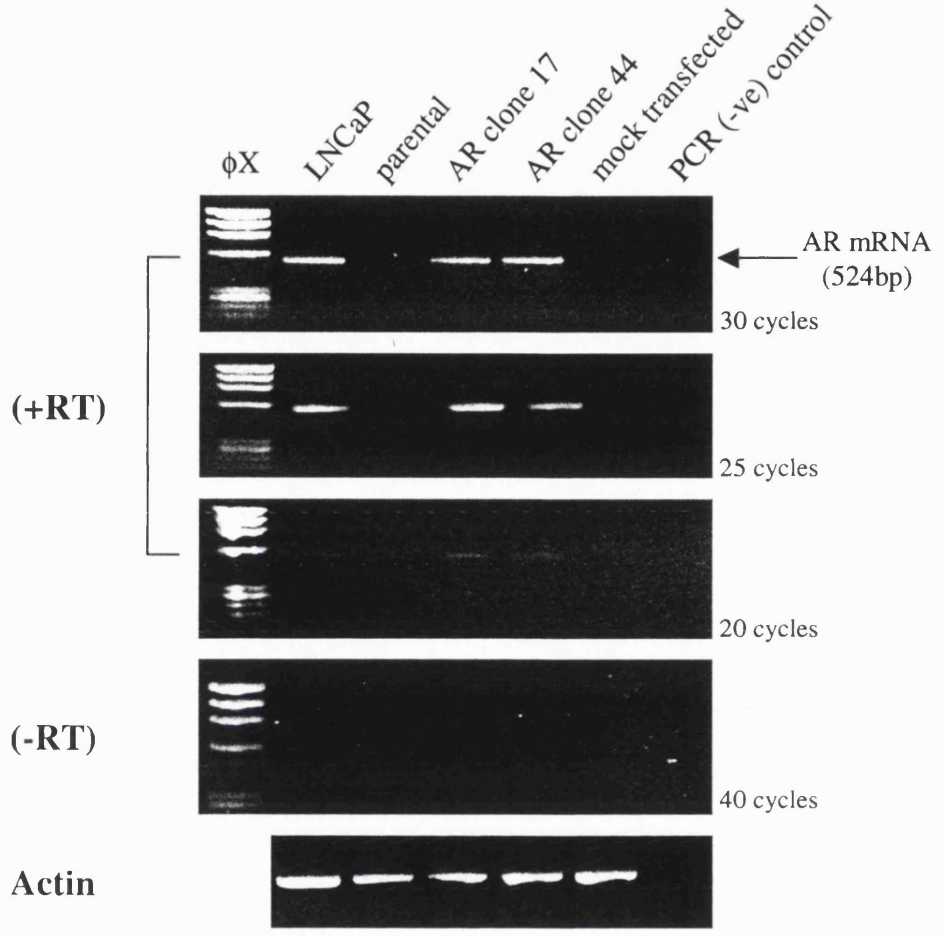


Figure 6.6 **RT-PCR analysis of DUSF cells stably transfected with the constitutive pCMVhAR plasmid.**

EtBr-stained gels showing the RT-PCR analysis with ARAs and ARDa primers. Total RNA was isolated from exponentially growing cells and reverse transcribed into cDNA which was then subjected to PCR analysis. Total RNA from mock-transfected and non-transfected (parental) cells was included as negative control. The expected PCR product (524bp) was detected in the LNCaP cells that were included as positive control and in both the AR clones, as expected. There was no difference in the AR mRNA expression levels between the two AR clones when subjected to varying number of PCR cycles (as indicated by the intensity of the bands). No band was visible from the PCR analysis of the mock reverse transcription reaction (-RT) indicating that total RNA was free from plasmid DNA contaminants. Equal loading and integrity of the RNA was confirmed by amplification of actin mRNA (a representative gel is shown). The reaction mixture containing all components except for the template DNA was used as negative control for the PCR reaction. φX: *Hae*III fragments of phage φX174 DNA.

of androgens was analysed. Specifically, androgen effects on AR mRNA expression of the two AR clones, clone 17 and clone 44, expressing low and high AR protein levels, respectively, were investigated.

The effect of androgen exposure on AR mRNA levels was examined by growing cells (3×10^5 cells/well in 6-well plates) in medium containing 5nM of the synthetic androgen mibolerone; cells grown in the absence of mibolerone were grown in medium containing the ethanol vehicle (0.05% v/v). Mock-transfected and non-transfected (parental) cells, with and without mibolerone, were grown in parallel as negative controls. LNCaP cells grown in medium containing 8% (v/v) dextran-coated charcoal-treated stripped serum (DCC-FBS) in the absence and presence of 5nM mibolerone were used as positive control (for preparation of stripped serum see section 2.20). At 24 and 48hrs of incubation, cells were harvested and assessed for AR mRNA expression by RT-PCR. Specifically, total RNA was isolated (see section 2.15) and reverse transcribed into cDNA (see section 2.17.1). cDNA was purified (see section 2.17.2) to remove contaminants from the RT reaction (i.e. reverse transcriptase, unicorporated nucleotides, salts) and subjected to PCR analysis as described in section 6.8.

RT-PCR analysis of the AR-transfectants incubated for 24hrs with 5nM mibolerone indicated that there was no difference between cells grown in the absence (-) and cells grown in the presence (+) of the androgen. Moreover, there was no difference between the high AR-expressing cells (clone 44) and the low AR-expressing cells (clone 17) grown in the absence (-) or presence (+) of the androgen. Also, no difference between LNCaP cells cultured in the absence (-) or presence (+) of 5nM mibolerone was apparent. As expected, no AR transcript was detected in non-transfected (parental), and mock-transfected cells (Figure 6.7a).

However, RT-PCR analysis of the AR-transfectants incubated for 48hrs with 5nM mibolerone indicated an up-regulation of AR mRNA in the high AR-expressing cells (clone 44), while down-regulation of AR mRNA was apparent in LNCaP cells. No difference was detected in the low AR-expressing cells (clone 17) cultured in the

absence (-) or presence (+) of 5nM mibolerone. As expected, no AR transcript was detected in non-transfected (parental), or mock-transfected cells (Figure 6.7b).

Androgen receptor mRNA expression of LNCaP and high AR-expressing (clone 44) cells was also examined in medium containing 50nM mibolerone. Specifically, cells were incubated for 24 and 48hrs with and without 50nM mibolerone and were assayed for AR mRNA expression by RT-PCR (Figure 6.8).
RT-PCR analysis of LNCaP cells incubated for 24hrs with 50nM mibolerone indicated that there was no difference between cells grown in the absence (-) and cells grown in the presence (+) of the androgen.
RT-PCR analysis of LNCaP cells incubated for 48hrs with 50nM mibolerone indicated a down-regulation of AR mRNA.
RT-PCR analysis of high AR-expressing cells (clone 44) incubated for 48hrs with 50nM mibolerone indicated an up-regulation of AR mRNA.

The experiments described in this section indicated that following 24hrs androgen exposure, no difference in the AR mRNA levels between cells grown in the absence (-) and cells grown in the presence (+) of the androgen was observed. This result is in agreement with a previous study where little or no change in AR mRNA within the first 24hrs of androgen treatment was reported (Krongrad et al., 1991). However, 48hrs incubation with 5 or 50nM mibolerone induced a down- and up-regulation of AR mRNA in LNCaP and high AR-expressing cells (clone 44), respectively. No difference was apparent in the low AR-expressing cells (clone 17) following 48hrs androgen treatment.

6.10 Androgen effects on AR protein expression

To investigate whether the differences detected on AR mRNA levels of cells exposed to mibolerone would parallel effects on AR protein levels, cells (3 x 10^5 cells/well in 6-well plates) were grown for 48hrs in the absence and presence of 5nM mibolerone

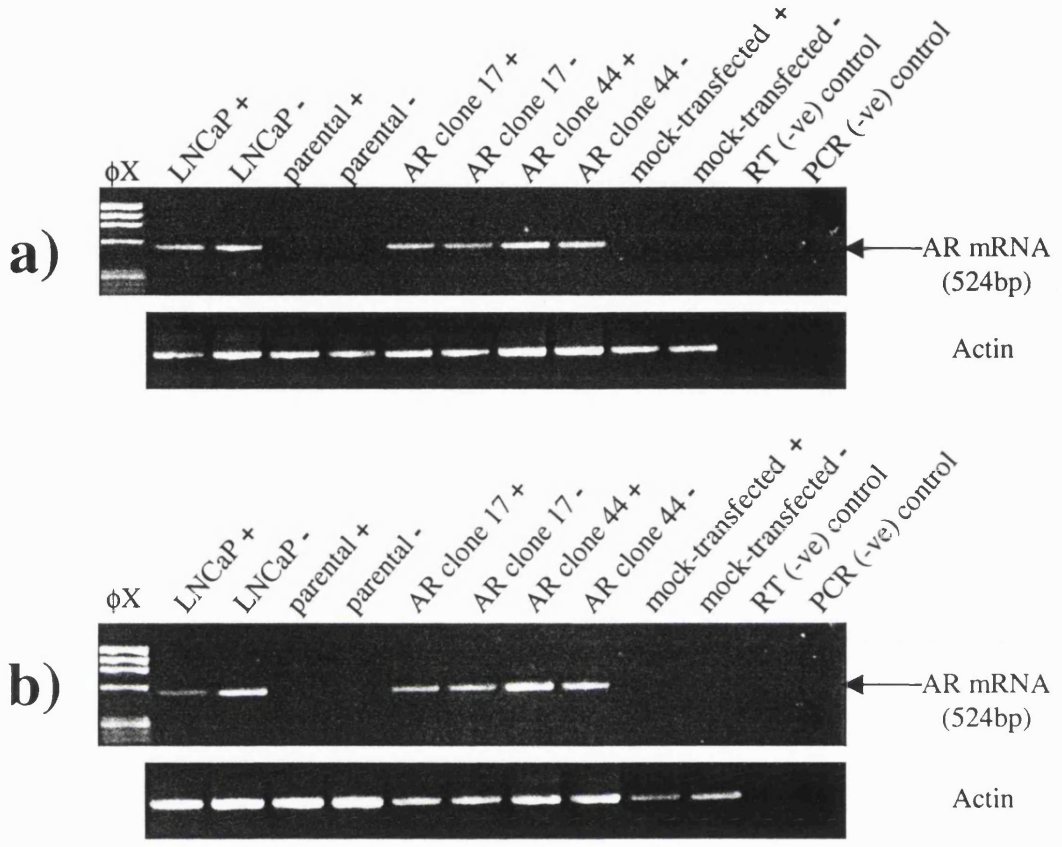


Figure 6.7 **Androgen effects (+/- 5nM miborelone) on AR mRNA of DUSF cells stably transfected with the constitutive pCMVhAR plasmid.**

EtBr-stained gels showing the RT-PCR analysis of cells grown in the absence and presence of 5nM mibolerone. Total RNA was reverse transcribed into cDNA which was then subjected to PCR analysis (ARAs and ARDa primers). Mock-transfected and non-transfected (parental) cells were included as negative control. The expected PCR product (524bp) was detected in the LNCaP cells that were included as positive control and in both the AR clones, as expected. There was no difference in the AR mRNA expression levels of cells grown for 24hrs in the absence and presence of 5nM mibolerone. Down- and up-regulation was apparent in LNCaP cells and AR clone 44, respectively, grown for 48hrs in the presence of 5nM mibolerone. Equal loading and integrity of the RNA was confirmed by amplification of actin mRNA. The reaction mixture containing all components except for the template RNA (DNA) was used as negative control for the RT (PCR) reaction. φX: *Hae*III fragments of phage φX174 DNA.

a) RT-PCR analysis of cells grown for 24 hrs in the absence and presence of 5nM mibolerone.

b) RT-PCR analysis of cells grown for 48 hrs in the absence and presence of 5nM mibolerone.

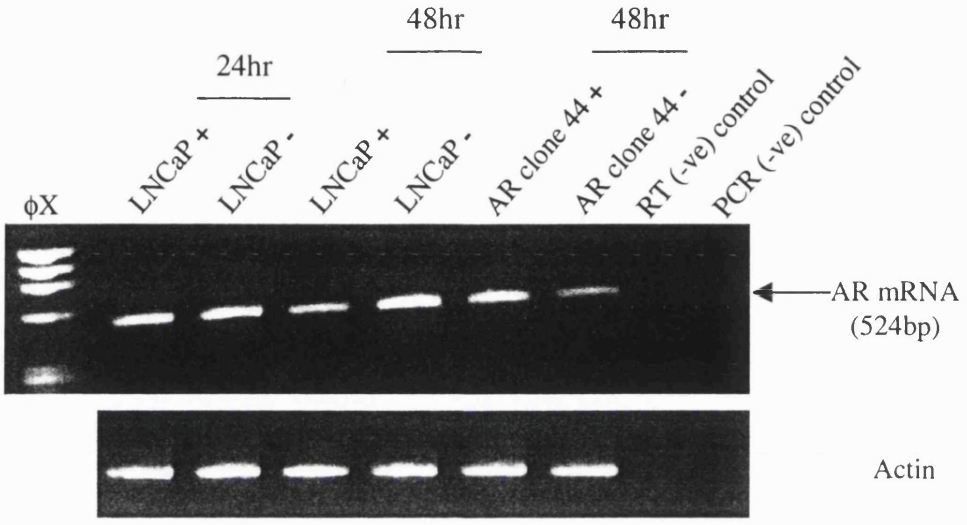


Figure 6.8 **Androgen effects (+/- 50nM mibolerone) on AR mRNA of LNCaP cells and AR clone 44.**

EtBr-stained gels showing the RT-PCR analysis of cells grown in the absence and presence of 50nM mibolerone. Total RNA was reverse transcribed into cDNA which was then subjected to PCR analysis (ARAs and ARDa primers). There was no difference in the AR mRNA expression levels of LNCaP cells grown for 24hrs in the absence and presence of 50nM mibolerone. Down- and up-regulation was apparent in LNCaP cells and AR clone 44, respectively, grown for 48hrs in the presence of 50nM mibolerone. Equal loading and integrity of the RNA was confirmed by amplification of actin mRNA. The reaction mixture containing all components except for the template RNA (DNA) was used as negative control for the RT (PCR) reaction. φX: *Hae*III fragments of phage φX174 DNA.

and assayed for AR protein expression by Western analysis. As no difference was observed at the AR mRNA levels of cells analysed after 24hrs mibolerone exposure, the 24hrs time point was excluded from the experiment. Cell lysates from mock-transfected and non-transfected (parental) cells, with and without mibolerone, were grown in parallel as negative controls. LNCaP cells grown in medium containing 8% (v/v) dextran-coated charcoal-treated stripped serum (DCC-FBS) in the absence and presence of 5nM mibolerone were used as positive control (for preparation of stripped serum see section 2.20).

As indicated by Western analysis, up-regulation of AR protein was apparent in both AR-transfected clones (clone 17 and clone 44) incubated for 48hrs with 5nM mibolerone. No AR transcript was detected in non-transfected (parental), and mock-transfected cells, as expected (Figure 6.9).
Down-regulation of AR protein was apparent in LNCaP cells incubated for 48hrs with 5nM or 50nM mibolerone. Up-regulation of AR protein was apparent in high-AR expressing (clone 44) cells incubated for 48hrs with 50nM mibolerone (Figure 6.10).

6.11 Androgen effects on PSA mRNA expression

After establishing two androgen-sensitive AR-expressing clones, their ability to induce transcription of the endogenous prostate-specific antigen (PSA) gene was tested. The promoter of the PSA gene contains androgen-responsive elements (ARE) and PSA expression has been shown to be regulated by androgens (Riegman et al., 1991; Young et al., 1991; Young et al., 1992).

To assess the transcriptional activity of the transfected AR, androgen effects on PSA mRNA expression of the two AR clones were analysed. Cells (3×10^5 cells/well in 6-well plates) were grown for 48hrs in the absence and presence of 5nM mibolerone and assayed for PSA mRNA expression by RT-PCR analysis. Mock-transfected and non-transfected (parental) cells were grown as negative controls; while LNCaP cells grown in DCC-FBS-containing medium were used as positive control.

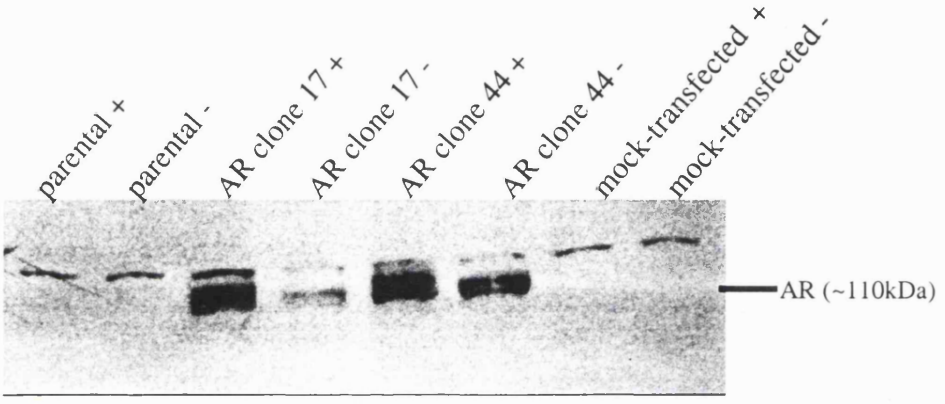


Figure 6.9 **Androgen effects (+/- 5nM mibolerone) on AR protein of DUSF cells stably transfected with the constitutive pCMVhAR plasmid.**

Cells grown for 48hrs in the absence and presence of 5nM mibolerone were assayed for AR protein expression by Western analysis. Mock-transfected and non-transfected (parental) cells were included as negative control. Androgenic up-regulation was apparent in both the low (clone 17) and high (clone 44) AR-expressing transfectants. Equal loading was confirmed by Ponceau S staining (data not shown). The presence of a non-specific band of slightly higher molecular weight than AR is apparent in all samples tested (see section 3.9 for details).

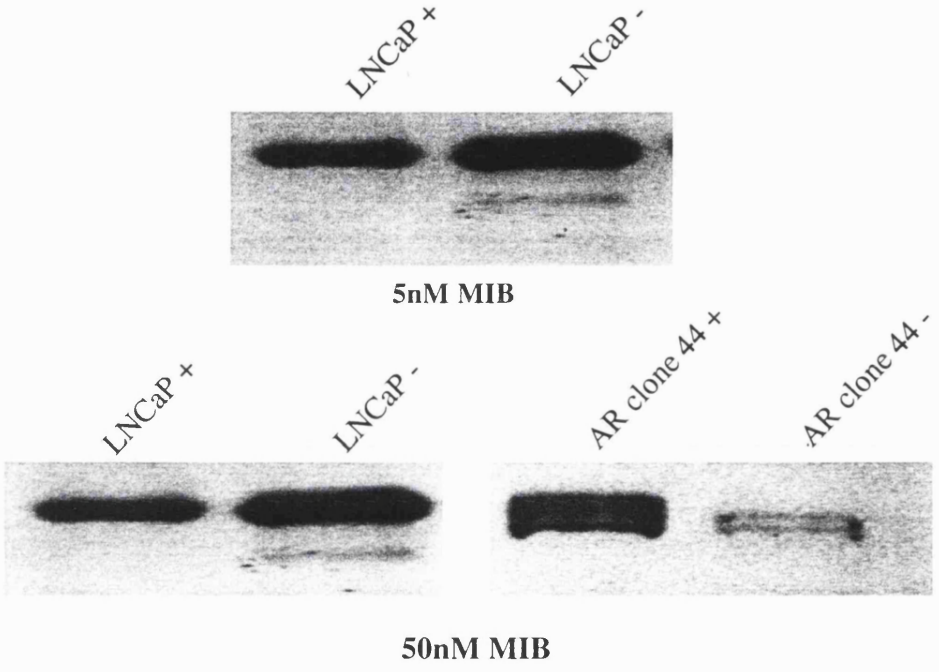


Figure 6.10 **Androgen effects (+/- 5 or 50nM mibolerone) on AR protein of LNCaP cells and AR clone 44.**

Cells grown for 48hrs in the absence and presence of 5 or 50nM mibolerone were assayed for AR protein expression by Western analysis (F39.4.1 antibody). Down-regulation was apparent in LNCaP cells grown in the presence of 5 or 50nM mibolerone. Up-regulation was apparent in AR clone 44 grown in the presence of 50nM mibolerone. Equal loading was confirmed by Ponceau S staining (data not shown).

Total RNA was isolated (see section 2.15) and reverse transcribed into cDNA (see section 2.17.1). cDNA was purified (see section 2.17.2) to remove contaminants from the RT reaction (i.e. reverse transcriptase, unicorporated nucleotides, salts) and subjected to PCR analysis. Primers (PSAs and PSAa) hybridising to different exons (Exon 2/3 and Exon 4) of the PSA gene were used to exclude amplification of contaminating genomic DNA. Actin mRNA was amplified as a control for relative amounts and integrity of the mRNA. The reaction mixture containing all components except for the template RNA was used as negative control for the RT-reaction [(-ve) RT control]. The reaction mixture containing all components except for the template DNA was used as negative control for the PCR-reaction [(-ve) PCR control]. The primers sequences and their thermal cycling conditions are shown in section 2.17.3, Table 2.4.

RT-PCR analysis of LNCaP cells incubated for 48hrs with 5 or 50nM mibolerone indicated an up-regulation of PSA mRNA (Figure 6.11).

RT-PCR analysis of AR-transfectants incubated for 48hrs with 5nM mibolerone, detected no difference between cells grown in the absence (-) and those grown in the presence (+) of the androgen (Figure 6.12). A range of PCR cycles was evaluated. A product of the expected size (362bp) appeared at 35 PCR cycles. Surprisingly, the PSA transcript was present in all DUSF cells tested (non-transfected (parental), AR-, and mock-transfected).

The DUSF cell line being a DU145 subline, is expected, like its parent line, to lack AR and PSA expression. The AR transcript and protein are absent from DUSF cells, as indicated by Western and RT-PCR analysis (Figure 6.5 and Figure 6.6). Therefore, the PSA transcript was also expected to be absent from DUSF cells. RT-PCR analysis of DU145 and DUSF cells (in the absence of mibolerone) with PSA primers indicated that DUSF expressed PSA mRNA, despite the absence of AR protein. LNCaP cells were included as positive control (Figure 6.13a).

To ensure that the total RNA from DU145 cells used for the RT-PCR analysis was amplifiable and not degraded, RT-PCR analysis with COX-1 (cyclooxygenase-1) primers was performed. COX-1 was chosen randomly as an additional control.

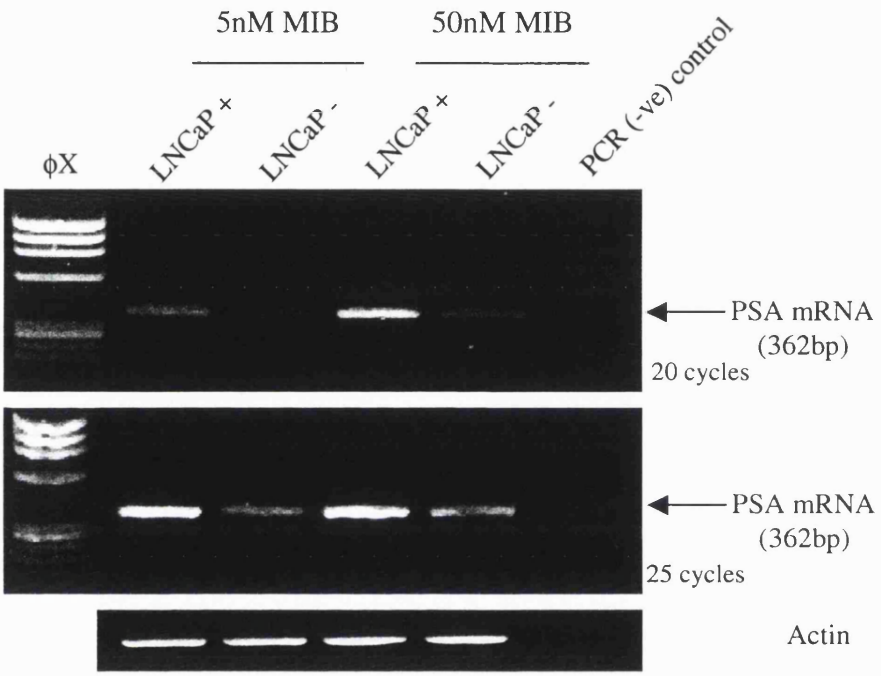


Figure 6.11 **Androgen effects (+/- 5 or 50nM mibolerone) on PSA mRNA of LNCaP cells.**

EtBr-stained gels showing the RT-PCR analysis of LNCaP cells grown for 48hrs in the absence or presence of 5 or 50nM mibolerone. Total RNA was reverse transcribed into cDNA which was then subjected to PCR analysis (PSAs and PSAa primers). Up-regulation was apparent in LNCaP cells grown for 48hrs in the presence of 5 or 50nM mibolerone. Equal loading and integrity of the RNA was confirmed by amplification of actin mRNA. The reaction mixture containing all components except for the template DNA was used as negative control for the PCR reaction. φX: *Hae*III fragments of phage φX174 DNA.

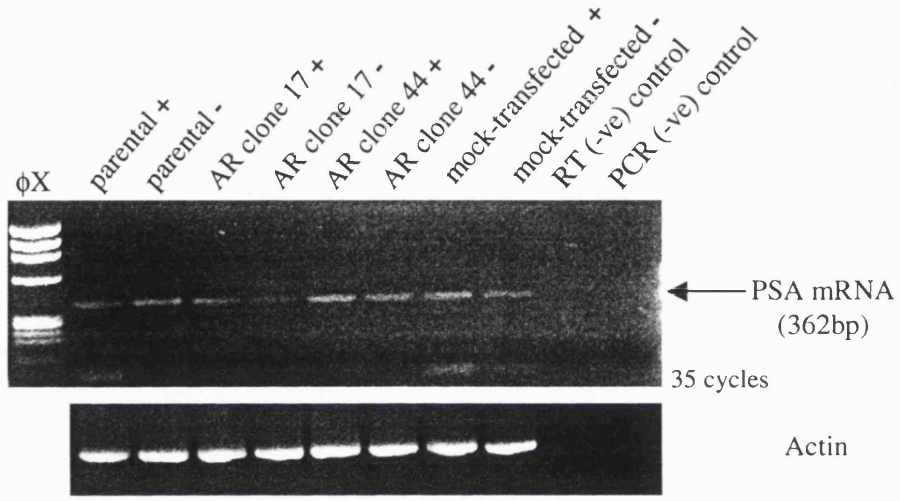


Figure 6.12 **Androgen effects (+/- 5nM mibolerone) on PSA mRNA of DUSF cells stably transfected with the constitutive pCMVhAR plasmid.**

EtBr-stained gels showing the RT-PCR analysis of cells grown for 48hrs in the absence and presence of 5nM mibolerone. Total RNA was reverse transcribed into cDNA which was then subjected to PCR analysis (PSAs and PSAa primers). Mock-transfected and non-transfected (parental) cells were included as negative control. The expected PCR product (362bp) was detected in all cells tested. There was no difference in the levels of PSA mRNA expression between cells grown in the absence and presence of 5nM mibolerone. Equal loading and integrity of the RNA was confirmed by amplification of actin mRNA. The reaction mixture containing all components except for the template RNA (DNA) was used as negative control for the RT (PCR) reaction. φX: *Hae*III fragments of phage φX174 DNA.

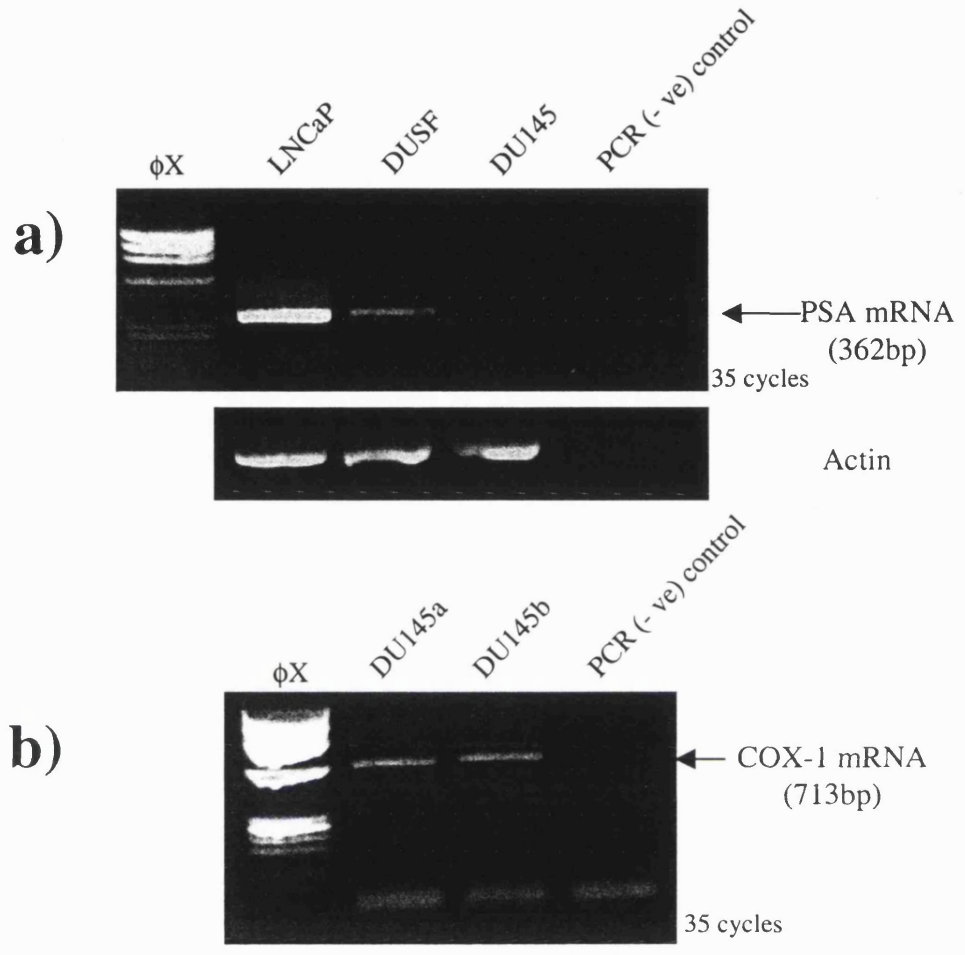


Figure 6.13 **PSA mRNA expression in DU145 and DUSF cells.**

EtBr-stained gels showing the RT-PCR analysis (in the absence of mibolerone) of DU145 and DUSF cells. Total RNA was reverse transcribed into cDNA which was then subjected to PCR analysis (PSAs and PSAa primers). Total RNA from LNCaP cells was included as positive control. PSA mRNA expression was detected in DUSF cells, despite the absence of PSA mRNA expression in their parental DU145 cell line. Equal loading and integrity of the RNA was confirmed by amplification of actin mRNA. Integrity of the DU145 mRNA was further confirmed by RT-PCR analysis using COX-1 primers. Two DU145 total RNA preparations (DU145a and DU145b) were tested. The reaction mixture containing all components except for the template DNA was used as negative control for the PCR reaction. φX: *Hae*III fragments of phage φX174 DNA.

a) EtBr-stained gel showing the RT-PCR product with PSA primers.

b) EtBr-stained gel showing the RT-PCR product with COX-1 primers

Two different RNA preparations (DU145a and DU145b) were tested. Both samples produced the expected size PCR product (713bp) (Figure 6.13b). This indicated that total RNA from DU145 cells used for RT-PCR with PSA primers was not degraded, and confirmed the authenticity of the results.

The experiments described in this section indicated that following 48hrs androgen exposure of AR-transfected cells, no difference in the PSA mRNA levels between cells grown in the absence (-) and cells grown in the presence (+) of the androgen was observed. However, 48hrs incubation with 5 or 50nM mibolerone induced an up-regulation of PSA mRNA in LNCaP. Finally, all DUSF cells tested, at 35 PCR cycles produced a PCR product of the expected size (362bp), suggesting that DUSF cells unlike their parent DU145 cell line are expressing low levels of PSA, independent of the presence of exogenous androgens.

6.12 Conclusions

The work described in this chapter was designed to establish an *in vitro* prostate cancer model where androgen effects on cells stably expressing a range of AR concentrations could be studied.

Specifically, the DUSF AR-negative prostate cancer cell line was stably transfected with a constitutive AR expression vector (pCMVhAR). The stable AR clones were screened for AR expression by ICC, Western and RT-PCR analysis. Two clones, clone 17 and clone 44, expressing relatively low and high levels of AR protein, respectively were further analysed to determine their androgen responsiveness. Expression of AR mRNA and protein was examined in transfected cells exposed to the synthetic androgen mibolerone.

Up-regulation of AR mRNA levels was detected 48hrs after androgen exposure in the high AR-expressing cells (clone 44). LNCaP cells, however, exhibited an androgenic down-regulation. This cell-specific and divergent regulation of AR mRNA is in accordance with previous studies which have reported that androgen exposure of the

AR-negative DU145 and PC-3 cells stably transfected with a constitutively active AR cDNA results in androgenic up-regulation of AR mRNA (Dai et al., 1996). In normal prostatic epithelium and in the androgen-sensitive LNCaP prostate cancer cells, exposure to androgens results in a down-regulation of AR mRNA (Quarmby et al., 1990; Shan et al., 1990; Krongrad et al., 1991; Hackenberg et al., 1992; Wolf et al., 1993; Burnstein et al., 1995). Androgen effects on AR protein levels paralleled the androgen effects on AR mRNA. Specifically, androgenic up-regulation of the AR protein was observed in both AR clones (clone 17 and clone 44). An up-regulation of AR protein has been described in the androgen-treated DU145/AR and PC-3/AR cells (Dai et al., 1996).

After establishing androgen responsiveness in the DUSF stable clones, the transcriptional activity of the AR protein was investigated. Hence, androgen-induced gene expression of the endogenous PSA gene was studied by RT-PCR analysis. PSA is silent in AR-negative prostate cancer cells and has been reported to be up-regulated by androgens in normal prostatic epithelium, LNCaP cells, and DU145 and PC-3 cells stably transfected with a constitutively active AR cDNA (Dai et al., 1996).

The experiments described in section 6.11 indicated that DUSF cells express low PSA mRNA levels. These results were surprising because DUSF cells do not express endogenous AR protein and therefore are not expected to transcribe the PSA gene. Moreover, the parent cell line, DU145, has a transcriptionally silent PSA gene due to lack of endogenous AR expression. It is tempting to speculate that transcriptional activation of the PSA gene in DUSF cells is not mediated by an AR-dependent mechanism. Moreover, addition of the androgen mibolerone to DUSF cells did not affect the levels of PSA mRNA, implying an androgen-independent PSA activation. Further evidence for the AR- and androgen-independent activation of the PSA gene was drawn from the fact that the androgenic up-regulation of the AR protein in the AR transfectants (clones 17 and 44) had no effect on PSA mRNA levels.

The androgen- and AR-independent PSA expression observed in DUSF cells needs to be further explored as it may provide insights in the progression of the prostate cancer into the androgen-independent state.

Chapter 7

General Discussion

Prostate cancer cells, like normal prostatic epithelium depend on androgens for growth and differentiation (Coffey and Isaacs, 1981; Cunha et al., 1987). The action of androgens on their target tissues is mediated by the androgen receptor (AR). AR is a ligand-dependent nuclear transcription factor that binds androgen and stimulates transcription of androgen-responsive genes, thereby regulating the growth of prostate cells (Lubahn et al., 1988a; Kallio et al., 1994). AR is expressed in normal prostatic epithelium and in cells from primary and recurrent prostatic tumours (Lubahn et al., 1988a; de Winter et al., 1991; de Winter et al., 1994; Hobisch et al., 1995; Visakorpi et al., 1995).

The growth of prostate cancer is known to be dependent on androgens. Based on this, androgen ablation is the primary treatment of advanced prostate cancer and induces remissions in the majority of patients (Lepor et al., 1982). Response rate is high (80%) but eventually prostate cancer cells overcome androgen dependence and acquire the ability to proliferate in the absence of androgen (Crawford et al., 1989; Brandstrom et al., 1994; McConkey et al., 1996). Although androgen-independent, the prostate cancer cells continue to express AR and AR-regulated genes, such as PSA (Prins et al., 1998; Polascik et al., 1999). Changes in the AR signalling pathway have been implicated in the transition from androgen-dependent to androgen-independent disease. However, the exact molecular mechanisms remain unknown.

7.1 Establishment of an *in vitro* model of prostate cancer

Most of the *in vitro* models of malignant human prostate lack AR and AR-regulated gene expression. Down-regulation of AR expression appears to be at the transcriptional level (Hayward et al., 1995; Sharrad, RM, personal communication), and only transfection of the cells with an AR cDNA can restore wild type AR protein to a measurable level (Yuan et al., 1993; Brass et al., 1995; Dai et al., 1996; Heisler et al., 1997).

The aim of the work described in this thesis was to establish an *in vitro* model of metastatic prostate cancer where the regulation of expression of the androgen receptor and AR-regulated genes (such as PSA) could be studied. To develop stable prostatic

cell lines expressing different levels of the human androgen receptor, two different approaches using an inducible and a constitutive gene expression system were employed. The gradual change of AR intracellular concentration was expected to reveal phenotypes and targets of interaction that remain obscure when AR is simply inactivated.

7.1.1 Establishment of the inducible TetOff™ gene expression system

To date, studies examining the quantitative relationship between gene transcription and specific functional outcomes have relied on comparisons of different stable clones. Because many genetic and epigenetic changes accumulate during the passage of cells, many of the clones consist of a heterogenous population of cells with different responses. Hence, clone heterogeneity complicates the interpretation of experimental results (DeWald et al., 1994).

The inducible TetOff™ gene expression system (CLONTECH) was chosen as it allows the analysis of one stable clone expressing different amounts of the protein of interest rather than analysing different cell clones, thereby eliminating the problem of clonal variation. In addition, the onset of transcription is reversibly controlled by the inducing stimulus, tetracycline (Tc), while modulated gene expression is regulated by varying the concentration of tetracycline.

The AR-negative DU145 cell line was stably transfected with pTetOff regulator plasmid and one highly inducible clone was isolated (DUTetOff cell line). Androgen receptor cDNA was subcloned into pTRE response plasmid (pTRE-AR) and its functionality was assayed by transient transfection into the inducible DUTetOff cell line, three human prostate cancer cell lines (DU145, PC-3, DUSF) and into a monkey kidney fibroblast cell line (COS-1).

From the transient transfection experiments, it was apparent that DUSF, PC-3 and COS-1 cells were tightly controlled by the Tc-responsive promoter. AR protein expression was clearly suppressed by tetracycline (Chapter 3), while AR mRNA levels were substantially higher in the induced (- Tc) state (Chapter 5). It is likely that the basal level of AR mRNA expression observed in the uninduced state (+ Tc) was

due to tTA-independent activity resulting from chromosomal integration of the minimal promoter (P_{minCMV}) near enhancers (referred to as "leakiness" of the promoter) (Freundlieb et al., 1997).

No AR protein expression was apparent from the transient transfection experiments in DUTetOff and DU145 cells. However, the presence of AR mRNA in the transfected DU145 cells indicated that the TetOff system is inducible in the DU145 cell line (Chapter 5). According to previous studies (Ackland-Berglund and Leib, 1995; Gossen and Bujard, 1995; Gossen et al., 1995; Howe et al., 1995) the efficiency of the TetOff system has been shown to be influenced by cell type. Different cell lines differ in their composition of transcription activator proteins and in their controls for plasmid integration and thus behave differently when used as hosts for the Tet-system (Yin et al., 1996). Based on this, it is likely that very low levels of AR, detectable only by the nested RT-PCR are inducted in DU145 cells.

The findings described in Chapter 3 and Chapter 5 are in agreement with published reports describing many difficulties of the TetOff systems' technology (Furth et al., 1994; Ackland-Berglund and Leib, 1995; Howe et al., 1995; Hofmann et al., 1996; Yin et al., 1996). Hence, even though the principle of the system is simple and there are several advantages over other gene expression systems (see section 3.1), in practice, the two-plasmid system is hampered for several reasons:

1. Its application is limited to certain types of cells with HeLa cells being the most stable Tc-responders (Gossen and Bujard, 1992; Ackland-Berglund and Leib, 1995; Yin and Schimke, 1996). In poorly transfectable cell lines (like DU145), the use of the system remains difficult.
2. A great deal of work is involved in establishing the system requiring months for the selection and testing of individual clones at each step.
3. The system is low in yield of inducible clones and the tTA expression in stable cell lines is also generally low (Gossen and Bujard, 1995). Strong expression of the tTA transactivator may be deleterious to the cells, as overexpression of transcription factors and particularly of VP16 is known to be toxic (Berger et al., 1992). This toxicity results from sequestering of transcription factors from other genes, a phenomenon described as squelching (Gill and Ptashne, 1988; Berger et

al., 1992). Based on this, for the generation of stable tTA-expressing cells, integration of the transfected DNA in chromosomal sites where the transactivator gene is only moderately expressed will be favoured.

Since the first generation of Tc-regulated vectors, several modifications of the basic tTA system have been described (Shockett et al., 1995; Hofmann et al., 1996; Baron et al., 1997; Kringstein et al., 1998). Because retroviral gene delivery is more rapid and efficient than stable transfection, many researchers have turned to tet-retroviral vectors. With retroviruses, genes can be introduced with >90% efficiency into tens of thousands of cells, yielding polyclonal populations with diverse integration sites within a week (Hofmann et al., 1996; Kringstein et al., 1998). In order to prevent toxic effects of the transactivator in the uninduced state and allow for higher levels of transactivator after induction, autoregulatory tTA expression vectors were created (Shockett et al., 1995; Hofmann et al., 1996). Also, novel Tc-controlled transactivators containing VP16-derived minimal activation domains were generated (Baron et al., 1997). By reducing the size of the activation domain, the new transactivators were tolerated at higher intracellular concentrations.

7.1.2 Establishment of a constitutive gene expression system

For the development of stable prostatic cell lines expressing different levels of the human androgen receptor (AR), the constitutive pCMVhAR plasmid was used to introduce AR cDNA into the AR-negative DUSF cells. In order to establish an *in vitro* model that reflects the conditions in androgen-ablated patients, DUSF cells, a clonal derivative of the DU145 cell line were used. DUSF cells permit androgen studies in a completely defined, serum-free medium, eliminating complications arising from the use of stripped-serum.

Varying levels of AR protein were detected in the DUSF stable clones (5/34) as determined by ICC and Western analysis (Chapter 6). Androgen sensitivity of two AR-transfectants (clone 17 and clone 44) was assayed by RT-PCR and Western analysis. Results showed AR up-regulation in the AR-transfectants (clone 17 and clone 44), and AR down-regulation in LNCaP cells incubated for 48hrs with 5nM

mibolerone. Ligand-dependent regulation of AR mRNA and protein is in agreement with previous studies (Quarmby et al., 1990; Krongrad et al., 1991; Dai et al., 1996) and indicated the presence of an androgen-responsive receptor in the DUSF transfectants. Ligand-dependent regulation also demonstrated that the DUSF cells have retained the mechanisms required for responding to androgen.

The differential androgenic regulation of AR expression observed in DUSF (up-regulation) and LNCaP (down-regulation) cells has been reported to be transcriptional and cell line-specific (Dai and Burnstein, 1996; Dai et al., 1996). It has been shown that androgen regulates the levels of AR mRNA in most cells and tissues containing the AR and that the AR gene is transcriptionally regulated by AR (autoregulation) (Burnstein et al., 1995; Dai and Burnstein, 1996; Dai et al., 1996). Autoregulation is a control mechanism common to several proteins of the steroid/thyroid hormone receptor superfamily. Both the GR and ER contain sequences within their coding region that are involved in autoregulation (Burnstein et al., 1990; Kaneko et al., 1993; Burnstein et al., 1994). In experiments where expression of the human AR cDNA is driven by a heterologous promoter (P_{CMV}), ARE sequences have been identified within the AR cDNA and not in CMV promoter sequences (Burnstein et al., 1995; Dai and Burnstein, 1996). Androgen receptor protein binds to these sequences in the presence of hormone. It is not known whether the same sequences mediate AR up- and down-regulation. Putative AREs located within the coding region may be silent in the absence of essential regulatory elements (cell-specific) (Burnstein et al., 1995). Hence, the differential androgenic regulation observed in DUSF and LNCaP cells is consistent with the hypothesis that other factors, in addition to AR, may influence androgen-mediated regulation.

Transcriptional activity of the transfected AR in DUSF cells was assayed by measuring the expression of the endogenous PSA gene in the presence of 5nM mibolerone. These studies revealed that:

a) in the absence of transfected AR, DUSF cells expressed a low level of PSA
b) androgens did not have any effect on PSA gene expression in the DUSF transfectants (androgen-independent)

c) changes in the AR protein levels did not induce a change in the PSA gene expression (AR-independent)

Androgen ablation leads to reduction in the levels of circulating PSA (Catalona et al., 1991). However, in androgen-resistant prostate cancer, PSA regulation escapes androgen control and becomes elevated in the absence of androgen (Stamey et al., 1989). Little is known about the androgen-independent regulation of PSA expression in hormone-refractory prostate cancer due to limited availability of androgen-independent, PSA-producing prostate cancer cell lines. The androgen-dependent LNCaP cell line and its androgen-independent subline, C4-2, produce high amounts of PSA in the absence of androgen stimulation (Thalmann et al., 1994; Wu et al., 1994). PSA production in LNCaP cells is further up-regulated by treatment with androgens (Young et al., 1991; Henttu et al., 1992; Montgomery et al., 1992). The androgen-independent cells DU145 and PC-3 do not produce PSA, and androgens have no effect on PSA production (Blok et al., 1995).

Most studies have focused mainly on the androgen regulation of the PSA promoter. (Young et al., 1991; Montgomery et al., 1992; Murtha et al., 1993; Ablin, 1997; Trapman and Cleutjens, 1997). PSA expression has been shown to be regulated by the proximal promoter and a strong upstream enhancer region (Cleutjens et al., 1997a; Cleutjens et al., 1997b; Zhang et al., 1997). Both regulatory regions contain binding sites for AR (androgen responsive elements, AREs) and are essential for androgen-induced activation of the PSA gene (Young et al., 1991; Montgomery et al., 1992; Murtha et al., 1993; Ablin, 1997; Trapman and Cleutjens, 1997). Three AREs within the 5.8kb PSA promoter have been identified (Riegman et al., 1991; Cleutjens et al., 1996; Cleutjens et al., 1997b). ARE-I and ARE-II are located within the proximal region of the promoter, whereas ARE-III is contained within a 440bp strong enhancer element core (AREc) located at -4.2kb of the promoter (Schuur et al., 1996; Cleutjens et al., 1997a; Zhang et al., 1997). Additional non-consensus AREs have been identified within the AREc enhancer element (Huang et al., 1999). ARE-I and ARE-III closely resemble the ARE consensus sequence, while ARE-II deviates

considerably from the consensus sequence and has a low binding affinity for AR (Riegman et al., 1991; Cleutjens et al., 1996; Cleutjens et al., 1997b).

Recently, two distinct regions (AREc and pN/H) involved in androgen-independent activation of PSA gene expression were identified (Yeung et al., 2000). The two regions suggest two different pathways involved in the up-regulation of PSA promoter activity. One pathway is mediated by AREc and requires AR (AR possibly activated through a ligand-independent pathway). The second pathway is mediated by pN/H (a region 150bp upstream of the TATA box) and is AR- and androgen-independent.

Androgen-independent transcriptional activation of the PSA promoter was also described by Oettgen et al. (2000). A Prostate-Derived Ets Factor (PDEF) acts as an AR coactivator and enhances PSA promoter activity in the absence of androgen. PDEF expression is restricted to luminal epithelial cells within the prostate and LNCaP cells, the same cells that express AR and PSA. Several putative Ets binding sites are located in both the promoter and enhancer region of the PSA gene. Ets factors are involved in the regulation of genes involved in haematopoiesis, angiogenesis, organogenesis, and specification of neuronal connectivity (Dittmer and Nordheim, 1998; Graves and Petersen, 1998; Wasylyk et al., 1998).

PSA gene expression in the absence of androgens suggests that either a) the AR can be activated in the absence of androgen (androgen-independent AR activation) to elevate PSA gene expression through AREs on the PSA gene, or b) transcription factors other than AR are stimulated and activate the PSA promoter.

Androgen-independent AR activation by elevation of intracellular levels of cAMP (Culig et al., 1994; Nazareth and Weigel, 1996; Sadar, 1999), compounds such as butyrate (Gleave et al., 1998; Sadar and Gleave, 2000), and growth factors (Culig et al., 1994; Nazareth and Weigel, 1996; Sadar, 1999) provides evidence for androgen-independent PSA gene expression mediated by AR.

Evidence for AR-independent PSA expression can be drawn from the following:

1. An androgen-independent regulatory element (RI) identified in the proximal PSA promoter has been shown to bind a transcription factor (p45) only in PSA-positive prostate cancer cells (LNCaP and C4-2). p45 is absent in PC-3 cells known to lack

PSA expression (Yeung et al., 2000). It is suggested that p45 may represent a new class of androgen-independent prostate-specific transcription factors that regulate PSA expression independent of androgen and AR.

2. Resveratrol, a polyphenol present in red wines, peanuts and mulberries has been shown to down-regulate PSA expression in LNCaP cells (Hsieh and Wu, 2000). No down-regulation in AR expression is observed, suggesting an AR-independent mechanism for regulation of PSA expression.
3. Hisatake et al. (2000) have also reported down-regulation of PSA expression in an AR-independent manner. Troglitazone, a ligand for Peroxisome Proliferator-Activated Receptor γ (PPARγ) inhibits transactivation of AREs in the PSA promoter without inhibiting AR activation. PPARγ is a member of the nuclear receptor superfamily highly expressed in fat cells (Chawla et al., 1994). PPARγ behaves as a tumour suppressor inducing differentiation while inhibiting growth of prostate (Kubota et al., 1998), breast (Elstner et al., 1998; Mueller et al., 1998), colon (Sarraf et al., 1998), and gastric cancer cells (Takahashi et al., 1999). Reporter gene studies showed that troglitazone inhibits androgen-induced PSA production without suppressing AR expression.

7.2 Future directions

The lack of AR protein expression in the inducible DUTetOff cells needs be further investigated as it may provide insights into the AR regulation in tumour cells. Knowing that AR protein is stabilised in the presence of androgen (Kemppainen et al., 1992), it would be of interest to assess AR mRNA and protein expression in the presence and absence of androgens in the inducible DUTetOff cell line. It would also be interesting to carry out reporter gene assays in DUTetOff cells using a response plasmid carrying another gene, instead of AR, downstream of the minimal P_{minCMV} promoter. Transient transfection assays with a reporter gene such as Green fluorescent protein (pTRE-GFP) may provide evidence for selective translational silencing of the transfected AR in DU145 prostate cancer cells.

The constitutive gene expression system developed in DUSF cells is a valuable tool for the study of hormone-refractory disease, as it resembles more the androgen-ablated conditions in the patient compared to previous models. Expression of PSA in an androgen-independent manner detected in DUSF cells compares with the situation *in vivo* where PSA production in androgen-resistant disease escapes androgen control.
It would be interesting to assess the androgen-mediated cell growth of the stable DUSF clones, expressing high and low AR levels, and to compare the results with previous similar studies in the androgen-independent PC-3 cells (Yuan et al., 1993; Brass et al., 1995; Dai et al., 1996; Heisler et al., 1997). PC-3/AR cells exhibit a paradoxical inhibition of cell growth in the presence of androgen. A similar, androgen-mediated growth repression is observed in androgen-independent derivatives of LNCaP cells (Umekita et al., 1996; Gao et al., 1999). Assessment of cellular proliferation of the DUSF/AR cells might provide insights into the mechanisms for paradoxical androgen-mediated growth inhibition.

The ability of the transfected AR to transactivate expression of androgen-responsive genes together with the androgen- and AR-independent pattern of endogenous PSA expression also needs further investigation.
It would be interesting to determine the effect of AR on the transcriptional activation of an MMTV promoter fused to a luciferase reporter gene (MMTV-luciferase). MMTV-LTR (mouse mammary tumour virus-long terminal repeat) promoter has been shown to contain androgen responsive elements and is androgen-sensitive (Otten et al., 1988). Hence, it would be possible to further examine the functional status of the transfected AR by its ability to transactivate the MMTV-luciferase reporter plasmid in transient transfection assays. Alternatively, DUSF cells can be transfected with a PSA (-630/+12) promoter-luciferase reporter plasmid (PSA-luciferase). This region of the PSA promoter contains the essential AREs for androgen induction. A marked increase of luciferase transactivation in the presence of androgens would confirm transactivation activity.
It would also be important to examine the promoter and enhancer of the endogenous PSA gene for mutations, and also assay DUSF cells for expression of the p45

transcription factor suggested to play a role in AR-independent PSA activation in C4-2 cells.

In conclusion, this thesis has provided a valuable *in vitro* model for hormone-relapse prostate cancer where the AR signalling pathway can be studied and the androgen-independent regulatory elements involved in PSA gene regulation can be identified. Understanding of the expression and regulation of the PSA gene in prostate cancer might signify the involvement of the gene during disease progression. Understanding of AR regulation is of fundamental and potentially therapeutic significance.

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