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DEVELOPMENT OF A TISSUE-SPECIFIC PRODRUG ACTIVATION APPROACH TO PROSTATE CANCER GENE THERAPY

A thesis submitted for the degree of

Doctor of Philosophy

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B.A. (Genetics) Hons.

Trinity College, University of Dublin

October 2005

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In memory of my father

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DECLARATION

I hereby certify that this thesis, submitted for examination for the degree of Doctor of Philosophy to the University of Dublin, has not been previously submitted for a degree or diploma to this or any other university. The work presented here is entirely my own, except where stated.

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.....Ruth Foley.....

Ruth Foley

October 2005

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SUMMARY

Prostate cancer is the most common fatal cancer in Irish men after lung and colorectal cancer. Since the risk of prostate cancer rises with age, its impact is likely to increase as the population ages. Standard treatments fail to prevent disease progression in some patients, particularly those with advanced disease, and many novel therapies are therefore being investigated. Gene therapy is emerging as a promising adjuvant to conventional treatments. Several Phase I clinical trials have been carried out with generally acceptable safety profiles and evidence in some cases of biological activity against prostate cancer.

The most promising approaches to gene therapy for prostate cancer have employed the prodrug activation gene strategy, or alternatively, vaccination with tumour antigens. The prodrug activation systems most extensively researched for their potential application in prostate cancer have involved a thymidine kinase gene derived from herpes simplex virus, and a bacterial gene, cytosine deaminase (CD). In many of these studies, the prodrug activation gene has been regulated by a constitutive promoter, while a smaller proportion have employed tissue-specific promoters for added safety. This PhD project aimed to combine the CD prodrug activation system with a prostate-specific promoter, and to provide proof of principle of its cytotoxicity and selectivity for prostate tissue. While the majority of experiments involved cell line models of prostate cancer, a primary culture system was established and employed.

To achieve prostate-specific expression of the CD gene, promoter and enhancer sequences from the human prostate-specific antigen (PSA) gene have been cloned upstream of the CD gene. Expression plasmids have been constructed, containing the CD gene downstream of three variant PSA promoters (PSA-CD constructs). To enable modulation of PSA transcriptional regulation, the PSA promoter variants were produced by site-directed mutagenesis to mimic natural mutations that may be associated with increased expression of PSA *in vivo*, but did not alter gene expression levels under the conditions of this study. An expression plasmid in which the CD gene is regulated by a constitutive promoter, pCMV-CD1.10, has also been generated.

Transient transfection using liposomes was employed to deliver these plasmid constructs to prostate cancer cell lines. Transfection procedures were optimised for each cell line by testing a variety of reagents and conditions, followed by microscopic analysis of the expression of the green fluorescent protein reporter gene. A preliminary assessment of the suitability of this transfection technique for primary human prostate cancer cells was also performed. Western blotting was carried out to confirm the presence of CD protein in cells transfected with CD expression plasmids. Finally, cytotoxicity was assessed in transfected cells treated with 5-fluorocytosine, the prodrug activated by the CD gene.

Transient transfection of primary prostate cultures with a reporter gene, as a first step towards using such cultures as an additional model system, resulted in up to 10% transfection efficiency. Primary cultures from six further patients were grown *in vitro*, characterized and tested for prodrug activation resulting in cell kill. Variability in transfection efficiency, and the limited numbers of cells available, may have contributed to the lack of cytotoxic prodrug activation observed in primary cultures.

CD was detected in PSA-positive prostate cancer cells following transfection with PSA-CD plasmids, and sensitised these cells to prodrug doses which had no intrinsic toxicity to parental cells, but killed 60-70% of transfectants. In contrast, transient transfection with PSA-CD constructs did not influence sensitivity to prodrug in a PSA-negative prostate cancer cell line and a colon cell line. Transfection of pCMV-CD1.10 brought about CD expression, and when combined with prodrug treatment, resulted in cell death in all cell lines tested. Analysis of the mechanism of cell death showed that the prodrug activation system induced apoptosis.

These results demonstrate that the CD-based prodrug activation system causes the desired cytotoxic effect, and indicate that the PSA promoter constructs generated in this study retain the tissue-specific behaviour of the endogenous PSA gene, though more detailed experiments are required to confirm this conclusion. Future studies will examine the potential synergy of this gene therapy strategy with radiotherapy, investigate its efficacy in other model systems, and focus on the development of second-generation vectors with more efficient and specific expression of the therapeutic gene.

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ABBREVIATIONS

5-FC	5-fluorocytosine
5-FU	5-fluorouracil
AAV	adeno-associated virus
AD	androgen dependent
adH_2O	autoclaved deionised water
AI	androgen independent
AP	alkaline phosphatase
APS	ammonium persulfate
AR	androgen receptor
ARCA	attenuated replication-competent adenovirus
ARE	androgen response element
ATP	adenosine triphosphate
bp	base pairs
BSA	bovine serum albumin
CAR	cocksackie and adenovirus receptor
CD	cytosine deaminase
cDNA	complementary DNA
CDS	coding DNA sequence
CGH	comparative genomic hybridisation
CIP	calf intestinal phosphatase
CMV	cytomegalovirus
CRC	colorectal cancer
DC	dextran/charcoal
$DD3^{PCA3}$	differential display code 3 (prostate cancer 3)
DHT	dihydrotestosterone
DME	drug metabolising enzyme
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease

dNTP	deoxyribonucleoside triphosphate
EBAO	ethidium bromide/acridine orange
EBNA-1	Epstein-Barr virus nuclear antigen 1
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EZH2	enhancer of zeste homologue 2
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage colony stimulating factor
GPAT	genetic prodrug activation therapy
gpt	guanine phosphoribosyl transferase
HBSS	Hanks' balanced salt solution
hK2	human kallikrein 2
HPC1	hereditary prostate cancer 1
HRP	horseradish peroxidase
IC	inhibitory concentration
IE	immediate early
IGF	insulin-like growth factor
IPTG	isopropyl-beta-D-thiogalactoside
kb	kilobases
kD	kilodaltons
LB	Luria-Bertrani
MAPK	mitogen-activated protein kinase
mRNA	messenger RNA
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NK	natural killer
NSC	neural stem cell
NTR	nitroreductase
OC	osteocalcin

ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDEF	prostate-derived Ets factor
PDMAEMA	poly 2-(dimethylamino) ethyl methacrylate
PEG	polyethylene glycol
PEI	polyethyleneimine
PI	propidium iodide
PNP	purine nucleoside phosphorylase
PSA	prostate-specific antigen
PSMA	prostate-specific membrane antigen
rATP	ribosomal adenosine triphosphate
RNA	ribonucleic acid
RNase	ribonuclease
RNASEL	ribonuclease L
ROI	region of interest
RT-PCR	reverse-transcriptase PCR
SCID	severe combined immunodeficiency
SDS	sodium dodecyl sulfate
STE	Saline-Tris-EDTA
TAE	Tris-acetate-EDTA
TBS	Tris-buffered saline
TE	Tris-EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
TFO	triplex-forming oligonucleotides
TGF-β	transforming growth factor β
TK	thymidine kinase
T_m	melting temperature
TMB	3,3',5,5'-tetramethylbenzidine
TRAMP	transgenic adenocarcinoma of the mouse prostate

tRNA	transfer RNA
TSTA	two-step transcriptional activation
UPRT	uracil phosphoribosyltransferase
VEGF	vascular endothelial growth factor
X-Gal	5-bromo-4-chloro-3-indolyl β–D-galactopyranoside

CHAPTER 1: INTRODUCTION

1.1. INTRODUCTION TO PROSTATE CANCER

1.1.1. Incidence

Prostate cancer is a significant cause of illness and death in the Irish male population, accounting for over a tenth of all diagnosed cancers (National Cancer Registry Ireland 2003). With 1500 new cases and over five hundred deaths per year, the condition is exceeded only by lung and colorectal cancer as a cause of cancer mortality in Irish men (National Cancer Registry Ireland 2003). Since the disease is more common among older men, its incidence is expected to increase as the population ages (Kirby *et al* 2001).

Considerable geographic variation in the occurrence of prostate cancer has been noted, ranging from 60 per 100,000 in Scandinavia to 4 per 100,000 in China and Japan (Kirby *et al* 2001). This is presumably due to variations in some of the environmental and genetic differences discussed below, in addition to possible differences in detection rates.

1.1.2. Clinical course

Prostate cancer patients may present with bladder outflow obstruction or symptoms of metastatic disease such as bone pain or weight loss, though presymptomatic detection has been facilitated by measurement of serum prostate-specific antigen (PSA) levels. This is combined with findings from digital rectal examination and biopsy specimens for diagnosis of the condition. PSA screening for prostate cancer has been extensively used in the USA, though certain medical and social issues involved in this approach are currently a matter of debate (Jacobsen 2000). The benefits of more frequent early detection need to be assessed, and balanced with the impact of screening on quality of life (particularly where false positive results are obtained), and the financial costs involved.

Computed tomography, magnetic resonance imaging and radionuclide bone scans are further used to determine the extent and stage of the disease. In addition, the Gleason scoring system or similar systems are used to grade the tumour (Bostwick & Dundore

1997). Clinicians have used combinations of Gleason score, T stage and PSA to define patients with varying degrees of risk for disease progression and relapse. Reverse-transcriptase PCR (RT-PCR) of PSA mRNA in the bone marrow has been investigated as a potentially sensitive way to detect subclinical metastases (Wood *et al* 1994), which occur mainly in bone and in lymph nodes, in addition to lung, liver, adrenal gland and other sites (Catalona 1984). However, this assay has not yet entered routine clinical laboratory practice.

1.1.3. Risk factors

The most important risk factors for prostate cancer are age, race and family history. Prostate cancer becomes increasingly more common with age, from being very rare in men under the age of fifty to its highest prevalence in the 75-79 age group (Kirby *et al* 2001). Ethnic origin also clearly influences the risk of prostate cancer, with incidences per 100,000 of 212 for African-Americans, 128 for Caucasians and 78 for Asians in the USA in 1998 [http://seer.cancer.gov]. This variation is probably due to several factors. The incidence of prostate cancer in Japanese men living in the USA has been shown to be more than double the incidence in their counterparts remaining in Japan, though still lower than that of American Caucasians (Cook *et al* 1999). This and similar studies suggest that prostate cancer risk is influenced by environmental, in addition to genetic, factors. Selenium levels (Brooks *et al* 2001) and dietary fat intake (Kolonel *et al* 1999) have been implicated.

A positive family history for prostate cancer is a strong risk factor (Grönberg *et al* 1996), particularly with an early age of onset (Carter *et al* 1992). Efforts to isolate familial prostate cancer genes by linkage analysis have been complicated by the high frequency of sporadic cases, and by different modes of inheritance in different families (Cui *et al* 2001). However, several chromosomal loci linked to prostate cancer have recently been proposed (Simard *et al* 2002). The first was HPC1 on chromosome 1q24-25 (Smith *et al* 1996), where RNASEL, a candidate gene which encodes a ribonuclease, has been identified (Casey *et al* 2002).

The possible significance in prostate cancer development of polymorphisms in the androgen receptor (AR) has been investigated (Ingles *et al* 1997, Stanford *et al* 1997, Correa-Cerro *et al* 1999), in addition to the steroid 5-α reductase gene, which activates testosterone to dihydrotestosterone (Makridakis *et al* 1997), and the receptor for the steroid hormone vitamin D (Ingles *et al* 1997). However, none have been reproducibly found to have a major impact on prostate cancer risk.

1.1.4. Genes involved in prostate cancer development

Prostate tumours, though slower-growing than most tumours, vary widely in their aggressiveness (Chodak *et al* 1994). Extensive efforts are ongoing to characterise molecular lesions which may distinguish between latent tumours which will not progress and aggressive tumours which require immediate treatment. High-throughput techniques including microarrays and serial analysis of gene expression (SAGE) have been instrumental towards this aim (Rhodes *et al* 2002, Waghray *et al* 2001). Resources such as the Cancer Genome Anatomy Project [http://cgap.nci.nih.gov] and the Gene Expression Atlas [http://expression.gnf.org] have made considerable amounts of gene expression data available (Su *et al* 2002, Boon *et al* 2002).

Not surprisingly, many genes associated with cell cycle regulation and cell proliferation have been implicated. p53 mutations are common in prostate cancer, particularly in advanced disease (Meyers *et al* 1998). Aberrant nuclear accumulation of p53 protein is a negative prognostic factor for disease-free survival (Leibovich *et al* 2000, Quinn *et al* 2000). The tumour suppressor gene PTEN encodes a phosphatase that negatively regulates cell cycle progression, and is frequently deleted or inactivated in advanced prostate tumours (Cairns *et al* 1997, McMenamin *et al* 1999). PTEN loss has been associated with risk of clinical recurrence (Halvorsen *et al* 2003).

The c-met proto-oncogene encodes the hepatocyte growth factor/scatter factor receptor. Over 90% of 88 metastatic specimens were positive for c-met, in contrast to half of the 90 primary prostate cancer samples examined (Knudsen *et al* 2002). Interestingly, c-met expression was particularly high in bone metastases and also occurs in normal bone

stroma, raising the question of whether it is partly responsible for the tendency of prostate tumours to metastasise to bone sites (Knudsen *et al* 2002). EZH2 encodes a homolog of a *Drosophila* transcriptional repressor gene, and is highly overexpressed in metastatic prostate cancer compared to localised disease (Varambally *et al* 2002). It represses a wide range of genes, at least some of which function in prostate cancer cell proliferation (Varambally *et al* 2002).

Microarray experiments have helped reveal some trends in the molecular aberrations in prostate cancer. A meta-analysis of four microarray studies on prostate cancer showed that biosynthesis of polyamines and of purines was consistently dysregulated in prostate cancer (Rhodes *et al* 2002). Almost a quarter of the genes found to be most overexpressed or underexpressed during prostate cancer progression were associated with regulation of gene expression (LaTulippe *et al* 2002).

Genes associated with cell adhesion, oxidative stress response, fatty acid metabolism and other functions have also been implicated by microarray studies and other techniques. E-cadherin was less active in tumours than in normal matched tissue in paired samples from 18 patients (Rashid *et al* 2001), and levels of the transmembrane glycoprotein KAI1 affect metastasis development in a rat model (Dong *et al* 1995). Glutathione-S-transferase, part of a pathway protecting cells from oxidative damage, shows a strong association between inactivation by hypermethylation and prostate cancer development (Goessl *et al* 2001). Fatty acid synthase (Dhanasekaran *et al* 2001) and another fatty acid metabolism enzyme, human alpha-methylacyl-CoA racemase (Xu *et al* 2000) were also shown to be overexpressed in prostate tumours, and were significantly overexpressed in a meta-analysis (Rhodes *et al* 2002).

PSA is well established as the biochemical marker of choice in prostate cancer diagnosis and prognosis (Small & Roach 2002). However, its sensitivity and specificity are limited (Kirby *et al* 2001), and other markers have been investigated. Recent large-scale gene expression analyses have identified possible alternatives, including $DD3^{PCA3}$ and hepsin. $DD3^{PCA3}$, a gene identified in a differential display study, has shown potential for use in

prostate cancer diagnosis (Schalken *et al* 2003). Analysis of $DD3^{PCA3}$ as a prostate cancer marker in urine samples yielded a negative predictive value of 90% (Hessels *et al* 2003). Overexpression in prostate cancer of hepsin, a transmembrane serine protease, has been validated by RT-PCR (Welsh *et al* 2001) and tissue microarrays (Dhanasekaran *et al* 2001), in addition to DNA microarrays (Rhodes *et al* 2002). When benign prostatic hyperplasia and Gleason grade 4 or 5 prostate cancer were compared using cDNA arrays of almost seven thousand genes, hepsin was the gene that was most upregulated in prostate cancer (Stamey *et al* 2001).

1.1.5. Development of androgen-independence

Many prostate tumours, though initially androgen-dependent, become androgen-independent (AI) and refractory to hormone withdrawal therapy, presenting a significant problem in the treatment of the disease. Elucidating the basis for progression to androgen independence has been a focus of prostate cancer research. It is not clear whether AI prostate tumours derive from pre-existing AI prostate cells, or, more likely, from prostate cells which acquire the ability to proliferate in the absence of androgen. Prostate growth is normally dependent on androgen receptor (AR) activation. On binding dihydrotestosterone or similar steroid hormones, the nuclear AR dissociates from heat shock proteins and binds to androgen response elements (AREs) in various promoters, stimulating expression of androgen-regulated genes. This ability is acquired by both AR-dependent and AR-independent mechanisms.

Most AI prostate tumours express the AR (van der Kwast *et al* 1991). It is activated in androgen-deprivation conditions by at least three mechanisms: overexpression due to gene amplification, cross-activation by agents other than androgens, and mutations that alter its specificity.

Gene amplification is probably the most common mutational mechanism for the AR. This event occurs in 22-30% of tumours which recur following endocrine therapy (Visakorpi *et al* 1995, Koivisto *et al* 1997, Bubendorf *et al* 1999a). AR gene

amplification was associated with a positive response to combined androgen blockade after failure of endocrine mono-therapy in a prospective study of 92 patients (Palmberg *et al* 2000).

Several non-androgen agents have been found to cross-activate the AR in the absence of androgen, including insulin-like growth factor I (Culig *et al* 1994), interleukin-6 and a related cytokine oncostatin-M, and the breast cancer oncogene HER2/neu. Oncostatin-M (Godoy-Tundidor *et al* 2002) and the transcription factor HER2/neu (Yeh *et al* 1999) also modulate the response of the AR to the antiandrogen hydroxyflutamide and to very low androgen concentrations, respectively, suggesting possible mechanisms by which tumours evade control by endocrine therapy. Various signalling pathways have been implicated in AR cross-activation, particularly the mitogen-activated protein kinase (MAPK) pathway (Yeh *et al* 1999, Ueda *et al* 2002, Franco *et al* 2003), which is activated in 70% of high-grade prostate tumours (Gioeli *et al* 1999).

The clinical significance of AR mutations remains to be clarified, since estimates of their frequency vary from 6% to 44% (Tilley *et al* 1996, Wallen *et al* 1999). One point mutation in the hormone-binding domain, which alters the response to anti-androgens[1] (Veldscholte *et al* 1990), has been reported to occur in five of sixteen patients following combined androgen blockade (Taplin *et al* 1999), though it was not detected in two other studies of hormone-refractory tumours (Visakorpi *et al* 1995, Koivisto *et al* 1997).

Alternatively, the AR pathway may be bypassed altogether in certain tumours by other mechanisms which regulate the balance of proliferation and apoptosis. Protein levels of the anti-apoptotic proto-oncogene bcl-2 have been correlated with progression to AI status in human tumours (McDonnell *et al* 1992), and bcl-2 stable transfection enabled the AD prostate cancer cell line LNCaP to survive androgen depletion *in vitro* and *in vivo* (Raffo *et al* 1995). c-myc mRNA levels were higher in prostate tumours than in benign

[1] Anti-androgens behave as agonists, activating the mutant AR which harbours this threonine to alanine substitution at codon 877.

prostate tissue (Fleming *et al* 1986, Dhanasekaran *et al* 2001), and in addition, 11% of 62 metastatic prostate cancer specimens, but none of 223 primary tumours, showed c-myc gene amplification (Bubendorf *et al* 1999a).

$p21^{WAF\text{-}1/CIP1}$, an effector of the p53 protein, negatively regulates the cell cycle, and its overexpression inhibits AD and AI prostate cancer cell proliferation *in vitro* and tumorigenicity *in vivo* (Gotoh *et al* 2003). However, it is expressed more frequently in AI than in AD prostate cancer (Baretton *et al* 1999, Fizazi *et al* 2002), suggesting that the G1/S cell cycle checkpoint may be aberrantly regulated in some AI prostate tumours (Baretton *et al* 1999). p53 status may also influence responsiveness to androgens. *In vitro* experiments demonstrated that four p53 mutations common in prostate cancer each enabled the androgen-dependent prostate cancer cell line LNCaP to grow in an androgen-independent manner (Nesslinger *et al* 2003).

A cDNA microarray study on the androgen-dependent human prostate cancer xenograft CWR22 and its AI derivatives showed that insulin-like growth factor binding protein 2, insulin receptor and insulin-like growth factor-II (IGF-II) were all overexpressed in AI conditions (Bubendorf *et al* 1999b). This pathway as a whole clearly shows dysregulation in prostate cancer (Culig *et al* 1994, Chan *et al* 1998, Rhodes *et al* 2002), and further work is needed to clarify its role in progression to androgen independence.

1.1.6. Prostate cancer treatment

Prostate cancer is conventionally treated by radical prostatectomy (for localised disease), external beam radiation therapy or brachytherapy, various androgen withdrawal regimens, or combinations of these. Chemotherapy with mitoxantrone, cyclophosphamide or doxorubicin has also been employed but encounters the problems of acquired drug resistance and patient tolerance. Recently, a Phase III clinical trial of docetaxel and prednisone showed significantly improved survival for patients with AI prostate cancer (Eisenberger *et al* 2004).

Gene therapy is emerging as a promising adjuvant to these strategies. Several approaches to gene therapy have been developed and are described in Section 1.2.2 below. Encouraging results have also been obtained in phase I clinical trials of immunotherapy strategies, with autologous dendritic cells exposed to prostatic acid phosphatase protein or prostate-specific membrane antigen peptides (Lodge *et al* 2000, Fong *et al* 2001).

1.2. GENE EXPRESSION AS A THERAPEUTIC TARGET

1.2.1. Current status of gene therapy

Since the first clinical trial of gene therapy, for adenosine deaminase-deficient severe combined immunodeficiency (SCID) in 1990 (Muul *et al* 2003), over six hundred phase I to phase III trials have been initiated, involving over three thousand patients [http://www.wiley.co.uk/genetherapy]. Monogenic diseases including SCID, cystic fibrosis and haemophilia, multigenic diseases such as cancer and atherosclerosis, and HIV/AIDS have all been subject to efforts to bring about clinical improvement by introducing exogenous nucleic acids of specific sequence and function.

Successful gene therapy requires an appropriate therapeutic gene, efficiently delivered and expressed at therapeutic levels for a sufficient time, with minimal effects on tissues other than the target tissue, and minimal short-term or long-term toxicity. Many genetic strategies have been developed, including several for cancer (Section 1.2.2). Cancer gene therapy protocols comprise 63% of current gene therapy trials [http://www.wiley.co.uk/genetherapy]. A wide variety of both viral and nonviral delivery methods is now available (Section 1.2.3). Efforts have also been made to ensure tissue-specific activity of the therapeutic gene (Section 1.4).

Gene therapy has exciting potential as an addition to the present armoury of therapies available, either as an adjuvant or possibly in the future as a single agent. However, as new possibilities emerge, they also raise complex ethical, social and regulatory issues (Nevin 1999).

1.2.2. Gene therapy strategies in cancer

Numerous approaches to cancer gene therapy have been explored so far. Therapies may be targeted to specific signal transduction proteins, mRNAs or genes associated with malignancy. The monoclonal antibody Herceptin (Kumar *et al* 2000) and the ATP inhibitor imatinib mesylate (Mauro & Druker 2001) have been developed to inhibit the HER2/*neu* and bcr-abl tyrosine kinases, which are associated with breast cancer and chronic myeloid leukaemia respectively. Alternatively, vector-based strategies that introduce genes encoding tumour suppressor proteins, pro-apoptotic proteins, cytokines, tumour-specific proteins as vaccines, anti-angiogenic proteins or prodrug activation enzymes, have also been developed.

A number of targeted approaches have been used in *in vitro* models of prostate cancer, and less commonly in animal models or phase I clinical studies. The AR is a key therapeutic target, as it stimulates prostate cancer cell proliferation by activating transcription of target genes. This process has been competitively inhibited by transfection of double-stranded DNA "decoy" fragments containing AREs (Kuratsukuri *et al* 1999). The AR has also been targeted at the mRNA level using an antisense strategy (Eder *et al* 2002), as have the proto-oncogenes bcl-2 (Tolcher 2001) and c-myc (Steiner *et al* 1998a). Antisense oligonucleotides inhibit gene expression by binding to a specific target mRNA with a complementary nucleotide sequence, resulting in degradation and/or inhibition of translation of the target gene.

RNA may also be targeted by the small interfering RNA (siRNA) approach. siRNAs are short RNA duplexes which mediate sequence-specific mRNA degradation and have recently been shown to be involved in gene silencing in eukaryotes (Shi 2003). siRNA directed against the EZH2 gene inhibited cell proliferation in prostate cancer cell lines (Varambally *et al* 2002). EZH2, a homologue of a *Drosophila* transcription regulation gene, was selected for this approach as previous microarray studies had identified it as the gene most significantly upregulated in metastatic prostate cancer, relative to localised prostate cancer (Varambally *et al* 2002).

Triplex-forming oligonucleotides (TFOs) offer another mechanism for sequence-specific gene silencing, but have fewer potential target sequences than antisense or siRNA approaches. They bind to double-stranded DNA to form a triple helix structure, and can inhibit transcription of genes such as the transcription factor Ets2, which has been implicated in prostate cancer development (Carbone *et al* 2003). All of the above strategies harness the ability to identify genes that are upregulated in prostate cancer or in prostate cancer progression, and use gene targeting approaches to interfere with or specifically ablate target gene expression.

Other strategies aim to bring about cancer cell death by means unrelated to the genetic lesions responsible for their malignancy. Prodrug activation or "suicide" gene therapy involves introducing into tumour cells a gene which will convert a nontoxic prodrug to a toxic derivative, thereby killing the tumour cells (discussed in detail in Section 1.3). Alternatively, genes which cause toxicity without additional factors have been used. The pro-apoptotic gene Bax (Lowe *et al* 2001) and diphtheria toxin A (Zheng *et al* 2003) are examples. Similarly, replication-competent adenoviral vectors capable of tissue-specific adenoviral replication and cell death (Yu *et al* 1999a, DeWeese *et al* 2001) have been designed (see Section 1.2.3).

A tumour's microenvironment presents other therapeutic targets in addition to the tumour itself. There has been considerable interest in the role of angiogenesis in tumour growth. Genes encoding anti-angiogenic proteins, and antisense mRNA against genes including vascular endothelial growth factor (VEGF) (Nguyen *et al* 1998), may indirectly inhibit tumour growth.

Activation of an immune response against tumour cells may be achieved by expression of a tumour protein such as prostate-specific antigen (Sanda *et al* 1999, Eder *et al* 2000, Gulley *et al* 2002), or transfection of tumour cells with cytokines including granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin-2 to stimulate an immune response (Simons *et* al 1999, Belldegrun *et al* 2001). A recent Phase I clinical trial combined both these approaches by administering a vaccinia vector encoding the

tumour antigen MUC-1 and interleukin-2 (Pantuck *et al* 2004). Shah and colleagues used a therapeutic gene to indirectly enhance the ability of the immune system to respond to challenges such as prostate cancer. Mice were treated with a gene encoding a mutant form of the transforming growth factor β (TGF-β) receptor, which rendered bone marrow cells insensitive to the normal immunosuppressive effects of TGF-β and inhibited metastasis of prostate cancer xenografts (Shah *et al* 2002).

1.2.3. Vectors for delivering therapeutic genes

Crucial to any strategy that relies on the introduction of foreign genetic material is the ability to deliver genes to the appropriate cell or tissue in sufficient quantities to achieve a therapeutic effect. A wide variety of vectors have been developed for this purpose, and are summarized in Table 1.1. Viral vectors are designed to harness the attributes of viruses as delivery agents; this involves the removal of many virally encoded genes and introduction of the therapeutic gene(s). Vectors derived from retroviruses, adenoviruses, vaccinia virus, adeno-associated virus and herpes simplex virus have been extensively used (Bonnet *et al* 2000). Nonviral methods of delivering genes include cationic liposomes, polymers, and various physical methods (Nishikawa & Hashida 2002). Cellular methods are also being developed, in which therapeutic genes are delivered *in vitro* to cell types which subsequently target tumours *in vivo*. These include bacteria (Pawelek *et al* 1997) and neural stem cells (Brown *et al* 2003).

Retroviruses (Günzburg & Salmons 1999) are the most frequently used vectors in gene therapy clinical trials [http://www.wiley.co.uk/genetherapy], and have been used in two prostate cancer clinical trials (Steiner *et* al 1998b, Simons *et al* 1999). Retroviral vectors transduce only dividing cells, an advantage in some settings for targeting malignant rather than nonmalignant cells. Lentiviral vectors derived from HIV-1 (Lever 1999, Zheng *et al* 2003) are an exception, transducing both dividing and nondividing cells.

	Methods	Advantages	Disadvantages
Viral	Retrovirus Adenovirus AAV HSV Vaccinia	Efficient gene transfer Long-term expression (integrating viruses)	Immunogenicity Possible pathogenicity
Chemical	Cationic liposomes Cationic polymers	Non-immunogenic	Inefficient gene transfer
Physical	Naked DNA injection Electroporation Biolistic acceleration	Low toxicity Possibly efficient enough (electroporation)	Suitable only for specific sites Generally low efficiency
Cellular	*Salmonella typhimurium* Neural stem cells	Capable of tumour localisation	Safety and efficacy remain to be established

Table 1.1. Summary of vectors developed for gene therapy. No ideal vector has been found to date, despite much research on viral and chemical gene delivery in particular. However, these vectors continue to be refined to overcome their disadvantages. More novel *in vivo* gene transfer methods, including electroporation and the use of genetically modified tumour-targeting cells, may also contribute much to the search for efficient and safe gene therapy vectors. AAV, adeno-associated virus; HSV, herpes simplex virus.

The single-stranded RNA genome of a retrovirus integrates stably into the cellular genome following reverse transcription, allowing stable expression for up to ten years (Muul *et al* 2003). Integration can also result in serious adverse events if insertional mutagenesis activates a proto-oncogene. This event was recently reported for the first time in a retroviral gene therapy clinical trial, when two cases of uncontrolled T cell proliferation were described among ten patients receiving γc receptor gene therapy for X-linked SCID disorder (Hacein-Bey-Abina 2003), raising serious concerns about ensuring the safety of gene therapy. The utility of retroviruses is also limited by promoter

silencing (Xu *et al* 1989), variable transduction efficiency, small transgene size, immunogenicity (Patel & Belldegrun 2000) and difficulty in obtaining high titres during production (Yu *et al* 2001a).

Adenoviral vectors (Connelly 1999) have also been extensively studied. Adenovirus type 5 is the most frequently used vector in clinical trials of gene therapy for prostate cancer. These double-stranded DNA parvoviruses are transiently maintained, as they do not integrate into the genome. Their transduction efficiency is generally superior to that of retroviruses (Patel & Belldegrun 2000). Immune resistance to adenoviruses has posed significant problems both for repeated injections of the vector (necessary in settings where sustained expression is required) and for patient toxicity. Adenoviral vectors have been extensively modified to reduce their immunogenicity, including the development of "gutless" vectors in which no adenoviral genes remain (Chen *et al* 1997). Such developments also alleviate the limited transgene size (Patel & Belldegrun 2000).

Both replication-deficient (Herman *et al* 1999, Teh *et al* 2001, Miles *et al* 2001, Kubo *et al* 2003, Trudel *et al* 2003, van der Linden *et al* 2005) and replication-competent (DeWeese *et al* 2001, Freytag *et al* 2002a) adenoviruses have been used in prostate cancer gene therapy. Yu and colleagues (Yu *et al* 1999a) have developed attenuated replication-competent adenoviruses (ARCAs) in which the viral E1A and E1B genes, essential for viral replication, are under the control of prostate-specific promoters. This strategy targets virus replication and cytotoxicity to prostate tissue.

Adeno-associated viruses (AAVs) are small single-stranded DNA viruses of the parvovirus family. Genetically engineered AAVs transduce only dividing cells, though wild-type viruses can also transduce non-dividing cells (Patel & Belldegrun 2000). Long-term expression is facilitated by stable integration into the genome (Nakai *et al* 1999), which does not require the presence of a helper virus, unlike replication (Patel & Belldegrun 2000). Recombinant AAVs efficiently transduce a range of human tissues including brain, colorectal and liver cancer (Nguyen *et al* 1998). However, production of

high titres can be difficult, and the virus is also constrained by a transgene size limit of 4.8kb (Patel & Belldegrun 2000). Hybrid vectors containing AAV and insect baculovirus sequences have been designed to overcome this limitation (Palombo *et al* 1998).

Vectors based on herpes simplex virus type I (Soares *et al* 1999) are large double-stranded DNA viruses of the herpesvirus family. They are maintained in neurons as latent episomes, and due to their natural tropism, have been particularly used to deliver genes to the central nervous system (Ruffini *et al* 2001). Stable expression has been obtained only at low levels under the control of latency-associated promoters, though transient expression can be more efficient (Soares *et al* 1999).

Vaccinia virus, a large non-integrating DNA poxvirus, has emerged more recently as a promising gene therapy vector. It efficiently transduces both dividing and nondividing cells, and can carry over 30kb of foreign DNA, but is not stably maintained and, like adenoviruses and retroviruses, can evoke an immune response (Patel & Belldegrun 2000, Bonnet *et al* 2000). Modified vaccinia viruses have been used in three prostate cancer gene therapy clinical trials to deliver a PSA vaccine (Sanda *et al* 1999, Eder *et al* 2000, Gulley *et al* 2002), and also to deliver the tumour antigen MUC-1 and interleukin-2 in a phase I clinical trial of advanced prostate cancer patients (Pantuck *et al* 2004).

Liposomes are cationic lipids, or more usually a combination of cationic and neutral lipids, which have been used for cellular delivery of drugs (Ishida *et al* 2001) as well as therapeutic genes. Their positive charge facilitates the formation of complexes with DNA, and their lipid structure allows them to cross cell membranes. Liposomes have generally been less efficient than viral vectors, and other disadvantages have included cytotoxicity and sensitivity to serum. Improved formulations have been developed, to enhance uptake and reduce the problem of vectors being sequestered in endosomes. One such formulation allowed a liposome to effectively release fluorescent oligonucleotides into the nucleus, although the lipid component was retained in endosomal compartments (Shi *et al* 2001). Liposomes complexed with virion proteins from the haemagglutinating

virus of Japan (Otomo *et al* 2001) and with fragments of an antibody to the oncogene HER2/neu (Meyer *et al* 1998) have also been used to bypass the endosomal pathway. Although most genes delivered by liposomes are inserted in plasmids and transiently expressed, the ability of liposomes to complex with much larger DNA constructs than viruses has also been harnessed to allow more long-term maintenance. The oriP origin of replication and the EBNA-1 gene of Epstein-Barr virus facilitate episomal replication and have been incorporated into plasmids (Otomo *et al* 2001). Mammalian artificial chromosomes (Vos 1998) have also been delivered using liposomes, and maintained in 70% of cells *in vitro* for two months without selection (Mejía *et al* 2001). Liposomes have frequently been used to deliver therapeutic genes in prostate cancer preclinical studies (Miyake *et al* 1999, Pang 2000, Iyer *et al* 2001), and in one prostate cancer clinical trial (Belldegrun *et al* 2001).

Cationic polymers also bind to nucleic acids and facilitate their intracellular delivery. The cationic polymer polyethyleneimine (PEI) has been used for prostate cancer cell transfection *in vitro* (Frønsdal *et al* 1998). Other polymers have also been developed, such as PDMAEMA (poly 2-(dimethylamino) ethyl methacrylate), which efficiently transfects ovarian cancer cells *in vitro* (Fonseca *et al* 1999). Nanoparticles have recently been found to facilitate chemical methods of gene delivery (Panyam & Labhasetwar 2003). A vector composed of poly-L-lysine and iron oxide nanoparticles delivered a reporter gene to 60% of lung cells *in vivo*, with low toxicity, though delivery to other tissues was less efficient (Xiang *et al* 2003).

Naked plasmids have been delivered to cells or tissues by a number of physical techniques. Naked DNA injection in certain tissues such as muscle (Kim *et al* 1998) and skin (Pandha *et al* 1999) can be taken up at sufficient levels not to require any carrier. A recent Phase I clinical trial treated patients with androgen-independent prostate cancer with intramuscular and intradermal injections of a PSA-expressing plasmid, inducing cellular and humoral immune responses (Pavlenko *et al* 2004). Alternatively, DNA-coated gold or tungsten particles, accelerated using biolistic equipment ("gene gun"), have brought about transient luciferase expression in mouse skin, liver and ear (Williams *et al* 1991). Plasmids have also been delivered to prostate cancer cells (Pang *et al* 1997)

and other tissues using electroporation, in which cell membranes are temporarily permeabilised by exposure to a strong electrostatic field. This approach, though mainly used in cell culture systems, has also been employed to deliver transgenes to subcutaneous prostate tumour xenografts in mice (Mikata *et al* 2002).

1.2.4. Current status of gene therapy in prostate cancer

Prodrug activation gene therapy for prostate cancer has been investigated in several clinical trials, using either the thymidine kinase (TK) or cytosine deaminase (CD) genes. Treatment of recurring nonmetastatic disease with the adenovirus-TK approach and subsequent prodrug administration caused sustained serum PSA decreases, from six weeks to over a year, in three of eighteen patients in a Phase I trial, though one grade 4 thrombocytopenia was observed at the highest dose (Herman *et al* 1999). In a Phase I/II trial, TK gene therapy was performed in combination with radiotherapy (Teh *et al* 2001). Another Phase I/II trial of TK therapy in patients with local recurrence following radiotherapy achieved partial PSA reduction in 78% of patients (Miles *et al* 2001). Most interestingly, a similar cohort of patients was treated with a replication-competent adenovirus containing a CD-TK fusion gene (Freytag *et al* 2002a). In this Phase I trial, no dose-limiting toxicities were observed, and two of 18 patients showed no evidence of prostate cancer a year after treatment. The CD/5-fluorouracil system alone has also been shown to be effective against prostate cancer in a mouse model (Yoshimura *et al* 2002). A recent Phase I clinical trial treated eleven patients with metastatic or locally recurrent prostate cancer with the TK gene, combined with the prodrug valacyclovir (Kubo *et al* 2003). The adenoviral vector, in which the TK gene was controlled by an osteocalcin promoter, was injected into individual lesions, resulting in temporary disease stabilisation in one patient and toxicity ranging from grade 1 to grade 3 (Kubo *et al* 2003).

Freytag *et al*, using a mouse xenograft system, have assessed trimodal therapy for prostate cancer. The three modes consisted of a replication-competent adenovirus vector, a CD-TK fusion gene and prodrugs activated by both of the enzymes it encodes (see section 1.3 for the prodrug activation strategy), and radiation treatment (Freytag *et al* 2002b). They found that trimodal therapy (Figure 1.1) improved the cure rate and

decreased the incidence of metastases, although they noted that virus combined with external beam radiation treatment was associated with mild but synergistic toxicity. Patients with newly diagnosed nonmetastatic prostate cancer have been treated with trimodal therapy in a Phase I clinical trial, resulting in a maximum of Grade 3 toxicity (11 events) among the 16 subjects (Freytag *et al* 2003).

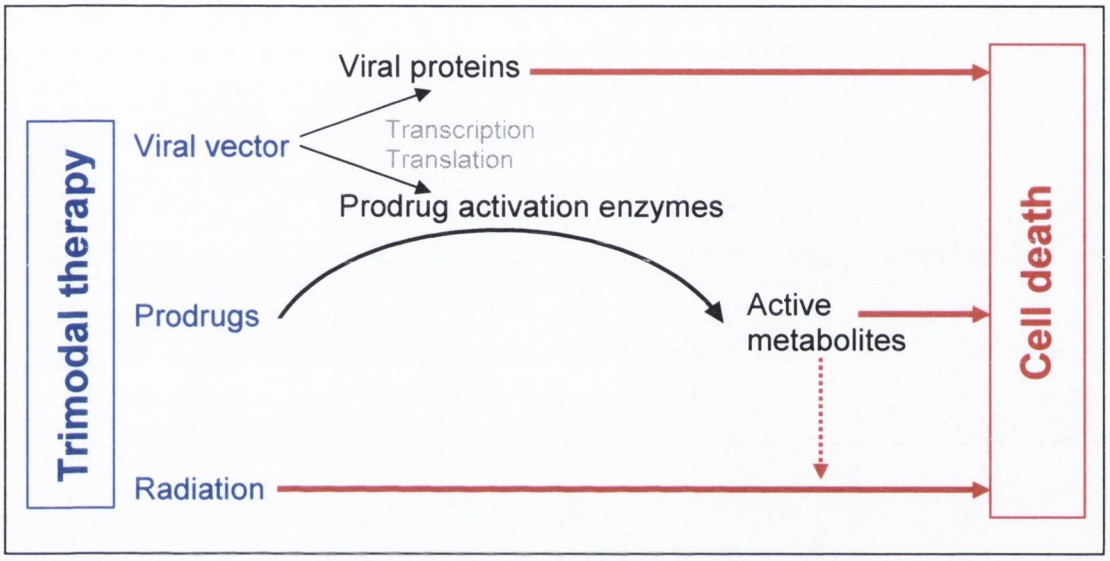


Figure 1.1. Trimodal therapy for prostate cancer. Subjects are treated with three agents: (A) an attenuated replication-competent viral vector, (B) prodrugs activated by the CD and TK enzymes inserted in the vector, and (C) radiation. Cell death is therefore induced by three pathways: (A) viral replication resulting in cell lysis, (B) drug-related toxicity and (C) radiation damage. Furthermore, cells are radiosensitised by both the CD and TK prodrug activation systems (dotted arrow).

In addition to prodrug activation gene therapy studies, a variety of other means of manipulating cell cycle control and apoptosis have been investigated. An ARCA (see Section 1.2.3) has achieved a dose-dependent serum PSA reduction in a Phase I clinical trial (DeWeese *et al* 2001). Tumour regression has been observed in mice with skeletal prostate cancer xenografts, following treatment with an ARCA controlled by an osteocalcin promoter, which cotargets tumour and bone stroma (Matsubara *et al* 2001). Survival of mice with LNCaP xenografts has been promonged by treatment with a prostate-specific inducible caspase 9 gene (Xie *et al* 2001a). The diphtheria toxin A (Peng *et al* 2002) and Fas ligand (Nakanishi *et al* 2003) genes have brought about growth inhibition in prostate cancer xenografts in mice and merit further study as therapeutic agents.

The therapeutic potential of several tumour suppressor genes has been investigated. The tumour suppressor gene BRCA1 has been administered to advanced prostate cancer patients in a Phase I clinical trial, by intraprostatic injection of retrovirus, with no significant toxicity (Steiner *et al* 1998b). p53 and p21 transgene expression reduced the growth and tumorigenicity of androgen-independent human prostate cancer cells *in vivo* (Yang *et al* 1995, Gotoh *et al* 2003).

Antisense approaches have also shown promise. A phase I/II clinical trial is currently underway to ascertain the combined efficacy and safety of bcl-2 antisense oligonucleotide and docetaxel in progressive metastatic prostate cancer. Four of twelve patients showed decreases in serum PSA, though one patient experienced dose-limiting toxicity (Tolcher 2001). In mouse model studies, antisense to c-myc has been shown to bring about prostate tumour regression (Steiner *et al* 1998a), while antisense targeted to the androgen receptor has inhibited tumour growth (Eder *et al* 2002). AR antisense inhibits the androgen-independent cross-activation of the AR by IGF-I *in vitro* (Hamy *et al* 2003).

Various prostate cancer gene therapy strategies which target the immune system have also been explored. A PSA vaccine delivered by a modified vaccinia virus has shown low toxicity and varying degrees of efficacy against advanced prostate cancer in three Phase I clinical trials (Sanda *et al* 1999, Eder *et al* 2000, Gulley *et al* 2002), following promising studies in animals (Kim *et al* 1998). Treatment by PSA vaccination combined with GM-CSF protein brought about disease stabilisation for up to 25 months (Sanda *et al* 1999), and PSA-specific immune responses (Eder *et al* 2000, Gulley *et al* 2002), in some patients. Three further prostate cancer Phase I clinical trials have involved GM-CSF or interleukin-2 gene therapy, and have demonstrated lymphocyte responses to tumour cells (Simons *et al* 1999, Belldegrun *et al* 2001, Trudel *et al* 2003). In a mouse model of prostate cancer, a gene encoding a modified TGF-β receptor improved survival by overcoming suppression of bone marrow immune function (Shah *et al* 2002).

Finally, genetic strategies have shown inhibition of angiogenesis in preclinical models of prostate cancer, by introducing the anti-angiogenic gene thrombospondin-1 (Jin *et al* 2000), and antisense oligonucleotides targeted against VEGF (Nguyen *et al* 1998) and matrix metalloproteinase 9 (London *et al* 2003).

1.3. THE PRODRUG ACTIVATION STRATEGY

1.3.1. Introduction

The prodrug activation strategy (also known as the "suicide gene" or genetic prodrug activation therapy (GPAT) strategy) involves the introduction of a gene for a drug metabolising enzyme (DME) into target cells, such that these cells can activate a subsequently administered non-toxic prodrug, converting it to its toxic form. Only cells transfected with the appropriate DME gene, therefore (and "bystander" cells discussed below), are killed by the administration of the prodrug. This is illustrated in Figure 1.2.

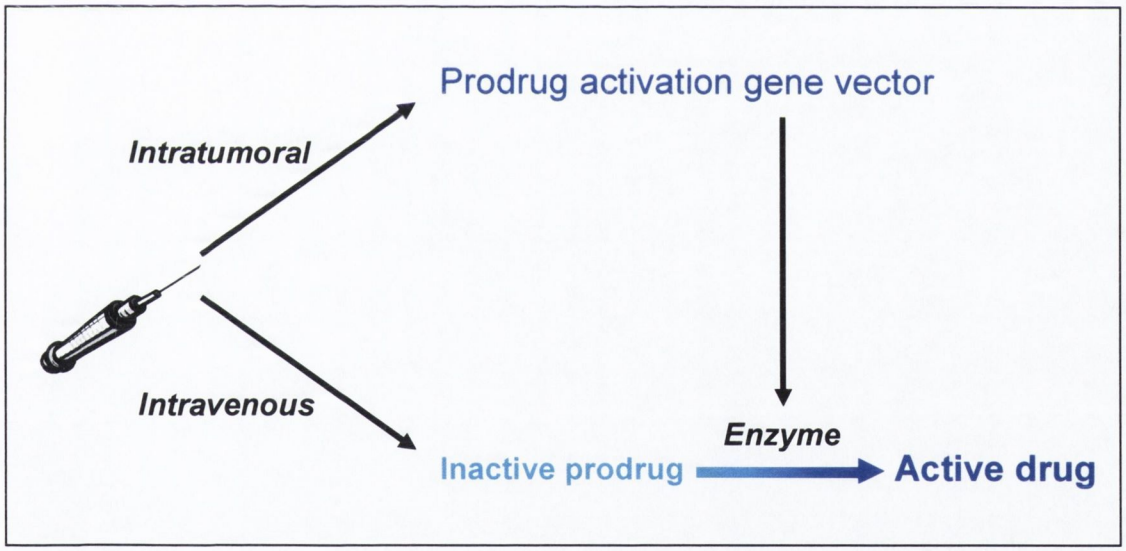


Figure 1.2. The prodrug activation strategy. This approach aims to kill tumour cells by combined treatment with a prodrug and a therapeutic gene. A prodrug is administered intravenously, but requires a specific prodrug activation enzyme to convert it to an actively cytotoxic derivative. This enzyme is produced in the tumour following intratumoral injection of a prodrug activation gene.

This concept has been extensively tested in the thymidine kinase DME/ganciclovir prodrug system, in which the thymidine kinase (TK) gene from herpes simplex virus type I converts ganciclovir (or acyclovir) to its monophosphate form. This can then be further

phosphorylated by cellular factors, and in its triphosphate form inhibits DNA replication, causing cell death. An early study using this system showed complete regression of rat glioma tumours when retrovirally transduced with TK and treated with prodrug (Culver *et al* 1992). This combination has been widely used in prostate and other cancers, and is the subject of two prostate cancer clinical trials (Herman *et al* 1999, Teh *et al* 2001).

The cytosine deaminase/5-fluorouracil system selected for this project has also been well characterised, and is an attractive choice as a therapeutic strategy for a number of reasons (Section 1.3.2). Numerous other enzyme/prodrug combinations have been assessed *in vitro* and *in vivo*, and the most promising systems are summarised in Table 1.2.

1.3.2. The cytosine deaminase/5-fluorouracil prodrug activation system

The *Escherichia coli* cytosine deaminase (CD) gene was cloned and sequenced by Austin and Huber (1993, Genbank accession number S56903). It was demonstrated to be, in combination with 5-fluorocytosine (5-FC), capable of inducing cell death in malignant mammalian cell lines (Mullen *et al* 1992). CD converts the nontoxic prodrug 5-fluorocytosine to 5-fluorouracil (5-FU), which is phosphorylated by cellular enzymes to 5-fluorouridine-5'-triphosphate and 5-fluoro-2'-deoxyuridine 5'-monophosphate, resulting in inhibition of RNA and DNA synthesis and cell death. Most studies of the CD/5-FC system, to date, have used the bacterial cytosine deaminase gene, though a CD gene from *Saccharomyces cerevisiae* has been cloned (Erbs *et al* 1997) and shown recently to have higher activity than the bacterial gene (Kievit *et al* 2000). Though CD has generally been used as a single therapeutic gene, a fusion gene has been designed to encode CD and uracil phosphoribosyltransferase (UPRT), and has shown *in vivo* efficacy (Erbs *et al* 2000). UPRT phosphorylates 5-fluorouracil and thus enhances its metabolism (Erbs *et al* 2000).

Prodrug activation gene	*Prodrug*	*Mechanism*	*Comments*	*Reference*
Nitroreductase (NTR) (*Escherichia coli*)	CB1954	Alkylation	Complete regression of large liver tumours in mice Strong bystander effect	Djeha *et al* 2000
Methionine α,γ lyase (*Pseudomonas putida*)	Seleno-methionine	Reactive oxygen species production	Survival increase in rat hepatoma Strong bystander effect	Miki *et al* 2001
Guanine phosphoribosyl transferase (gpt) (*E. coli*)	6-thioxanthine or 6-thioguanine	Inhibition of purine metabolism	Glioma tumour volume reduced in nude mice	Ono *et al* 1997
Purine nucleoside phosphorylase (PNP) (*E. coli*)	6-methylpurine deoxyribose	Inhibition of RNA, DNA and protein synthesis	Growth suppression of prostate tumours in mice Strong bystander effect	Martiniello-Wilks *et al* 2002
Carboxypeptidase G2 (*Pseudomonas*)	Mustard prodrugs	Alkylation	No cellular enzymes required for toxicity Survival increase in mice with breast tumours	Friedlos *et al* 2002
Carboxylesterase (Rabbit)	CPT-11	Inhibition of topoisomerase I	Survival increase in mice with cervical tumours	Stubdal *et al* 2003

Table 1.2. Other prodrug activation systems. Prodrug activation genes with *in vivo* efficacy are derived from a variety of sources and work by several different mechanisms.

The bystander effect is a characteristic feature of the prodrug activation approach, in which not only cells transfected with the therapeutic gene but also nontransfected neighbouring cells are killed on administration of the prodrug. This is a particularly useful property *in vivo*, where high transfection efficiencies can be difficult to achieve. The CD system exhibits a strong bystander effect. In a rat glioma model *in vitro*, transduction of 10% of cells with CD was as potent as 100% transduction in inhibiting proliferation (Ichikawa *et al* 2000). Regression of colorectal tumours in nude mice occurred when even 4% of tumour cells were transduced with CD (Huber *et al* 1994).

Some prodrug activation systems such as TK (Denning & Pitts 1997) require intracellular gap junctions for an efficient bystander effect, but this is not the case for CD. In mouse xenografts of WiDr colorectal tumours, which do not contain gap junctions, 4% transduction with CD brought about complete regression in three of five animals, an efficacy not obtained with TK unless 50% or more of the tumour cells were transfected (Trinh *et al* 1995). Greater efficiency and bystander effect in CD compared to TK has also been documented in lung adenocarcinoma (Hoganson *et al* 1996). 5-FU appears to freely diffuse into nontransfected cells from the medium conditioned by CD-transfected cells (Ichikawa *et al* 2000). Since this could also affect nonmalignant cells, it is important that DMEs should be used along with intratumoral injection and/or tissue-specific promoters to minimise damage to normal tissues.

CD/5-FC, like TK/ganciclovir (Vile *et al* 1994), can activate an immune response not only against transfected tumour cells, but also against nontransfected parental cells. When liver metastases of CD-transfected colon cancer regressed in rats treated with 5-FC, natural killer (NK) cells were shown to be recruited to the tumour in a 5-FU dependent manner, and wild-type cancer cells subsequently injected in the other liver lobe failed to grow (Pierrefite-Carle *et al* 1999).

Colorectal cancer (CRC) is an obvious setting in which to employ CD/5-FC as anticancer agents, as 5-FU is the drug of choice in the treatment of metastatic CRC (Vaughn & Haller 1993). The CD system was shown to have potent antitumour activity in CRC

(Huber *et al* 1993, Huber *et al* 1994), in addition to efficacy in prostate (Anello *et al* 2000, O'Keefe *et al* 2000, Uchida *et al* 2001, Yoshimura *et al* 2002), breast (Wolff *et al* 1998, Pandha *et al* 1999), glioma (Ichikawa *et al* 2000) and other cancers. The production of 5-FU by this prodrug activation system has been shown to sensitise tumours to radiation, as discussed later (Section 7.4.3). It is interesting to note that hypoxia, which has been demonstrated in human prostate tumours (Cvetkovic *et al* 2001), enhances the toxicity of CD/5-FC *in vitro* (Dachs *et al* 1997).

1.4. ACHIEVING TISSUE-SPECIFIC GENE EXPRESSION

Several approaches have aimed to achieve tissue-specific expression of the therapeutic gene. In prostate cancer gene therapy, *ex vivo* transfection (Simons *et al* 1999) and intraprostatic injection (Herman *et al* 1999, Teh *et al* 2001, Miles *et al* 2001, DeWeese *et al* 2001, Freytag *et al* 2002a, Trudel *et al* 2003) have been employed for this purpose. However, *ex vivo* transfection is not always practically suitable, and intraprostatic injection has been shown in animal models to allow some vector dissemination to other tissues (Steiner *et al* 1999, Adams *et al* 2002). Alternative strategies involve regulating transgene activity at the levels of transcription or gene delivery. The latter can be achieved by conjugating the vector with a ligand to a target cell receptor; for example, a HER2/*neu* antibody fragment has been used to enhance transfection of breast cancer cells (Meyer *et al* 1998). Tissue-specific gene promoters, such as the tyrosinase promoter which is specifically active in melanoma cells (Vile & Hart 1993), have demonstrated that transcriptional regulation can be a very useful means of restricting expression at effective levels to the target tissue. Much research has focused on developing such promoters for use in prostate cancer gene therapy, including the challenge of achieving high levels of promoter activity without sacrificing specificity. In addition to the PSA promoter discussed below, several other promoters have shown potential for use in prostate cancer gene therapy, and are discussed later (Section 7.6.3). The ability of a promoter to distinguish the target tissue from liver and bladder may be particularly important, since in preclinical murine and canine models, vectors have been noted to accumulate at high levels in the liver (Adams *et al* 2002) and in the bladder (Steiner *et al* 1999) after intraprostatic injection.

1.4.1. Prostate-specific antigen

Prostate-specific antigen (PSA) is the major "tumour-specific" marker of prostate cancer, and is used in the early detection of disease and as a marker of response and relapse. The PSA gene, also known as human kallikrein 3, encodes a 35kDa serine protease which is involved in cleaving seminal vesicle proteins. Serum PSA level measurement plays a vital role as a clinical marker of prostate cancer (Small & Roach 2002). The promoter region has been extensively characterised (Section 1.4.2), and modified by several strategies to optimise its activity (Section 7.2). Its expression is highly prostate-specific (Section 7.6.3), and closely regulated by androgens via the androgen receptor, though recent work has also identified androgen-independent control pathways (Section 1.4.3). Several functional protein binding sites have been described (Section 1.4.4). The promoter has been widely used to direct expression of therapeutic genes in cell lines and in animal models of prostate cancer (Section 1.4.5).

1.4.2. PSA transcriptional control

The PSA 5' regulatory region (Figure 1.3) consists of a proximal promoter and an enhancer over 3kb upstream. It contains six functional androgen response elements (AREs). These are 15bp sequence motifs consisting of two 6bp half-sites and 3bp between them, which bind to the androgen receptor, and are usually a variation on the consensus sequence GGTACAnnnTGTTCT.

The proximal promoter region contains one strong ARE at positions -170 to -156 (ARE-I). It has been confirmed to be active and androgen-inducible by generating deletion mutants (Riegman *et al* 1991, Zhang S *et al* 1997). A second ARE occurs at -393 to -379. This element, ARE-II, is less active than ARE-I in binding AR and in androgen-dependent activation of a basal thymidine kinase promoter (Cleutjens *et al* 1997a). The region surrounding ARE-II is also active in the reverse orientation (Cleutjens *et al* 1996).

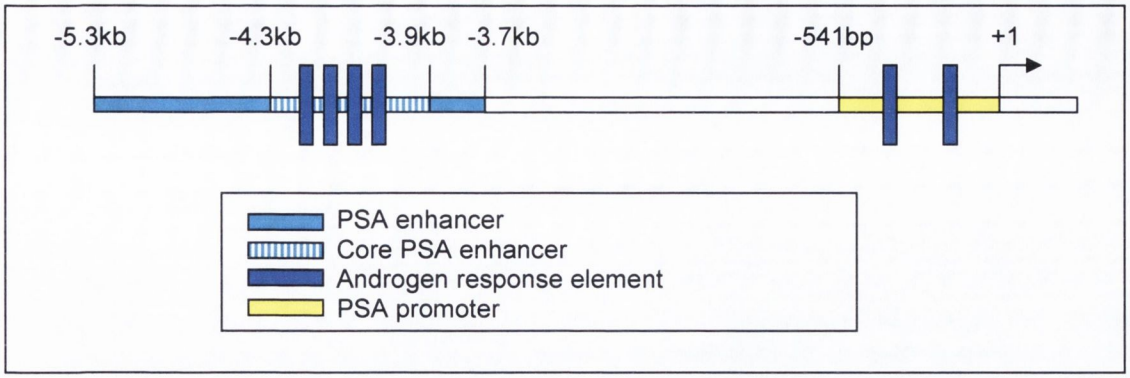


Figure 1.3. Schematic summary of PSA 5' regulatory sequences. The key sequences are the proximal promoter immediately upstream of the transcriptional start site (+1), and the upstream enhancer, particularly the core enhancer sequence, which contains four AREs.

However, a much stronger enhancer is found further upstream, which dramatically increases the proximal promoter efficiency (Cleutjens *et al* 1997a, Zhang S *et al* 1997). It contains four functional androgen response elements (Huang *et al* 1999). Although only the strongest one, ARE-III at positions -4148 to -4134, was initially identified (Cleutjens *et al* 1997a, Zhang S *et al* 1997), it was clear that the region between -5.3kb and -3.7kb contained additional positive regulatory elements, as adding this region conferred higher activity than the proximal promoter alone even when ARE-III was inactivated (Zhang S *et al* 1997). The core enhancer (Figure 1.3), the smallest region with high androgen-inducible expression, was defined by deletion mutations as a 440bp fragment between -4324 and -3884 (Cleutjens *et al* 1997a). Both this enhancer and a 2.2kb fragment encompassing it displayed orientation-independent behaviour (Cleutjens *et al* 1997a), as did a 1.4kb enhancer (-5.3kb to -3.9kb) isolated from a prostate cancer patient with high serum PSA levels (Pang *et al* 1997). An 822bp fragment of this latter enhancer contained nine sequence changes, and in addition to efficient enhancer properties, could function as a weak promoter (Pang *et al* 1997, Iyer *et al* 2001).

Deletion studies revealed ARE-III to be a crucial component of androgen-inducible PSA promoter expression (Cleutjens *et al* 1997a, Zhang S *et al* 1997). In a 6kb promoter construct, inactivation of ARE-III reduced expression more than inactivating ARE-I or ARE-II, though ARE-I bound the AR with comparable affinity (Cleutjens *et al* 1997a). Recently, five further putative AREs close to ARE-III were identified by DNaseI

footprinting (Huang *et al* 1999). Three of these (designated IIIa, IV and V) enhance transcription in LNCaP, though two others do not appear to be functionally important (Huang *et al* 1999). Synergy between ARE-III and the neighbouring AREs is strongly suggested both by deletion mutants and by the large DNaseI footprint over the region (Figure 1.5) (Huang *et al* 1999). Co-operativity also occurs between ARE-I, ARE-II and ARE-III (Cleutjens *et al* 1997a). Two classes of ARE are now known.[1] ARE-III and other typical AREs belong to class I, while ARE-IIIa and ARE-V fall into class II (Reid *et al* 2001).

1.4.3. Androgen-dependent regulation of PSA promoter and enhancer

Reductions in PSA expression in LNCaP and patient prostate cancer cells has been noted on hormone withdrawal (Israeli *et al* 1994, Wright *et al* 1996). This is in keeping with the behaviour of the PSA promoter *in vitro*, which is well documented to be induced by androgens. However, reported induction factors vary (Schuur *et al* 1996, Pang *et al* 1997, Cleutjens *et al* 1997a, Rodriguez *et al* 1997), and progress has also been made in elucidating androgen-independent regulation of PSA transcription (discussed in Section 7.6). Among the most androgen-inducible constructs tested was a 6kb native enhancer-promoter which was 3000-fold enhanced by 1nM R1881, an androgen analogue (Cleutjens *et al* 1997a). A 822bp enhancer juxtaposed to a 620bp promoter from a prostate cancer patient was over 1000-fold induced by 10nM dihydrotestosterone (DHT) to be almost as active as the nonspecific cytomegalovirus (CMV) immediate early gene promoter (Cleutjens *et al* 1997a, Pang *et al* 1997).

As would be expected, androgen response elements play a key role in PSA expression. Mutations in ARE-I, ARE-II and ARE-III reduce androgen stimulation to 50%, 20% and dramatically to 1% of that of the wild-type 6kb enhancer-promoter, respectively (Cleutjens *et al* 1997a).

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[1] Class I AREs have the consensus sequence RGAACA-NGN-TGTNCT and do not show hypersensitivity to guanine methylation. Class II AREs have a slightly different consensus sequence, RGGACA-NNA-AGCCAA, and the underlined sites are hypersensitive to guanine methylation, indicating that the local DNA structure is altered by binding AR (Reid *et al* 2001). Although class I AREs have a higher affinity for the AR, it appears that class II AREs also play an important role.

The PSA 5' region contains four functional binding sites for the prostate-specific Ets transcription factor PDEF (Oettgen *et al* 2000). This protein stimulates the activity of a 7kb PSA enhancer-promoter 11-fold in LNCaP in the absence of androgen. However, it can physically interact with the AR, and activated AR and PDEF stimulate PSA promoter activity synergistically (Oettgen *et al* 2000).

1.4.4. Proteins which bind to the PSA promoter and enhancer

DNaseI footprints are seen in the proximal promoter, protecting the TATA box (-16bp to -30bp) and GC box (-45bp to -70bp) (Riegman *et al* 1991). The GC box overlaps with the binding site of a 45kDa protein possibly involved in androgen-independent expression (Yeung *et al* 2000). This protein is not SP-1, contrary to suggestions from DNA sequence analysis, and is more abundant in androgen-independent C4-2 than in androgen-dependent LNCaP (Yeung *et al* 2000). A large DNaseI footprint has been observed at and upstream of ARE-I (Riegman *et al* 1991). ARE-II also has a large protein binding site upstream, which was defined as precisely as possible by deletion studies to a 140bp region and may contain multiple weak binding sites (Sun *et al* 1997). This site binds an AR-associated protein of the fos family, though c-Fos has been excluded (Sun *et al* 1997). Figure 1.4 illustrates these sites.

Numerous functional protein binding sites have now been described in the PSA 5' upstream sequence, particularly in the region around ARE-III, and are illustrated in Figure 1.5. PDEF binds to four sites and acts as an androgen-independent positive regulator of transcription (Oettgen *et al* 2000). PDEF differs to other Ets transcription factors (TFs) in binding preferentially to a nonconsensus Ets binding site (Oettgen *et al* 2000).

Three functional GATA-2 transcription factor binding sites are also present, each consisting of two adjacent or overlapping consensus sites (Perez-Stable *et al* 2000). Interestingly, these binding sites and four AREs are located in the same 0.2kb region around -4.1kb, and two overlaps between AREs and GATA binding sites occur (Figure 1.5). However, in LNCaP extracts in which no functional GATA TFs were found, a

putative novel TF, GAGATA, bound to a 6bp site overlapping two GATA binding sites at –4045bp (Wang *et al* 2003). Mutation of this novel binding site in a reporter plasmid completely abolished DHT-dependent transcription, indicating that it may play an important role. This site does not interact with the AR, supporting the idea that a complex of proteins is involved in transcriptional regulation of androgen-dependent genes (Wang *et al* 2003).

One GATA binding site also corresponds to a DNAseI footprint in LNCaP, PC-3 and U937 cells which displays sequence-specific protein binding activity (Farmer *et al* 2001). In the same study, an 8bp DNAseI footprint was identified just upstream of ARE-III which shows both sequence and tissue specificity. This site forms a protein complex with extracts from PSA-positive but not PSA-negative cell lines, and contains two consensus binding sites for downstream effectors of TGF-β (Farmer *et al* 2001). Though binding by the two effectors could not be confirmed, mutations of the binding sites significantly reduced transcription of a downstream reporter (Farmer *et al* 2001).

Recently, a protein binding site has been identified at –3.9kb, on the border of the core enhancer. Both the AR and the TF NF-κB bind to this site, and it has been suggested that NF-κB activation may be involved in the inhibition of AR-regulated genes in advanced prostate cancer (Cinar *et al* 2004).

In addition, the core enhancer contains a region, around the upstream AREs and GATA binding sites, which is protected by a protein complex in LNCaP but not MCF-7 cells (Schuur *et al* 1996). Numerous lines of evidence therefore show this to be a crucial region for protein regulation of PSA transcription.

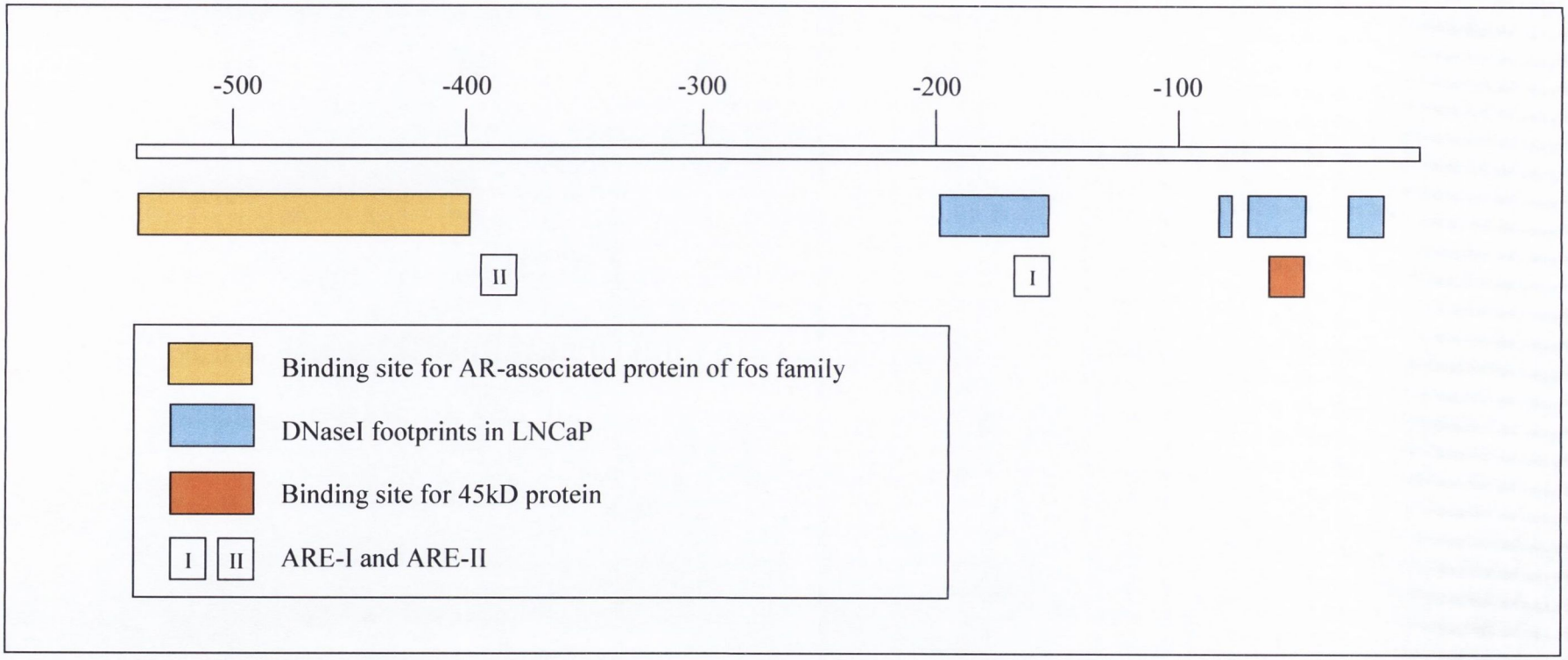


Figure 1.4. Protein binding sites in the proximal PSA promoter. See text (Section 1.4.4) for details and references. Sizes and positions of binding sites and footprints are shown to scale. Distance from the transcription start site is indicated in bp.

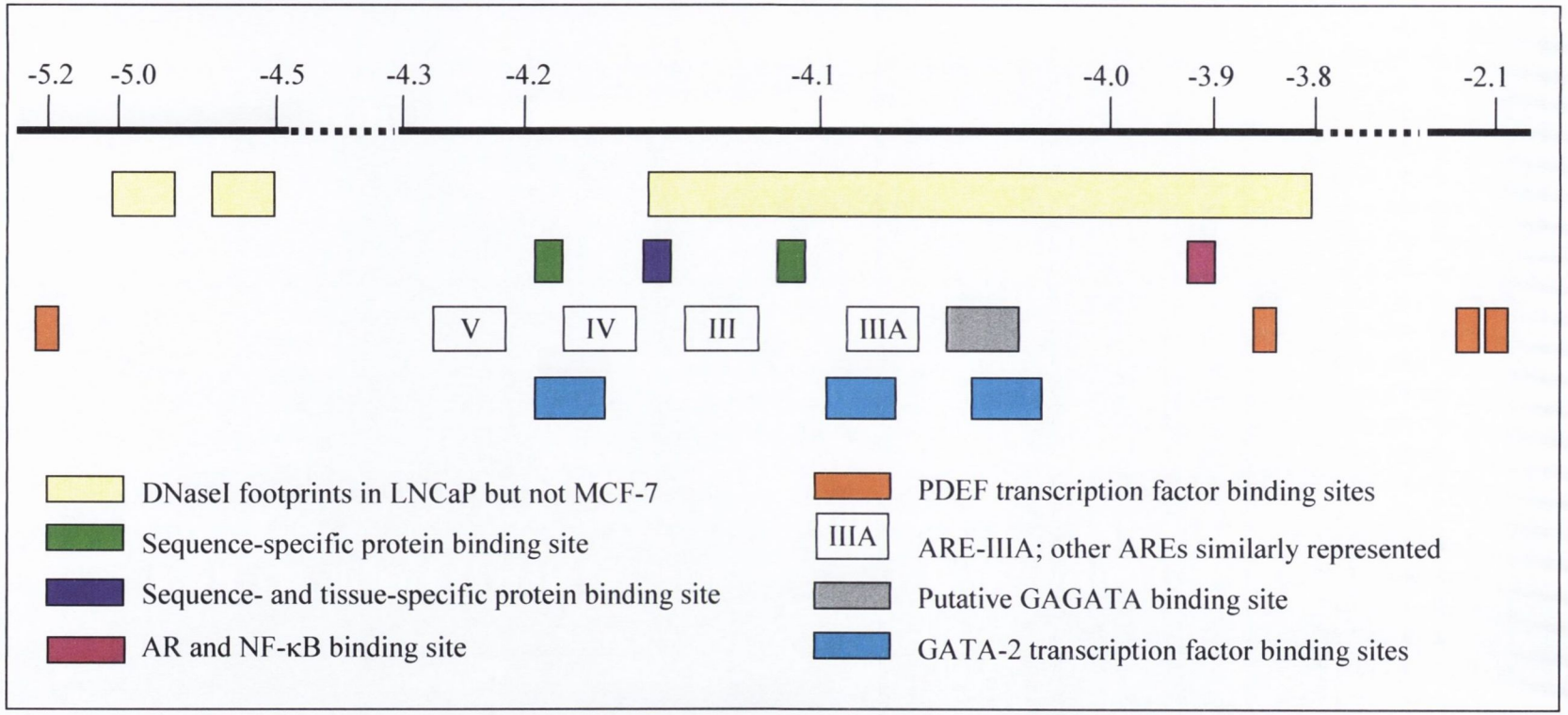


Figure 1.5. Protein binding sites in the PSA upstream enhancer region. Distances from the transcription start site (not to scale) are indicated in kb. Androgen response elements are centred at -4227, -4178, -4141 and -4080bp. GATA-2 binding sites are centred at -4190, -4085 and -4045bp. Sequence-specific protein binding sites (green and purple boxes) are located at [-4195 to -4187], [-4164 to -4156] and [-4116 to -4108]. See text (Section 1.4.4) for discussion and references.

1.4.5. Use of the PSA promoter *in vitro*, *in vivo* and in clinical trials

PSA regulatory sequences have been used in the following viral/vector gene therapy systems to achieve the cytotoxic and/or therapeutic effects. *In vitro*, LNCaP sensitivity to the prodrug activated by the nitroreductase gene was enhanced (Latham *et al* 2000). A prostate cancer patient-derived PSA promoter and enhancer was shown to express diphtheria toxin A in LNCaP cells with 95% toxicity (Pang 2000), and this treatment resulted in tumour regression in mice (Zheng *et al* 2003). Regression of LNCaP tumours *in vivo* has also been brought about by an ARCA (CV706) containing PSA sequences upstream of the adenoviral E1A gene (Rodriguez *et al* 1997), and by PSA promoter-driven expression of sodium iodide symporter, causing the prostate cells to take up radioactive iodine (Spitzweg *et al* 2000). CV706 is currently being tested in a clinical trial (DeWeese *et al* 2001), as is a similar ARCA which also contains rat probasin promoter elements (Yu *et al* 2001b). CV706 treatment brought about over 50% reductions in serum PSA in five of twenty patients with locally recurrent prostate cancer (DeWeese *et al* 2001).

CHAPTER 2: MATERIALS AND METHODS

2.1. CLONING PLASMIDS

2.1.1. PCR of PSA DNA sequence

2.1.1.1. Primer design

PCR primers were designed to amplify two regions of the PSA 5' regulatory region. This strategy was based on published studies by Schuur *et al* (1996) and Rodriguez *et al* (1997). These papers demonstrated that a 1.6kb upstream region between –5322 and –3738, juxtaposed to a proximal promoter region as small as 541bp, was as active as a 5.8kb promoter. Like the parent promoter, the deleted sequence was active in LNCaP cells but not in the PSA-negative DU145 cell line.

Throughout this work, sequence positions in the PSA promoter are described either relative to the transcription start site as negative or positive numbers (e.g. –682 to +16), or as their position in the Genbank PSA promoter sequence, accession number U37672 (e.g. 5655-5669), unless stated otherwise.

Primers PSAP1F and PSAP1R were selected, based on a paper by Zhang S *et al* (1997), to amplify the region between –5316 and –3744. PSAP1F and PSAP1R differed to the published primer in having 8bp and 5bp less PSA sequence respectively, and in having 6bp linker sequence and a *Bgl*II restriction site at their 5' ends. Primers PSAP2F and PSAP2R were generated to amplify the PSA sequence between –682 and +16. PSAP2F was derived from the Genbank PSA promoter sequence and contained 6bp linker and a *Sac*I site at the 5' end. PSAP2R was altered from a primer in Schuur *et al* (1996) to have 4bp more PSA sequence (+12 to +16, derived from the human PSA complete CDS, Genbank accession number M27274, residues 646-649) and a different linker sequence and restriction site (*Sac*I). Oligonucleotides were purchased from MWG Biotech (Germany). Their sequences are detailed in Table 2.1.

PRIMER	SEQUENCE (5' TO 3')
PSAP1F	CGGAATAGATCTAATCTAGCTGATATAGTGTGGC
PSAP1R	CGGAATAGATCTGGATCCTGAGCGCTGCCTTATT
PSAP2F	CGGAATGAGCTCGCACAGATAGAGTGTACAGG
PSAP2R	CGGAATGAGCTCTGGTAAGCTTGGGGCTGGGG

Table 2.1. Primers used for PCR amplification of the human PSA promoter.

2.1.1.2. PCR conditions

An anonymised human genomic DNA sample was used as template. 50µl reactions contained 4ng template, 10X magnesium free PCR buffer (Invitrogen, UK), final concentration of 0.2mM each dNTP (Roche, UK), recombinant Taq DNA polymerase (0.5u) and primers and magnesium chloride as discussed below. Amplification was carried out on a Trio Thermoblock (Biometra), in which case mineral oil was added to prevent evaporation, or on a Peltier PTC-200 (MJ Research). Taq DNA polymerase was added after a hot start at 94°C for 5 minutes.

PSAP1F/PSAP1R

These primers were used at 1µM final concentration. Reactions were optimised for magnesium ion final concentration between 0.5 and 3.5mM, by adding appropriate volumes of $MgCl_2$ (Invitrogen, UK). For these primers, 3.5mM $MgCl_2$ was optimal. Thermocycling conditions were:

94°C, 5 mins

80°C, 10 mins

[94°C, 1 min; 64°C, 1 min; 72°C, 1.5 min] x 30

PSAP2F/PSAP2R

Reactions were optimised for magnesium ion concentration, primer concentration and annealing temperature. Magnesium ion final concentrations between 0.5 and 3.5mM were tested. The PCR products which were finally ligated were produced using 2-3.5mM $MgCl_2$. Final primer concentrations from 0.5μM to 4μM were tested, with 0.5-1μM being sufficient for discrete amplification products. PSAP2F and PSAP2R, with annealing temperatures of 70.8°C and 74.6°C respectively, were annealed at 64 - 68°C, successfully at 64 - 65°C. Amplification was carried out under these conditions:

94°C, 5 mins

80°C, 10 mins

[94°C, 1 min; 64 - 65°C, 1 min; 72°C, 1 min] x 30

2.1.1.3. Purification of PCR products

PCR reactions were electrophoresed on an agarose gel as in Section 2.1.1.5. The product bands were excised from the gel using a sterile scalpel (Swann-Norton, UK). The agarose slices were loaded on GenElute agarose spin columns (Sigma, UK) and processed as recommended by the manufacturer. The eluted DNA was ethanol precipitated as in Section 2.1.1.6.

2.1.1.4. Control PCR for TA cloning

A 50μl control PCR was set up containing 100ng template, 0.1μg each of primers 1 and 2, 10X buffer, and 0.5M each dNTP (all supplied by Invitrogen, UK). 0.5u Taq DNA polymerase were added. PCR was carried out under conditions of 2 minutes at 94°C; (1 minute at 94°C, 1 minute at 55°C, 1 minute at 72°C) x 25; 7 minutes at 72°C.

2.1.1.5. Agarose gel electrophoresis

Agarose (Invitrogen, UK) gels were electrophoresed in TAE buffer, containing 2µl 10mg/ml ethidium bromide (Oncor, UK) per 25ml agarose, using a Horizon 58 gel electrophoresis apparatus (Invitrogen, UK). Samples were loaded with 5X agarose gel loading dye. Size standards (Invitrogen, UK) were also loaded. For DNA fragments longer than 1kb, a 1% agarose gel and the 1kb Plus DNA ladder were used; for shorter DNA fragments, a 2% agarose gel and the 100bp DNA ladder were used.

2.1.1.6. Ethanol precipitation

2.5 volumes of 100% ethanol, one-tenth volume of 3M sodium acetate at pH5.3, and 2µl 20mg/ml glycogen (Roche, UK; optional) were added per 100-400µl DNA sample. Samples were incubated for at least 15 minutes at -20°C or at least 10 minutes at -70°C, and centrifuged at 12,000–15,000g for 15–30 minutes at 4°C, in a Micromax RF centrifuge (IEC, USA). The supernatant was removed and 500µl 70% ethanol added. Samples were centrifuged for 5 minutes at 12,000–15,000g, and supernatant was removed. Pellets were dried for 5–10 minutes at 55°C on a heatblock (Driblock DB-1M, Techne), or for 5 minutes at high heat in a vacuum centrifuge (DNA Speedvac 110, Savant), and resuspended in autoclaved deionised water (adH_2O) or TE (pH 8.0).

2.1.2. Vector and insert preparation

2.1.2.1. Restriction digests

Table 2.2 shows the suppliers and buffers of the restriction enzymes used for preparative digests during the cloning processes. An example of these is the digestion of pCD1 with *Not*I as the first step in producing the insert for pCMV-CD1.10 and pMinusCD1.24. The following components were assembled:

pCD1	2.3µl (7.5µg)
Buffer D	2.5µl
BSA, 1mg/ml (NEB, UK)	2.5µl
AdH_2O	16.2µl
*Not*I, 10u/µl	1.5µl (15u, 2X excess)

The digest was incubated at 37°C for 3 hours before electrophoresing 0.2µl (60ng) on an agarose gel with undigested plasmid and size marker to check for complete digestion.

Enzyme	**Supplier**	**Buffer**
*Bgl*II	Roche, UK	Buffer M
*Dde*I	NEB, UK	Buffer 2
*Eco*RI	Invitrogen, UK	React3
*Eco*RV	Roche, UK	Buffer B
*Hin*dIII	Invitrogen, UK	React2
I-*Ppo*I	Promega, UK	I-*Ppo*I buffer
*Nhe*I	Sigma, UK	Buffer SM
*Not*I	Promega, UK	Buffer D
*Sgf*I	Promega, UK	Buffer M (Roche)
*Xba*I	Invitrogen, UK	React2
*Xho*I	Invitrogen, UK	React2

Table 2.2. Restriction enzymes used, suppliers and buffers.

Where a plasmid was digested with two enzymes which use the same buffer (e.g. pCD1/*Xho*I/*Hin*dIII), this was done sequentially to confirm double rather than single digestion. The digest was electrophoresed on an agarose gel after digestion with the first enzyme. A control digest containing the plasmid and only the second enzyme was performed along with the second digest to ensure correct digestion patterns.

Where a plasmid was digested with two enzymes using different buffers (e.g. pCD1/*Not*I/*Eco*RI), a single digest was diluted fivefold and appropriate amounts of the second buffer and enzyme were added, and the controls above were also performed. NotI was heat-inactivated by incubating at 70°C for 15 minutes before adding a second enzyme (this step was omitted during digestion of pCD1 for pMinusCD1.24 as complete double digestion was demonstrated on an agarose gel without it).

Phenol extraction

To phenol extract, samples were made up to 400µl if necessary with water, and an equal volume of phenol: chloroform: isoamyl alcohol 25:24:1 (Sigma, UK) was added. The mixture was vortexed and centrifuged at 12,000 x g for 5 minutes. The upper aqueous phase was carefully removed and transferred to a clean tube. This process was repeated.

2.1.2.2. Gel isolation of restriction fragments

Following digestion, cloning inserts and some vectors were gel isolated. The band of interest was electrophoresed on an agarose gel and excised. For improved recovery, low melting point agarose (Invitrogen, UK) was used and melted at 70°C for 15 minutes after excision. DNA was extracted using GenElute columns as in Section Section 2.3.1.3, or Wizard DNA purification system (Promega, UK), as recommended by the manufacturers. After GenElute extractions, DNA was ethanol precipitated.

2.1.2.3. Calf intestinal phosphatase treatment

Vector backbones cut with a single enzyme were treated with calf intestinal phosphatase (CIP) (Invitrogen, UK) to prevent self-ligation. 10X reaction buffer and an appropriate amount of 1u/μl CIP were added to the DNA. The units of CIP required were calculated as (0.072 x μg linearised plasmid/plasmid length in kb), assuming 0.08pmol 5' phosphates per 100ng linear 4.5kb plasmid, and 0.02u CIP per pmol of protruding 5' ends. This was double the manufacturer's recommendation, since a significant number of colonies grew from cells transformed with CIP-treated vector only. The reaction was incubated at 37°C for 30 minutes, followed by phenol extraction to remove CIP, and ethanol precipitation.

2.1.2.4. Blunt ending with T4 DNA polymerase

After digestion with I-*Ppo*I and *Bgl*II, pALTER-MAX was treated with T4 DNA polymerase to generate blunt ends which would allow self-ligation. The following reaction was incubated at 11°C for 20 minutes.

5X buffer (Roche, UK)	5μl
T4 DNA polymerase, >1u/μl (Roche, UK)	5μl
pALTER-MAX/I-*Ppo*I/*Bgl*II	9.5μl (1μg)
each dNTP, 2.5mM (Roche, UK)	1μl
adH_2O	1.5μl

The reaction was phenol extracted and ethanol precipitated before ligation.

2.1.2.5. Summary of vector and insert preparation

Table 2.3 shows how individual vectors and inserts were prepared.

Plasmid	Vector preparation	Insert preparation
pPSA-CD1.8	pCD1 *Xho*I, *Hin*dIII	pPSA2.5 *Xho*I, *Hin*dIII Gel isolation (0.7kb), GenElute Ethanol precipitation
pPSA-CD4.19	pPSA-CD1.8 *Bgl*II Gel isolation (5.2kb), GenElute Ethanol precipitation CIP, phenol extraction Ethanol precipitation	pPSA1.3 *Bgl*II Gel isolation (1.6kb), GenElute Ethanol precipitation
pPPE-CD2.15	pAMdPE1.1 *Not*I, heat-inactivation CIP, phenol extraction Ethanol precipitation	pPSA-CD4.19 *Not*I Gel isolation (3.9kb), GenElute Ethanol precipitation
pCMV-CD1.10	pALTER-MAX *Not*I, heat-inactivation *Eco*RI Wizard	pCD1 *Not*I, heat-inactivation *Eco*RI Gel isolation (1.5kb), Wizard
pMinus-CD1.24	pAMdPE1.1 *Not*I, heat-inactivation *Eco*RI Wizard	pCD1 *Not*I, *Eco*RI Gel isolation (1.5kb), Wizard

Table 2.3. Preparation of individual vectors and inserts for cloning. pPSA-CD1.8 was gel-isolated due to contamination with pCD1 from which it was derived.

2.1.3. Ligations

2.1.3.1. TA ligations

Reactions contained 10ng pCR2.1-TOPO vector (Invitrogen, UK), PCR products as below, and sterile water to 5µl. 60ng PSAP1F/R or 10ng PSAP2F/R gel-isolated PCR products, or 1µl control PCR product, was added to the vector.

Reactions were incubated for 5 minutes at room temperature. After being centrifuged briefly, the reactions were placed on ice and transformed into TOP10 *Escherichia coli* cells.

2.1.3.2. Cohesive end ligations

15µl reactions were assembled containing vector backbone and insert as below, 1mM rATP, 10X buffer and 2u T4 DNA ligase (all supplied by Stratagene, UK). Reactions were set up with 100ng vector, and insert at molar ratios of 1:1, 1:5 and 1:10 of vector: insert, or no insert. Where CIP was used, 100ng vector treated as for vector plus insert ligations, except without CIP digestion, was ligated as a control. A positive control for ligation efficiency was included in each series: either *Hin*dIII-cut pUC18 (Stratagene, UK), or the vector backbone linearised with one restriction enzyme. Ligations were incubated at 16°C overnight (16 hours).

2.1.3.3. Blunt end ligations

To compensate for the inefficiency of blunt end ligations, more ligase was used and PEG-6000 was included to increase the effective concentration of DNA. The reaction mix was composed of 100ng plasmid (restriction-digested and treated with T4 DNA polymerase), 1mM rATP, 10X buffer (Roche, UK), 15% PEG-6000, and 15u T4 DNA ligase (Roche, UK). 100ng *Hin*dIII-cut pUC18 was ligated as a positive control. The ligations were incubated at 16°C for 16 hours, followed by 65°C for 10 minutes to terminate the reactions.

2.1.4. Transformation into *E. coli*

2.1.4.1. Transformation into TOP10 cells

50µl chemically competent TOP10 cells (Invitrogen, UK) were thawed on ice and transferred to 1.5ml polypropylene eppendorf tubes (Anachem, UK). Initially 2µl 0.5M β-mercaptoethanol was added until Invitrogen recommended that it was no longer necessary. DNA was added and mixed by stirring or flicking the tube gently. 2µl TA ligation reaction, 5µl cohesive end ligation reaction, 10pg supercoiled pUC18 (Invitrogen, UK) as a positive control, or no DNA as a negative control, was added. The reactions were incubated on ice for half an hour, then heat-shocked at 42°C for 30 seconds in a Type JB1 water bath (Grant Instruments, UK). 250µl SOC (at room temperature) was added. The cells were shaken horizontally at 37°C, 150rpm, for 30 minutes during TA cloning, and 1h otherwise.

Suitable volumes of cells were plated on LB agar (preheated to 37°C) in 85mm petri dishes (Bibby Sterilin, UK) using a glass spreader sterilised by dipping in ethanol and flaming in a bunsen burner. LB agar was supplemented as appropriate with 50µg/ml ampicillin or 34µg/ml chloramphenicol, and during TA cloning with 1mg/ml 5-bromo-4-chloro-3-indolyl β–D-galactopyranoside (X-Gal; Sigma, UK). For TA cloning, 50µl and 10µl were plated from each transformation reaction. Otherwise, 150µl and 30µl were plated from each reaction, except those containing supercoiled plasmid (10µl only) and no DNA (150µl only). Where 10µl cells were plated, this was premixed with 20µl SOC or LB broth for ease of spreading. Plates were incubated overnight at 37°C.

2.1.4.2. Transformation into JM109 cells

This was performed using a protocol similar to TOP10 transformation. DNA (<10µl) was added to 100µl chemically competent JM109 cells (Promega, UK) and mixed. A maximum of 50ng DNA was added, with the same amount of vector DNA in all reactions (27 – 39ng). In addition to ligations and ligation controls, cells were transformed with supercoiled parent vector and no DNA as transformation controls.

Reactions were incubated on ice for 10 min before heat-shocking at 42°C for 45–50s and incubating on ice for 2 min. 900µl SOC (4°C) was added and cells were shaken horizontally at 37°C, 150rpm, for 1h. For each transformation reaction, 100µl cells, undiluted and diluted 1:10 in LB broth (except cells transformed with no DNA, from which 100µl undiluted cells were plated), were spread on LB agar in 85mm petri dishes and grown overnight.

2.1.5. Plasmid DNA preps

2.1.5.1. Bacterial cultures

LB broth and LB agar bacterial cultures for all bacterial procedures were supplemented with 50µg/ml ampicillin (Sigma, UK), 34-170µg/ml chloramphenicol (Sigma, UK) or 50µg/ml kanamycin (Sigma, UK) depending on which antibiotic resistance gene was expressed by the plasmid (see Appendix 3). LB agar in 85mm plates was inoculated with bacteria either from transformation reactions (Section 2.1.4) or from glycerol stocks (Section 2.1.6). Plates were incubated overnight (12-16h), inverted, in a 37°C incubator (Laboratory Thermal Equipment, UK). LB broth cultures were shaken at 37°C, 150rpm, in a model G25 shaker incubator (New Brunswick Scientific Co., New Jersey).

2.1.5.2. Minipreps

5ml overnight cultures of LB broth in 50ml screw cap tubes (Sarstedt, Germany) were inoculated with single colonies using a metal inoculating loop sterilised in a bunsen flame. These were shaken overnight at 150rpm, 37°C.

3ml culture was centrifuged in an eppendorf tube at 12,000g, 4°C, for 30s, and the supernatant was removed. The pellet was dried in a vacuum centrifuge for 5 minutes at high heat and resuspended by pipetting or vortexing in 200µl ice-cold Solution I. 400µl Solution II (freshly made up) was added, mixed by inversion, and incubated on ice for 5 minutes. 300µl ice-cold Solution III was added, mixed by vortexing or

inversion, and incubated on ice for 5 minutes. Samples were centrifuged at 12,000g for 15 minutes at 4°C to pellet the cell debris, and 600µl supernatant was transferred to a new tube. (See Appendix 1 for details of solutions.)

600µl phenol: chloroform: isoamyl alcohol (25:24:1) was added and vortexed, then centrifuged at 12,000g for 5 minutes. 500µl of the upper phase was carefully transferred to a new tube. Phenol extraction was repeated, removing 400µl supernatant. The samples were ethanol precipitated and resuspended in 50µl adH_2O or TE (pH 8.0). (If cultures had not grown to log phase, samples were resuspended in 10-30µl to compensate.) DNase-free RNase (Roche, UK) was added to 5µg/ml and samples were incubated at 37°C for 30 minutes in a water bath or on a thermocycler. 1µl was electrophoresed on a 1% agarose gel and minipreps were screened to check for presence of plasmids of the correct size.

2.1.5.3. Rapid DNA isolation from colonies (cracking colonies)

Colonies grown overnight were transferred both to preheated new LB agar plates and to eppendorf tubes, using sterile pipette tips. The plates were incubated at 37°C while DNA was prepared. 20-25µl 10mM EDTA at pH 8.0 (Sigma, UK) and an equal volume of 2X cracking buffer (freshly made up) were added, vortexing after each addition. Samples were incubated at 70°C for 5 minutes in a water bath, and cooled to room temperature for 15 minutes. 0.6-0.75µl 4M KCl (Sigma, UK) and 0.5µl 0.4% bromophenol blue (Sigma, UK) were added and vortexed. (Volumes of EDTA, cracking buffer and KCl were reduced proportionally from 50µl and 1.5µl in the original protocol due to low yields.) Samples were incubated on ice for 5 minutes and centrifuged for 3 minutes at 12,000g, 4°C. 10-15µl supernatant was electrophoresed on a 0.7% agarose gel and compared with the parent plasmid. For clones which contained the desired insert, overnight 5ml LB broth cultures were set up from the corresponding plate as for standard minipreps.

2.1.5.4. Sequencing grade plasmid minipreps

LB broth cultures were inoculated from colonies and grown overnight. 3ml culture was processed using the Qiaprep Spin Midiprep kit (Qiagen, UK) as recommended by the manufacturer, including the optional wash step and centrifuging for the longest recommended times. DNA was eluted in 50µl adH_2O after a 5 minute incubation.

2.1.5.5. Birnboin plasmid maxiprep

A 2-5ml LB broth starter culture was inoculated from a freshly streaked LB agar plate, and grown through the day (7-10h). 0.5ml starter culture was inoculated into 25ml LB broth culture in a conical flask or 200ml bottle, and shaken overnight at 37°C.

20ml culture was transferred to a 28ml polyallomer centrifuge tube and pelleted at 5,000rpm, 4°C, for 5 minutes. The pellet was resuspended in 10ml ice-cold STE, centrifuged as before, and resuspended in 3ml ice-cold Solution I. After incubating for 5 minutes at room temperature, 6ml Solution II was added, mixed gently, and incubated for 5 minutes on ice. 4.5ml Solution III was added, mixed gently, and incubated for a further 5 minutes on ice.

The cell debris was pelleted by centrifuging for 30 minutes at 4°C and 10,000rpm. The supernatant (about 12ml) was transferred to a clean 28ml tube, mixed with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and centrifuged for 10 minutes at 4°C and 5,000rpm. The aqueous phase was transferred to two clean 28ml tubes.

After adding two volumes of 100% ethanol and incubating for 10 minutes at room temperature, samples were centrifuged for 5 minutes at 10,000rpm. The supernatant was replaced with 3ml 70% ethanol and samples were centrifuged for for 5 minutes at 10,000rpm. The supernatant was removed and the pellet was air-dried and resuspended thoroughly in 250µl TE (pH 8.0).

For each prep, DNA was re-pooled in an eppendorf tube, and incubated at 37°C for 30 minutes with 5µg/ml DNase-free RNase (Roche, UK). DNA was then precipitated by adding 300µl 20% PEG-6000/2.5M NaCl (PEG-6000 supplied by BDH, UK; NaCl by Sigma, UK), incubating for 1h on ice, and centrifuging for 10 minutes at 10,000rpm. The resulting pellet was washed with 500µl 70% ethanol, centrifuged for 5 minutes at 10,000rpm, and dried for 5 minutes at high heat in a vacuum centrifuge. The pellet was finally dissolved in 50µl TE (pH 8.0).

2.1.5.6. Preparation of transfection grade DNA

2ml LB broth starter cultures were inoculated from colonies on LB agar plates and shaken through the day (7-10h) at 37°C, 150rpm. Starter cultures were diluted 1/500 or 1/1000 into 10-40ml LB broth cultures in 200ml glass bottles (Schott, Germany) and shaken overnight at 37°C, 150rpm.

Cells were pelleted at 5,000 x g for 10 minutes in 28ml polyallomer centrifuge tubes (Nunc, Denmark), using a GS-15R centrifuge (Beckman). DNA was prepared using the GenElute Endotoxin-free Plasmid Midiprep kit (Sigma, UK). Pellets were resuspended thoroughly in 1.2ml resuspension solution. 1.2ml lysis solution was added, mixed by inversion and incubated at room temperature for up to 5 minutes until the solution was clear and viscous. 0.8ml neutralisation solution was added and mixed by inversion. The cell debris was pelleted by centrifuging for 15 minutes at 4°C, 15,000 x g. This was repeated after transferring the supernatant to a clean 28ml tube.

The supernatant was mixed with 300µl endotoxin removal solution in a 15ml conical tube (Sarstedt, Germany), and incubated on ice for 5 minutes, mixing twice. After incubating for 5 minutes in a 37°C water bath, samples were centrifuged at 3700rpm in an Allegra 6KR centrifuge (Beckman, UK). The upper phase was transferred to a clean 15ml tube. Endotoxin removal was repeated.

0.8ml DNA binding solution was added and mixed by inversion. Samples were loaded in GenElute columns and centrifuged at 3700rpm for 2 minutes. 2ml optional wash solution was added to the column, and this spin was repeated. After addition of 3ml wash solution, columns were centrifuged at 3700rpm for 5 minutes. DNA was eluted in 0.8-1ml endotoxin free water at 3700rpm for 5 minutes.

Alternatively, transfection grade plasmid DNA was isolated using Wizard PureFection plasmid DNA purification system (Promega, UK), as recommended by the supplier, using ammonium acetate supplied by Sigma and ethanol from Merck (Germany), and resuspending in 1ml ultrapure water.

2.1.6. Making glycerol stocks of transformed bacteria

0.85ml broth culture of *E. coli* transformed with the desired plasmid was mixed on ice with 0.15ml glycerol (Sigma, UK). The mixture was vortexed and stored immediately at -70°C. To grow cultures for preparation of more DNA, glycerol stocks were streaked on LB agar using a flame-sterilised metal inoculating loop, and the resulting colonies were inoculated in LB broth as in Section 2.1.5.

2.2. MUTAGENESIS

2.2.1. Primers

Mutagenic primers were selected to meet the criteria in Section 2.3.3.1, and to have at least 8 bases on each side of the mismatch annealing to the target plasmid pPPE-CD2.15. Primer mut158G was designed to change A to G at –158 in the PSA promoter sequence, and extends from Genbank positions 5676 to 5659 (reverse sequence). This alteration fortuitously restores an *Nhe*I restriction site, which facilitated screening for successful mutagenesis. Mut155G changes A to G at position –155 in the PSA promoter sequence, and extends from residues 5679 to 5656. These primers were obtained from Sigma-Genosys (UK), phosphorylated at the 5' end (as were the other primers used during mutagenesis).

The pPPE-CD2.15 sequence contains an inactive ampicillin resistance gene, which can be made functional by mutagenesis using an ampicillin repair primer (Promega, UK). By annealing the plasmid with this selectable primer and an excess of mutagenic primer, ampicillin could be used to select colonies likely to contain both changes.

As a positive control, a mutagenic primer which inactivates the LacZ gene, and a selectable primer that renders the ampicillin resistance gene also resistant to an antibiotic selection mix, were annealed to the pGEM plasmid (all supplied by Promega, UK).

Sequences of all oligonucleotides used during mutagenesis are in Table 2.4.

Primer	**Sequence (5' to 3')**	**Functional effect**
Mut158G	GAG AGC TAG **C**AC TTG CTG	Increase in PSA promoter activity?
Mut155G	AGG GAG AGC **C**AG TAC TTG CTG TTC	Increase in PSA promoter activity?
Ampicillin Repair Primer	GTT GCC ATT GCT GCA GGC ATC GTG GTG	Resistance to ampicillin
LacZ Bottom Primer	GGG TAA CGC CAG GGT TAT TCC CAG TCA CGA CG	LacZ knocked out
Bottom Select Primer	CCG CGA GAC CCA CCC TTG GAG GCT CCA GAT TTA TC	Resistance to antibiotic cocktail

Table 2.4. Sequences of mutagenic and selectable oligonucleotides used during mutagenesis.

2.2.2. Denaturation reactions

0.5-2pmol plasmid was incubated at room temperature for 5 minutes in 1M NaOH (BDH, UK), 50mM EDTA (20-40µl reaction volume). 1/10 original volume 2M ammonium acetate at pH 4.6 (Sigma, UK), and 3.75 volumes 100% ethanol, were

added. Reactions were incubated at -70°C for 30 minutes and centrifuged at 12,000rpm, 4°C, for 15 minutes. The supernatant was removed, 200µl 70% ethanol was added and samples were centrifuged as before. After removing the supernatant, pellets were dried in a vacuum centrifuge at high heat for 5 minutes and resuspended in 50µl TE (pH 8.0). The denatured plasmids were electrophoresed on a 0.8% agarose gel.

Since the first attempt at mut158G mutagenesis was not successful, this primer was denatured before annealing by heating at 90°C for 5 minutes, then placing on ice, to exclude secondary structure problems.

2.2.3. Annealing and synthesis reactions

Reactions initially contained 0.025pmol denatured plasmid, 0.25pmol selection primer and 1.25pmol mutagenic primer (1:10:50) per 25µl, in addition to 1X annealing buffer (Promega, UK). After improving the yield from the denaturation step, this was adjusted to 0.05pmol plasmid, 0.025pmol selection primer and 1.25pmol mutagenic primer (1:5:25) per 20µl.

Reactions were heated to the temperatures below for 5 minutes on a PTC-200 thermocycler or Minicycler (both MJ Research), and slowly cooled (1.5°C per minute) to 37°C on the Minicycler. Primer mut155G, with an estimated annealing temperature of 67.7°C, was heated in three different reactions to 87.7°C (20°C above its T_m), 75°C (recommended for the ampicillin repair primer), and 81.4°C. Primer mut 158G was heated to 76.3°C (20°C above its T_m) and 75°C. The mutagenesis positive control reaction was heated to 75°C.

Once the reactions were cooled to 37°C, synthesis reactions were set up by adding 3µl 10X synthesis buffer, 1µl T4 DNA ligase (1-3u/µl), 1µl T4 DNA polymerase (5-10u/µl), and sterile water as required per 30µl reaction (all supplied by Promega, UK). Reactions were incubated at 37°C for 90 minutes.

2.2.4. Transformation into *mutS* cells

This was performed in a similar fashion to TOP10 transformation (Section 2.3.4.1). 1.5-3µl synthesis reaction (2-14ng), or 1µl 0.1ng/µl supercoiled pGEM was added to 100µl *E. coli* BMH71-18 *mut*S cells (Promega, UK). Cells were incubated on ice for 10 minutes, heatshocked at 42°C for 45-50s, and immediately replaced on ice for 2 minutes. 0.9ml medium (LB for mutagenesis reactions, SOC for pGEM control) was added, and cells were shaken at 37°C for 1h.

For mutagenesis reactions involving the ampicillin selection primer, 4ml LB broth and ampicillin to 100µg/ml were added. For the positive control mutagenesis reaction, 4ml LB broth and 100µl antibiotic selection mix were added. These cultures were shaken overnight at 37°C in 28ml glass tubes (Lennox, Ireland), and plasmid DNA was prepared the following day as in Section 2.3.5.2.

100µl cells transformed with supercoiled pGEM (positive control for transformation) were spread on each of 2 LB-ampicillin agar plates and incubated overnight at 37°C, for colony counting the following day.

2.2.5. Transformation into JM109 cells

This was performed using a similar protocol to JM109 transformation in Section 2.3.4.2, except in the amount of DNA, and in incubating on ice for 30 minutes instead of 10 minutes. For each mutagenesis reaction, 5ng and 10ng plasmid DNA from *mut*S cells was transformed, with 10ng pGEM as a positive control.

Mutagenesis reactions involving the ampicillin selection primer were plated on LB-ampicillin agar. Positive control mutagenesis reactions were plated on LB agar containing 100µg/ml ampicillin, 80µg/ml X-gal (Promega, UK), 0.5mM IPTG (Sigma, UK) and 150µl antibiotic selection mix per 20ml agar. pGEM-transformed cells were plated on LB-ampicillin agar and LB-ampicillin agar containing 80µg/ml X-gal and 0.5mM IPTG. 100µl and 10µl volumes were plated.

Plasmid DNA preps from the resulting colonies was screened for the correct mutation by digestion with *Nhe*I or sequencing.

2.3. CONFIRMATION OF PLASMID STRUCTURE

2.3.1. PCR

After TA cloning, the resulting plasmids pPSA1.3 and pPSA2.5 (and others generated at the same time) were PCR amplified in 25µl reactions to ensure they contained the correct insert.

Reactions were carried out with the primers used to amplify the inserts, and other PCR materials and conditions as before. pPSA1.3 was amplified using primers PSAP1F and PSAP1R, with 3.5mM $MgCl_2$, and 0.2-1µl plasmid per reaction. pPSA2.5 was amplified using PSAP2F and PSAP2R, with 3mM $MgCl_2$ and 0.25-1µl plasmid per reaction, and an annealing temperature of 65°C.

PCRs were also set up using the M13 forward (-20) and reverse primers (Invitrogen, UK), and other PCR materials as before, to amplify all TA-cloned plasmids across the cloning junction. 25µl reactions contained 2.5mM $MgCl_2$, 50ng/µl each primer, and 0.1-1µl plasmid. Conditions for amplification were:

94°C, 5 minutes
80°C, 10 minutes
(94°C, 1 minute; 55°C, 1 minute; 72°C, 1 minute) x 25

2.3.2. Restriction digests

Restriction digests were set up using the enzymes in Table 2.5 (see Table 2.2 for enzyme suppliers and buffers) to detect the presence or absence of the desired insert or deleted fragment, and the orientation of the insert if necessary. 20µl digests were set up containing typically 2µl 10X buffer, 0.1µg BSA, 2-10u enzyme, and sufficient plasmid to see all predicted fragments. Digests were incubated at 37°C for 1-3h, after which approximately 5µl was electrophoresed on a 1% agarose gel. This procedure was also used to check the integrity of plasmids after each new plasmid prep, and to detect whether mutagenesis had successfully generated a restriction site.

Plasmid	Enzyme	Diagnostic pattern	Fragment sizes (bp)
pPSA1.3	*Eco*RI	Presence of insert	Present 1617, 3888 Absent 20, 3888
	*Eco*RV	Orientation of insert	Forward 334, 5171 Reverse 1296, 4209
pPSA2.5	*Eco*RI	Presence of insert	Forward 73, 668, 3889 Reverse 68, 673, 3889 Absent 20, 3888
	*Bgl*II	Orientation of insert	Forward 1534, 3096 Reverse 1119, 3511
pPSA-CD1.8	*Eco*RI	Presence of insert	Present 68, 651, 4470 Absent 4465
pPSA-CD4.19	*Bgl*II	Presence of insert	Present 1573, 5195 Absent 5189
	*Eco*RV	Orientation of insert	Forward 862, 1444, 4462 Reverse 482, 1824, 4462
pAMdPE1.1	*Xho*I and *Hin*dIII	Absence of deleted fragment	Absent 1281, 3400 Present 335, 1281, 3917
pPPE-CD2.15	*Not*I	Presence of insert	Present 3871, 4681 Absent 4681
	*Xba*I	Orientation of insert	Forward 3881, 4671 Reverse 23, 8529
pCMV-CD1.10	*Xba*I and *Xho*I	Presence of insert	Present 1540, 5499 Absent 5533
pMinusCD1.24	*Xba*I and *Xho*I	Presence of insert	Present 1540, 4647 Absent 4681

pPCM1.24	*Nhe*I	*Nhe*I site produced by mutagenesis	Restored 185, 2198, 2813, 3356 Not restored 2383, 2813, 3356
pALTER-MAX	*Sgf*I and *Bgl*II	Checking structure	669, 4864
pCD1	*Dde*I	Checking structure	166, 409, 540, 779, 914, 1657

Table 2.5. Restriction enzymes used to screen plasmids. Enzymes were identified which would clearly distinguish between the desired plasmid structure and other possible structures. For example, if the insert in pPSA1.3 was present, 3.9kb and 1.6kb *Eco*RI fragments would be expected; if the insert was absent, 3.9kb and 20bp *Eco*RI fragments would be expected. Alternative insert orientations in pPSA1.3 and pPSA2.5 were arbitrarily designated forward or reverse. For pPSA-CD4.19, PSA enhancer inserts were considered to be in the forward orientation if they were in the same orientation to the PSA promoter into which they were inserted as in the natural PSA promoter. For PPE-CD2.15, the insert contained the PSA enhancer, PSA promoter, and CD gene in that order, and was considered to be in the forward orientation when the CD gene was adjacent to the poly(A) sequence in the vector.

2.3.3. Sequencing

2.3.3.1. Primer design

Oligonucleotides were designed to sequence all cloning junctions in pPPE-CD2.15, pCMV-CD1.10 and pMinusCD1.24, as well as the three androgen response elements in the PSA 5' region, and the PSA promoter (Table 2.6).

Primer sequences were selected to be approximately 20bp long, to avoid either 3 G/C residues or a T residue at the 3' end, to have 40-60% GC content, and to exclude ≥3bp of self-complementarity, unless the complementary regions were less than 3bp apart. Secondary structure was checked to ensure that ΔG values were greater than zero (i.e. that primer self-annealing was thermodynamically unfavourable) using the DNA mfold server at http://bioinfo.math.rpi.edu/~mfold/dna/form1.cgi (SantaLucia 1998).

Oligonucleotides were obtained lyophilised from Sigma-Genosys (UK) and rehydrated by adding 1-2ml ultrapure water, washing the sides of the tube, incubating for 10 minutes on ice and vortexing.

Primer	Sequence (5' to 3')	Derived from
A1F1	CTT TTG CTG GCC TTT TGC TC	pALTER-MAX: 5496-5515
A1F2	TTA TAG TCC TGT CGG GTT TC	pALTER-MAX: 5379-5398
A1R1	CAG CAC AAA TCA CAC CGT TA	PSA: 541-522
A1R2	GTG AGG GAG ACT GTG CAA C	PSA: 780-762
A1R3	CAC GTG ACC ACA TTT GAT CG	PSA: 813-794
B1F1	AGC CTT TGT CTC TGA TGA	PSA: 1613-1630
B1R5	TTC TTT TCC TTG CAC TCC CA	PSA: 5355-5336
C1F1	AAG TTC TAG TTT CTG GTC TC	PSA: 5400-5419
C1F2	GAT TCT GGG TTG GGA GTG CA	PSA: 5326-5345
C1R1	CGG GCG TTA ATA ATT GTT TG	CD: 53-34
C2F1	ACT CTT GCG TTT CTG ATA GG	pALTER-MAX: 958-977
D1F3	AAC CGC GCA TTC CTC CAA CG	CD: 1460-1479
D1R2	ACA TCT CCC CCT GAA CCT GA	pALTER-MAX: 1350-1331

Table 2.6. Sequencing primers. The complete CD sequence is given in Appendix 4. PSA sequence positions refer to Genbank accession number U37672. For reverse primers (designated _ _ R _, e.g. A1R1), the sequence position of the 5' end of the primer is given first.

2.3.3.2. Sequencing reactions

Reactions contained 0.6μg plasmid, 3.2pmol primer, 8μl terminator ready reaction mix (BigDye terminator cycle sequencing kit v2.0, Applied Biosystems, UK) and ultrapure water to 20μl. Plasmids used for sequencing were prepared as in Sections 2.1.5.4. or 2.1.5.6 (Sigma kit method). Control reactions contained pGEM and M13 forward (-21) primer (BigDye kit). Plasmid DNA and water were mixed in 0.2ml PCR tubes (Anachem, UK), and denatured at 95°C for 5 minutes on a PTC-200 thermocycler. Primers and reaction mix were added on ice. Reactions were subjected to the following thermocycling conditions on a GeneAmp 2400 PCR system (Applied Biosystems, UK:

25 cycles of:

1°C/sec to 96°C

96°C for 10s

1°C/sec to 50°C

50°C for 5s

1°C/sec to 60°C

60°C for 4 min

1°C/sec to 4°C

2.3.3.3. Removing unincorporated dye terminators

Free dye terminators were removed using DyeEx spin columns (Qiagen, UK), as recommended by the manufacturer. Samples were dried in a vacuum centrifuge at medium heat for 40 minutes.

2.3.3.4. Electrophoresis of sequencing reactions on 310 Genetic Analyser

Samples were resuspended in 25µl template suppression reagent (Applied Biosystems, UK), transferred to 0.5ml PCR tubes, denatured at 95°C for 5 minutes, and placed on ice. The reactions were transferred to 0.5ml sequencing tubes (Applied Biosystems, UK), vortexed, and placed on ice again, ensuring that the sample was at the bottom of the tube.

The 310 sequencer was set up with fresh 1X 310 Genetic Analyser buffer (Applied Biosystems, UK) at each end of the capillary, and samples and water assembled in the sample tray. A sample sheet and injection list were prepared, setting the detector length to 50cm and the sequencing module to SeqPOP6(1ml)E, before starting the run.

Operation of the instrument is described in detail in its manual[1].

[1] ABI Prism 310 Genetic Analyser Users Manual [Applied Biosystems, UK].

2.3.3.5. Electrophoresis of sequencing reactions on 3100 Genetic Analyser

Samples were resuspended in 10µl deionised formamide (Applied Biosystems, UK), transferred to 0.5ml PCR tubes, and denatured as above. After denaturation, samples were transferred to a 96 well plate (Applied Biosystems, UK) and loaded on the 3100 sequencer, set up with a 50cm capillary array (Applied Biosystems, UK).

A plate record was set up, selecting dye set E, mobility file DT3100 POP6{BD}v2.mob, analysis module BC-3100_SeqOffFtOff.saz, and run module StdSeq50_POP6 (default) or StdSeq50_POP6(10s). These run modules were identical except for their 20s and 10s injection times.

Details of operation of the instrument are in its manual[1].

2.3.4. Identification of potential promoter sequences

Plasmid DNA sequence was imported into the PromoterScan program for identifying putative promoter regions (Prestridge 1995).

2.4. CELL CULTURE

2.4.1. Cell lines

The four human prostate cancer cell lines used in this study, LNCaP, 22Rv1, PC-3 and DU145, were obtained from the American Type Culture Collection. LNCaP (Horoszewicz *et al* 1983), PC-3 (Kaighn *et al* 1979) and DU145 (Stone *et al* 1978) were established from prostate cancer metastatic sites: lymph node, bone and brain respectively. The 22Rv1 cell line was derived from a primary prostate cancer sample propagated as a xenograft by serial transplantation in mice (Pretlow *et al* 1993). Castration brought about regression and subsequent relapse of the CWR22 xenograft, and the 22Rv1 cell line was derived from a relapsed tumour after further transplantation in mice (Sramkoski *et al* 1999). LNCaP and 22Rv1 were PSA-positive, while PC-3 and DU145 were PSA-negative (van Bokhoven *et al* 2003).

[1] ABI Prism 3100 Genetic Analyser Users Manual [Applied Biosystems, UK].

The benign human prostate cell line PWR-1E was obtained from A.M. McCrohan (Department of Surgery, University College Dublin). This cell line was established by transformation of normal adult prostate cells with an adenovirus-12/simian virus-40 hybrid virus (Webber *et al* 1996). H929 myeloma cells (Gazdar *et al* 1986) used as controls in cell adhesion assays were provided by E. Maginn (Cancer Research Laboratory, Institute of Molecular Medicine).

In addition, the colon cancer cell line HCT116 (Brattain *et al* 1981) was kindly provided by E. Caraher (Department of Clinical Medicine, Trinity College, Dublin).

2.4.2. Cell line maintenance

Prostate cancer cells were cultured in RPMI1640 with Glutamax, supplemented with 10% fetal calf serum (FCS), 50u/ml penicillin and 5μg/ml streptomycin (complete medium; all components supplied by Invitrogen, UK). Complete medium also contained 2.5μg/ml amphotericin B (Sigma, UK) during later experiments. PWR-1E cells were cultured in keratinocyte serum-free medium (KSFM), supplemented with 10% FCS, 50u/ml penicillin, 5μg/ml streptomycin, 5ng/ml epidermal growth factor and 25μg/ml bovine pituitary extract (all supplied by Invitrogen, UK). HCT116 cells were cultured in McCoy's medium (Invitrogen, UK), supplemented with 10% foetal calf serum, 2mM L-glutamine (Invitrogen, UK), 50u/ml penicillin, 5μg/ml streptomycin and 2.5μg/ml amphotericin B.

Cultures were grown in an incubator at 37°C and 5% CO_2 (Forma Scientific, USA), and handled in a Class II laminar flow cabinet (Holten, UK). Cells were generally passaged weekly as below and also fed once weekly as required by replacing half the medium with fresh medium. Mycoplasma testing was carried out regularly as in Appendix 2 using the Mycoplasma PCR ELISA kit (Roche, UK). No mycoplasma contamination was detected.

2.4.3. Passaging cells

Medium was removed from cells. PC-3, DU145, 22Rv1 and PWR-1E cells (not LNCaP, as they were more weakly adherent) were washed with phosphate buffered saline (PBS; Invitrogen, UK). 0.25% trypsin (Invitrogen, UK) was added, 1.5ml or

2.5ml per T25 or T75 flasks (Nunc, Denmark). Cells were incubated for 5-10 minutes at 37°C. Trypsin was neutralised by adding the medium removed from the cells earlier, to a total volume of 5ml per flask (except for PWR-1E cells, for which trypsin was neutralised with RPMI1640 medium containing 10% FCS). A further 5ml of this medium was added and cells were transferred to 30ml sterile tubes (Bibby Sterilin, UK). Cells were pelleted at 1500rpm for 5 minutes in a Centra GP8 centrifuge (IEC, USA). Most of the supernatant was poured off and cells were resuspended in the remaining 0.2ml, then in 1-3ml fresh complete medium. An appropriate volume of cells was transferred to 10ml or 20ml fresh complete medium in T25 or T75 flasks.

HCT116 cells were passaged similarly, except that they were washed before trypsinisation with a sterile solution of 0.25% trypsin/0.03% ethylenediaminetetraacetic acid (EDTA; Sigma, UK) and trypsinised with the same trypsin/EDTA solution. DU145 and PC-3 cells were initially trypsinised by an alternative procedure, in which the cells were not washed with PBS and were removed from the surface of the flask using sterile scrapers (Nunc, Denmark).

35mm wells were trypsinised similarly, adding to each well 0.8ml trypsin, 0.8ml previously used medium to neutralise trypsin, and 3ml previously used medium to wash out wells, instead of the above volumes. $2cm^2$ wells were trypsinised using 60µl, 60µl and 380µl corresponding volumes.

LNCaP and 22Rv1 cells were routinely passaged at a 1:3 to 1:6 ratio. PC-3, DU145 and HCT116 cultures were passaged at 1:10 to 1:20 ratios for routine maintenance. PWR-1E cells were passaged at 1:5 to 1:10 ratios.

2.4.4. Freezing cell lines

Cultures were grown to 70-80% confluence in T75 flasks and trypsinised as in Section 2.1.3, except that flasks were washed with 10ml PBS or Hanks' balanced salt solution (HBSS; Invitrogen, UK) and the washings also centrifuged. Cells were resuspended in complete medium (1ml per flask) and placed on ice. Ice-cold 2X freeze medium was added dropwise. Cells were aliquoted into prelabelled 2ml cryovials (Nunc,

Denmark) on ice (1ml per vial), and immediately stored at -70°C in a polystyrene box previously at room temperature, allowing them to cool at -1°C per minute. The cells were stored in liquid nitrogen within three days of freezing.

2.4.5. Thawing cells

Frozen cells were thawed at room temperature or 37°C until they had almost become liquid. The vial was placed on ice and cells were transferred to a 20ml sterile tube on ice. RPMI1640 medium with Glutamax containing 20% FCS was added, dropwise for twice the original volume, then at normal speed, to 10ml. Cells were centrifuged for 5 minutes at 1500rpm. The supernatant was poured off and cells were resuspended in the remaining volume. 10ml fresh complete medium was added and the cells were transferred to a T25 flask.

2.4.6. Counting cells

Cells were diluted 1:10 or 1:20 in ethidium bromide-acridine orange solution (Oncor, UK and Sigma, UK), in a 200µl volume. A 22 x 22mm or 22 x 40mm coverslip (Chance Propper, UK) was placed on an Improved Neubauer haemacytometer (Hawksley, UK) and about 10µl cells/EBAO mix was loaded in each chamber. Cells in the central 25 squares were counted under fluorescent light using a Dialux 20EB microscope (Leitz, Germany). The number of cells per chamber was averaged over at least four chambers. Cell concentration per ml was calculated as

$$\{\text{Cells per chamber} \times 10^5\}$$

for cells diluted 1:10, and as

$$\{\text{cells per chamber} \times 2 \times 10^5\}$$

for cells diluted 1:20.

2.5. OPTIMISATION OF TRANSIENT TRANSFECTION CONDITIONS

2.5.1. GFP reporter plasmid

The 4.7kb plasmid pEGFP-NI expresses a variant of wild type GFP protein, enhanced for expression in human cells, under the control of the CMV immediate early promoter. pEGFP-NI was kindly provided by T. Robson, School of Biomedical Sciences, University of Ulster. Transfection grade plasmid DNA was prepared as in Section 2.1.5.6.

2.5.2. Seeding cell lines for transfection optimisation

Cells were trypsinised, resuspended in HBSS and transferred to 96-well plates, 24-well plates, 6-well plates or 90mm dishes (Nunc, Denmark) containing 22 x 22mm or 22 x 40mm coverslips (Chance Propper, UK). Relevant sizes and volumes for these vessels are shown in Table 2.7.

In initial experiments, cells were plated 1-7 days before transfection, and subsequently plated the day before transfection. Cells were generally 50-90% confluent before plating and 60-90% confluent before transfection. DU145 cells were counted and plated at a density of 5×10^4 cells per $2cm^2$ well, or plated at empirically determined split ratios. PC-3 cells were plated similarly to DU145, and also in 96-well plates at a density of 1.5×10^4 or 2.25×10^4 cells per well during GeneJuice and Transfast optimisation. 22Rv1 cells were seeded in 96-well plates during transfection optimisation, using 2.5×10^4, 5×10^4 or 1×10^5 cells per well. 2.5×10^4 or 5×10^4 HCT116 cells per well were seeded in 96-well plates for transfection optimisation. LNCaP cells were difficult to count reliably due to their tendency to form clusters of many cells. 60-90% confluent cultures were seeded at split ratios between 3.5:1 and 1.5:1 to produce 60-70% confluent cultures the following day. 3.2×10^4 primary prostate cancer cells per well were plated in a 96-well plate 24h before transfection.

Vessel	Area per well/dish	Culture volume	Transfection volume
96-well plate	0.33 cm^2 (25X smaller than 35mm well)	0.2ml	30-40µl
24-well plate	2cm^2 (5X smaller than 35mm well)	1ml	0.2ml (except Superfect: 0.2-0.25ml)
6-well plate	9.6cm^2 (35mm well)	5ml	1ml (except Superfect: 0.7-0.75ml)
90mm dish	63.6cm^2 (6.6X larger than 35mm well)	8ml	6.6ml

Table 2.7. Tissue culture vessels used for transfection optimisation. Transfection procedures below give volumes for one culture vessel; in some cases different vessels were used with volumes adjusted proportionately.

2.5.3. Transfection procedures

2.5.3.1. LipofectAmine (6-well plate format)

1-4µg DNA, and 2-25µl LipofectAmine (Invitrogen, UK) were diluted separately to 100µl each in OPTIMEM-I reduced serum medium (Invitrogen, UK), mixed together, and incubated at room temperature for 15-45 minutes, during which cells were washed with OPTIMEM-I. The DNA/LipofectAmine mix was made up to 1ml with OPTIMEM-I and added to the wells. Cells were incubated at 37°C and 5% CO_2 for 3-24h. Transfections were ended by adding 1ml 20% FCS in RPMI, except 24h transfections. At this point, medium in all wells was replaced with complete medium. Cells were fixed or scored for GFP expression after 24-72 hours.

2.5.3.2. LipofectAmine Plus (6-well plate format)

1-4µg DNA and 6-20µl LipofectAmine Plus were diluted together to 100µl in OPTIMEM-I and incubated for 15 minutes at room temperature. 4-20µl LipofectAmine (Invitrogen, UK) was diluted separately to 100µl in OPTIMEM-I. These were mixed together, and incubated at room temperature for 15 minutes. 0.8ml transfection medium (OPTIMEM-I or 10% FCS in RPMI) was added to the wells, followed by 0.2ml DNA/LipofectAmine/LipofectAmine Plus mix. Cells were incubated for 3-4h at 37°C and 5% CO_2, after which 1ml 20% FCS in RPMI was

added. 24h after transfection, medium in all wells was replaced with complete medium if cells were not being fixed at this point. Cells were fixed or scored for GFP expression after 24 or 72 hours.

2.5.3.3. Lipofectin (24-well plate format)

0.4-4µl Lipofectin (Invitrogen, UK) was diluted to 20µl in OPTIMEM-I and incubated at room temperature for 30 minutes. 0.2-0.4µg DNA was diluted separately to 20µl in OPTIMEM-I. These were mixed together and incubated at room temperature for 15 minutes, during which cells were washed with 0.5ml OPTIMEM-I. The DNA/Lipofectin mix was made up to 200µl with OPTIMEM-I and added to the wells. Cells were incubated at 37°C and 5% CO_2 for 5h, after which transfection medium was replaced with complete medium. Cells were scored for GFP expression after 48 and 72 hours.

2.5.3.4. Superfect (24-well plate format)

0.2-2µg DNA was diluted to 60µl RPMI1640 medium (serum-free and antibiotic-free) or OPTIMEM-I. 2-10µl Superfect per µg DNA was added. The mixture was incubated at room temperature for 10 minutes, during which the cells were washed with PBS. 350µl complete medium was added to the DNA/Superfect mix and it was transferred to the wells. Cells were incubated at 37°C and 5% CO_2 for 3h, after which they were washed with PBS and 1ml complete medium was added. GFP-positive cells were counted after 24 hours.

2.5.3.5. Transfast (24-well plate format)

0.1-1µg DNA was diluted in pre-warmed RPMI1640 medium (serum-free and antibiotic-free) and vortexed. 0.4-6µl Transfast (Promega, UK) was vortexed and added to the diluted DNA to a final volume of 200µl. The mixes were immediately vortexed and incubated at room temperature for 15 minutes. Cells were washed with PBS (optional). The DNA/Transfast mixes were vortexed briefly and added to the cells. After 1-3 hours at 37°C and 5% CO_2, 1ml warm complete medium was added.

Transfection medium was replaced with complete medium 24 hours after transfection if cells were cultured for longer times. Cells were fixed or scored for GFP expression 24-72 hours after transfection.

2.5.3.6. GenePorter2 (24-well plate format)

1.2-12µl GenePorter2 (Gene Therapy Systems, US) was diluted in 20µl OPTIMEM-I or RPMI1640. 0.24-2µg DNA were diluted separately in 20µl OPTIMEM-I or DNA diluent (Gene Therapy Systems, US). The mixes were incubated for 10 minutes at room temperature, combined, and incubated at room temperature for a further 10 minutes. 0.16ml OPTIMEM-I or 10% FCS in antibiotic-free RPMI1640 was added to the cells, followed by 40µl GenePorter2/DNA mix. Cells were incubated for 3-5 hours at 37°C and 5% CO_2, after which 0.2ml FCS in RPMI1640 was added to a final concentration of 10% FCS. After 24 hours, cells were fixed or scored for GFP expression.

Alternatively, for LNCaP cells, 1.5-12µl GenePorter2 was diluted 1:4 in OPTIMEM-I, and DNA was diluted in DNA diluent to 25µl per µg DNA. The mixes were incubated for 1 minute at room temperature, combined, and incubated at room temperature for a further 15 minutes. GenePorter2/DNA mixes were made up to 500µl with OPTIMEM-I and added to the cells. After incubating the cells for 4 hours at 37°C and 5% CO_2, 500µl 20% FCS in RPMI1640 was added. Medium was replaced with 1ml complete medium after 24 hours and GFP-positive cells were counted after 48 hours. GenePorter2 was tested on 22Rv1 cells using the same procedure, except that the DNA and lipid dilutions were incubated for 1 minute, the combined DNA and lipid mixes were incubated for 10 minutes, and the transfection time was 3h.

2.5.3.7. GeneJuice (96-well plate format)

0.1-0.9µl GeneJuice (Novagen, UK) was added to 4µl serum-free medium and vortexed thoroughly. The mix was incubated at room temperature for 5 minutes. 50-150ng DNA was added and mixed gently, and incubated at room temperature for a

further 15 minutes. The mix was added to each well, in 200μl normal growth medium or serum-free medium. The volume of mix added per well was the total volume of GeneJuice, DNA and 4μl medium and therefore varied between wells during optimisation.

2.5.4. Optimised transient transfection procedures

The following procedures were subsequently used for transfecting cell lines prior to transfection-related toxicity studies, Western blots, MTS assays and annexin V labelling. Cells were plated 24h before transfection. For transfection efficiency controls, pEGFP-NI was co-transfected with the plasmid(s) of interest at 1:4 or 1:9 ratios in separate wells. All plasmids used for transfection were prepared as described in Section 2.1.5.6.

2.5.4.1. LNCaP and 22Rv1: Transfast

LNCaP cells were transfected in 6-well plates for toxicity studies, Western blots and annexin V labelling, and in 24-well plates for GFP transfection efficiency controls. 22Rv1 cells were transfected in 6-well plates for toxicity studies, T25 flasks for Western blots, and 96-well plates for MTS assays and GFP controls.

DNA was diluted in pre-warmed RPMI1640 medium (serum-free and antibiotic-free) and vortexed. Transfast was vortexed and added to the diluted DNA. The mixes were immediately vortexed and incubated at room temperature for 15 minutes. The DNA/Transfast mixes[a] were vortexed briefly and added to the cells after removing growth medium. After 3 hours at 37°C and 5% CO_2, warm complete medium[b] was added. Transfection medium was replaced with complete or hormone-supplemented medium 24 hours after transfection. Details of amounts used are in Table 2.8.

	Cell line	96-well plate	24-well plate	6-well plate	T25 flask
Number of cells plated	LNCaP	ND	See legend	See legend	ND
	22Rv1	2.5×10^4	ND	1×10^6	2.5×10^6
Transfast	LNCaP	ND	3µl	15µl	ND
	22Rv1	0.3µl	ND	7.5µl	15µl
DNA	LNCaP	ND	1µg	5µg	ND
	22Rv1	0.1µg	ND	2.5µg	5µg
DNA/Transfast mix[a]	All	40µl	200µl	1ml	2ml
Complete medium[b]	All	200µl	1ml	5ml	10ml

Table 2.8. Details of optimised Transfast transfection procedure. Amounts shown are per well or per flask. Before transfection, 22Rv1 cells and LNCaP cells were 50-60% and 60-70% confluent respectively. Because LNCaP cells were difficult to count reliably due to their tendency to form clusters of many cells, 60-90% confluent cultures were seeded at split ratios between 3.5:1 and 1.5:1. ND, not done.

2.5.4.2. PC-3: GenePorter2

PC-3 cells were transfected using GenePorter2 in 6-well plates for toxicity studies, T75 flasks for Western blots, and 96-well plates for MTS assays and GFP tranfection efficiency controls.

GenePorter2 was diluted in OPTIMEM-I to an appropriate volume[a] (Table2.3). DNA was diluted separately in OPTIMEM-I to the same volume.[a] The mixes were incubated for 10 minutes at room temperature, combined, and incubated at room temperature for a further 10 minutes. Growth medium was removed from the cells and OPTIMEM-I[b] was added, followed by the GenePorter2/DNA mix.[c] Cells were incubated for 3 hours at 37°C and 5% CO_2, after which 20% FCS in RPMI1640[d] was added. After 24 hours, cell supernatant was replaced with complete medium (except where cells were harvested at this time). Details of amounts used are in Table 2.9.

	96-well plate	6-well plate	T75 flask
Number of cells plated	1.5×10^4	4.4×10^5	4×10^6
GenePorter2	1.2µl	36µl	270µl
DNA	0.2µg	6µg	45µg
Dilution volume[a]	3.3µl	100µl	750µg
OPTIMEM-I on cells[b]	26.7µl	800µl	6ml
GenePorter2/DNA mix[c]	6.6µl	200µl	1.5ml
20% FCS[d]	33.3µl	1ml	7.5ml

Table 2.9. Details of optimised GenePorter2 transfection procedure. Amounts shown are per well or per flask. PC-3 cells were 70-80% confluent before transfection.

2.5.4.3. PC-3 and HCT116: GeneJuice

PC-3 cells were transfected using GeneJuice in 35mm wells for toxicity studies and Annexin V labelling, and in 96-well plates for GFP transfection efficiency controls. HCT116 cells were transfected in 35mm wells for toxicity studies and in 96-well plates for MTS assays and GFP controls.

GeneJuice was added to OPTIMEM-I reduced serum medium and vortexed thoroughly. The mix was incubated at room temperature for 5 minutes. DNA was added and mixed gently, and incubated at room temperature for a further 15 minutes. The mix was added to the normal growth medium in each well. The volume of mix added per well was the total volume of GeneJuice, DNA and serum-free medium and therefore varied as different plasmids were used. Amounts used are noted in Table 2.10.

	Cell line	96-well plate	6-well plate
Number of cells plated	PC-3	1.5×10^4	4.4×10^5
	HCT116	2.5×10^4	7.3×10^5
GeneJuice	PC-3	0.4µl	10µl
	HCT116	0.3µl	7.5µl
DNA	PC-3	0.1µg	2.5µg
	HCT116	0.15µg	3.75µg
OPTIMEM-I	All	4µl	100µl

Table 2.10. Details of optimised GeneJuice transfection procedure. Amounts shown are per well. PC-3 and HCT116 cells were 70-80% and 50% confluent before transfection, respectively.

2.5.5. Assessing transfection-related toxicity

Cells were transfected with pEGFP-NI using optimised procedures (Section 2.5.4). Three control samples were also plated with the same number of cells (Tables 2.8, 2.9, 2.10). They contained cells treated with liposome but no plasmid, mock-transfected cells treated with neither liposome nor plasmid but subjected to the same procedure, and untreated cells.

24 hours after transfection, cells were trypsinised as in 2.13 and counted as in 2.16. Ethidium bromide (EB) and acridine orange (AO) are both fluorescent dyes which intercalate into DNA, and the ability of intact cells to exclude EB allows live and dead cells to be identified and counted. After staining with EB/AO, live cells contain AO and fluoresce bright green, but exclude EB. Dead cells, in contrast, take up EB as well as AO and therefore show orange fluorescence. Dead and live cells were distinguished by this principle and counted. The toxicity of the transfection methods optimised for LNCaP, PC-3, 22Rv1 and HCT116 cell lines was assessed in two independent experiments, with four chambers per sample counted in each experiment.

2.5.6. Paraformaldehyde fixation

Cells (on coverslips in 35mm wells) were washed three times with PBS. 2ml fresh 4% paraformaldehyde (Sigma, UK) in PBS was added. Cells were incubated at room temperature for 30 minutes and washed twice with PBS. A drop of fluorescent mounting medium (Dako, UK) was placed on a glass slide (Chance Propper, UK).

The coverslip was removed from the well and gradually laid down in the mounting medium (cells down). Excess mounting medium was removed. Slides were dried for 30 minutes at room temperature and stored in the dark at 4°C.

2.5.7. Counting GFP-positive cells

Cells were examined for GFP fluorescence either as paraformaldehyde-fixed slides, or *in situ* in multiwell plates. Fluorescent cells were counted on an Eclipse TE300 microscope (Nikon, USA), using a blue filter and 20X or 40X magnification. GFP fluorescence was distinguished from nonspecific fluorescence by switching to a green filter which removed GFP-derived emissions around its 507nm emission maximum. Cells were photographed using a DC100 digital camera (Leica, Germany).

2.6. PSA PROTEIN DETECTION

2.6.1. Enzyme-linked immunosorbent assay (ProStatus kit)

70% confluent LNCaP cells were plated in a 24-well plate at a 1:3 split ratio, and grown for 6 days in hormone-supplemented media. Dihydrotestosterone (DHT; Sigma, USA) was added to DC-FCS-RPMI (medium containing FCS treated with dextran and charcoal to remove steroid hormones), at concentrations from 1nM to 1mM. Supernatants from these cells was removed and assayed for PSA by the enzyme-linked immunosorbent assay (ELISA) technique, using the ProStatus PSA Free/Total AutoDELFIA kit (Wallac, Finland). The assay was kindly performed by the Department of Endocrinology, St. James' Hospital, Dublin. After harvesting the supernatant, cells were trypsinised and counted as in Section 2.4.6 (although accurate counting was difficult due to the presence of large clumps of cells).

2.6.2. ELISA (PSA EIA kit)

5 x 10^4 22Rv1 cells per well were plated in 24-well plates, and grown for 6 days in DC-FCS-RPMI supplemented with DHT (1nM to 10μM). Supernatants from these cells was removed and assayed for PSA using the PSA EIA kit (CanAg, Sweden) according to the manufacturer's instructions. Standards and samples were incubated with a biotinylated anti-PSA mouse monoclonal antibody in a streptavidin-coated 96-well plate, thus binding PSA to the plate surface. The strips were washed, incubated

with a horseradish peroxidase-labelled anti-PSA mouse monoclonal antibody and washed again. Hydrogen peroxide (substrate for horseradish peroxidase) and 3,3', 5,5' tetra-methylbenzidine (chromogen) were added. The reaction was stopped by adding 0.12M hydrochloric acid. Absorbance at 405nm was read using a Victor 1420 multilabel counter (Wallac, Finland). A standard curve of absorbance against PSA concentration (0-60ng/ml) was plotted and used to derive the PSA concentration of the samples. Cells were trypsinised and counted as in Section 2.4.6 immediately after removing supernatants for the ELISA assay.

2.6.3. Immunohistochemistry

1.2×10^7 LNCaP and 22Rv1 cells, cultured for three days in complete medium, were harvested as pellets. Subsequent steps were kindly carried out by the Department of Histology, St. James' Hospital, Dublin (essentially as in this department's procedure for PSA immunohistochemistry on clinical samples). Paraffin-embedded sections were made, and slices of the sections were cut. Immunohistochemistry was carried out using the Peroxidase Vectastain Elite ABC kit (Vector Laboratories, UK), a polyclonal rabbit anti-human PSA primary antibody (Dako, UK) and a biotinylated swine anti-rabbit secondary antibody (Dako, UK).

2.7. CD PROTEIN DETECTION

2.7.1. Polyacrylamide gel electrophoresis (PAGE)

Denaturing polyacrylamide gels were poured in a Dual Minislab gel rig (Atto, Japan). For 8% gels used to optimise the anti-CD primary antibody, the resolving gel contained (per gel):

3.22ml adH_2O

1.82ml 30% acrylamide/bis-acrylamide, mix ratio 37.5:1 (Sigma, UK)

1.82ml 1.5M Tris, pH 8.8 (Sigma, UK)

70µl 10% (w/v) SDS (Sigma, UK)

70µl 10% (w/v) APS (Sigma, UK)

4.2µl TEMED (Sigma, UK)

For 15% gels used in subsequent experiments, the resolving gel contained (per gel):

1.54ml adH_2O
3.5ml 30% acrylamide/bis-acrylamide, mix ratio 37.5:1 (Sigma, UK)
1.82ml 1.5M Tris, pH 8.8 (Sigma, UK)
70µl 10% (w/v) SDS (Sigma, UK)
70µl 10% (w/v) APS (Sigma, UK)
2.8µl TEMED (Sigma, UK)

This was overlaid with 1ml isopropanol and allowed to set for 15 minutes. The isopropanol was washed off with water. A stacking gel made up as below was poured and allowed to set for 20 minutes.

1.4ml adH_2O
330µl 30% acrylamide/bis-acrylamide
250µl 0.5M Tris, pH 6.8
20µl 10% SDS
20µl 10% APS
2µl TEMED

Samples were mixed with an equal volume of 2X protein dissociation buffer and boiled for 5 minutes at 100°C, and loaded using extended pipette tips (Bio-Rad, UK). For $\alpha_5\beta_3$ integrin analysis, samples were mixed with 2X non-reducing protein buffer. Kaleidoscope protein size standards (Bio-Rad, UK) were also loaded. The gel was electrophoresed in 1X running buffer at a constant current of 20mA per gel on a Horizon 58 gel electrophoresis apparatus.

2.7.2. Transfer to nitrocellulose membrane

Following electrophoresis, gels were transferred to nitrocellulose membrane (Amersham Pharmacia Biotech, UK) in a Mini Trans Blot wet transfer apparatus (Bio-Rad, UK) filled with 1X transfer buffer. The transfer was assembled as follows (from negative electrode to positive electrode): sponge, 3 layers of 3mm

chromatography paper (Whatman, UK), gel, membrane pre-wet in transfer buffer, 3 layers of filter paper, and sponge. Proteins were transferred at 60V for 90 minutes.

2.7.3. Western blotting

The membrane was blocked for 1h in 50ml blocking reagent (5% Marvel powdered milk in TBS-Tween), rocking at room temperature on a STR6 platform shaker. (For blots using CD primary antibody, Tween concentration in TBS-Tween was increased to 0.1% to reduce background.) Primary antibody was diluted in 5ml blocking reagent per membrane. Anti-CD primary antibody (MTM Laboratories, Germany) was diluted at various factors during optimisation and 1:1200 during subsequent experiments. Monoclonal mouse primary antibodies specific for actin (Sigma, UK), vimentin (Sigma, UK), pan-cytokeratin (Santa Cruz, UK) and $\alpha_5\beta_3$ integrin (Chemicon, UK) was diluted 1:5000, 1:100, 1:500 and 1:30 respectively. The membrane and primary antibody were assembled in plastic bags sealed with a TEW Impulse Sealer (Packworld, USA), and rocked for 90 minutes. The membrane was washed three times for three minutes in 50ml TBS-Tween, before rocking for 1h in 50ml HRP-conjugated rabbit anti-mouse secondary antibody (Dako, UK), diluted 1:1000 in blocking reagent. The membrane was washed as before, and incubated with 1.5ml luminol/enhancer and 1.5ml stable peroxide (Pierce, USA) for 5 minutes. The membrane was placed in a development folder inside a cassette (Agfa, Ireland) and exposed to Curix Blue HC-S Plus X-ray film (Agfa) for 1 second to 30 minutes. The film was developed in a Curix 60 developer (Agfa) using fixer and developer solutions also supplied by Agfa.

2.7.4. Anti-CD antibody optimisation

2.7.4.1. Preparation of RNase-free CD expression plasmid

To linearise pCMV-CD1.10 with *Hin*dIII, the following digest was set up and incubated for 3h at 37°C.

pCMV-CD1.10	40µg
BSA, 1mg/ml	40µl
10X React 2 buffer	40µl
adH_2O	to 400µl
*Hin*dIII, 10u/µl	20µl

Approximately 100ng digested and undigested plasmid was electrophoresed on a 1% agarose gel to confirm linearisation. The digest was cleaned using the Wizard DNA Clean-up system (Promega, UK) as recommended by the manufacturer (5ml syringes supplied by B.Braun, Germany; isopropanol by Alkem, Ireland). 5μg DNA was loaded per column and eluted in 30μl adH_2O after a 5-minute incubation.

A Proteinase K digest was carried out for 30-60 minutes at 37°C to ensure no RNases were present. This contained plasmid (digested and cleaned), 10X Proteinase K buffer, Proteinase K (Sigma, UK) to 50μg/ml, and adH_2O to 270μl. (A different buffer was used initially but changed because it tended to precipitate.)

After Proteinase K digestion, the plasmid was assumed to be RNase-free, and appropriate care was taken not to reintroduce RNases. Where solutions were made up in water, nuclease-free water (Sigma, UK) was used. The Proteinase K digest was terminated by adding EDTA (pH 8.0) to 2mM, and phenol extracted twice, ethanol precipitated and resuspended in 6μl nuclease-free water.

2.7.4.2. *In vitro* transcription and translation of CD protein

Reactions were assembled containing master mix (TNT T7 quick coupled transcription/translation system, Promega, UK), 20μM methionine (Promega, UK), plasmid template, Transcend biotinylated lysyl tRNA (Promega, UK) and nuclease-free water, in that order. To transcribe and translate CD, plasmid template was prepared as in Section 2.5.1.1, and 1μg was used per 50μl reaction. After initial difficulty detecting any product, twice the standard amount of Transcend was used (4μl instead of 2μl per 50μl reaction). As a positive control, luciferase was also transcribed and translated from a linearised plasmid (supplied in the T7 transcription/translation system), of which 0.5μg per 50μl was found to be sufficient. For negative control reactions, no plasmid was added. 40μl master mix was used per 50μl. Reactions were incubated at 30°C for 90 minutes and assayed for luciferase activity or electrophoresed on a polyacrylamide gel.

Figure 2.1 below shows in detail the region of pCMV-CD1.10 relevant for the transcription and translation of CD.

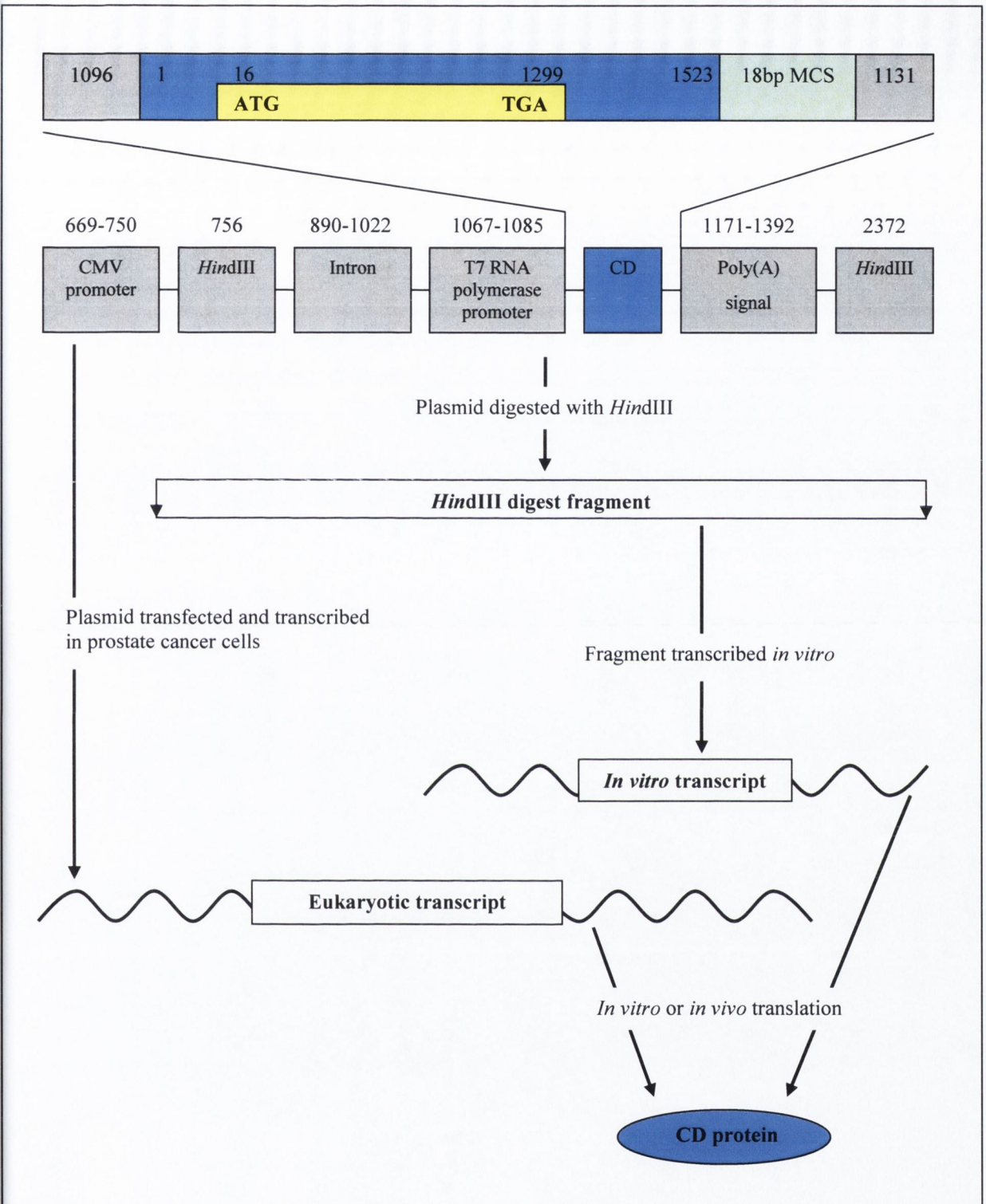


Figure 2.1. Transcription and translation of CD from pCMV-CD1.10. The structure of the relevant region of pCMV-CD1.10 is shown, with grey regions derived from pALTER-MAX and the CD open reading frame in yellow. In eukaryotic cells, CD is expressed from a CMV promoter-driven transcript also containing an intron and polyadenylation signal to maximise expression. The complete CD DNA sequence in Appendix 4 details the changes made by Mullen *et al* (1992) for more efficient transcription in eukaryotic systems. *In vitro*, CD expression from a 3.2kb restriction fragment is controlled by a T7 promoter. The transcription and translation process, *in vitro* and *in vivo*, is outlined above.

2.7.4.3. Luciferase activity assay

2.5µl *in vitro* transcribed and translated luciferase (Section 2.7.4.2.), or recombinant luciferase protein diluted in luciferase assay buffer (both supplied by Promega, UK) was added to 50µl luciferase assay reagent (Promega, UK) in a black 96-well plate (Nunc, Denmark). 50µl luciferase assay reagent alone served as a negative control. Luciferase activity was immediately measured in relative light units using a LuminoSkan EL luminometer (Thermo Labsystems, UK). The luminometer was programmed to read integral measurements over 10s, with 2s delay before readings, at maximum sensitivity. For detailed operation of the luminometer, refer to its manual.[1]

2.7.4.4. Detection of biotinylated CD protein

Proteins immobilised on nitrocellulose membrane were detected using the streptavidin AP Lumiblot kit (Novagen, UK), as recommended by the manufacturer. A STR6 platform shaker (Stuart Scientific, UK) was used to shake the membrane. Background was reduced when all washes after the streptavidin AP incubation were carried out for 5 minutes. X-ray film was exposed to the membrane and developed as in Section 2.7.3.

2.7.5. Detection of CD protein in extracts from transfected cell lines

2.7.5.1. Transfection of cell lines for Western blotting

Cells were seeded in 6-well plates (LNCaP), T25 flasks (22Rv1) or T75 flasks (PC-3) and transfected as described in Section 2.5.4. For PC-3 cells, 1.2 x 10^6 cells per T75 were plated for untransfected controls, and protein was extracted 24h after transfection. 22Rv1 and LNCaP cells were fed with DC-FCS-RPMI medium supplemented with 0.1µM and 1µM DHT respectively, 24h after transfection, and cultured for a further 3-4 days before extracting protein.

2.7.5.2. Protein extracts from cell lines

5 x 10^5 cells were spun down at 1500rpm for 5 minutes, and resuspended in 15µl lysis buffer. Samples were incubated on ice for 15 minutes and centrifuged for 20 minutes at 12,000rpm, 4°C. The supernatant was removed, assayed for protein concentration and stored in aliquots at -70°C.

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[1] Luminoskan Operating Instructions [Thermo Labsystems, USA].

2.7.5.3. Bradford assay for protein concentration

Standard solutions of BSA Fraction V (Sigma, UK) over the range 1-32μg/ml were prepared, containing 0.1% lysis solution. Samples were diluted 1:1000 in water. An equal volume of Bradford reagent (Sigma, UK) was added and mixed by inversion. 200μl was transferred to a 96-well plate (Nunc, Denmark). Absorbance at 595nm was read on a SpectraFluor Plus fluorometer (Tecan, UK). An Excel graph was plotted to compare the samples to the standards.

2.7.5.4. Stripping Western blots for re-probing

Nitrocellulose membranes were stored in cling-film at -20°C after Western blotting with anti-CD primary antibody was completed. This antibody was removed by stripping the blot prior to re-probing for actin. 35μl β-mercaptoethanol (Sigma, UK) was added to 5ml stripping buffer to make stripping solution. The membrane and stripping solution were assembled in plastic bags sealed with a TEW Impulse Sealer, and incubated for 30 minutes in a water bath at 50°C, protein side up. The membrane was washed for five minutes three times in 100ml 1X TBS, and for five minutes three times in 50ml TBS-Tween. The membrane was blocked for 30 minutes in 50ml blocking reagent, rocking at room temperature on a STR6 platform shaker. Anti-actin primary antibody was then added and the Western blotting procedure in Section 2.7.3 was followed (omitting the initial blocking step).

2.7.5.5. Densitometry

The intensity of CD and actin bands on the autoradiographs produced by Western blotting was quantitated using the Image Station 440CF digital imaging system (Kodak, UK). Images of the autoradiographs were acquired using the Kodak ID v3.5.5.B program. Bands were selected as regions of interest (ROI). Intensity, displayed as the sum of the intensity within the ROI, was normalised to the median of the ROI perimeter.

2.8. MODIFIED MTS ASSAY FOR CELL VIABILITY

2.8.1. Cell culture and 5-FC treatment prior to viability assay

Cells were seeded in 96-well plates and transfected as described in Section 2.5.4, then treated as appropriate for each of the cell lines tested (22Rv1, PC-3 and HCT116).

24h after transfection, 22Rv1 cells were fed with DC-FCS-RPMI medium supplemented with 100nM DHT. A further 24h later, 22Rv1 cells were treated with various concentrations of 5-FC (Fluka, Switzerland), in DC-FCS-RPMI medium containing 100nM DHT.

Cells were assayed for viability after three or six days of 5-FC treatment. In the case of cells treated for six days, the treatment was repeated four days after the initial addition of 5-FC.

PC-3 cells were processed identically to 22Rv1 cells, except that complete medium was used instead of hormone-supplemented DC-FCS-RPMI.

HCT116 cells were treated with 5-FC (in complete McCoy's medium) 24h after transfection, and assayed for viability after treatment with 5-FC for 24h.

2.8.2. Measurement of cell viability

Medium was removed from wells to a new 96-well plate, and 100µl used medium was returned to each well. 20µl assay reagent from the CellTiter 96 AQueous One Solution Cell Proliferation kit (Promega, UK) was added. Cells were incubated for 2h or 4h at 37°C and 5% CO_2 with the assay reagent. This reagent contains a tetrazolium compound which is reduced by living cells to a coloured formazan product. Absorbance at 490nm was measured in a Victor 1420 multilabel counter. Results shown represent the mean of two independent experiments performed in triplicate wells.

2.9. ANNEXIN V/ PROPIDIUM IODIDE ASSAY

2.9.1. Cell culture and 5-FC treatment prior to assay

Cells were seeded in 6-well plates and transfected as described in Section 2.5.4. PC-3 cells were also seeded at a lower density, 1.5 x 10^5 per well, for untransfected controls. PC-3 cells (transfected with pCMV-CD1.10 and untransfected) were treated with 1mM 5-FC in complete medium, 24h after transfection. LNCaP cells (transfected with pCMV-CD1.10 and pPPE-CD2.15, and untransfected) were treated with 1mM 5-FC in DC-FCS-RPMI medium containing 1μM DHT. The treatments were repeated five days after transfection. Cells were harvested, labelled with annexin V and propidium iodide, and assayed by flow cytometry after six days (PC-3) or seven days (LNCaP) of 5-FC treatment.

2.9.2. Labelling cells with annexin V and propidium iodide

1 x 10^5 – 2 x 10^6 cells per sample were washed with 1ml 1X annexin V binding buffer (Biosource, Belgium) and resuspended in 100μl 1X annexin V binding buffer. Samples were transferred to 5ml polystyrene round bottom tubes (Becton Dickinson, US). 3μl annexin V FITC conjugate (IQ Products, Holland) was added to samples labelled with annexin V, and vortexed briefly. Cells were incubated for 20 minutes on ice in the dark, then washed again with 1ml 1X annexin V binding buffer. Samples were resuspended in 125μl 1X annexin V binding buffer. For samples labelled with propidium iodide (PI; Sigma, UK), 125μl PI (diluted to 250ng/ml in PBS) was added and vortexed briefly. For samples not labelled with PI, 125μl PBS was added. Cells were placed on ice in the dark, and assayed immediately by flow cytometry.

2.9.3. Detection of labelled cells by flow cytometry

After cells were labelled with annexin V and PI, they were loaded on a FACSCalibur flow cytometer (Becton Dickinson, USA), and processed as recommended in the manufacturer's manual[1], using the CellQuest software and settings optimised for each cell line (Section 2.9.4).

[1] CellQuest Software Reference Manual [Becton Dickinson, USA].

2.9.4. Optimisation of flow cytometry parameters

PC-3 cells were treated for five days with geneticin (G418; Sigma, UK). LNCaP cells were grown in DC-FCS-RPMI for three days without hormone supplementation (treated), and in complete medium (untreated). Treated cells were labelled with annexin V only, PI only, and both annexin V and PI. Untreated cells were labelled with both annexin V and PI. These samples, and treated and untreated cells with neither label, were analysed on a FACSCalibur flow cytometer to set appropriate compensation parameters and voltages for each cell line.

2.10. PRIMARY PROSTATE CELL CULTURE

2.10.1. Sample collection

Fresh prostate tissue was collected from prostate cancer patients following radical prostatectomy. The patients gave informed consent and the study was approved by the Ethics Committee of St. James' Hospital. The prostate gland was immersed in transport medium on removal from the patient and brought from the operating theatre in St. James' Hospital to the Department of Histopathology, St. James' Hospital. Transport medium contained RPMI1640 with Glutamax, supplemented with 10% FCS, 50u/ml penicillin and 5µg/ml streptomycin and 0.5mg/ml amphotericin B.

After weighing the gland and labelling right and left lobes with ink, a consultant pathologist cut a sample of tissue from a region of the gland in which tumour had been confirmed by biopsy, and a sample from a region of the gland found to be benign at biopsy. These samples were again immersed in transport medium and stored at 4°C until initiation of *in vitro* culture (3-14 days). The remainder of the prostate gland was stored in formalin and processed in the Department of Histopathology according to standard procedures.

A culture of human primary prostate epithelial cells was obtained from A.M. McCrohan (Department of Surgery, University College Dublin). Tissue had been collected following radical prostatectomy as above. Cells had been grown *in vitro* for two passages prior to transfection with pEGFP-NI (Section 2.10.6).

2.10.2. Tissue disaggregation

Tissue samples were diced into pieces of approximately $1mm^3$ using sterile scalpels, and digested using 1500 units Type IV collagenase (Worthington, UK) per gram of tissue in a total volume of 7.5ml per gram. Collagenase digests were incubated at 37°C for 16-20 hours with moderate shaking.

2.10.3. Separation of stromal and epithelial compartments

Following overnight collagenase digestion, the enzyme was inactivated by adding transport medium to a total volume of 20ml. Samples were centrifuged at 770 x g for 10 minutes. The supernatant was discarded and cells were resuspended in 20ml fresh transport medium and centrifuged at 770 x g for 10 minutes. This step was repeated. Cells were then resuspended in 10ml transport medium and incubated at room temperature for 15 minutes. Epithelial cells sedimented during this incubation were removed using a Pasteur pipette. The supernatant was centrifuged at 120 x g for 20s. After removing the epithelial cell pellet, the supernatant was centrifuged at 770 x g for 10 minutes to pellet stromal cells.

2.10.4. Culture conditions

For each sample, a culture of epithelial cells and a culture of stromal cells was initiated in 2.5ml epithelial or stromal cell growth media in $75cm^2$ flasks, and incubated for 24h at 37°C and 5% CO_2 for 24h. 2ml further growth medium was then added. After 48h in culture, medium was removed and replaced with 10ml fresh medium. Cells were subsequently fed with 10ml fresh medium every 2-4 days. Epithelial cells were grown in WAJC404 medium (detailed in Appendix I), and stromal cells in stromal medium at 37°C and 5% CO_2. Stromal medium contained RPMI1640 with Glutamax, supplemented with 10% FCS, 50u/ml penicillin and 5µg/ml streptomycin.

2.10.5. Cell adhesion assay

White 96-well plates (Nunc, Denmark) were coated with fibronectin, vitronectin or BSA (Sigma, UK) at concentrations of 3µg/ml, 3µg/ml and 3% respectively, for 2h at 37°C. Plates were subsequently blocked for 1h at 37°C with 0.5% BSA.

Primary prostate cells or cell lines were harvested non-enzymatically using 5mM EDTA (Sigma, UK) in Dulbecco's PBS containing calcium and magnesium (Invitrogen, UK). Cells were removed from the flask surface with sterile cell scrapers (Nunc, Denmark) and counted as in Section 2.4.6. 2.5×10^4 cells per well were plated for cell adhesion assays and allowed to adhere at 37°C and 5% CO_2 for 2h. Cells were then gently washed twice with PBS and 5μM calcein AM (Molecular Probes, USA) was added. After a further 1h incubation at 37°C and 5% CO_2, fluorescence was measured in a Victor 1420 multilabel counter at an excitation wavelength of 485nm and an emission wavelength of 535nm. For each cell line, the fluorescence of standard numbers of cells was measured, following incubation for 1h with calcein AM as above, and standard curves were generated.

2.10.6. Primary culture transfection

2.10.6.1. LipofectAmine 2000/pEGFP-NI

For transfection with pEGFP-NI, one culture of primary prostate cells obtained from A.M. McCrohan in a T75 flask were trypsinised using 5ml 0.25% trypsin for 10 minutes at 37°C. 10ml transport medium was added, and the cells were transferred to a tube containing 15ml used PrEBM medium (Clonetics, UK). The cells were centrifuged for 5 minutes at 1500rpm, resuspended in fresh PREBM medium, counted as in Section 2.4.6 and seeded in 96-well plates for transfection the following day.

0.4-0.75μl LipofectAmine 2000 (Invitrogen, UK) and 0.2-0.25μg DNA were diluted separately in OPTIMEM-I, to a total volume of 25μl per well for each mix. These mixes were incubated for 5 minutes at room temperature, then the diluted DNA and lipid were combined and incubated for a further 20 minutes at room temperature. Medium was removed from the cells, and 100μl OPTIMEM-I or fresh PrEBM medium was added to the cells, followed by 50μl lipid/DNA mix. After 4h incubation at 37°C, 5% CO_2, medium was replaced by 200μl PrEBM medium.

2.10.6.2. GenePorter2/CD expression plasmids

Primary cultures were harvested and counted as in 2.10.5, and transiently transfected in 96-well plates. 0.5μl GenePorter2 and 0.1μg DNA were diluted separately in KSFM and DNA Diluent (Gene Therapy Systems, USA), to a total volume of 2.5μl

per well for each mix. CD expression plasmids or pAMdPE1.1 control were mixed with pEGFP-NI at a 4:1 ratio. These mixes were incubated for 5 minutes at room temperature, then the diluted DNA and lipid were combined and incubated for a further 10 minutes at room temperature. 100µl KSFM per well was added to each complex. Medium was removed from the cells, and replaced by 105µl lipid/DNA mix. After 3h incubation at 37°C, 5% CO_2, medium was replaced by 200µl WAJC404 medium.

2.10.7. Primary culture prodrug treatment and cell death assay

Transfected cells were allowed to recover for 24h prior to prodrug treatment. Medium was removed and 100µl 10mM 5-FC, in KSFM medium supplemented with 30ng/ml cholera toxin (Sigma, USA), was added per well. Untreated cells were fed with the same medium without 5-FC. Cells were returned to the incubator and grown for 72h. 15µl MTT dye solution (Promega, UK) was added and incubated at 37°C, 5% CO_2, for 4h. 100µl solubilisation reagent (Promega, UK) was added and the cells continued to incubate overnight. Absorbance at 595nm was then measured in a SpectraFluor Plus fluorometer.

2.11. STATISTICAL ANALYSIS

Significance testing on results in Chapters 6-7 was performed using the paired two-tailed t-test in Microsoft Excel.

CHAPTER THREE: DEVELOPMENT OF PSA-CD PLASMIDS AND CONTROLS

INTRODUCTION

Since this project involved the development of a strategy for treatment of prostate cancer cells with the suicide gene cytosine deaminase, a series of expression plasmids containing this gene was essential. As well as tissue-specific constructs containing PSA 5' regulatory elements, negative and positive control plasmids were also produced.

After sequencing the PSA promoter and comparing the results to a number of published promoter sequences, two further plasmids with slightly different PSA sequences were generated by site-directed mutagenesis, to begin optimising prostate-specific transcriptional regulation in our system.

RESULTS

3.1. Cloning

The plasmids summarised in Table 3.1 below were constructed, through the steps shown in Figures 3.1 and 3.2. Maps of the plasmids are shown in Appendix 5.

Plasmid	Promoter and/or ORF	Backbone
pPSA1.3	PSAE	pCR2.1-TOPO
pPSA2.5	PSAP	pCR2.1-TOPO
pCD1	CD	pBluescript
pPSA-CD1.8	PSAP-CD	pBluescript
pPSA-CD4.19	PSAE-PSAP-CD	pBluescript
pALTER-MAX	CMVPE	pALTER-MAX
pAMdPE1.1	-	pALTER-MAX
pPPE-CD2.15	PSAE-PSAP-CD	pALTER-MAX
pPCM1.24	PSAE-PSAP-CD	pALTER-MAX
pPCM2.3	PSAE-PSAP-CD	pALTER-MAX
pCMV-CD1.10	CMVPE-CD	pALTER-MAX
pMinusCD1.24	CD	pALTER-MAX

Table 3.1. Summary of plasmid structures. Abbreviations: ORF, open reading frame; PSAE, 1.6kb PSA enhancer; PSAP, 0.7kb PSA promoter; CD, cytosine deaminase ORF; CMVPE, cytomegalovirus immediate early promoter and enhancer.

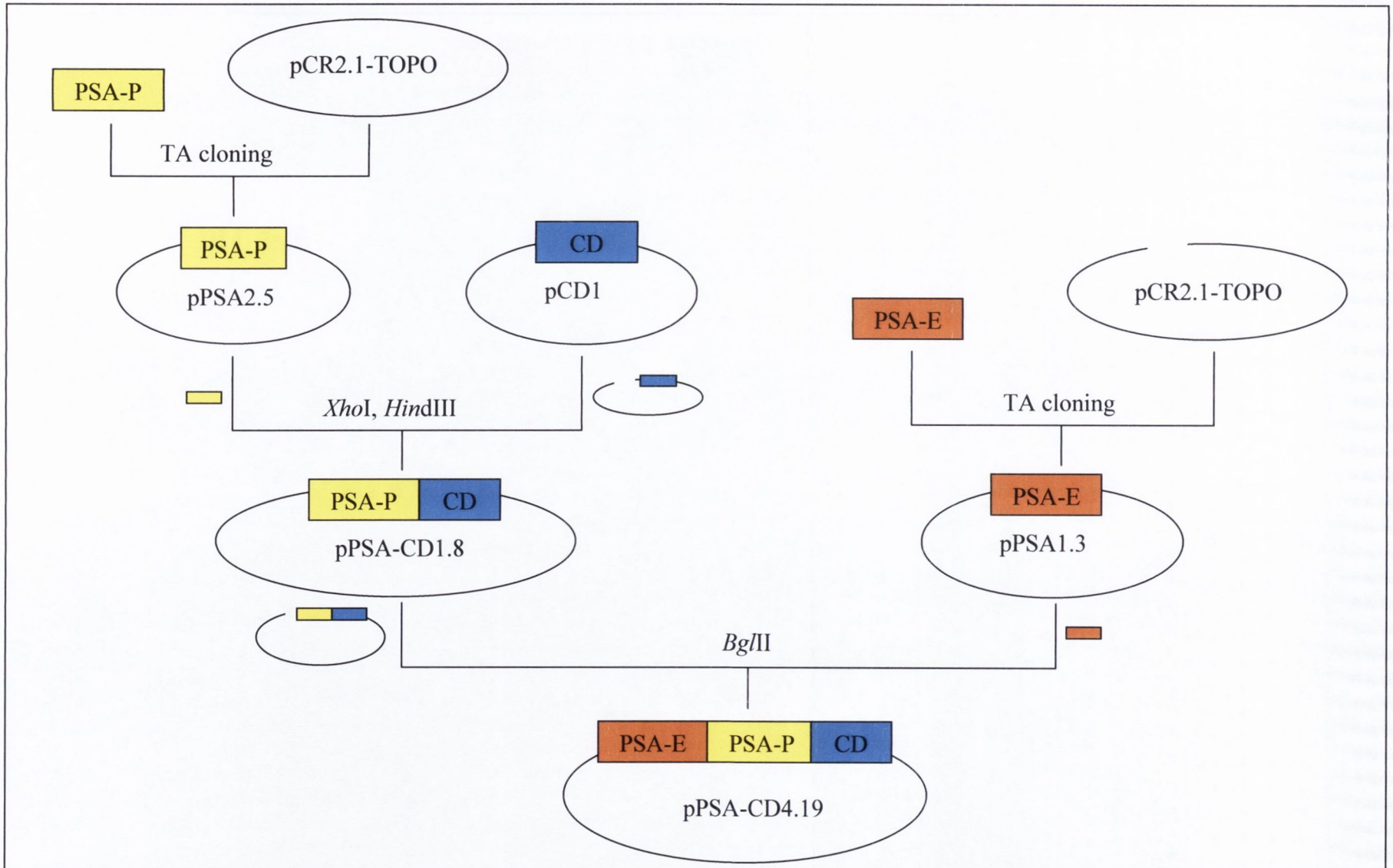


Figure 3.1. Construction of PSA-CD cassette. Abbreviations: PSA-P, PSA promoter; PSA-E, PSA upstream enhancer; CD, cytosine deaminase open reading frame.

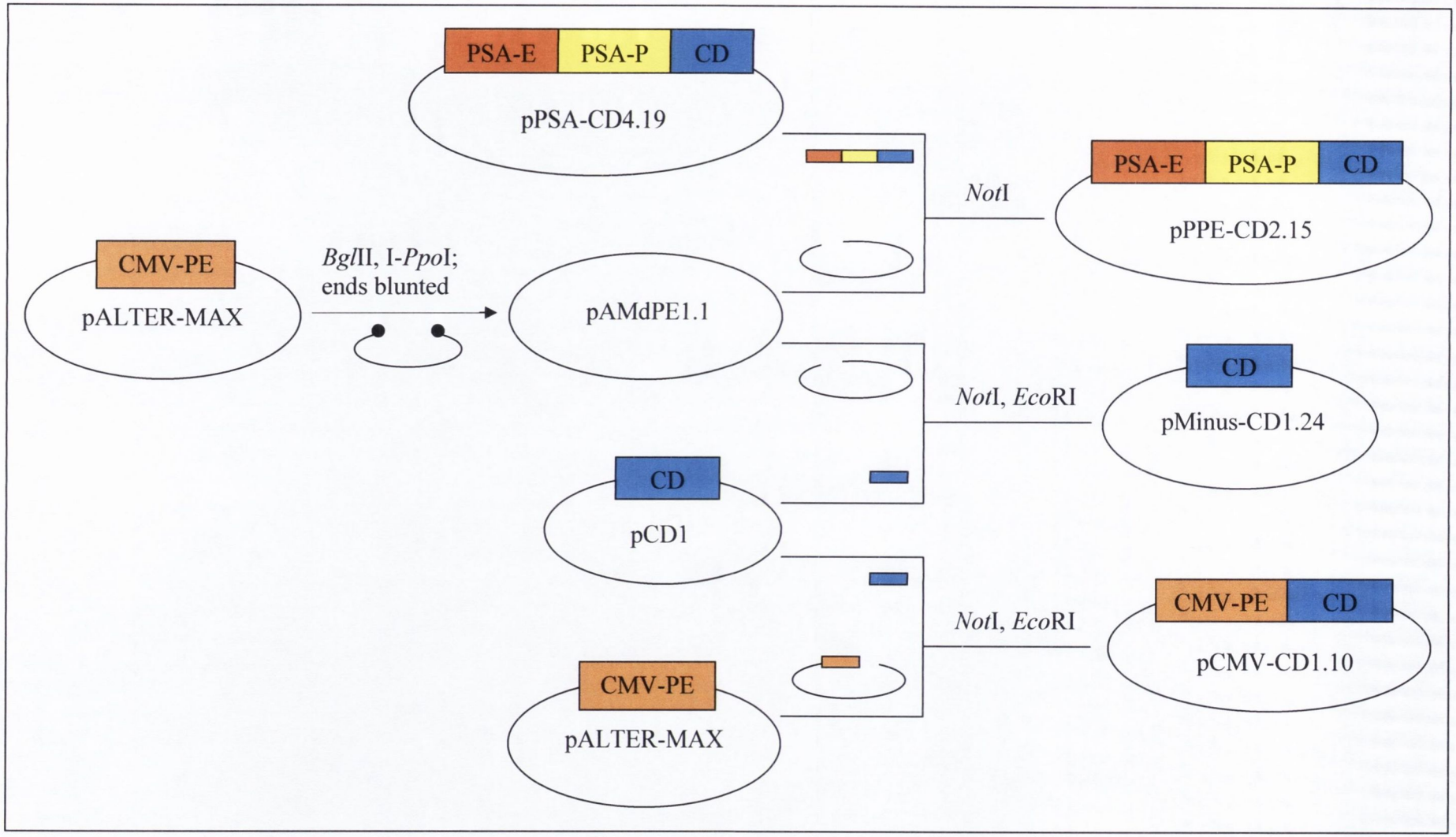


Figure 3.2. Construction of eukaryotic CD expression plasmids. Abbreviations: PSA-P, PSA promoter; PSA-E, PSA upstream enhancer; CD, cytosine deaminase open reading frame; CMV-PE, CMV IE gene promoter and enhancer.

3.1.1. PCR amplification of PSA promoter and enhancer

Based on previous studies of PSA promoter structure and function (Rodriguez *et al* 1997, Schuur *et al* 1996), we decided to use two separate regions of the PSA 5' regulatory region to drive CD expression. A 0.7kb fragment of promoter sequence and a 1.6kb fragment of upstream enhancer sequence were PCR amplified from human genomic DNA.

Figure 3.3 shows optimisation of PCR conditions for amplifying the 0.7kb promoter fragment, and amplification of the 1.6kb enhancer fragment and control PCR product.

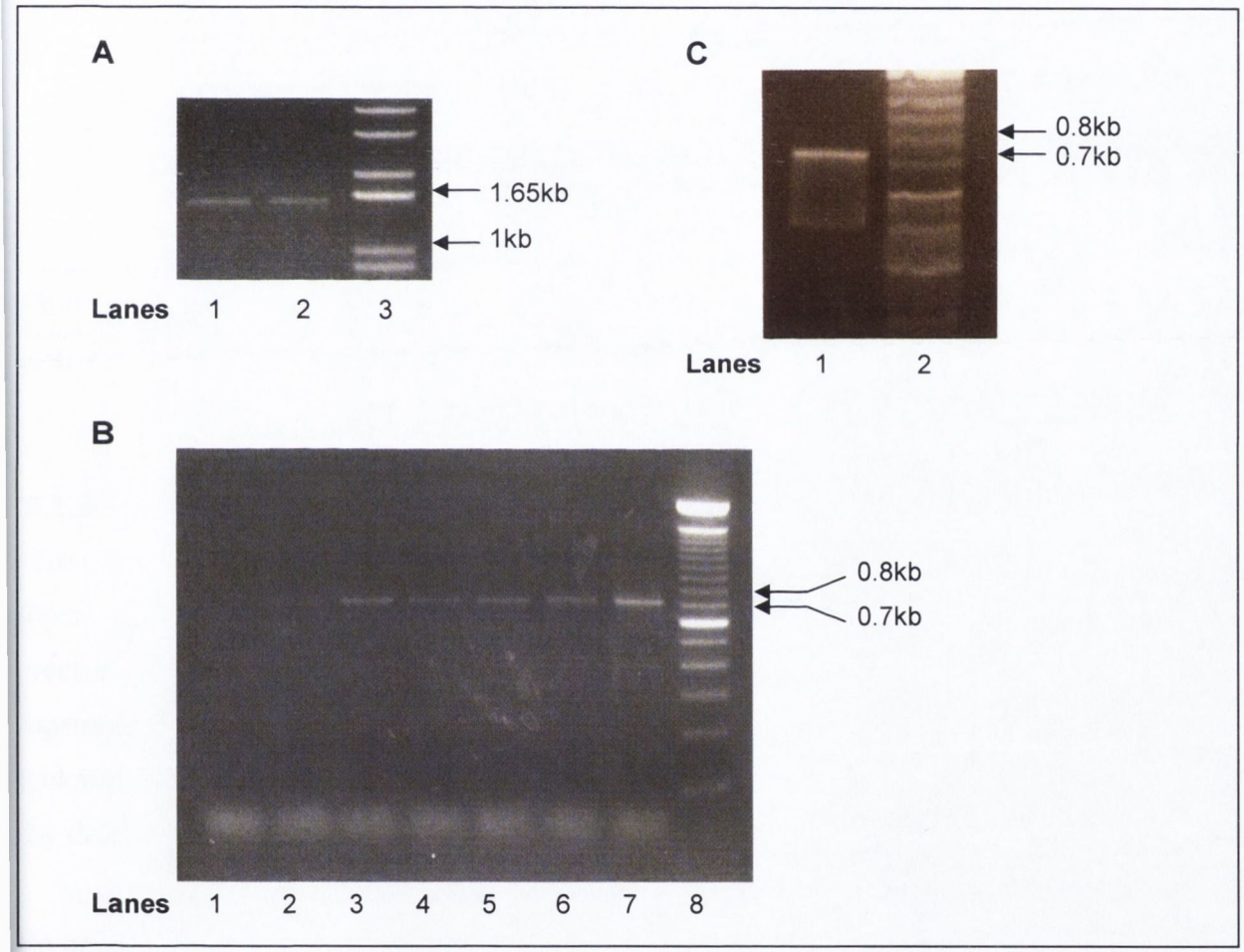


Figure 3.3. PCR of PSA fragments and control insert. A. PSA enhancer. Lanes 1-2: 1.6kb PSA enhancer amplified using PSAP1F and PSAP1R. Lane 3: 1kb Plus size standard. **B.** 722bp PSA promoter amplified using PSAP2F and PSAP2R at the indicated magnesium concentrations. Lane 1: 2mM. Lanes 2-3: 2.5mM. Lanes 4-5: 3mM. Lanes 6-7: 3.5mM. Lane 8: 1kb Plus size standard. **C.** Control for TA cloning. Lane 1: 750bp control PCR product. Lane 2: 1kb Plus size standard.

After ligating the PCR products into a TA cloning vector, pPCR2.1-TOPO, the vector was transformed into *Escherichia coli* TOP10 cells with a transformation efficiency of 9×10^6 cfu/μg (further details of colony counts are provided in Appendix 6).

DNA from six single colonies was prepared by miniprep analysis and screened for the presence of each PCR-amplified PSA fragment, using the restriction enzymes in Table 2.8. pPSA1.3 and four other plasmids contained the PSA enhancer, while pPSA2.5 and four others contained the PSA promoter. This was confirmed by PCR analysis as outlined in Section 2.3.1. The 0.7kb PSA promoter (Figure 3.4) or 1.6kb PSA enhancer were PCR amplified from all plasmids and showed the restriction digest pattern predicted for successful clones.

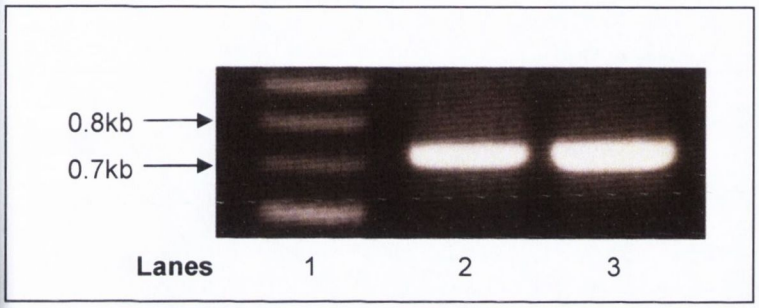


Figure 3.4. PCR of PSA promoter fragment from transformed plasmid. Lane 1: 1kb Plus size standard. Lanes 2-3: 722bp PSA promoter fragment, amplified using primers PSAP2F and PSAP2R from plasmid pPSA2.5, following TA cloning.

3.1.2. Subcloning further plasmid constructs

The PSA promoter was subcloned into pCD1, a pBluescript-derived vector containing the CD open reading frame, making pPSA-CD1.8. The PSA enhancer was then inserted into this vector to produce pPSA-CD4.19, in which PSA enhancer and promoter sequences lie upstream of the CD ORF. This PSA-CD cassette was excised and cloned into pAMdPE1.1, a eukaryotic expression vector, resulting in pPPE-CD2.15. pAMdPE1.1 had been constructed by deleting the CMV promoter and enhancer from pALTER-MAX.

In order to provide a negative clone for subsequent studies, the CD ORF alone was subcloned from pCD1 to pAMdPE1.1 to produce pMinusCD1.24. The CD ORF was also excised from pCD1 and inserted in pALTER-MAX downstream of the CMV promoter as a positive control plasmid, pCMV-CD1.10.

Vectors and inserts were prepared for subcloning these plasmids as described in Section 2.1.2. Figures 3.5 and 3.6 show, as an example, the preparation of vector and insert for cloning pMinusCD1.24.

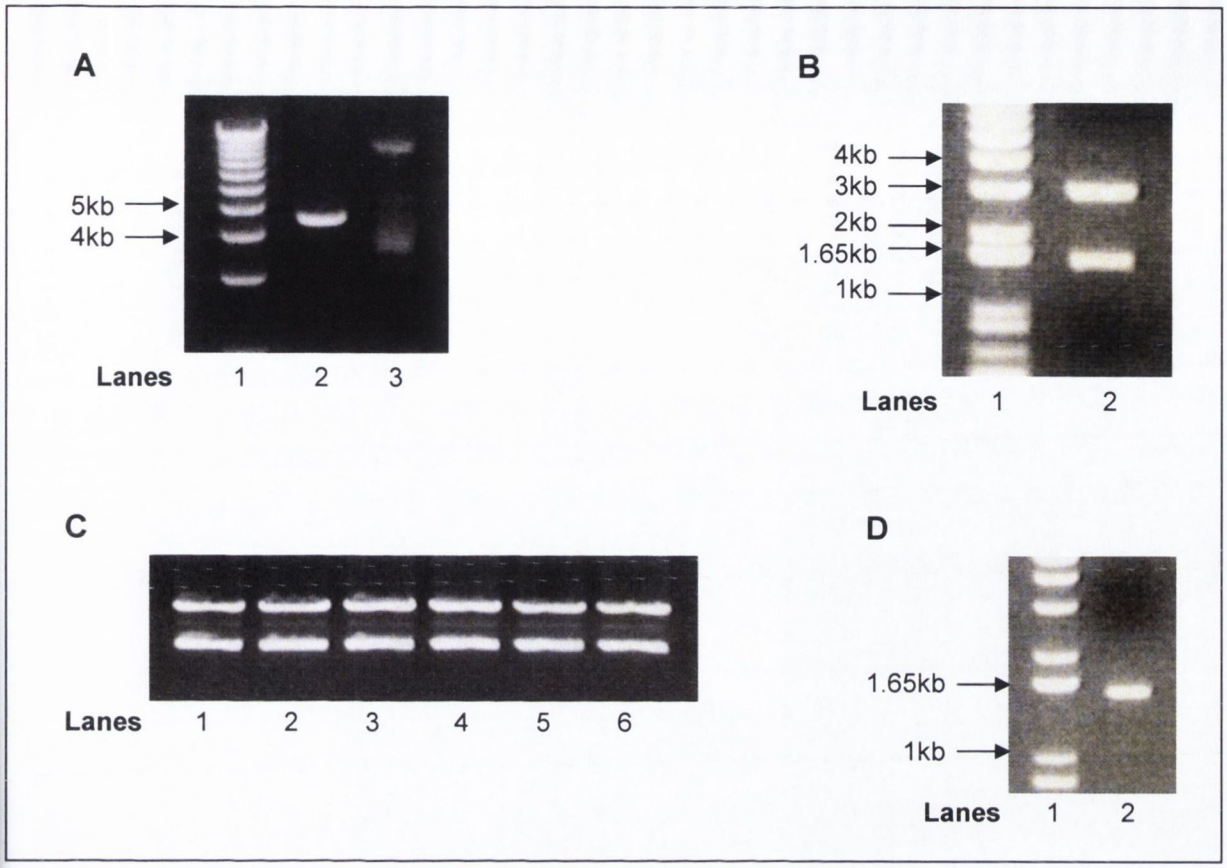


Figure 3.5. Preparation of insert for cloning pMinusCD1.24. A. *Not*I digest of pCD1. Lane 1: 1kb Plus size standard. Lane 2: pCD1, digested with *Not*I to yield a 4.5kb linearised plasmid. Lane 3: pCD1, undigested plasmid. **B.** *Eco*RI digest of pCD1 following digestion with *Not*I. Lane 1: 1kb Plus size standard. Lane 2: pCD1, further digested with *Eco*RI to produce 2.9kb and 1.6kb fragments. **C.** Gel isolation of insert. Lanes 1-6: 2.9kb and 1.6kb *Eco*RI-*Not*I fragments of pCD1, prior to gel isolation of the 1.6kb fragment. **D.** Purification of gel-isolated insert. Lane 1: 1kb Plus size standard. Lane 2: 1.6kb *Eco*RI-*Not*I fragment of pCD1, cleaned using the Wizard DNA clean-up kit.

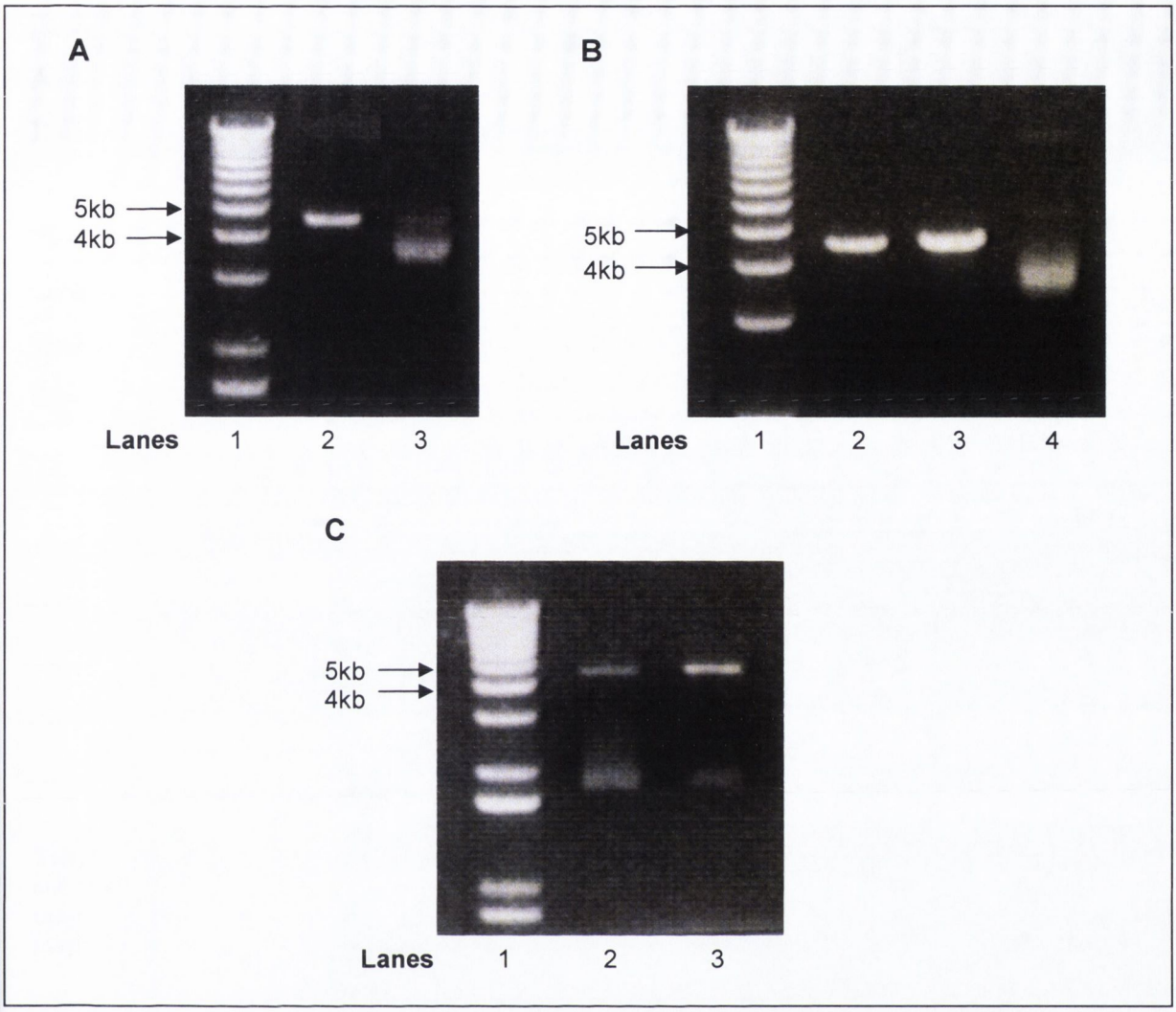


Figure 3.6. Preparation of vector for cloning of pMinusCD1.24. A. Digestion of pAMdPE1.1 with *Not*I. Lane 1: 1kb Plus size standard. Lane 2: pAMdPE1.1, digested with *Not*I to yield a 4.7kb linearised plasmid. Lane 3: pAMdPE1.1, undigested plasmid. **B.** Digestion of pAMdPE1.1 with *Eco*RI, following digestion with *Not*I. Lane 1: 1kb Plus size standard. Lane 2: pAMdPE1.1, further digested with *Eco*RI to produce 4.7kb and 35bp fragments. Lane 3: pAMdPE1.1 digested with *Eco*RI alone. Lane 4: pAMdPE1.1, undigested plasmid. **C.** Purification of vector. Lane 1: 1kb Plus size standard. Lanes 2-3: 4.7kb *Not*I-*Eco*RI fragment from pAMdPE1.1, cleaned using the Wizard DNA clean-up kit.

The origin of the lower band in Figure 3.6C, lane 2 is uncertain. Such a band, electrophoresing faster than the predicted band, was observed to appear several times after ethanol precipitation or processing with the Wizard DNA clean-up system. It migrates too fast to be undigested plasmid, but appears related to the main band. For example, on processing pCD1 (4.5kb) and a 1.6kb restriction fragment of the same plasmid, bands of about 1.6kb and 0.8kb respectively were detected. It is possible that some form of secondary structure is responsible. The presence or absence of this band was seen to vary even between samples processed in parallel. Its presence, however, did not prevent successful cloning of pPPE-CD2.15 and pMinusCD1.24, or preparation of functional *Hin*dIII-digested pCMV-CD1.10 for *in vitro* transcription and translation.

Ligation and transformation into *E. coli* were carried out with the results in Appendix 6. Average transformation efficiency was 5 x 10^7 cfu/μg for TOP10 cells and 2 x 10^5 cfu/μg for JM109 cells.

Plasmid clones with the desired structure were identified by digesting a series of minipreps with suitable restriction enzymes, listed in Table 2.8. 10-24 minipreps were carried out to yield 1-5 correct clones (Table 3.2), except for pAMdPE1.1. Following blunt-end ligation of this vector, all four colonies produced were screened and found to be correct.

Plasmid	Number of colonies screened	Number of correct clones
pPSA-CD1.8	10	1
pPSA-CD4.19	16	5
pPPE-CD2.15	14	1
pCMV-CD1.10	10	1
pMinusCD1.24	24	1

Table 3.2. Screening plasmid minipreps. For each plasmid, a series of single colonies was cultured overnight and plasmid minipreps were carried out, followed by restriction digests to identify clones containing the desired insert (in the desired orientation if relevant). Other clones were self-ligated parent vectors or contained the insert in the alternative orientation, except four clones where digest results were inconclusive.

pMinusCD1.24, for example, was identified using *Eco*RI, *Xba*I and *Xho*I (Figure 3.7).

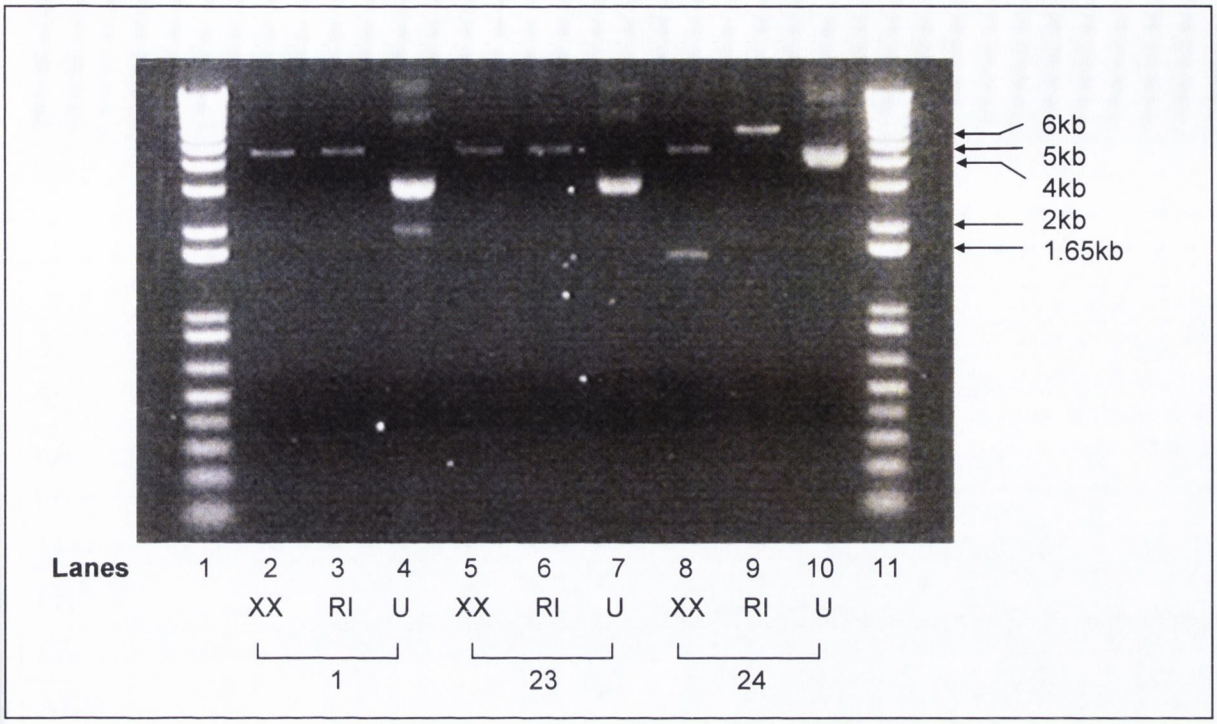


Figure 3.7. Screening pMinusCD clones. Minipreps 1, 23 and 24 were digested with *Xho*I and *Xba*I (XX), or *Eco*RI (RI). Undigested plasmid (U) was also electrophoresed.
Lanes 1 and 11: 1kb Plus size marker. Lane 2: Miniprep 1, digested with *Xho*I and *Xba*I. Lane 3: Miniprep1, digested with *Eco*RI. Lane 4: Miniprep 1, undigested plasmid. Lane 5: Miniprep23, digested with *Xho*I and *Xba*I. Lane 6: Miniprep 23, digested with *Eco*RI. Lane 7: Miniprep 23, undigested plasmid. Lane 8: Miniprep 24, digested with *Xho*I and *Xba*I. Lane 9: Miniprep 24, digested with *Eco*RI. Lane 10: Miniprep 24, undigested plasmid.
Clones in which the vector had self-ligated were expected to yield one 4.7kb band on digestion with either *Xho*I and *Xba*I, or *Eco*RI. Clones containing the insert were predicted to yield 4.7kb and 1.6kb fragments after *Xho*I-*Xba*I digestion, and a 6.2kb band after *Eco*RI digestion.

3.1.3. Sequencing plasmids

Having generated plasmids containing the CD suicide gene downstream of the PSA promoter (pPPE-CD2.15), downstream of the CMV promoter (pCMV-CD1.10), and in a promoterless plasmid (pMinusCD1.24), all the cloning junctions involved were sequenced. The androgen receptor binding sites ARE-I, ARE-II and ARE–III in the PSA promoter and enhancer were also sequenced. Forward and reverse primers were designed for each target.

Plasmids were sequenced using fluorescent dye terminator technology, initially on a 310 Genetic Analyser, but subsequently on a 3100 Genetic Analyser when this was acquired for sequence analysis.

Sequences of cloning junctions in pPPE-CD2.15 were as predicted, except that 1bp (5533T) was deleted during blunt-end cloning. One difference from the Genbank PSA sequence was noted in ARE-I at position 5667 (-158 relative to the transcription start site). Table 3.3 below summarises pPPE-CD2.15 sequencing results.

Target	Primer	Result
*Bgl*II/I-*Ppo*I (see pALTER-MAX map)	A1F2	5533T deleted
5507-866	A1R1	5533T deleted
pALTER-MAX/pCR2.1-TOPO/PSAP2F/PSA promoter	A1F1	As expected
1118 - 5169	A1R1	As expected
PSA promoter/*Bgl*II/ PSAP1F/ PSA enhancer	A1F1	As expected
5271 - 538	A1R3	As expected
ARE-III	B1F1	As expected
1668 - 1699	B1R5	As expected
PSA enhancer/PSAP1R/*Bgl*II/PSA promoter	B1F1	As expected
2050 - 5300	B1R5	As expected
ARE-II	C1F2	As expected
5420 - 5458	C1R1	As expected
ARE-I	C1F1	5667G→A
5643 - 5681		5667G→A
	C1R1	5667G→A
		5667G→A
PSA promoter/pBluescript/pCD1	C1F1	As expected
5821 - 17	C1R1	As expected
pCD1/pBluescript/pALTER-MAX	D1F3	As expected
1513 - 1150	D1R2	As expected

Table 3.3. Results of sequencing pPPECD2.15. Plasmid sequence spanning the target was read between the sequence positions indicated. Where sequence is derived from more than one original plasmid, the sequence positions refer to the first and last source. See Appendix 5 for plasmid maps. PSA sequences were compared to Genbank accession number U37672. pCD1 sequence is provided in Appendix 4 since the native sequence of the CD ORF was altered to improve expression in eukaryotic cells (Mullen *et al* 1992).

An electropherogram from the 3100 Genetic Analyser (Figure 3.8) shows the sequence of pPPE-CD2.15 at ARE-I.

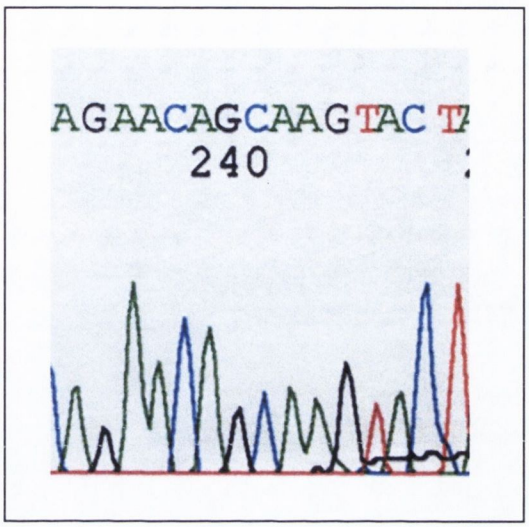


Figure 3.8. Plasmid sequencing data. The sequence of ARE-I (-170 to –156, or 5655-5669) in pPPECD2.15, with the G→A substitution, was obtained using primer C1F1.

Sequences of cloning junctions in pCMV-CD1.10 (Table 3.4) were as predicted.

Target	Sites	Primer	Result
pALTER-MAX/pCD1	1086 - 17	C2F1	As expected
		C1R1	As expected
pCD1/pBluescript/ pALTER-MAX	1513 - 1150	D1F3	As expected
		D1R2	As expected

Table 3.4. Results of sequencing pCMV-CD1.10. Plasmid sequence spanning the target was read between the sequence positions indicated. Where sequence is derived from more than one original plasmid, the sequence positions refer to the first and last source. See Appendix 5 for plasmid maps. pCD1 sequence is provided in Appendix 4 since the native sequence of the CD ORF was altered to improve expression in eukaryotic cells (Mullen *et al* 1992).

Sequences of cloning junctions in pMinusCD1.24 (Table 3.5) were as predicted, except that 1bp (5533T) was deleted during blunt-end cloning.

Target		Primer	Result
*Bgl*II/I-*Ppo*I (see pALTER-MAX map)	5507 - 866	A1F2	5533T deleted
		C1R1	5533T deleted
pALTER-MAX/pCD1	1086 - 17	C2F1	As expected
		C1R1	As expected
pCD1/pBluescript/ pALTER-MAX	1513 - 1150	D1F3	As expected
		D1R2	As expected

Table 3.5. Results of sequencing pMinusCD1.24. Plasmid sequence spanning the target was read between the sequence positions indicated. Where sequence is derived from more than one original plasmid, the sequence positions refer to the first and last source. See Appendix 5 for plasmid maps. pCD1 sequence is provided in Appendix 4 since the native sequence of the CD ORF was altered to improve expression in eukaryotic cells (Mullen *et al* 1992).

The entire PSA promoter region from –541 forward was also sequenced in duplicate as three contigs (Figure 3.9).

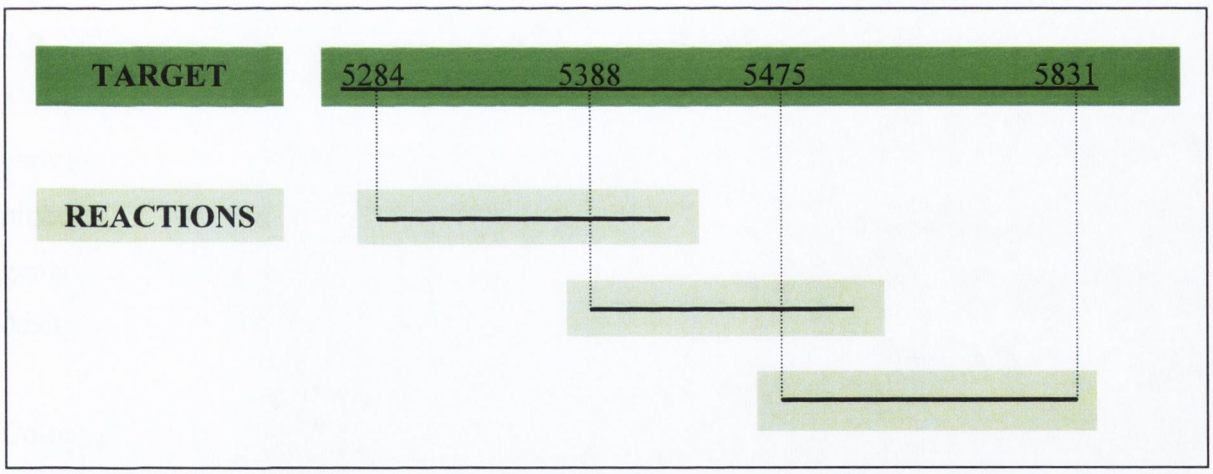


Figure 3.9. Contigs used to sequence PSA promoter. Target positions refer to Genbank sequence U37672 (from -541 to +6). Overlapping sequence at least 12bp long was obtained where each contig joined the next.

When the sequence obtained was compared to the Genbank PSA promoter sequence, the changes detailed in Figure 3.10 were observed.

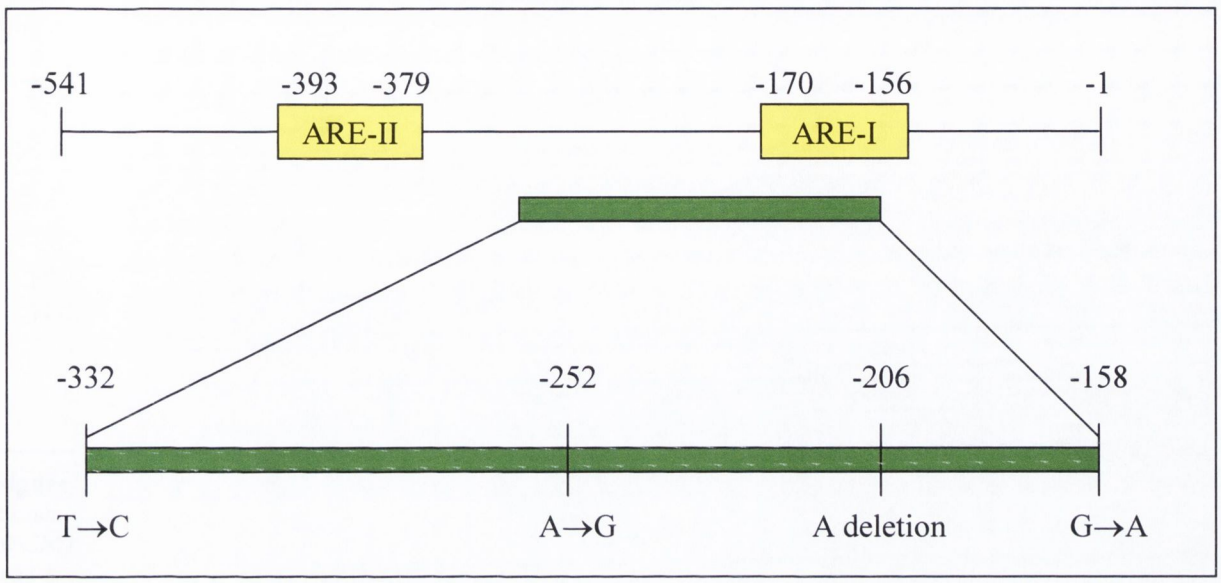


Figure 3.10. Sequence changes identified in PSA promoter region. Four changes were observed between –158 and –332.

3.2. Mutagenesis

As outlined in "Discussion" below, some variation exists among PSA promoter sequences at various sites, including, interestingly, ARE-I. Since the effect of these polymorphisms or mutations on promoter activity is not known, we carried out site-specific mutagenesis to generate two derivatives of pPPE-CD2.15, differing only by 1bp in or adjacent to the ARE-I, designated pPCM2.3 and pPCM1.24 (see Table 3.6).

Colonies were counted after each series of transformations with the results in Appendix 6. Transformation efficiency averaged 4 x 10^5 cfu/μg for JM109 cells and 2 x 10^7 cfu/μg for BMH71-18 *mut*S cells.

The restoration of an *Nhe*I restriction site was used to screen for successful mutagenesis at position –158 (Figure 3.11). Twenty-four colonies were miniprepped and digested, with one (pPCM1.24) containing the restored site.

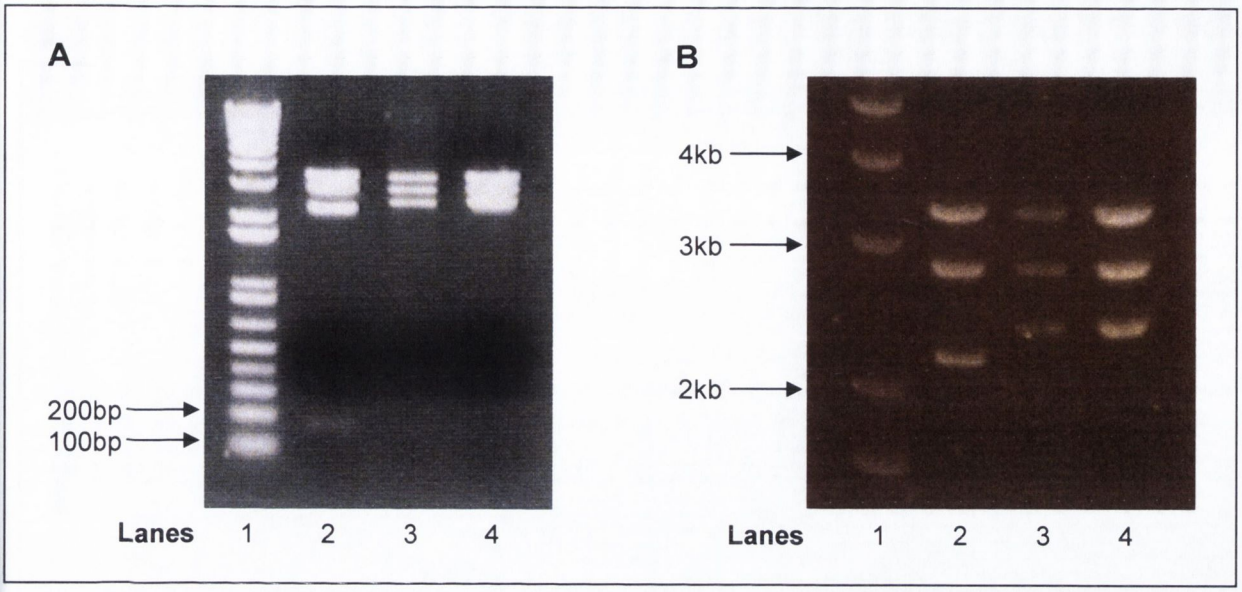


Figure 3.11. Restoration of restriction site by mutagenesis. Plasmids were digested with *Nhe*I to screen for clones which had undergone mutagenesis. Lane 1: 1kb Plus size marker. Lane 2: Successful mutagenesis of pPCM1.24 resulted in 185bp, 2.2kb, 2.8kb and 3.2kb fragments. Lane 3: pPPE-CD2.15, from which pPCM1.24 was derived, was digested to yield 2.4kb, 2.8kb and 3.2kb fragments. Lane 4: pPCM1.12, in which mutagenesis was unsuccessful, showed the restriction pattern of the parental plasmid. The same gel is shown after electrophoresis for times suitable for clearly visualising the 185bp fragment (**A**) and fragments between 2.2kb and 3.2kb (**B**).

pPCM2.3 was identified as the only clone containing the desired A→G substitution at position -155 by sequencing plasmid midipreps of six colonies.

The predicted sequence changes were confirmed by sequencing pPCM1.24 and pPCM2.3 using forward and reverse primers in duplicate at ARE-I (Table 3.6).

Source	-158	-155
pPPE-CD2.15	A	A
pPCM1.24	**G**	A
pPCM2.3	A	**G**
Genbank U37672	G	A

Table 3.6. PSA promoter sequence at positions -158 and -155. Changes during mutagenesis are in bold.

Figure 3.12 shows the sequence of ARE-I in the three plasmids containing the PSA promoter.

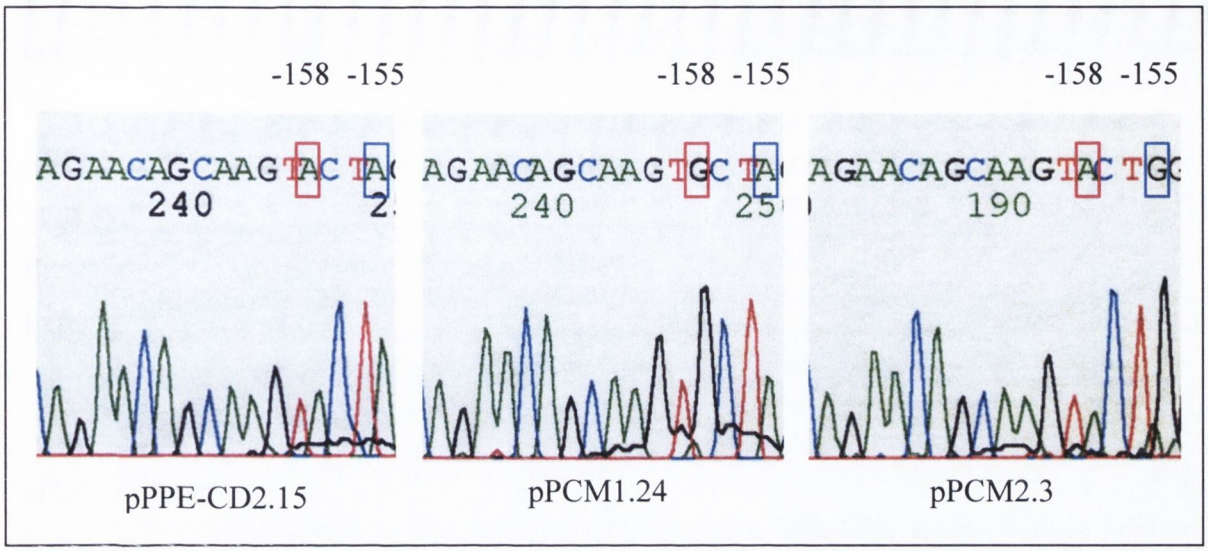


Figure 3.12. PSA promoter sequence data. Readouts from the 3100 Genetic Analyser of ARE-I (-170 to –156) and the –155 polymorphism are shown for each PSA promoter variation.

DISCUSSION

0.7kb of PSA promoter sequence and 1.6kb of PSA upstream enhancer sequence were PCR amplified from human genomic DNA and ligated into cloning vectors. Further subcloning and mutagenesis generated CD expression plasmids driven by three different PSA promoters, and control plasmids with CD downstream of a constitutive promoter and in a promoterless construct. Fluorescent dye-terminator sequencing confirmed that the cloning junctions in these plasmids were as expected and revealed previously reported and unique differences between the PSA sequence obtained and the Genbank PSA sequence.

When the PSA sequence obtained was compared to the Genbank sequence, four changes (Figure 3.10) were observed. Other authors have scanned this region for mutations, with some similar findings. -332T→C was the only substitution detected by sequencing but not found in any published paper. It is not clear how much effect the region in which it is situated has on PSA promoter activity. Zhang J *et al* (1997) report that deletion of positions –342 to –320 reduces activity twofold, while Cleutjens *et al* (1996) found that the presence or absence of fragments encompassing positions –375 to –324 made no significant difference to promoter activity.

An A/G polymorphism at -252 has been described in panels of prostate cancer (Yang *et al* 2001) and breast cancer (Yang *et al* 2000) patients. An A/AA polymorphism at -205 also occurs among breast and prostate cancer patients and controls (Yang *et al* 2000, Yang *et al* 2001). Incidentally, there appears to be a mutation hotspot between -209 and –205, where at least four variations exist. Linkage has been observed between polymorphisms at -252 and -205 (Yang *et al* 2000, Yang *et al* 2001).

The best studied polymorphic site in the PSA promoter, possibly due to its location in the key transcriptional control region of ARE-I, is at –158, and this A/G polymorphism may be functionally significant (Xue *et al* 2000). Adjacent to ARE-I, an A→G mutation at -155 was detected in two breast tumours which overexpressed PSA (Majumdar & Diamandis 1999). We generated the plasmids pPCM1.24 and pPCM2.3 by site-directed mutagenesis of pPPE-CD2.15 at -158 and -155. This set of plasmids, in which variant PSA promoters regulate the CD gene, was designed to allow the effect of these sequence alterations to be examined *in vitro* (Chapters 5-6). PSA promoter polymorphisms are discussed further in Chapter 8.

CHAPTER FOUR: OPTIMISATION OF TRANSFECTION CONDITIONS

INTRODUCTION

Since this study required the expression of the transgene CD in human prostate cancer cells, transfection of CD expression plasmids into human cells *in vitro* was of key importance. At the time of these experiments, no detailed data was available on optimal transfection reagents or conditions for this purpose. A significant number of experiments was therefore necessary to establish suitable transfection conditions for all the cell lines used (Table 4.1). Transfection conditions were also optimised for the DU145 cell line, though it was not used for further experiments in this study. It was acquired as one of very few human prostate cancer cell lines, either PSA-positive or negative, which were commercially available at the time. However, when the PSA-positive 22Rv1 cell line subsequently became available, it offered a more suitable model than PSA-negative DU145 cells for experiments involving the PSA promoter.

Cell line	Origin	PSA status	AR status
LNCaP	Lymph node metastasis of prostate cancer	+	+
22Rv1	Primary prostate cancer	+	+
PC-3	Bone metastasis of prostate cancer	-	-
DU145	Brain metastasis of prostate cancer	-	-
HCT116	Primary colon cancer	-	-

Table 4.1. Properties of cell lines. Transfection conditions were optimised for the cell lines above, which were all derived from human tumours at the indicated sites. PSA and AR status in several human prostate cancer cell lines, including those listed above, has recently been examined (van Bokhoven *et al* 2003). The published literature has not reported the PSA and AR status of HCT116 cells. However, the CGAP website (Boon *et al* 2002) indicates that HCT116 cells do not express either AR or PSA as shown by SAGE analysis.

Cells were transiently transfected using a variety of liposomes (LipofectAmine and related products, Transfast, GenePorter2, Lipofectin) or cationic polymers Superfect and GeneJuice (described in Table 4.2). Liposomes consist of one or more lipids which form multimolecular particles when suspended in media. Cationic lipids

facilitate the formation of complexes with negatively charged DNA. Neutral lipids such as L-dioleoyl phosphatidylethanolamine (DOPE) are also used in conjunction with cationic lipids to increase their efficacy (Felgner *et al* 1994). The lipid bilayer structure of the complexes allows them to cross the cell membrane.

Cationic polymers with various compositions and structures are available. Polylysine (Xiang *et al* 2003) has a straight backbone, while polyethyleneimine (Frønsdal *et al* 1998) consist of a highly branched but not precisely defined network. Dendrimers are macromolecules with branches radiating out of a central core to form sphere-like structures, and can be designed with positively charged amino or other groups at the ends of the many branches to produce polycationic molecules such as polyamidoamine (Nakanishi *et al* 2003). Cationic polymers are capable of binding both to DNA and to cell surface receptors. They can also reduce endosomal degradation of DNA, and can be linked to ligands for cell surface receptors such as extracellular growth factor (Ogris *et al* 2003), to enhance uptake into cells or to target specific cells.

Table 4.3 summarises the reagents tested in each cell line. Transfections were performed under different assay conditions to optimise DNA concentration, carrier concentration, dilution reagent, transfection time and assay time. For each cell line, a reagent was required which would transfect cells efficiently and with minimal damage. During optimisation, cells were transfected with pEGFP-NI, a plasmid which constitutively expressed GFP (Tsien 1998), and examined by fluorescent microscopy to provide a quantitative measure of transfection efficiency.

Reagent	Description
Transfast	Cationic lipid (+)-N,N [bis (2-hydroxyethyl)-N-methyl-N-[2,3-di(tetradecanoyloxy)propyl] ammonium iodide Neutral lipid DOPE
GenePorter2	Proprietary cationic lipid Neutral lipid DOPE
LipofectAmine	Cationic lipid 2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propaniminium trifluoroacetate (DOSPA) Neutral lipid DOPE
LipofectAmine 2000	Proprietary lipid formulation
LipofectAmine Plus	Proprietary lipid formulation
Lipofectin	Cationic lipid N-[1-(2,3-dioleyloxy)propyl]-n,n,n-trimethylammonium chloride (DOTMA) Neutral lipid DOPE
GeneJuice	Cationic polyamine dendrimer complexed with cellular protein
Superfect	Cationic polyamidoamine dendrimer (PAMAM)

Table 4.2. Composition of transient transfection reagents used. Detailed information on GenePorter2, LipofectAmine 2000, LipofectAmine Plus and GeneJuice was not available from the manufacturers as it was considered proprietary.

	LNCaP	22Rv1	PC-3	DU145	HCT116	Primary cells
Transfast	*	*	#	#	#	-
GenePorter2	#	#	*	#	-	-
LipofectAmine 2000	-	-	-	-	-	#
LipofectAmine	#	-	#	#	-	-
LipofectAmine Plus	-	-	#	#	-	-
Lipofectin	#	-	-	-	-	-
GeneJuice	-	-	*	-	*	-
Superfect	#	-	#	#	-	-

Table 4.3. Summary of transfection reagents used in each cell line. In each cell line, each reagent is classed as tested (#), optimised and used in later experiments (*), or not tested (-).

RESULTS

4.1. Optimisation of conditions for transfection of DU145 cell line

4.1.1. LipofectAmine and LipofectAmine Plus

Initial studies focused on two parameters, the concentration of LipofectAmine (2, 10 or 25µl per well) and timing of incubation. Results indicated a maximum of 7.5% transfection efficiency (Figure 4.1).

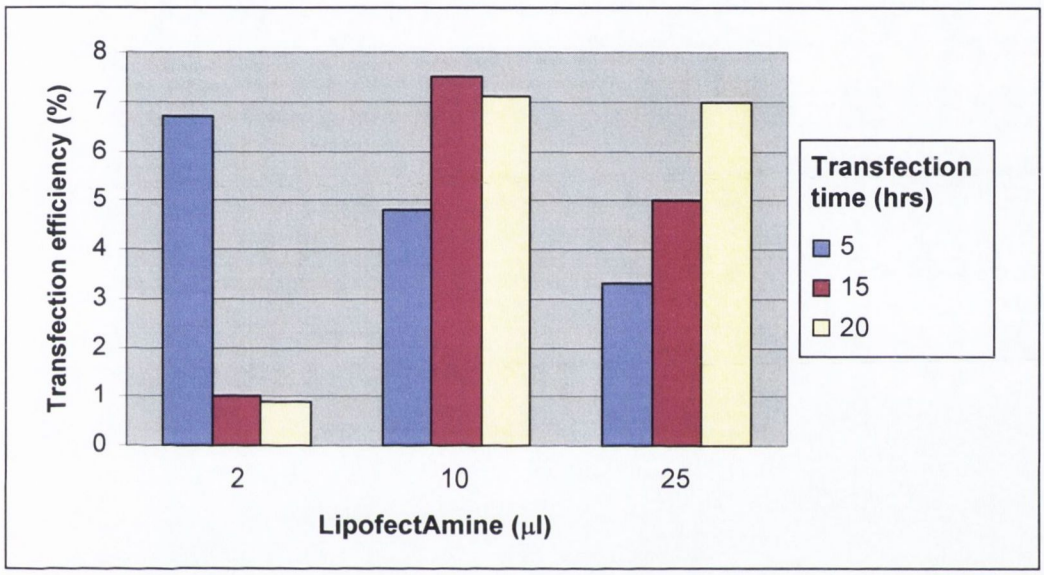


Figure 4.1. Transfection of DU145 cells using LipofectAmine. 80% confluent cells were transfected in 35mm wells with 1µg pEGFP-N1 and the indicated volumes of LipofectAmine, for the indicated times. Cells were fixed 72h after beginning transfection.

Subsequently, DU145 cells were transfected with LipofectAmine and LipofectAmine Plus (which does not facilitate transfection on its own but is designed to enhance the efficiency of LipofectAmine). Amounts of both reagents and of plasmid pEGFP-NI were varied, in addition to the time for which cells were grown between transfection and fixation. 19-24% efficiency was obtained in cells transfected with 1μg DNA, 20μl LipofectAmine and 6-20μl LipofectAmine Plus, and fixed 72h after transfection, compared to up to 12.5% in other wells (Figure 4.2A). Two to threefold more cells were GFP-positive after 72h than after 24h.

In order to increase transfection efficiency and to develop a more convenient assay, the procedure was modified, using more confluent cells (80% rather than 60%), and performing the transfection in $2cm^2$ wells rather than in 35mm wells on coverslips. Using this approach, there was an increase in transfection efficiency to 20.9% for cells transfected with LipofectAmine alone (Figure 4.2B), although combination of the two transfection reagents did not lead to an increase in transfection efficiency in this assay system.

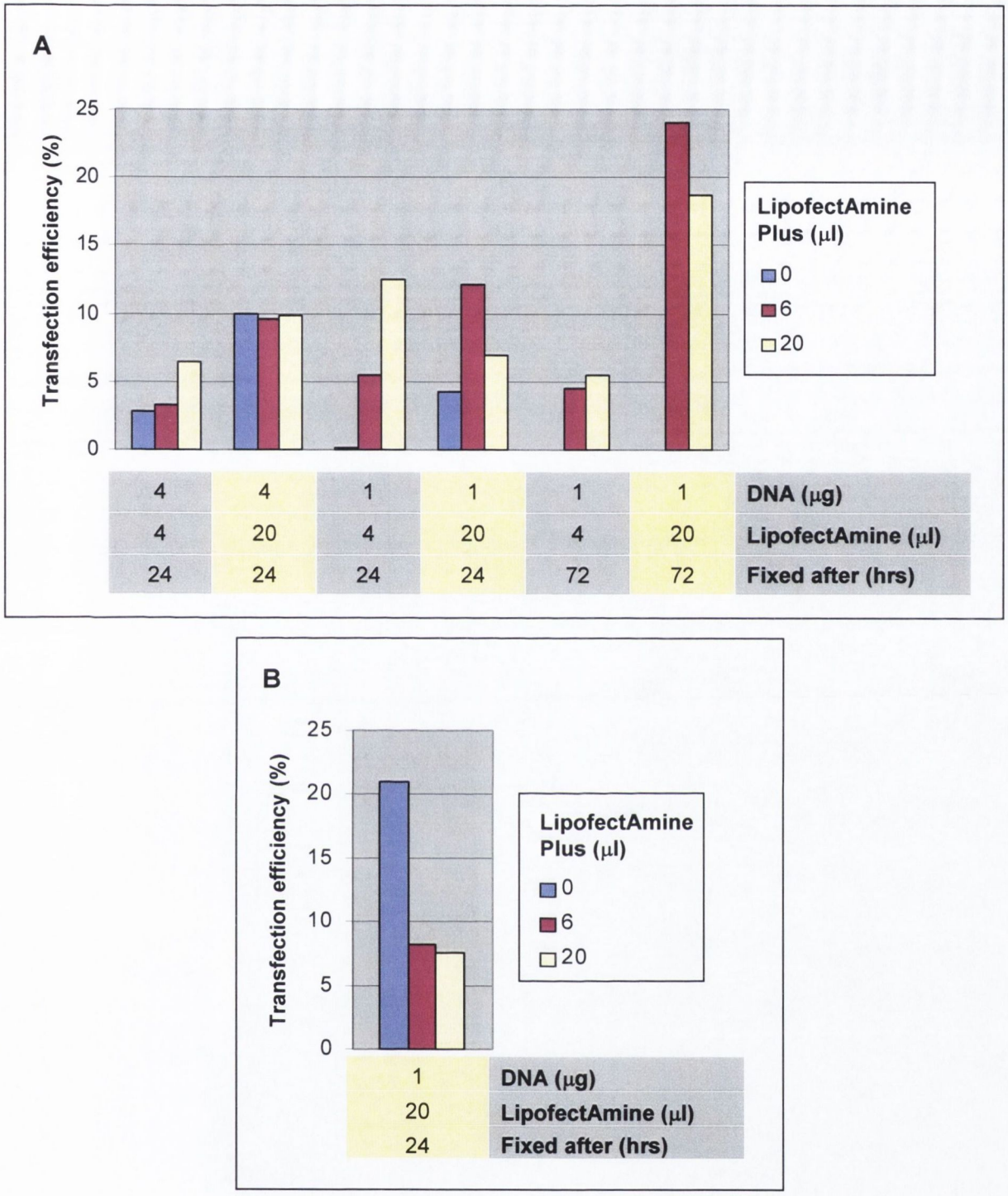


Figure 4.2. Transfection of DU145 cells using LipofectAmine and LipofectAmine Plus. A. 60% confluent cells were transfected in 35mm wells, for 3 hours, under various conditions as indicated above. All cells fixed after 72h were transfected using both LipofectAmine and LipofectAmine Plus. **B.** 80% confluent cells were transfected in $2cm^2$ wells, for 3 hours, under the conditions indicated above. Reagent amounts shown above were per 35mm well for ease of comparison with Figure 4.2A, though the actual volumes used per $2cm^2$ wells were scaled down proportionately.

4.1.2. GenePorter2

A small-scale transfection of DU145 cells using GenePorter2 and varying the plasmid concentration gave transfection efficiencies of less than 2% (Figure 4.3). This reagent was not tested further with this cell line, due to the extremely low efficiency.

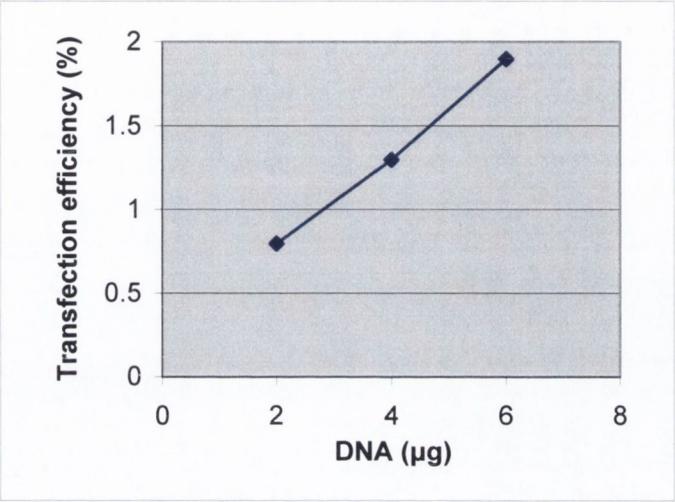


Figure 4.3. Transfection of DU145 cells usingGenePorter2. 70% confluent cells were transfected with pEGFP-NI in 35mm wells for 5 hours. 4.5µl GenePorter2 (recommended range 3-6µl) per µg DNA was used. Cells were fixed 24h after beginning transfection.

4.1.3. Superfect

This reagent initially transfected up to 8% of DU145 cells with pEGFP-N1 (Figure 4.4). Transfection under identical conditions with 2µg plasmid and 10µl Superfect resulted in 10.3% efficiency. Increasing amounts of DNA and Superfect generally improved efficiency but were also more toxic to the cells. 27% fewer cells were GFP-positive after 72h than after 24h, in contrast to results obtained during LipofectAmine/LipofectAmine Plus optimisation (Figure 4.2A).

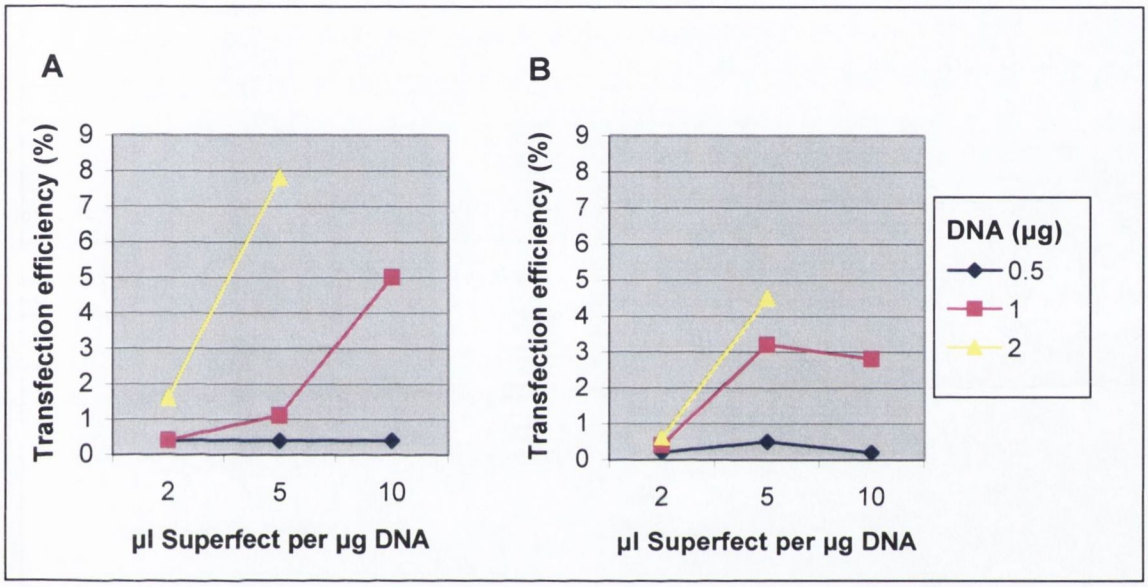


Figure 4.4. Transfection of DU145 cells using Superfect. 80% confluent cells were transfected in 2cm^2 wells, for 3 hours, under various conditions as indicated above, and scored for GFP expression after 24h (**A**) and 72h (**B**).

4.1.4. Transfast

This reagent initially transfected up to 6% of DU145 cells with pEGFP-N1 (Figure 4.5). 17% fewer cells were GFP-positive after 72h than after 24h (as for Superfect though not for LipofectAmine). Best results were obtained using 1μg plasmid and 6μl Transfast. This combination was repeated twice in duplicate under identical conditions, except that transfection was extended from 1h to 3h, to give dramatically increased efficiency of 30.5% and 33.1% after 24h.

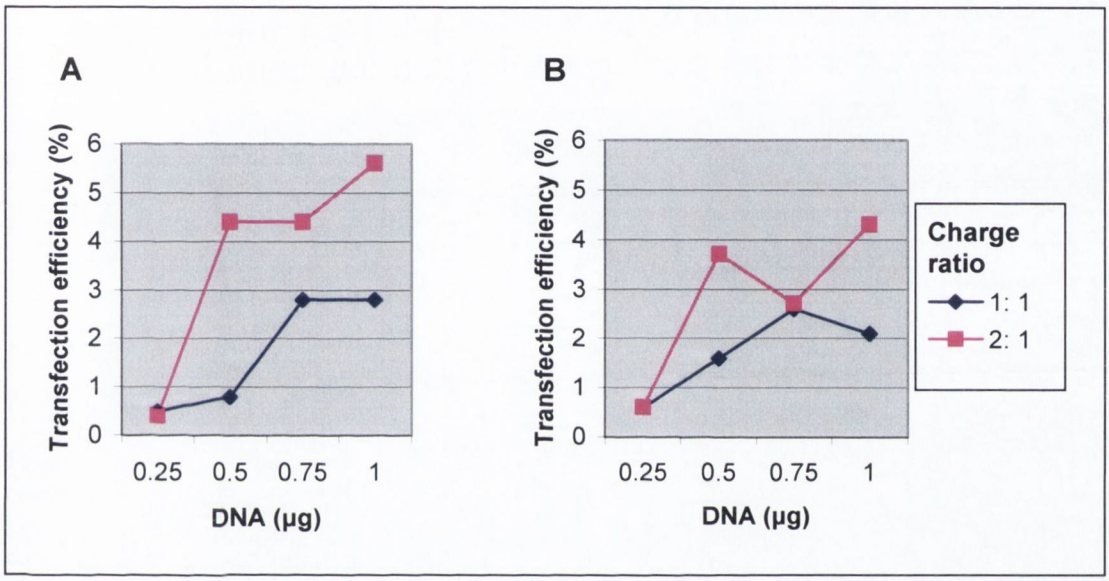


Figure 4.5. Transfection of DU145 cells usingTransfast. 80% confluent cells were transfected in 2cm^2 wells, for 1 hour, under various conditions as indicated above, and scored for GFP expression after 24h (**A**) and 72h (**B**). Charge ratios of 1:1 and 2:1 corresponded to 3μl and 6μl respectively of Transfast per μg of DNA.

Having established optimum conditions for this cell line, DU145 cells were then transfected with reporter plasmid pEGFP-NI and various CD expression plasmids at a 1:9 ratio (Figure 4.6). 0.1μg pEGFP-NI was transfected, unexpectedly, less efficiently on its own than in the presence of other plasmids, though 1μg of reporter plasmid again transfected over 30% of cells.

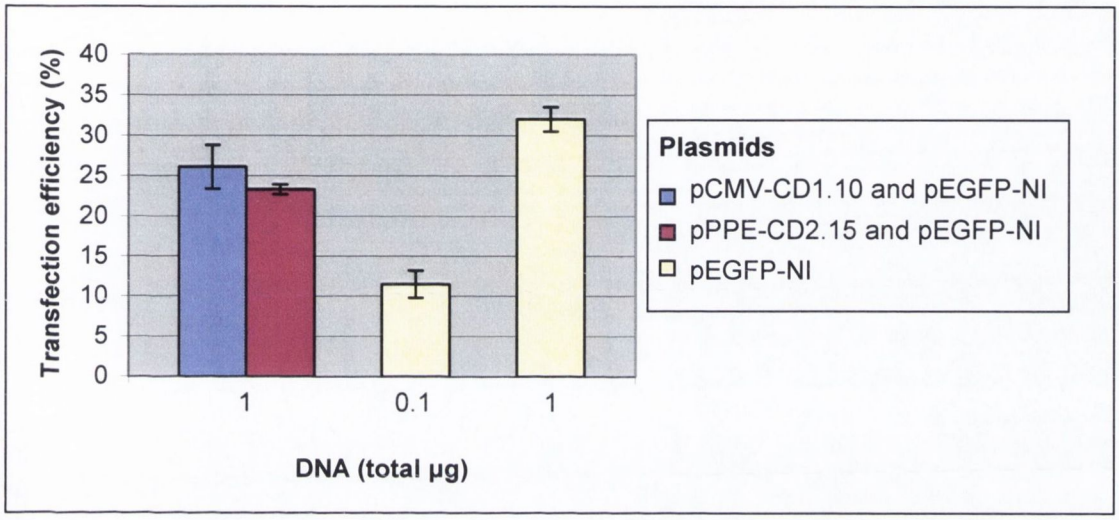


Figure 4.6. Transfection of DU145 cells with reporter and expression plasmids. 80% confluent cells were transfected in $2cm^2$ wells for 3 hours, using 6μl Transfast and the indicated amounts of plasmid in duplicate, and scored for GFP expression after 24h. Reporter plasmid pEGFP-NI was combined with each expression plasmid at a 1:9 ratio, i.e. 0.1μg reporter plasmid and 0.9μg expression plasmid per well.

4.2. Optimisation of conditions for transfection of PC-3 cell line

4.2.1. LipofectAmine and LipofectAmine Plus

PC-3 cells were transfected using LipofectAmine, varying the volume of liposome and transfection time as in Figure 4.7. Optimal transfection efficiency, 19%, was obtained using 10µl LipofectAmine with a transfection incubation time of 14h.

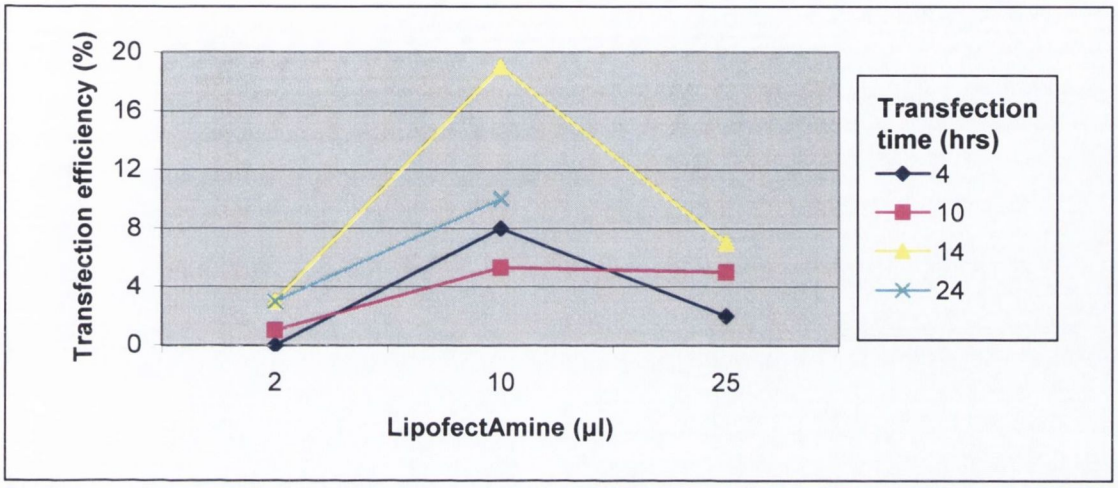


Figure 4.7. Transfection of PC-3 cells using LipofectAmine. 80% confluent cells were transfected in 35mm wells with 1µg pEGFP-N1 and the indicated volumes of LipofectAmine, for the indicated times. Cells were fixed 48h after beginning transfection. Data was not obtained for 25µl LipofectAmine transfected for 24h due to toxicity.

Transfection of PC-3 using various amounts of LipofectAmine and LipofectAmine Plus resulted in up to 10% efficiency (Figure 4.8).

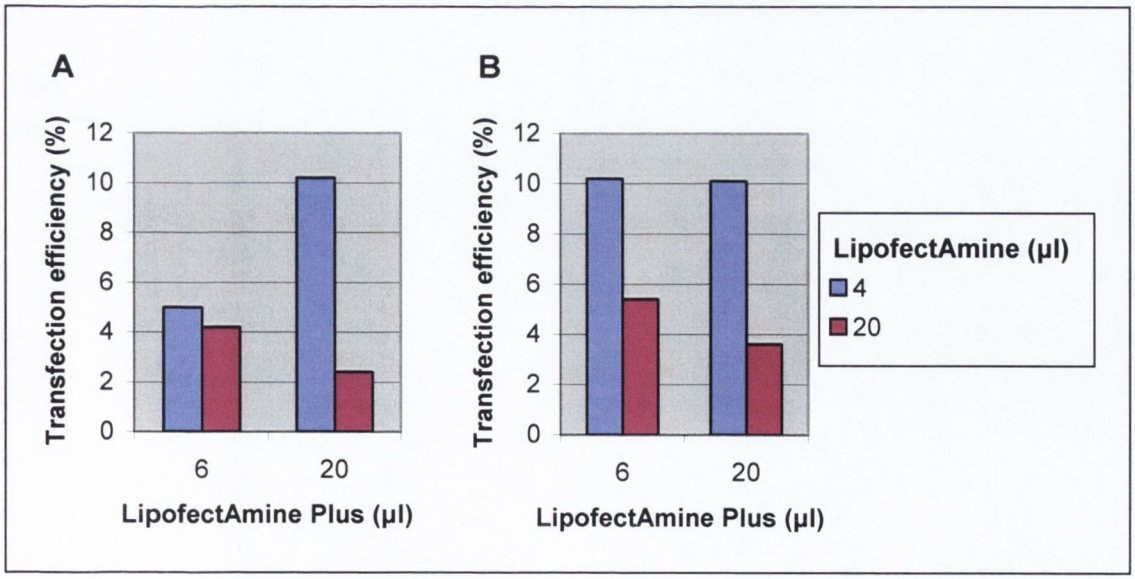


Figure 4.8. Transfection of PC-3 cells using LipofectAmine and LipofectAmine Plus. 80% confluent cells were transfected in 35mm wells with 1µg (**A**) or 4µg (**B**) pEGFP-N1 and the indicated reagent volumes, for 4h. Cells were fixed 24h after beginning transfection.

4.2.2. Superfect

Up to 7% of cells were transfected with pEGFP-N1 using this reagent (Figure 4.9). Higher doses of DNA and Superfect generally increased efficiency, except at the maximum dose of both plasmid and transfection reagent.

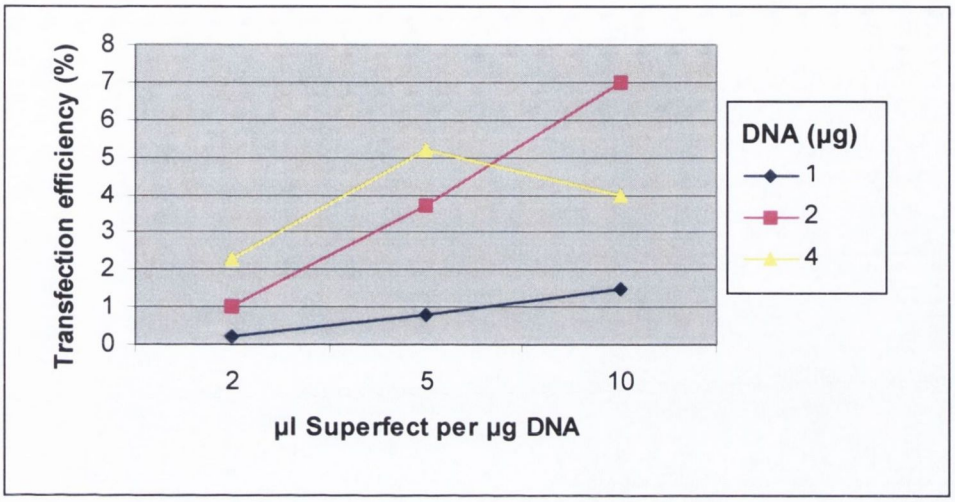


Figure 4.9. Transfection of PC-3 cells using Superfect. 80% confluent cells were transfected in 35mm wells for 3h, using the volumes of plasmid and reagent indicated above. Cells were fixed 24h after beginning transfection.

4.2.3. Transfast

Transfast brought about 20-30% transfection of PC-3 cells, over a range of DNA and liposome concentrations and two different plating densities (Figure 4.10). However, significant toxicity was associated with most of the conditions tested. Cells seeded at the lower density and transfected with 50ng plasmid and 0.3µl Transfast were 40% confluent after 48h, compared to 90% for untransfected control cells, and larger amounts of reagents were even more toxic.

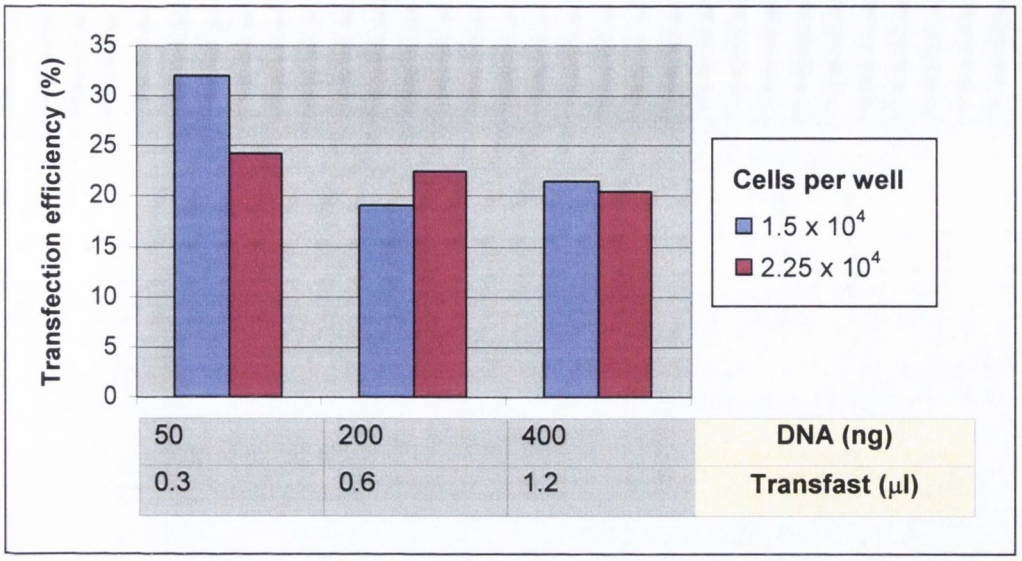


Figure 4.10. Transfection of PC-3 cells using Transfast. 70% and 80% confluent cells were transfected in 96-well plates for 3h, using the amounts of plasmid and Transfast indicated above in duplicate. GFP expression was examined 48h after transfection.

4.2.4. GenePorter2

PC-3 cells were transfected with various amounts of GenePorter2 and pEGFP-N1 as in Figure 4.11A, with up to 29% efficiency. The three best combinations were then repeated in duplicate (Figure 4.11B), two of which transfected 19-25% of cells. Best results were obtained using 6µg plasmid and 36µl liposome per 35mm well (corresponding to 1.2µg and 7.2µl per $2cm^2$ well).

Cells transfected with 2µg plasmid and 6µl carrier were counted 72h as well as 24h after transfection, resulting in threefold higher efficiency (20%), but also greater toxicity (over 70% of cells had de-adhered from the growth surface).

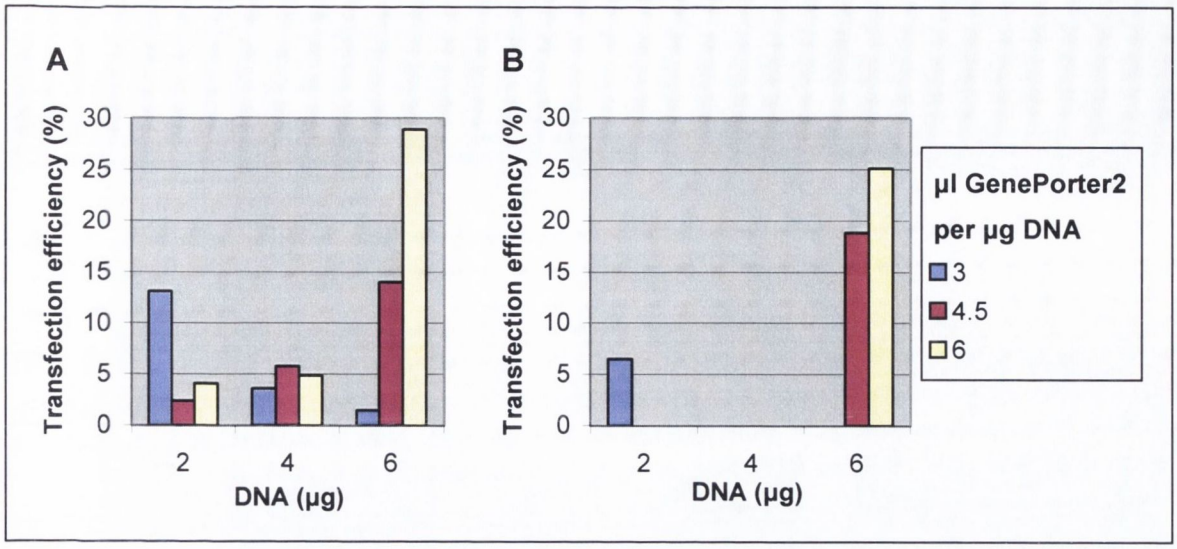


Figure 4.11. Transfection of PC-3 cells usingGenePorter2. 80% confluent cells were transfected in 35mm wells for 3h, using the volumes of plasmid and reagent indicated above. **A.** Three lipid-DNA ratios and three DNA amounts were tested. **B.** The most efficient combinations were repeated. Cells were fixed 24h after beginning transfection.

Having established optimum conditions for this cell line, PC-3 cells were then transfected with reporter plasmid pEGFP-NI and various CD expression plasmids at a 1:9 ratio. Transfection efficiency of pEGFP-NI was increased from almost 30% using 6µg in 35mm wells (Figure 4.11) to over 40% using 1.2µg in $2cm^2$ wells (Figure 4.12), under otherwise identical conditions. 0.12µg pEGFP-NI was transfected slightly more efficiently on its own than in the presence of other plasmids.

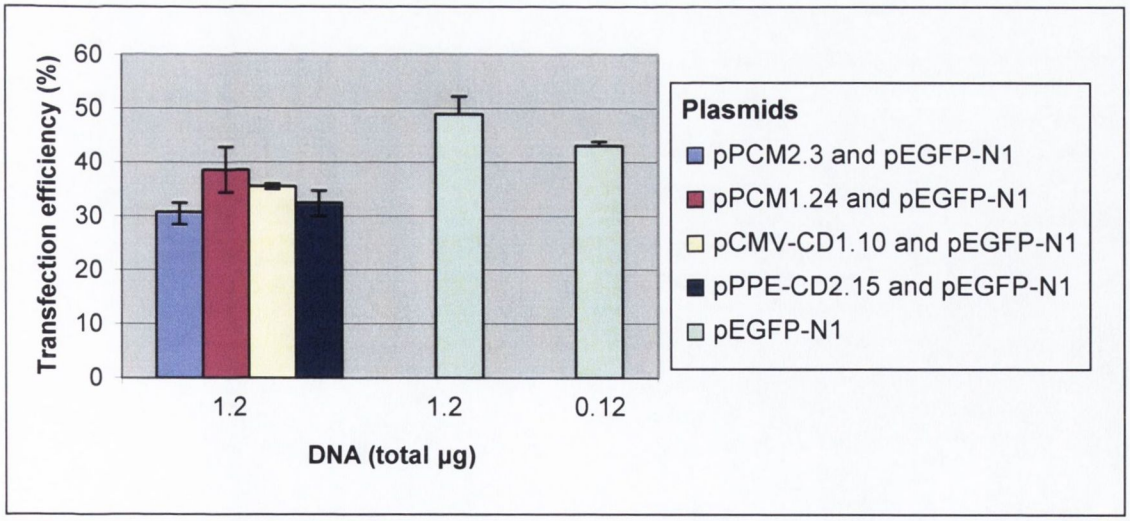


Figure 4.12. Transfection of PC-3 cells with expression and reporter plasmids. 80% confluent cells were transfected in $2cm^2$ wells for 3h, using 7.2µl GenePorter2 and the amounts of DNA indicated above in duplicate. Cells were assayed for GFP expression 24h after beginning transfection. Reporter plasmid pEGFP-NI was combined with each expression plasmid at a 1:9 ratio, i.e. 0.12µg reporter plasmid and 1.08µg expression plasmid per well.

Two different diluent combinations for PC-3 transfection were tested. OPTIMEM-I, which had been used during previous optimisation experiments, was found to be slightly superior (Figure 4.13).

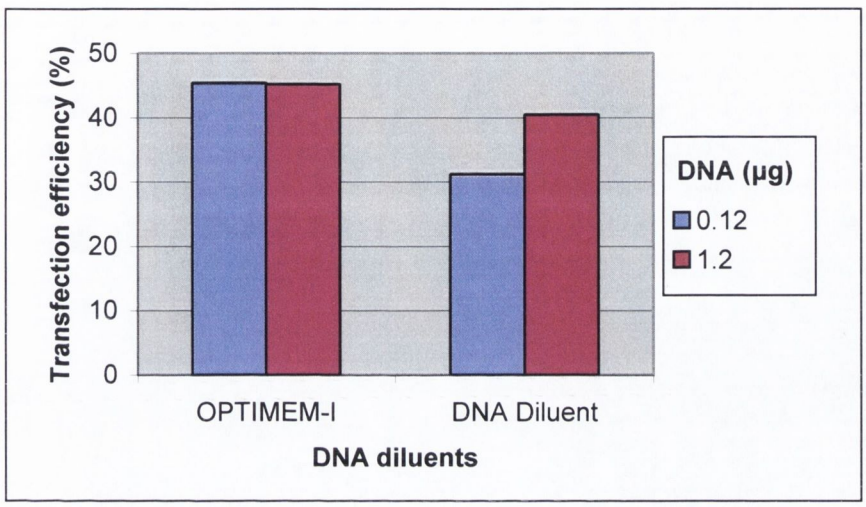


Figure 4.13. Transfection of PC-3 cells usingGenePorter2 and varying diluents. 80% confluent cells were transfected in 2cm^2 wells for 3h, using 7.2µl GenePorter2 and the amounts of plasmid indicated above in duplicate. Cells were fixed 24h after beginning transfection. OPTIMEM-I was used to dilute both plasmid and liposome. DNA Diluent, supplied with GenePorter2, was used as recommended to dilute plasmid only, while GenePorter2 was diluted in RPMI1640 medium.

4.2.5. GeneJuice

PC-3 cells were transfected with GeneJuice using the amounts of cells, DNA and liposome shown below (Figure 4.14).

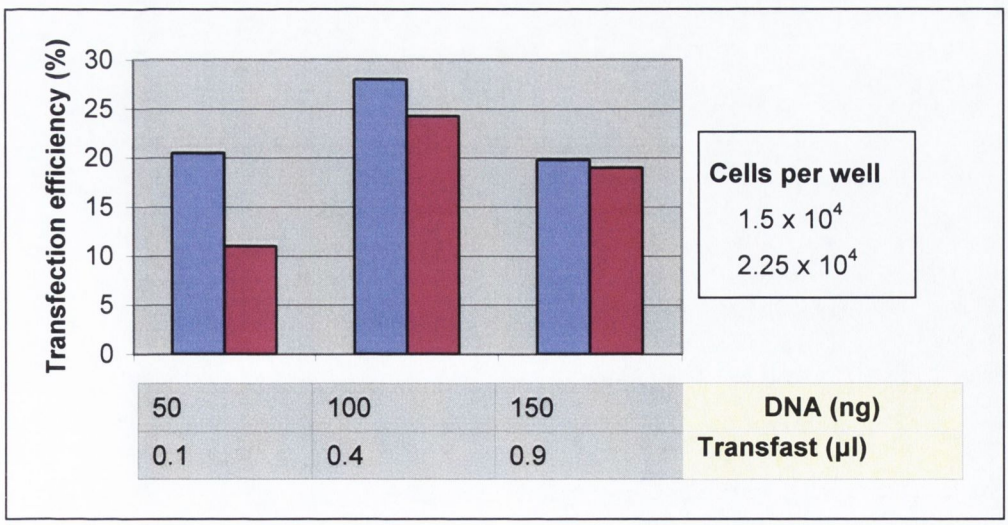


Figure 4.14. Transfection of PC-3 cells usingGeneJuice. 70% and 80% confluent cells were transfected in 96-well plates, using the amounts of plasmid and GeneJuice indicated above in duplicate. Cells were transfected in the presence of serum. Reagents were diluted in OPTIMEM-I. GFP expression was examined 48h after transfection.

Optimal transfection (28%) was achieved by 100ng plasmid and 0.4µl GeneJuice after seeding 1.5 x 10^4 cells per well. Under these conditions, no reduction in confluence compared to untransfected controls was seen by light microscopy, suggesting that GeneJuice causes very little toxicity unlike most such reagents.

PC-3 cells were then co-transfected with the reporter plasmid pEGFP-NI and the CD expression plasmid pCMV-CD1.10, at a 1:4 ratio, using GeneJuice. This resulted in similar transfection efficiency to pEGFP-NI alone at the optimised amount of 100ng DNA per well (Figure 4.15).

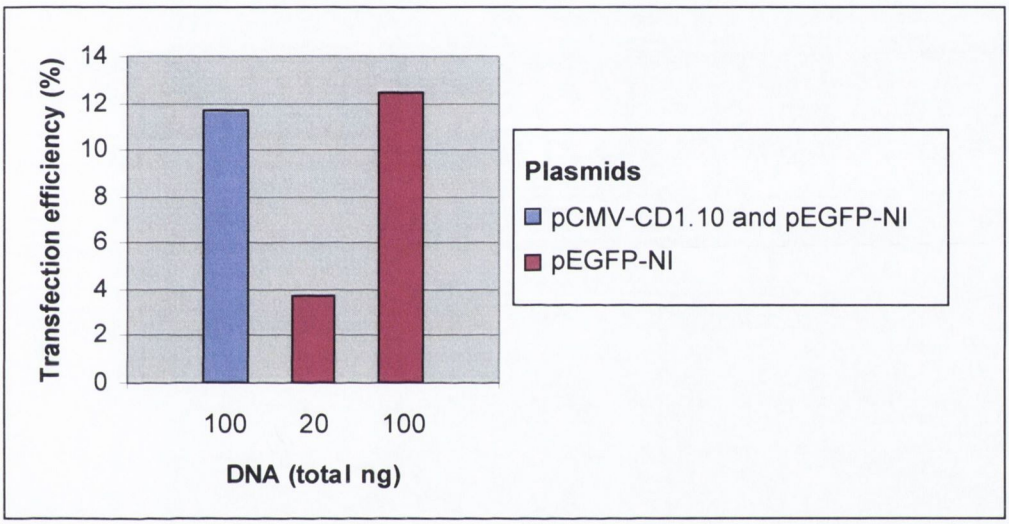


Figure 4.15. Transfection of PC-3 cells with reporter and expression plasmid using GeneJuice. 80% confluent cells were transfected in 96-well plates, using 0.4µl GeneJuice and the indicated amounts of plasmid in duplicate, in the presence of serum. Cells were scored for GFP expression after 48h. Reporter plasmid was combined with expression plasmid at a 1:4 ratio.

4.3. Optimisation of conditions for transfection of LNCaP cell line

4.3.1. LipofectAmine

LipofectAmine was much less efficient in LNCaP cells than in the other two cell lines. Cells transfected with 0.2-0.4μg plasmid and 0.4-5μl liposome were 0.1-3.1% GFP-positive (Figure 4.16).

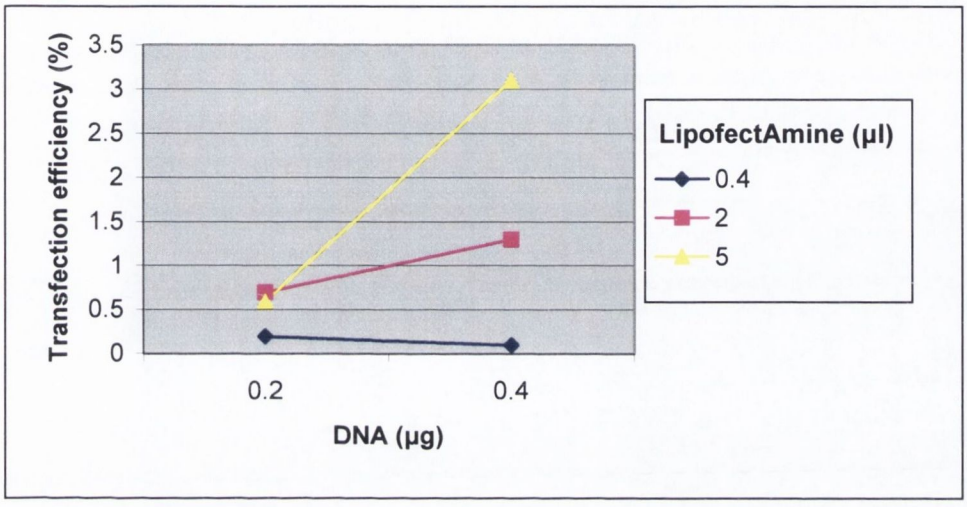


Figure 4.16. Transfection of LNCaP cells using LipofectAmine. 60% confluent cells were transfected in 2cm^2 wells for 5h, using the amounts of DNA and LipofectAmine indicated above in duplicate. Cells were assayed for GFP expression 72h after beginning transfection.

4.3.2. Lipofectin

LNCaP transfection efficiency was slightly higher than with LipofectAmine but still very low compared to other cell lines (Figure 4.17). On average, 17% more cells were GFP-positive after 72h than after 48h, and treatment with 0.4μg plasmid resulted in more GFP-positive cells than treatment with 0.2μg plasmid.

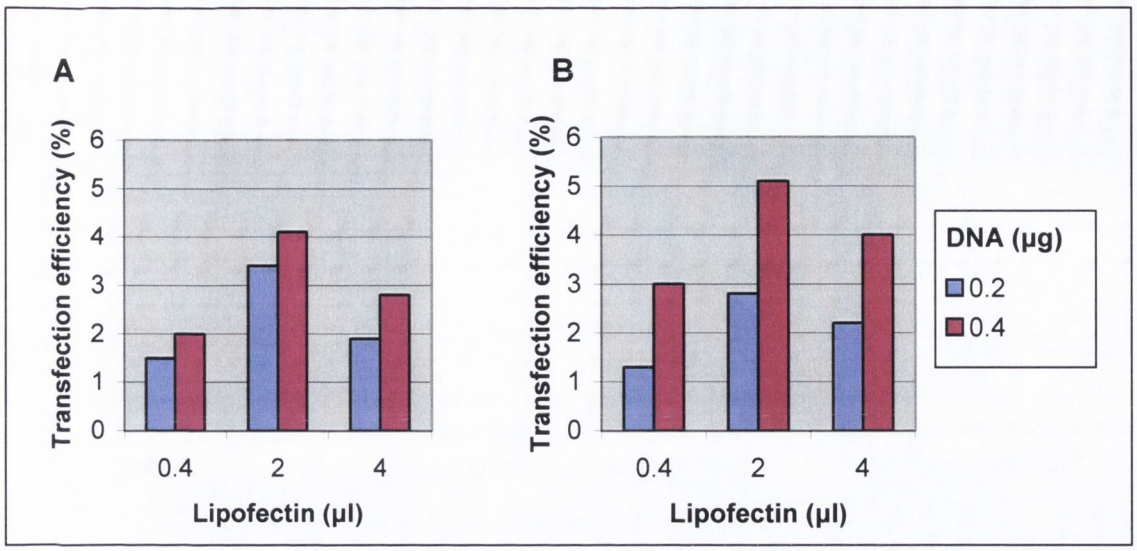


Figure 4.17. Transfection of LNCaP cells using Lipofectin. 60-70% confluent cells were transfected in 2cm^2 wells for 5h, using the amounts of pEGFP-NI and Lipofectin indicated above in duplicate. Cells were assayed for GFP expression 48h (**A**) and 72h (**B**) after beginning transfection.

4.3.3. GenePorter2

GenePorter2 transfected LNCaP cells more efficiently with greater amounts of pEGFP-NI and carrier, but maximum efficiency using this carrier was less than 5% (Figure 4.18).

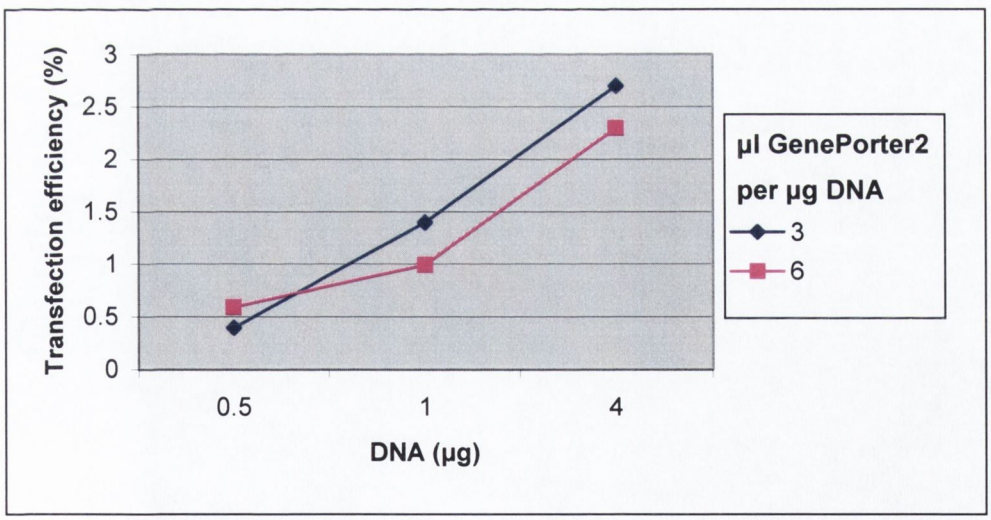


Figure 4.18. Transfection of LNCaP cells using GenePorter2. 70% confluent cells were transfected in 2cm^2 wells for 4h, using the amounts of plasmid and reagent indicated above in duplicate. Cells were assayed for GFP expression 48h after beginning transfection.

4.3.4. Transfast

High levels of transfection (28-35%), over a range of conditions, were obtained in LNCaP cells using Transfast (Figure 4.19).

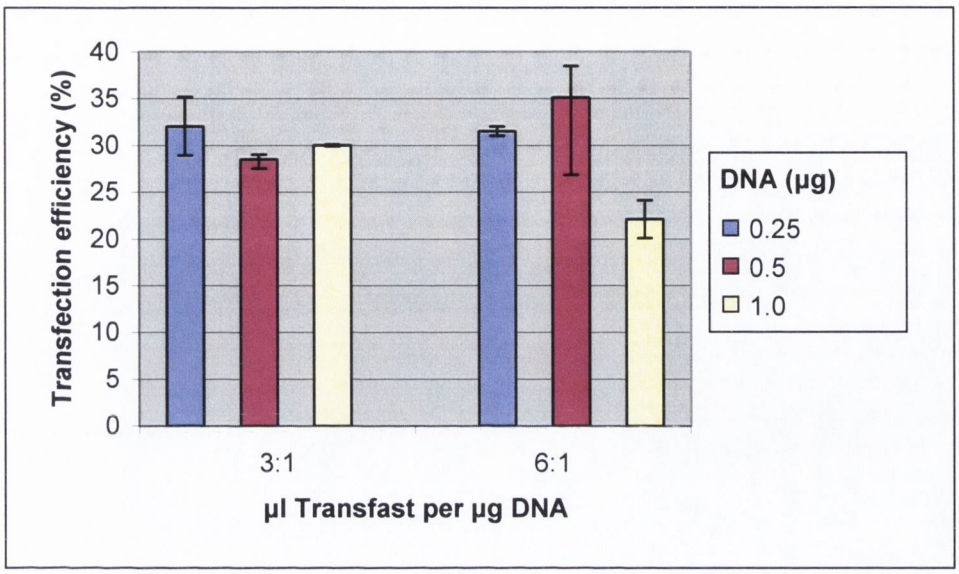


Figure 4.19. Transfection of LNCaP cells using Transfast. 60% confluent cells were transfected in $2cm^2$ wells for 3h, using the amounts of pEGFP-NI and reagent indicated above in duplicate. Cells were assayed for GFP expression 72h after beginning transfection.

Having optimised conditions for transfection of these cells, LNCaP cells were then transfected (Figure 4.20) with a mixture of CD expression plasmid and GFP reporter plasmid at a 9:1 ratio, using two of the conditions used successfully in Figure 4.19. Best results, with 41% transfection efficiency, were achieved with 1µg pEGFP-NI and 3µl Transfast per well.

Proportionally the same amounts of pEGFP-NI and Transfast (5µg and 15µl) were also used to transfect LNCaP cells, under the same conditions except in 35mm wells instead of 2cm wells, in parallel. Transfection was slightly less efficient in 35mm wells (35.0% over two wells).

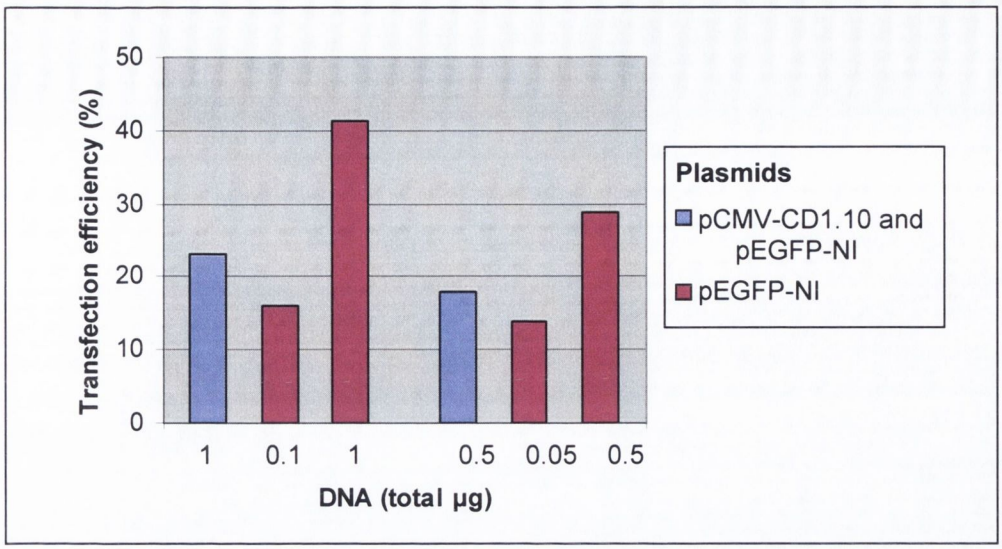


Figure 4.20. Transfection of LNCaP cells with expression and reporter plasmids. 60-70% confluent cells were transfected in $2cm^2$ wells for 3h, using 3µl Transfast and the amounts of DNA indicated above in duplicate. pCMV-CD1.10 and pEGFP-NI were co-transfected at a 9:1 ratio. Cells were assayed for GFP expression 48h after beginning transfection.

4.4. Optimisation of conditions for transfection of 22Rv1 cell line

4.4.1. GenePorter2

70% confluent 22Rv1 cells were transfected using GenePorter2 with various amounts of DNA and 3µl or 6µl carrier per µg DNA. Two dilution media supplied with GenePorter2 were compared with OPTIMEM-I reduced serum medium for diluting DNA. However, transfection was inefficient under the conditions tested, not exceeding 3% (Figure 4.21). This reagent was not optimised further in 22Rv1 cells.

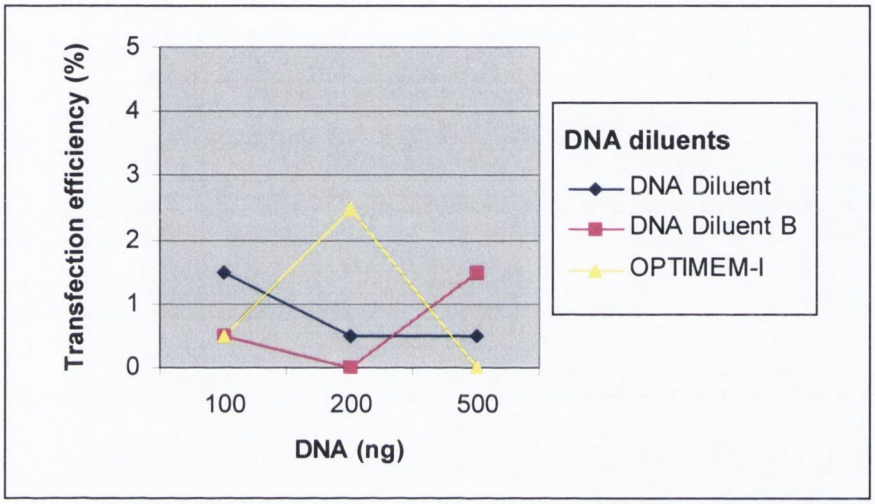


Figure 4.21. Transfection of 22Rv1 cells using GenePorter2. 5 x 10^4 cells per well were seeded in 96-well plates and transfected for 3h, using the amounts of plasmid and the dilution media indicated above. Cells were assayed for GFP expression 24h after transfection. Results shown are from wells treated with 6µl GenePorter2 per µg DNA. Results for 3µl GenePorter2 per µg DNA (not shown) were similar.

4.4.2. Transfast

70% confluent 22Rv1 cells, transfected with various amounts of DNA and Transfast, showed efficient plasmid uptake (up to 44% of cells). Increasing the amount of Transfast improved transfection of 50ng DNA, but not 100ng or 200ng (Figure 4.22).

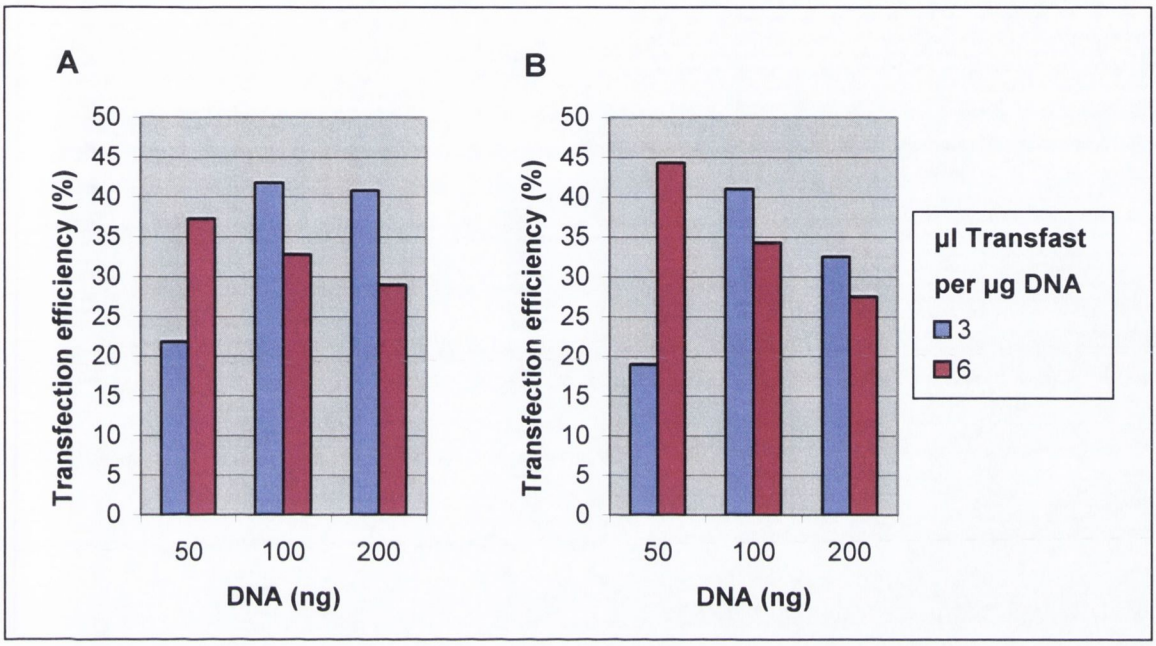


Figure 4.22. Transfection of 22Rv1 cells using Transfast. 5 x 10^4 cells per well were seeded in 96-well plates and transfected for 3h, using the amounts of plasmid and Transfast indicated above in duplicate. Cells were assayed for GFP expression 24h, 48h (**A**) and 72h (**B**) after transfection. Results after 24h revealed similar trends but lower percentages of GFP-expressing cells.

Transfection time and cell density were then optimised for the two most efficient combinations of DNA and Transfast. Varying cell density did not significantly alter transfection efficiency. Optimal transfection (46%) was achieved by transfecting 100ng DNA for 3 hours (Figure 4.23).

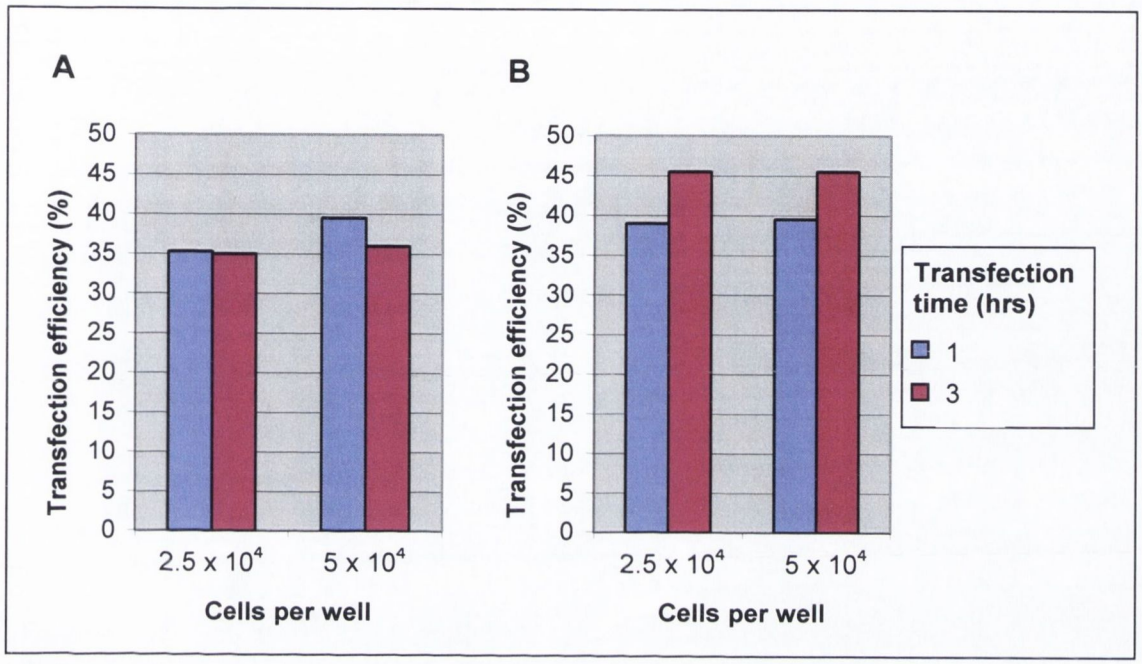


Figure 4.23. Transfection of 22Rv1 to optimise transfection time and cell density. 60% and 80% confluent cells were transfected in duplicate in 96-well plates, using 0.3µl Transfast and 50ng (**A**) or 100ng (**B**) plasmid. Cells were assayed for GFP expression 48h after transfection.

Co-transfection of 80ng of another plasmid with 20ng pEGFP-NI resulted in transfection rates comparable to 20ng pEGFP-NI alone in 22Rv1 cells (Figure 4.24). Though co-transfection of two plasmids yielded half as many GFP-positive cells as transfections using the optimised amount of 100ng pEGFP-NI per well, the overall percentage of cells which have taken up DNA is likely to be higher than the percentage of GFP-positive cells used as an estimate of transfection efficiency.

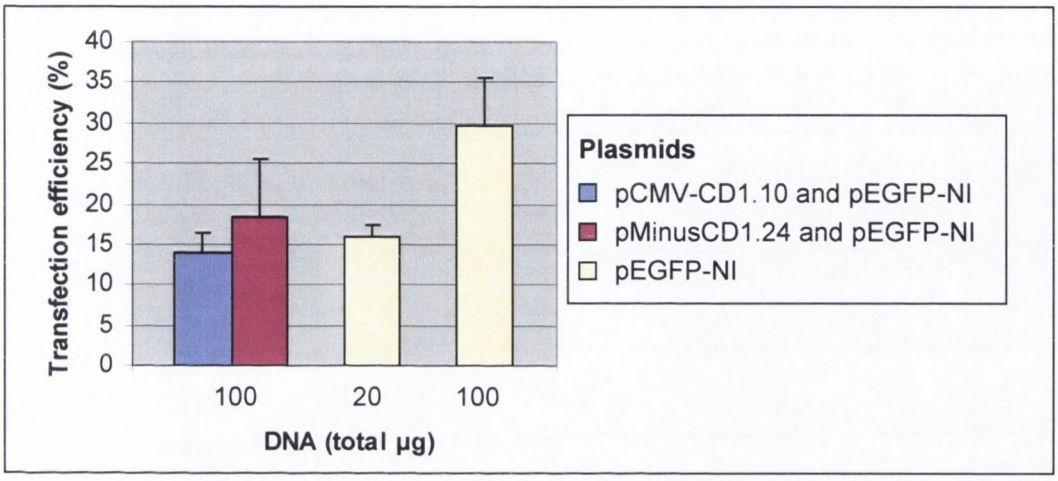


Figure 4.24. Co-transfection of 22Rv1 cells with reporter and CD plasmids. 2.5 x 10^4 cells per well were seeded in 96-well plates, and transfected for 3h using 0.3µl Transfast and the indicated plasmids. Co-transfections used 20ng pEGFP-NI and 80ng CD plasmid. Results above are from two independent experiments carried out in duplicate. Cells were scored for GFP expression after 48h.

4.5. Optimisation of conditions for transfection of HCT116 cell line

4.5.1. Transfast

In addition to testing the efficacy of the CD/5-FU system in killing prostate cancer cells, it was important to determine if use of the PSA promoter would allow the system to function only in prostate cells, and not in surrounding tissues. To begin assessing whether the CD gene was likely to be active in the colon under the regulation of the PSA promoter, transient transfection conditions for the colon cancer cell line HCT116 were optimised.

Various amounts of Transfast and DNA were transfected into 50% confluent HCT116 cells. Higher volumes of Transfast slightly increased transfection efficiency, but also inhibited cell proliferation to a greater extent. 3µl Transfast per µg DNA was clearly more effective than 6µl Transfast per µg DNA. Up to 17% transfection was achieved (Figure 4.25).

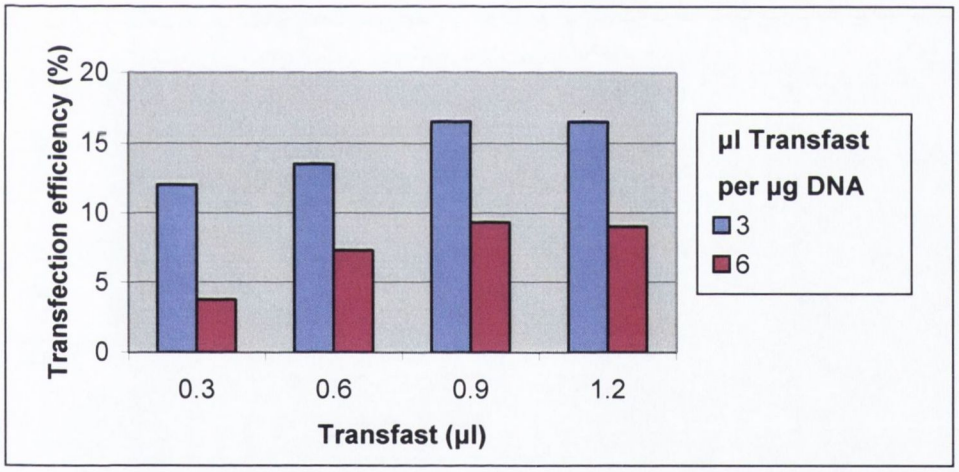


Figure 4.25. Transfection of HCT116 cells using Transfast. 2.5 x 10^4 cells per well were seeded in 96-well plates, and transfected in duplicate for 3h using the indicated amounts of Transfast and DNA. Cells were scored for GFP expression after 72h.

HCT116 cells (50% confluent) were then transfected using Transfast with and without serum, and for varied times. The presence of serum in the culture medium during transfection alleviated toxicity. Cells were 60% confluent two days after transfection with serum, in contrast to cells transfected without serum, which were 30% confluent. However, transfection was up to twice as efficient in the absence of serum (Figure 4.26). Shorter transfection times slightly reduced transfection efficiency under serum-free conditions, but were not observed to increase viability.

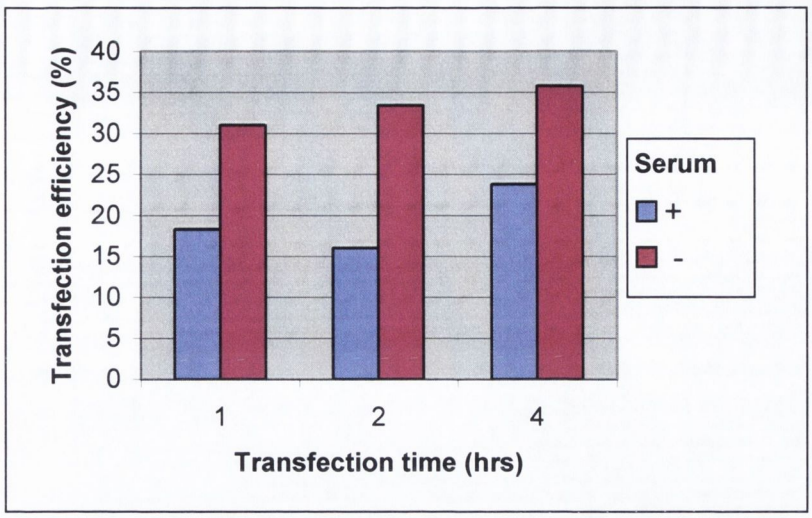


Figure 4.26. Transfection of HCT116 cells using Transfast: efforts to reduce toxicity. 2.5 x 10^4 cells per well were seeded in 96-well plates, and transfected in duplicate using 0.9µl Transfast and 0.3µg DNA per well. Cells were scored for GFP expression 48h after transfection.

4.5.2. GeneJuice

Various amounts of DNA and GeneJuice were used for initial optimisation of this reagent for HCT116 cells. Under the conditions tested, transfection efficiency was low, 6% at most (Figure 4.27). However, due to the very low toxicity of the GeneJuice reagent compared to other reagents such as Transfast, optimisation was continued. Transfection time was varied, and transfection was carried out in the presence of serum, for two of the more promising GeneJuice/DNA combinations from the first optimisation experiment. The presence of serum trebled the transfection efficiency, compared to cells transfected in serum-free conditions for four hours (Figure 4.28). Transfection efficiency was not assessed in wells seeded with 5 x 10^4 cells and transfected using GeneJuice in the presence of serum, since they were 100% confluent two days after transfection. This would not be ideal for the MTT assays for which transfection of this cell line was optimised (Chapter 6).

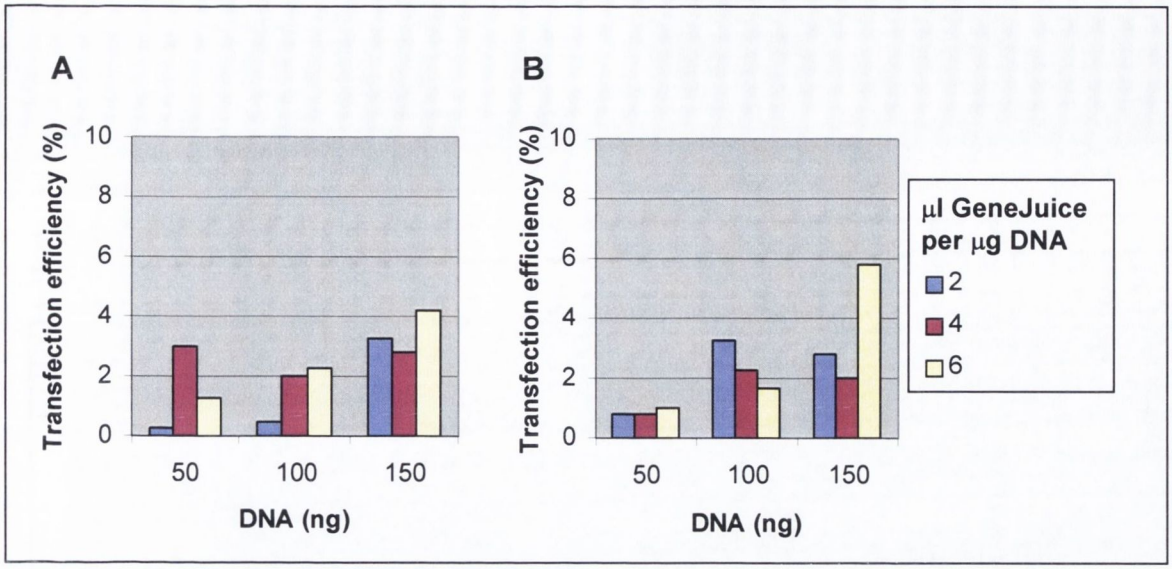


Figure 4.27. Transfection of HCT116 cells using GeneJuice. 2.5 x 10^4 cells (**A**) or 5 x 10^4 cells (**B**) per well were seeded in 96-well plates. 60% and 80% confluent cultures were transfected in duplicate for 6h using the indicated amounts of Transfast and DNA, under serum-free conditions. Cells were scored for GFP expression 48h after transfection.

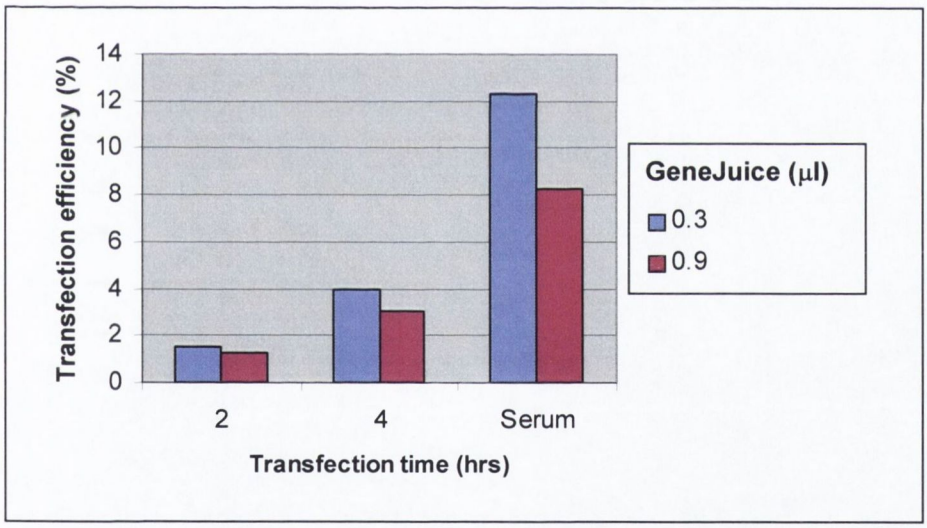


Figure 4.28. Transfection of HCT116 cells using GeneJuice with and without serum. 2.5 x 10^4 cells per well were seeded in 96-well plates. 50% confluent cells were transfected in duplicate for the indicated times in the absence of serum, and in the presence of serum (which does not involve a specific duration of transfection). Cells were treated with 150ng plasmid and 0.3µl or 0.9µl GeneJuice, and scored for GFP expression 48h after transfection.

Transfection of HCT116 using 0.3µl GeneJuice and 150ng pEGFP-NI, the most efficient combination in the presence of serum, was repeated. In addition, other plasmids were co-transfected with pEGFP-NI at a 4:1 ratio, using a total amount of 150ng DNA. Both the co-transfections and the repeated optimised conditions achieved 20% GFP-positive cells (Figure 4.29), with minimal toxicity, importantly.

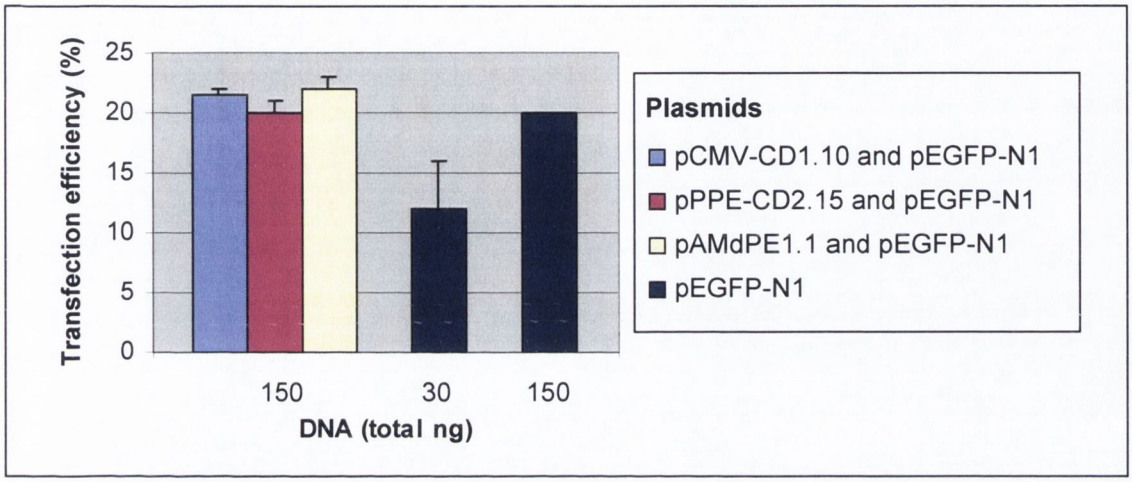


Figure 4.29. Co-transfection of HCT116 cells with two plasmids. 2.5 x 10^4 cells per well were seeded in 96-well plates, and transfected in duplicate using 0.3µl GeneJuice in the presence of serum. Co-transfections contained 30ng pEGFP-NI and 120ng of the second plasmid in each case. Cells were scored for GFP expression 48h after transfection.

4.6. Reliability of counting GFP-positive cells

Negative controls (cells transfected without plasmid or without carrier) were always negative for GFP expression. Such cells rarely showed fluorescence above the background level of the culture medium. When they did, the fluorescence was visible using a green filter in addition to the blue filter used to detect GFP, and therefore nonspecific. In contrast, GFP-expressing cells were not detectable using a green filter, indicating that background fluorescence did not pose problems for GFP detection.

Counting GFP-positive cells was reproducible in duplicate wells. The mean and standard deviation of transfection efficiency were calculated, for five sets of duplicate wells transfected in each of two independent experiments in each cell line. From this data, the average standard deviation between duplicate wells ranged from 8% to 12% of the transfection efficiency, depending on the cell line.

4.7. Toxicity of optimised transfection methods

The toxicity associated with each of the optimised transfection procedures used for further experiments was assessed by ethidium bromide/acridine orange staining 24h after transfection. Most of the transfection procedures had little impact on cell viability (Figure 4.30). The exception was the Transfast procedure used for LNCaP. In this case, cell viability was reduced from 55% to 30% when cells were treated with DNA and liposome, and also when they were mock transfected using the same procedure but omitting DNA and liposome. These data suggest that the transfection-related toxicity was associated either with the process of removing and adding media to the cells, or with the absence of serum for three hours, rather than the specific reagent used.

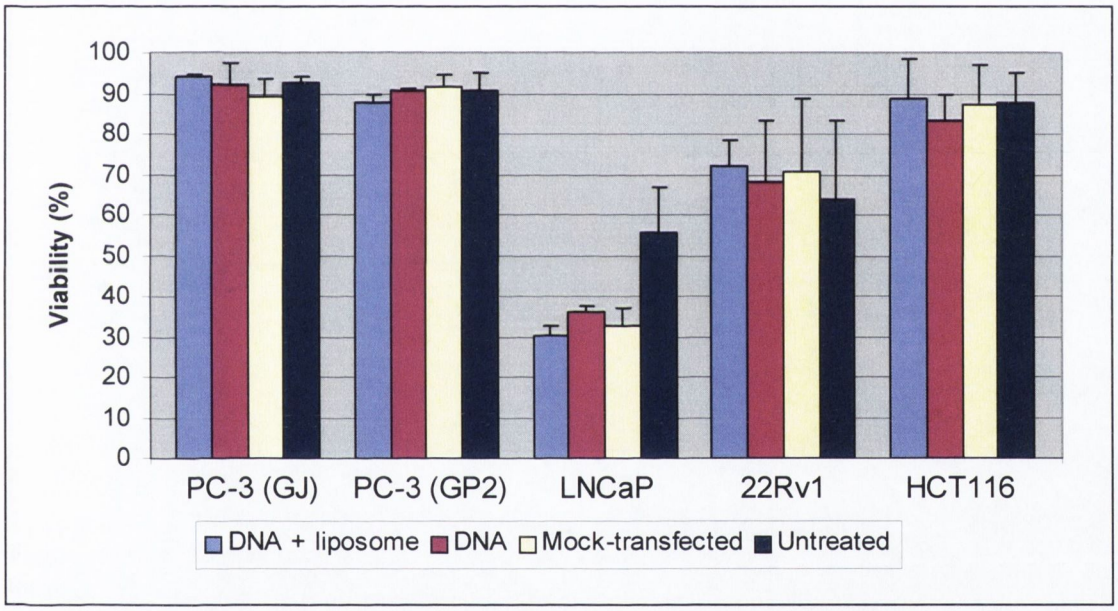


Figure 4.30. Toxicity of optimised transfection procedures. Cells were transfected as described in Section 2.5.4, stained with ethidium bromide and acridine orange and counted under ultraviolet light. Results represent mean and standard deviation values from two independent experiments in each cell line. PC-3 cells were transfected with both GeneJuice (GJ) and GenePorter2 (GP2).

4.8. Summary of transfection efficiency optimisation

Figure 4.31 summarises the transfection rates achieved in the five cell lines studied. The activity of most individual reagents in different cell lines varied widely. In DU145 cells, Transfast was slightly more efficient than LipofectAmine (with or without LipofectAmine Plus). For LNCaP and PC-3 cell lines, Transfast and GenePorter2 respectively were much more efficient than any other reagent tested for the same cell line.

Transfast was the most efficient reagent tested in DU145, LNCaP, 22Rv1 and HCT116 cell lines, and also effectively transfected PC-3 cells. Its activity therefore appears less cell line-dependent than other reagents such as GenePorter2 and LipofectAmine, transfecting over 30% of all five cell lines tested. GenePorter2 transfected PC-3 cells with high efficiency, but showed very little activity in DU145, LNCaP and 22Rv1 cells, while the moderate efficiency of LipofectAmine in DU145 and PC-3 cells was not reproduced in LNCaP cells.

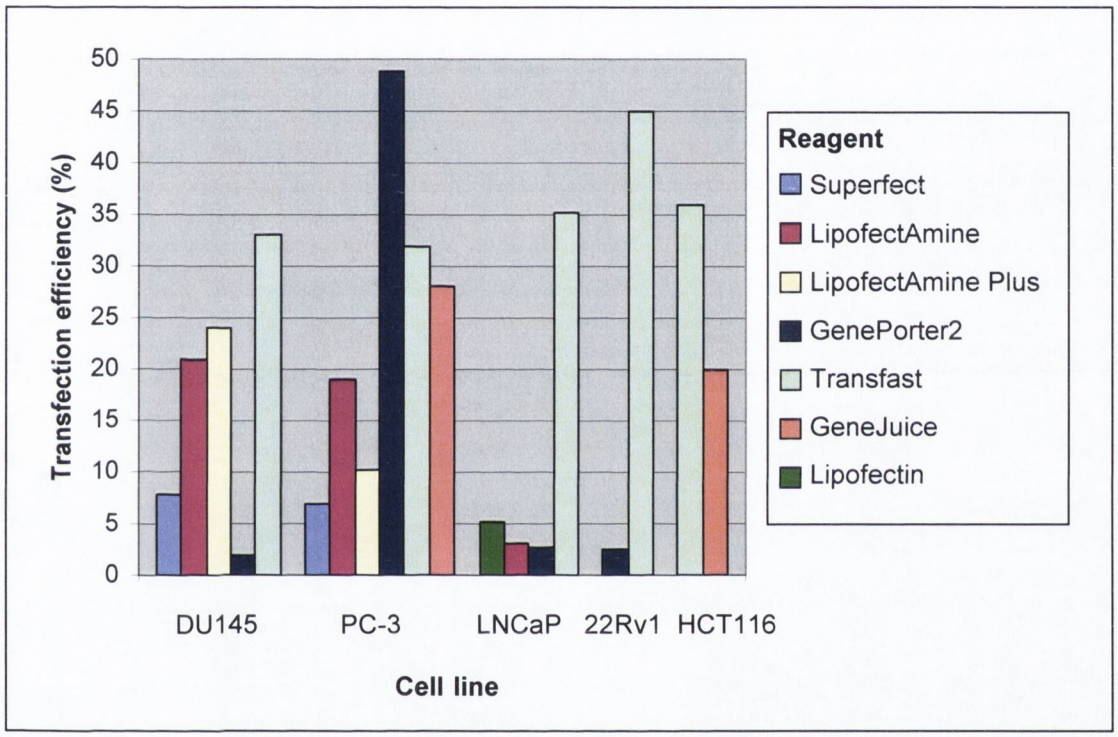


Figure 4.31. Summary of transfection efficiency of each reagent tested for each cell line. Optimal transfection efficiency is indicated. Not all reagents were tested in all cell lines.

4.9. Fluorescent micrographs of transfected cells

Figures 4.32 and 4.33 shows fluorescent micrographs of prostate cancer cell lines and the HCT116 colon cancer cell line, respectively, following transfection with pEGFP-NI. All cell lines show effective transfection, with clear definition of GFP-positive cells.

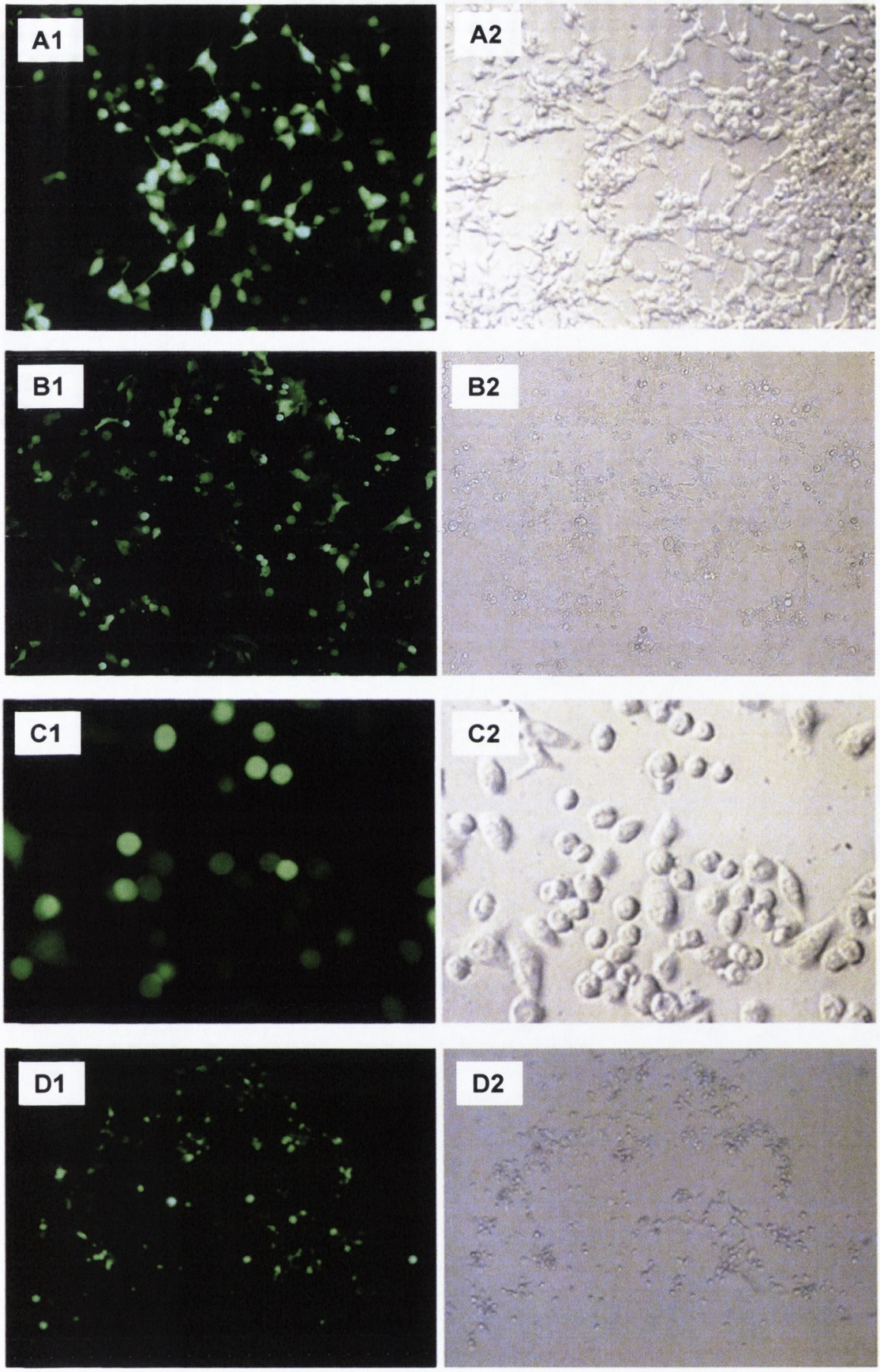


Figure 4.32. Prostate cancer cell lines transfected with GFP reporter plasmid. LNCaP (**A**), 22Rv1 (**B**), PC-3 (**C**) and DU145 (**D**) cells at 20X, 20X, 40X and 10X magnification respectively. Cells were transfected using Transfast, except PC-3 cells, which were transfected using GenePorter2. The same fields are shown under fluorescent (**A1-D1**) and white (**A2-D2**) light.

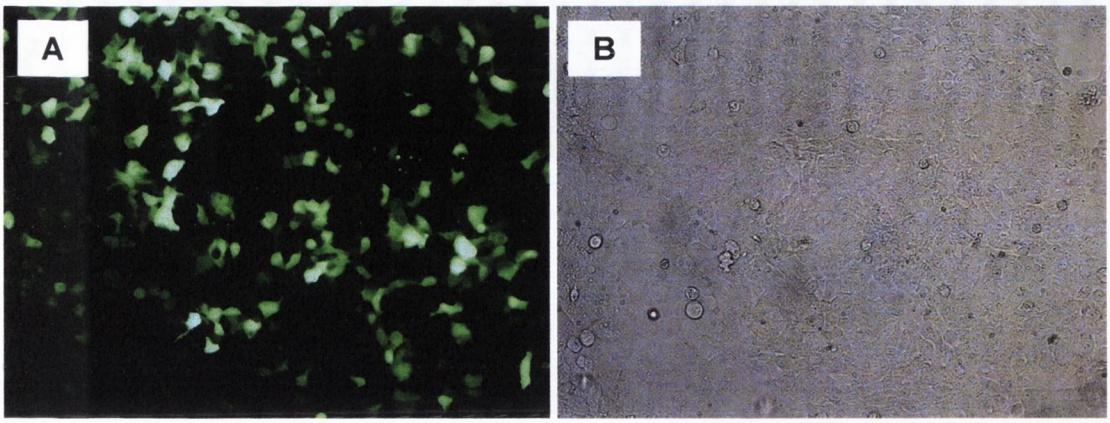


Figure 4.33. Colorectal cancer cell line HCT116 transfected with GFP reporter plasmid using GeneJuice. Panels **A** and **B** show the same field under fluorescent and white light, respectively, at 20X magnification.

DISCUSSION

Transfection conditions were optimised for use in further studies in the project, for each of four prostate cancer cell lines and a colon cancer cell line. As expected, considerable variation was seen among cell lines in their response to individual transfection reagents.

Optimal transfection of DU145 cells (33% efficiency) was obtained with 80% confluent cells transfected for 3h with 1μg plasmid and 6μl Transfast per $2cm^2$ well, and assayed 24h after transfection. Optimal transfection conditions (41% efficiency) for LNCaP cells were selected to be the transfection of 60-70% confluent cells for 3h with 1μg plasmid and 3μl Transfast per $2cm^2$ well (5μg and 15μl per 35mm well), assaying 48h after transfection. Transfast was also highly efficient in 22Rv1 cells, achieving 46% transfection when 50-60% confluent cells were treated for 3h with 100ng plasmid and 0.3μl liposome per well in 96-well plates, and assayed 48h later.

PC-3 cells were most optimally transfected (49% efficiency) at 80% confluence for 3h with 1.2μg plasmid and 7.2μl GenePorter2 per $2cm^2$ well (6μg and 36μl per 35mm well), and assayed 24h after transfection. However, when GenePorter2-transfected cells were used for annexin V assays, problems were encountered with toxicity over the course of the several days required for these experiments.[1] Further optimisation was therefore carried out, and GeneJuice, although less efficient than GenePorter2, was selected as a low-toxicity carrier suitable for annexin V experiments in PC-3 cells. 80% confluent cells were transfected at 28% efficiency using 100ng plasmid and 0.4μl GeneJuice per well in 96-well plates, in the presence of serum, and assayed 48h after transfection.

[1] For initial annexin V assays, PC-3 cells plated in T25 flasks and exposed to both pCMV-CD1.10 and GenePorter2 were observed to be significantly less viable than cells exposed to either DNA or vector alone, in the absence of 5-FC.

For the HCT116 colon cancer cell line, as for PC-3 cells, transfection efficiency and toxicity were both considered as factors in finding an optimal transfection reagent. Though GeneJuice brought about lower transfection efficiencies than Transfast in this cell line, the observation that Transfast but not GeneJuice inhibited its growth meant that GeneJuice was selected for use in subsequent MTT experiments. 20% of HCT116 cells (50% confluent) were transfected in the presence of serum with 150ng plasmid and 0.3µl GeneJuice per well in 96-well plates, and assayed 48h later.

Each cell line was also transfected with a combination of CD expression plasmid and pEGFP-NI as a reporter plasmid at a 9:1 or 4:1 ratio (Figures 4.6, 4.12, 4.15, 4.20, 4.24, 4.29). Under these conditions, 12-40% of cells were transfected with the reporter plasmid, and possibly a greater percentage with the expression plasmid. The presence of another plasmid in excess of pEGFP-NI inhibited its transfection slightly in PC-3 cells, possibly due to competition, though this was not observed in other cell lines. Indeed, in co-transfections involving LNCaP, DU145, HCT116 and PC-3 (GeneJuice procedure), suboptimal amounts of pEGFP-NI were taken up more efficiently in the presence of an excess of another plasmid. This effect may be associated with the extent to which transfection efficiency depends on the absolute DNA amount and on the ratio of liposome volume to DNA amount.

Factors found to influence transfection efficiency were transfection reagent choice, reagent volume, amount of plasmid(s), transfection time, cell density before transfection, medium used to dilute DNA and carrier, presence or absence of serum during transfection, time of assay, and culture vessel. The first four factors in particular were manipulated until satisfactory levels of transfection were obtained in each cell line.

When GFP expression was assayed at different times during the same experiment, the effect of extending the assay time varied. In one case (Figure 4.2A), much higher levels of GFP expression were observed in DU145 cells after 72h than after 24h, but otherwise levels were slightly lower after 72h than after 24h in the same cell line (Figures 4.4-4.5). Possibly the higher cell density at transfection observed in Figures 4.4-4.5 than in Figure 4.2A resulted in cells being too dense 72h later to function optimally. In LNCaP cells, efficient transfection was observed after both 48h and 72h

(Figures 4.19-4.20). In 22Rv1 cells, this was also the case (Figure 4.22), though the same cells observed *in situ* at different time points showed lower percentages of GFP-positive cells 24h after transfection.

Though it was not anticipated that the vessel in which cells were cultured would affect transfection efficiency, slightly higher levels of GFP expression (29% in PC-3 cells, 18% in LNCaP cells) were observed in cells grown in $2cm^2$ wells, than in cells cultured in parallel on coverslips in 35mm wells to facilitate fixation.

In conclusion, transfection efficiencies have been significantly improved by judicious choice of assay condition and transfection reagent, allowing an increase in transfection efficiency of 3- to 18-fold in the five different cell lines under study.

CHAPTER FIVE:
CD PROTEIN EXPRESSION

INTRODUCTION

Having generated a number of plasmids (summarised in Table 3.1) by cloning and mutagenesis, in which the coding sequence of the CD prodrug activation gene was regulated by a variety of promoters, it was necessary to ensure that each of these plasmids fulfilled their function of expressing CD. The plasmids were therefore transfected into appropriate cell lines, and protein extracts from transfected and parental cells were analysed by Western blotting, to confirm that the transfection of CD expression plasmids resulted in the presence of the CD enzyme.

As a positive control for CD antibody function, biotinylated CD protein was produced by *in vitro* transcription and translation, in addition to luciferase protein as a control. Proteins produced *in vitro* were separated by PAGE, transferred to nitrocellulose membranes and assayed for biotin, to verify that CD protein was present. This protein was used to optimise the Western blotting conditions for a primary antibody against bacterial CD (Haack *et al* 1997). CD protein was then detected by Western blotting in extracts from cell lines transfected with various CD expression plasmids, following PAGE and transfer to nitrocellulose membranes.

The CD expression plasmids pPPE-CD2.15, pPCM1.24 or pPCM2.3, in which CD expression is regulated by the PSA promoter and enhancer, were transfected into the cell lines LNCaP and 22Rv1. Hormone concentrations in growth media were expected to be a factor in CD expression from PSA promoters in LNCaP cells (Schuur *et al* 1996, Rodriguez *et al* 1997). Though the 22Rv1 cell line was derived from a tumour which relapsed after castration of the mouse xenograft host, its growth was weakly stimulated by DHT (Sramkoski *et al* 1999), suggesting that DHT could also influence PSA transcription in this cell line. Androgen-dependence of endogenous PSA expression in LNCaP and 22Rv1 cells was therefore assessed by ELISA, to select appropriate growth media for subsequent experiments involving PSA promoter constructs. In experiments where growth media were supplemented with a specific concentration of DHT, hormone-depleted FCS was substituted for standard FCS, since standard FCS contained undefined amounts of steroid hormones.

RESULTS

5.1. Anti-CD antibody optimisation

5.1.1. Production of biotinylated CD protein *in vitro*

Biotinylated luciferase and CD were successfully detected by a streptavidin-alkaline phosphatase enzyme conjugate (Figure 5.1). No evidence of protein bands at the appropriate positions was visible in the negative control reaction. CD and luciferase both migrated more slowly than expected. This probably reflects the presence of the biotin moiety.

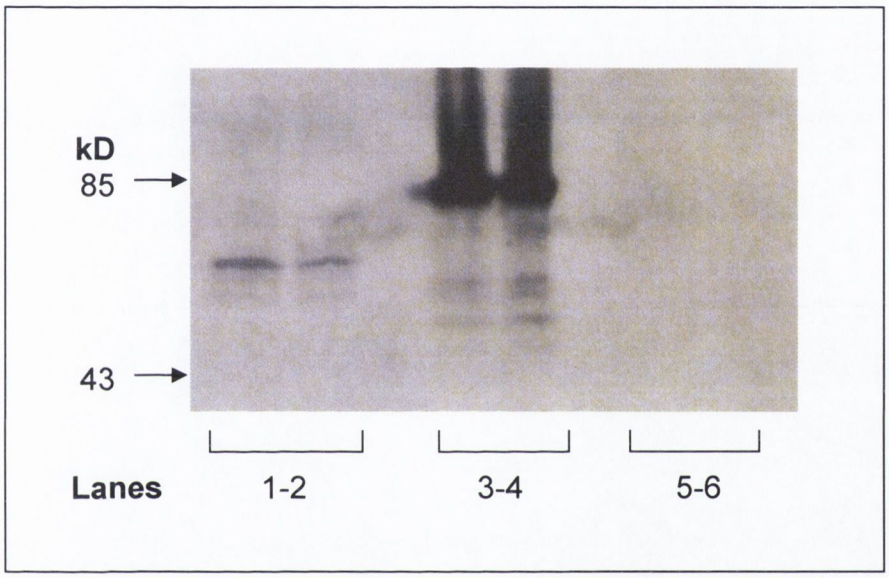


Figure 5.1. Autoradiogram of biotinylated proteins. Biotin-labelled CD (47kD) and luciferase (61kD) were transcribed and translated from plasmids *in vitro*, electrophoresed and transferred to nitrocellulose for biotin detection. Lanes 1-2: CD. Lane 3-4: luciferase. Lanes 5-6: negative control (no plasmid).

5.1.2. Anti-CD antibody dilution curve

Western blotting was carried out on *in vitro* transcription and translation products as described in Section 2.7.3, with anti-CD antibody diluted 1:400, 1:800 and 1:1200. Figure 5.2 shows that a strong CD band was detected using a 1:1200 antibody dilution. A band of comparable size but much weaker intensity was also detected in the control lanes, in which the CD expression plasmid was omitted from the transcription and translation reaction.

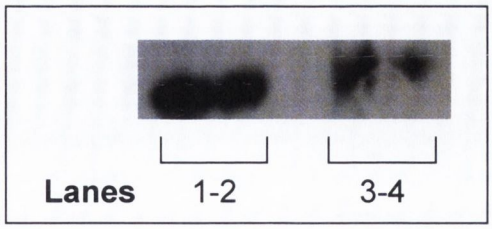


Figure 5.2. Western blot of *in vitro*-produced CD protein. Products of the *in vitro* transcription and translation reactions indicated below were assayed by Western blotting, using anti-CD primary antibody (diluted 1:1200) and rabbit anti-mouse secondary antibody. Lanes 1-2: pCMV-CD1.10. Lanes 3-4: negative control (no plasmid). Similar results were obtained in an independent experiment.

5.2. CD protein expression in transfected PC-3 cells

Protein was extracted from PC-3 cells transfected with CD expression plasmids and assayed for CD expression by Western blotting. CD was only expressed in cells transfected with pCMV-CD1.10, which contains the cytomegalovirus immediate early gene promoter upstream of the CD open reading frame (Figure 5.3). No CD was detected in cells transfected with the negative control plasmid pMinusCD1.24, in which the CMV promoter was deleted, or in untransfected control cells.

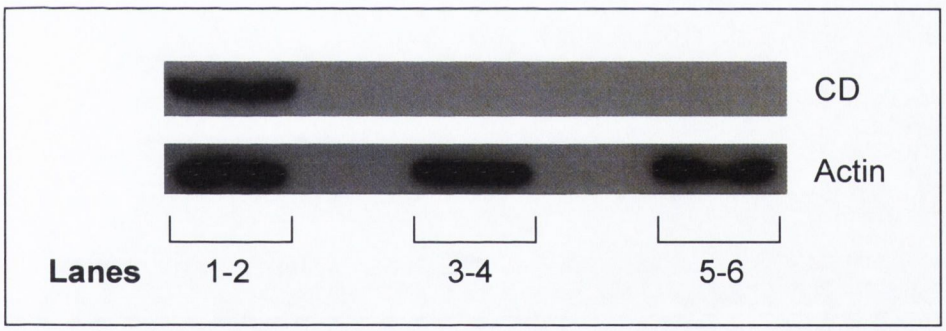


Figure 5.3. CD protein expression in PC-3 transfectants. Protein was extracted from PC-3 cells transiently transfected with the plasmids indicated below. CD expression was assessed by Western blotting. Blots were then stripped and re-probed for actin as a loading control. Lanes 1-2: pCMV-CD1.10. Lanes 3-4: pMinusCD1.24. Lanes 5-6: untransfected control.

5.3. Selection of suitable growth media for PSA-positive cell lines

5.3.1. Selection of DHT concentration for LNCaP experiments

PSA expression by LNCaP cells grown in several DHT concentrations was compared (Figure 5.4). PSA secretion in LNCaP cells peaked at 1μM DHT under the conditions tested, and this concentration was used in subsequent experiments for Western blotting and flow cytometry.

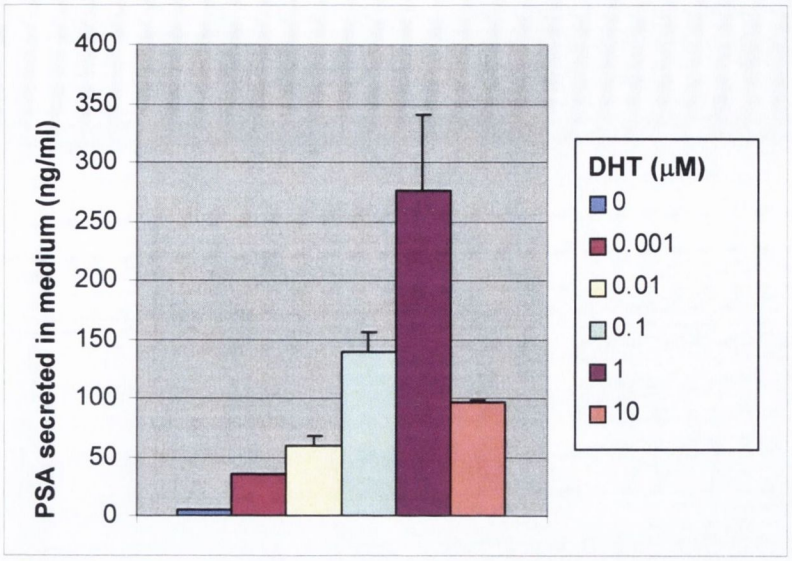


Figure 5.4. Response of PSA expression to hormone concentration in LNCaP cells. Cells were grown for six days in medium containing 10% charcoal-dextran treated FCS (DC-FCS) and DHT as indicated above. The medium was then assayed for PSA by ELISA (Wallac kit). Data represent mean and standard deviation values from cells grown in duplicate wells.

5.3.2. Selection of DHT concentration for 22Rv1 experiments

The effect of hormone concentration on PSA expression in 22Rv1 cells was assessed (Figure 5.5). 22Rv1 cells produced most PSA when grown in standard medium, and when grown in medium with a defined hormone concentration, 0.1μM DHT was optimal. Standard medium was therefore used for initial Western blotting experiments in 22Rv1 cells.

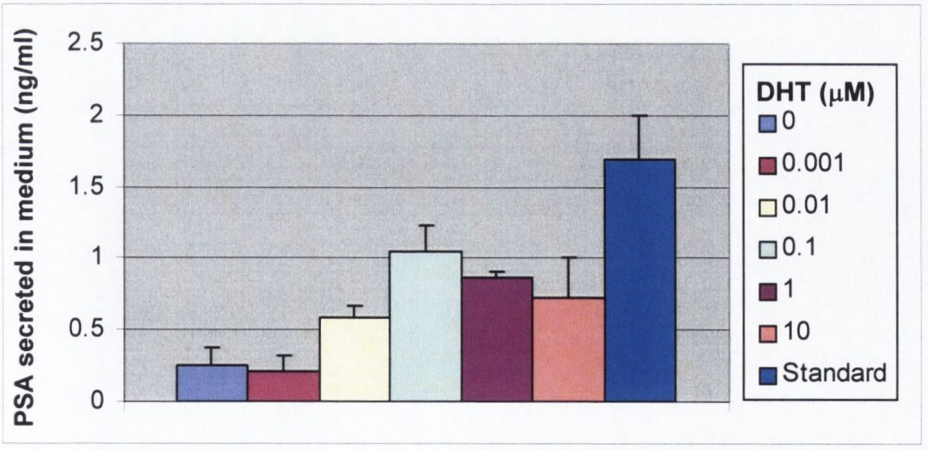


Figure 5.5. Response of PSA expression to hormone concentration in 22Rv1 cells. Cells were grown for six days in medium containing DHT as indicated above, or in medium containing standard FCS without DHT supplementation. The medium was then assayed for PSA by ELISA (CanAg kit). Data represent mean and standard deviation values from cells grown in duplicate wells.

5.3.3. Effect of medium on CD expression in transfected 22Rv1 cells

Protein extracts from 22Rv1 cells transfected with CD expression plasmids were assayed for CD expression by Western blotting. Cells cultured in standard growth medium and transfected with pPPE-CD2.15, pPCM1.24 and pPCM2.3 failed to produce detectable CD. Cells were then grown in standard medium and in medium supplemented with 0.1μM DHT, and transfected with CD expression plasmids containing PSA promoters. Surprisingly, Western blotting detected CD protein only in extracts from cells cultured in DHT-supplemented medium (Figure 5.6), though endogenous PSA production was higher in standard medium (Figure 5.5).

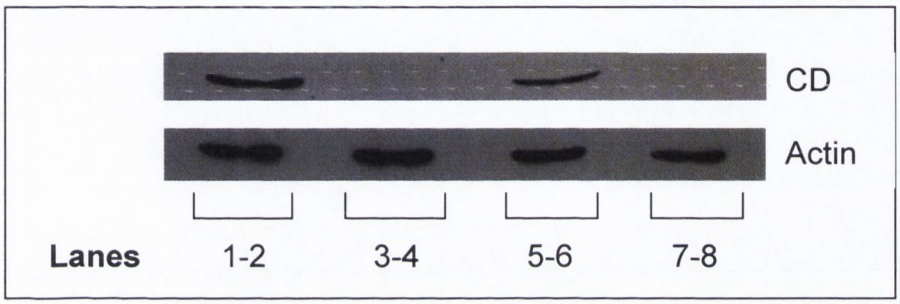


Figure 5.6. Growth medium influences CD expression from PSA promoters in 22Rv1 transfectants. Protein was extracted from 22Rv1 cells transiently transfected and cultured in medium supplemented with 0.1μM DHT or standard medium, as indicated below. CD expression was assessed by Western blotting. Blots were then stripped and re-probed for actin as a loading control. Lanes 1-2: pPPE-CD2.15, 0.1μM DHT. Lanes 3-4: pPPE-CD2.15, standard medium. Lanes 5-6: pPCM2.3, 0.1μM DHT. Lanes 7-8: pPCM2.3, standard medium. This result was reproduced in an independent experiment on pPPE-CD2.15.

Though it was anticipated that the expression pattern of a CD transgene regulated by a PSA promoter would reflect the hormonal regulation of endogenous PSA expression in 22Rv1 cells, it would appear that hormone concentrations regulate the transgene differently to the endogenous PSA (see Discussion below). However, for subsequent Western blotting experiments and MTS experiments, 22Rv1 cells were cultured in medium containing 0.1μM DHT on the basis of these results.

5.3.4. 22Rv1 cells express less PSA than LNCaP

It was noted that PSA secretion by 22Rv1 cells (Figure 5.5) appeared much lower than by LNCaP cells (Figure 5.4). However, since the two cell lines were assayed separately by different ELISA kits, medium from LNCaP cells grown in 1μM DHT and from 22Rv1 cells grown in 0.1μM DHT were assayed in parallel by the same

method used for 22Rv1 cells alone, and yielded 27 ± 5ng/ml and 1 ± 0.1ng/ml PSA concentrations respectively. Since little published data was available on PSA expression in 22Rv1 cells, immunohistochemistry was employed to compare PSA expression in LNCaP and 22Rv1 cells. Strong PSA staining was detected in LNCaP cells, while 22Rv1 cells stained weakly positive (Figure 5.7).

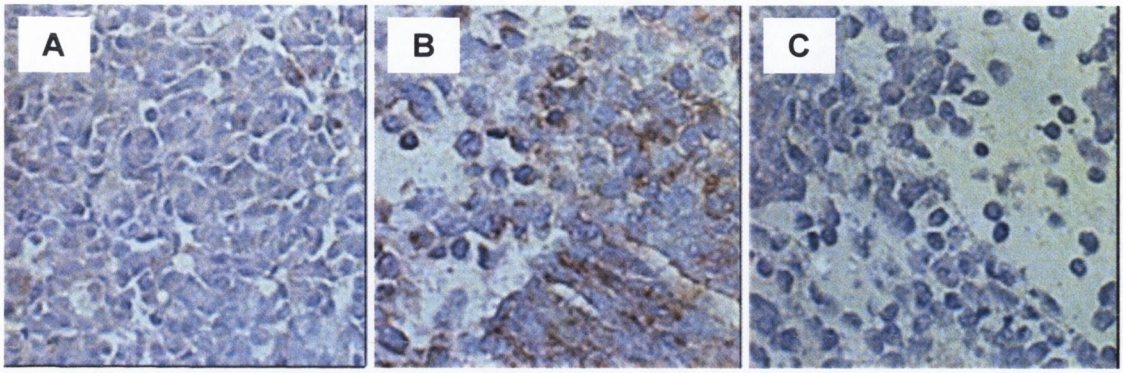


Figure 5.7. PSA expression in 22Rv1 and LNCaP cells. Cells were cultured in standard medium for three days before harvesting for immunohistochemistry. **A.** 22Rv1, anti-PSA antibody. **B.** LNCaP, anti-PSA antibody. **C.** LNCaP, negative control.

5.4. CD protein expression in transfected LNCaP cells

LNCaP cells were transiently transfected with CD expression plasmids. Protein was extracted from the transfectants and assayed for the presence of CD protein by Western blotting. CD expression was observed, as expected, in cells transfected with pCMV-CD1.10, but not in pMinusCD1.24 transfectants or parental LNCaP cells (Figure 5.8). CD was present in cells transfected with all three PSA-CD constructs, pPPE-CD2.15, pPCM1.24 and pPCM2.3, confirming that each of the PSA promoters can facilitate detectable levels of transgene expression.

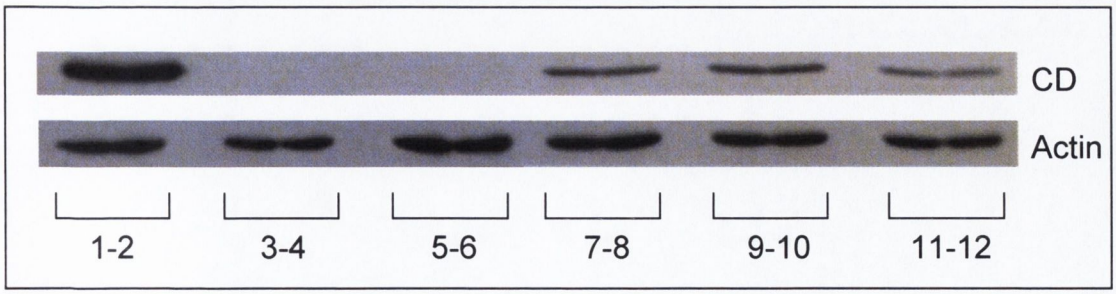


Figure 5.8. CD protein expression from PSA promoters in LNCaP cells. Protein was extracted from LNCaP cells following transfection with the plasmids below, and from untransfected control cells. CD expression was assessed by Western blotting. Blots were then stripped and re-probed for actin as a loading control. Lanes 1-2: pCMV-CD1.10. Lanes 3-4: pMinusCD1.24. Lanes 5-6: untransfected. Lanes 7-8: pPPE-CD2.15. Lanes 9-10: pPCM1.24. Lanes 11-12: pPCM2.3. The data are representative of two independent experiments.

5.5. CD protein expression in transfected 22Rv1 cells

Protein extracts were made from transfected 22Rv1 cells and used to assay for CD expression by Western blotting. High levels of CD were present in cells transfected with pCMV-CD1.10 (Figure 5.9). CD was also expressed in cells transfected with pPPE-CD2.15, pPCM1.24 and pPCM2.3, as in LNCaP cells. No expression was detected in cells containing the negative control plasmid pAMdPE1.1, or in untransfected cells.

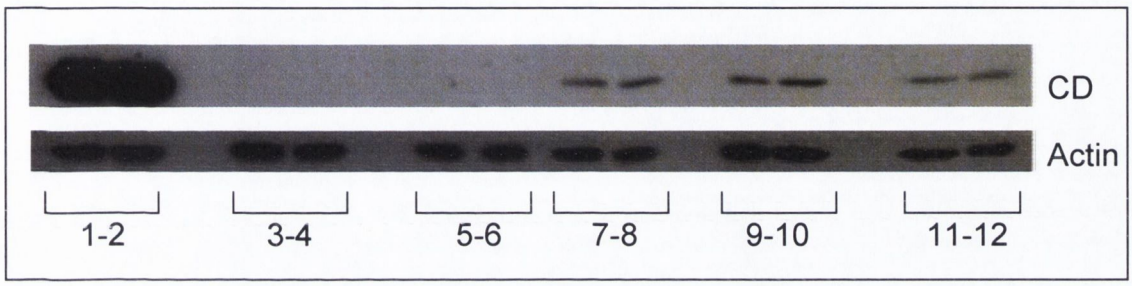


Figure 5.9. CD protein expression from PSA promoters in 22Rv1 cells. Protein extracts from 22Rv1 cells transfected with the plasmids above, and from untransfected 22Rv1 cells, were used for Western blotting for CD expression. Blots were stripped and re-probed for actin as a loading control. Lanes 1-2: pCMV-CD1.10. Lanes 3-4: pAMdPE1.1. Lanes 5-6: untransfected. Lanes 7-8: pPPE-CD2.15. Lanes 9-10: pPCM1.24. Lanes 11-12: pPCM2.3. The findings were reproduced in two independent experiments.

The plasmid pAMdPE1.1 was substituted in 22Rv1 experiments for pMinusCD1.24, the negative control plasmid used for Western blotting in other cell lines, due to apparent low-level detection of CD in 22Rv1 cells transfected with pMinusCD1.24 (Figure 5.10). pMinusCD1.24 contains the CD open reading frame but not the CMV promoter, while pAMdPE1.1 contains neither of these elements. This is discussed further in Section 6.5.

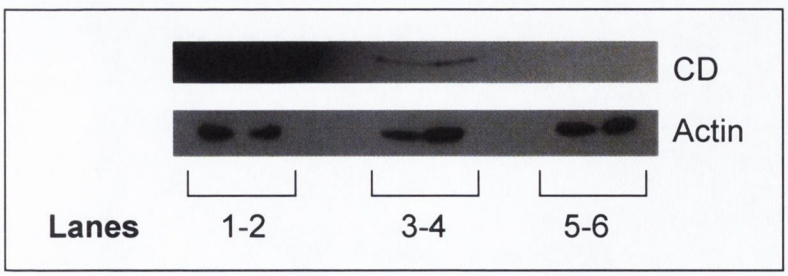


Figure 5.10. Western blot of extracts from 22Rv1 cells transfected with pMinusCD1.24. Protein extracts from 22Rv1 cells, transfected as indicated above, were used for Western blotting for CD expression. Blots were stripped and re-probed for actin as a loading control. Lanes 1-2: pCMV-CD1.10. Lanes 3-4: pMinusCD1.24. Lanes 5-6: untransfected. Similar results were observed in two independent experiments.

5.6. Quantitation of CD protein expression in transfected cells

CD expression was compared in cells transfected with pPPE-CD2.15, pPCM1.24 and pPCM2.3. The CD open reading frame is downstream of the PSA enhancer and promoter in each of these plasmids, but they differ in sequence at androgen response element I (ARE-I). The PSA promoter sequence in pPPE-CD2.15 contains the nucleotide A at both position –158 in ARE-I, and position –155 adjacent to ARE-I. The corresponding sequences in pPCM1.24 and pPCM2.3, generated by site-directed mutagenesis of pPPE-CD2.15, are -158G/-155A and –158A/-155G respectively. Mutagenesis was carried out with the aim of increasing PSA promoter efficiency, based on published data on the PSA promoter (Majumdar & Diamandis 1999, Xue *et al* 2000). However, it does not appear that these particular substitutions affect PSA promoter activity in this setting, since CD expression from all three promoters is similar in both cell lines tested (Figure 5.11). The three plasmids also sensitise cells to 5-FC to a comparable extent (Chapter 6).

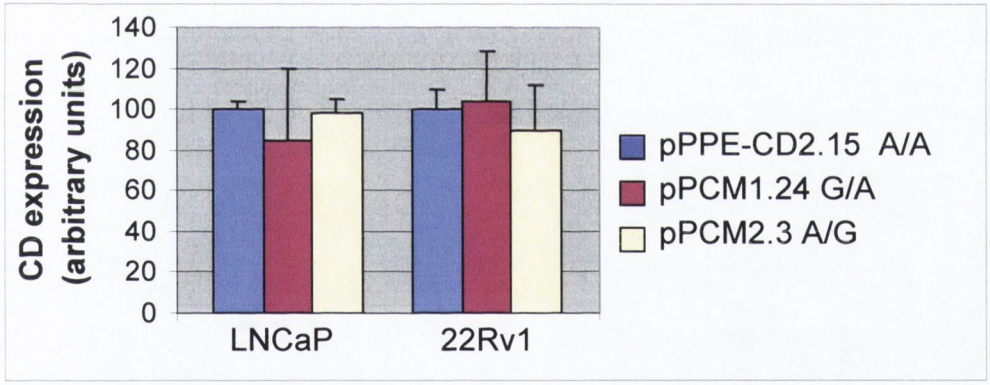


Figure 5.11. Densitometry on CD expression in cells transfected with PSA-CD constructs. Protein was extracted from LNCaP and 22Rv1 cells containing the plasmids indicated above, in which CD transcription was controlled by PSA promoters with sequence variations (see text). The intensity of CD bands on autoradiograms from Western blots were used to quantitate CD expression. Expression in pPPE-CD2.15 transfectants was standardised to 100%. Data represent mean and standard deviation from two independent experiments with wells loaded in duplicate during PAGE.

DISCUSSION

DNA sequencing was initially used to verify that the desired plasmid vectors encoding the CD prodrug activation gene had been successfully generated by cloning and site-directed mutagenesis (Chapter 3). The above data further confirmed that transfection of CD expression plasmids into human prostate cancer cell lines resulted in CD protein expression, from each of the plasmids designed to do so.

High levels of CD were detected in PC-3, LNCaP and 22Rv1 cells transfected with pCMV-CD1.10 (Figures 5.3, 5.8 and 5.9), as expected, since strong activity of the CMV immediate early gene promoter has been demonstrated in a range of cell types (Boshart *et al* 1985, Philip *et al* 1994, Segawa *et al* 1998). Each of the plasmids encoding CD under the control of PSA promoter and enhancer sequences, pPPE-CD2.15, pPCM1.24 and pPCM2.3, also demonstrated CD expression in both the PSA-positive cell lines tested, LNCaP and 22Rv1 (Figures 5.8 – 5.9). PC-3 cells were not transfected with these plasmids for Western blotting, since this cell line has been demonstrated to be PSA-negative (Sokoloff *et al* 1996, van Bokhoven *et al* 2003), and previous studies of PSA promoter constructs have found them to be inactive in PC-3 cells (Pang *et al* 1997, Gotoh *et al* 1998, Segawa *et al* 1998).

While determining suitable DHT concentrations in which to culture 22Rv1 cells, it was noted that optimal conditions for endogenous PSA secretion were less appropriate for expression of a transgene regulated by the PSA promoter and enhancer. Standard growth medium allowed higher levels of PSA secretion than medium supplemented with 0.1μM DHT, as measured by ELISA on the culture supernatant (Figure 5.5). However, though cells transfected with PSA-CD constructs and grown in standard medium failed to produce detectable CD, cells transfected in parallel but grown in 0.1μM DHT showed CD expression (Figure 5.6). Though this was unexpected, several explanations are possible. The endogenous PSA promoter in 22Rv1 cells may differ from that of the plasmid construct. The ELISA technique measures secreted PSA protein, and therefore could be influenced by post-translational processing steps not required for the CD enzyme. Alternatively, CD mRNA or protein could be destabilised by a factor present in standard medium but not in hormone-supplemented medium.

22Rv1 cells were reported to be PSA-positive, due to the increased serum PSA of mice bearing 22Rv1 xenograft tumours (Sramkoski *et al* 1999). However, unlike LNCaP (Cleutjens *et al* 1997a, Rodriguez *et al* 1997, Latham *et al* 2000), no published data was available on previous use of PSA promoter constructs in 22Rv1 cells. Initial observations suggested that 22Rv1 cells expressed significantly lower (over 100-fold) levels of PSA than LNCaP cells under similar conditions (grown for six days in 24-well plates using the same materials and range of DHT concentrations), though using different ELISA kits (Figures 5.4 – 5.5). This raised the question of whether the 22Rv1 cell line was suitable for studies requiring PSA promoter activity. A further ELISA assay of the two cell lines using the same assay method was performed to clarify the issue. When grown in optimal defined hormone concentrations and assayed in parallel by the same method, LNCaP cells secreted 27-fold more PSA than 22Rv1 cells. Immunohistochemistry showed that 22Rv1 cells stained weakly positive for PSA (Figure 5.7). Assays on PSA-CD constructs were continued in 22Rv1 cells, partly due to the technical difficulties involved in growing LNCaP cells. Results from Western blots (Figures 5.6 and 5.9) and MTS assays (Chapter 6) show that the 22Rv1 can serve as an adequate model system for this study. Prostate cancer model systems are discussed further in Chapter 8.

Densitometric assessment of the CD Western blots showed that mutagenesis of the PSA promoter at two sites at ARE-I did not increase promoter activity. A mean 16% reduction in CD expression was observed in LNCaP cells transfected with pPCM1.24, compared with pPPE-CD2.15 transfectants, though no reduction was detected in 22Rv1 cells. Otherwise, PSA promoter activity after mutagenesis, as measured by CD densitometry, was 90-104% of that of the original plasmid (Figure 5.11). MTS assays (Chapter 6) yielded similar findings. The effect of the substitutions introduced by mutagenesis is likely to depend on interactions with other regulatory sequences in the PSA promoter, and on interactions with specific transcription factors which the cell lines tested may lack.

CHAPTER 6: INDUCTION OF CELL DEATH BY CD/5-FC PRODRUG ACTIVATION SYSTEM

INTRODUCTION

To assess the CD/5-FU prodrug activation system in the context of an *in vitro* model of prostate cancer, procedures were optimised for delivering the therapeutic gene to cells (Chapter 4) and plasmid constructs were generated (Chapter 3) which expressed the CD protein (Chapter 5). The experiments below proceed to address the crucial issue of cell death induction by this prodrug activation approach. Viability and apoptosis were measured in cell lines treated with CD expression plasmids and 5-FC. The ability of the PSA promoter to confer tissue-specific gene expression, and the possible effect of sequence variations in the PSA promoter, were also assessed.

Three methods were employed to measure cell death. Firstly, MTS assays were carried out. A tetrazolium compound, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] (MTS), was added to culture medium, where it was reduced to a coloured formazan product. This reaction required dehydrogenase enzymes expressed by viable cells. The amount of reaction product therefore depended on the number of viable cells present, and was assayed by measuring the absorbance at 490nm of the culture medium. The assay reagent differed slightly from the conventional MTS reagent in that its product was soluble in culture medium, making it unnecessary to carry out a solubilisation step after adding the reagent. This technique allowed quantitative data on cell viability to be obtained from many samples simultaneously.[1] However, this method did not provide any information about the mechanism of cell death. Flow cytometry was carried out for this purpose.

[1]96-well MTS assays were not suitable for LNCaP cells, due to the difficulty of controlling for random cell loss during transfection and treatment procedures.

Flow cytometry evaluates the proportion of a cell population which contains a fluorescent label, by counting the total number of single cells analysed and the measuring the fluorescent properties of each cell. In this study, cells were labelled with annexin V (fluorescently tagged with FITC) and propidium iodide. Annexin V is an anticoagulant protein which binds to phospholipids such as phosphatidyl serine. During the early stages of apoptosis, cytoplasmic membrane structure is disrupted so that phosphatidyl serine is relocated to the surface of the cell and becomes accessible to annexin V. Cells which bind annexin V are therefore considered to be undergoing apoptosis. Propidium iodide (PI) is taken up by late apoptotic or necrotic cells, but excluded by cells with intact cytoplasmic membranes.

RESULTS

6.1. Effects of CD/5-FC treatment on PC-3 cells

6.1.1. MTS assays

Transfection of PC-3 cells with pCMV-CD1.10 increased their sensitivity to 5-FC in a dose-dependent manner (Figure 6.1). After treatment for six days, 50% of pCMV-CD1.10 transfectants were killed by 1mM 5-FC, a concentration which had no effect on untransfected cells and caused slightly reduced viability in cells transfected with other plasmids (Figures 6.1 – 6.2). When treatment time was reduced to three days, only pCMV-CD1.10 transfectants were killed by 1mM 5-FC, though to a lesser extent than after six days (Figure 6.3).

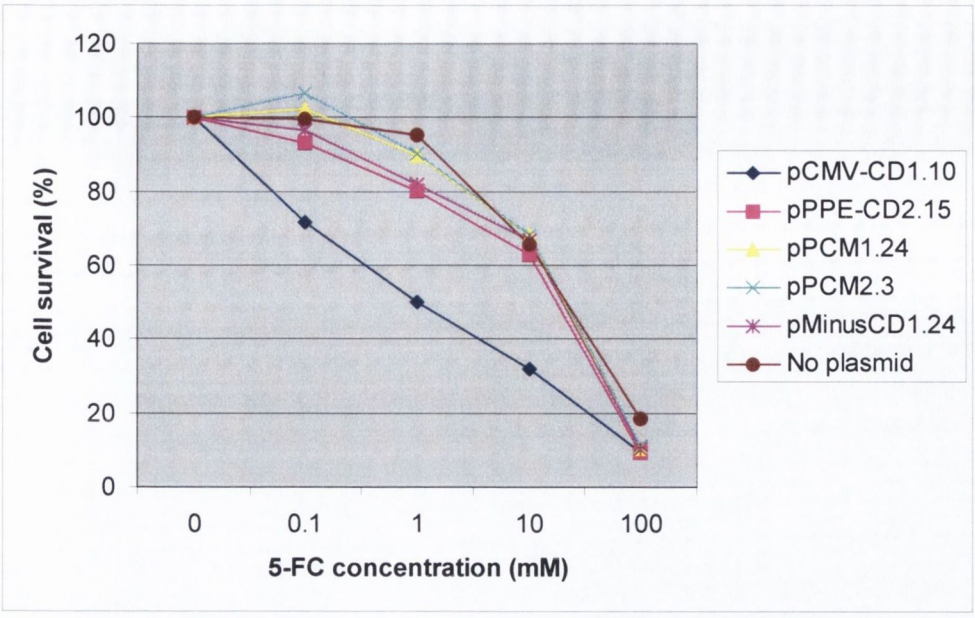


Figure 6.1. 5-FC induces dose-dependent cell death in PC-3 transfectants. Cells were transfected with plasmids and treated for six days with 5-FC as indicated above. Viability was normalised to 100% for untreated cells. The data represent mean values from two independent experiments done in triplicate.

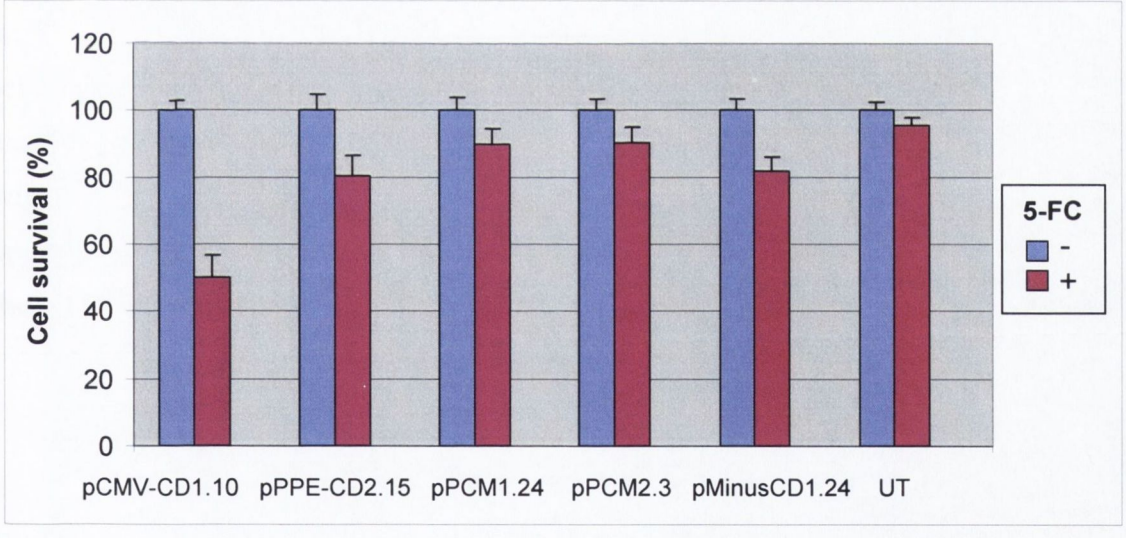


Figure 6.2. Cell kill in PC-3 transfectants after 5-FC treatment for six days. PC-3 cells were treated with 1mM 5-FC after transfection with the indicated plasmids. Cell survival was calculated relative to that of untreated cells containing the same plasmid. Absorbance readings of untreated cells in MTS assays was standardised to 100%. The data represent mean and standard deviation values from two independent experiments done in triplicate. UT, untransfected.

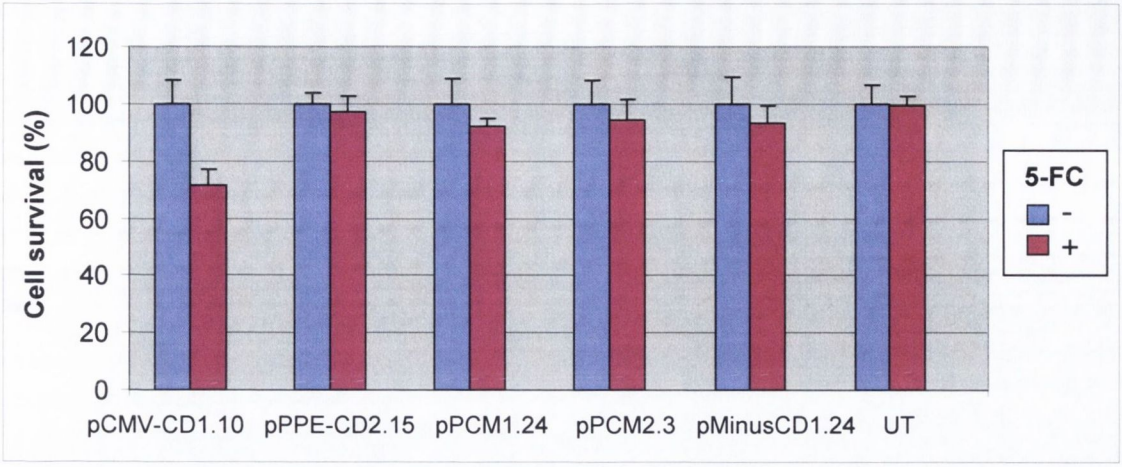


Figure 6.3. Cell kill in PC-3 transfectants after 5-FC treatment for three days. Cells were transfected with the plasmids above and treated with 1mM 5-FC for three days. Viability was calculated as a percentage of the MTS assay absorbance readings of untreated cells transfected with the same plasmid. The data represent mean and standard deviation values from two independent experiments done in triplicate. UT, untransfected.

6.1.2. Annexin V/propidium iodide flow cytometry

Flow cytometry was carried out on PC-3 cells after transfection with pCMV-CD1.10 and treatment for six days with 1mM 5-FC. Data was prepared for analysis by selecting the main populations of cells according to their size (forward scatter axis) and granularity (side scatter axis) (Figure 6.4A). Unlabelled PC-3 cells with and without transfection and 5-FC treatment were also analysed, in order to define appropriate fluorescence intensity thresholds to distinguish cells which had acquired the annexin V and/or PI labels from cells which had not (Figure 6.4B).

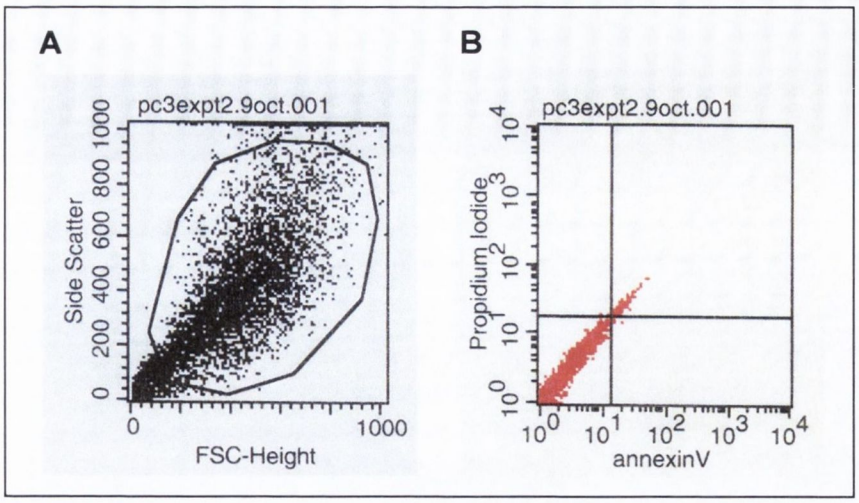


Figure 6.4. Preparation of PC-3 flow cytometry data for analysis. A. Cells were gated to select the main population of cells and exclude debris. **B.** Thresholds for annexin V and PI positivity were set using CellQuest software and cells not exposed to annexin V or PI, such that >95% of unlabelled PC-3 cells were negative.

Figure 6.5, 6.6 and 6.12 show flow cytometry histograms in which the number of cells is plotted against intensity of fluorescence at the appropriate wavelength for detection of annexin V-FITC or propidium iodide. Transfection of PC-3 cells with pCMV-CD1.10 combined with 5-FC treatment significantly increased annexin V labelling in the experiment shown in Figure 6.5. 5-FC treatment had no effect on untransfected cells, and similar trends were seen for PI (Figure 6.6). The proportion of annexin V-positive cells in cultures exposed to both pCMV-CD1.10 and 5-FC was 2.5-fold higher than in transfected but untreated cells, and 5-FC also increased PI uptake in transfected but not untransfected cells. Further experiments are required to confirm the reproducibility of these results (Figure 6.7). However, they suggest that 5-FC treatment of PC-3 cells expressing the CD gene causes apoptosis, and that this is due to the CD transgene, since parental PC-3 cells are not sensitive to 5-FC treatment under the same conditions. These findings concur with those obtained in MTS assays on PC-3 cells (Figure 6.2).

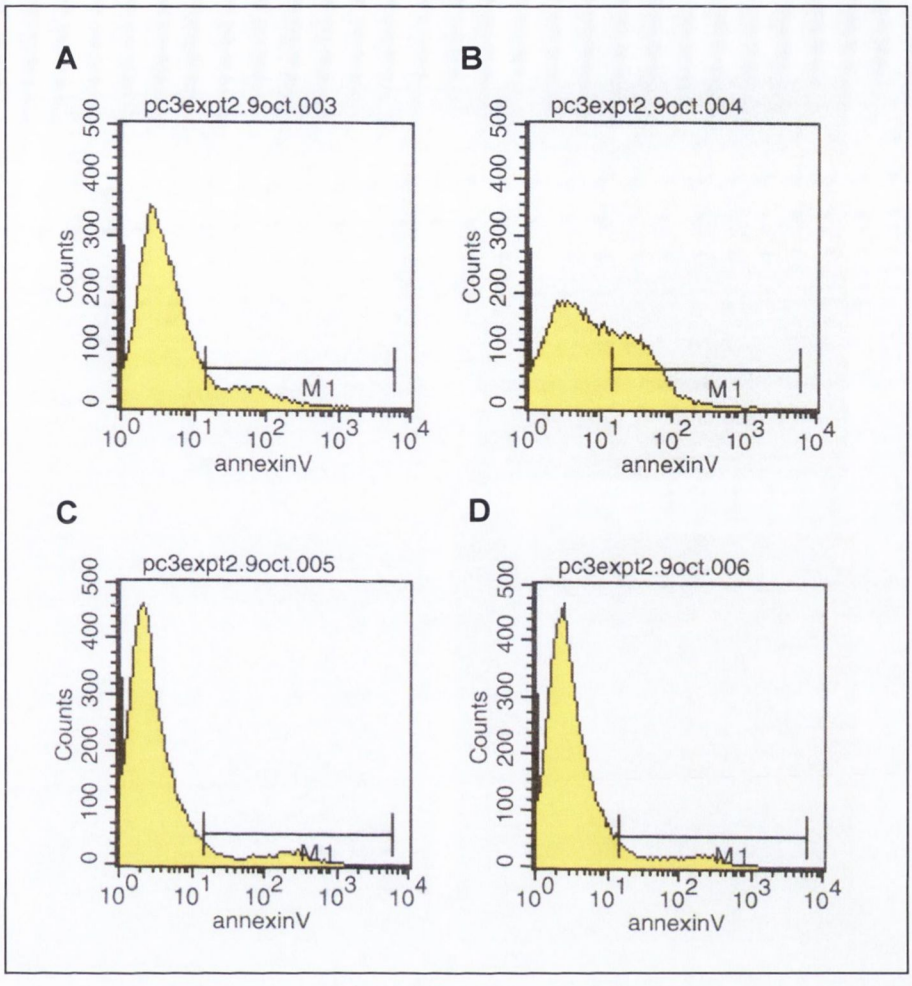


Figure 6.5. Increased annexin V staining in PC-3 transfectants treated with 5-FC. Parental PC-3 cells and pCMV-CD1.10 transfectants were treated for six days with 1mM 5-FC. **A.** pCMV-CD1.10, untreated. **B.** pCMV-CD1.10, treated. **C.** Untransfected, untreated. **D.** Untransfected, treated. Markers for annexin V positive cells (indicated as M1) were inserted, and excluded >95% of unlabelled PC-3 cells (transfected and untransfected).

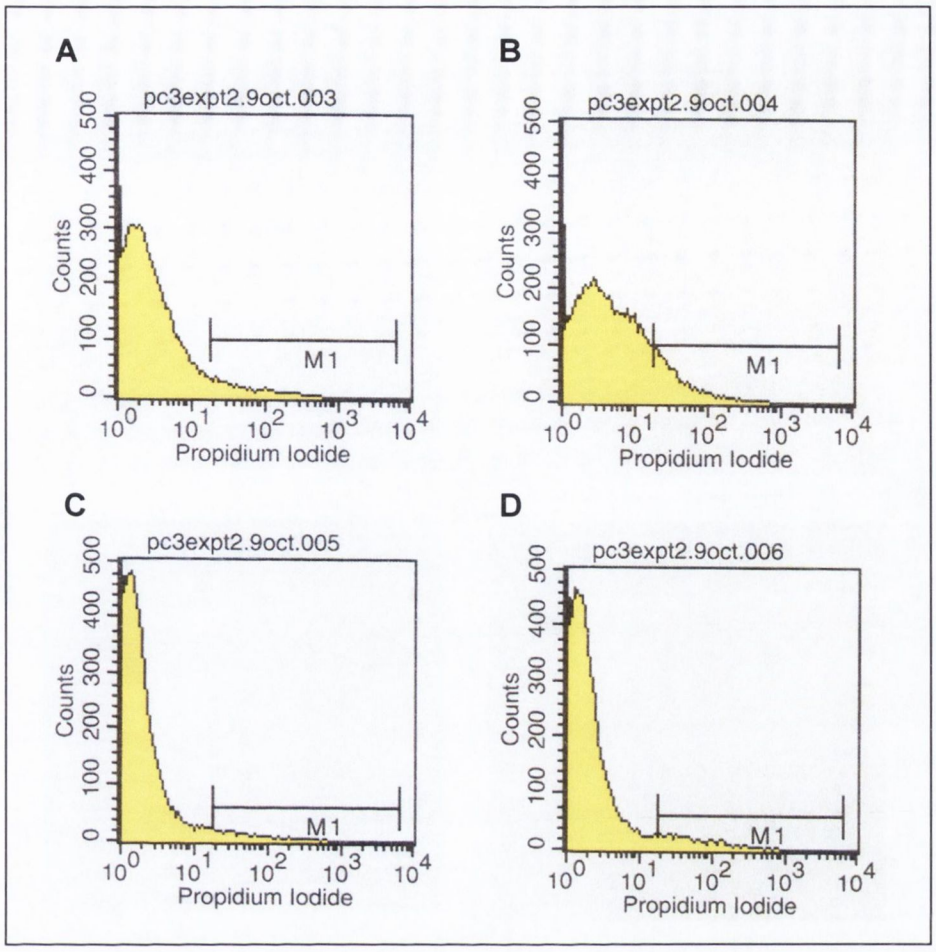


Figure 6.6. Propidium iodide staining in PC-3 transfectants treated with 5-FC. PC-3 cells transfected with pCMV-CD1.10 and untransfected control cells were treated with 1mM 5-FC for six days. **A.** pCMV-CD1.10, untreated. **B.** pCMV-CD1.10, treated. **C.** Untransfected, untreated. **D.** Untransfected, treated. Markers (shown as M1 above) were inserted to indicate cells which had taken up PI, at a position excluding 95% of unlabelled cells.

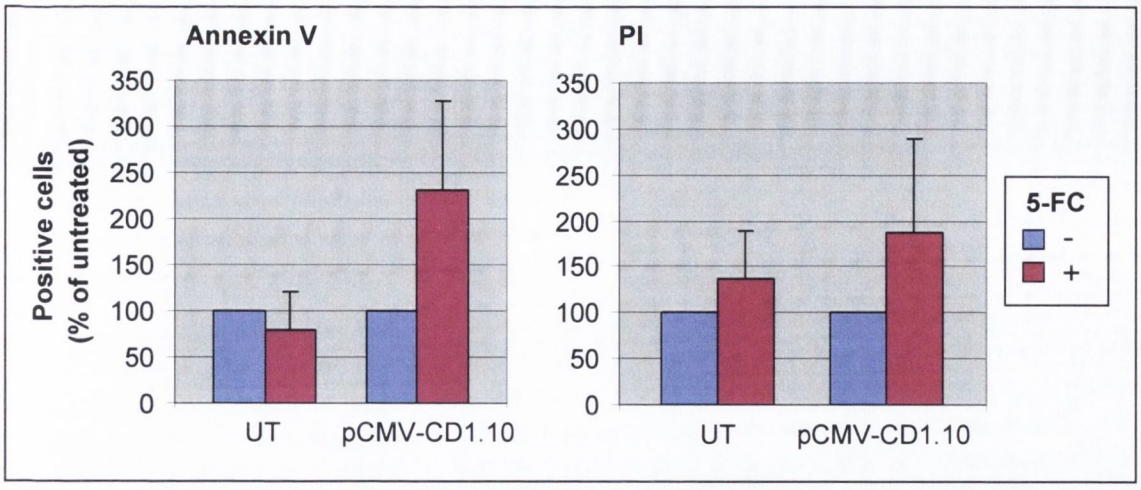


Figure 6.7. Summary of annexin V and propidium iodide data in PC-3 cells. Untransfected (UT) and pCMV-CD1.10-transfected PC-3 cells were treated for six days with 1mM 5-FC. Cells were gated and thresholds were set as in Figure 6.4. The proportion of untreated cells which were positive for each fluorescent label was normalised to 100%. Data represent mean and standard deviation of two independent experiments.

6.2. Effects of CD/5-FC treatment on 22Rv1 cells

22Rv1 cells also showed dose-dependent responses to 5-FC following transfection with CD expression plasmids.[1] 1mM 5-FC was sufficient to kill almost 80% of pCMV-CD1.10 transfectants after six days, but had little effect on cells containing other plasmids (Figure 6.8). Increasing the 5-FC concentration to 10mM had little additional effect on pCMV-CD1.10 transfectants, but in contrast, brought about 60-70% cell kill in cells transfected with pPPE-CD2.15, pPCM1.24 and pPCM2.3. This was clearly due to CD transgene expression facilitated by the PSA promoter in these plasmids, since neither parental 22Rv1 cells nor cells transfected with the negative control plasmid pAMdPE1.1 were killed by treatment with 10mM 5-FC for six days (Figures 6.8 and 6.9). 22Rv1 cells were also treated with 5-FC for three days, but the resulting data was less consistent that obtained after six days of treatment (Figure 6.10).

[1] See Section 8.4.4 for discussion of 5-FC concentrations used *in vitro* and in clinical practice.

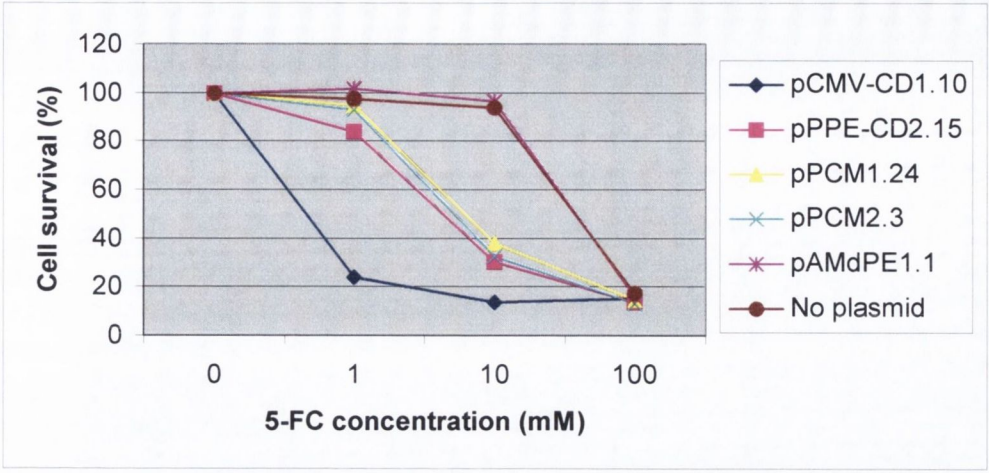


Figure 6.8. 5-FC induces dose-dependent cell death in 22Rv1 transfectants. Cells were transfected and treated with 5-FC for six days as indicated above. Cell survival, as measured by modified MTS assay, was normalised to 100% for untreated cells. The values shown are the mean of two independent experiments.

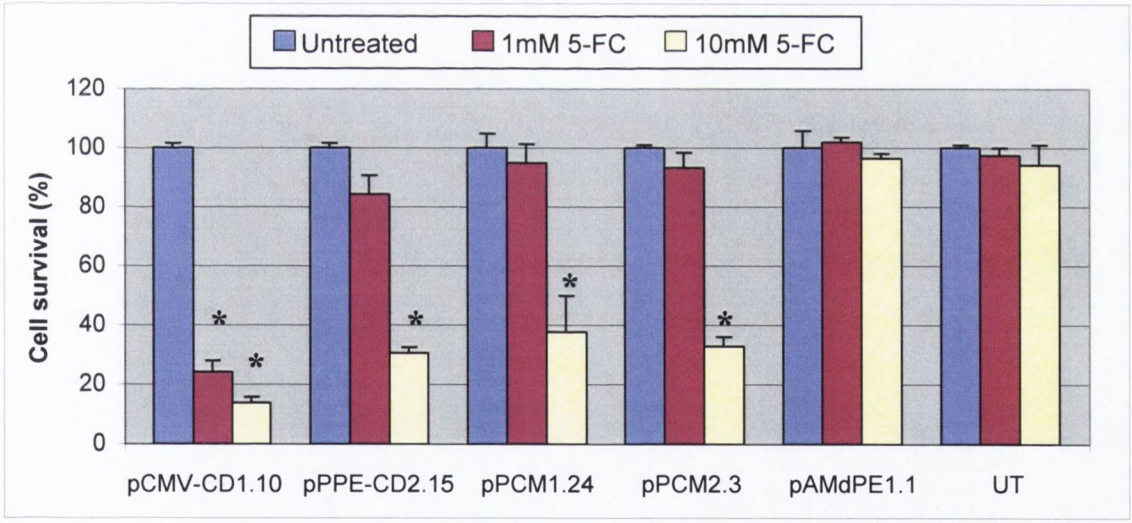


Figure 6.9. Cell kill in 22Rv1 transfectants after 5-FC treatment for six days. Cells were transfected with the plasmids above and treated with 5-FC. Cell viability was assessed by a modified MTS method, and compared with untreated cells transfected with the same plasmids, in which viability was normalised to 100%. The data represent mean and standard deviation values from two independent experiments performed in triplicate. UT, untransfected. *Survival of treated cells differed significantly from untreated controls, $p<0.001$.

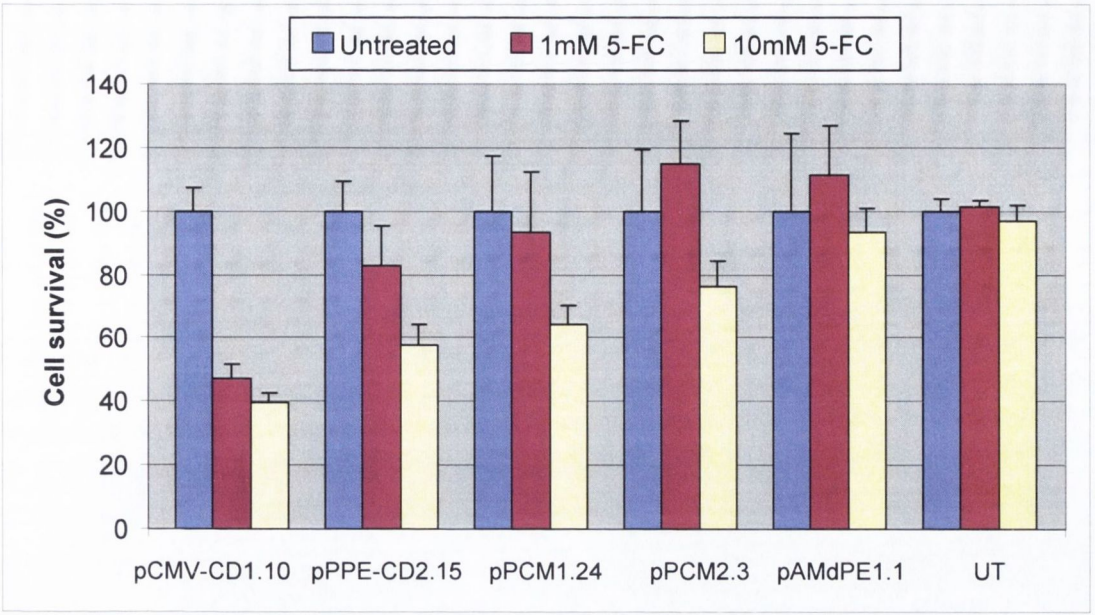


Figure 6.10. Cell kill in 22Rv1 transfectants after 5-FC treatment for three days. Cells transfected with the plasmids above were treated with 5-FC, and assessed for viability by a modified MTS method. Viability of untreated cells transfected with the same plasmids was normalised to 100%. The data represent mean and standard deviation values from two independent experiments performed in triplicate. UT, untransfected.

6.3. Effects of CD/5-FC treatment on LNCaP cells

As for PC-3 (Figure 6.4), LNCaP flow cytometry data was initially prepared by gating the cell population and setting thresholds for annexin V and propidium iodide (Figure 6.11).

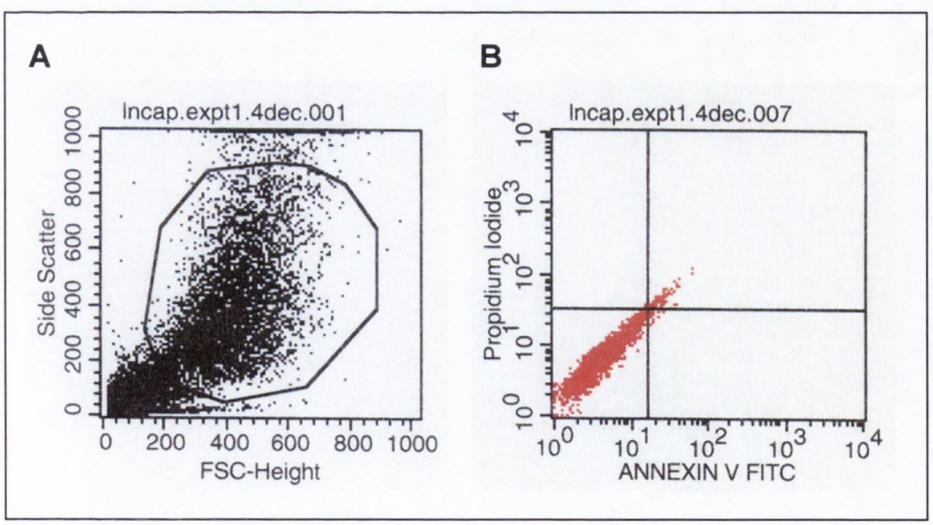


Figure 6.11. Preparation of LNCaP cells for flow cytometry analysis. A. Cells were gated to select the main population of cells and exclude debris. **B.** Thresholds for annexin V and PI positivity were set using CellQuest software, such that >95% of unlabelled LNCaP cells were negative.

LNCaP cells were transfected with pCMV-CD1.10 and with pPPE-CD2.15, treated with 1mM 5-FC for six days, labelled with annexin V-FITC and propidium iodide, and assayed by flow cytometry. The intensity of annexin V-FITC labelling was slightly higher in transfected and treated cells than untreated transfectants on histogram plots (Figure 6.12). This increase appeared as a small shift of the main peak to the right, in contrast to the large increase in annexin V intensity in transfected and treated PC-3 cells (Figure 6.5). 5-FC treatment slightly increased PI labelling in both transfected and untransfected LNCaP cells, with the greatest increase of 1.7-fold in pCMV-CD1.10 transfectants (Figure 6.13).

Statistical analyses of two independent experiments indicated that in pPPE-CD2.15 and pCMV-CD1.10 transfectants, addition of 5-FC increased the proportion of annexin-V positive cells threefold and 2.5-fold respectively (Figure 6.14). Under the same conditions, 5-FC treatment did not increase annexin-V labelling of untransfected cells. These data suggest that CD expression directed by either the CMV promoter or the PSA promoter in these plasmids sensitises LNCaP cells to apoptosis induced by 5-FC. It was not expected that the PSA promoter would be as effective as the CMV promoter, and further experiments are warranted to clarify whether this is the case.

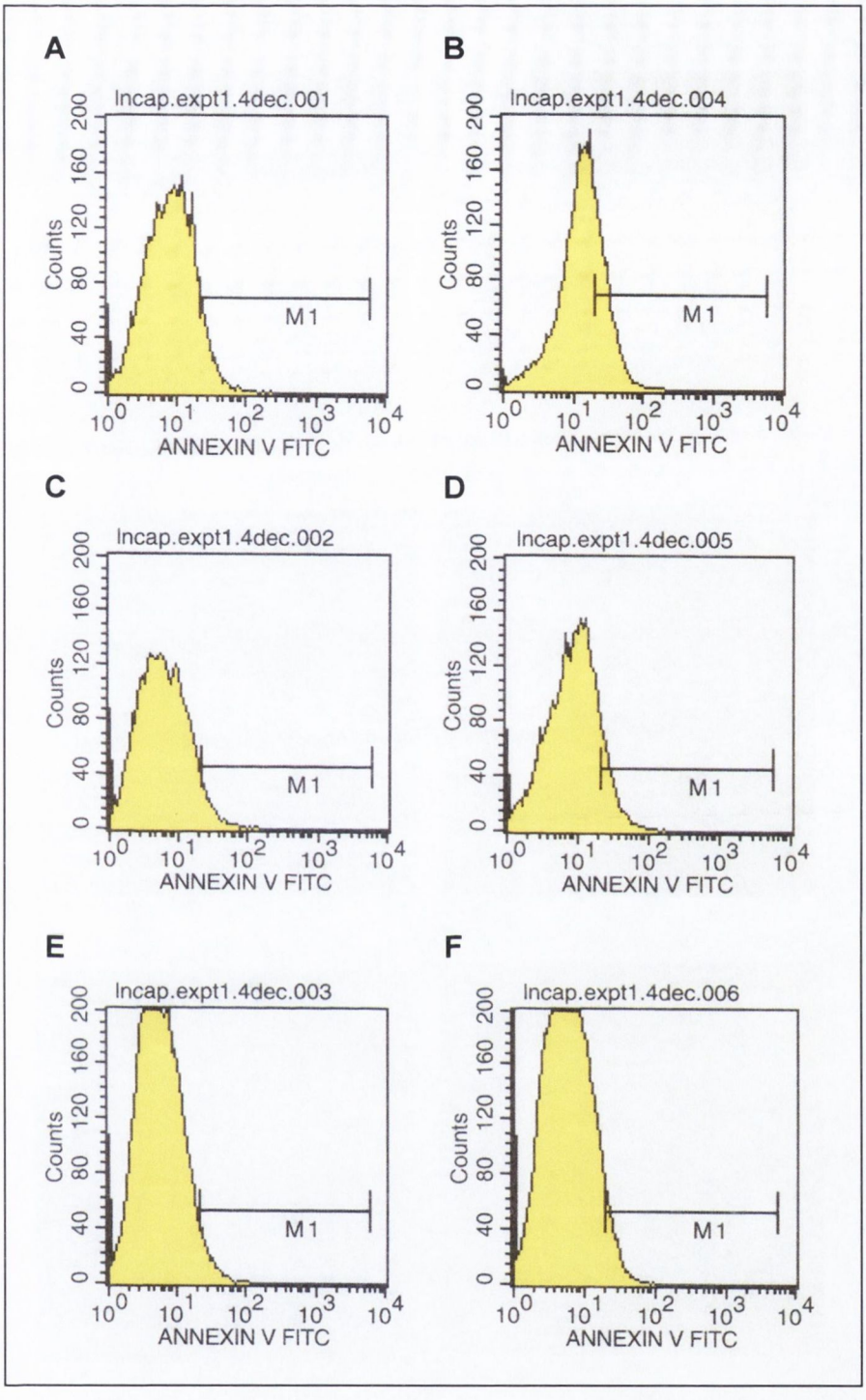


Figure 6.12. Increased annexin V staining in LNCaP transfectants treated with 5-FC. Parental PC-3 cells and pCMV-CD1.10 transfectants were treated for six days with 1mM 5-FC, or grown as untreated controls. **A.** pCMV-CD1.10, untreated. **B.** pCMV-CD1.10, treated. **C.** pPPE-CD2.15, untreated. **D.** pPPE-CD2.15, treated. **E.** Untransfected, untreated. **F.** Untransfected, treated. Markers for annexin V positive cells (indicated as M1) were inserted, and excluded >95% of unlabelled LNCaP cells (transfected and untransfected).

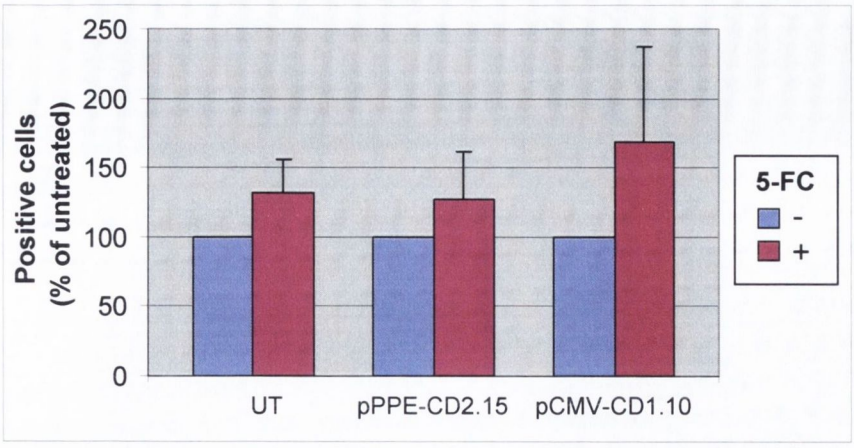


Figure 6.13. Summary of PI data in LNCaP cells. Transfected and untransfected (UT) LNCaP cells were treated for six days with 1mM 5-FC. Cells were gated and thresholds were set as in Figure 6.11. The proportion of untreated cells which were positive for PI was normalised to 100%. Data represent mean and standard deviation of two independent experiments.

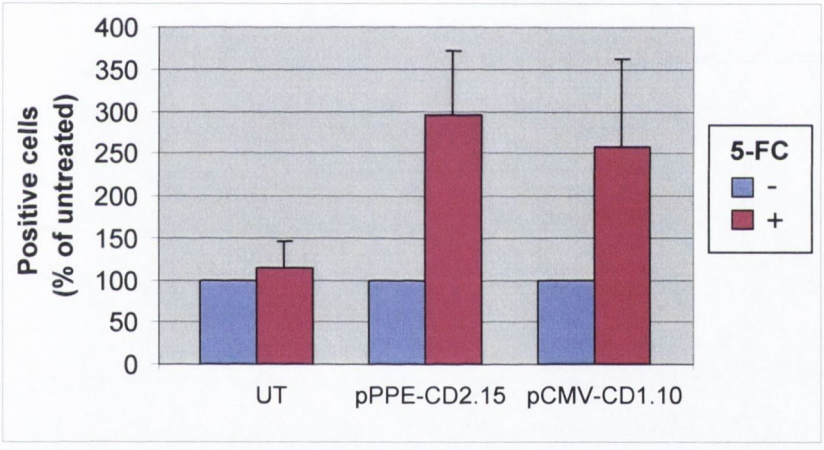


Figure 6.14. Summary of annexin V data in LNCaP cells. Untransfected (UT) and pCMV-CD1.10-transfected LNCaP cells were treated for six days with 1mM 5-FC, or grown as untreated controls. Cells were gated and thresholds were set as in Figure 6.11. The proportion of untreated cells positive for annexin V was normalised to 100%. Data represent mean and standard deviation of two independent experiments.

6.4. Effects of CD/5-FC treatment on HCT116 colon cancer cells

The colon cancer cell line HCT116 was employed to investigate the activity of the PSA promoter and enhancer in tissues other than prostate, since normal tissue injury following radiotherapy to the prostate primarily affects the colon and rectum. Although cells were plated at a relatively low density to be 50% confluent at transfection, cells transfected with plasmids other than pCMV-CD1.10 and treated with low doses of 5-FC rapidly became confluent. To avoid limiting the growth of control cultures due to confluence, treatment was therefore carried out for a period of 24 hours in this cell line. After this time, compared to untreated cells, 26% of pCMV-CD1.10 transfectants were killed by 1mM 5-FC, while the same dose killed <3% of cells transfected with other plasmids (Figures 6.15 – 6.16). At higher doses, cell kill by 5-FC in cells transfected with PSA-CD constructs occurred to the same extent as in cells containing negative control plasmid pAMdPE1.1 and in parental HCT116 cells (Figure 6.15). These results demonstrate that transfection of pCMV-CD1.10 sensitised HCT116 cells to 5-FC, but that transfection of PSA-CD constructs pPPE-CD2.15, pPCM1.24 and pPCM2.3 did not.

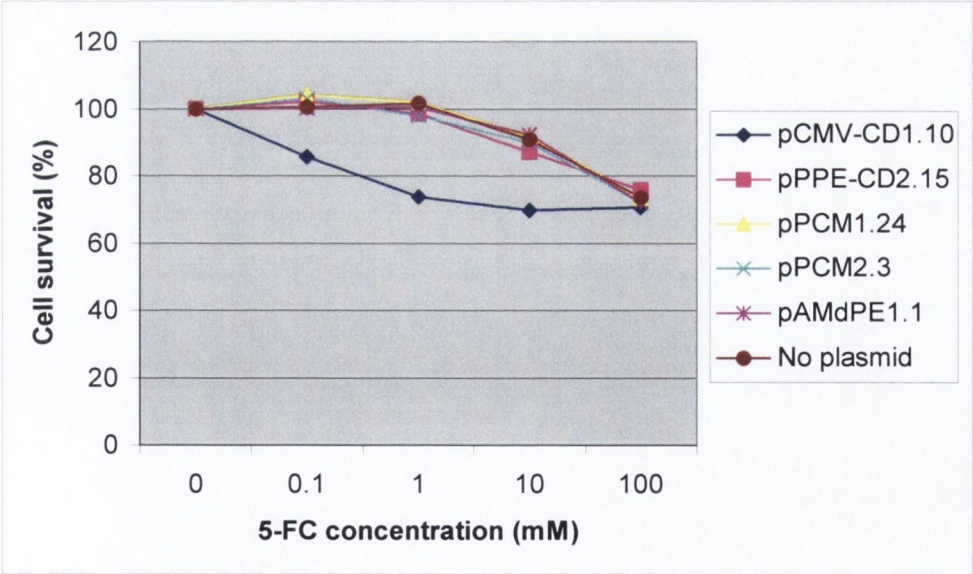


Figure 6.15. 5-FC induces cell death in HCT116 cells after pCMV-CD1.10 transfection. Cells transfected with the plasmids indicated above were treated for one day with several concentrations of 5-FC. Viability was normalised to 100% for untreated cells. The data shown are mean values from two independent experiments done in triplicate.

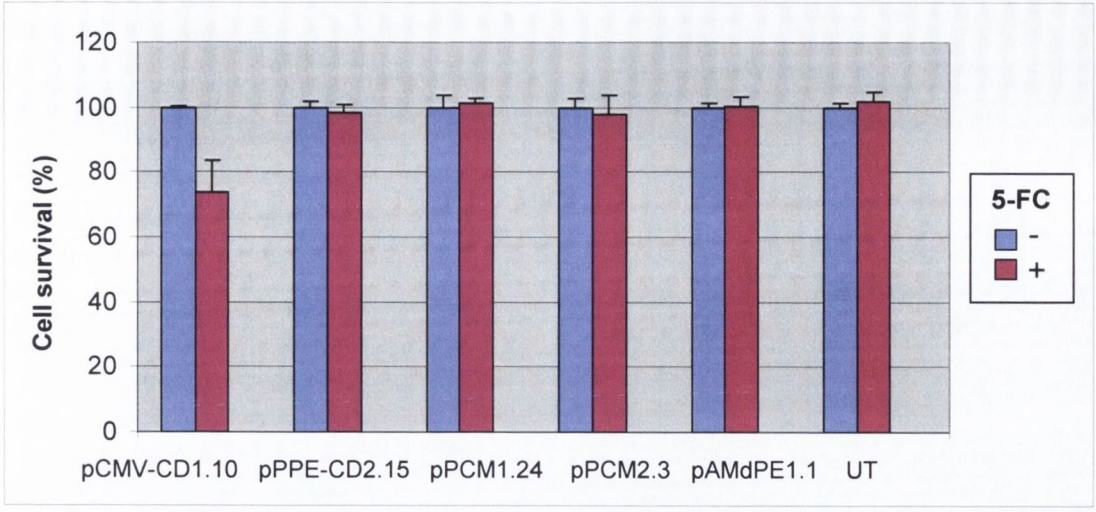


Figure 6.16. Cell kill in HCT116 transfectants after 5-FC treatment for twenty-four hours. Cells transfected with the plasmids above were treated with 5-FC. Viability of untreated cells transfected with the same plasmids was normalised to 100%. The data represent mean and standard deviation values from two independent experiments performed in triplicate. UT, untransfected.

6.5. pAMdPE1.1 substituted for pMinusCD1.24 as negative control

The negative control plasmid pAMdPE1.1 was substituted for pMinusCD1.24 in MTS assays on 22Rv1 and HCT116 cells. Cells transfected with pMinusCD1.24 and treated with 5-FC showed approximately 20% more cell death than similarly treated pAMdPE1.1 transfectants (Figure 6.17). The apparent ability of pMinusCD1.24 to sensitise cells to 5-FC (Figure 6.17) and to express CD protein (Figure 5.10) raised the concern that it was contaminated with pCMV-CD1.10, though it is not known how this could have occurred. *Hin*dIII restriction digests were performed and yielded fragments of the expected sizes for both pCMV-CD1.10 and pMinusCD1.24, and did not show contamination of pMinusCD1.24 with pCMV-CD1.10 (Figure 6.18).

The web-based bioinformatic program PromoterScan (Prestridge 1995) was employed to analyse the sequence upstream of the CD open reading frame in pMinusCD1.24. The program predicted a weak promoter in this region, with two Sp1 binding sites 330bp upstream of the CD sequence and another promoter-associated motif a further 150bp upstream.

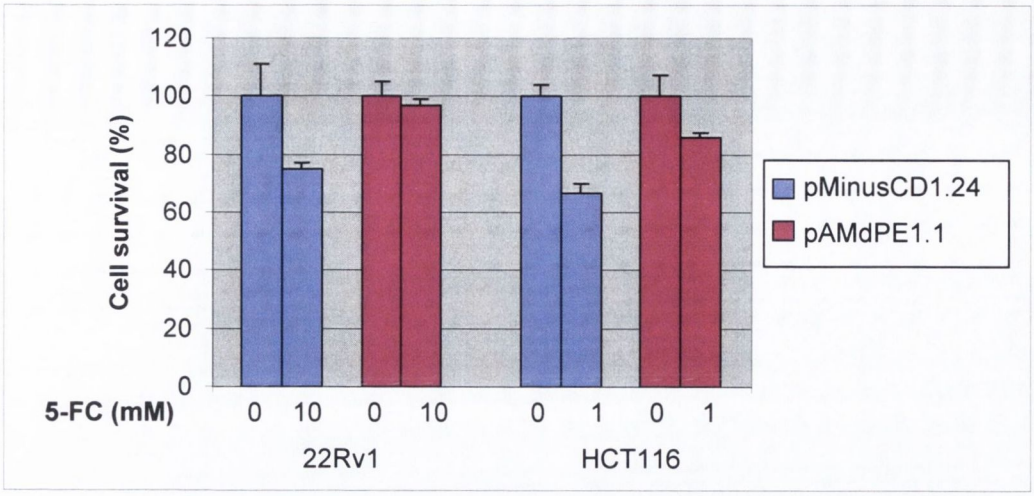


Figure 6.17. Negative control plasmids for MTS assays in 22Rv1 and HCT116 cells. The indicated cell lines were transfected with pMinusCD1.24 and pAMdPE1.1, and treated with 5-FC for one day (HCT116) or three days (22Rv1). Survival of untreated cells was normalised to 100%. The above data show mean and standard deviation values from triplicate wells.

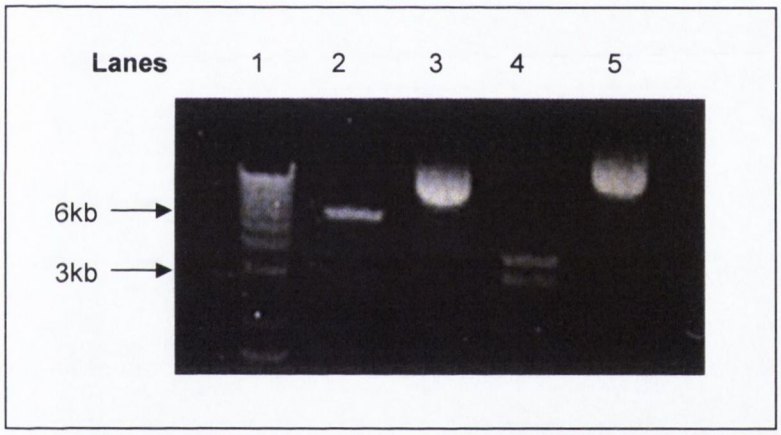


Figure 6.18. Restriction digest analysis of pMinusCD1.24. Restriction digests of the relevant plasmids were electrophoresed on a 1% agarose gel. Lane 1: size marker. Lane 2: pMinusCD1.24 digested with *Hin*dIII. Lane 3: undigested pMinusCD1.24. Lane 4: pCMV-CD1.10 digested with *Hin*dIII. Lane 5: undigested pCMV-CD1.10.

DISCUSSION

The CD/5-FC prodrug activation system selected for this study achieved cell kill in all the cell lines tested (LNCaP, 22Rv1, PC-3 and HCT116) when cells were transfected with the CD transgene under the control of the constitutive CMV promoter. In contrast, constructs in which the CD expression was regulated by the PSA promoter caused sensitisation to the prodrug 5-FC in LNCaP (Figure 6.14) and 22Rv1 cells (Figure 6.9), but not HCT116 colon cancer cells (Figure 6.15), or PC-3 cells (Figures 6.2 – 6.3, see below).

PC-3 cells transfected with PSA-CD constructs showed 10-20% lower survival after treatment with 1mM 5-FC for six days (Figures 6.1 and 6.3). However, this appears more likely to be associated with the effect of transfection on the cells, than with CD enzyme expression. 18% of cells transfected with pMinusCD1.24 were also killed by 1mM 5-FC after six days, though Western blots (Chapter 5) detected no CD enzyme in these cells. Also, PC-3 cells transfected using the liposome reagent GenePorter2 and used for initial flow cytometry assays showed transfection-related toxicity in the absence of 5-FC, and a less toxic liposome reagent was later substituted.

Despite low levels of endogenous PSA expression in 22Rv1 cells, 5-FC treatment killed up to 70% of cells containing PSA-CD constructs, in a dose-dependent manner (Figure 6.9). Under the same conditions, 97% of cells transfected with the negative control plasmid pAMdPE1.1 survived, demonstrating that transfection alone did not sensitise 22Rv1 cells to 5-FC and that the PSA-CD constructs were effective in causing cell kill in combination with 5-FC. After treatment of 22Rv1 cells for six days, LD_{50} values for 5-FC in cells transfected with pCMV-CD1.10, pPPE-CD2.15 transfectants and parental 22Rv1 cells were 0.7mM, 6.7mM and 61.8mM respectively (Figure 6.8). Expression of CD by PSA promoters therefore made 22Rv1 cells nine times more sensitive to 5-FC.

The PSA promoters in the plasmids pPPE-CD2.15, pPCM1.24 and pPCM2.3, although varying in sequence due to mutagenesis, did not differ significantly in their ability to express CD and thereby sensitise cells to 5-FC (Figure 6.9). Among these plasmids, highest levels of cell kill following 5-FC treatment for six days (69%) were

observed in cells transfected with pPPE-CD2.15, but pPCM1.24 and pPCM2.3 also facilitated over 60% cell death. Results after treatment for three days suggested that pPPE-CD2.15 was more effective (Figure 6.10). However, cell viability readings at this time point were less consistent, in contrast to the good reproducibility of the data obtained after six days (Figure 6.9).

Preliminary flow cytometry data from LNCaP transfectants also suggest that the PSA-CD construct in pPPE-CD2.15, combined with 5-FC, can bring about cell kill. This infers that the PSA promoter produces sufficient CD for a biological effect. 5-FC treatment increased the proportion of apoptotic cells among pPPE-CD2.15 transfectants by a factor of 3 ± 0.8, while the corresponding increase in parental LNCaP cells was 1.1 ± 0.3 (Figure 6.14). However, further studies are required to confirm these results and assess the possibility that this effect was caused by transfection rather than CD expression. In both LNCaP and PC-3 cell lines, 5-FC treatment of CD-expressing cells was more clearly associated with increased annexin V labelling than with elevated PI uptake (Figures 6.7, 6.13 and 6.14), indicating that that the CD/5-FC system induces apoptosis in prostate cancer cell lines.

The plasmid pMinusCD1.24 was generated as a negative control, identical in structure to the plasmids in which PSA or CMV promoters controlled CD expression, except for the absence of either of these promoters. Its apparent ability to express low levels of CD (Figure 5.10) and sensitise cells slightly to 5-FC (Figure 5.17) was unexpected, and led to its replacement by pAMdPE1.1 as a negative control plasmid. pAMdPE1.1 was derived from the commercial vector pALTER-MAX by deleting the CMV promoter and enhancer, and pMinusCD1.24 was generated by inserting the CD open reading frame into pAMdPE1.1. pMinusCD1.24 therefore contained the CD coding sequence but not the CMV promoter in the original vector. pAMdPE1.1 contained neither of these elements, and appeared inactive in 22Rv1 cells (Figure 5.9, Figure 6.9). Contamination of the pMinusCD1.24 plasmid stock with pCMV-CD1.10 was not indicated by restriction digests (Figure 6.18), but bioinformatic analyses showed a putative weak promoter in the plasmid vector sequence upstream of the deleted CMV promoter. It appears possible that factors in 22Rv1 and HCT116 cells may have activated this weak promoter, though Western blots of LNCaP and PC-3 cells

transfected with pMinusCD1.24 showed no trace of CD expression (Chapter 5). In any case, pMinusCD1.24 was required only as a negative control plasmid, and this function was fulfilled satisfactorily by pAMdPE1.1.

The intrinsic sensitivity of the various cell lines to the CD/5-FC system varied. Treatment of pCMV-CD1.10 transfectants with 1mM 5-FC for six days killed 50% of PC-3 cells (Figure 6.1) and almost 80% of 22Rv1 cells (Figure 6.8). This variation may be due to cellular enzymes such as thymidine phosphorylase which metabolise 5-FU (Longley *et al* 2003). A 26% reduction in viability was observed in HCT116 cells transfected with pCMV-CD1.10 after treatment with 1mM 5-FC, but these cells were only treated for 24 hours (Figure 6.15).[1] While results are indicative of a prostate-specific cell kill using this system, a more comprehensive screening of HCT116 and other non-prostate cell lines needs to be completed. In 22Rv1 cells transfected with PSA-CD constructs, cell kill increased from 10-20% to 60-70% when the 5-FC concentration was raised from 1mM to 10mM. Though intermediate concentrations have not been tested to date, it is likely that lower doses than 10mM may also be effective.

[1] HCT116 cells were transfected for MTS assays using GeneJuice. Due to the low toxicity of this reagent and the rapid growth of this cell line, cells which were not treated with 5-FC were fully confluent three days after transfection, though only 50% confluent at transfection. Cell death was therefore initially assayed two days after transfection and one day after treatment. An alternative transfection reagent may be required to obtain MTS results in HCT116 cells after longer 5-FC treatment times.

CHAPTER 7: PRODRUG ACTIVATION IN PRIMARY PROSTATE CULTURES

INTRODUCTION

In addition to testing the PSA-CD prodrug activation system in prostate cancer cell lines (Chapter 6), experiments were undertaken towards growing human primary prostate cancer cells *in vitro* for use as an additional model system. This presents a more challenging goal, not least because of the scarcity of data on transfection of primary cultures in general. However, investigation of the CD/5-FU system, or other gene-based therapeutic approaches, in a primary culture model of prostate cancer would be much more relevant to the clinical setting than cell lines cultured for long periods under artificial conditions.

A further challenge in establishing a primary prostate cancer model system is the sourcing of relevant prostate cancer tissue samples. A system was initiated by which tissue specimens were collected from the operating theatre, sampled for use in research by a consultant pathologist, and stored in the laboratory while awaiting confirmation that the samples were not required for patient diagnosis (see Section 2.10.1). However, prostate cancer occurs in a multifocal manner within the prostate gland, and is difficult to identify macroscopically, compared to solid tumours in other organs. This compromises the establishment of robust primary prostate cancer cultures. Thus it is important to liaise with the pathologist in the identification of benign and tumour material. Based on transrectal ultrasound results for each patient, regions of the prostate were selected for sampling malignant and benign tissue. Results of subsequent histological analysis were obtained and correlated with the location of the samples taken from each patient. A total of ten patients donated samples for research. Full work up was performed for all of these tissue samples. Material from 6 patients gave informative results which could be used for the assessment of the potential for gene directed approaches in primary prostate tissue (Table 7.1).

Patient number	Results
IMM0015	Results not informative
IMM0016	Not viable in second passage
IMM0017	Not viable in second passage
IMM0018	Results not informative
IMM0019	Results presented
IMM0021	Results presented
IMM0022	Results presented
IMM0023	Results presented
IMM0024	Results presented
IMM0025	Results presented

Table 7.1. Summary of patient samples. The patients' samples were anonymised and identified by the reference numbers IMM0019-IMM0025. Results are presented in the remainder of this chapter from six of the ten patients involved.

In order to create a reliable primary prostate cancer culture system, it is necessary to characterise the material that is promulgated in the culture system. A detailed examination of the literature in this area does not indicate common markers of prostate cancer that are used routinely in evaluating prostate cancer models, but after due consideration, we selected a number of parameters to distinguish benign and malignant prostate tissue including tissue morphology, epithelial and stromal marker expression, cell adhesion function and PSA expression.

Primary cultures were characterised *in vitro* in terms of morphology, epithelial and stromal marker expression, cell adhesion function and PSA expression. Zheng *et al* (1999) reported that malignant but not benign primary prostate cells adhered to vitronectin, due to their preferential expression of the cell surface receptor $\alpha_5\beta_3$ integrin, while both cell types adhered to fibronectin. A cell adhesion assay was therefore optimised using cell lines, including the benign human immortalised cell line PWR-1E

and a suspension cell line H929, and performed on paired samples taken from anticipated malignant and benign regions of patients' prostate glands. $\alpha_5\beta_3$ integrin expression was examined in the same cell lines by Western blotting.

Primary cultures were transiently transfected with CD expression plasmids. Due to low PSA expression levels *in vitro* in the first set of cultures grown, cells were cultured in keratinocyte serum-free medium supplemented with cholera toxin, epidermal growth factor and bovine pituitary extract after transfection and during prodrug treatment, as this medium was reported to permit PSA expression in primary prostate cultures (Maitland *et al* 2001). Initial transfections employed Lipofectamine 2000, and in one primary tumour sample transfected with a GFP reporter gene under various conditions, successful DNA uptake was acheieved at a level of 15%. However, after further samples analysed showed very poor viability after transfection with this carrier, it was substituted with GenePorter2 which was used for all subsequent studies. Cell death after 5-FC treatment was assayed using an MTT method suitable for small numbers of cells.

RESULTS

7.1. Patient details

Table 7.2 shows the clinical details of the patients from whose samples results are reported. In the case of IMM0025, the biopsy report indicated extensive tumour foci, and no benign sample was attempted. This patient's histology report indicated that tumour occupied 60% of the prostate gland. The percentage of tumour was considerably lower ($\leq$30%) in all other patients. On macroscopic examination of the prostate gland of patient IMM0022, tumour tissue was clearly visible, and this was sampled for use in primary culture. However, for the other patients, the location of the tumour was not clear macroscopically. Malignant histology was confirmed in all of the samples taken as tumour, with the exception of patient IMM0019, for whom tumour occupied less than 10% of the total volume of the prostate. Three of five samples taken as matching benign tissue for patients IMM0021, IMM0022 and IMM0024 also contained tumour in adjacent sections.

Patient number	Tumour sample		Benign sample		Gleason score	% tumour
	Histology	Location	Histology	Location		
IMM0019	Normal	Centre, right lobe	Normal	Centre, left lobe	3 + 4	<10%
IMM0021	Tumour/Normal	Centre, left lobe	Tumour/Normal	Centre, right lobe	3 + 2	<10%
IMM0022	Tumour/Normal	Apex, right lobe	Tumour/Normal	Apex, left lobe	3 + 4	30%
IMM0023	Tumour	Base, right lobe	Normal	Centre, right lobe	3 + 3	10%
IMM0024	Tumour/Normal	Base, right lobe	Tumour/Normal	Base, left lobe	3 + 3	15%
IMM0025	Tumour	Apex, right lobe	Not taken	N/a	5 + 3	60%

Table 7.2. Patient characteristics. Clinical details are shown for each patient sample for which informative results were obtained. Histological analysis of slides adjacent to the samples was performed by a consultant pathologist.

7.2. Morphology

Samples were separated into stromal and epithelial fractions on initiation of culture. Stromal cultures contained highly elongated cells aligned in the same direction as neighbouring cells (Figure 7.1A, 7.1B). Epithelial cultures displayed a characteristic cobblestone-like morphology (Figure 7.1C, 7.1D). No morphological differences were observed between samples taken from malignant and benign regions. Low-level stromal contamination occurred in some epithelial cultures but accounted typically for less than 20% of the cells present.

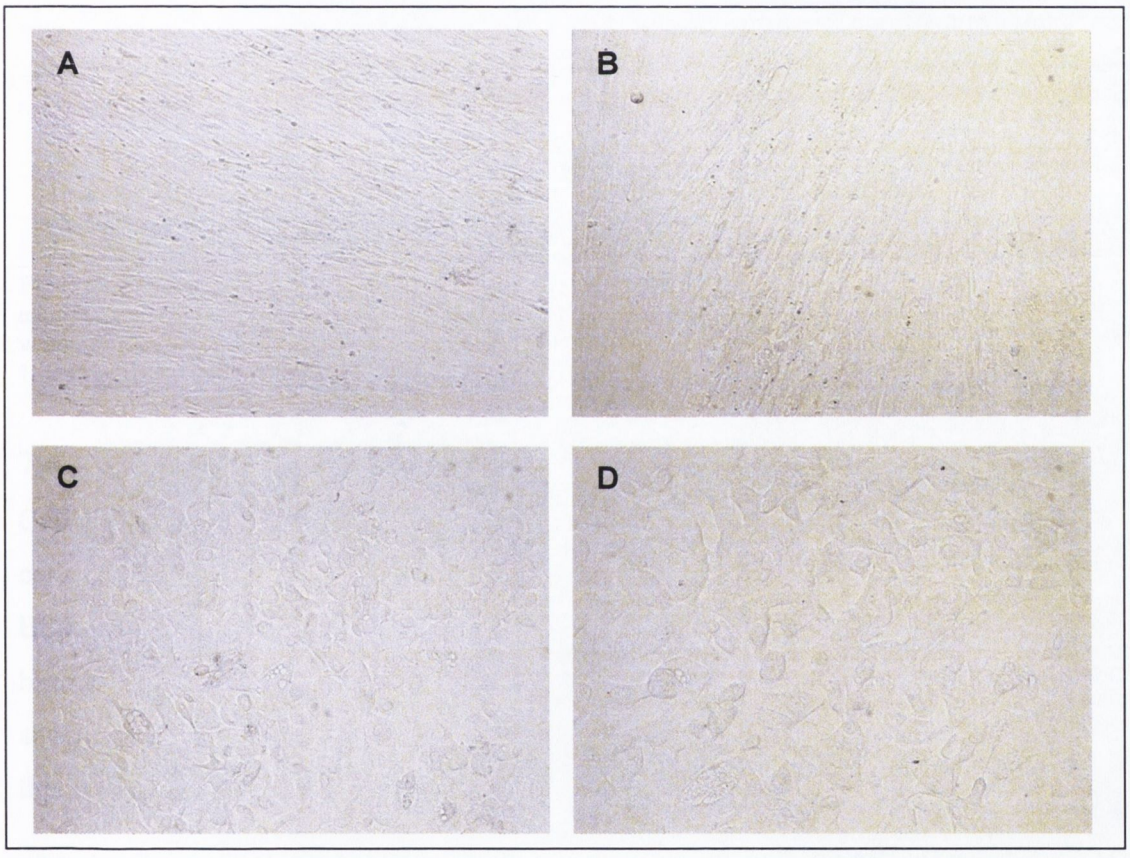


Figure 7.1. Morphological appearance of primary prostate cells. **A.** IMM0021, tumour stroma. **B.** IMM0022, benign stroma. **C.** IMM0024, benign epithelium. **D.** IMM0025, tumour epithelium. Cells are shown at 20X magnification.

7.3. Stromal and epithelial cell markers

In addition to morphological features, stromal and epithelial cultures were expected to differ in their expression of intermediate filament proteins. Protein extracts from primary cultures were examined by Western blotting for expression of cytokeratins characteristic of epithelial cells, and for the presence of the stromal cell marker vimentin. Figure 7.2 shows cytokeratin expression in epithelial cell protein extract and vimentin expression in stromal cells. Three distinct species of cytokeratin were detected by the pan-cytokeratin primary antibody against cytokeratins 4, 5, 6, 8, 10, 13 and 18.

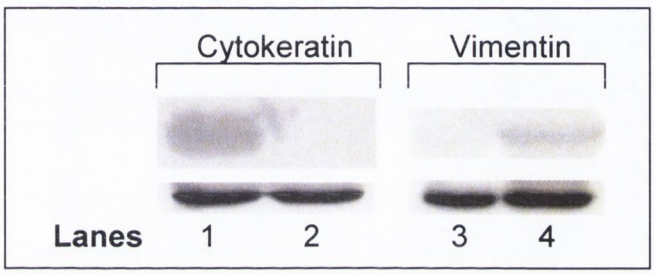


Figure 7.2. Expression of stromal and epithelial markers in prostate primary cell culture. Protein was extracted from the primary cultures indicated below. Cytokeratin and vimentin expression (upper bands) were assessed by Western blotting. Blots were probed for actin (lower bands) as a loading control. Lanes 1: IMM0023, benign epithelium. Lanes 2 and 4: IMM0021, tumour stroma.

7.4. Tumour cell markers

Cell adhesion assays were performed on five cell lines (Figure 7.3). DU145 and PC-3 cells both bound to fibronectin and vitronectin with very low non-specific binding to BSA-coated wells. LNCaP also bound to both fibronectin and vitronectin, but showed higher non-specific binding. The myeloma cell line H929, which grows in suspension, showed less than 10% adhesion and was unaffected by the coated surface. Surprisingly, this was also the case for the immortalized prostate cell line PWR-1E.

These cell lines were also investigated for expression of $\alpha_5\beta_3$ integrin by Western blotting (Figure 7.4). Expression was detected in both DU145 and PC-3 cells, consistent with the functional cell adhesion assay results. No $\alpha_5\beta_3$ integrin was detected in other cell lines.

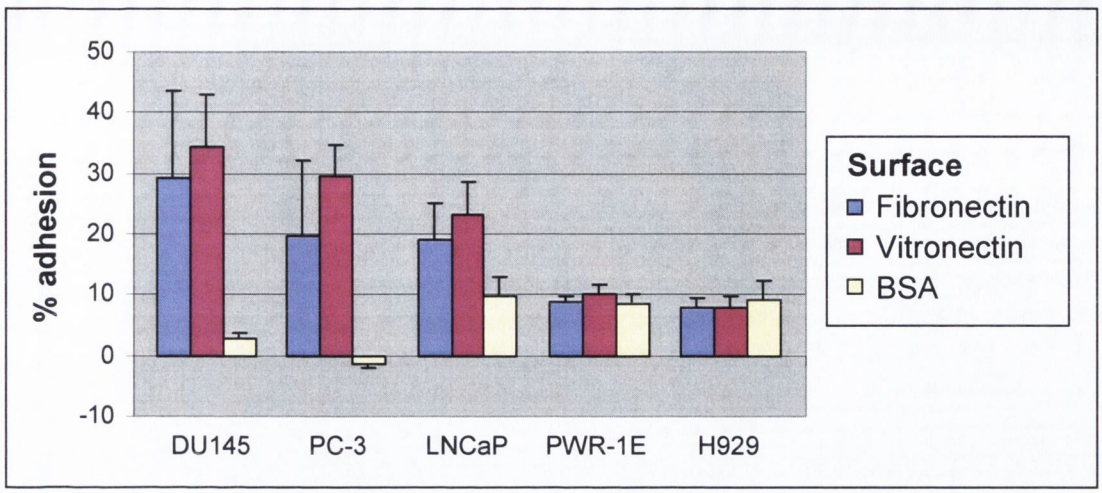


Figure 7.3. Adhesion of cell lines to fibronectin and vitronectin. The cell lines above were allowed to adhere for 2h on each of the indicated coated surfaces, after which the cells were washed twice in PBS, calcein AM was added and fluorescence was measured. A standard curve of the fluorescence of known numbers of cells was constructed for each cell line, and used to calculate the percentage of adhered cells relative to the 2.5 x 10^4 cells plated in each well. Data represent the mean and standard deviation of two independent experiments done in triplicate.

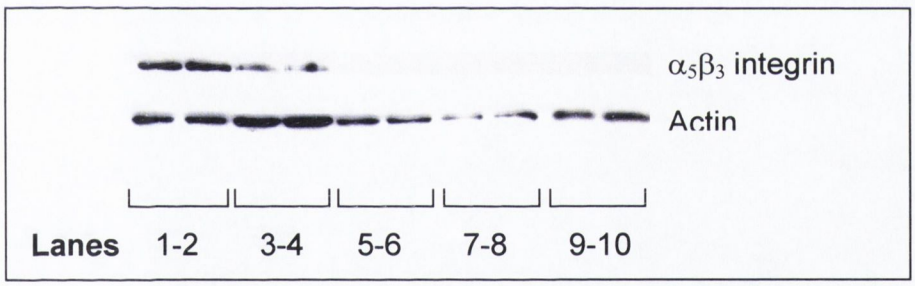


Figure 7.4. Expression of $\alpha_5\beta_3$ integrin in cell lines. The cell lines indicated below were harvested non-enzymatically as in Section 2.10.5 and protein was extracted. $\alpha_5\beta_3$ integrin expression was assessed by Western blotting. Blots were probed for actin as a loading control. Lanes 1-2: DU145. Lanes 3-4: PC-3. Lanes 5-6: LNCaP. Lanes 7-8: PWR-1E. Lanes 9-10: H929.

Cell adhesion assays in primary cultures did not distinguish clearly between tumour and benign cultures (Figure 7.5B), except for patient IMM0023. In the case of this patient, adhesion to vitronectin was significantly lower (50%) than adhesion to fibronectin in the benign culture, whereas this was not the case in the tumour culture (Figure 7.5A). This finding suggests a different functional cell adhesion phenotype in paired samples from the same patient. Interestingly, histological analysis of adjacent sections confirmed both the malignant and benign nature of the corresponding samples taken from this patient, in

contrast to other paired samples. Due to the use of available tumour cells for transfection and prodrug treatment, primary cultures were not subjected to Western blotting for $\alpha_5\beta_3$ integrin expression.

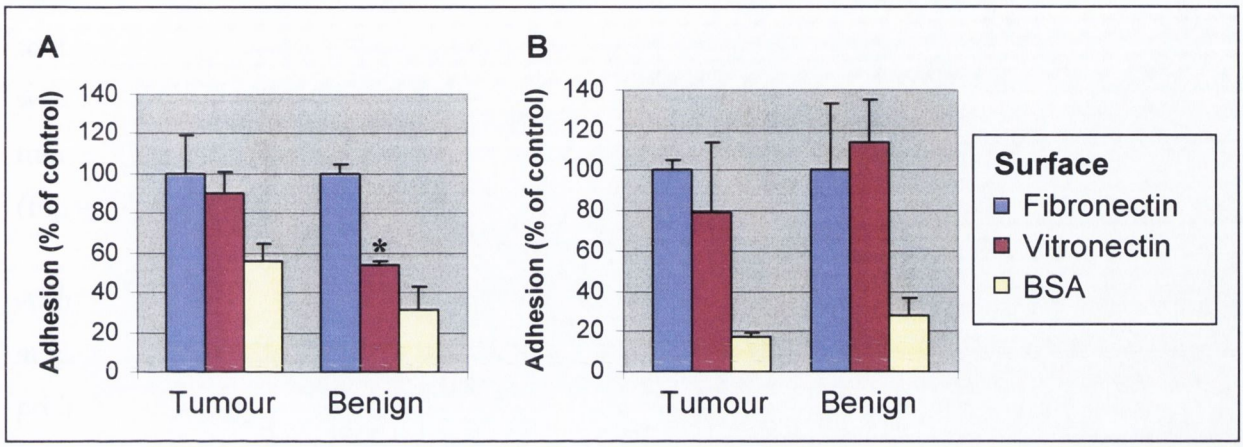


Figure 7.5. Adhesion of primary prostate cells to fibronectin and vitronectin. A. IMM0023. **B.** IMM0019. The indicated primary cultures were allowed to adhere for 2h on the coated surfaces above, after which the cells were washed twice in PBS, calcein AM was added and fluorescence was measured. Adhesion to fibronectin was set at 100% for each culture. Data represent the mean and standard deviation of triplicate wells. *Adhesion to vitronectin differed significantly from adhesion to fibronectin, p=0.028.

7.5. PSA expression in primary cultures

PSA expression was tested by enzyme immunoassay of conditioned medium in which primary cultures were grown. Initial assays showed low levels of PSA expression (Table 7.3). However, this varied considerably between samples from different patients.

Patient number	PSA expression (ng/10^7 cells/day)
IMM0022	0 ± 2
IMM0023	25 ± 3
IMM0024	397 ± 110
IMM0025	149 ± 5

Table 7.3. PSA expression in primary prostate cell cultures. Cell culture medium in which cells had been growing for 3 days was collected, and tested by PSA enzyme immunoassay as in Section 2.6.2. PSA concentration was corrected for cell number and time in culture. Data represent the mean and standard deviation of duplicate assays.

7.6. Primary culture transfection and 5-FC treatment

One culture of primary cells obtained at passage 2 from our collaborators was transfected using various amounts of DNA and LipofectAmine 2000 (see Figure 7.7), and two alternative serum-free media. PrEBM, the medium in which the primary culture was routinely grown, did not contain serum or antibiotics which might inhibit the carrier, and was therefore suitable for use during transfections, along with OPTIMEM-I. 8-15% transfection efficiency was achieved under all of the eight transfection conditions tested (Figures 7.6, 7.7).

Although some debris was observed in all transfected wells, the cells remained viable for at least 6 days after transfection, as observed under a light microscope. These preliminary results suggested that human primary prostate cultures could be useful as a model system for gene-based therapeutic approaches.

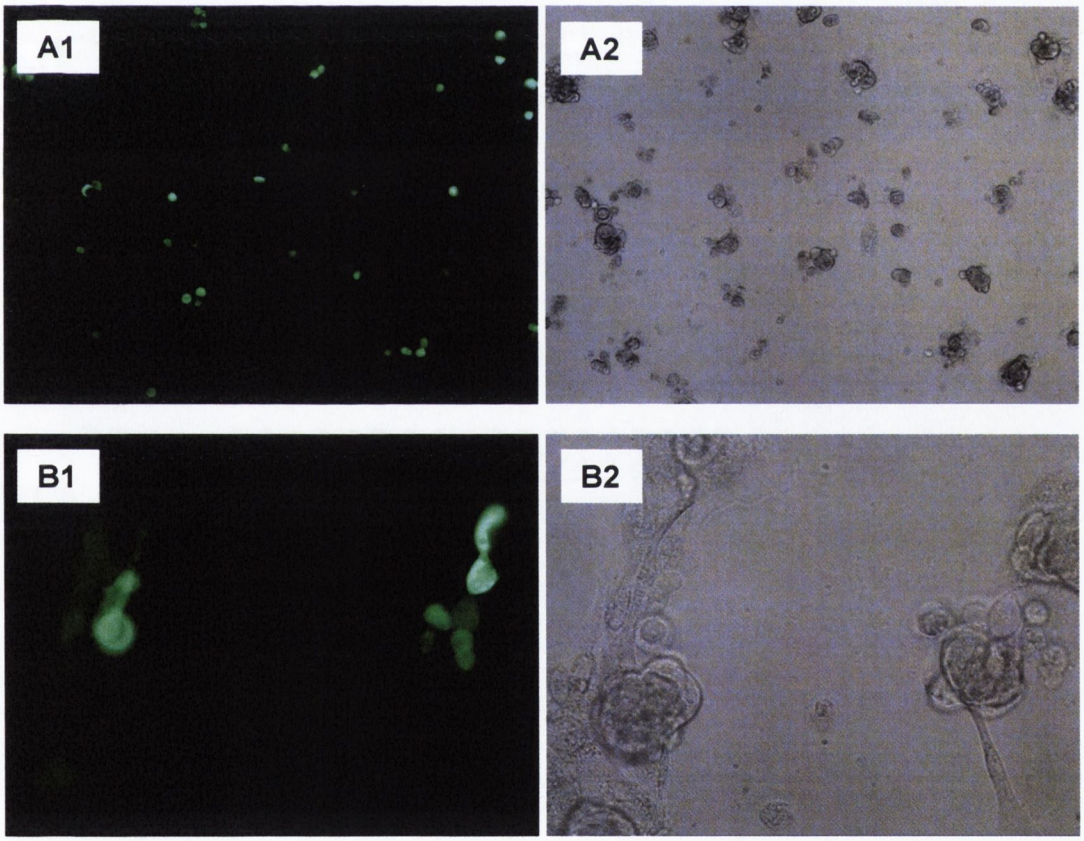


Figure 7.6. Human prostate primary cells transfected with GFP reporter plasmid. A1, A2: 10X magnification. **B1, B2:** 40X magnification. The same fields are shown under fluorescent (**A1, B1**) and white (**A2, B2**) light. Cells were transfected using 250ng plasmid and 0.5µl LipofectAmine 2000.

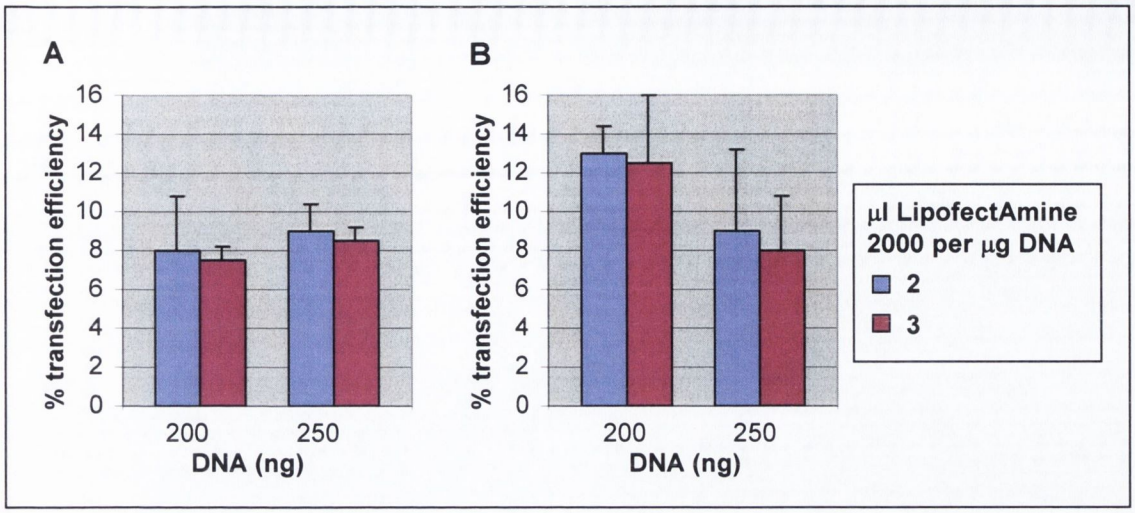


Figure 7.7. Transfection of human primary prostate cancer cells. 3.2 x 10^4 cells were plated in a 96-well plate, and transfected as in Section 2.10.6.1 24 hours later, by which time the cultures were 40% confluent. The amounts of LipofectAmine 2000 and DNA used are shown above. Cells were incubated for 4h during transfection in PrEBM (**A**) or OPTIMEM-I (**B**) media. GFP-positive cells were counted 48h after transfection. The mean and standard deviation of duplicate wells are shown.

Two tumour epithelium cultures were subsequently transfected using 200ng DNA and 2µl LipofectAmine 2000 per µg DNA. However, viability following transfection was minimal. Further tumour epithelium cultures were transfected using GenePorter2 under conditions which permitted low transfection-related toxicity in cell lines (L. Marignol, personal communication). Although viability was improved in primary cultures transfected using GenePorter2, transfection efficiency as shown by expression of the GFP reporter gene was <1% in cultures from patients IMM0023, IMM0024 and IMM0025.

Figure 7.8 shows cell survival data following transient transfection and 5-FC treatment from patients IMM0023 and IMM0025. In the case of culture IMM0023, 5-FC treatment killed the majority of cells, while transfection with any plasmid did not affect viability. For culture IMM0025, transfection reduced survival in untreated cells, and prodrug-related toxicity was noted in untransfected cells, though not in transfected cells. However, no evidence was observed that transfection with pCMV-CD1.10 or pPPE-CD2.15 rendered cells more sensitive to prodrug treatment in the cultures tested.

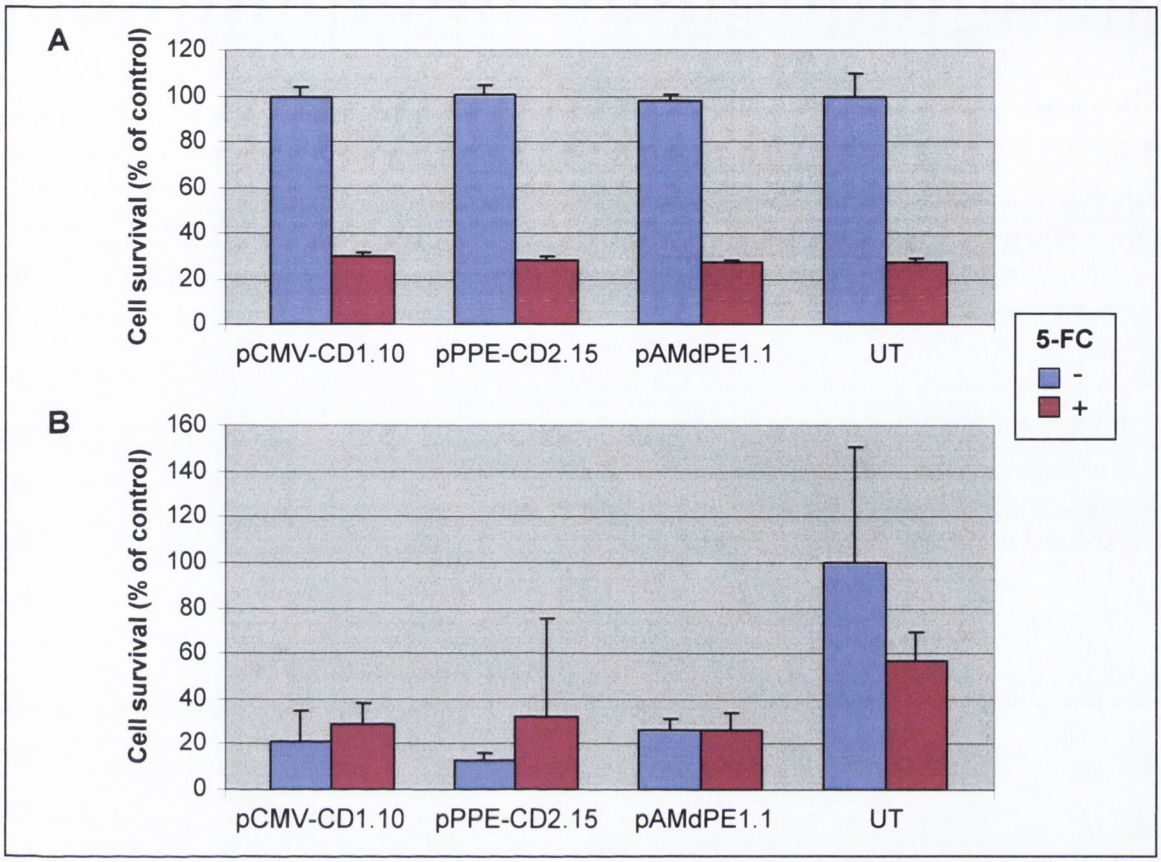


Figure 7.8 Cell death in primary prostate cells following transfection and 5-FC treatment. 2.5 x 10^4 cells from tumour epithelium cultures of patients IMM0023 (**A**) and IMM0025 (**B**) were plated in a 96-well plate, and transfected as in Section 2.10.6.2 24 hours later. The following day, cells were treated with 10mM 5-FC for three days. Cell survival was measured using the MTT method in Section 2.10.7. Survival was normalised to 100% for untransfected and untreated cells. Data represent the mean and standard deviation of triplicate wells. *UT*, untransfected.

DISCUSSION

While the use of primary prostate tumour cells offers a more relevant model system for clinical disease, it also poses a number of significant challenges. Prostate tissue for the experiments above was obtained from patients with localised disease undergoing radical prostatectomy, since those with advanced or extensive disease would not be candidates for curative surgery, contributing to the difficulty of collecting samples.

Growth rates and morphology in culture did not differ significantly between tumour and benign samples from the same gland. Cell adhesion assays demonstrated functional differences between tumour and benign samples from patient IMM0023, though this difference was not detectable in other samples (Figure 7.5). Sampling of appropriate malignant and benign regions of the prostate was successful in this patient, although only 10% of the prostate volume was malignant (Table 7.2). Samples taken to obtain tumour tissue were histologically confirmed as malignant in five of six cases. However, macroscopic identification of matched benign regions proved more difficult. This is perhaps not surprising, given the heterogenous and multifocal nature of prostate cancer. Selection of patients with higher Gleason scores and/or with transrectal ultrasound biopsy reports indicative of multifocal tumour could further enhance the correct sampling of tumour tissue. However, this would reduce the number of specimens available, as such patients would frequently be treated by radiotherapy rather than radical prostatectomy.

Cell adhesion assays in cell lines compared three prostate cancer cell lines with the benign prostate cell line PWR-1E (Figure 7.3). Although the latter cell line grows in an adherent fashion, it failed to adhere to either fibronectin or vitronectin above background levels. Adhesion of PC-3 and DU145 to vitronectin confirmed previously published findings (Zheng *et al* 1999), though this publication did not observe adhesion of LNCaP cells to vitronectin, as in Figure 7.3. However, specific adhesion to vitronectin by LNCaP cells was reduced compared to PC-3 and DU145, which display more aggressive and invasive behaviour as *in vivo* tumours, and LNCaP did not express detectable $\alpha_5\beta_3$ integrin by Western blotting (Figure 7.4).

PSA secretion varied widely between primary cultures from different patients (Table 7.3). However, even lower levels were comparable to 22Rv1 cells, which secreted 30 ± 5ng/10^7 cells/day under the conditions used for transfection and 5-FC treatment in Chapter 6. This suggests that PSA promoter activity *in vitro* may not be an obstacle to the use of primary cultures as a model system for testing prodrug activation genes regulated by the PSA promoter.

Variability was also observed in the efficiency and toxicity of transient transfection of primary cultures. In addition to the expected variations between patients, the small number of cells available hindered optimisation of transfection conditions or introduction of prodrug activation genes under multiple transfection conditions. Reduction of the 5-FC concentration might also decrease prodrug-derived toxicity as a confounding factor. Further refinement of the *in vitro* culture method could yield higher numbers of cells and facilitate a greater variety of assay conditions for prostate cancer primary cultures.

CHAPTER 8: DISCUSSION

8.1. INTRODUCTION

Prostate cancer has become an increasingly significant health burden as the population age profile rises (National Cancer Registry Ireland 2003). While localised prostate cancer can often be treated effectively by surgery and radiotherapy, disease progression in some patients results in an androgen-independent state which is resistant to androgen withdrawal and other currently available therapeutic options. Several approaches to gene therapy for prostate cancer have therefore been investigated, and some have shown promise in recent phase I clinical trials (Eder *et al* 2000, Freytag *et al* 2002a, Kubo *et al* 2003).

In this study, the prodrug activation approach to gene therapy has been combined with a tissue-specific gene expression strategy, and assessed in *in vitro* models of prostate cancer. This goal first required appropriate vectors to be constructed. Plasmids were generated encoding the cytosine deaminase prodrug activation gene, under the control of the constitutive CMV promoter and the tissue-specific PSA promoter. CD expression by these plasmids in prostate cancer cell lines was successfully demonstrated by Western blotting. The CMV promoter expressed higher quantities of transgene than the PSA promoter, as reported previously (Segawa *et al* 1998), but lacked tissue specificity and was active in colon as well as in prostate cells (Figures 6.14 – 6.15). The PSA promoter was subjected to site-directed mutagenesis with the aim of improving its expression. Although no improvement was observed, several recent studies (Section 8.2) have indicated that PSA promoter activity can be improved in a variety of ways without sacrificing tissue-specificity (Wu L *et al* 2001, Sato *et al* 2003).

Transfection conditions were optimised for four prostate cancer cell lines and one colon cancer cell line. Although not the most efficient transfection method available, the liposome-based procedure used offered a relatively simple means of introducing the CD

gene into cell lines to demonstrate proof of principle for the tissue-specific prodrug activation approach. Other transduction or transfection methods could facilitate higher levels of cell kill (Section 8.3).

However, the tissue-specific expression plasmids developed in this study caused up to 60% cell death when combined with 5-FC. These results with a first-generation vector, delivered using liposomes, demonstrate the promise of this strategy. Further vector development may increase cell kill and reduce the dose of 5-FC required (Section 8.4).

In addition to the use of cell lines, preliminary experiments were performed to test the prodrug activation system under study in human primary prostate cancer cultures, and to characterise the cultures. Assays of therapeutic genes in primary cells are clearly a more relevant and informative model than the small selection of prostate cancer cell lines available. Evidence from several other studies suggests that it may be possible to overcome the technical obstacles to this approach (Section 8.5).

8.2. AN IDEAL TRANSGENE CONSTRUCT?

8.2.1. Modification of the PSA promoter sequence by substitution

Mutagenesis of the PSA promoter did not significantly change either CD expression levels (Chapter 5) or the capacity of PSA-CD constructs to sensitise cells to 5-FC (Chapter 6). Sites were selected for mutagenesis, based on the emerging evidence of polymorphisms in the PSA promoter with possible functional relevance. The G and A alleles at position -158 occurred at equal frequency in 156 normal control individuals, but the G allele, when combined with a polymorphism in the androgen receptor gene, significantly increased prostate cancer risk (Xue *et al* 2000). This substitution lies just inside ARE-I, a region crucial for promoter function, and therefore seemed a good candidate for modifying activity. pPCM1.24 was therefore generated, varying from pPPE-CD2.15 only in substituting –158G for –158A.

Recently, several publications have investigated the A/G polymorphism at position –158 and its association with serum PSA levels and prostate cancer risk. Frequencies of the two alleles in healthy populations were almost equal (Xue *et al* 2000, Cramer *et al* 2003). The AA genotype has been significantly associated with higher serum PSA (Xue *et al* 2001, Medeiros *et al* 2002), though other reports have not found this to be the case (Xu *et al* 2002, Rao *et al* 2003). The AA genotype has also been linked to higher risk of prostate cancer (Gsur *et al* 2002). However, another study reported that the GG genotype, when combined with a polymorphism in the androgen receptor gene, increased prostate cancer risk five-fold (Xue *et al* 2000). The data on the effect of this polymorphism is therefore not entirely consistent, and will require further studies for clarification.

Sequences of two of a series of nine breast tumours had an A replaced by G at position –155, just outside ARE-I. It was intriguing that effectively the same sequence change occurred by different mechanisms in two of four PSA-overexpressing tumours in this series (Majumdar & Diamandis 1999). In one case, a single base substitution was involved, while one base pair was deleted in the other. We hypothesised that if –155G contributed to the high activity of the PSA promoter in these two tumours, it might also improve the activity of our promoter, so we generated this substitution by site-directed mutagenesis in pPCM2.3.

A number of possible reasons could explain the absence of any functional difference between pPPE-CD2.15 and pPCM2.3. The PSA overexpression observed in tumours containing –155G may have been coincidental. Alternatively, the mutation may have played a role, but in association with factors present in the breast tumours in which it was reported, and absent in prostate cancer cells. It is also possible that interactions with other cis-elements in the PSA promoter or enhancer were involved, as both the tumours in question harboured additional mutations in the enhancer region (Majumdar & Diamandis 1999). ARE-I is known to function co-operatively with other promoter and enhancer elements (Cleutjens *et al* 1997a).

The effect of small sequence changes in the PSA promoter is likely to depend on the surrounding sequence, and on the hormone receptor status and other biochemical properties of the cell. However, some of the polymorphisms identified in the PSA promoter and enhancer have been shown to influence promoter activity. Three linked single nucleotide polymorphisms in the PSA enhancer have been associated with both serum PSA levels, and PSA promoter activity in reporter gene assays (Cramer *et al* 2003). The –4643G allele, and the combination of –5412C and –5429G, result in higher PSA promoter activity in LNCaP cells than their more common variants (Cramer *et al* 2003).

Two further promoter polymorphisms, -252A/G and –205AA/A, showed linkage disequilibrium in a population of prostate cancer patients. At least one -252A/-205AA allele has been associated with a much higher likelihood of prostate cancer patients having serum PSA protein levels higher than 10ng/ml, though not with prostate cancer risk (Yang *et al* 2001). This variant was also associated with PSA RNA expression in a higher percentage of breast cancer patients (Yang *et al* 2000). However, the –252 polymorphism was not associated with serum PSA in a larger healthy population (Xue *et al* 2001).

8.2.2. Modification of the PSA promoter structure

Alterations to the structure of the PSA promoter may be a more robust strategy than targeting single bases. The simplest means by which PSA transcriptional activity has been improved is by deleting unnecessary sequences. This may both bring elements which act synergistically closer together, or remove possible negative regulators. A construct containing sequences from -5.3kb to -3.7kb juxtaposed to the proximal promoter at -632kb was twice as active as the 6kb region from which it was derived (Cleutjens *et al* 1997a). Deletion of sequences between -3.7kb and -541bp similarly produced higher activity than the entire 5.3kb regulatory region (Schuur *et al* 1996). 2- to 9-fold increased expression was observed in three separate constructs when 0.9-1.1kb (between -3935 and -2855) was deleted from the 3' end of the enhancer (Wu L *et al* 2001).

Inserting additional copies of positive regulatory regions has also been successful, though interestingly, too many copies may not be helpful. Tandem duplication of the 440-bp core enhancer increased activity by up to 19-fold, though three to four copies were less efficient than two (Wu L *et al* 2001). Similarly, Latham and coworkers inserted one to three copies of the enhancer (-5322bp to -3869bp) upstream of the proximal promoter at -541bp, and obtained optimal expression with two copies (Latham *et al* 2000). This promoter was 4-5% as active as the CMV promoter in the absence of androgen, which stimulated its activity up to 40-fold (Latham *et al* 2000).

In a systematic study of modified PSA constructs with potentially high activity, the most efficient was PSE-BC, in which the region between -5322bp and -3744bp, with the core enhancer duplicated in tandem, was ligated to the proximal promoter at -541bp (Wu L *et al* 2001). Another efficient variant contained the same structure, except that the enhancer sequence downstream of the duplicated core enhancer was replaced by four AREs identical to ARE-I (Wu L *et al* 2001). All the PSA constructs described above retained their tissue specificity *in vitro* (Latham *et al* 2000, Wu L *et al* 2001), and *in vivo* (Wu L *et al* 2001).

A hybrid enhancer has been generated from regions of the PSA and PSMA promoters, which appears to maintain the tissue-specificity of the PSA sequences while being less androgen-dependent (Lee SJ *et al* 2002). This bidirectional enhancer has been used to regulate the adenoviral E1a and E4 genes in an attenuated replication-competent vector, resulting in inhibition of prostate tumour growth in mice (Li X *et al* 2005).

8.2.3. Amplification of tissue-specific transcription by two-step activation

Vectors containing PSA promoter and enhancer constructs have also been modified outside the PSA sequences in efforts to increase activity. The two-step transcriptional activation (TSTA) system outlined in Figure 8.1 caused up to 700-fold increase in gene expression, rendering a 5.3kb PSA enhancer and promoter more active than the CMV promoter, while maintaining tissue-specificity *in vitro* (Segawa *et al* 1998). Yoshimura

et al (2002) overcame the androgen-dependence of the PSA promoter by using the Cre recombinase system from bacteriophage P1 (Figure 8.2). This system was as active, even in the absence of androgen, as was the PSA promoter alone after stimulation with androgens. Sato *et al* (2003) developed a strategy which combined the improved PSA structure of PSE-BC (see above), and a third TSTA system (Figure 8.3). This promising combination elicited higher reporter gene expression than the CMV promoter *in vivo*, without loss of tissue specificity (Sato *et al* 2003). Expression of therapeutic genes from tissue-specific promoter with these qualities could be expected to have efficient antitumour activity.

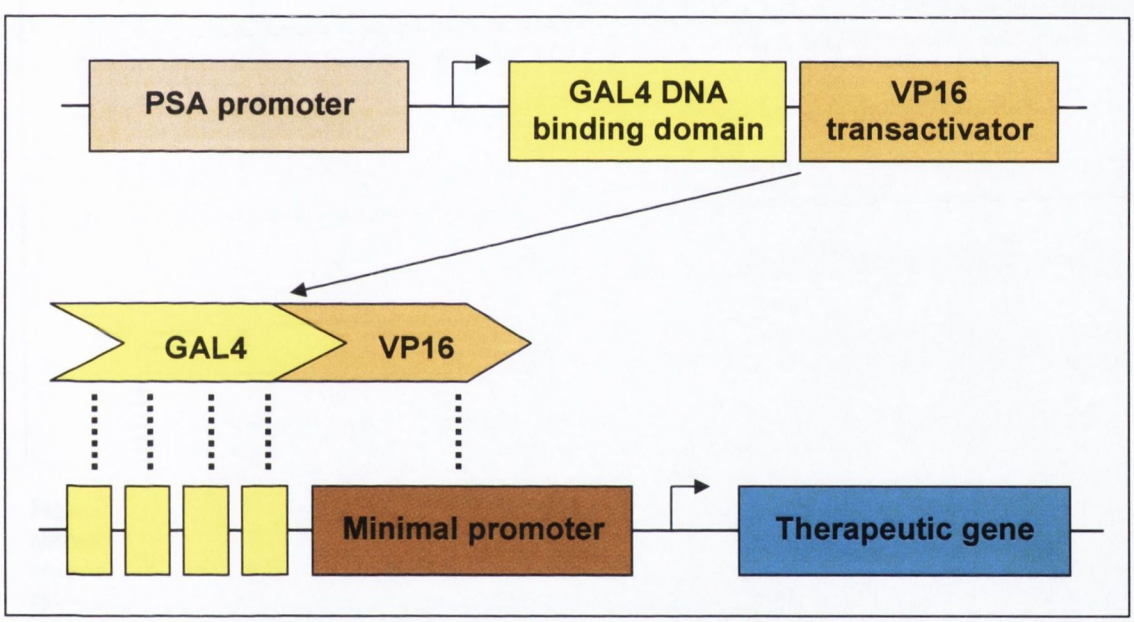


Figure 8.1. Two-step transcriptional activation by GAL4-VP16 system. In the first step, the PSA promoter expresses a fusion protein with sequences from the yeast DNA binding protein GAL4 and the herpes simplex virus transcriptional activator VP16. The fusion protein then binds to four GAL4 response elements upstream of a minimal promoter and stimulates transcription of a therapeutic gene (Segawa *et al* 1998).

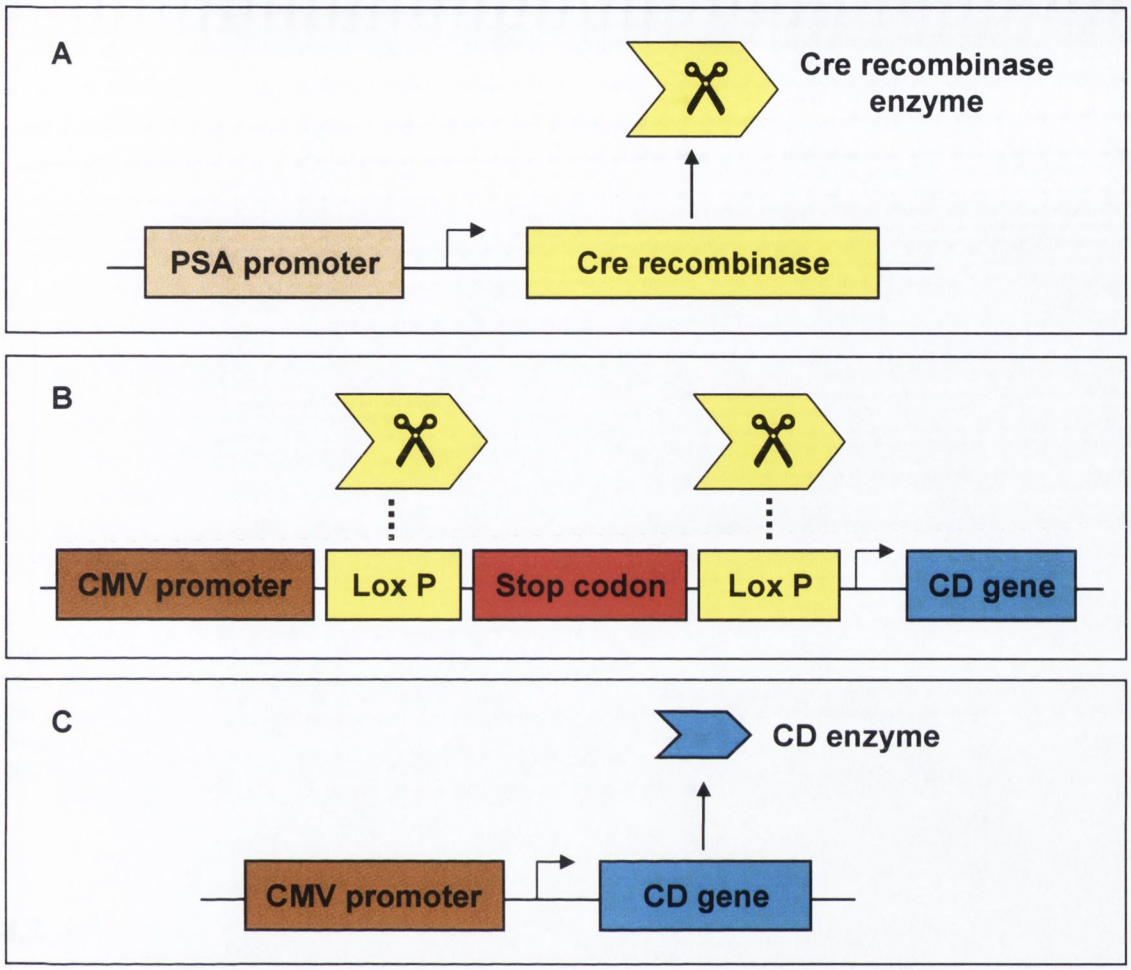


Figure 8.2. Two-step transcriptional activation by Cre recombinase system. A. The PSA promoter allows expression of the Cre recombinase enzyme in a prostate-restricted manner. **B.** The Cre recombinase enzyme recognises loxP sites and excises the intervening sequences from a second construct. **C.** The cytosine deaminase (CD) prodrug activation gene is now expressed at high levels from a constitutive cytomegalovirus (CMV) promoter (Yoshimura *et al* 2002).

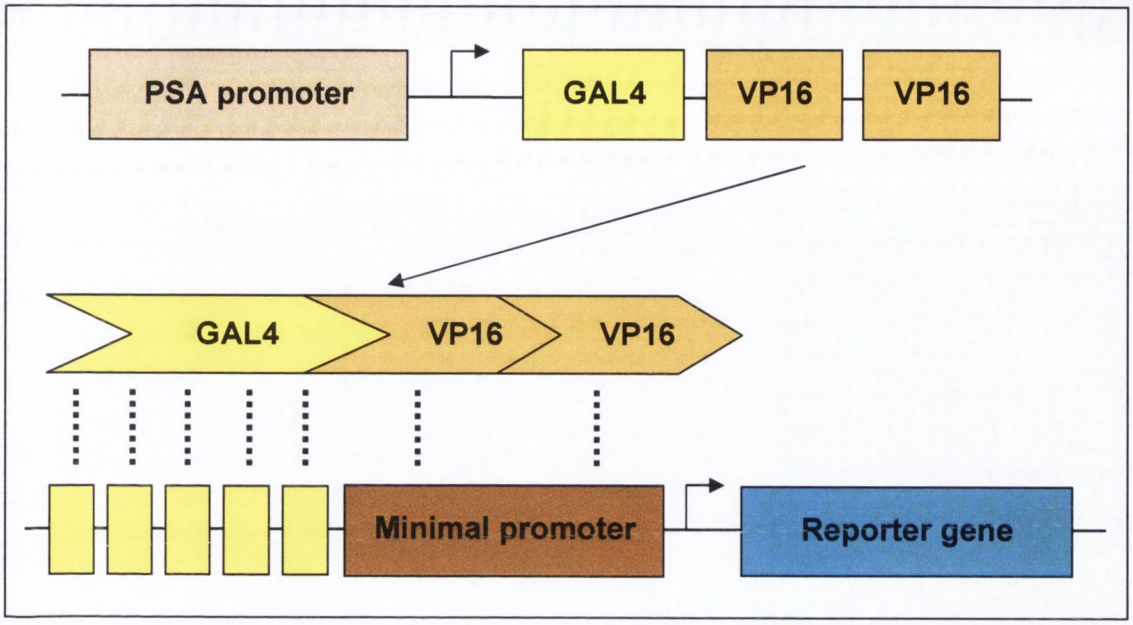


Figure 8.3. Two-step transcriptional activation by optimised GAL4-VP16 system. In the first step, the PSA promoter expresses a fusion protein with the DNA binding domain from the yeast protein GAL4 and the herpes simplex virus transcriptional activator VP16. The fusion protein then binds to four GAL4 binding sites upstream of a minimal promoter and stimulates transcription of a downstream gene (Sato *et al* 2003).

8.2.4. Tissue-specificity and other means of tumour targeting

In addition to tissue-specific promoters (discussed in detail in Section 8.6.3), other transcriptional strategies are available for targeting transgene expression to tumours. Introducing hypoxia response elements from the VEGF gene into a minimal promoter resulted in strong induction of gene expression in hypoxic conditions (Shibata *et al* 1998). Prostate and other tumours are known to contain hypoxic regions (Cvetkovic *et al* 2001). Similarly, CArG sequence elements from radiation-responsive promoters, upstream of an appropriate transgene, can increase radiation-induced cytotoxicity (Scott *et al* 2000). Radiation-responsive promoters could thus target expression of pro-apoptotic transgenes to the radiation field directed at a tumour during radiotherapy. There is potential for a combination of some of the above strategies to permit optimally efficient and tumour-specific transgene expression.

8.3. AN IDEAL VECTOR?

For gene therapy of prostate cancer, the principal requirements of a vector are that it should efficiently deliver genes to prostate cancer cells, and that it should be safe for patients. In addition, the vector should ideally target tumour cells rather than surrounding healthy tissue. Since the desired outcome is to eliminate the tumour, long-term duration of gene expression is not essential, in contrast to other applications of gene therapy such as correction of inherited enzyme deficiencies.

Adenoviruses have been the vector of choice for clinical trials of gene therapy for prostate cancer. Adenoviral vectors have been used in eight of the fifteen clinical trials reported to date (Herman *et al* 1999, Teh *et al* 2001, Kubo *et al* 2003), in addition to numerous preclinical studies (Yoshimura *et al* 2002, Sato *et al* 2003, Furuhata *et al* 2003). These vectors offer efficient transduction of prostate cancer cells, up to 90-100% (Blackburn *et al* 1998, Xie *et al* 2001a) and high titres can be readily prepared (Herman *et al* 1999, Freytag *et al* 2002a). Prostate cancer cells frequently express cocksackie and adenovirus receptor (CAR), unlike other malignant cell types (Pandha *et al* 2003).

However, adenoviruses elicit a strong immune response, partly due to the high prevalence of wild-type adenovirus infection (Piedra *et al* 1998). The death of one patient treated with an adenoviral gene therapy vector has highlighted the safety implications of this issue (Barbour 2000), though it should be noted that this patient's treatment violated the clinical trial's protocol ("Gene therapy" 2000). Adenoviral vectors have been extensively modified to reduce their immunogenicity, by deleting the E1 gene and other genes including E2 and E3 (Herman *et al* 1999, Kubo *et al* 2003). All adenoviral genes can potentially be deleted from the vector genome, and provided during production by packaging cell lines (Chen *et al* 1997).

Clinical trials in which prostate cancer patients were injected with replication-deficient adenoviral vectors (without other therapies) have to date treated fifty-nine patients, and recorded 6 grade 3 toxicities and one grade 4 thrombocytopenia. The frequency of adverse events of grade 3 severity has been somewhat higher in patients treated only with

replication-competent adenoviral vectors, 14 events in 36 patients. Since hepatotoxicity is a particular concern when administering adenoviral vectors, it is worth noting that the vast majority of adverse events affecting the liver were of Grade 2 severity or less, with one Grade 3 event among the 95 prostate cancer patients treated. One study found that patients temporarily shed infectious virus particles in their urine after intraprostatic injection with replication-competent adenovirus (DeWeese *et al* 2001). Infectious virus shedding may have been due to the viral dose and the injection regimen, since two other Phase I clinical trials involving replication-competent adenoviral vectors did not detect infectious adenovirus in blood (Freytag *et al* 2002a, Freytag *et al* 2003) or urine (Freytag *et al* 2002a). Alternatively, the 5-FC and ganciclovir prodrug regimen used in these studies may have inhibited viral replication. Overall, data from the Phase I or Phase I/II clinical trials of adenoviral gene therapy for prostate cancer suggest that the use of adenoviral vectors should be continued, and that replication-deficient vectors, as would be expected, have a better safety profile.

Vectors derived from vaccinia virus, encoding PSA as a tumour antigen, have been used in three prostate cancer Phase I clinical trials (Sanda *et al* 1999, Eder *et al* 2000, Gulley *et al* 2002). Vaccinia virus can elicit a rapid and sustained humoral immune response (Gnant *et al* 1999a). This immune response has not been associated with undesired side-effects to the same extent as in adenoviruses, possibly due to modulation by vaccinia virus-encoded genes. Among 71 prostate cancer patients treated with vaccinia-derived gene therapy vectors (without additional treatment), 96% of the adverse events reported were Grade 1 or 2, with only three Grade 3 toxicities recorded (Sanda *et al* 1999, Eder *et al* 2000, Gulley *et al* 2002). Vaccinia vectors have been used most frequently in immunotherapy approaches to prostate cancer gene therapy. However, a vaccinia virus encoding the CD prodrug activation gene combined with 5-FC has increased the survival of mice with metastatic colon cancer (Gnant *et al* 1999b). Vaccinia-based vectors may therefore be an alternative to adenoviruses, though maximum doses can be limited by production yields (Sanda *et al* 1999).

Liposomes do not pose major safety concerns, but their efficiency is limited. We have achieved liposomal transfection efficiencies of 20-50% following reagent optimisation (Chapter 4). Transfection of plasmids encoding the CD prodrug activation gene under these conditions, combined with 5-FC prodrug treatment, has been sufficient to cause cell death *in vitro* (Chapter 6). Prodrug activation and cell death have similarly been achieved using even lower liposomal transfection efficiencies of 8-15% *in vitro* (O'Keefe *et al* 2000). Liposomes have been successfully used to deliver antisense oligonucleotides (Miyake *et al* 1999) and therapeutic genes (Jin *et al* 2000) to prostate tumours *in vivo*. The only prostate cancer Phase I clinical trial to date in which liposomes were employed, to deliver the interleukin-2 gene, resulted in no toxicities greater than grade 2 (Belldegrun *et al* 2001). Though liposomes are safer and easier to use than adenoviruses, and have been efficient enough in our study to demonstrate proof of principle for the PSA-CD system, the superior transduction efficiency of viral vectors must be a factor in selecting vectors for future studies.

Adeno-associated viruses (AAV) have not been widely used in research on gene therapy approaches to prostate cancer. AAVs have possibly the best safety profile among the viruses widely used to date, with low immunogenicity and toxicity, and no known association with any human disease (Bonnet *et al* 2000). However, these advantages are offset by the difficulty of producing high yields, and by the 4.5kb upper limit on transgene size (Bonnet *et al* 2000). This would prevent the insertion of long regulatory sequences or multiple genes, such as those required for the Cre-LoxP system (Section 8.2.3). However, AAV inverted terminal repeat sequences, required for integration into the genome, have been inserted into plasmids. These plasmids, complexed with liposomes, successfully transfected human prostate cancer primary cultures, and were delivered more efficiently than plasmids without AAV sequences in cell lines (Vieweg *et al* 1995).

Salmonella typhimurium and similar bacterial strains may have potential for gene delivery. *S. typhimurium* grows well in anaerobic conditions, can be eliminated by antibiotics if required, and contains a large and well-characterised genome. An

attenuated *S. typhimurium* strain, which was selectively amplified in tumour tissue in mice, expressed sufficient herpes simplex virus thymidine kinase (HSV-TK) to bring about ganciclovir-mediated tumour reduction, though bacteria without the TK prodrug activation gene also had significant antitumour effects (Pawelek *et al* 1997). An attenuated strain of *S. typhimurium* which expresses the *Escherichia coli* prodrug activation gene cytosine deaminase, accumulates preferentially in the extracellular region of tumours. This novel vector has generated antitumour effects in mice, which were enhanced by administration of 5-fluorocytosine (Cunningham & Nemunaitis 2001), and a small Phase I clinical trial in advanced cancer patients has been performed (Nemunaitis *et al* 2003).

An alternative approach to cell-based gene delivery involves neural stem cells (NSCs), which can migrate from distant sites to accumulate in tumours (Aboody *et al* 2000, Brown *et al* 2003). Murine NSCs stably transduced with the cytosine deaminase gene, combined with 5-FC, inhibited tumour growth in a mouse gliobastoma model (Aboody *et al* 2000). In addition, NSCs stably transduced with the CD gene were recently reported to be capable of accumulating in non-neural tumours, following systemic injection (Brown *et al* 2003). Though further investigations are needed, this system has considerable potential as a vehicle to deliver anti-tumour genes.

Vectors which preferentially infect dividing cells, such as retroviruses (Bonnet *et al* 2000), can be an advantage in cancer gene therapy in order to target tumours and avoid benign tissue. However, this strategy may not be as beneficial in prostate cancer, since many prostate tumours grow very slowly (Schmid *et al* 1993). Similarly, the ability of retroviruses to maintain long-term transgene expression (Muul *et al* 2003) is more useful in gene therapy for diseases other than cancer. Retroviruses have been used in two clinical trials of gene therapy for prostate cancer (Steiner *et al* 1998b, Simons *et al* 1999). However, a recent report of oncogenesis following retroviral insertion into the genome in a SCID gene therapy trial (Hacein *et al* 2003) has raised significant safety concerns. Overall, it seems unlikely that retroviruses will play a significant role in the development of prostate cancer gene therapy.

8.4. DEFINING THE OPTIMAL CONDITIONS FOR A SUCCESSFUL CD/5-FC APPROACH

8.4.1. 5-fluorouracil in cancer treatment

The development of the CD/5-FC prodrug activation system follows extensive use of 5-FU, the product of this system, in cancer therapy at various disease sites. 5-FU has been the drug of choice in chemotherapy of colorectal cancer for four decades (Midgley & Kerr 1999), and has also been frequently used during adjuvant chemotherapy for breast cancer (Han *et al* 2003). Many clinical trials have been carried out to assess the benefits and risks of 5-FU, and to develop combined chemotherapy regimens involving 5-FU and other drugs including irinotecan, epirubicin, cisplatin and α-interferon (Daliani *et al* 1995, Chao *et al* 1997, Douillard *et al* 2000). In prostate cancer treatment, 5-FU-based chemotherapy has been employed in the palliative setting for patients with androgen-independent disease (Chao *et al* 1997, Daliani *et al* 1995). In one phase II clinical trial in advanced prostate cancer patients, 5-FU caused significant toxicity and two treatment-related deaths (Daliani *et al* 1995), though the drug was well tolerated in a Phase I dose-escalation trial of 5-FU and brachytherapy in locally advanced prostate cancer patients (See *et al* 1996).

8.4.2. Prodrug activation or drug delivery?

Genetic prodrug activation therapy offers the possibility of intratumoral 5-FU production, which would be expected to be less toxic than the systemic administration of 5-FU during standard chemotherapy, because of reduced systemic distribution of drug. In CD-transduced LNCaP tumours in mice, 5-FC and 5-FU brought about comparable growth inhibition, but significant weight loss was observed in the mice treated with 5-FU (Kato *et al* 2002). In this model system, the CD/5-FC system resulted in intratumoral 5-FU levels twice as high as those achieved by systemic 5-FU administration, as measured by high-performance liquid chromatography of tumour extracts (Kato *et al* 2002). These findings indicate that the prodrug activation strategy could deliver more effective intratumoral 5-FU doses than those which can be tolerated by systemic administration. The success of such a strategy depends on efficient vector delivery and therapeutic gene

expression. Though these factors have limited the clinical utility of gene therapy to date, developments in vector technology and the design of therapeutic gene constructs (as discussed in Sections 8.2-8.3) suggest that these hurdles may be overcome.

8.4.3. 5-FU and CD/5-FC as radiosensitising agents

5-FU and radiation have exhibited synergistic cytotoxicity *in vitro* and *in vivo*, possibly due to the impact of 5-FU on DNA repair after damage by radiation (Byfield *et al* 1982, Berrada *et al* 2002). Clinical studies have also demonstrated that 5-FU treatment improves the efficacy of radiotherapy. Randomised controlled trials in patients with pancreatic cancer (Moertel *et al* 1981) and head and neck cancer (Browman *et al* 1994) observed significantly better survival and response rates in patients treated with 5-FU and radiotherapy, than in those treated with radiotherapy alone. Combinations of 5-FU chemotherapy and radiotherapy have also been beneficial in other malignancies, including rectal and oesophageal cancer (O'Connell *et al* 1994, Heath *et al* 2000). Whether this is the case in prostate cancer remains to be determined, though prostate cancer patients have also been treated with combined radiotherapy and 5-FU (See *et al* 1996).

However, many investigators have assessed the effect of combining the CD/5-FC prodrug activation system with radiation in preclinical models of prostate and other cancers, and the CD/5-FC system has been employed as part of a two- or three-pronged approach in phase I prostate cancer clinical trials (Freytag *et al* 2002a, Freytag *et al* 2003). The CD/5-FC system and radiation have shown synergistic levels of cytotoxicity in mouse prostate cancer and human AI prostate cancer cells *in vitro* (Freytag *et al* 1998, Anello *et al* 2000). Though low doses of radiation alone were not effective, CD/5-FC treatment and low-dose radiation caused additive growth inhibition of LNCaP tumours in mice (Kato *et al* 2002). Radiosensitisation of tumours by CD/5-FC has also been shown in squamous carcinoma in mice (Hanna *et al* 1997), and in gliosarcoma cells (Rogulski *et al* 1997).

8.4.4. 5-FC concentration

The prodrug dose must be considered when assessing the efficacy of a prodrug activation system. Our results indicate IC_{50} values for 5-FC of 61mM, 6mM and 0.6mM in parental 22Rv1 cells and in 22Rv1 cells transfected with PSA-CD constructs and pCMV-CD1.10, respectively (Chapter 6). LNCaP cells, which displayed a slightly higher IC_{50} value for 5-FC of 15mM, were transfected using liposomes with a PSMA promoter-CD plasmid (O'Keefe *et al* 2000). Transient transfection with this construct reduced the IC_{50} value of 5-FC to 2mM in LNCaP cells (O'Keefe *et al* 2000), a reduction comparable to that achieved in our study. Using LNCaP cells transduced with the CD gene, in an adenoviral vector containing a PSA promoter and enhancer and the Cre/LoxP transcriptional amplification system (Figure 8.2), 0.8μM 5-FC was required to kill 20% of cells (Yoshimura *et al* 2002). 5-FC concentrations greater than 0.8μM were not tested in this study; however, since approximately 700μM 5-FC was required to kill 20% of LNCaP cells transfected with a PSMA promoter-CD plasmid (O'Keefe *et al* 2000), it would appear that this AdPSA-TSTA transcription system (Yoshimura *et al* 2002) was considerably more efficient than the former construct. Though the relative activity of the PSA and PSMA promoters has not been studied, it seems likely that adenoviral delivery and the use of a TSTA system contributed substantially to the superior efficiency of the AdPSA-TSTA strategy.

The biologically available dose of 5-FC in patients must also be borne in mind. Prostate cancer patients have been treated with 150mg/kg/day 5-FC (Freytag *et al* 2002a, Freytag *et al* 2003), the same dose as that used routinely as an antifungal treatment (Summers *et al* 1997). This dose has been reported to result in a peak serum concentration of 80-100μg/ml (Ostergaard *et al* 1995), and it has been recommended that serum 5-FC concentrations should be maintained at a maximum of 100μg/ml or 0.8mM (Stamm *et al* 1987). Patients with metastatic breast cancer have been treated safely with the CD gene and 5-FC, at the higher dose of 200mg/kg/day (Pandha *et al* 1999), but clinical trials to date have not measured serum concentrations of 5-FC prodrug in cancer patients.

While pCMV-CD1.10 expresses sufficient CD protein to sensitise cells to biological doses of 5-FC, the first-generation PSA-CD vector pPPE-CD2.15 may not. It may be significant that the 22Rv1 cell line used to test pPPE-CD2.15 expresses relatively low levels of endogenous PSA (see Section 8.5.1). Transfection of the PSA-CD constructs developed in this study into other prostate cancer cells would be expected to yield more CD expression, which would probably require lower 5-FC concentrations for cytotoxic effects. In the LNCaP cell line, preliminary results indicated that 1mM 5-FC caused apoptosis in prostate cancer cells transfected with pPPE-CD2.15. However, further experiments are necessary to verify this data. Regardless of the model used, more efficient second-generation vectors are an essential next step, an issue discussed in Sections 8.2-8.3. In addition, the radiosensitising ability of pPPE-CD2.15 combined with 5-FC has yet to be tested.

8.4.5. CD/5-FC: from *in vitro* to clinical use

A number of authors have reported the development of the CD/5-FC prodrug activation system in cell lines, and subsequently in mouse models of prostate cancer. Yoshimura and colleagues (2002) found that although this system was less potent *in vitro* under the control of the AdPSA-TSTA system than when regulated by the CMV promoter, LNCaP tumour growth inhibition occurred in mice using both promoters, to a similar extent. Significant growth inhibition of LNCaP xenografts was also achieved by 5-FC treatment and stable transfection of a CD expression plasmid, which apparently required very high 5-FC concentrations (800mM) to kill over 50% of cells *in vitro* (Kato *et al* 2002).

A PSMA promoter-CD plasmid decreased the IC_{50} OF 5-FC in C4-2 prostate cancer cells to 0.2mM *in vitro*, and when combined with 5-FC, brought about regression of C4-2 tumours in nude mice (Uchida *et al* 2001). The mice in this study were treated with 1200 mg/kg/day 5-FC, though most similar studies in mice have used a lower 5-FC dose of 500mg/kg/day (Yoshimura *et al* 2002, Kato *et al* 2002, Freytag *et al* 2002b).

A replication-deficient adenoviral vector expressing a CD/TK fusion gene, controlled by the CMV promoter, reduced the IC_{50} of 5-FC in DU145 cells to 0.3mM (Freytag *et al* 2002b). In mice with DU145 xenograft tumours, the prodrugs 5-FC and ganciclovir were

added to a regimen consisting of a replication-competent adenovirus expressing the CD/TK gene, and radiotherapy (Freytag *et al* 2002b). Though the addition of prodrugs slightly increased the rate of tumour cure from 29% to 44%, the authors concluded that trimodal therapy depended more on the effects of radiation and of viral replication than on prodrug activation in this setting (Freytag *et al* 2002b). The adenovirus used expresses a truncated viral E1B protein, which allows preferential though not exclusive replication in cells lacking functional p53 (Freytag *et al* 1998).

Patients with locally recurrent prostate cancer after radiotherapy were treated in a Phase I clinical trial with replication-competent adenovirus encoding the CD/TK gene, and the prodrugs 5-FC and ganciclovir (Freytag *et al* 2002a). Three patients exhibited over 50% reductions in serum PSA for up to four months, and biopsies from two patients showed no evidence of adenocarcinoma one year after treatment (Freytag *et al* 2002a). However, nine Grade 3 adverse events were observed among the sixteen patients in this trial. Subsequently, a Phase I clinical trial of trimodal therapy (replication-competent adenovirus, CD/TK prodrug activation gene and prodrugs, and radiotherapy) was performed, involving 15 patients with newly diagnosed nonmetastatic prostate cancer (Freytag *et al* 2003). After a median follow-up of 9 months, 11 incidences of Grade 3 toxicity had been recorded, and all patients on this study had serum PSA levels of less than 1.5ng/ml. The CD/5-FC system has also been tested in metastatic skin lesions in breast cancer patients. In this Phase I clinical trial, the therapy was well tolerated, and CD expression was demonstrated in the injected lesions (Pandha *et al* 1999).

Since a small number of Phase I clinical trials involving the CD/5-FC strategy have taken place to date, with the primary goal of examining the safety profile of the novel therapeutics, its antitumour efficacy in prostate cancer patients remains to be established. However, when the CD/TK double suicide gene and relevant prodrugs were used in combination with radiotherapy and a replication-competent adenovirus, longer prodrug courses caused more rapid reductions in serum PSA, though viral DNA persisted in at least some patients after prodrug treatment (Freytag *et al* 2003). Though PSA half-life is not a prognostic factor, its rate of response and absolute nadir in after treatment settings

(such as 3D conformal radiotherapy) are predictive of patient response and outcome. The findings in this study suggest that prodrug activation contributed to the antitumour effect of this trimodal therapy. The contributions of 5-FC and ganciclovir to cytotoxicity in cells containing the CD/TK double suicide gene have been found to be comparable in human prostate cancer cells *in vitro* (Freytag *et al* 1998, Lee YJ *et al* 2002).

8.5. PROSTATE CANCER MODEL SYSTEMS

8.5.1. Cell lines

Cell lines derived from human tumours offer a useful and readily available first-line model for testing novel therapies, without the ethical and practical issues associated with animal studies and particularly with clinical trials. However, they present their own set of challenges. Prostate tumours are particularly difficult to propagate *in vitro* (Simons *et al* 1999), are vulnerable to overgrowth by surrounding stromal cells (Sramkoski *et al* 1999), and like cell lines in general, can become contaminated even after establishment (van Bokhoven *et al* 2003).

The extent to which cell lines resemble clinical tumours must also be considered. PC-3 and DU145 cell lines, widely used as models of AI prostate cancer, lack the AR and do not express PSA. This behaviour is not representative of clinical AI prostate tumours, which do express AR (van der Kwast *et al* 1991) and PSA, since increasing serum PSA levels correlates with disease progression (Brawer & Meyer 2000, Kirby *et al* 2001). Recently, androgen receptor status was examined in seven pairs of isogenic androgen-dependent and androgen-independent prostate cancer cell lines (Chen *et al* 2004). Interestingly, AR expression levels were higher in the AI cell lines, and this resulted not only in hypersensitivity to androgens, but in activation by ligands such as oestrogen.

LNCaP has a mutant androgen receptor, which binds hormones other than androgens (Veldscholte *et al* 1990). This is perhaps not surprising, since this cell line was derived from a lymph node metastasis in a patient with hormone-resistant disease. In addition, the constitutive level of PSA expression by LNCaP cells is approximately a tenth of that observed in many human prostate tumours (J. Isaacs, personal communication).

The 22Rv1 cell line harbours a mutation in the ligand-binding domain of the AR, and an exon 3 duplication in the same gene, which arose during the passaging of xenografts in mice (van Bokhoven *et al* 2003). The frequency of AR mutations in patients is not clear, though they have been recorded (Taplin *et al* 1999, Marcelli *et al* 2000). 22Rv1 cells also have low PSA levels as detected by RT-PCR, compared to other PSA-positive cell lines, which showed a wide range (250-fold) of different PSA expression levels (Denmeade *et al* 2003). Another study found similar variability in PSA expression among prostate cancer cell lines, and failed to detect PSA protein in the supernatant of 22Rv1 cells (van Bokhoven *et al* 2003).

8.5.2. Primary cells

Despite difficulties in growing primary prostate cancer cells in culture, reproducible techniques have now been developed (Peehl *et al* 2005). The slow growth of the cultures obtained in this study *in vitro* meant that the CD/5-FC prodrug activation system could be assessed only under limited conditions (Section 7.6). Alternative transfection conditions, prodrug concentrations and/or prodrug treatment times could possibly yield more information about the effectiveness of the prodrug activation approach in primary cultures. Due to the multifocal nature of prostate tumours, macroscopic identification of malignant and benign regions to sample for primary culture poses a significant challenge. However, in five of six radical prostatectomy patients in this study, tumour samples were successfully obtained (Section 7.1). 59 of 76 specimens from radical prostatectomies, pelvic lymph node dissections or transurethral resections were successfully grown *in vitro* (König *et al* 1998). Such cultures would be expected to more reliably resemble the tumours from which they were derived, than cell lines grown for long periods under artificial conditions. The need to grow primary cultures for long enough to perform meaningful assays must therefore be balanced with minimising the likelihood of *in vitro* changes.

To test the CD/5-FC system in this model, techniques to introduce therapeutic genes are required. A number of reports have indicated that this can be achieved. Short-term primary prostate cancer cultures have been transfected with a plasmid containing AAV

inverted terminal repeats (Vieweg *et al* 1995). Transfection efficiency was not directly measured in this study, but transgene expression levels were comparable to those of retrovirally transduced cell lines. A foreign gene was also introduced into human primary prostate cancer cells, using a retrovirus expressing GM-CSF, during a Phase I clinical trial of prostate cancer gene therapy (Simons *et al* 1999). Most prostate cancer cell lines have been established by growth in specialised media, and have not required viral transduction (Kaighn *et al* 1979, Narayan & Dahiya 1992, Navone *et al* 1997). However, in one case a cell line (957E/hTERT) was established from a radical prostatectomy sample, by growing it *in vitro* and transducing it with a retrovirus encoding the human telomerase gene (Yasunaga *et al* 2001).

Primary cells from other tissues have also been transfected. Liposomes were employed to introduce an AAV-based plasmid encoding IL-2 into human primary cultures (Philip *et al* 1994). Short-term cultures derived from human human colorectal tumours were transduced with both adenoviral and retroviral vectors (Diaz *et al* 1998). Most interestingly, short-term cultures obtained from human gliomas have been transduced with adenoviral vectors encoding the TK prodrug activation gene (Maleniak *et al* 2001). TK transduction and ganciclovir treatment induced apoptosis in this model system, demonstrating that primary cultures of human tumours can be used to test prodrug activation gene therapy strategies. Liposomal transfection of a human primary prostate cultures (Section 7.6) resulted in 10% of cells expressing the GFP reporter gene, although this proved difficult to reproduce. Improved transfection techniques, either by optimising the liposomal procedure or employing viral vectors, should make it possible to test the CD/5-FC approach in prostate cancer primary cultures in addition to cell lines.

8.5.3. Spheroids

Another strategy for developing more realistic *in vitro* models of prostate cancer involves the production of spheroids. When prostate cancer cell lines are grown in three dimensional media, they form sphere-like structures. This has been achieved in agar (Mitrofanova *et al* 2003) and in Matrigel, a matrix containing the basement membrane factors which surround epithelial cells in organs (Lang *et al* 2001). In the case of PC-3

cells, the spheroids consist of a lumen surrounded by a single layer of cells, though this structure can vary (Lang *et al* 2001). Significantly, spheroids exhibit greater radiosensitivity than the same cells grown as monolayers. DU145 cells, stably transduced with the sodium iodide symporter gene, were treated with the prodrug ^{131}I. This treatment killed 70% of cells in monolayers, but resulted in complete destruction of three-dimensional spheroids (Mitrofanova *et al* 2003).

8.5.4. Animal models

Animal models, mainly in mice, are employed for a number of purposes in the development of novel prostate cancer therapeutics. Numerous studies have injected human prostate cancer cell lines into mice and treated the resulting xenograft tumours with various therapies (Uchida *et al* 2001, Xie *et al* 2001a, Peng *et al* 2002, Freytag *et al* 2002b). In other cases, human prostate tumours have been propagated in mice by serial transplantation instead of establishing cell lines *in vitro*. These include the human CWR22 model (Pretlow *et al* 1993). In addition, transplantable Shionogi tumours (Miyake *et al* 1999), and the AI Dunning rat tumour model (Oades *et al* 2002) have been used to test potential therapies for prostate cancer. Though Shionogi tumours originate from a mouse mammary carcinoma, their castration-induced regression and subsequent relapse *in vivo* mimics the progression of human prostate cancer (Miyake *et al* 1999).

Animal models have also proved valuable for ascertaining the tissue-specificity of various promoters used to direct therapeutic gene expression to the prostate. Transgenic mice have been produced which expressed the human PSA gene from its native promoter (Wei *et al* 1997), or the chloramphenical acetyl transferase reporter gene regulated by the rat probasin promoter (Zhang *et al* 2000). More often, reporter gene constructs under the control of tissue-specific promoters have been delivered *in vivo* to animals. Constructs containing the luciferase gene downstream of the PSA promoter have been injected in mice (Wu L *et al* 2001, Adams *et al* 2002). A powerful method of non-invasively measuring luciferase levels in live mice has been developed. This has facilitated quantitative analyses of transgene expression in various tissues and at several time points after vector delivery (Adams *et al* 2002, Sato *et al* 2003). Adenoviral vectors

containing the lacZ reporter gene, and various promoters with prostate selectivity, have been injected in dogs (Steiner *et al* 1999). Dogs, unlike rodents, naturally develop prostate cancer with a similar disease course to that observed in humans, and canine models of prostate cancer have been favoured by some investigators for that reason. However, mice can also be induced to form prostate tumours. TRAMP transgenic mice which express the SV40 large T antigen regulated by the rat probasin promoter all spontaneously develop prostate cancer, and have been used to investigate gene therapy approaches to the disease (Martiniello-Wilks *et al* 2004).

8.6. POTENTIAL OF PSA PROMOTER FOR USE IN ADVANCED PROSTATE CANCER

Patients with advanced disease represent the major therapeutic challenge in prostate cancer. Disease progression is characterised both by acquired resistance to hormonal therapies, and by the development of skeletal and lymph node metastases, and their associated complications (Catalona 1984). It is therefore particularly relevant to consider whether therapeutic genes regulated by the AR-dependent PSA promoter are likely to be of benefit to this group of patients.

8.6.1. Androgen-independent disease

Studies on PSA expression in AI prostate cancer, and on the molecular mechanisms of androgen independence, suggest that PSA promoter-based therapies can be of benefit to at least some AI patients. Most AI prostate tumours express the AR (van der Kwast *et al* 1991), and some mechanisms involved in the development of androgen independence require a functional AR (see Section 1.1.5). Indeed, AR gene amplification has been demonstrated in 13% of prostate tumours which relapsed after hormone therapy (Palmberg *et al* 2000).

The fact that rising serum PSA levels correlate with disease progression (Brawer & Meyer 2000, Kirby *et al* 2001) indicates that advanced prostate tumours also continue to express PSA. Comparison of PSA expression by localised and AI prostate tumours using microarrays showed that AI prostate cancer cells express 20% more PSA (Dhanasekaran *et al* 2001). The AD human prostate cancer cell line LAPC-4, and a derived AI subline, have been examined for endogenous PSA expression, and for luciferase expression from an optimised PSA promoter and enhancer. Luciferase expression was ten times higher in AI cells in this model, and similar results were obtained for endogenous PSA expression (Adams *et al* 2002).

A subset of advanced prostate cancer patients, who escape hormonal control by AR-independent mechanisms, may display both minimal circulating androgen levels and low levels of active AR. Such patients are less likely to benefit from gene therapy involving the PSA promoter, since this promoter is mainly dependent on activated AR (Schuur *et al* 1996, Pang *et al* 1997, Cleutjens *et al* 1997a). Despite this, low levels of PSA promoter activity in the absence of androgen have been considerably increased by two-step transcriptional activation (Segawa *et al* 1998). Recently, progress has been made in identifying PSA sequences and binding proteins involved in androgen-independent PSA expression (Yeung *et al* 2000). However, the frequency of AR-independence among AI prostate cancer patients, and the effect of AR-independence on PSA expression, remain to be clarified.

8.6.2. Metastatic disease

The prospect of using PSA-based gene therapy to treat metastatic prostate cancer faces some challenges, but deserves consideration in view of the very limited number of other effective therapies for this group of patients. Adenoviral vectors in which the PSA promoter directed luciferase expression were injected systemically in mice to study the distribution of luciferase expression *in vivo*. Unexpectedly, low levels of luciferase expression were detected in metastatic lesions in the lung and spine, as well as in human prostate cancer xenografts at their original site (Adams *et al* 2002). Though these results suggest the possible future development of systemic gene therapy for metastatic prostate

cancer, a number of issues must clearly be addressed if this goal is to be pursued. Vectors should be sufficiently safe to be administered systemically, for which purpose vaccinia virus or liposomal delivery might be more acceptable than adenoviruses. Alternatively, adenoviral vectors are being developed to avoid accumulating in the liver and to target other tissues rather than liver. Vectors based on adenovirus serotype 35 showed much reduced liver targeting compared to conventional serotype 5 adenoviruses, while maintaining efficient transduction of human cells (Seshidhar Reddy *et al* 2003).

Therapeutic gene expression should be targeted to malignant cells as much as possible. Optimisation of a tissue-specific promoter to ensure minimal transcription in non-prostate tissues (see Section 8.6.3 below) would therefore be imperative. As an alternative approach to tissue-targeting, antibody conjugates have been developed to link adenoviral capsids to an anti-PSMA antibody (Kraaij *et al* 2005). The resulting PSMA-targeted vectors showed enhanced delivery to two prostate cancer cell lines and reduced efficiency in four non-prostate cell lines.

Polypeptide motifs inserted in virion proteins have also been employed to increase adenoviral transduction of specific cell types (Xia *et al* 2000). Similar approaches could be used to target prostate cancer cells transductionally as well as transcriptionally, and potential prostate cancer cell surface targets such as PSMA are currently being evaluated (Lupold & Rodriguez 2004).

8.6.3. Transcriptional targeting of prostate cancer

Extensive research has confirmed that PSA transcription displays a high degree of prostate specificity. Immunohistochemical staining has shown highly specific PSA staining, with weak expression only in the kidney, parotid gland and pancreas out of 34 nonmalignant human tissues (Alanen *et al* 1996). PSA has also been detected in breast cancer (Yu *et al* 1995a, Alanen *et al* 1996), ovarian cancer (Yu *et al* 1995b), and by reverse-transcriptase PCR in blood samples from both males and females (Smith *et al* 1995). Though PSA staining is reduced in malignant prostate cells relative to normal

prostate (Bright *et al* 1997, Darson *et al* 1997), increases in serum PSA levels are well documented to correlate with disease progression (Brawer & Meyer 2000, Kirby *et al* 2001). This discrepancy is presumably due to the greater number of cells present in cancer patients, as it seems unlikely that a subpopulation of strongly expressing cells would not have been detected if present. The human Expression Atlas, in a large-scale analysis of gene expression patterns in many human tissues, found that both benign and malignant prostate produce high levels of PSA, with slightly lower levels in malignant samples. Twenty-six other non-malignant tissues displayed a median of 80-fold lower PSA expression, though the salivary gland expressed a tenth as much PSA as prostate (Su *et al* 2002).

Numerous studies in which PSA promoter constructs have been placed upstream of reporter genes or therapeutic genes have further demonstrated tissue specificity. *In vitro*, activity has been shown in the PSA-positive prostate cancer cell lines LNCaP (Pang *et al* 1997, Cleutjens *et al* 1997a, Rodriguez *et al* 1997, Latham *et al* 2000) and C4-2 (Yeung *et al* 2000), but not in PSA-negative prostate cancer cell lines (Pang *et al* 1997, Cleutjens *et al* 1997a) or in cell lines from other tissues (Rodriguez *et al* 1997, Latham *et al* 2000), except low levels in breast cancer cell line T47D (Cleutjens *et al* 1997a). In mouse models, expression of downstream genes has similarly been restricted to PSA-positive prostate cancer cells, relative to non-prostate cell lines (Yu *et al* 2001a) or PSA-negative prostate cells (Rodriguez *et al* 1997). In a dog model, adenoviral delivery of a PSA promoter-reporter construct by intraprostatic injection resulted in vector DNA detection in several tissues, but gene expression only in prostate (Steiner *et al* 1999). Little adenoviral uptake and no PSA promoter activity was observed in the liver in this study (Steiner *et al* 1999).

In a further reporter gene study, mice were injected systematically with an adenoviral vector expressing luciferase under the control of a modified PSA promoter, PSE-BC (Wu L *et al* 2001). Luciferase measurements *in vivo* showed that PSA expression was 10- to 30-fold higher in prostate than in other tissues, except liver, which expressed half as much luciferase as the prostate (Wu L *et al* 2001). The authors estimated, however, that

viral delivery was 1000-fold more efficient in the liver than other tissues, a problem which may in future be overcome by improved adenoviral vector design (see Section 8.3). Luciferase transcription from the PSE-BC promoter, amplified by a GAL4-based TSTA system, maintained prostate-specific regulation after intratumoral injection *in vivo*, though absolute levels exceeded those obtained using the CMV promoter (Sato *et al* 2003).

Two transgenic mouse models using the human PSA promoter have been tested and shown prostate-specific PSA transgene expression (Cleutjens *et al* 1997b, Wei *et al* 1997). In a transgenic mouse model containing a 14kb fragment of human genomic DNA spanning the PSA gene, PSA expression was at least 250-fold higher in prostate tissue than in twelve other tissues tested, in which PSA was not detected (Wei *et al* 1997).

Other promoters have also been investigated. Human kallikrein 2 (hK2), a PSA homologue, has shown similar androgen-inducible (Murtha *et al* 1993) and tissue-specific expression. Structurally modified hK2 promoter constructs have brought about prostate-specific transcription in cell lines (Yu *et al* 1999b, Latham *et al* 2000, Xie *et al* 2001b) and in mice with prostate cancer xenografts (Xie *et al* 2001b). Recent microarray data have shown that relative to PSA, hK2 shows slightly higher prostate specificity, and lower salivary gland expression. However, hK2 expression in prostate cancer samples, like PSA, was slightly greater in normal prostate than in prostate cancer (Su *et al* 2002).

The specificity of the rat probasin promoter for prostate tissue has been shown in cell lines (Lowe *et al* 2001, Xie *et al* 2001a), and in animal models (Steiner *et al* 1999, Wu X *et al* 2001), though low levels of probasin promoter activity were detected in reproductive organs in a transgenic mouse model (Wu X *et al* 2001). Like PSA, it is androgen-inducible (Xie *et al* 2001a). A recombinant probasin promoter expressed sufficient pro-apoptotic caspase 9 to prolong survival in mice bearing LNCaP tumours (Xie *et al* 2001a).

The prostate-specific membrane antigen (PSMA) gene is expressed in prostate and absent in most other human tissues, except low levels in small intestine, brain, salivary gland (Israeli *et al* 1994), colon, and kidney (Silver *et al* 1997). Interestingly, PSMA is upregulated in the absence of androgens, both in LNCaP and in patients on androgen ablation therapy (Wright *et al* 1996). Constructs in which the PSMA promoter directed expression of a prodrug activation gene have been developed, and shown antitumour activity in an animal model (Uchida *et al* 2001). However, PSMA promoter constructs have shown variable degrees of tissue specificity *in vitro* (Good *et al* 1999, O'Keefe *et al* 2000). More importantly, microarray experiments have shown significant expression in human brain, spinal cord, salivary gland and kidney (Su *et al* 2002), and it seems unlikely that PSMA promoter constructs will prove useful in prostate cancer. However, a hybrid construct of sequences from PSMA and PSA enhancers showed prostate-specific activity *in vitro* and in a murine model, and merits further investigation (Lee SJ *et al* 2002).

The $DD3^{PCA3}$ gene is transcribed to an apparently untranslated RNA of unknown function (Bussemakers *et al* 1999). $DD3^{PCA3}$ mRNA levels, measured by quantitative PCR, were 34-fold higher in malignant prostate than in normal prostate or benign prostatic hyperplasia (BPH), and very low or absent in non-prostate tissues (de Kok *et al* 2002). However, the prostate specificity of $DD3^{PCA3}$ appears to be regulated both during transcription and post-transcriptionally. A 0.2kb promoter from $DD3^{PCA3}$ conferred higher expression of a reporter gene in LNCaP cells than in non-prostate cells, but only 3-6-fold higher (Verhaegh *et al* 2000), and an alternative $DD3^{PCA3}$ transcript is expressed in several non-prostate tissues (Gandini *et al* 2003).

Another tissue-specific promoter, that of the mouse osteocalcin gene, has been investigated specifically for its utility in advanced prostate cancer. Osteocalcin (OC) is strongly expressed in metastatic prostate cancer cells, and also present in primary prostate cancer cells and osteoblasts (Matsubara *et al* 2001). The risk of bone damage by expressing antitumour genes from an osteocalcin promoter may be alleviated by the low adenoviral transduction efficiency of normal human bone (Matsubara *et al* 2001). Patients with locally recurrent and metastatic prostate cancer, were treated in a phase I

clinical trial with an OC promoter-driven TK gene, after the failure of one or more prior therapies (Kubo *et al* 2003). Though evidence of antitumour activity was seen in some patients, the authors suggested that identification of patients with higher OC and CAR expression might improve the response rate to OC-TK gene therapy. An adenoviral vector which expressed the viral E1 gene under the control of the OC promoter, combined with an anti-angiogenic gene, was employed to target both prostate tumours and the tumour endothelium, resulting in complete regression of prostate tumour xenografts in three of ten mice (Jin *et al* 2005).

Although few tumour-specific promoters have been identified to date, this approach may present another option in transcriptional targeting in the future. The use of one such promoter, from the rat progression-elevated 3 gene, to express a pro-apoptotic gene prevented the growth of prostate tumours in immunodeficient mice (Su *et al* 2005). Targeting tumours and sparing normal tissue clearly offers advantages for both safety and efficacy of gene therapy.

Of the tissue-specific promoters investigated in preclinical models of prostate cancer gene therapy, the PSA, hK2, probasin and osteocalcin promoters offer the most promise for translation into clinical practice. Although recent data suggest that the native hK2 promoter is at least as prostate-specific as PSA, its ability to express therapeutic levels of transgene *in vivo* is less well characterised. The tissue-specificity of the rat probasin and mouse osteocalcin promoters in human tissues also requires further examination. A disadvantage of the PSA promoter is its activity in benign as well as malignant prostate cells. However, among the genes discussed in Section 8.6.3, only the $DD3^{PCA3}$ gene is known to be significantly overexpressed in malignant prostate, and this appears to be due to post-transcriptional as well as transcriptional regulation. It therefore seems reasonable to continue to pursue the development of PSA promoter-regulated approaches to prostate cancer gene therapy.

8.7. FUTURE DIRECTIONS

Prostate-directed prodrug activation therapy undoubtedly has the potential to complement and improve current treatments for prostate cancer. The realisation of this goal depends on the continued development of prostate-targeting techniques, efficient gene expression *in vivo*, gene delivery methods, preclinical models of prostate cancer, and strategic combinations of gene therapy with other treatments such as radiotherapy.

At present, transcriptional regulation offers the best prospects for restricting the activity of a therapeutic gene to the prostate. Constructs based on PSA promoter and enhancer sequences have been extensively studied and refined. An optimised PSA construct known as PSE-BC (Figure 8.4A) emerged from a systematic analysis of the effects of duplicating key regions (Wu L *et al* 2001). This construct displayed prostate-specificity in mice when reporter gene expression was analysed by luminescent imaging of the entire bodies of live mice (Adams *et al* 2002). To date, this construct has not been employed to express a therapeutic gene. The combination of PSE-BC and the CD prodrug activation gene is therefore a priority for future development.

One limitation of tissue-specific promoters such as the PSA promoter is low activity. This has been alleviated most effectively by two-step transcriptional activation (Figures 8.1, 8.2 and 8.3). A GAL4-VP16 TSTA system containing two VP16 domains and five GAL4 binding sites has been optimised (Zhang *et al* 2002). This system has brought about high-level, tissue-specific luciferase expression in mice when combined with the PSE-BC construct above (Sato *et al* 2003), and will be pursued as a promising means to bring about therapeutic levels of tissue-specific gene expression.

Another limitation of the PSA promoter is that its activity may be reduced in some androgen-independent prostate tumours. A PSA-PSMA chimeric enhancer upstream of a minimal promoter showed surprisingly high tissue-specificity and reduced dependence on androgens (Figure 8.4B, Lee SJ *et al* 2002). A 0.2kb fragment of the PSA core enhancer was included in this construct, and it is likely that inclusion of other PSA promoter and enhancer sequences could result in improved chimeric designs for androgen-independent patients.

Gene delivery in this project was achieved using cationic liposomes and polymers. While these techniques were sufficient to show proof of principle for the CD prodrug activation system, more efficient delivery will be important to achieve a stronger therapeutic effect. Adenoviral transduction has been the method of choice for delivering genes to prostate cancer cells *in vivo* and in clinical trials. Recombinant adenoviral vector designs are well established, high yields of the virus can be produced, and efficient transduction is possible. PSA-CD constructs should therefore be transferred from the plasmid vectors generated during this project to adenoviral vectors for future use.

Further development of the PSA-CD strategy will require the use of *in vitro* and *in vivo* models of prostate cancer. A panel of non-prostate cell lines is to be acquired to assess the tissue-specificity of PSA promoter constructs, with emphasis on colorectal, bladder and liver tissues, since they are at the highest risk of accidental exposure to gene therapy vectors. Additional PSA-positive prostate cancer cell lines such as MDA PCA-2b will be tested, and three-dimensional cultures of prostate cancer cell lines in Matrigel will provide a more realistic model than mololayer cultures. Development of primary cultures of human prostate cancer samples will also be further pursued. However, animal models will be crucial for testing the ability of the PSA-CD system to inhibit prostate tumour growth. Xenografts of human prostate cancer cell lines in mice will be treated with this prodrug activation system. The TRAMP model of spontaneous prostate cancer in mice may also be employed.

Gene therapy is likely to be combined with other treatments for prostate cancer for maximum results. The CD/5-FU prodrug activation system is particularly suitable for use in conjunction with radiation, due to the activity of 5-FU as a radiosensitising agent, and this concept will be tested *in vitro* using the PSA-CD vectors constructed to date. Vectors will be modified by insertion of CArG elements to allow induction of transcription by radiation, to further improve the interaction between radiation and gene therapy strategies. Hypoxia response elements will also be inserted, to target therapeutic gene expression to regions of tumours which are most resistant to current therapies.

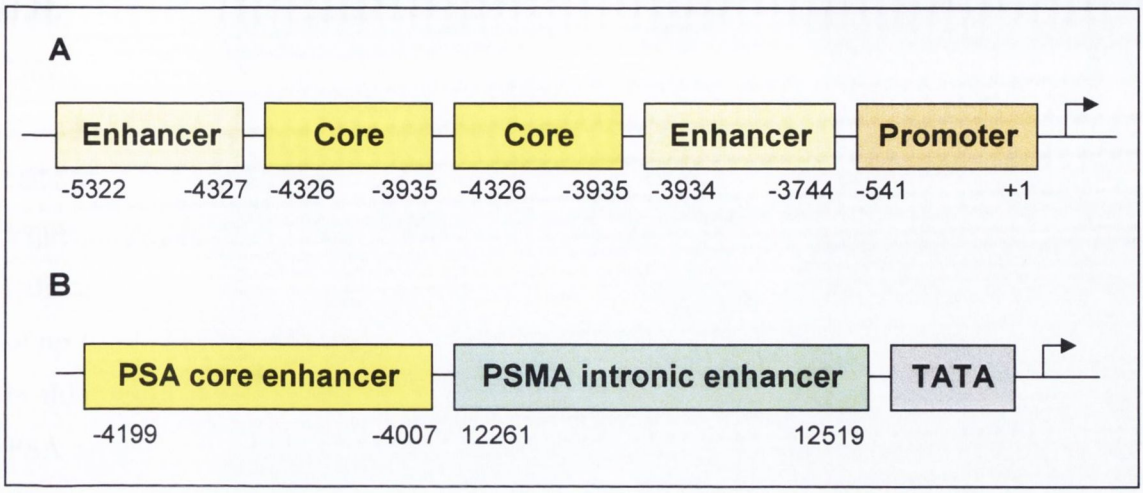


Figure 8.4. Modified PSA promoter constructs. A. Optimised PSA construct PSE-BC (Wu L *et al* 2001). 391bp of core enhancer sequence was duplicated in tandem within the PSA enhancer upstream of the PSA promoter. The duplicated region contains ARE-IIIA, ARE-III, ARE-IV, ARE-V (Huang *et al* 1999), and three GATA-2 binding sites (Perez-Stable *et al* 2000). **B.** PSA-PSMA chimeric enhancer (Lee SJ *et al* 2002). 193bp of the PSA core enhancer was ligated to 259bp of the transcriptional enhancer element located in the third intron of the PSMA gene, upstream of a synthetic TATA box (Lee SJ *et al* 2002). Sequence positions in the PSMA gene refer to Genbank accession number NT009237. The PSA sequence in this construct contains the same protein binding sites as PSE-BC, except ARE-V.

8.8. CONCLUSION

This proof of principle study has demonstrated that the CD prodrug activation gene, expressed under the control of the tissue-specific PSA promoter, kills prostate cancer cells in combination with the prodrug 5-FC. More efficient CD expression will be required for this strategy to be clinically useful. Results by other researchers clearly indicate that this can be achieved, by two means. Firstly, viral transduction efficiencies of up to 100% have been noted, in contrast to the maximum of 50% transfection obtained in this study. Secondly, PSA transcription has been manipulated both by altering the PSA sequence and by amplifying transcription using TSTA systems. A combination of these strategies resulted in greater promoter activity than that of the CMV promoter *in vivo*, an extremely encouraging indication that tissue-specificity and efficiency can be achieved simultaneously. Even without amplification, PSA promoters have expressed enough therapeutic transgene to bring about regression in prostate tumours in mice, and dose-dependent serum PSA reductions in a Phase I clinical trial. Antitumour activity by the CD/5-FC system in animal models has already been shown in a number of investigations, including one which used a PSA promoter. However, the optimal use of this strategy is likely to be in combination with radiotherapy. 5-FU enhances sensitivity to radiation *in vitro*, *in vivo* and in patients, and the intratumoral production of 5-FU by the CD/5-FC system also has this effect in preclinical models of prostate and other cancers. Much evidence therefore exists to suggest that PSA-CD gene therapy could be effective. It is equally relevant to note that the CD/5-FC strategy has not been associated with significant toxicity problems in animal models. Prostate cancer patients have been treated with the CD/5-FC system (in addition to other modalities), and with an antitumour transgene regulated by the PSA promoter, in separate Phase I clinical trials. All three trials have maintained acceptable safety profiles even using replication-competent adenoviral vectors. In conclusion, it is to be both hoped and expected that prostate cancer patients in the future will benefit from the introduction of this or similar approaches to prostate cancer gene therapy.

APPENDICES

APPENDIX 1: SOLUTIONS USED

5X agarose gel loading dye

0.25% bromophenol blue (Sigma, UK)

0.25% xylene cyanol (Sigma, UK)

15% Ficoll type 400 (Sigma, UK)

2X cracking buffer (per 50ml)

2ml 5M NaOH

2.5ml 10% SDS

10g sucrose (Sigma, UK)

DC-FCS-RPMI

500ml RPMI1640 with Glutamax

50ml dextran-charcoal treated FCS (Hyclone, USA)

50u/ml penicillin

5μg/ml streptomycin

2.5μg/ml amphotericin B

Ethidium bromide/acridine orange solution

16μg/ml ethidium bromide (Sigma, UK)

10μg/ml acridine orange (Sigma, UK)

Add ethidium bromide and acridine orange to phosphate buffered saline (Invitrogen, UK).

2X freezing medium

40% foetal bovine serum

40% RPMI1640 medium

20% dimethylsulfoxide (DMSO) (Sigma, UK)

Add DMSO last, on ice, as its addition is exothermic.

LB agar

Prepare as for LB broth and add 15g/L agar (Oxoid, UK) before autoclaving.

LB broth

10g/L tryptone (Oxoid, UK)

5g/L yeast extract (Oxoid, UK)

10g/L sodium chloride

Adjust pH to 7.0 to 7.2 using 5M NaOH (BDH, UK). Autoclave.

Lysis buffer for protein extracts

10mM TrisHCl, pH 7.4

50mM sodium chloride

10mM sodium pyrophosphate (Sigma, UK)

50mM sodium fluoride

1% IGEPAL CA-360 (Sigma, UK)

1μg/ml leupeptin (Sigma, UK)

1mM phenylmethylsulfonylfluoride (PMSF; Sigma, UK)

1mM sodium orthovanadate (Sigma, UK)

0.1% aprotinin (Sigma, UK)

2X non-reducing protein buffer

100mM TrisHCl, pH6.8

2% SDS

20% glycerol

0.2% bromophenol blue (Sigma, UK)

2X protein dissociation buffer (per 30ml)

1.5M TrisHCl, pH 6.8	3.3ml
10% SDS	10ml
1M EDTA	0.2ml
Glycerol (Sigma, UK)	10ml
Bromophenol blue, 2.5%	5ml
β-mercaptoethanol (Sigma, UK)	1.2ml

10X proteinase K buffer (per 500µl)

TrisHCl, pH 7.8, 1M	50µl
SDS, 10%	250µl
Calcium chloride (BDH, UK), 1M	5µl

10X running buffer for polyacrylamide gels

0.25M Tris base

1.92M glycine (Sigma, UK)

2% SDS

Saline-Tris-EDTA (STE) buffer

0.1M NaCl (Sigma, UK)

10mM TrisHCl, pH 8.0 (Sigma, UK)

1mM EDTA, pH 8.0 (Sigma, UK)

SOC medium

Dissolve in 1L deionised water:

20g/L tryptone

5g/L yeast extract

0.5g/L sodium chloride

Add potassium chloride (Sigma, UK) to 2.5mM final concentration. Adjust pH to 7.0 with 5M NaOH and autoclave. After cooling, add 20ml/L 1M sterile glucose (Sigma, UK). Before use, add 5ml/L sterile 2M magnesium chloride (Sigma, UK). Sterilise glucose and magnesium chloride solutions by filtration through 0.2µ filters (Sartorius, Germany).

Solution I

25mM TrisHCl, pH 8.0 (Sigma, UK)

10mM EDTA, pH 8.0

50mM glucose

Solution II

0.2M NaOH

1% SDS (sodium dodecyl sulfate; Sigma, UK)

Make up fresh on day of use.

Solution III

600ml/L 5M potassium acetate (Sigma, UK)

115ml/L glacial acetic acid

Stripping buffer

62.5mM TrisCl

2% SDS

Adjust pH to 6.7.

TBS-Tween

5% 20X TBS

0.05% Tween 20 (polyoxyethylenesorbitan monolaurate; Sigma, UK)

Tris-EDTA (TE) buffer, pH 8.0

10mM TrisHCl, pH 8.0

1mM EDTA, pH 8.0

10X transfer buffer

144g/L glycine

30g/L Tris base

10g/L SDS

For 1X transfer buffer, mix 80ml 10X buffer, 720ml water and 200ml methanol.

Tris-acetate-EDTA (TAE) buffer (50X)

242g/L Tris base (Sigma, UK)

20.2g/L EDTA (Sigma, UK)

57.1ml/L glacial acetic acid (Scharlau, Spain)

20X Tris-buffered saline (TBS) buffer

0.2M Tris base

3M sodium chloride

Adjust pH to 8.0.

WAJC404 epithelial cell medium

WAJC404 powder (Invitrogen, UK)	11.04g/L
HEPES	28mM
Sodium hydrogen carbonate (Sigma, UK)	14mM
FCS	10%
Penicillin	25u/ml
Streptomycin	25μg/ml
Amphotericin B	250μg/ml
Cholera toxin (Sigma, USA)	20ng/ml
Insulin (Sigma, UK)	25ng/ml
Dexamethasone (Sigma, UK)	1μM
Hydrocortisone (Sigma, UK)	2.5nM

pH was adjusted to 7.6 using 4M NaOH.

APPENDIX 2: MYCOPLASMA TEST

Cell lines were grown for two passages in antibiotic-free medium prior to mycoplasma testing using a mycoplasma PCR ELISA kit (Roche, UK). (All materials below, except water and where otherwise indicated, were obtained in this kit). 1ml cell culture supernatant was centrifuged at 200 x g for 10 minutes at room temperature. The supernatant was removed and centrifuged at 13,000 x g for 10 minutes at 4°C to sediment mycoplasma. After discarding the supernatant, any pellet present was resuspended in 10µl sterile water (autoclaved deionised water, sterilised by ultraviolet light), and 10µl lysis solution. At least two positive and negative controls were included. Positive controls consisted of 10µl positive control DNA and 10µl lysis solution. Negative controls contained 10µl sterile water and 10µl lysis solution. Samples and controls were incubated at 37°C for 1 hour, after which 30µl neutralisation solution was added.

PCR reactions were carried out using the cell extracts and controls. 10µl each was added to 25µl PCR mix and 15µl sterile water, and amplified on a PTC-200 thermocycler under the following conditions:

95°C for 5 min
39 cycles of
    94°C for 30s
    62°C for 30s
    72°C for 1 min
72°C for 10 min

Positive control PCR products were electrophoresed on a 1% agarose gel (as described in 2.1.1.5), to ensure that the 520bp product had been successfully amplified before proceeding to the ELISA step.

40µl denaturation reagent was added to 10µl PCR product and incubated for 10 minutes at room temperature. 450µl hybridisation reagent (containing biotinylated

probe) was added to each sample and mixed thoroughly. 200µl of each sample was transferred to a 96-well plate. The plate was sealed with adhesive foil and covered in aluminium foil (Sparks, Ireland), and shaken at 150rpm, 37°C, for 3h in a Stuart Scientific SI50 incubator (Bibby Sterilin, UK). Wells were washed three times with 250µl washing buffer. 200µl anti-DIG-peroxidase, prewarmed to room temperature, was added. The plate was covered as before, and shaken at 150rpm and room temperature for 30 minutes. Wells were washed five times with 250µl washing buffer. 100µl TMB (3,3',5,5'-tetramethylbenzidine) substrate solution was added. The plate was covered as before, and shaken at 150rpm and room temperature for 20 minutes, after which 100µl stop solution was added.

Absorbance at 450nm and reference absorbance at 690nm were measured using a SpectraFluor Plus fluorimeter. $A_{450} - A_{690}$ was greater than 1.2 for positive controls and less than 0.25 for negative controls; otherwise, the test was repeated. Samples for which

$$(A_{450} - A_{690}) - (\text{mean } A_{450} - A_{690} \text{ of negative controls})$$

was below 0.2 were regarded as mycoplasma-negative. The cell lines used in this study consistently tested negative for mycoplasma contamination.

APPENDIX 3: ANTIBIOTIC SELECTION FOR PLASMIDS

Plasmid	Antibiotic resistance genes
pPSA1.3	Ampicillin and kanamycin
pPSA2.5	Ampicillin and kanamycin
pPSA-CD1.8	Ampicillin
pPSA-CD4.19	Ampicillin
pAMdPE1.1	Chloramphenicol
pPPE-CD2.15	Chloramphenicol
pCMV-CD1.10	Chloramphenicol
pMinusCD1.24	Chloramphenicol
pPCM1.24	Chloramphenicol and ampicillin
pPCM2.3	Chloramphenicol and ampicillin
pALTER-MAX	Chloramphenicol
pCD1	Ampicillin
pEGFP-NI	Kanamycin

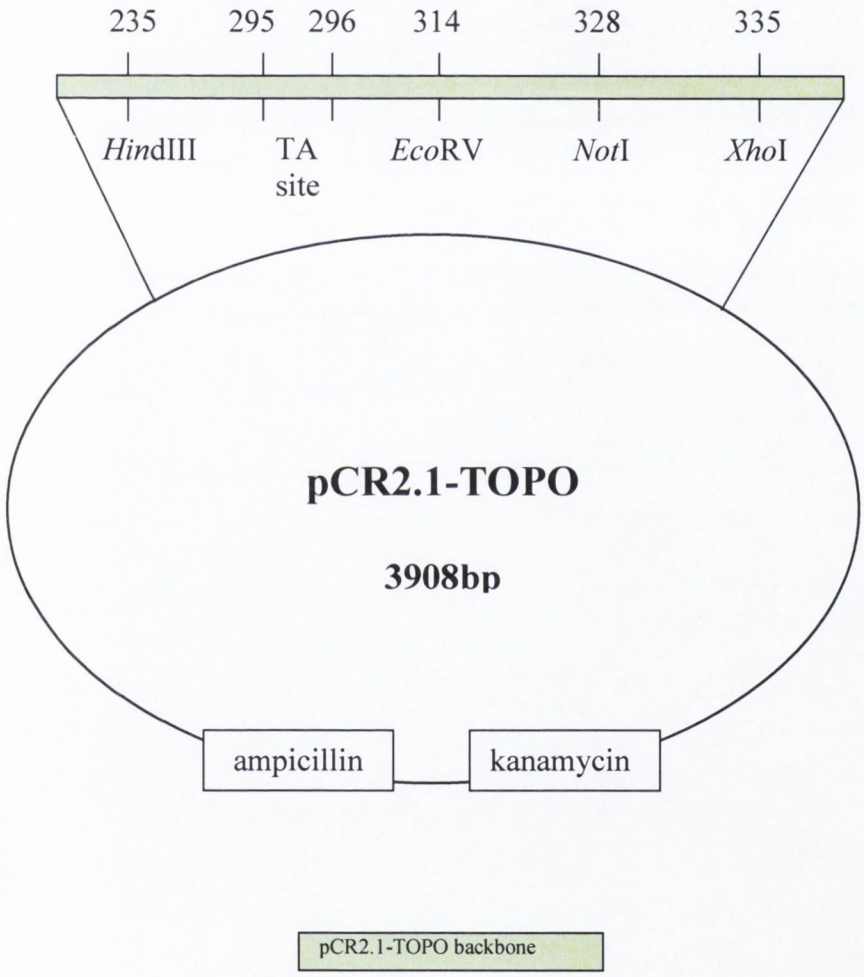
APPENDIX 4: CYTOSINE DEAMINASE SEQUENCE

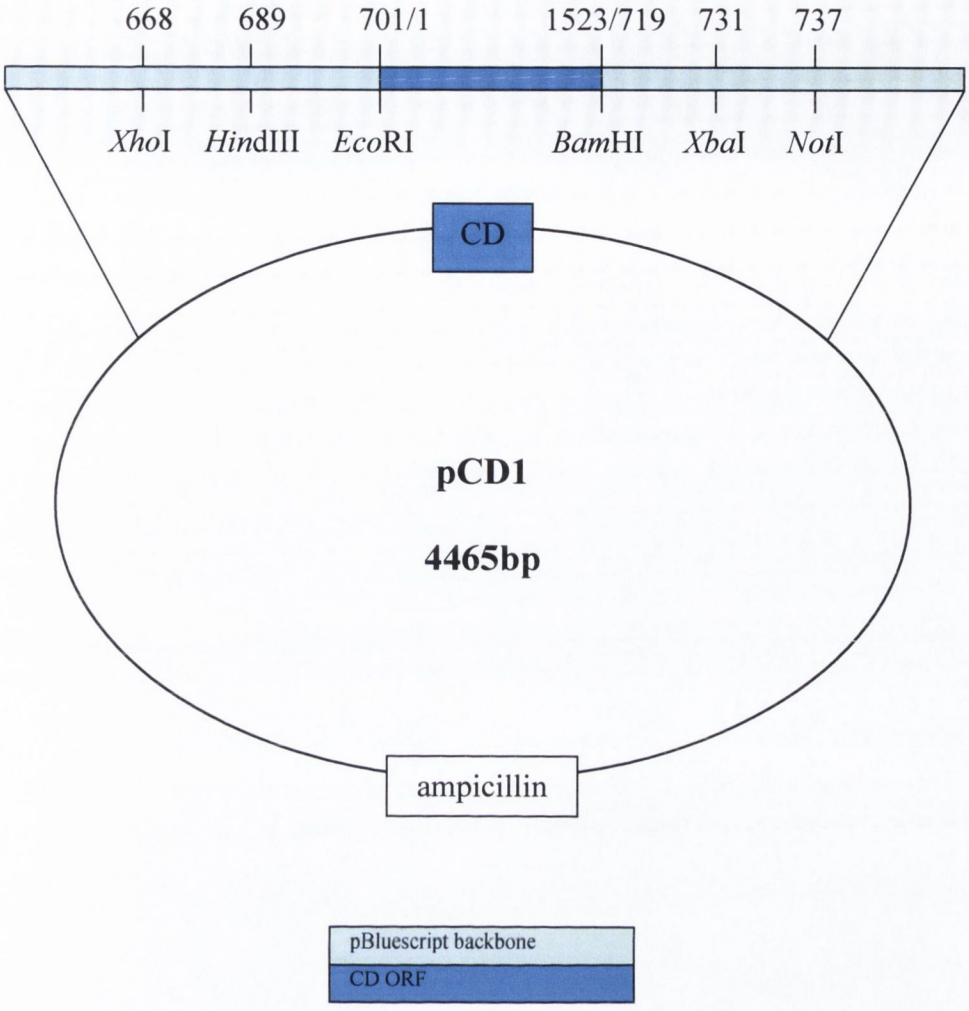
The cytosine deaminase gene and its sequence, shown below, was received from Lesley-Anne Martin, as an *Eco*RI-*Bam*HI fragment in the plasmid pBluescript SK+. Positions 17-1519 of this 1528bp sequence correspond to positions 131-1633 of the Genbank sequence (accession number S56903). The translation start site (ATG at position 16) was previously modified to improve expression in eukaryotic cells (Mullen *et al* 1992).

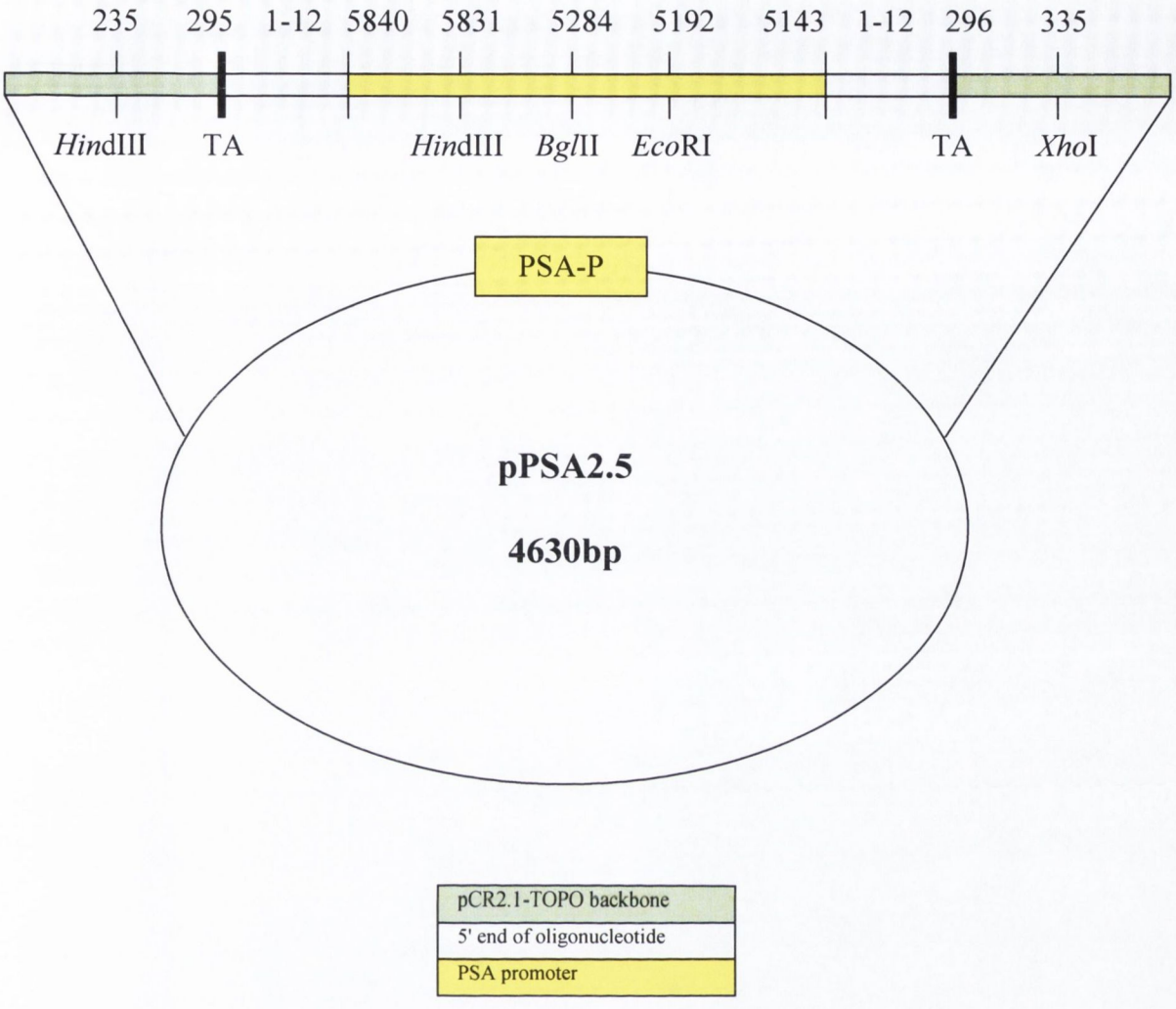
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1       GAATTCAGGC TAGCAATGTC GAATAACGCT TTACAAACAA TTATTAACGC
51      CCGGTTACCA GGCGAAGAGG GGCTGTGGCA GATTCATCTG CAGGACGGAA
101     AAATCAGCGC CATTGATGCG CAATCCGGCG TGATGCCCAT AACTGAAAAC
151     AGCCTGGATG CCGAACAAGG TTTAGTTATA CCGCCGTTTG TGGAGCCACA
201     TATTCACCTG GACACCACGC AAACCGCCGG ACAACCGAAC TGGAATCAGT
251     CCGGCACGCT GTTTGAAGGC ATTGAAGGCT GGGCCGAGCG CAAAGCGTTA
301     TTAACCCATG ACGATGTGAA ACAACGCGCA TGGCAAACGC TGAAATGGCA
351     GATTGCCAAC GGCATTCAGC ATGTGCGTAC CCATGTCGAT GTTTCGGATG
401     CAACGCTAAC TGCGCTGAAA GCAATGCTGG AAGTGAAGCA GGAAGTCGCG
451     CCGTGGATTG ATCTGCAAAT CGTCGCCTTC CCTCAGGAAG GGATTTTGTC
501     GTATCCCAAC GGTGAAGCGT TGCTGGAAGA GGCGTTACGC TTAGGGGCAG
551     ATGTAGTGGG GGCGATTCCG CATTTTGAAT TTACCCGTGA ATACGGCGTG
601     GAGTCGCTGC ATAAAACCTT CGCCCTGGCG CAAAAATACG ACCGTCTCAT
651     CGACGTTCAC TGTGATGAGA TCGATGACGA GCAGTCGCGC TTTGTCGAAA
701     CCGTTGCTGC CCTGGCGCAC CATGAAGGCA TGGGCGCGCG AGTCACCGCC
751     AGCCACACCA CGGCAATGCA CTCCTATAAC GGGGCGTATA CCTCACGCCT
801     GTTCCGCTTG CTGAAAATGT CCGGTATTAA CTTTGTCGCC AACCCGCTGG
851     TCAATATTCA TCTGCAAGGA CGTTTCGATA CGTATCCAAA ACGTCGCGGC
901     ATCACGCGCG TTAAAGAGAT GCTGGAGTCC GGCATTAACG TCTGCTTTGG
951     TCACGATGAT GTCTTCGATC CGTGGTATCC GCTGGGAACG GCGAATATGC
1001    TGCAAGTGCT GCATATGGGG CTGCATGTTT GCCAGTTGAT GGGCTACGGG
1051    CAGATTAACG ATGGCCTGAA TTTAATCACC CACCACAGCG CAAGGACGTT
1101    GAATTTGCAG GATTACGGCA TTGCCGCCGG AAACAGCGCC AACCTGATTA
1151    TCCTGCCGGC TGAAAATGGG TTTGATGCGC TGCGCCGTCA GGTTCCGGTA
1201    CGTTATTCGG TACGTGGCGG CAAGGTGATT GCCAGCACAC AACCGGCACA
1251    AACCACCGTA TATCTGGAGC AGCCAGAAGC CATCGATTAC AAACGTTGAA
1301    CGACTGGGTT ACAGCGAGCT TAGTTTATGC CGGATGCGCG GTGAACGCCT
1351    TATCCGGCCT ACGTAGAGCA CTGAACTCGT AGGCCTGATA AGCGTAGCGC
1401    ATCAGGCAAT TCCAGCCGCT GATCTGTGTC AGCGGCTACC GTGATTCATT
1451    CCCGCCAACA ACCGCGCATT CCTCCAACGC CATGTGCAAA AATGCCTTCG
1501    CAGCGGCTGT CTGCCAGCTA GAGGATCC
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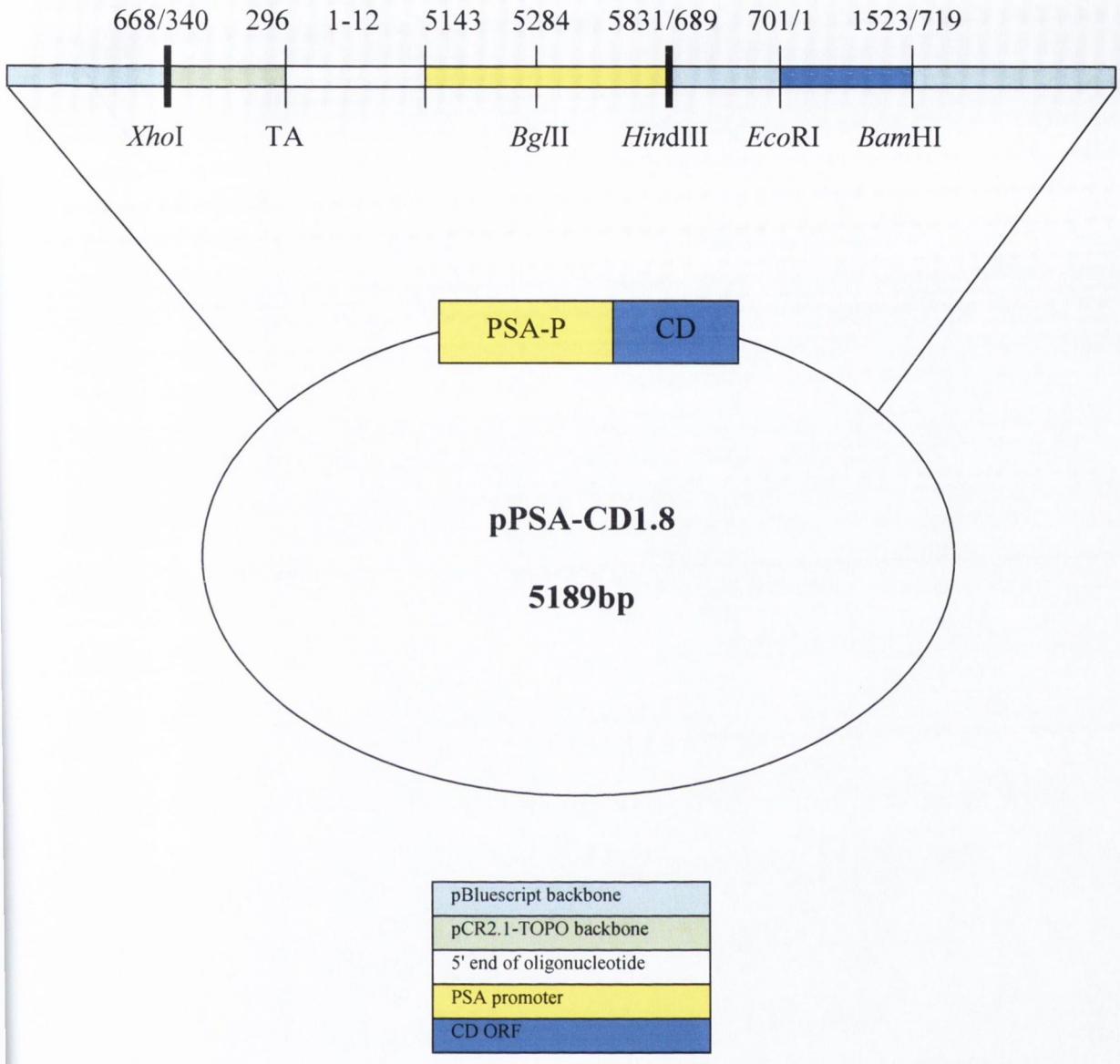
APPENDIX 5: PLASMID MAPS

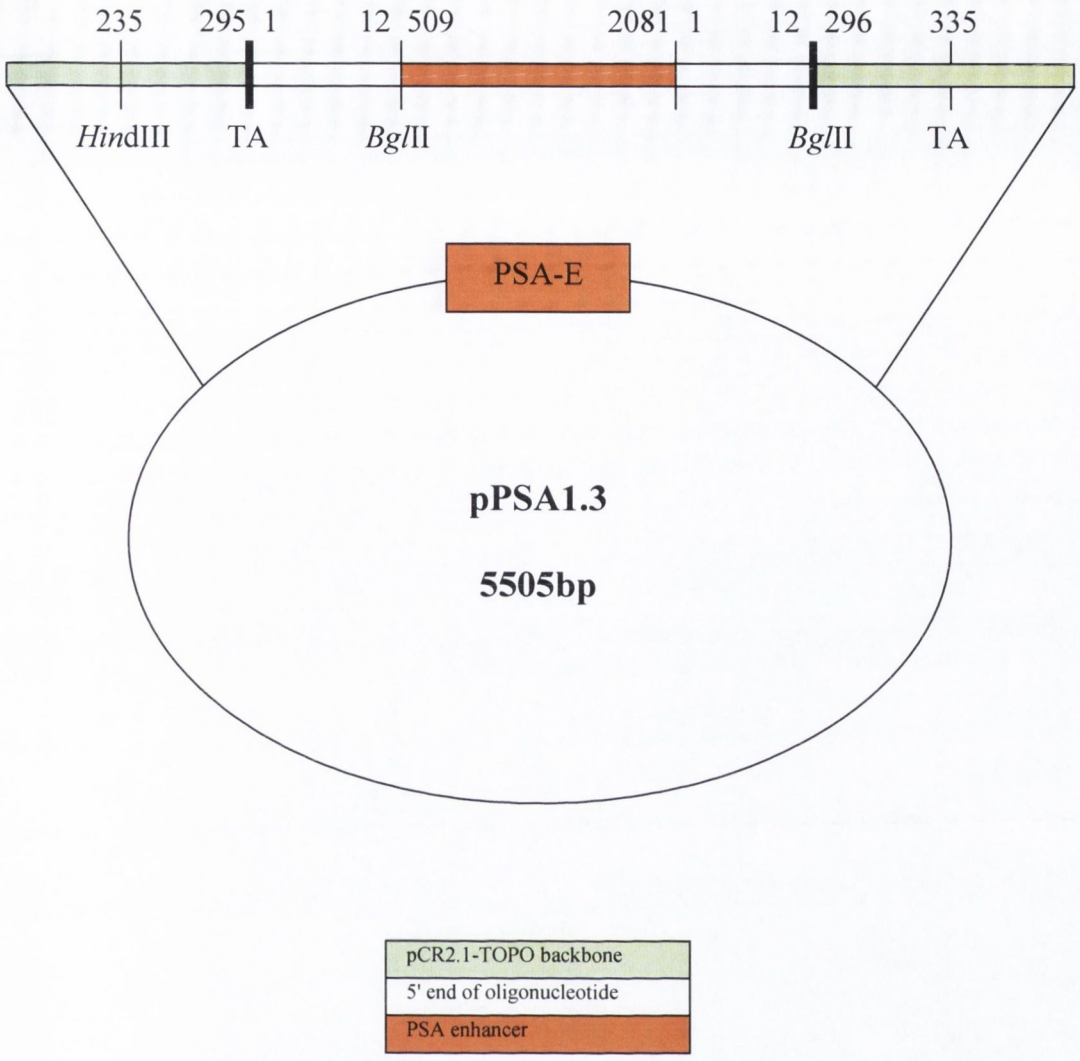
This appendix contains maps of all plasmids used for cloning during this project. Table 3.1 provides a summary of their structures. Antibiotic resistance genes are indicated in the plasmids pCR2.1-TOPO, pCD1 and pALTER-MAX. The remaining plasmids are derived from these and from PSA sequences amplified using PCR, as outlined in Figures 3.1 and 3.2. Cloning junctions in plasmids generated during this work are represented by bold lines.

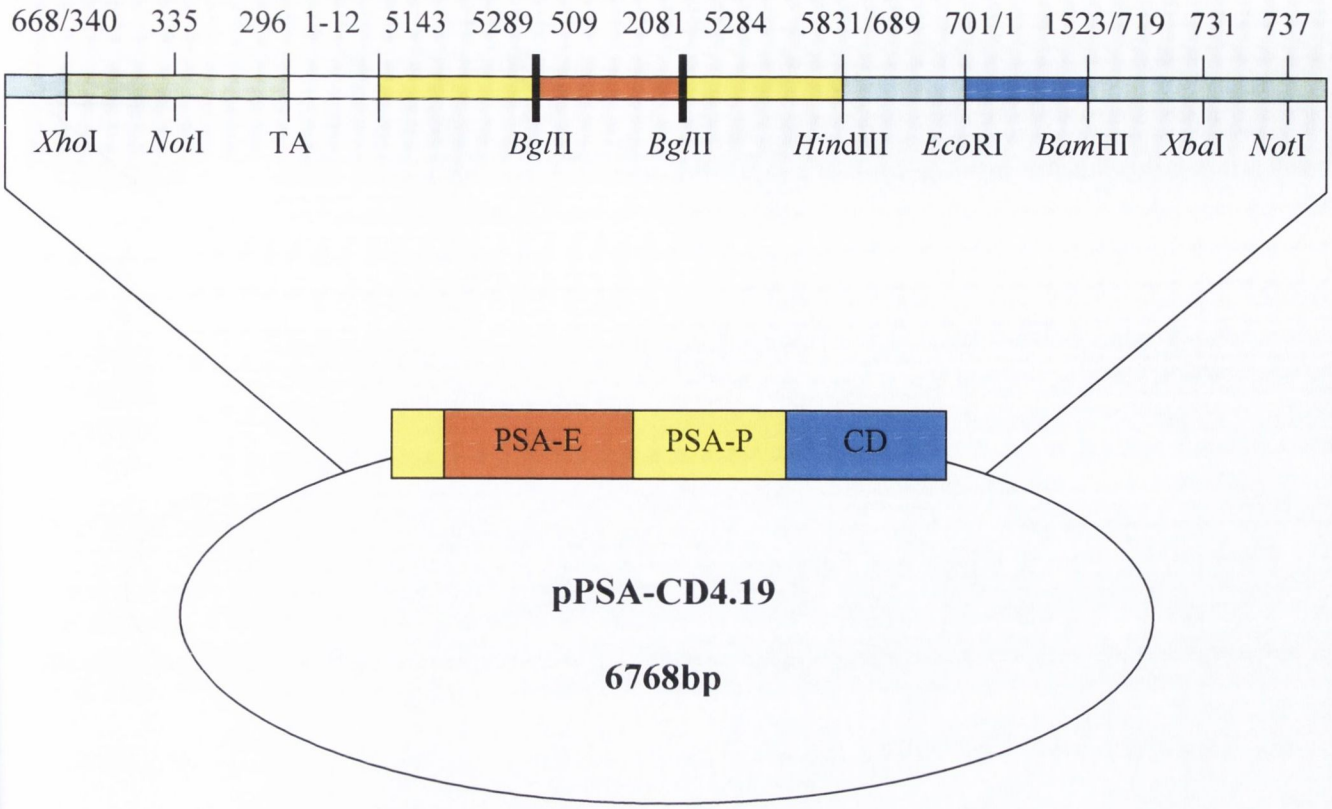



668
689
701/1
1523/719
731
737
XhoI
HindIII
EcoRI
BamHI
XbaI
NotI
CD
pCD1
4465bp
ampicillin
pBluescript backbone
CD ORF

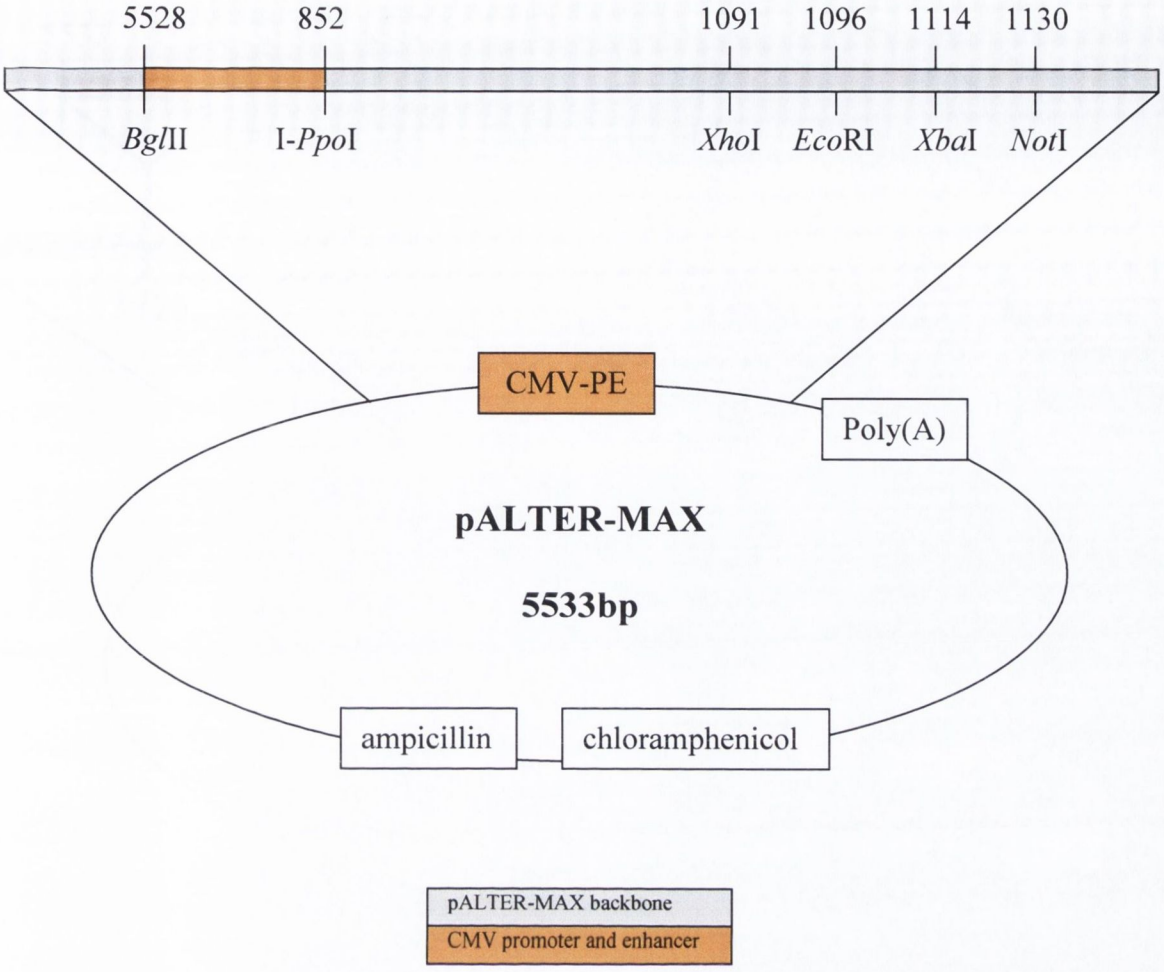

235
295
1-12
5840
5831
5284
5192
5143
1-12
296
335
HindIII
TA
HindIII
BglII
EcoRI
TA
XhoI
PSA-P
pPSA2.5
4630bp
pCR2.1-TOPO backbone
5' end of oligonucleotide
PSA promoter

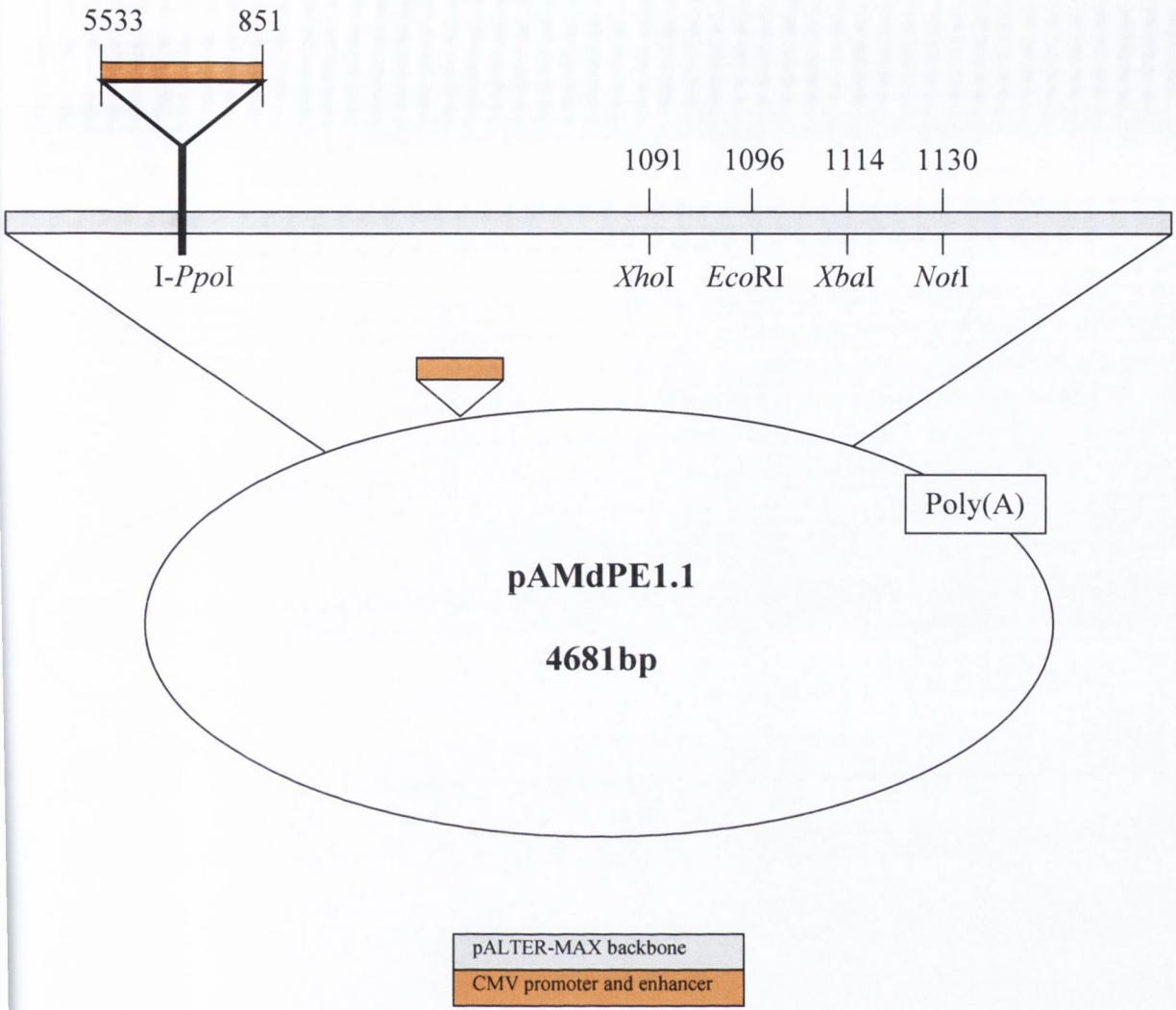

668/340
296
1-12
5143
5284
5831/689
701/1
1523/719
XhoI
TA
BglII
HindIII
EcoRI
BamHI
PSA-P
CD
pPSA-CD1.8
5189bp
pBluescript backbone
pCR2.1-TOPO backbone
5' end of oligonucleotide
PSA promoter
CD ORF

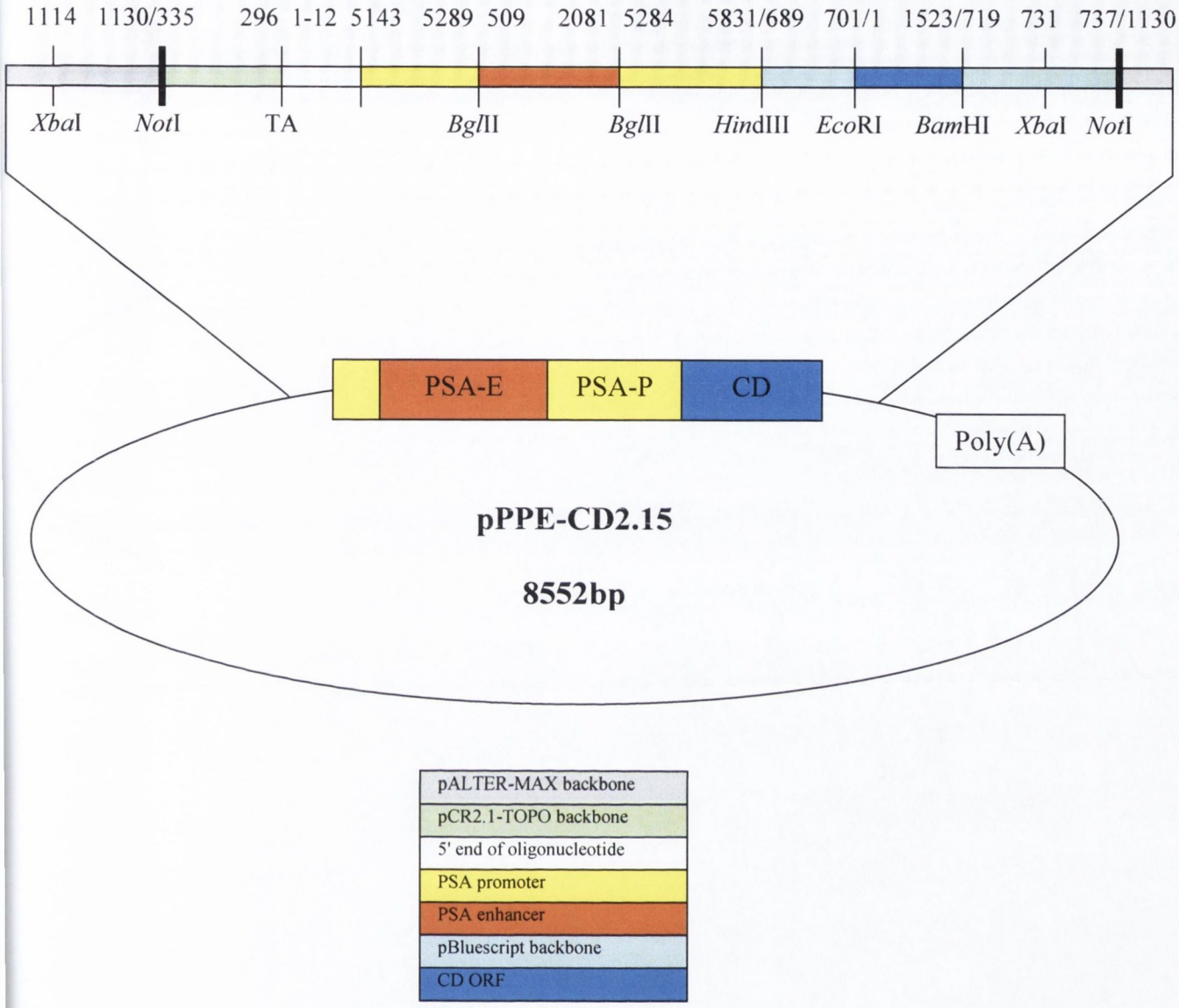

235
295
1
12
509
2081
1
12
296
335
HindIII
TA
BglII
BglII
TA
PSA-E
pPSA1.3
5505bp
pCR2.1-TOPO backbone
5' end of oligonucleotide
PSA enhancer

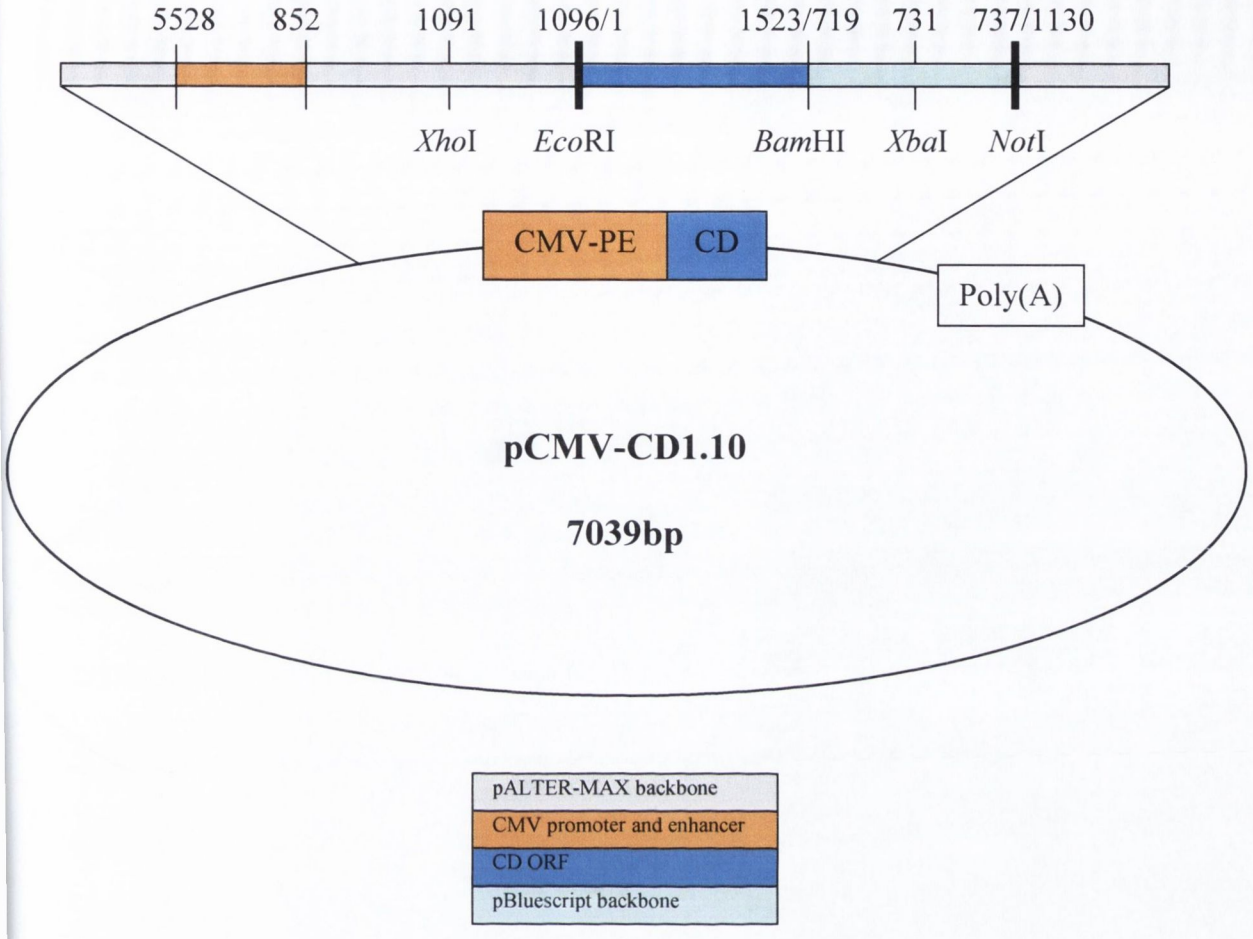


pBluescript backbone
pCR2.1-TOPO backbone
5' end of oligonucleotide
PSA promoter
PSA enhancer
CD ORF

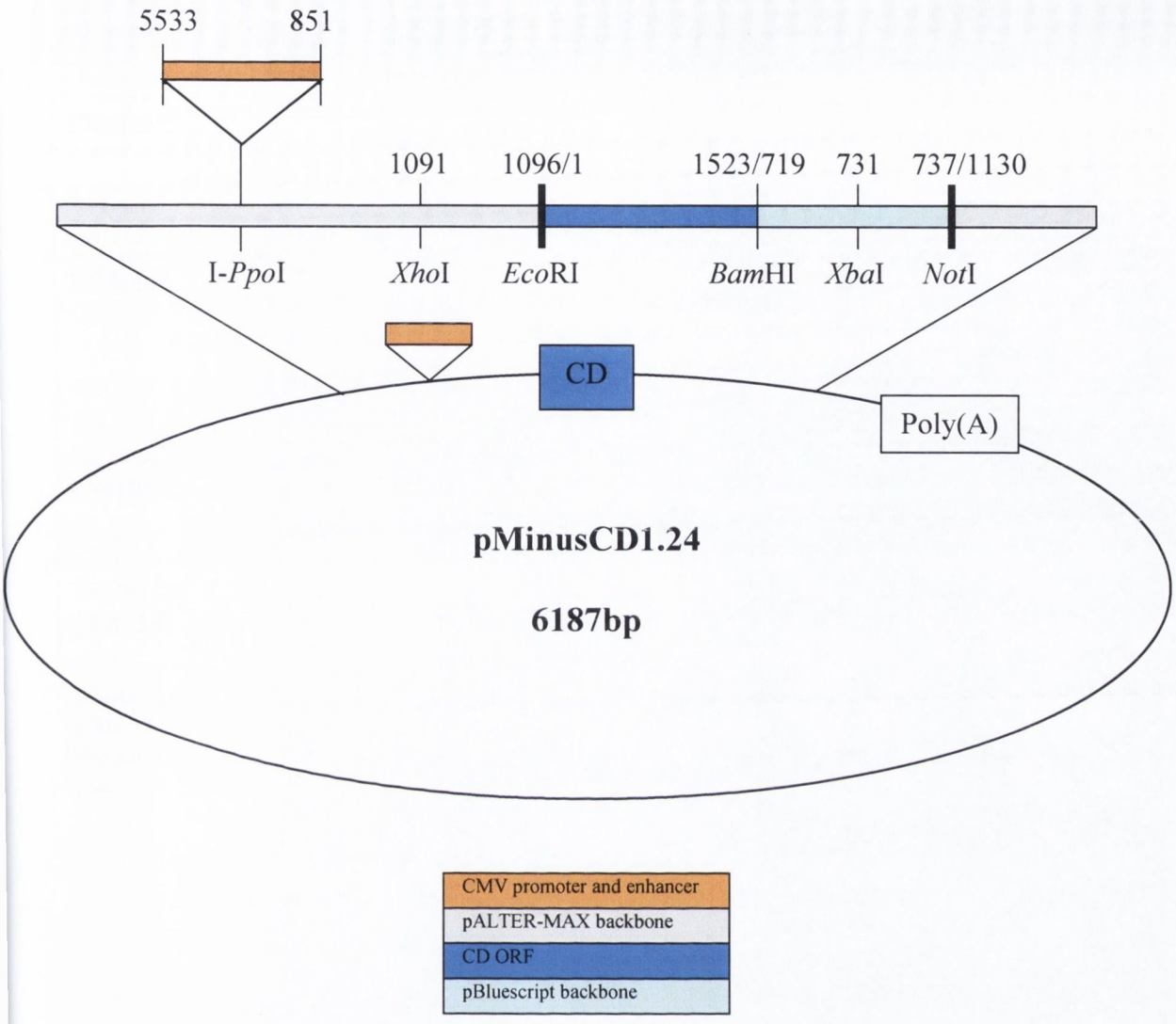

5528
852
1091
1096
1114
1130
BglII
I-PpoI
XhoI
EcoRI
XbaI
NotI
CMV-PE
Poly(A)
pALTER-MAX
5533bp
ampicillin
chloramphenicol
pALTER-MAX backbone
CMV promoter and enhancer


5533
851
1091
1096
1114
1130
I-PpoI
XhoI
EcoRI
XbaI
NotI
Poly(A)
pAMdPE1.1
4681bp
pALTER-MAX backbone
CMV promoter and enhancer


1114
1130/335
296
1-12
5143
5289
509
2081
5284
5831/689
701/1
1523/719
731
737/1130
XbaI
NotI
TA
BglII
BglII
HindIII
EcoRI
BamHI
XbaI
NotI
PSA-E
PSA-P
CD
Poly(A)
pPPE-CD2.15
8552bp
pALTER-MAX backbone
pCR2.1-TOPO backbone
5' end of oligonucleotide
PSA promoter
PSA enhancer
pBluescript backbone
CD ORF


5528
852
1091
1096/1
1523/719
731
737/1130
XhoI
EcoRI
BamHI
XbaI
NotI
CMV-PE
CD
Poly(A)
pCMV-CD1.10
7039bp

pALTER-MAX backbone
CMV promoter and enhancer
CD ORF
pBluescript backbone



CMV promoter and enhancer
pALTER-MAX backbone
CD ORF
pBluescript backbone

APPENDIX 6: COLONY COUNTS AFTER BACTERIAL TRANSFORMATIONS

Plasmid	pPSA1.3		pPSA2.5	
	Colonies	White colonies/ total colonies (%)	Colonies	White colonies/ total colonies (%)
PSA insert	19	74	11[1]	100
			3[1]	100
	1	100	2[2]	100
			0[2]	-
Control insert	500-2000	99	500-2000	99
	100-200	98	300-1000	100
Supercoiled pUC18	40	25	200	0
	4	100	20	100

Table A.1. Colony counts from TOP10 TA cloning transformations. 50µl and 10µl cells were plated for each sample. Total numbers of colonies per 90mm plate and blue/white screening results are shown. For the PSA promoter insert in pPSA2.5, cloning reactions were set up with 1µl[1] and 0.5µl[2] insert.

Plasmid	pPSA-CD1.8		pPSA-CD4.19	
Vector + insert	1:1	500-2000	1:1	0
		>2000		18
	1:5	500-2000	1:3	1
		>2000		2
	1:10	500-2000	1:6.5	4
		>2000		8
Vector only (treated as for vector + insert)	200-500		2	
	>2000		2	
Vector only No CIP treatment	Not done		200	
			400	
Linearised pUC18	500-2000		Almost lawn	
	Almost lawn		Almost lawn	
Supercoiled pUC18	50-100		400	

Table A.2. Colony counts from TOP10 transformations. Vector: insert molar ratios are shown in columns two and five, otherwise figures refer to colonies per 90mm plate. 30µl and 150µl cells were plated, except for supercoiled pUC18, where 10µl cells were plated. "Almost lawn" refers to plates on which growth was too dense to distinguish individual colonies but not a complete lawn of bacteria. 1:3 and 1:6.5 ratios of vector to insert were used for PPSA-CD4.19 instead of 1:5 and 1:10 due to difficulty obtaining sufficiently concentrated insert.

Plasmid	pPPE-CD2.15	pCMV-CD1.10	pMinus-CD1.24
Vector + insert	11	24	1400
1:1	0	0	21
Vector + insert	13	1	850
1:5	0	2	12
Vector + insert	2	0	540
1:10	0	1	4
Vector only	5	1	1350
(treated as for vector + insert)	0	1	42
Vector only	1332	Not done	Not done
No CIP treatment	131		
Ligation positive control	36	0	8
	2	0	0
Transformation positive control	Almost lawn	12	2000
	784	0	147
Cells only	Lawn	Almost lawn	Almost lawn

Table A.3. Colony counts from JM109 transformations. 100µl and 10µl cells were plated for each reaction, except for the cells-only reaction, where 100µl cells were plated. "Almost lawn" refers to plates on which growth was too dense to distinguish individual colonies but not a complete lawn of bacteria.

Plasmid	Volume plated (μl)	Colonies
pALTER-MAX (CMV promoter deleted, blunt ended)	20	0
	100	4
Linearised pUC18	20	500
	100	1000
Supercoiled pUC18	20	1000
	100	Almost lawn
Cells only	20	Lawn
	100	Lawn

Table A.4. Colony counts from pAMdPE1 transformation following blunt-end ligation. "Almost lawn" refers to plates on which growth was too dense to distinguish individual colonies but not a complete lawn of bacteria.

Cells	mut155G		mut158G	
	ng plasmid	Colonies	ng plasmid	Colonies
JM109/PSA mutagenesis	5	125[2]	5	840[1]
	10	250[2]	1	280[1]
JM109/control mutagenesis	5*	440[2] (92-98% white)	5	15[1] (100% white)
JM109/pGEM	10	1625[2]	1	670[1]
BMH71-18*mutS* /pGEM	0.1	1425[2]	0.1	1700[2]

Table A.5. Colony counts from transformations during mutagenesis. Colonies were counted on one[1] or two[2] plates, each plated with 100μl cells. Average colonies are shown where two plates were used. *Results for the control mutagenesis reaction carried out alongside mut155G mutagenesis were obtained after the main series of JM109 transformations, as 5ng and 10ng DNA initially failed to yield any colonies. Probably the medium used (M9 agar supplemented with X-Gal, IPTG, ampicillin and antibiotic selection mix) was faulty. Plasmid DNA subjected to mutagenesis with the control lacZ mutagenic oligonucleotide, miniprepped from BMH71-*18mutS* cells alongside plasmid treated with mut155G, was re-transformed into JM109 cells and plated on LB agar supplemented with the same reagents to give the colony counts above. Colony counts for PSA mutagenesis refer to annealing reactions at 87.7°C and 76.3°C for mut155G and mut158G respectively, since these reactions yielded the desired mutants.

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