Development of a gene therapy strategy for prostate cancer

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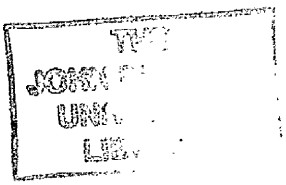

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Abstract

A number of studies have shown that controllable or tissue specific promoters can be used to selectively express foreign genes in tumour cells. However, to date, the use of this strategy to deliver therapeutic genes to a specific tissue (or tumour) has had limited success. One of the main problems is the inability of most if not all tissue specific promoters to express the therapeutic gene at sufficiently high levels and for sufficient time to have therapeutic value. Recently, a molecular switch has been developed providing a solution to this problem. In this system, a tightly controlled or highly specific promoter is used to drive low level expression of Cre recombinase. Once expressed, Cre is able to excise a silencing cassette inserted between a highly active promoter and a therapeutic gene. This enables a persistent and high level of expression of the gene in a controllable or tissue specific manner. The aim of this project is to identify either a controllable or cell-specific promoter able to drive the molecular switch producing therapeutic levels of a pro-drug activating enzyme, principally HSVtk, within prostate cancer. Initially, the investigation focussed on the use of various radiation responsive promoter constructs for use in the molecular switch. Four synthetic and one wild-type radiation responsive promoter constructs were used to drive the expression of the reporter gene, GFP. The results of these experiments showed that even in the absence of radiation, each of the constructs tested were constitutively active, i.e. no increase in the expression levels of GFP was observed after irradiation, even under growth conditions involving low oxygen or low serum. To test the suitability of prostate specific promoters a total of seven promoter enhancer constructs were tested in both prostate and non prostate cell lines. In LNCaP cells each of the constructs were responsive to androgen treatment showing a fold increase in GFP expression of between 34 and 276. Two PSA enhancers coupled to either a DD3 or PSA promoter were then used to drive the molecular switch. Due to problems consistently transfecting LNCaP cells with the two molecular switch vectors and the insensitivity of LNCaP cells to HSVtk activated GCV, prostate specific activation of the molecular switch has yet to be determined. Nevertheless, the molecular switch was proved to be functional in PC-3 and H460 cells, allowing for future developments of this strategy.

Declaration

Nor portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institutes of learning.

Copyright

Abbreviations

5-FC	5-fluorocytosine
5-FdUMP	5-fluorodeoxyuridine-5′-monophosphate
5-FdUTP	5-fluorodeoxyuridine-5′-triphosphate
5-FU	5-fluorouracil
5-FUTP	5-fluorouridine-5′-triphosphate
ACV	Acyclovir
AFP	α-fetoprotein promoter
Amp^r	Ampicillin resistance
AP1	Activated protein-1
APS	Ammonium persulfate
AR	Androgen receptor
ARE	Androgen response element
ARR	Androgen responsive region
ATCC	American Tissue Culture Collection
BGH	Bovine growth hormone
bp	Basepair
BSA	Bovine serum albumin
BVDU	(e)-5-(2-bromovinyl)-2′-deoxyuridine
CAG	Beta-actin promoter
CB1954	5-(1-Aziridinyl)-2,4-dinitrobenzamide
CD	Cytosine deaminase
CEA	Carcinoembryonic antigen promoter
CMV	Cytomegalovirus IE enhancer-promoter
Cre	Cre recombinase
CyFP	Cyan fluorescent protein
DD3	Differential display code 3
DHT	Dihydrotestosterone
DMEM	Dulbecco's Modification of Eagles Medium:F12
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DT-diaphorase	NADPH dehydrogenase
DTT	1,4- dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylenediamine tetra acetic acid
Egr-1	Early growth response gene-1
Epo	Erythropoietin

ERK	Extracellular signal-related kinases
FACS	Fluorescence activated cell sorter
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FIAU	2′-fluoro-2′-deoxy-1-β-D-arabinofuranosyl-5-iodo-uracil
GDEPT	Gene directed enzyme prodrug therapy
GFP	Green fluorescent protein
GCV	Gancyclovir
Hams-F12	Hams nutrient medium F12
HIF-1	Hypoxia inducible factor
HIS	Histidine
hK2	Human kallikrein 2
HRE	Hypoxia regulatory elements
HRP	Horseradish peroxidase
HRPC	Hormone refractory prostate cancer
HSVtk	Herpes simplex virus thymidine kinase
IAA	Indole acetic acid
IE	Immediate early
IPTG	Isopropyl β-D-1-thiogalactopyranoside
JNK	c-Jun amino-terminal kinase cascade
Kan	Kanamycin resistance
kb	Kilobase
kDa	Kilodalton
LB	Luria-Bertani
LoxP	Locus of crossing over
MAPK	Mitogen activated protein kinase
MCS	Multiple cloning site
MOI	3-methylene-2-oxinodole
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADPH	Nicotine adenine dinucleotide phosphate
neo	neomycin phosphotransferase
NFκB	Nuclear factor κB
NTR	Nitroreductase
OD	Optical density
ORF	Open reading frame
OSC	Osteocalcin
PAGE	Polyacrylamide gel electrophoresis
PB	Probasin
PBS	Phosphate buffered saline

PCV	Pencyclovir
PET	Positron emission tomography
PGK1	Phosphoglycerate kinase-1
PMSF	Phenylmethylesulfonylflouride
PolyA	polyadenylation stop signal
PS	Precision plus protein dual colour standard
PSA	Prostate specific antigen
PSE	Prostate specific antigen enhancer
PSMA	Prostate specific membrane antigen
RIT	Radioimmunotherapy
RNA	Ribonucleic acid
ROI	Reactive oxygen intermediates
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
SAPK	Stress-activated protein kinase
SDS	Sodium dodecylsulphate
SE	Standard error
siRNA	Short interfering RNA
Sp1	S protein-1
SRE	Serum response element
SRF	Serum response factor
TEMED	N,N,N',N'-Tetramethylethylenediamine
TG	Thyroglobulin
Tm	Melting temperature
TNFα	Tumour necrosis factor α
TNM	Tumour, nodes and metastases
UV	Ultra violet
VCV	Valacyclovir
VEGF	Vascular endothelial growth factor
WT	Wild-type
X-Gal	5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside

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Chapter 1

1.0 Introduction

1.1 Prostate cancer – where are we now?

The prostate is a gland; present only in males and located just below the bladder. Its function is to produce nutrients for the sperm in semen and to regulate its viscosity. It also produces the prostaglandin hormones. The prostate is divided into peripheral, central and transitory zones with ducts and acini lined by an epithelial sheet. The epithelium, consisting of a bi-layer of basal cells beneath secretory luminal cells interspersed with neuroendocrine cells, is surrounded by fibromuscular stroma. The basal cells are mainly androgen receptor (AR) negative stem cells which differentiate into AR-positive luminal cells and the AR-negative neuroendocrine cells. The growth, differentiation and maintenance of the prostate is closely controlled by androgens, mainly testosterone (Mason 2003; Kopper and Timar 2005).

The development of prostate cancer is a multi-step process through a series of morphologically distinct lesions initiated by genetic and epigenetic changes. These lesions, known as high grade prostatic intraepithelial neoplasia (PIN), are characterised by the proliferation of the luminal epithelium within the acini and ducts of the peripheral zone of the prostate. PIN lesions are frequently seen in the prostate of men in their 30's and it is thought that these lesions slowly progress to malignant prostate cancer over decades. PIN is present in more then 85% of cases of prostate cancer. Prostate tumour growth is largely dependent on the male sex hormone, testosterone. Interestingly, men who underwent castration before puberty and those with congenital abnormalities in androgen metabolism, do not develop prostate cancer. Moreover, androgen deprivation by 5α-reductase inhibitors is an effective method of treating early stage cancer (DeMarzo *et al.,* 2003; Rubin and De Marzo 2004; Calvo *et al.,* 2005; Kopper and Timar 2005). In the UK male population, death caused by prostate cancer is second only to that of lung cancer. In 2003 there were 10,164 deaths from prostate cancer; this accounts for approximately 13% of the deaths from cancer in males. In men aged 85 and over, this increases to 25% of all the male deaths from cancer making prostate cancer the most common cause of cancer deaths in this age group.

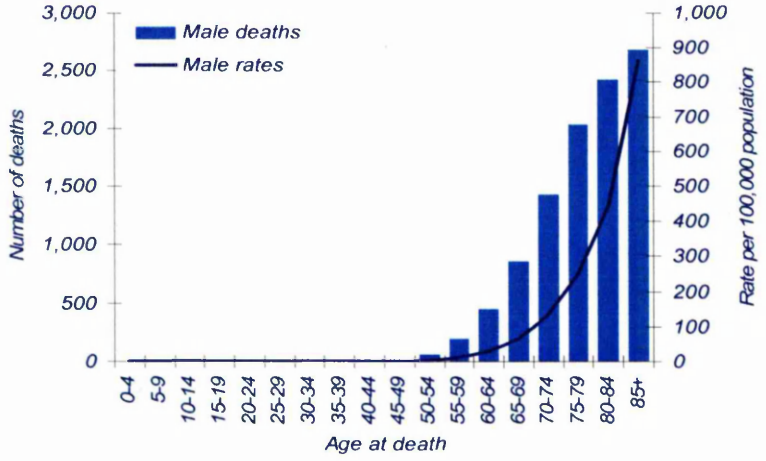
The risk of being diagnosed with prostate cancer is strongly related to age; very few new cases are diagnosed in men under 50 and more then 63% of cases occur in men over 70 years old (figure 1.1A). The largest number of cases is diagnosed in the 70-74 and 75-79 age groups (figure 1.1B). It is estimated that 33% of men over 50 have a small cancer in the prostate, rising to 50% by the age of 80. However, only 1 in 25 men (4%) will die from this

disease. This means that men are more likely to die *with* prostate cancer than *from* it. Over the last 30 years, prostate cancer mortality rates have steadily increased, reaching a peak in the early 90's with a slight fall off until the end of the century. However, the last six years have seen an up turn in the death rates. Since the start of data collection, the incidence has increased dramatically (figure 1.1C), mainly due to the increased use of the prostate specific antigen (PSA) test to screen for early prostate cancer. (These data are from Cancer Research UK http://info.cancerresearchuk.org, the Prostate Cancer Charity http://www.prostate-cancer.org and the Office for National Statistics http://www.statistics.gov.uk).

In addition to age, both hereditary and environmental factors can contribute to the development of prostate cancer. Family history is a strong and consistent risk factor. A man with a first degree relative with prostate cancer has a risk of 2, with a second-degree relative a risk of 1.4 and with a first and second degree relative an 8.8 relative risk of developing prostate cancer. In addition, the risk of prostate cancer is increased 1.4 times when a man is a first or second degree relative of a woman affected with breast cancer. Hereditary is usually defined as a degree as no associated genes have yet been firmly identified. Some prostate cancer susceptibility loci have been identified, including Xq27-28 and 20q13, and some candidate genes include the breast cancer susceptibility gene BRCA1 (17q21) and BRCA 2 (13q12.30) which confer a risk of prostate cancer of 3.0 and 2.6-7.0 respectively. However, the problem of studying familial prostate cancer is that as the incidence of the disease in the genetic population is so high there is a high rate of sporadic cases amongst the familial cluster. In addition, confirmatory studies show weak or no linkage between these regions suggesting that hereditary prostate cancer is a heterogeneous disease for which no single gene is responsible for the high incidence in certain families (Rubin and De Marzo 2004; Bott *et al.,* 2005; Cancel-Tassin and Cussenot 2005; Kopper and Timar 2005).

Figure 1.1 UK Prostate cancer statistics. A) The number of deaths and age specific mortality rate per 100,000 people from prostate cancer in 2003. B) Number of new cases diagnosed with prostate cancer in 2003 C) Incidence and mortality rates of prostate cancer in the UK over the period 1971 to 2003. Data by Cancer Research UK (http://info.cancerresearchuk.org) and the Office for National Statistics (http://www.statistics.gov.uk/).

A)



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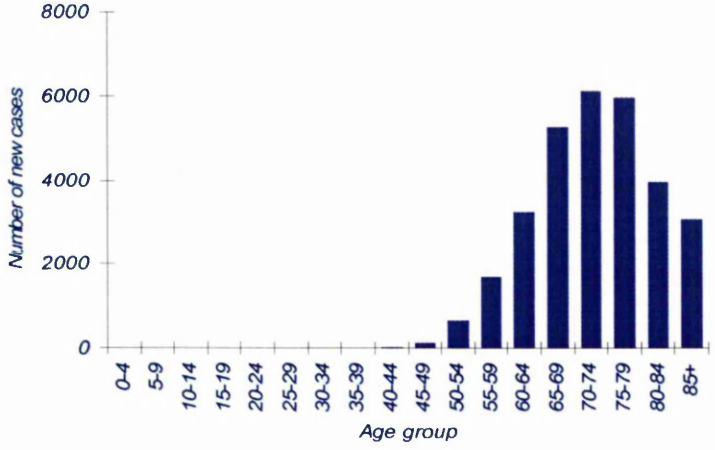

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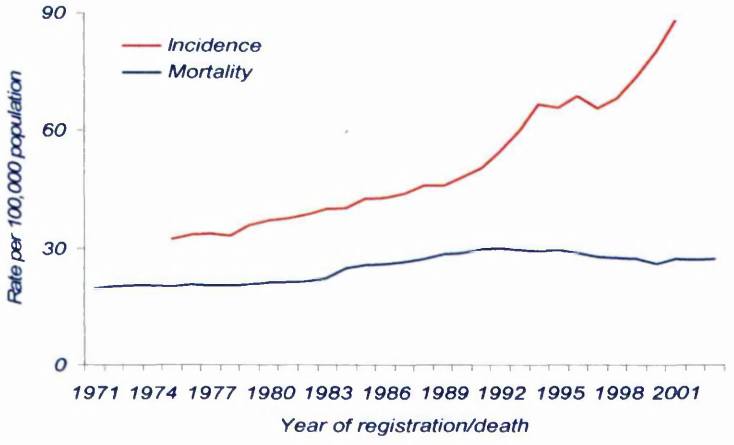


Environmental factors are clearly involved as well. Although the exact exposures that increase prostate cancer risk are unclear; diet, industrial chemicals, sexually transmitted disease and chronic prostatitis have been implicated to varying degrees. In particular, dietary fat, mainly from red meat and dairy products, may be responsible for the higher prostate cancer risk in the western world. The association between dietary fat and prostate cancer stems from the observation that men who consume less fat have lower testosterone levels suggesting that fat may be affecting androgen levels. In addition, the breakdown of fatty acids within the prostate generates hydrogen peroxide, which may be a source of carcinogenic oxidative damage to prostate DNA. In contrast, vegetarian diets, diets rich in vitamin E and selenium (an essential trace element found in grains, fish and meat), soybean foodstuffs containing isoflavones and lycoprene (present in raw and processed tomato products) may reduce the risk of prostate cancer (Crawford 2003; Rubin and De Marzo 2004; Freedland and Aronson 2005; Sonn *et al.,* 2005).

1.2 Diagnosis and treatment

Broadly speaking there are three different types of prostate cancer staged using the tumour, nodes and metastases (TNM) system (Schroder *et al.,* 1992). T1 and T2 tumours are confined within the prostate and are defined as 'early' disease. When the tumour invades beyond the prostate gland to surrounding tissue it is known as 'locally advanced' disease and is staged as T3. T4 tumours are those that have spread to remote regions of the body, mainly bones and lymph nodes; this is called metastatic cancer. Metastatic cancer is further classified using the scales N0 to N3 to describe the extent of spread to the lymph nodes and M0, M1, M1a, M1b and M1c to represent the spread of metastasis around the body. Tumours are also graded according to the histological pattern of arrangement of carcinoma cells using the Gleason system. The Gleason grading system is the most frequently used grading system for prostate cancer as it takes into account both the most predominant (primary) pattern of cancer and that of the second most predominant (secondary) pattern, thus taking into account the considerable heterogeneity of most prostate cancers. The primary and secondary patterns are identified and each is graded 1 (most differentiated) to 5 (least differentiated) and the two grades are added together. If only one grade is in the tissue sample then the grade is multiplied by two. The Gleason grade, sometimes called score, thus ranges from 2 to 10. In general, the lower the grade the less likely the tumour is going to progress and the better the prognosis (Gleason 1966, 1992; DeMarzo *et al.,* 2003; Humphrey 2004).

If prostate cancer is caught early in its development, the prognosis is very good. Unfortunately, despite an increasing trend towards early detection of prostate cancer, about

half the men who are diagnosed with prostate cancer are diagnosed at a late stage when the disease is less curable. Although there is no consensus on the best way of treating prostate cancer, the management options for localized prostate cancer include radical prostatectomy, radiotherapy (brachytherapy, external beam and conformal), and watchful waiting (Mason 2003; Moul *et al.,* 2003; Norderhaug *et al.,* 2003; Shaffer and Scher 2003). For younger patients (70 and under), radical prostatectomy is the most common treatment followed by radiotherapy and watchful waiting. However, older patients will, in general, undergo watchful waiting. This is based on the premise that elderly patients will have a relatively short life expectancy and that their prostate cancer is likely to progress very slowly, may not cause symptoms and may not be the cause of their death. While the ten year survival for all four treatment options is 65-90%, these curative options have significant side-effects including urinary incontinence, bowel problems and erectile dysfunction (Mason 2003; Moul *et al.,* 2003).

Locally advanced cancer is a more serious condition. Because it has progressed outside the prostate gland, surgery is unlikely to remove the entire tumour and there is a higher risk of developing metastasis at a later date. Treatment options are therefore restricted to radiotherapy and/or testosterone deprivation (Skala *et al.,* 2005). While androgen deprivation does not usually eradicate the tumour it can keep the tumour under control for some time, maybe for some years. For patients with metastatic cancer there is as yet no curative option. The most important consideration is to improve or maintain the quality of life. This is usually done through androgen deprivation therapy, radiotherapy and chemotherapy (Higano 2005; Skala *et al.,* 2005).

At all stages of prostate cancer, the use of androgen deprivation therapy can lead to the development of hormone refractory prostate cancer (HRPC), characterised by disease progression and metastases. HRPC is an incurable disease from which most men will die within a relatively short period. Current treatment options include chemotherapy, radiotherapy and further hormone manipulation; however these provide palliative benefits at best. Most treatment is therefore focussed on improving the quality of life of the patient (Feldman and Feldman 2001; Clarke 2003; Gulley and Dahut 2003; Shaffer and Scher 2003; Sternberg 2003; Clarke and Wylie 2004; Berry 2005; Petrylak 2005).

These facts highlight two main problems; a need to detect prostate cancer early and methods for effectively treating localised and metastatic cancer without significant side effects. This project aims to tackle the second problem through a gene therapy approach in which

controllable or prostate specific promoters drive the expression of a therapeutic gene. Promoters that generate high levels of expression are rarely controllable or tissue specific, however controllable or tissue specific promoters express target genes at levels below therapeutic effectiveness. A solution to this would be to create a molecular switch in which a tightly controlled or highly specific promoter is used to drive the expression of Cre recombinase (Cre). The low levels of Cre protein produced result in the excision of a silencing cassette inserted between a highly active promoter and a therapeutic gene enabling high levels of the therapeutic gene to be expressed in a controllable/tissue specific manner. The following sections will describe this molecular switch and then examine the features of the controllable or tissue specific promoters and the therapeutic genes that would be employed in this treatment strategy.

1.3 Cre-*LoxP* recombination

A number of bacterial and yeast genes encode recombinase enzymes that recognise certain DNA sequences and catalyse site specific DNA rearrangements. In particular, widespread use has been made of the bacteriophage P1 Cre recombinase which catalyses reciprocal recombination at a specific locus of crossing over (*loxP*) with no requirement for accessory proteins. This Cre-*loxP* system requires two well-characterised components, the 38kDa recombinase protein, Cre, and the 34-bp *loxP* target sequences. The *loxP* sequence is composed of two 13-base-pair (bp) inverted repeats separated by an 8-bp spacer region. Cre binds cooperatively to the *loxP* sites, with one Cre monomer contacting each of the two 13-bp inverted repeats, and catalyses precise recombination between the asymmetric 8bp core regions of two 34-bp *loxP* target sequences (see figure 1.1). Recombination between two parallel (directly repeated) *loxP* sites as defined by the core region, in for example, a plasmid, results in the excision of the intervening sequence, producing two recombination products each containing one *loxP* site, whereas recombination between anti-parallel sites inverts the bracketed fragment (see figure 1.2) (Sauer and Henderson 1989; Kilby *et al.*, 1993; Snaith *et al.*, 1995; Gorman and Bullock 2000; Ghosh and Van Duyne 2002). Since the Cre-*loxP* system functions reversibly in both bacteria and eukaryotic cells it has been extensively exploited for the excision and/or integration of fragments into cellular and viral genomes and for the control of tissue specific gene expression, or deletion of genes to produce conditional knockout mice (Sauer and Henderson 1989; Lakso *et al.*, 1992; Anton and Graham 1995; Stricklett *et al.*, 1999; Tronche *et al.*, 2002; Van den Plas *et al.*, 2003; Leow *et al.*, 2005). More recently its potential to be used as a molecular switch to enhance the efficacy of tissue specific gene therapy has been explored.

Figure 1.1 Cre recombinase binding to a single *loxP* site. Cre recombinase (Cre) specifically recognises the *loxP* site and a Cre monomer binds to each of the two 13-base-pair (bp) inverted repeats (black) that are separated by an 8-bp spacer region (red). Recombination results in cleavage and crossing over in the 8-bp spacer region.

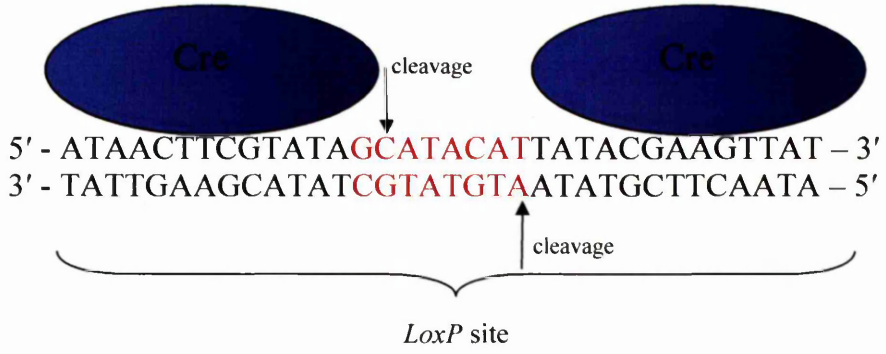
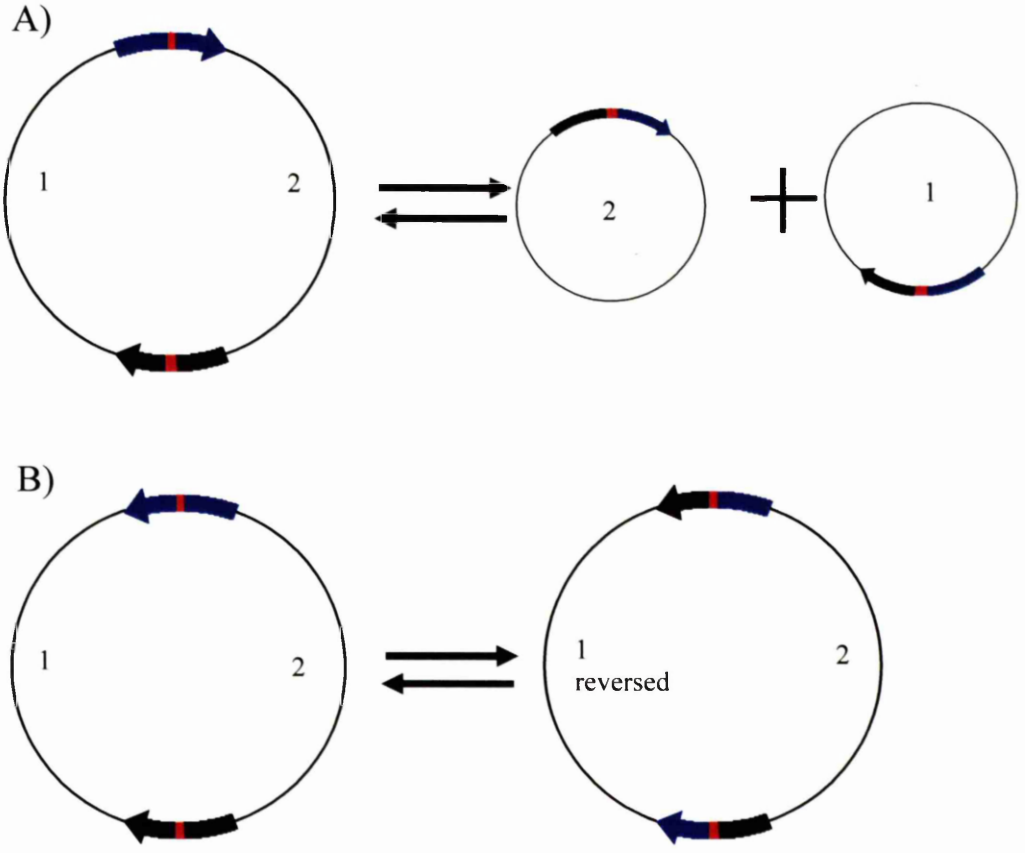


Figure 1.2 Cre mediated recombination. The *loxP* sites are represented in two colours, black and blue, with the 8-bp spacer region in red. A) Recombination between two parallel loxP sites leads to the excision of the intervening DNA and the creation of two plasmids each containing one *loxP* site that is a combination of *loxP* blue and *loxP* black. The reaction is reversible and so two monomers can recombine to form a co-integrate. B) Recombination between two anti-parallel *loxP* sites inverts the bracketed fragment (1).

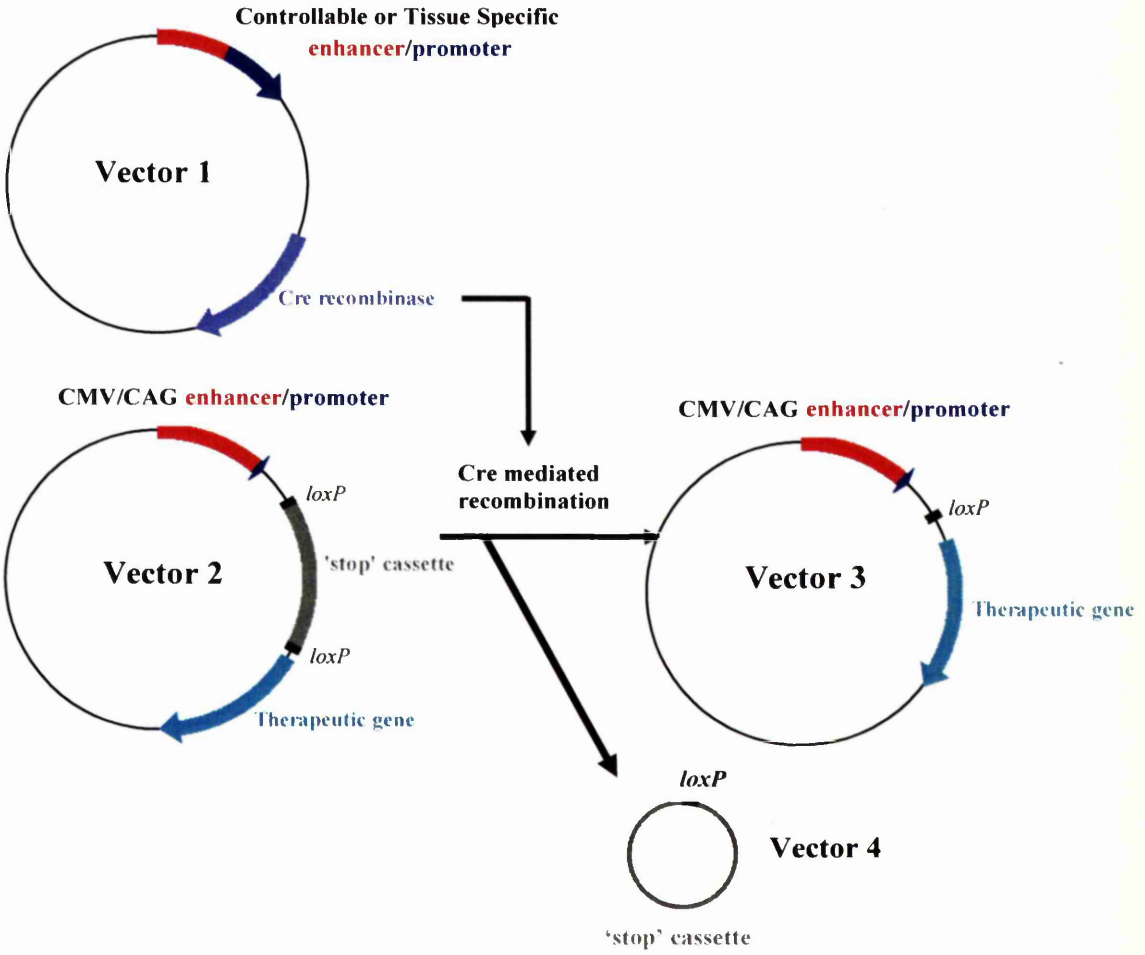


1.4 Exploitation of the molecular switch in gene therapy

Controlled or tissue specific gene expression is crucial to achieving success in suicide gene therapy. However, the expression levels from such promoters are generally low. Powerful promoters that enable much higher levels of therapeutic gene expression are not tissue specific and would thus not differentiate between normal and tumour cells. Several groups have therefore developed molecular switches that exploit the Cre-*loxP* system to enhance therapeutic gene expression levels while maintaining tissue specificity. The molecular switch consists of a controllable/tissue specific promoter which conditionally controls the expression of the Cre recombinase gene. Cre recombinase then activates a 'silenced' expression cassette consisting of a strong promoter (e.g. Cytomegalovirus immediate early (IE) promoter-enhancer; CMV) upstream of a tumour sensitising gene from which expression is silenced by an intervening 'stop' cassette flanked by parallel *loxP* sites. Recombination at the loxP sites results in the removal of the stop cassette and activation of transcription (figure 1.3).

The first switch system to be developed for therapy used the hepatocarcinoma-specific α-fetoprotein (AFP) promoter to drive Cre recombinase expression from one vector, and the potent CMV enhancer combined with the beta-actin promoter (CAG) was used to drive LacZ expression in a second vector. A double transfection of the two vectors led to a 50 fold enhancement in gene expression compared to using the AFP promoter alone while maintaining strict specificity to AFP producing cells (Sato *et al.*, 1998). Substitution of the AFP promoter in the molecular switch with the carcinoembryonic antigen (CEA) promoter (Ueda *et al.*, 2000) and radiation responsive CArG (Scott and Marples 2000; Scott *et al.*, 2000) elements have also been shown to induce similar fold induction in reporter gene expression. Subsequently, the tissue specific promoters; AFP, CEA, thymoglobulin (TG), prostate specific antigen (PSA) enhancer/promoter, prostate specific membrane antigen (PSMA) enhancer/promoter and the radiation responsive CArG elements, have all been used successfully in a molecular switch to enhance the expression of the tumour sensitising genes; herpes simplex virus thymidine kinase (HSVtk) and cytosine deaminase (CD), leading to between 3 and 300 fold increase in cell death compared to using the promoters alone (Kijima *et al.*, 1999; Nagayama *et al.*, 1999; Sakai *et al.*, 2001; Ueda *et al.*, 2001; Ikegami *et al.*, 2002; Marples *et al.*, 2002; Yoshimura *et al.*, 2002; Ikegami *et al.*, 2004).

Figure 1.3 Cre/loxP switch scheme. Cre recombinase is under the control of a controllable or tissue specific enhancer/promoter (vector 1). The constitutively active CMV or CAG promoter drives the expression of the therapeutic gene; however, gene expression is silenced by an intervening 'stop' cassette flanked by parallel loxP sites (vector 2). Cre mediated recombination between the two loxP sites excises the 'stop' cassette from vector 2 generating vector 3, which expresses the therapeutic gene under the control of the CMV/CAG enhancer/promoter and vector 4 containing the 'stop' cassette and a single loxP site.



1.5 Promoter-capture gene therapy

1.5.1 Radiation responsive elements

Ionising radiation generates reactive oxygen intermediates (ROI) within cells that directly damage DNA leading to growth arrest, which in turn may activate DNA repair mechanisms (Datta *et al.*, 1993; Hallahan 1996). Recently radiation has received considerable interest in the gene therapy field as the ROI produced lead to the activation of certain immediate-early genes, such as the c-jun, early growth response gene-1 (Egr-1), c-fos and nuclear factor κB (NFκB) gene families (Prywes *et al.*, 1988; Sherman *et al.*, 1990; Hallahan *et al.*, 1991b; Datta *et al.*, 1992; Weichselbaum *et al.*, 1994a). The Egr-1 gene, also known as *zif*/268, TIS-8, NFG1-A and K*rox*-24, encodes a 533-amino acid nuclear phosphoprotein that contains three tandem-repeat Cys2-His2 zinc-finger motifs. The EGR-1 protein binds to the DNA sequence CGCCCCCGC in a zinc-dependent manner to regulate the transcription of other genes encoding growth factors, such as basic fibroblast growth factor (FGF), and cytokines, for example tumour necrosis factor α (TNFα), to repair radiation induced damage of tissues (Hallahan *et al.*, 1991b; Datta *et al.*, 1992; Datta *et al.*, 1993; Weichselbaum *et al.*, 1994a; Weichselbaum *et al.*, 2002).

There are many regulatory elements in the full length Egr-1 promoter (700bp), including two S protein-1 (Sp1) sites, an activated protein-1 (AP1) site, two cAMP response elements and an Egr-1 binding site (Christy and Nathans 1989; Sakamoto *et al.*, 1991; Schwachtgen *et al.*, 2000). However, activation of Egr-1 in response to radiation occurs by the binding of Elk1, in concert with other transcription factors, mainly p68/serum response factor (SRF), to CC $(A/T)_6GG$ (CArG) domains (Rolli *et al.*, 1999; Meyer *et al.*, 2002). There are five CArG domains within the Egr-1 promoter, present in the region -404 to -71 (Christy and Nathans 1989; Sakamoto *et al.*, 1991; Schwachtgen *et al.*, 2000), and for gene therapy purposes, a core promoter of 490bp (nucleotides -425 to +65 relative to the putative transcription start) has been described to be sufficient for radio-activation (Weichselbaum *et al.*, 1994b; Hallahan *et al.*, 1995a). Although the precise activation mechanism of the CArG elements has not been fully elucidated, there is growing evidence that both a protein kinase C dependent pathway and mitogen activated protein kinase (MAPK) pathways, probably through extracellular signal-related kinases 1 and 2 (ERK1/2) and c-jun amino terminal-kinase and stress-activated protein kinase (JNK/SAPK), are involved (Joseph *et al.*, 1988; Jamieson *et al.*, 1989; Hallahan *et al.*, 1991a; Hallahan *et al.*, 1991b; Adler *et al.*, 1995;

Garrington and Johnson 1999; Rolli *et al.*, 1999; Schaeffer and Weber 1999; Meyer *et al.*, 2002; Quinones *et al.*, 2003).

CArG elements are not only activated by irradiation, they were originally identified as highly conserved motifs within serum response elements (SRE) to which SRF binds in response to a variety of extracellular signals, in particular serum and growth factors (Treisman 1985; Gilman *et al.*, 1986; Treisman 1986, 1987; Qureshi *et al.*, 1991; Alexandropoulos *et al.*, 1992; Croissant *et al.*, 1996; Soulez *et al.*, 1996; Spencer and Misra 1996; Spencer *et al.*, 1999a; Spencer *et al.*, 1999b; Spencer and Misra 1999). Activation of CArG elements by SRF is thought to occur through the p38MAPK/SAPK2 pathway as opposed to the ERK1/2 and SAPK/JNK pathways that are activated in response to irradiation and in response to chemotherapeutic drugs and DNA damaging agents, such as cisplatin (Meyer *et al.*, 2002; Quinones *et al.*, 2003; Greco *et al.*, 2005b).

The radiation-responsive CArG domains within the Egr-1 promoter have been exploited experimentally to activate transcription of downstream target genes in response to irradiation. One such strategy involved the use of a truncated Egr-1 promoter containing 5 CArG elements to activate the radiosensitising and tumouricidal cytokine, tumour necrosis factor α (TNFα), in human epithelial tumour xenografts in nude mice. A reduction in tumour volume was observed without an increase in normal tissue damage, representing a novel method of localised and temporal regulation of gene-based medical treatment (Weichselbaum *et al.*, 1992; Weichselbaum *et al.*, 1994b; Hallahan *et al.*, 1995a; Mauceri *et al.*, 1996; Weichselbaum *et al.*, 2002). Due to the success of these preliminary studies, TNFα combined with radiotherapy has now entered phase I clinical testing (Hallahan *et al.*, 1995b; Sharma *et al.*, 2001; Mundt *et al.*, 2004; Senzer *et al.*, 2004). While these studies used high does of radiation, almost exceeding clinical limits (20-50Gy), other studies have been conducted in which the Egr-1 promoter was used successfully to drive the expression of reporter genes and sensitise cells to the effects of the therapeutic genes; HSVtk and CYP4B1, in response to considerably lower doses of radiation (2-10Gy) (Takahashi *et al.*, 1997; Manome *et al.*, 1998; Kawashita *et al.*, 1999; Hsu *et al.*, 2003).

The studies described above only induced low level gene expression for a limited period of time, thus potentially reducing the effectiveness of such a treatment. Therefore, Marples *et al.*, (2000; 2002) and Scott *et al.*, (2002), developed synthetic radiation responsive enhancers consisting of between 4 and 12 directly repeating CArG elements and the CMV minimal promoter. It was found that increasing both the number and arrangement of CArG elements

enhanced transcription levels of the reporter gene, green fluorescent protein (GFP), above the Egr-1 wild type promoter, in MCF-7 breast adenocarcinoma cells and U87-MG glioma cells, but only to a maximum of 9 consecutive CCTTATTTGG sequences. Since CArG elements are functionally interchangeable with serum response elements (SRE), subsequent studies found that altering the core A/T sequences to ATATAA led to a greater positive response as SRF has a higher affinity for ATATAA then the previously used TTATTT sequence (Scott *et al.*, 2002). However, altering the spacing between the CArG elements had little effect and neither did the addition of an Sp-1 transcription factor binding site, a factor previously seen to enhance self-regulation of SRF in the native EGR-1 gene promoter (Spencer and Misra 1996; Scott *et al.*, 2002). So far the CArG elements have been exposed to a 60 Cobalt γ-ray external source producing maximal expression of a downstream reporter gene after 5 Gy, which is comparable to 5 doses of 1 Gy (Marples *et al.*, 2000). At present, patient treatments usually consist of some 30 or more daily 2 Gy exposures. These CArG elements thus offer an advantage over truncated promoters as lower doses of radiation can be used to activate the downstream therapeutic genes.

Although radiation responsive promoters have yet to be tested in the prostatic environment, the prostate is an ideal organ for radiotherapy. Considerable advances have been made in delivering radiotherapy, either as external beam radiation, in which a high dose of x-rays are given directly to the prostate gland, or as brachytherapy, in which radioisotopes emitting short range radiation are implanted directly into the tumour, while limiting the damage to surrounding tissues (Mason 2003; Norderhaug *et al.*, 2003). However, radiation is never 100% specific to the target tissue and damage to normal tissues occurs, possibly leading to long-term side effects. Interestingly, it has been possible to activate the radiation inducible Egr-1 promoter using radioisotopes, such as iodine-125 and Ga-67-citrate (Takahashi *et al.*, 1997; Manome *et al.*, 1998). Such radioisotopes could be used to label monoclonal antibodies specific for the target tissues, in a radioimmunotherapy (RIT) context, thus overcoming the problem of radiation dose limiting toxicity induced by damage to surrounding normal tissues (Goldenberg 1993; Essand *et al.*, 1995; Essand *et al.*, 1996; Rydh *et al.*, 1997; O'Donnell *et al.*, 1998; Rydh *et al.*, 1999; Britton *et al.*, 2000; O'Donnell *et al.*, 2000; Carter 2001; Rydh *et al.*, 2001; O'Donnell *et al.*, 2002).

1.5.2 Prostate specific promoters

An alternative to using radiation responsive promoters to restrict effective gene expression to the target tissue would be to use tissue specific promoters. An ideal prostate specific promoter needs to have sufficiently strong transcriptional activity and a high degree of tissue

or tumour specificity. In the prostate, many promoters are active in normal tissue but are up-regulated in benign and malignant tissues. This level of specificity is acceptable as treatment is generally necessary only for the over 60's at which stage the prostate is no longer an essential organ (Satoh *et al.*, 2005). To date, many promoters have been investigated but they often display a lack of activity, specificity or both. Discussed below and listed in table 1.1 are some that still remain promising.

Prostate specific antigen (PSA)

PSA is expressed exclusively in normal, hyperplastic and malignant prostatic epithelium. The highly tissue specific expression pattern of PSA is due in part to its transcriptional regulation by androgen via the androgen receptor (AR). The serum level of PSA is a useful clinical marker for the diagnosis and assessment of prostate cancer as it is proportional to tumour volume and correlates positively with the clinical stage of the disease. Androgen ablation therapy of prostate cancer leads to a reduction in cancer progression and in serum PSA levels (Stamey *et al.*, 1987; Young *et al.*, 1991; Young *et al.*, 1992; el-Shirbiny 1994; Lilja 2003). Because of the clinical importance of PSA the regulation of PSA expression has been extensively studied. The PSA gene (also known as human kallikrein 3) is one of three kallikrein genes; the other two members are the pancreatic/salivary/renal kallikrein (hK1) and the human kallikrein 2 (hK2), all of which are located as a cluster on chromosome 19q13.2-13.4. Hk2 is located 12 kb downstream from the PSA gene in a head-to-tail fashion, wheras hK1 is located 30 kb upstream of PSA in a head-to-head fashion. The promoter of the PSA gene were first characterised by Riegman *et al.*, (1988; 1989b) who identified the presence of regulatory sequences upstream of the PSA gene including a TATA-box (at position -28 bp to -23 bp with respect to the start of transcription), a GC-box (-53 bp to -48 bp), a CACCC-box (-129 bp to -125 bp) and the sequence AGAACAGCAAGTGCT (-170 bp to -156 bp) which closely resembles the reverse complement of the consensus sequence for binding of the AR. This region was later confirmed to be an androgen response element (ARE) to which the AR interacts to control the expression of PSA in response to androgen. In addition, a 35 bp androgen responsive region (ARR) starting at -400 bp (GTGGTGCAGGGATCAGGGAGTCTCACAATCTCCTG) contains a functional active low affinity AR binding site and cooperates with the ARE in androgen induction of the PSA promoter (Riegman *et al.*, 1991a; Riegman *et al.*, 1991b; Cleutjens *et al.*, 1996; Schuur *et al.*, 1996; Shin *et al.*, 2005). Work by several groups has concluded that a minimal promoter of 642 bp (-630 bp to +12 bp) is required for maximal promoter activity and tissue specificity.

Upstream of the PSA promoter is an enhancer region located between -5824 bp and -3738 bp containing six AREs between -4243 bp and – 4065 bp relative to the start of transcription (Schuur *et al.*, 1996; Cleutjens *et al.*, 1997a, b; Pang *et al.*, 1997; Huang *et al.*, 1999; Yeung *et al.*, 2000; Farmer *et al.*, 2001). Of this enhancer the region -5322 bp to -3870 bp has been identified as the minimal PSA enhancer (PSE) conferring maximum androgen responsiveness and prostate tissue specificity when linked to the PSA promoter (Schuur *et al.*, 1996; Pang *et al.*, 1997; Brookes *et al.*, 1998; Yeung *et al.*, 2000; Wu *et al.*, 2001). The PSE resides on a DNA segment distinct from the PSA promoter but requires a promoter to initiate transcription. In addition, the PSE can be moved relative to the promoter and its orientation reversed without affecting activity (Schuur *et al.*, 1996; Pang *et al.*, 1997). While the PSA promoter alone is weak and relatively promiscuous, showing high activity in breast cancer, bladder cancer and human embryonic kidney cell lines, when combined with one or more PSE, gene expression is considerably increased (10 to 100-fold), highly prostate specific, and can be further enhanced by the addition of androgen. However, there is little or no activity in the AR negative prostate cell lines; PC-3 and DU145, with or without androgen (Pang *et al.*, 1995; Schuur *et al.*, 1996; Pang *et al.*, 1997; Brookes *et al.*, 1998; Latham *et al.*, 2000; van der Poel *et al.*, 2001; Wu *et al.*, 2001; Tsui *et al.*, 2004).

The high level of activity and strong prostate tissue specific activity of PSE and the PSA promoter has made them attractive choices in gene therapy strategies. The PSE/PSA has been used in a phase I clinical trial (DeWeese *et al.*, 2001) as it has been shown both in vitro and in vivo to regulate the expression of therapeutic genes in a tissue specific manner. These include; the pro-drug activating enzymes, purine nucleoside phosphorylase, cytosine deaminase, nitroreductase and thymidine kinase (Gotoh *et al.*, 1998; Martiniello-Wilks *et al.*, 1998; Latham *et al.*, 2000; Shirakawa *et al.*, 2000; Yoshimura *et al.*, 2002; Park *et al.*, 2003; Foley *et al.*, 2004b; Hsieh *et al.*, 2004), the E1A/B and E4 genes that control the replication of a cytotoxic adenovirus (Rodriguez *et al.*, 1997; Yu *et al.*, 1999b; Nettelbeck *et al.*, 2000; Li *et al.*, 2005; Satoh *et al.*, 2005) and the diphtheria toxin A (Pang 2000; Yu *et al.*, 2001a), to name just a few. In addition, PSE/PSA has been used successfully to express anti-sense oligonucleotides and small interfering RNA (siRNA) to down regulate the expression of genes, such as DNA polymerase-α and topoisomerase IIα which are involved in cell replication, specifically within prostate cells (Lee *et al.*, 1996; Song *et al.*, 2004).

Human kallikrein 2 (hK2)

Expression of hK2 incrementally increases from benign to high grade malignant cancer. However, it is also present in normal prostate epithelium and breast cells (Yu *et al.*, 1999b; Magklara *et al.*, 2002). The regulatory regions of the hK2 gene and the PSA gene share 78% to 80% sequence similarity and many of the same characteristics such as regulation by androgens due to the presence of AREs in the promoter (-622 bp to +25 bp) and enhancer (-5155 bp to -3387 bp) regions (Riegman *et al.*, 1988; Riegman *et al.*, 1989a; Riegman *et al.*, 1991a; Riegman *et al.*, 1992; Murtha *et al.*, 1993; Yu *et al.*, 1999b). In addition, expression is restricted to LNCaP prostate cells with very low levels in non-prostate cells lines (Brookes *et al.*, 1998; Yu *et al.*, 1999b; Latham *et al.*, 2000; Xie *et al.*, 2001; Tsui *et al.*, 2004). The tissue specificity and high activity of the hK2 enhancer promoter, although not quite as strong as PSA (Latham *et al.*, 2000), has made it an attractive tool for gene therapy approaches. Combinations of one or more hK2 enhancers linked to an hK2 promoter have been used successfully to drive tissue specific expression of the adenovirus E1A/B gene, leading to significant viral replication specifically in LNCaP cells (Yu *et al.*, 1999b). The only drawback of both the PSE/PSA and hK2 enhancer/promoters is that because their activation is strongly regulated through androgens, in patients with hormone refractory prostate cancer, who, in general, already underwent androgen ablation, such androgen-regulated promoters may have limited efficacy. A solution to this problem could be the development of a gene therapy strategy in which the AR is expressed in conjunction with the PSA/hK2 driven gene therapy. Several groups have shown that co-transfecting AR negative prostate; PC-3 and DU145, and non-prostate Hek293 cells with the AR cDNA driven by the CMV promoter and cDNAs of a reporter gene driven by PSA or hK2 enhancer/promoters, increased activity 5 to 60-fold compared to transfection with a vector containing only the PSA/hK2 enhancer/promoter and the reporter gene (Brookes *et al.*, 1998; Suzuki *et al.*, 2001; Xie *et al.*, 2001).

Differential display code 3 (DD3)

The DD3 gene is one of the most prostate cancer specific genes described to date. It is highly expressed in prostate cancer tissue and its levels of expression increase as the cancer develops to malignancy (Bussemakers *et al.*, 1999; de Kok *et al.*, 2002; Gandini *et al.*, 2003; Hessels *et al.*, 2003; Schalken *et al.*, 2003). Interestingly, no open reading frame for DD3 has been identified, suggesting that DD3 may function as non-coding RNA (Bussemakers *et al.*, 1999). The 5′ flanking sequence of the DD3 gene (-433 to + 62) has been isolated and

although no obvious promoter elements, such as TATA-boxes, CAAT-boxes, or GC-rich regions, have been identified at consensus positions, it is capable of inducing weak expression of a reporter gene (human growth hormone receptor) in LNCaP and PC-3 cells. In addition, truncated constructs showed increased transcriptional activity, suggesting the presence of a silencer that negatively regulates the expression of DD3 in the region -433 to -152 (Verhaegh *et al.*, 2000). Furthermore, activity and tissue specificity of the minimal DD3 promoter (-152 to +62) can be further enhanced by the addition of the PSA enhancer (PSE) while maintaining the lowest basal activity in non-prostate cell lines of all the prostate specific enhancer/promoter combinations tested to date (van der Poel *et al.*, 2001).

Probasin (PB)

Expression of the PB gene is highly restricted to prostate epithelium and as such the promoter region (- 426 bp to + 28 bp) has been extensively characterised and shown to contain two ARE conferring high androgen inducibility on the promoter (Greenberg *et al.*, 1994; Kasper *et al.*, 1994). In addition, important enhancer/regulatory elements have also been identified in an 11,000 bp region at the 5′ end of the promoter contributing to the tissue specific expression of the PB gene and greatly increasing levels of transgene expression induced by androgens (Yan *et al.*, 1997). The high degree of prostate specificity of the PB promoter makes it an attractive promoter for gene therapy. Various constructs have been designed in which either multiple copies of the ARE or the PSA enhancer (PSE) have been placed upstream of the PB promoter to successfully enhance the activity and tissue specificity of the promoter alone (Yu *et al.*, 1999a; Zhang *et al.*, 2000; van der Poel *et al.*, 2001; Yu *et al.*, 2001b; Kakinuma *et al.*, 2003; Wen *et al.*, 2003; Yu *et al.*, 2004). The PB promoter region has also been modified to enable activation by the retinoids–retinoid receptor complex instead of the androgen-AR complex. As a result, transgenes can be expressed in response to retinoids in both androgen-dependent (LNCaP) and androgen-independent (PC-3) prostate cancer cell lines (Furuhata *et al.*, 2003).

Osteocalcin (OSC)

OSC is a noncollagenous bone matrix protein expressed prevalently in prostate cancer epithelial cells, adjacent fibromuscular cells, osteoblasts in locally recurrent prostate cancer and prostate cancer bone metastasis (Matsubara *et al.*, 2001; Foley *et al.*, 2004b; Satoh *et al.*, 2005). The promoter has therefore been used in gene therapy strategies enabling specific activation of the therapeutic gene not only in the primary tumour but also in metastatic lesions (Eder *et al.*, 2005). OSC promoter driven adenoviral mediated gene delivery has been

shown to be highly effective in both androgen-dependent and androgen-independent prostate cancer cells (e.g. LNCaP, PC-3 and DU145) *in vitro* and *in vivo* (Koeneman *et al.*, 2000; Shirakawa *et al.*, 2000; Matsubara *et al.*, 2001; van der Poel *et al.*, 2001; Eder *et al.*, 2005). This treatment has also been shown to be well-tolerated in patients with locally recurrent prostate cancer (Herman *et al.*, 1999), and in patients with lymph node and bone metastasis of hormone refractory prostate cancer (Kubo *et al.*, 2003).

Prostate specific membrane antigen (PSMA)

PSMA is expressed predominantly in normal prostate epithelial cells but also in most adenocarcinomas of prostate cancer and virtually all prostate cancer metastases. In addition, PSMA expression is not induced by androgens, indeed expression is generally elevated in late-stage prostate cancer and in patients undergoing androgen deprivation or ablation therapies (Israeli *et al.*, 1994; Wright *et al.*, 1996; Troyer *et al.*, 1997). It therefore has the potential to be a useful diagnostic factor for the detection of prostate cancer and in the treatment of prostate cancer. The promoter (- 1283 bp to - 39 bp) and enhancer (+ 11,958 bp to + 13,606 bp) regions have been characterised (Good *et al.*, 1999; Watt *et al.*, 2001) and subsequently been used to drive the expression of reporter and therapeutic genes successfully *in vitro* in LNCaP cells; there is conflicting data on activity in PC-3 cells, and *in vivo* in tumour xenografts and in the normal mouse prostate. In general, PSMA activity has been found to be highly tissue specific with minimal to undetectable expression in non-prostate cell lines, such as breast, colorectal, liver and lung. Furthermore, no expression was seen in the liver, spleen, lung, kidney and brain of mice injected either directly into the prostate or into the tail vein with an adenovirus encoding a PSMA driven luciferase reporter gene (O'Keefe *et al.*, 2000; Lee *et al.*, 2002a; Li *et al.*, 2005; Zeng *et al.*, 2005).

Table 1.1 Additional prostate specific promoters.

Prostate specific promoters	References
Caveolin-1 (Cav-1)	(Cui *et al.*, 2001; Pramudji *et al.*, 2001; Ebara *et al.*, 2002)
Progression-elevated gene-3 (PEG-3)	(Su *et al.*, 2005)
Prostate secretory protein of 94 amino acids (PSP94)	(Ochiai *et al.*, 1995; Xuan *et al.*, 1997; Gabril *et al.*, 2002)
Prostate stem call antigen (PSCA)	(Gu *et al.*, 2000; Jain *et al.*, 2002; Watabe *et al.*, 2002; Lam *et al.*, 2005)
Prostatic acid phosphatase (PAP)	(Banas *et al.*, 1994; Zelivianski *et al.*, 1998; Zelivianski *et al.*, 2000; Zelivianski *et al.*, 2002)
Survivin	(Pennati *et al.*, 2004; Zhu *et al.*, 2004; Van Houdt *et al.*, 2006)
T cell receptor gamma-chain alternative reading frame protein (TARP)	(Wolfgang *et al.*, 2001; Cheng *et al.*, 2003; Cheng *et al.*, 2004; Maeda *et al.*, 2004)
Prostatic transglutaminase (pTGase)	(Dubbink *et al.*, 1996; Dubbink *et al.*, 1998; An *et al.*, 1999; Dubbink *et al.*, 1999a; Dubbink *et al.*, 1999b)

Hypoxic promoters

Hypoxia or reduced oxygen tension is common in many solid tumours and is an independent marker of poor treatment outcome as it is associated with a more malignant phenotype affecting genomic stability, apoptosis, angiogenesis and metastasis. Not only does it adversely affect the outcome of radio- and chemo-therapy but it also represents a physiological difference between tumour and normal tissue that has the potential to be exploited therapeutically (Dachs and Tozer 2000; Wouters *et al.*, 2002; Bachtiary *et al.*, 2003; Begg 2003; Bottaro and Liotta 2003; Bussink *et al.*, 2003). Many genes such as erythropoietin (Epo), phosphoglycerate kinase-1 (PGK1) and vascular endothelial growth factor (VEGF), are up-regulated specifically in hypoxic regions due to the presence of hypoxia regulatory elements (HRE) located within the enhancer regions of their promoters (Greco *et al.*, 2000b; Koshikawa *et al.*, 2000). The HRE are activated by the binding of hypoxia inducible factor (HIF-1), a transcription factor produced only in hypoxic tissues, thus ensuring hypoxic tissue specific expression of the genes (Nettelbeck *et al.*, 2000; Wouters *et al.*, 2002; Bachtiary *et al.*, 2003; Begg 2003; Scott and Greco 2004). The HRE from PGK-1 and VEGF have been isolated and used to drive the expression of downstream genes in solid tumours (Dachs *et al.*, 1997a; Dachs *et al.*, 1997b; Dachs and Tozer 2000; Koshikawa *et al.*, 2000; Marples *et al.*, 2002; Wouters *et al.*, 2002; Chadderton *et al.*, 2005). In addition, these hypoxia responsive enhancer elements can also be activated by radiation by an as yet unknown mechanism (Chadderton *et al.*, 2005). Recent studies have shown the potential of combining HRE and CArG elements permitting either or both stimuli to drive therapeutic gene expression (Greco *et al.*, 2002b; Greco *et al.*, 2002d).

At present these studies indicate that there is no promoter that is 100% specific to prostate cancer and capable of operating in both androgen dependant and independent prostate cancer cell lines. Whilst these studies show promise for the use of combinations of radiation responsive and prostate specific promoters, expression levels are both low and transient compared to the activity achieved through constitutive viral promoters, such as the CMV immediate early (IE) promoter. Thus expression of downstream therapeutic genes will be limited. However, the highly tissue specific expression driven by these promoters can in principle be used to activate a Cre-*loxP* molecular switch, resulting in high level expression of therapeutic genes.

1.6 Gene directed enzyme prodrug therapy (GDEPT)

The aim of the molecular switch is to achieve therapeutic levels of expression of a tumour sensitising gene in a gene directed enzyme prodrug therapy (GDEPT) system. GDEPT involves the delivery to the target cells of a foreign gene encoding an enzyme which is able to convert a non-toxic compound (prodrug) into a potent cytotoxin. An ideal enzyme/pro-drug system requires the enzyme to have adequate catalytic activity under physiological conditions and efficient pro-drug activation. The selected pro-drug should be inert at high doses, be able to diffuse freely and stably throughout the tumour to enable localisation to the activating enzyme, and lead to the formation of a toxic drug that has a half-life enabling it to diffuse, or be transported, into the surrounding tumour tissues. This 'bystander' effect is an important feature of GDEPT (see below). In addition the cytotoxicity of the drug should be proliferation independent enabling it to kill a wide range of cells varying from actively dividing invasive metastases to slow growing tumours (Greco and Dachs 2001; Denny 2003; Dachs *et al.*, 2005). The most commonly used enzyme/pro-drug system is the herpes simplex virus thymidine kinase (HSVtk) / gancyclovir (GCV) combination. However, there are others which hold a lot of promise including cytosine deaminase (CD) / 5-fluorocytosine (5-FC), nitroreductase (NTR) / 5-(1-Aziridinyl)-2,4-dinitrobenzamide (CB1954) and horseradish peroxidase (HRP) / indole acetic acid (IAA).

1.6.1 The bystander effect

The bystander effect can be defined as an extension of the killing effects of the active drug to un-transfected neighbouring cells. It is generally accepted to mean that if only 5-10% of the target cells are transfected with the vector and express the therapeutic gene then tumour eradication is still possible. This is crucial for a successful GDEPT strategy as the vectors, delivery systems and protocols currently adopted in clinical trials lead to transfection efficiencies of less that 10% (Greco and Dachs 2001). A significant bystander effect has been observed for active GCV, meditated through gap junctions or the exchange of apoptotic vesicles and for 5-FU, CB1954 and IAA, the active metabolites of which are freely diffusible (Elshami *et al.*, 1996; Mesnil *et al.*, 1996; Bridgewater *et al.*, 1997; Denning and Pitts 1997; Dilber *et al.*, 1997; Dilber and Smith 1997; Duflot-Dancer *et al.*, 1998; Friedlos *et al.*, 1998; Lawrence *et al.*, 1998; Touraine *et al.*, 1998; Degreve *et al.*, 1999; Pierrefite-Carle *et al.*, 1999; Kievit *et al.*, 2000; Kawamura *et al.*, 2001; Greco *et al.*, 2002a; Wilson *et al.*, 2002; Benouchan *et al.*, 2003; Patterson *et al.*, 2003; Helsby *et al.*, 2004). However, in the case of HSVtk/GCV, relying on gap junctions is limiting, as intercellular gap junction

communication is often down regulated in tumours (Holder *et al.,* 1993; Duflot-Dancer *et al.,* 1998; Greco and Dachs 2001).

A strategy that enhances the bystander effect is the use of HSV 1 structural protein VP22. When expressed in transfected cells it can efficiently spread via a golgi independent pathway to surrounding un-transfected cells, irrespective of cell type, where it accumulates in the nucleus despite lacking a recognised nuclear localisation signal (Elliott and O'Hare 1997). Thus fusion genes encoding VP22-GFP and VP22-HSVtk result in significant spread of the fusion protein to non-transfected neighbouring cells (Dilber *et al.,* 1999; Elliott and O'Hare 1999; Ford *et al.,* 2001; Greco *et al.,* 2005a).

A distant bystander effect has also been demonstrated by all of the GDEPT systems mentioned above whereby distant tumours, with no contact with the transduced tumour, regressed after treatment with GDEPT (Elshami *et al.,* 1996; Mesnil *et al.,* 1996; Denning and Pitts 1997; Dilber and Smith 1997; Degreve *et al.,* 1999). It is hypothesised that this distant bystander effect is mediated by the immune response either by the infiltration of cytotoxic T cells or by diffusible factors such as cytokines (Kianmanesh *et al.,* 1997). Such a phenomenon may be of great importance as it would enable the treatment of disseminated primary tumours and metastatic cancers by suicide gene therapy.

1.6.2 Herpes simplex virus thymidine kinase/gancyclovir (HSVtk/GCV)

To date this is the most well studied enzyme/pro-drug strategy. GCV and related agents, such as acyclovir (ACV), pencyclovir (PCV), valacyclovir (VCV) and (e)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU), are poor substrates for the human nucleoside monophosphate kinase but can be converted 1000-fold more efficiently to the monophosphate by wild type HSVtk. The monophosphates are then converted by cellular enzymes into a number of toxic metabolites, the most toxic being the triphosphates. GCV-triphosphate competes with deoxyguanosine triphosphate for incorporation into elongating DNA during S-phase prior to cell division, resulting in the complete inhibition of DNA polymerase, causing "chain termination", making it a very efficient cell killer (Greco and Dachs 2001; Denny 2003; Fillat *et al.,* 2003; Dachs *et al.,* 2005).

One of the advantages of using HSVtk is that positron emission tomography (PET) can be used with a radio-labelled HSVtk substrate, such as GCV, PCV and more commonly FIAU (2′-fluoro-2′-deoxy-1-β-D-arabinofuranosyl-5-iodo-uracil), to repeatedly and non-invasively track gene expression in patients receiving gene therapy. To date several groups are

investigating the distribution, accumulation and imaging sensitivity of HSVtk substrates radiolabelled with positron emitting isotopes such as ^{131}I, ^{18}F and ^{124}I, in order to generate a quantitative assay to image reporter gene expression in human gene therapy (Haberkorn *et al.*, 1998; Gambhir *et al.*, 1999a; Gambhir *et al.*, 1999b; Gambhir *et al.*, 2000; Hackman *et al.*, 2002; Sharma *et al.*, 2002; de Vries *et al.*, 2003; Buursma *et al.*, 2004). However a major drawback of this system is that the activated GCV is an S-phase specific cytotoxin and so the target cells must be actively dividing at the time of exposure. In addition the highly charged triphosphate is insoluble in lipid membranes, this impairs diffusion of the drug making cell-to-cell contact through gap junctions necessary for bystander killing (Elshami *et al.*, 1996; Dilber *et al.*, 1997; Duflot-Dancer *et al.*, 1998; Touraine *et al.*, 1998; Greco and Dachs 2001; Fillat *et al.*, 2003; Dachs *et al.*, 2005).

In the last 15 years more than 600 papers have investigated the potential benefits of HSVtk/GCV for cancer GDEPT including successful preclinical studies on human, rat and mouse prostate cancers, colon carcinoma, glioblastomas, brain tumours, leukaemias, melanomas, lung carcinomas, and breast adenocarcinomas, to name just a few (Kim *et al.*, 1994; Eastham *et al.*, 1996; Hall *et al.*, 1997; Boucher *et al.*, 1998; Dewey *et al.*, 1999; Katabi *et al.*, 1999; Steffens *et al.*, 2000; Loimas *et al.*, 2001; Paquin *et al.*, 2001; Todryk *et al.*, 2001; Yoshimura *et al.*, 2001; Corban-Wilhelm *et al.*, 2002; Soling *et al.*, 2002; Corban-Wilhelm *et al.*, 2003; Dachs *et al.*, 2005). In addition it has also been possible to use the prostate specific promoters; PSA, OSC, and caveolin-1, to drive the expression HSVtk in human and mouse prostate cancer cell lines and *in vivo* mouse xenografts (Martiniello-Wilks *et al.*, 1998; Pramudji *et al.*, 2001; Hsieh *et al.*, 2004) and CArG elements in glioma, pancreatic tumours and MCF-7 breast adenocarcinoma cells (Joki *et al.*, 1995; Takahashi *et al.*, 1997; Marples *et al.*, 2000; Scott *et al.*, 2000; Scott *et al.*, 2002) resulting in increased sensitivity to GCV. Phase I clinical trails have also been conducted demonstrating the safety of an adenovirus mediated HSVtk gene therapy approach to prostate cancer (Herman *et al.*, 1999; Miles *et al.*, 2001; Shalev *et al.*, 2001; Kubo *et al.*, 2003). In addition, many phase I, II and III clinical trials have been conducted on a range of tumours including brain, leukaemias/lymphomas, malignant gliomas, metastatic colorectal carcinomas, melanomas and ovarian cancer (Ram *et al.*, 1997; Klatzmann *et al.*, 1998; Hasenburg *et al.*, 2000; Rainov 2000; Sandmair *et al.*, 2000; Trask *et al.*, 2000; Sung *et al.*, 2001; Fillat *et al.*, 2003; Dachs *et al.*, 2005). However, the clinical benefits in these trials are still modest. This is possibly due to low transfection efficiency, the slower growth of human tumours compared to xenografts used in the animal models, and also the limited dose of GCV that can be tolerated due to bone marrow toxicity (Greco and Dachs 2001; Dachs *et al.*, 2005).

There are numerous possibilities for improving the efficacy of HSVtk treatment, mainly through improving gene delivery and the bystander effect, but also by the development of nucleoside analogues with a higher affinity for HSVtk and fewer side effects than GCV (Balzarini *et al.*, 1994; Balzarini *et al.*, 1998; Degreve *et al.*, 1999; Thust *et al.*, 2000; Tomicic *et al.*, 2002) and of HSVtk mutants engineered to increase specificity and activity towards the prodrug (Black *et al.*, 1996; Kokoris *et al.*, 1999; Kokoris *et al.*, 2000; Kokoris and Black 2002; Pantuck *et al.*, 2002). Interestingly, the therapeutic effect of HSVtk/GCV gene therapy can also be significantly enhanced by combining with radiotherapy *in vitro*, *in vivo* and in a phase I/II clinical trial for the treatment of prostate cancer (Kim *et al.*, 1994; Kim *et al.*, 1995; Kim *et al.*, 1997; Kim *et al.*, 1998; Atkinson and Hall 1999; Chhikara *et al.*, 2001; Teh *et al.*, 2001; Satoh *et al.*, 2004; Teh *et al.*, 2004). These two therapies are potentially synergistic for two reasons. Firstly, radiation induces membrane damage which may facilitate the bystander effect of HSVtk gene therapy as the cytotoxic GCV-triphosphates can diffuse more rapidly to neighbouring un-transduced cells. Secondly, the cytotoxic GCV-triphosphates may also incorporate into DNA at the sites of radiation damage in addition to incorporation during S phase elongation prior to cell division (Kim *et al.*, 1995; Chhikara *et al.*, 2001).

1.6.3 Cytosine deaminase/5- flurocytosine (CD/5-FC)

Cytosine deaminase (CD), found in certain bacteria and fungi but not in mammalian cells, catalyses the hydrolytic deamination of cytosine to uracil. It converts the non toxic prodrug 5-FC to 5-fluorouracil (5-FU), which is then transformed by cellular enzymes into three potent pyrimidine antimetabolites; 5-fluorodeoxyuridine-5′-monophosphate (5-FdUMP), which irreversibly inhibits thymidylate synthase thus preventing DNA replication, and triphosphate (5-FdUTP) and 5-fluorouridine-5′-triphosphate (5-FUTP), both of which are incorporated into DNA preventing nuclear processing of ribosomal and messenger RNA (Ferguson *et al.*, 2001; Greco and Dachs 2001; Denny 2003; Dachs *et al.*, 2005). At present 5-FU alone is widely used cancer chemotherapy, especially against colon cancer, as it has both proliferation dependent and independent actions. However, it displays an array of side effects and a high dose level is required for a tumour response, thus limiting its use. The pro-drug strategy is a method to circumvent such problems by activation of the drug specifically in tumour cells (Kammertoens *et al.*, 2000). One of the main advantages of the CD/5-FC system is a strong bystander effect that does not require cell-to-cell contact, since 5-FU can diffuse in and out of cells by non-facilitated diffusion. However, the cells must be actively dividing for the toxic metabolites to incorporate into the DNA (Domin *et al.*, 1993).

The effectiveness of 5-FC in the killing of prostate carcinoma cells transduced with a vector containing the CD gene under the control of a variety of prostate specific promoters, has been demonstrated (Yu *et al.,* 1999b; Uchida *et al.,* 2001; Yoshimura *et al.,* 2002). In addition, CD has also been combined with uracil phosphoribosyltransferase, one of the mammalian cellular enzymes involved in the catalysis of 5-FU into 5-FdUMP, to enhance the conversion of 5-FC into toxic metabolites (Chung-Faye *et al.,* 2001b; Miyagi *et al.,* 2003). It has also been observed that 5-FU and its intermediary metabolites enhance the radiosensitivity of tumour cells. Combinations of CD/5-FC therapy and radiotherapy have therefore been used in the treatment of biliary epithelium carcinoma, bladder and prostate cancer with promising results (Khil *et al.,* 1996; Pederson *et al.,* 1997; Kato *et al.,* 2002; Zhang *et al.,* 2003). Although in some tumours, such as mammary carcinoma and cervical carcinoma, this pro-drug scheme has a higher cure rate *in vivo* compare to HSVtk/GCV, in others, i.e. gliosarcoma cells and prostate tumours, the anti-tumour effect induced by HSVtk/GCV system is superior to that of the CD/5-FC approach (Rogulski *et al.,* 1997a; Blackburn *et al.,* 1998; Kim *et al.,* 1998; Uckert *et al.,* 1998; Rogulski *et al.,* 2000; Corban-Wilhelm *et al.,* 2003). It is becoming clear that one single GDEPT strategy might not be optimal for tumour control. However, it is possible to combine GDEPT systems into one delivery vector to enhance anti-tumour activity. Such a system has been developed by several groups in which fusion genes of CD-HSVtk were delivered to prostate tumour, gliosarcoma, cervical carcinoma and mammary adenocarcinoma cells both *in vitro* and *in vivo.* This resulted in pro-drug sensitivity and radiosensitisation that was equivalent to or better than that observed for each system independently (Rogulski *et al.,* 1997a; Rogulski *et al.,* 1997b; Aghi *et al.,* 1998; Blackburn *et al.,* 1998; Kim *et al.,* 1998; Uckert *et al.,* 1998; Blackburn *et al.,* 1999; Rogulski *et al.,* 2000; Corban-Wilhelm *et al.,* 2002; Freytag *et al.,* 2002b; Lee *et al.,* 2002b; Corban-Wilhelm *et al.,* 2003; Corban-Wilhelm *et al.,* 2004). The success of these preclinical studies has led to the development of replication-competent adenovirus mediated CD/5-FC and HSVtk/GCV double suicide gene therapy in combination with conventional radiation therapy to phase I clinical studies. The results demonstrate that such an approach can not only be safely applied to humans but also shows signs of biological activity (Rogulski *et al.,* 2000; Freytag *et al.,* 2002a; Freytag *et al.,* 2003).

1.6.4 Nitroreductase/5-(1-Aziridinyl)-2,4-dinitrobenzamide (NTR/CB1954)

The prodrug CB1954 was originally used as a single agent and showed effectiveness in preclinical studies on the Walker rat carcinoma. On this basis a phase I clinical trial was carried out in the 1970s. Unfortunately there was no clinical benefit to the treated patients.

The cause of the success in the rat tumour model was the high levels of nicotine adenine dinucleotide phosphate (NADPH) dehydrogenase (DT-diaphorase) which converted CB1954 to the mustard metabolite 5-aziridynyl-4-hydroxylamino-2-nitrobenzamide, which, after further reactions with cellular thioesters, such as acetyl coenzyme A, is a potent DNA cross-linking agent. This causes cell death predominantly by caspase-dependent and p53-independent apoptosis (Roberts *et al.*, 1986; Knox *et al.*, 1988a; Knox *et al.*, 1988b; Bridgewater *et al.*, 1995; Cui *et al.*, 1999; Denny 2003; Hay *et al.*, 2003; Palmer *et al.*, 2003). CB1954 is a poor substrate for DT-diaphorase and subsequent studies identified the catalytically superior *E. coli* NTR enzyme (Anlezark *et al.*, 1992). The NTR/CB1954 combination is effective under hypoxic and anoic conditions and because the activated drug is a DNA cross-linking agent, it is able to kill both proliferating and non-proliferating cells (Greco and Dachs 2001; Searle *et al.*, 2004). An efficient bystander effect has also been demonstrated in a number of cell lines and in animal models regardless of cell-to-cell contact and gap junctional status, as the metabolites of NTR are membrane permeable (Bridgewater *et al.*, 1997; Friedlos *et al.*, 1998; Westphal *et al.*, 2000).

When used as a GDEPT strategy, virus mediated expression of NTR in several human and murine tumour cells has resulted in up to 2000 fold increase in sensitivity to CB1954 compared to the parental lines (Drabek *et al.*, 1997; Friedlos *et al.*, 1998; McNeish *et al.*, 1998; Djeha *et al.*, 2000; Latham *et al.*, 2000; Weedon *et al.*, 2000; Djeha *et al.*, 2001; Read *et al.*, 2003; Chen *et al.*, 2004; Djeha *et al.*, 2005; Lipinski *et al.*, 2005). As a result the NTR/CB1954 combination is currently being tested in four clinical trials in the UK. Initial data published in patients with liver cancer shows minimal side effects from the viral vector carrying NTR (Palmer *et al.*, 2004). CB1954 is also well tolerated in patients with gastrointestinal malignancies and sufficient serum/peritoneal levels were achieved for an enzyme/prodrug approach to be feasible (Chung-Faye *et al.*, 2001a). However, since these trials did not co-administer NTR and CB1954, future clinical data will show whether this enzyme/prodrug combination lives up to its promise.

1.6.5 Horseradish peroxidase/indole acetic acid (HRP/IAA)

HRP is an iron containing heme peroxidase isolated from the roots of the horseradish plant, and IAA is a non toxic auxin plant hormone involved in the regulation of plant cellular growth, division and differentiation. IAA is oxidised by HRP to a radical cation which rapidly fragments to form a skatole radical, this radical is converted by further steps to the toxin 3-methylene-2-oxinodole (MOI) (Wardman 2002; Denny 2003). Cell death, by DNA damage, thiol depletion and lipid oxidation, can occur under both oxic and hypoxic

conditions, and can be further enhanced by radiation (Greco *et al.*, 2001; Greco *et al.*, 2002d; Dachs *et al.*, 2005). IAA activation, by HRP, in human T24 bladder carcinoma cells was fast and efficient since cytotoxicity could be evoked within a 2 h exposure. A strong bystander effect was also induced as MOI is able to pass rapidly into neighbouring cells by passive diffusion without the need for cell to cell contact (Greco *et al.*, 2000a; Greco and Dachs 2001). HRP/IAA GDEPT has been successfully applied to MCF-7 mammary carcinoma cells and nasopharyngeal squamous carcinoma under oxic and anoxic conditions. In addition, cytotoxicity was enhanced (3.6-fold) when combined with irradiation (Greco *et al.*, 2002d). There is also the potential to target drug activation to regions of hypoxia by using hypoxia specific promoters and/or the use of radiation responsive CArG elements, to control the expression of HRP (Greco *et al.*, 2000b; Greco *et al.*, 2002b).

1.7 Delivery

The selection of an appropriate vehicle to deliver the genes of interest to the target cells is extremely important as cell uptake and gene expression of naked DNA is relatively inefficient. An ideal vector should be safe for the recipient and environment and deliver the DNA construct to the target tissue while being protected from degradation and immune attack. It should also express the therapeutic genes for as long as possible and in an appropriately regulated manner. The delivery system, be it viral, bacterial or synthetic, should be large enough to carry the required genes and easy to produce and purify in large quantities and appropriate concentrations (Greco *et al.*, 2002c; Collis *et al.*, 2003). As yet no "magic" vector complying with all the above requirements has been designed but there are some potential viral and non-viral delivery methods that hold promise.

1.7.1 Viral delivery systems

Viral vectors capitalise on the ability of viruses to efficiently enter cells through specific receptors and transfer their genome into host cells. However, for them to be suitable for gene therapy the pathogenicity of a specific virus must be eliminated while retaining the efficiency of gene transfer and expression. To date, only retroviruses and adenoviruses are commonly employed in gene therapy clinical trials. However, these and other viruses, such as lentiviruses, baculoviruses and adeno-associated viruses, are being manipulated in order to accommodate more foreign DNA, up to 35kb, and to improve the transfection efficiency, in particular to allow targeted transduction of specific cell types (Robbins and Ghivizzani 1998; Zhang 1999; Chinnasamy *et al.*, 2000; Monahan and Samulski 2000; Steiner and Gingrich 2000; McCormick 2001; Stanbridge *et al.*, 2003; Kraaij *et al.*, 2005). For the treatment of prostate cancer, although vaccinia and lentiviral vectors have been used (Eder *et al.*, 2000; Yu *et al.*, 2001a; Yu *et al.*, 2004), the vector of choice are adenoviruses whose extensive use has shown them to be easy to manipulate and efficient at transfecting prostate cells both in vitro and vivo (Eastham *et al.*, 1996; Hall *et al.*, 1997; Rodriguez *et al.*, 1997; Blackburn *et al.*, 1998; Boucher *et al.*, 1998; Gotoh *et al.*, 1998; Martiniello-Wilks *et al.*, 1998; Atkinson and Hall 1999; Blackburn *et al.*, 1999; Hall *et al.*, 1999; Nagayama *et al.*, 1999; Yu *et al.*, 1999a; Yu *et al.*, 1999b; Hassan *et al.*, 2000; Koeneman *et al.*, 2000; Latham *et al.*, 2000; Shirakawa *et al.*, 2000; Chhikara *et al.*, 2001; Djeha *et al.*, 2001; Matsubara *et al.*, 2001; Pramudji *et al.*, 2001; Wu *et al.*, 2001; Xie *et al.*, 2001; Yu *et al.*, 2001b; Freytag *et al.*, 2002b; Lee *et al.*, 2002a; Lee *et al.*, 2002b; Yoshimura *et al.*, 2002; Furuhata *et al.*, 2003; Kakinuma *et al.*, 2003; Miyagi *et al.*, 2003; Park *et al.*, 2003; Hsieh *et al.*, 2004; Kraaij *et al.*, 2005; Leow *et al.*, 2005; Li *et al.*, 2005). In addition, adenoviruses have been used in several

gene therapy trials of prostate cancer and shown to be effective and safe (Herman *et al.*, 1999; DeWeese *et al.*, 2001; Sharma *et al.*, 2001; Freytag *et al.*, 2002a; Freytag *et al.*, 2003; Kubo *et al.*, 2003; Satoh *et al.*, 2004). Nonetheless, challenges still exist, such as the problem of random genome integration leading to insertional mutagenesis of retroviruses, the immunogenicity of adenoviral vectors and the development of a high throughput packaging cell lines for the latest generation of lentiviral vectors (Kirn 2000; Trono 2000; Shalev *et al.*, 2001; Greco *et al.*, 2002c).

1.7.2 Non-viral delivery systems

Besides viruses, other live vehicles, primarily bacteria and macrophages, have been investigated for gene therapy (Griffiths *et al.*, 2000; Greco *et al.*, 2002c). Bacteria are particularly attractive as they can incorporate a large genome and are thus able to express multiple therapeutic transgenes, they are highly motile and, if necessary, their spread can be controlled by antibiotics. Examples of prokaryotic vectors include anaerobic bacteria of the genera *Clostridium*, gram positive non-pathogenic anaerobic *Bifidobacteria* and the tumour invasive *Salmonella typhimurium* (Greco *et al.*, 2002c; Liu *et al.*, 2002).

1.7.3 Synthetic delivery systems

Synthetic delivery systems would be particularly suitable for gene therapy as they are not pathogenic, are simple to use, elicit no immune responses, can be produced safely in large quantities and can be manipulated easily. These delivery systems can be broadly divided into physical (electroporation and gene gun) or chemical (lipids, DNA conjugates) techniques. To date, electroporation seems the most promising. It has been shown to effectively deliver plasmids encoding GFP to four histologically different tumour models (mouse melanoma, rat carcinosarcoma, human bladder carcinoma and mouse sarcoma) *in vitro* and *in vivo*, and to hypoxic cells (Dachs *et al.*, 2000; Cemazar *et al.*, 2002). In addition, gene delivery can be further enhanced by combining with liposome-DNA complexes (Somiari *et al.*, 2000; Cemazar *et al.*, 2002; Greco *et al.*, 2002c). Liposomal gene delivery can also be enhanced by conjugating the liposomes to defective viral particles or viral proteins that are able to disrupt the lysosome and/or increase DNA transport to the nucleus (Robbins and Ghivizzani 1998; Greco *et al.*, 2002c; Merdan *et al.*, 2002). However, despite being easy to control and well characterised the efficiency of synthetic gene transfer is very low (Prince 1998; Shalev *et al.*, 2001).

1.8 Aims of project

Aim 1 - To identify a tightly controlled enhancer/promoter that will drive the expression of GDEPT within prostate cancer.

Aim 2 - To determine the most effective GDEPT approach to use with prostate cancer.

Aim 3 - To develop a molecular switch to enhance GDEPT gene expression levels while maintaining tissue specificity.

In more detail, the aim of this project is to combine a radiation controllable molecular switch with a non-specific high activity promoter to achieve high level, persistent and tissue specific expression of a therapeutic gene in a GDEPT context in localised prostate cancer. Once developed this system could then be further manipulated by the incorporation of a number of different prostate specific promoters, to target metastatic cancer and/or hypoxic regions and administered using a number of gene therapy approaches.

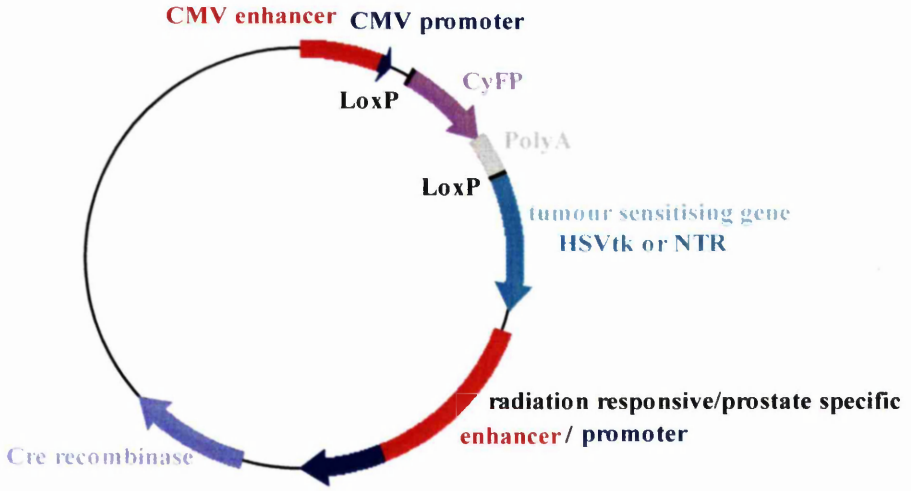
To this end, a pCI-neo based vector will be designed to enable the insertion of different synthetic radiation responsive enhancers and, later, prostate specific enhancers/promoters upstream of the green fluorescent protein (GFP) reporter gene. The vectors will then be assessed in a variety of prostate (PC-3, LNCaP, PNT2C2, PC-3AR) and non-prostate (MCF-7, HepG2, Hek293, H460) cell lines and FACS analysis will be used to determine the most suitable enhancer/promoter to drive the molecular switch.

In addition, the HSVtk/GCV and NTR/CB1954 enzyme/prodrug systems will be tested to determine the most efficient for induction of prostate cell death. Vectors will be designed in which the CMV enhancer/promoter will drive the expression of HSVtk or NTR in prostate and non-prostate cell lines. The prodrugs; GCV and CB1954, will then be added to the cells in the media post transfection. An MTT assay will be performed to determine cell growth inhibition and a western blot analysis of cell extract will determine protein expression.

The molecular switch will then be designed initially as two vectors but with the intention of combining into a single adenoviral delivery vector which has a maximum capacity for 8kb of foreign DNA. The switch will consist of the radiation responsive or prostate specific promoters controlling the expression of Cre recombinase. Within the same vector, the strong CMV IE promoter/enhancer will drive the expression of the tumour sensitising gene, HSVtk or NTR, the expression of which will be silenced by a 'stop' cassette. The 'stop' cassette will

consist of the cyan fluorescent protein (CyFP) reporter gene and a polyadenylation stop signal (PolyA), flanked by two *loxP* sites. Recombination between the *loxP* sites will result in the removal of the stop cassette and activation of transcription (see figure 1.4).

Figure 1.4 Map of the all-in-one molecular switch vector. The elements shown are: CMV enhancer (red), CMV promoter (dark blue), tumour sensitising gene (HSVtk or NTR; turquoise), radiation responsive or prostate specific enhancer (red) and promoter (dark blue), Cre recombinase (light blue) and the 'stop' cassette consisting of; two *LoxP* sites (black), the reporter gene (lilac) and a PolyA stop signal (grey). Cre mediated recombination between the two loxP sites excises the 'stop' cassette (CyFP and PolyA) enabling activation of the tumour sensitising gene (HSVtk or NTR) under the control of the CMV enhancer/promoter.



The molecular switch construct and control vectors containing the tumour sensitising gene under the control of the radiation responsive or prostate specific enhancer/promoters, will initially be tested *in vitro* in prostate and non-prostate cell lines by fluorescence activated cell sorter (FACS) analysis of CyFP expression, which will be lost as the 'stop' cassette is excised by Cre mediated recombination, and MTT growth inhibition assays. If the strategy is effective the long-term plan is that the constructs will also be tested *in vivo*. PC-3 and/or LNCaP xenografts will be propagated in BALB/c nude mice and, once the subcutaneous tumour has reached the size of 5mm in diameter, the adenovirus vectors will be administered by injection directly into the tumour and/or intravenously. To determine the tumour growth inhibition, tumour volume will be measured at regular intervals throughout the experiment until the tumour has reached the maximum size allowable. All the mice will then be sacrificed. Therapeutic gene expression will be assessed in the tumour and a variety of major organs (bladder, liver, heart, brain, lung, kidney, testis, skeletal muscle, stomach, intestine, and pancreas) by immunohistochemistry, fluorescence microscopy, or real-time RT-PCR.

It is envisaged that such a gene therapy approach will be fully adaptable depending on the type of cancer it is to treat. Not only could different promoters be used to drive the expression of Cre, but different GDEPT strategies could also be incorporated to enhance killing efficiency, such as fusion proteins of HSVtk with CD and/or HRP.

Chapter 2

2.0 Materials and Methods

2.1 Molecular biology

2.1.1 Enzymes

All enzymes were purchased from Promega (Southampton, UK), New England Biolabs (Hertfordshire, UK) or Roche Applied Sciences (East Sussex, UK). Enzymes were stored at -20°C and were used according to the conditions stated by the manufacturer.

2.1.2 Molecular biology kits

All molecular biology kits were obtained from Qiagen (West Sussex, UK). The kits used were QIAprep spin miniprep kit, Qiagen plasmid maxi kit, QIAquick gel extraction kit, QIAquick PCR purification kit and DNeasy tissue kit. All procedures were carried out as described in the appropriate protocol booklet.

2.1.3 Vectors

Listed below are the various vectors used in this project.

Vector	Description	Source
pCI-neo	Mammalian expression vector	Promega
pBS185	Cre expression vector	Invitrogen (Paisley, UK)
pEGFP-1	GFP expression vector	BD Biosciences Clontech
pORF-HSV1tk	HSVtk expression vector	InvivoGen (San Diego, California, USA)
pCyFP	CyFP expression vector	Flavia Moreira-Leite (PICR, Manchester, UK)
pDRIVE03-EGR-1(h) v02	WT Egr-1 expression vector	InvivoGen
pcDNA4/V5-His	Mammalian expression vector	Invitrogen
pShuttle2	Mammalian expression vector	BD Biosciences Clontech
pGem-T	Cloning shuttle vector	Promega
pUC18-PSE-2	PSE expression vector	Gerald Verhaegh (UMC, Nijmegen, The Netherlands)

2.1.4 Buffers and reagents

Listed below are the various buffers and reagents used in this project. All reagents were purchased from Sigma-Aldrich GmbH (Steinham, Germany) unless otherwise stated.

Reagent/Buffer	Constituents
10 x TE	0.1 M Tris-HCl, 10 mM EDTA (BDH, Poole, UK) pH 7.5, Autoclave
10 x TBE	0.9 M Tris-borate, 20 mM EDTA, pH 8.3
10 X TBS	0.5 M Tris-base, 1.5 M NaCl, pH 7.5.
1 X TBST	1 X TBS, 0.1% (v/v) Tween 20
Ethidium bromide	10 mg/ml stock solution in ddH_2O
Agarose gel DNA loading buffer	0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 30% (v/v) glycerol, 10 mM EDTA
SDS-PAGE loading buffer	385 mg DTT, 2.5 ml of 20% SDS, 2ml of 1 M Tris-HCl pH 6.8, 3.75 ml glycerol, 1ml of 1.5% (w/v) bromophenol blue
Non-denaturing loading buffer	30% (v/v) glycerol, 0.25% (w/v) xylene cyanol, 0.25% (w/v) bromophenol blue
Transfer Buffer	25 mM Tris-base, 190 mM glycine, 20% (v/v) methanol (BDH)
Buffer I	50 mM Tris-HCl, pH 8.3, 3 mM DTT, 1 mM EDTA
4 X Upper Tris	0.5 M Tris-HCl, 0.4% (w/v) SDS, pH 8.8
4 X Lower Tris	0.5 M Tris-HCl, 0.4% (w/v) SDS, pH 6.8
70% Ethanol	70% ethanol (BDH) (v/v), 30% (v/v) ddH_2O

2.1.5 Oligonucleotides.

Oligonucleotide sequences used for the PCR amplification of DNA are contained within the relevant results chapters. All oligonucleotides used in the amplification of genes were designed to add the Kozak optimal translation initiation sequence (ACC) (Kozak 1986) 5′ to the start codon (ATG). Over 100 primers were designed for DNA sequencing to determine correct insertion and sequence fidelity of cloned genes. These are not listed but were designed using the following criteria:

1. Primers were 17-28 bases in length.
2. Base composition was 50-60% G/C.
3. 3′ sequences ended in a GC clamp.
4. Melting temperature (Tm) of between 65-74°C was calculated using the formula: $Tm\ ^{o}C = 4(G + C) + 2(A + T)$.
5. Runs of three of more identical bases were avoided.
6. Self complementary primer sequences were avoided.

2.2 DNA protocols

2.2.1 Oligonucleotide annealing

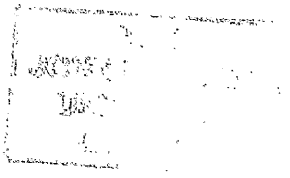
Annealing reactions were performed in 1 x TE buffer (section 2.1.4) using 1nmol of each oligonucleotide in a 50 µl reaction volume. The oligonucleotides were incubated in a heat block at 95°C for 5 min and then cooled slowly to room temperature (RT). Annealed oligonucleotides were analysed by non-denaturing polyacrylamide gel electrophoresis (section 2.2.8).

2.2.2 PCR amplification of DNA

Amplifications by polymerase chain reaction (PCR) were performed to generate products for gene cloning or to screen for the presence of an insert within a newly cloned construct. Mega mix blue taq polymerase (Flowgen, Leicestershire, UK) was used to screen for newly cloned inserts whilst high fidelity PCRs for gene cloning were carried out using $Vent_R$ DNA polymerase (New England Biolabs). A Hybaid Omni Gene thermal cycler (Franklin, Mass., USA) was used for all PCR amplifications. Amplification of 1-1.5 kb fragments employed 1 cycle for 2 min at 95°C, 35 cycles of 30 s at 95°C, 30 s / 500 bases at 55°C, 60 s at 72°C and 1 cycle of 5 min at 72°C. PCR products were purified using the QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions.

2.2.3 Restriction endonuclease digest

Restriction endonuclease digests of plasmid DNA or PCR products were set up according to the conditions stated by the manufacturer. Digested DNA molecules were then purified either by agarose gel electrophoresis (section 2.2.7 and 2.2.9), and phenol/chloroform extraction (section 2.2.4) followed by ethanol precipitation (section 2.2.5) or using the QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions.



2.2.4 Phenol/chloroform extraction

In order to remove proteins from DNA solutions after a restriction endonuclease digest, a phenol:chloroform extraction procedure was used. Equal volumes of phenol:chloroform:isoamylalcohol 25:24:1 (Sigma) and DNA solution were mixed thoroughly by shaking until an emulsion formed. The mixture was then centrifuged at 15,000 x g for 1 min at RT. The upper aqueous phase was then transferred to a fresh tube, the interface and organic phase were discarded. An equal volume of chloroform (Sigma) was then added, mixed thoroughly and then centrifuged at 15,000 x g for 1 min at RT. The upper aqueous phase was transferred to a fresh tube and the DNA recovered by standard precipitation with ethanol (section 2.2.5).

2.2.5 Ethanol precipitation

Ethanol precipitation was used to recover DNA from aqueous solutions. The DNA solution was mixed with 0.3 M sodium acetate (pH 5.2 Sigma) and exactly 2 volumes of ice-cold ethanol (BDH). The ethanolic solution was then stored at -20°C for 15 – 30 min. The DNA was then recovered by centrifugation at 13,000 x g for 10 min at 4°C. The supernatant was then removed, without disturbing the DNA pellet, 600 µl of 70% ethanol was added and the sample re-centrifuged at 15,000 x g for 10 min at 4°C. The supernatant was then removed and the tube left open at RT until the last traces of fluid had evaporated. The pellet was then dissolved in 1 x TE (section 2.1.4) and the DNA concentration determined using a NanoDrop ND 1000 spectrophotometer.

2.2.6 Shrimp Alkaline Phosphatase treatment

Dephosphorylation of restriction endonuclease digested DNA products was performed using shrimp alkaline phosphatase (Promega). Reactions were set up according to the manufacturer's instruction. After incubation for 1 h at 37°C the phosphatase was heat inactivated by incubation of the reaction for 15 min at 65°C. No further purification of the de-phosphorylated vector was necessary prior to ligation.

2.2.7 Agarose gel electrophoresis

Agarose gels were routinely used to resolve DNA molecules by molecular weight. Different percentage gels were made by dissolving the appropriate amount of agarose in 1 x TBE (section 2.1.4) containing 0.5 μg/ml ethidium bromide (section 2.1.4). For the resolution of low molecular weight DNA fragments (200 bp – 500 bp) a 3% metasieve gel (Flowgen, Nottingham, UK) was used and for high molecular weight fragments (500 bp – 6 kb) a 1-2% multi-purpose (MP) agarose gel (Roche Diagnostics GmbH, Mannheim, Germany) was used. Agarose gel loading buffer (section 2.1.4) was added to samples prior to loading and 1 kb or 100 bp DNA ladders (Promega) were used to estimate the size of separated DNA fragments. Electrophoresis was performed in 1 x TBE at 80 V for approximately 90 min. DNA bands within the gel were visualised by UV transillumination and digitally imaged using GeneSnap (Syngene, Cambridge, UK).

2.2.8 Non-denaturing polyacrylamide gel electrophoresis

Non-denaturing polyacrylamide gel electrophoresis was used to separate low molecular weight (<100bp) DNA molecules. Using the BioRad mini-protean gel apparatus (BioRad, Hemel Hempstead, UK) a 0.75 mm thick 40% (w/v) 19:1 acrylamide:bis-acrylamide (Accugel) gel was prepared. This consisted of 5 ml Accugel (National Diagnostics, Hessle, East Riding of Yorkshire, UK), 1 ml 10 x TBE (section 2.1.4), 100 μl 10% ammonium persulfate (APS; Sigma), 10 μl tetramethylethylenediamine (TEMED; Sigma) and ddH_2O to 10 ml. DNA samples were then mixed with non-denaturing loading buffer (section 2.1.4) and loaded onto the gel. Electrophoresis was carried out in 1 x TBE at a constant 150 V for approximately 60 min. The gel was then stained with 0.5 μg/ml ethidium bromide (section 2.1.4) in 1 x TBE and the DNA visualised by UV transillumination and digitally imaged using GeneSnap (Syngene, Cambridge, UK).

2.2.9 Isolation and purification of DNA fragments

Following agarose gel electrophoresis DNA bands were excised and the DNA isolated using a QIAquick gel extraction kit according to the manufacturer's instructions. The DNA was then ethanol precipitated and quantified (section 2.2.5).

2.2.10 Ligation reactions

Ligation into T/A cloning vectors:

PCR products were initially cloned into a T/A cloning vector (pGem-T, Promega; see appendix section 8.1) according to the manufacturer's instructions. This was to facilitate rapid cloning and confirmation of the fidelity of the sequence. Inserts of correctly sized products were excised using appropriate restriction endonucleases and re-cloned into the target vector.

Ligation into mammalian expression vectors:

Ligations into mammalian expression vectors (pCI-neo, pcDNA4/V5-His and pShuttle) were performed using 3 : 2 molar ratio of insert : vector totalling 50 ng. Ligations were routinely performed in a 20 µl reaction volume using T4 DNA ligase (New England Biolabs) according to the manufacturer's instructions.

2.2.11 Plasmid purification

Mini- and Maxi-preps were carried out to isolate plasmid DNA from transformed bacteria, according to the Qiagen kit protocols. For mini-preps: single colonies were picked and incubated overnight (37°C, 250 rpm) in 5 ml Luria-Bertani broth (LB-broth section 2.3.1) containing the appropriate antibiotic (Section 2.3.1). A QIAprep spin mini-prep kit was then used to isolate between 5 and 10 µg of plasmid DNA. For maxi-preps, a 10 ml culture was set up and incubated for 6-8 h (37°C, 250 rpm), 5 ml of this starter culture was then used to inoculate 400 ml of LB-broth (with antibiotics) and incubated overnight (37°C, 250 rpm). A Qiagen plasmid maxi kit was then used to isolate the DNA yielding approximately 500 ng of plasmid DNA. DNA concentrations were determined using a NanoDrop ND 1000 spectrophotometer and purity by restriction digest followed by agarose gel electrophoresis (section 2.2.7) and DNA sequencing (section 2.2.12).

2.2.12 DNA sequencing

Sequencing reactions were prepared using an ABI PRISM big-dye terminator kit (PE Biosystems, Warrington, UK) according to the requirements of the Paterson Molecular Biology Core Facility. Samples were analysed on an ABI 3730 capillary sequencer.

2.3 Bacteriology

2.3.1 Bacterial culture media

Media components were obtained from Difco Laboratories (West Moseley, UK) unless otherwise stated. Luria-Bertani (LB) broth and LB-agar were used for all bacterial preparations. All broth and agar was autoclaved at 121°C / 1.05 Kg/cm^2 for 10 min and stored at RT.

Media	Preparation
LB-broth:	10 g bacto-tryptone, 5g bacto-yeast extract, 10g NaCl in 1 L of ddH_2O
LB-Agar:	Same composition as LB-broth with the addition of 10 g bacto-agar
LB-Agar-IPTG/X-gal (Sigma)	Same composition as LB-Agar with the addition of: 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) : 40 mg/ml stock solution in formamide, added to a final concentration of 50 μg/ml. Isopropyl β-D-1-thiogalactopyranoside (IPTG) : 1 M stock solution in ddH_2O, added to a final concentration of 0.1 mM. Both reagents were added to media after autoclaving and cooling to below 55°C
Antibiotics (Sigma)	Ampicillin : 100 mg/ml stock soln. in 50% v/v ethanol in ddH_2O, added to a final concentration of 50 μg/ml Kanamycin : 25 mg/ml stock solution in ddH_2O, added to a final concentration of 50 μg/ml Antibiotics were added to media after autoclaving and cooling to below 55°C. Stock solutions were stored at -20°C

2.3.2 Transformation of bacteria

Competent DH5α cells (Invitrogen) were transformed according to the supplier's instructions. Transformed cells were plated at 1 x and 10 x dilutions on LB-Agar plates containing the appropriate antibiotic (section 2.3.1) and incubated overnight at 37°C. DH5α cells transformed with pGem-T constructs were plated on LB-Agar-X-Gal/IPTG plates with 50 μg/ml Ampicillin (section 2.3.1), enabling recombinant clones to be identified as white colonies due to the insertional disruption of the β-galactosidase gene. White colonies were then screened by PCR (section 2.2.2) to identify positive clones.

2.3.3 Preparation of bacterial cell extracts.

LB-broth (10 ml, section 2.3.1) was inoculated with a single DH5α bacterial colony and grown overnight at 37°C / 250 rpm. The overnight culture was then used to seed 100 ml of LB-broth to an optical density (OD_{600}) of 0.2 and incubated at 37°C with shaking until an OD_{600} of 0.6 was obtained. The culture was then centrifuged at 5,000 x g for 5 min at 4°C and the pellet was resuspended in 50 μl of ice cold buffer I (section 2.1.4) per 1 ml culture and sonicated in 3 x 10 s bursts. Immediately after sonication PMSF (phenylmethylesulfonylflouride, Sigma, 8.7 mg/ml in ethanol) was added to a final concentration of 0.5 mM and the samples were centrifuged at 13,000 x g for 10 min at 4°C. The supernatant was removed to a fresh tube and the samples stored on ice.

2.4 Protein protocols

2.4.1 Bradford assay for total protein determination

Protein standards (40 µl; BSA in ddH_2O) ranging in concentration from 0 - 0.1 mg/ml (increments of 0.01 mg) were pipetted in triplicate wells on a 96-well plate. Samples were pipetted (1 : 20, 1 : 40 and 1 : 80 dilutions in 40 µl) in triplicate on to the same plate. BioRad/CBG reagent (200 µl) was added to each well and the resultant colour change was measured at 620 nm using a TECAN plate reader (TECAN, Reading, UK). The protein concentration of each sample was determined from the standard curve.

2.4.2 SDS-Polyacrylamide gel electrophoresis

SDS-PAGE was used to separate proteins by molecular weight. The BioRad mini protein gel apparatus (BioRad) was used in this protocol. Protein samples were mixed with SDS-PAGE loading buffer (section 2.1.4) and heated to 95°C for 5 min, then centrifuged briefly to collect the sample. Protein samples and Precision Plus Protein size markers (BioRad) were loaded onto a 0.75 mm thick SDS-PAGE gel. This consisted of a 10% resolving gel (2.7 ml ProtoGel (National Diagnostics, Hull, UK), 2 ml 4 X Lower Tris (section 2.1.4), 60 µl 10% APS (Sigma), 6 µl TEMED (Sigma) and ddH_2O to 8 ml) lying below a 4.5% stacking gel (1 ml ProtoGel, 1.5 ml 4 X Upper Tris (section 2.1.4), 60 µl 10% APS, 6 µl TEMED and ddH_2O to 5 ml). Electrophoresis was carried out in 1 X TBS (Section 2.1.4) at a constant 200 V for 45 min.

2.4.3 Western blot analysis

Proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane (Hybond-C-super, Amersham Biosciences, Amersham, UK) by electroblotting, using a BioRad Mini Trans-blot apparatus. Transfer was carried out at 100 V for 1 h in western transfer buffer (section 2.1.4). After blotting the membrane was air dried and stored between two sheets of Whatmann 3 MM paper at 4°C until needed.

For processing, the membranes were washed with TBST (section 2.1.4) for 5 min and then blocked with 5% non-fat milk powder (Marvel made up in TBST) for 1 h. Membranes were then washed twice in TBST before adding the primary rabbit polyclonal His probe (H15) IgG antibody (1 : 2000 dilution, Santa-Cruz Biotechnology, Santa Cruz, California, USA) in 0.5% Marvel and incubating for a further 1 h. Membranes were then washed three times in TBST and incubated with secondary goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (1 : 2000 dilution; DakoCytomation, Ely, Cambs., UK) for a further 1 h. Membranes were then washed three times in TBST to remove any unbound antibodies. Bound secondary antibody was detected using Chemiluminescence blotting substrate (Roche Diagnostics GmbH) according to the manufacturer's instructions. Blots were exposed to X-ray film (Fujifilm, Bedford, UK) for between 10 s and 5 min and the film was developed manually under safe light, scanned (Epson Expression 1600 Pro), analysed with Adobe Photoshop version 6.0 and stored as a Tiff file.

2.5 Cell culture work

2.5.1 Tissue culture cell lines

Listed below are the various epithelial cell lines used in this project.

Cell line	Derivation	Source
H460	Lung adenocarcinoma	ATCC (Virginia, USA)
Hek293	Embryonic kidney	M. Watson (PICR, Manchester, UK)
HepG2	Hepatocellular carcinoma	ATCC
LNCaP	Metastatic prostate lymph node carcinoma	C. Hart (PICR, Manchester, UK)
MCF-7	Metastatic breast adenocarcinoma	ATCC
PC-3	Grade IV metastatic prostate prostate bone adenocarcinoma	ATCC
PC-3AR	Grade IV metastatic prostate bone adenocarcinoma stably transfected with the androgen receptor.	K. Burnstein (University of Miami, Florida, USA)
PNT2C2	Normal prostatic tissue	N. Maitland (University of York, York, UK)

2.5.2 Maintenance and subculture of cell lines

MCF-7, HepG2 and H460 cells were maintained in Roswell Park Memorial Institute 1640 medium (RPMI; Gibco) supplemented with 10% foetal calf serum (FCS; PAA Laboratories, Somerset, UK) and 2 mM L-glutamine (Sigma). LNCaP cells were maintained in RPMI complete medium supplemented with 1% v/v Hepes Buffer (Sigma) and 1% v/v Sodium Pyruvate (Sigma). PC-3AR cells were also maintained in RPMI complete medium supplemented with 350 μg/ml G418 (Sigma). PC-3 cells were maintained as adherent monolayer cultures in Hams nutrient medium F12 (Hams-F12; PAA Laboratories Ltd) supplemented with 7% FCS and 2 mM L-glutamine. Hek293 cells were maintained in Dulbecco's Modification of Eagles Medium:F12 (DMEM:F12; Invitrogen) supplemented with 10% FCS and 2 mM L-glutamine. From here onwards these media will be referred to as 'complete medium'. All cells were grown in T75 culture flasks at 37°C in humidified air containing 5% CO_2. Cells were routinely tested for mycoplasma infections by PCR using the VenorGeM mycoplasma detection kit according to the manufacturer's instructions (Minerva Biolabs GmbH, Berlin, Germany).

Cells were passaged (subcultured) by removing the culture medium and washing the monolayer with PBS. To harvest the cells, 1 x trypsin-EDTA (Sigma) was added to the culture flask and incubated at 37°C until the cells detached. Media was then added to neutralise the trypsin-EDTA and to wash the cells from the bottom of the flask. The cells were then centrifuged at 403 x g for 5 min at RT and resuspended in compete medium. H460, Hek293, and HepG2 cells were diluted 1 in 20, MCF-7 and PNT2C2 diluted 1 in 10, PC-3 and PC-3AR diluted 1 in 5 and LNCaP cells diluted 1 in 3 and used to re-seed complete medium in a new T-75 flask. The cells were then incubated as described above. All cell manipulations were carried out in a class II microbiology safety cabinet.

2.5.3 Charcoal stripped serum

For reducing the effects of hormones in media, charcoal stripped serum was used instead of FCS. Five g charcoal (Sigma) was added to 500 ml FCS and incubated at 50°C / 150 rpm for 1 h. The FCS/charcoal solution was then centrifuged at 2197 x g for 15 min at RT and the FCS transferred to a clean tube. In a class II microbiology safety cabinet the FCS/charcoal solution was then filtered through 0.4 μm and then 0.2 μm bottle top filters to remove all traces of charcoal and filter sterilise. The charcoal stripped serum was then stored at -20°C in 50ml aliquots.

2.5.4 Preparation of human cell extracts

Cell extracts were prepared for SDS-PAGE electrophoresis and western blotting. For extract preparation, two 6 well plates were plated with cells, transfected as described in section 2.6.1 and then incubated for 24 h at 37°C. The cells were then harvested (see section 2.5.1), pooled, centrifuged at 403 x g for 5 min at RT and washed with PBS. The cell pellet was stored at -20°C until required.

To isolate protein the cell pellet was resuspended in 400 µl of ice cold buffer I (section 2.1.4) with 0.5 mM leupeptin (Sigma) and 0.5 mM PMSF (Sigma). The cells were immediately sonicated using a Heat System XL ultrasonic processor (Misonix Inc., New York, USA), set at 4.5, using a 4 mm probe, for 3 x 5 s bursts, incubating on ice in-between each burst. The samples were centrifuged at 13,000 x g for 10 min at 4°C. The supernatant was removed to a fresh tube and the samples stored on ice. A Bradford assay (see section 2.4.1) was then performed to determine protein concentration.

2.6 Reporter gene assays

2.6.1 Transfection of cells

Transient transfections were carried out using Genejuice transfection reagent (Novagen/Merck Biosciences Ltd, Nottingham, UK) according to the manufacturer's instructions. A T-75 flask of human cells was grown to confluence and the cells harvested and counted using a haemocytometer. Triplicate 6 well plates were seeded with 2 x 10^5 cells per well in complete medium and incubated overnight at 37°C. The Genejuice:plasmid DNA mixture was then added drop-wise to the cells, distributed evenly by rocking and then incubated for 8 h at 37°C after which the medium was renewed.

2.6.2 Irradiation of cells

Irradiation of the cells was performed approximately 8 h after transfection. The cells were irradiated at 37°C using a ^{60}cobalt γ-ray source at a dose rate of 0.66 Gy/min until the required dose was achieved. In all experiments mock-irradiated controls were also used. Following radiation treatment, cells were re-incubated until the time of the reporter gene assay, approximately 40 h later.

2.6.3 FACS analysis

Cells were washed with PBS, and harvested as described in section 2.5.1 into 5 ml falcon tubes. The samples were then centrifuged at 403 x g for 3 min and re-suspended in 300 µl PBS for FACS analysis. For each sample 20,000 live cells were analysed on a Becton Dickinson FACScan flow cytometer (BD Biosciences) with an excitation wavelength of 488 nm to read both Green Fluorescent Protein (GFP, emission maxima 507 nm) and Cyan Fluorescent Protein (CyFP, emission maxima 475 nm). The level of GFP or CyFP expression in live cells was determined using the Becton Dickinson CellQuest programme.

Briefly, the distribution of GFP/CyFP fluorescence in the cell population was plotted against the cell number on a 4-log linear scale. Cells that exhibited GFP fluorescence above a specific intensity threshold of 10^2 (FL1-H 10^2 to 10^4) were considered to show promoter activity (see figure 2.1 for an example). The median GFP intensity or percent of GFP expressing cells within this gate was then determined. To calculate CyFP expression the total percentage of cells gated in R2 was measured (see figure 2.2). This is because the CyFP signal is neither as bright nor the protein as photostable as GFP. The fold increase in GFP/CyFP expression for each plasmid was calculated by dividing the average percentage/median intensity of GFP/CyFP positive cells in three test samples (e.g. irradiated)

by the corresponding value for the control samples (i.e. mock irradiated) transfected with the same plasmid.

Figure 2.1 Example of the method used for gating live cells exhibiting GFP fluorescence. A: R1 gate of live cells. B: R2 gate of cells positive for GFP expression. C: M2 gate of live cells expressing GFP above the specific intensity threshold of FL1-H 10^2.

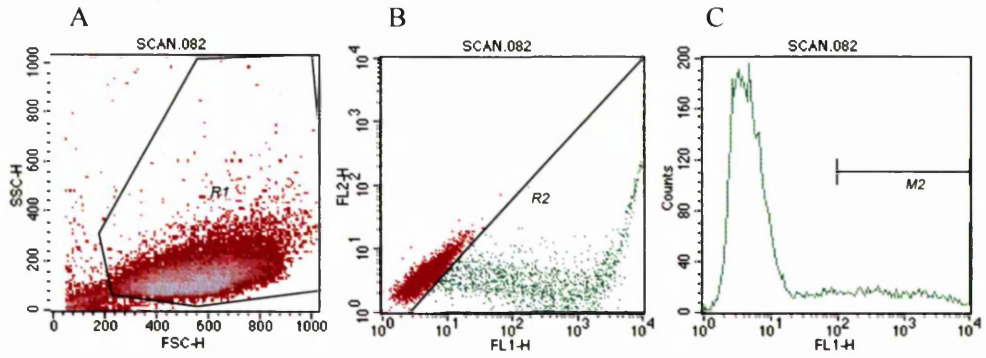
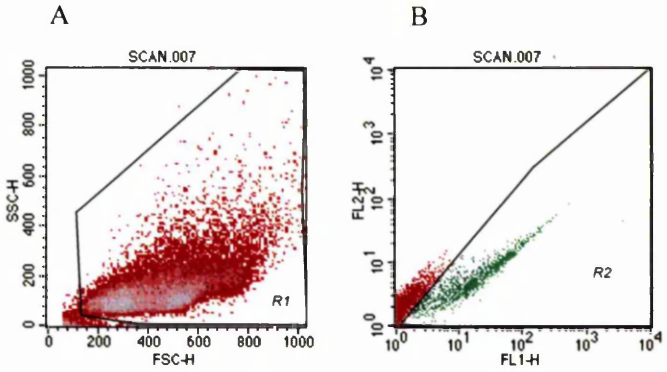


Figure 2.2 Example of the method used for gating live cells exhibiting CyFP fluorescence. A: R1 gate of live cells. B: R2 gate of cells positive for CyFP expression.



2.6.4 MTT assay

It is generally accepted that clonogenic assays are the best measure of true cell viability. However, such assays are very time consuming and have low throughput and due to the number of variables that needed to be tested, a more time-efficient assay was required. Therefore in order to determine cell growth inhibition after the administration of toxic agents to un-transfected and transfected cells, an assay was used based on the ability of a mitochondrial dehydrogenase enzyme in metabolising cells to cleave the tetrazolium rings of the pale yellow substrate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and form dark blue formazan crystals, which are largely impermeable to cell membranes, thus resulting in its accumulation only within healthy cells. Solubilisation of the cells by the addition DMSO results in the liberation of the crystals from the cells which then dissolve in the DMSO. The colour absorbance at 595 nm can then be measured by using a multiwell scanning spectrophotometer (TECAN reader) and the change in this absorbance is taken as an indicator of growth.

Duplicate 6 well plates were seeded with 2 x 10^5 cells per well in complete medium and incubated overnight at 37°C. The cells were then transfected as in section 2.6.1. After transfection, gancyclovir (GCV 0-1000 μg/ml in 0.1 M HCl, Sigma) or 5-(1-Aziridinyl)-2,4-dinitrobenzamide (CB1954 0-1000 μM in DMSO, Sigma) was added to the wells and the plates re-incubated at 37°C for 2 to 6 days depending on cell line and drug concentration. At the appropriate time, media was added to each well to a total volume of 6 ml and 1.5 ml MTT (3 mg/ml MTT in PBS, stored at 4°C, Sigma) was added and the plates incubated at 37°C for 3 h. For adherent cells, the media/MTT was removed and the formazan crystals dissolved in 2700 μl DMSO (Sigma). For non-adherent and LNCaP cells, which detach from the plate upon addition of MTT, the cells were pelleted by centrifuging at 1600 x g for 5 min, before dissolving in DMSO. The crystals were then diluted (1 : 2 to 1 : 4) with DMSO, to within the parameters of the plate reader (OD 0.1 to 2). Each well was then transferred in 200 μl aliquots to 8 wells of a 96 well plate and the OD_{595} read using a TECAN plate reader. The average OD_{595} of 8 wells was then calculated and averaged with the values obtained from the duplicate well in the 6 well plate. Due to clumping of LNCaP cells it was not possible to accurately plate 2 x 10^5 cells per well. Consequently, while within an experiment, equal numbers of cells were plated in each well, it was not possible to accurately plate exactly the same number in repeat experiments.

Therefore, in order to compare the inhibition of cell growth between repeat experiments, the % difference in cell growth with (w/) and without (w/o) drug in each individual experiment was calculated using the formula:

$$\% \text{ Cell Growth Inhibition} = \frac{\text{OD w/ Drug}}{\text{OD w/o Drug}} \text{ X } 100$$

All data analysis, including calculation of standard error (SE) and Student's two tailed T test assuming equal variance, was performed using Microsoft Office Excel 2003.

Chapter 3

3.0 Results: Radiation responsive promoters

3.1 Introduction

Initially the concept of controlling the molecular switch using radiation responsive enhancer/promoters was revisited. In order to assess the levels of transcription generated from radio-responsive enhancers, four different CArG element configurations and the wild type Egr-1 enhancer were engineered into pCI-neo (Promega; see figure 3.1 and the appendix section 8.2), upstream of the reporter gene GFP. These elements replaced the immediate/early enhancer of cytomegalovirus (CMV enhancer), but retained the basal 86 bp CMV promoter containing the transcription start site and TATA box. In addition, an enhancer-less construct was made (pLinker-CMV-GFP) in which the CMV enhancer was replaced with a short linker oligonucleotide (GACTAAAAAG). The four CArG configurations and linker fragment are shown in table 3.1. E4 contains four consecutive CArG elements of the sequence CCTTATTTGG. E4S is similar but an 8bp spacer (AGTTACGC) separates each CArG element. E6ns2 and E9ns2 contain 6 and 9 consecutive CArG elements of the sequence CCATATAAGG, believed to have a higher affinity for serum response factor (SRF) binding (Soulez *et al.*, 1996; Scott *et al.*, 2002). The wild type Egr-1 enhancer comprised of nucleotides -695 to -154 relative to the start of transcription of the Egr-1 gene; NCBI accession number AJ245926 (Sakamoto *et al.*, 1991; Marples *et al.*, 2000; Schwachtgen *et al.*, 2000).

Figure 3.1 pCI-neo vector map and multiple cloning site (MCS). pCI-neo was used as the host vector for testing radiation responsive enhancers. EcoRI/NotI restriction sites, used for the insertion of GFP, and the BglII/SgfI sites, used to insert the radiation responsive enhancers and linker thus replacing the CMV enhancer, are shown in blue. This vector also contains a chimeric intron between the 86 bp CMV promoter and MCS, an Amp^r marker for selection in *E. coli* and a neomycin phosphotransferase (neo) marker for selection in mammalian cells. A more detailed map is shown in the appendix section 8.2.

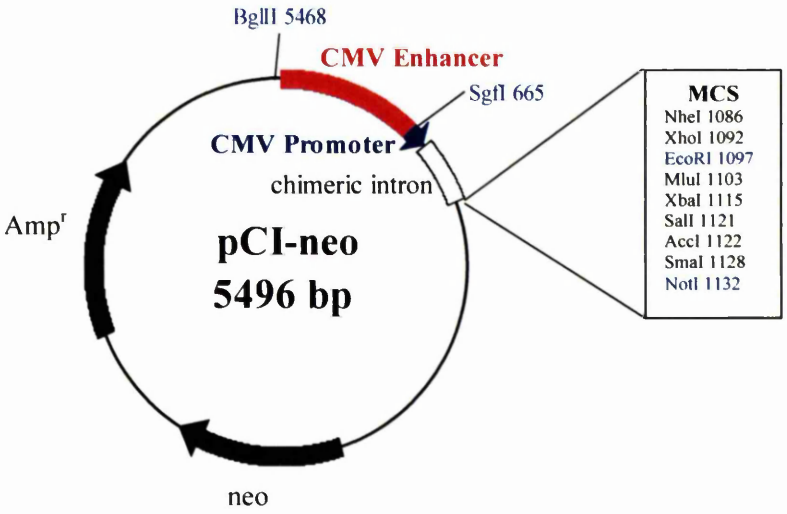


Table 3.1 The four CArG configurations; E4, E4S, E6ns2 and E9ns2, linker oligonucleotides and primers for PCR amplification of WT Egr-1. The CArG elements are highlighted in red and the digested/whole BglII and SgfI sites, required for cloning into pCI-neo, in light and dark blue respectively.

Synthetic enhancer	Sequence		Ref.
E4	Sense	5′ GATCT CCTTATTTGG CCTTATTTGG CCTTATTTGG CCTTATTTGG CGAT 3′	Marples *et al.*, 2000
	Anti-sense	5′ CG CCAAATAAGG CCAAATAAGG CCAAATAAGG CCAAATAAGG A 3′	
E4S	Sense	5′ GATCT CCTTATTTGG AGTTACGC CCTTATTTGG AGTTACGC CCTTATTTGG AGTTACGC CCTTATTTGG CGAT 3′	Scott *et al.*, 2002
	Anti-sense	5′ CG CCAAATAAGG GCGTAACT CCAAATAAGG GCGTAACT CCAAATAAGG GCGTAACT CCAAATAAGG A 3′	
E6ns2	Sense	5′ GATCT CCATATAAGG CCATATAAGG CCATATAAGG CCATATAAGG CCATATAAGG CCATATAAGG GCGAT 3′	Scott *et al.*, 2002
	Anti-sense	5′ CGC CCTTATATGG CCTTATATGG CCTTATATGG CCTTATATGG CCTTATATGG CCTTATATGG A 3′	
E9ns2	Sense	5′ GATCT CCATATAAGG CCATATAAGG CCATATAAGG CCATATAAGG CCATATAAGG CCATATAAGG CCATATAAGG GCGAT 3′	Scott *et al.*, 2002
	Anti-sense	5′ CGC CCTTATATGG CCTTATATGG CCTTATATGG CCTTATATGG CCTTATATGG CCTTATATGG CCTTATATGG A 3′	
Linker	Sense	5′ GATCT GACTAAAAAG GCGAT 3′	New England Biolabs
	Anti-sense	5′ CGC CTTTTTAGTC A 3′	
WT Egr-1	Sense	5′ GCAGATCTCAGCCGCTCCTCCCCCGC 3′	NCBI AJ245926
	Anti-sense	5′ CGGCGATCGCGCTGGGATCTCTCGCGACTCC 3′	

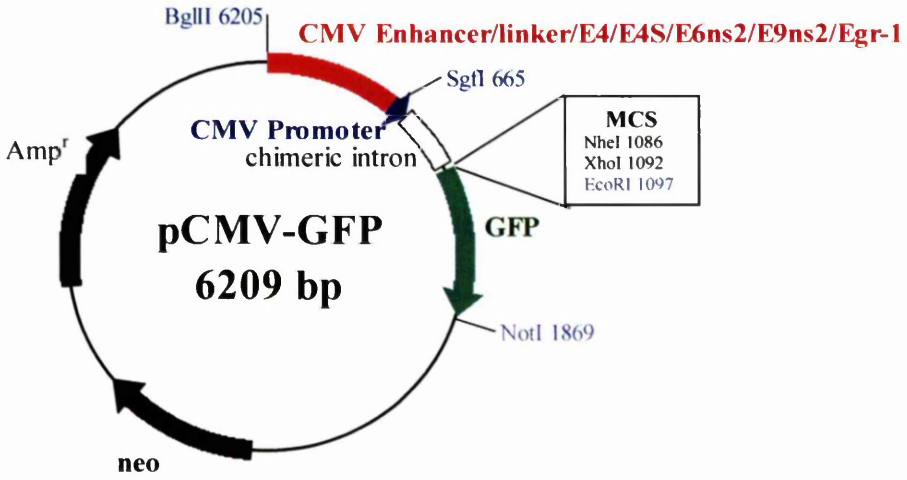
3.2 Creation of vectors

3.2.1 Control vectors pCMV-GFP and pLinker-CMV-GFP

pCMV-GFP was constructed to act both as a positive control for the irradiation experiments and also to act as a template for the addition of the putative radio-responsive enhancer regions in place of the CMV enhancer. GFP was excised from pEGFP-1 (see appendix section 8.3; Clontech) by EcoR1/Not1 restriction digest and cloned into the pCI-neo (see figure 3.1) multiple cloning site (MCS) using EcoR1/Not1 restriction sites. Correct insertion of GFP was initially confirmed by PCR and restriction digest and subsequently by DNA sequencing. The pCMV-GFP construct is shown in figure 3.2.

pLinker-CMV-GFP is an enhancer-less construct designed to act as a negative control for the irradiation experiments. The linker was generated from annealed synthetic oligonucleotides (see table 2.1) and introduced into pCMV-GFP using BglII/SgfI restriction sites thus replacing the whole of the CMV enhancer region leaving only the CMV promoter (see figure 3.2). Correct insertion of the linker was confirmed by PCR, restriction digest and DNA sequencing.

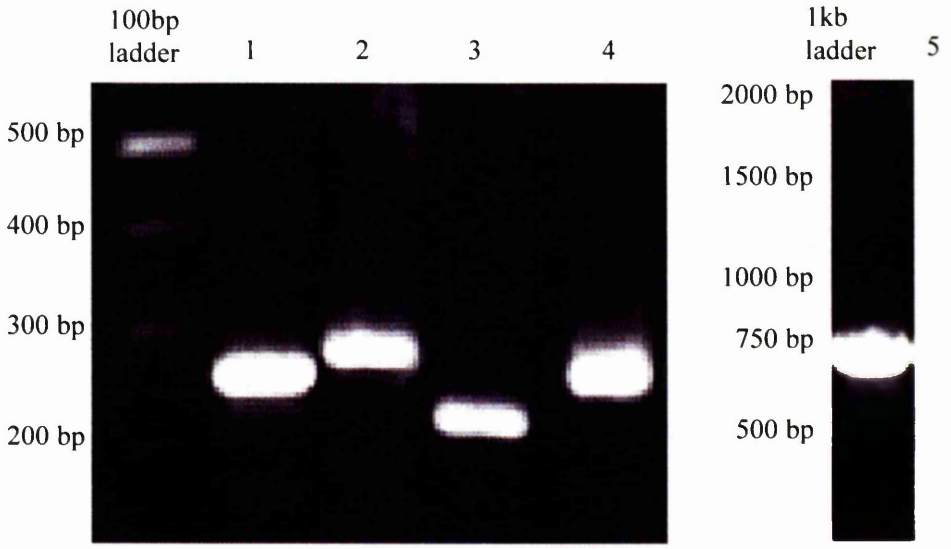
Figure 3.2 Generic vector map and MCS of pCMV-GFP, pLinker-CMV-GFP and the radiation responsive constructs. GFP was inserted into the MCS of pCI-neo using EcoR1/Not1 restriction sites (blue). To create pLinker-CMV-GFP, pE4-GFP, pE4S-GFP, pE6ns2-GFP, pE9ns2-GFP and pEgr-1-GFP, the linker and radiation responsive oligonucleotides were inserted into pCMV-GFP using BglII/SgfI restriction sites (blue) thus replacing the CMV enhancer. The vectors also contain a chimeric intron between the CMV promoter and MCS, an Amp^r marker for selection in *E. coli* and a neo marker for selection in mammalian cells.



3.2.2 Radiation responsive constructs

The radioresponsive elements E4, E4S, E6ns2 and E9ns2 were generated by annealing the appropriate synthetic oligonucleotides listed in table 3.1. The Egr-1 enhancer was generated by PCR amplification from pDRIVE03-EGR-1(h) v02 (see appendix section 8.4; InvivoGen) with primers designed to add BglII and SgfI restriction sites at the 5′ and 3′ ends respectively (see table 3.1). These elements and the Egr-1 enhancer were then introduced into the pCMV-GFP construct using BglII/SgfI restriction sites, replacing the CMV IE enhancer and generating five radioresponsive promoters pE4-GFP, pE4S-GFP, pE6ns2-GFP, pE9ns2-GFP and pEgr-1-GFP (see figure 3.2). Correct insertion of the radiation responsive elements was initially confirmed by PCR and agarose gel electrophoresis (see figure 3.3) and subsequently by DNA sequencing.

Figure 3.3 Agarose gel electrophoresis of PCR products from radiation responsive constructs. Lane 1; pE4S-GFP (261 bp), lane 2 pE9ns2-GFP (289 bp), lane 3 pE4-GFP (238 bp), lane 4 pE6ns2-GFP (259 bp) and lane 5 pEgr-1-GFP (780 bp). 100 bp and 1 kb ladders were used for determination of the size of the PCR products.

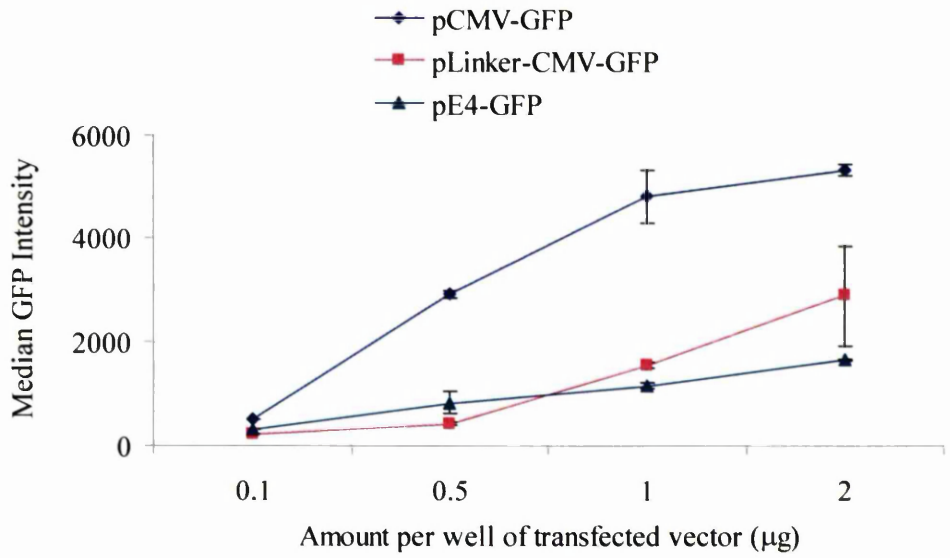


3.3 Assessment of the radiation responsive constructs

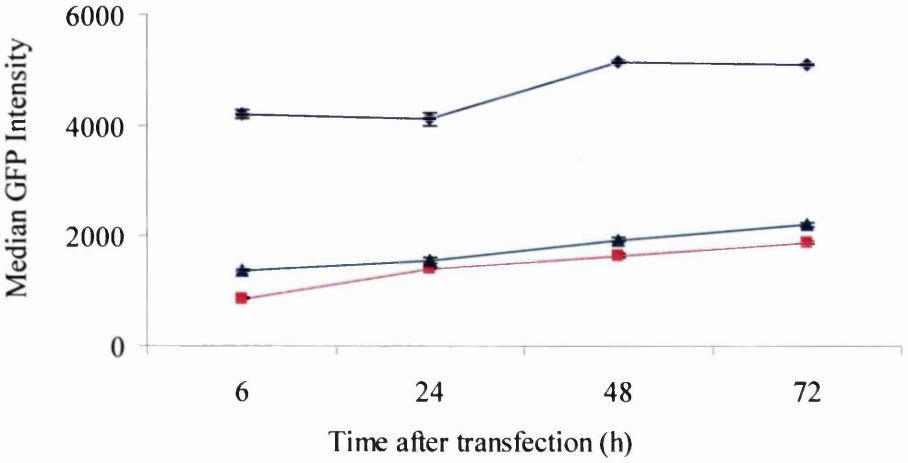
Initially the conditions of transfection and growth of the cells prior to irradiation were optimised. It was found that cells plated at a density of 2 x 10^5 cells/well (in a 6 well plate) and transfected with 1 μg DNA for 8 h was ideal for maximum transfection efficiency as determined by FACS analysis (see section 2.6.3 and figure 3.4 A). The cells were then irradiated with 5 Gy (see section 2.6.2); a dose that has been reported to be sufficient for activation of the radio-responsive promoters without causing excessive cell damage (Marples *et al.*, 2000). FACS analysis was then performed approximately 48 h after irradiation as this time point was optimal for maximum GFP expression (see figure 3.4 B). These conditions were maintained throughout subsequent experiments.

Figure 3.4 – Optimisation of GFP reporter gene expression. A) MCF-7 cells transfected with varying concentrations as indicated of the GFP reporter gene constructs pCMV-GFP, pLinker-CMV-GFP or pE4-GFP and FACS analysed 48 h later. B) GFP reporter gene assay performed 8 h, 24 h, 48 h and 72 h after irradiation of MCF-7 cells transfected with 1 μg of pCMV-GFP, pLinker-CMV-GFP and pE4-GFP. The vertical bars represent the standard error (SE) between triplicate samples.

A



B



3.3.1 Assessment of promoters in MCF-7, PC-3 and LNCaP cells

In the first series of experiments to assess the effectiveness of the radiation responsive promoters *in vitro,* MCF-7, PC-3 and LNCaP cells were cultured in oxic conditions (air i.e. 20% O_2) and transfected in triplicate with pCMV-GFP, pLinker-CMV-GFP, pE4-GFP, pE4S-GFP, pE6ns2-GFP, pE9ns2-GFP and pEgr-I-GFP (see section 3.2.1 and 3.2.2).

Figure 3.4 shows the median intensity of GFP expression for mock-irradiated and irradiated MCF-7 (A), PC-3 (B) and LNCaP (C) cells transiently transfected with the various constructs. In the absence of irradiation the removal of the enhancer region in pLinker-CMV-GFP led to a decrease in GFP expression of 67.5%, 37.7% and 91.8% in MCF-7, PC-3 and LNCaP cells respectively, when compared to pCMV-mediated GFP expression. Subsequent insertion of the radiation responsive enhancers into pLinker-CMV-GFP had either no significant effect, or decreased or increased GFP expression depending on the cell line (table 3.2). Interestingly, when compared with Linker-CMV mediated expression all four radio-responsive enhancers increased GFP expression in LNCaP cells by 20%, 4%, 7% and 2% for E4, E4S, E6ns2 and E9ns2 respectively. In contrast, increases in GFP expression of 17% and 55% were only seen with the Egr-1 enhancer in MCF-7 cells and the E4S enhancer in PC-3 cells respectively. Moreover, GFP expression induced by the E4S enhancer in PC-3 cells is comparable to that induced by the constitutively active CMV enhancer/promoter in pCMV-GFP. With the exception of Egr-1 in MCF-7 cells, E4S in PC-3 cells and E4 in LNCaP cells, many of these changes in GFP expression, while statistically significant, may not be biologically relevant. Interestingly, the enhancers E4, E6ns2 and E9ns2 markedly reduced GFP expression compared to Linker-CMV mediated GFP expression by 22%, 17% and 32% respectively (see table 3.2).

Irradiation of MCF-7 cells transfected with all seven constructs led to a significant reduction in GFP expression in comparison with the corresponding mock-irradiated controls (figure 3.5A). This was the equivalent to a fold change in GFP expression between mock irradiated and irradiated transfected cells of between 0.7 and 0.9 (figure 3.6). Irradiation of transfected PC-3 and LNCaP cell lines had no significant effect on GFP expression (figure 3.5B and C). The exception to this was LNCaP cells transfected with pE4-GFP in which GFP expression was enhanced 1.2 fold compared to mock irradiated cells (figure 3.6).

Figure 3.5 GFP expression in mock-irradiated (blue) and irradiated (red) A) MCF-7, B) PC-3 and C) LNCaP cells, cultured under oxic conditions. Before irradiation with a single dose of 5 Gy cells were transfected in triplicate with plasmids containing the GFP reporter gene controlled by the CMV enhancer (pCMV-GFP), the CMV promoter alone (pLinker-CMV-GFP) or the synthetic radiation responsive enhancers (pE4-GFP, pE4S-GFP, pE6ns2-GFP, pE9ns2-GFP and pEgr-1-GFP). The vertical bars represent the standard error (SE) between triplicate samples. * represents a significant difference compared to pLinker-CMV-GFP ($P < 0.01$ Two-tailed students T test assuming equal variance).

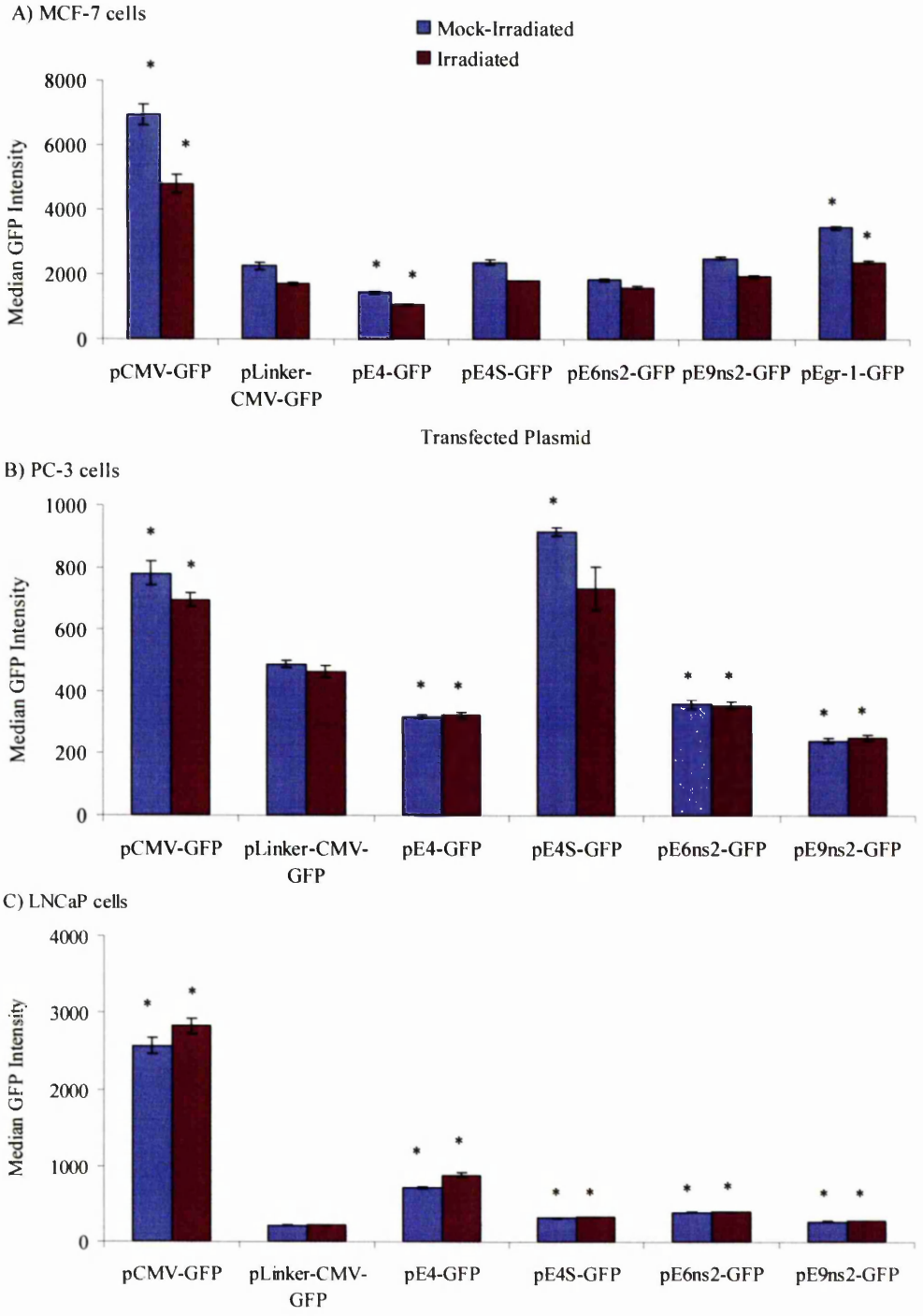
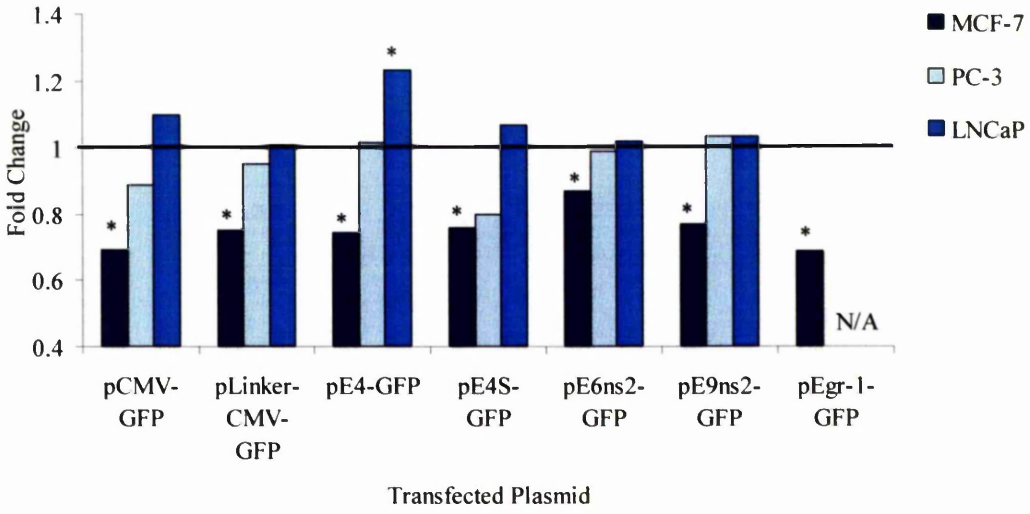


Table 3.2 Pre-irradiation changes in GFP expression induced by the insertion of the radiation responsive enhancers: E4, E4S, E6ns2, E9ns2 and Egr-1, into the enhancer-less construct pLinker-CMV-GFP ($P < 0.01$). The change in GFP expression is presented as a % of the expression from the CMV enhancer/ promoter which represents 100% activity. Marked increases in GFP expression are highlighted in red. N/S no significant difference, N/A data not available.

			Radiation responsive enhancers (versus Linker-CMV)				
Cell line	CMV	Linker-CMV (vs CMV)	E4	E4S	E6ns2	E9ns2	Egr-1
MCF-7	100%	33%	- 12%	N/S	N/S	N/S	+ 17%
PC-3	100%	62%	- 22%	+ 55%	- 17%	- 32%	N/A
LNCaP	100%	8%	+ 20%	+ 4%	+ 7%	+ 2%	N/A

Figure 3.6 Fold change in GFP expression induced by irradiation of MCF-7 (dark blue), PC-3 (Pale blue) and LNCaP (turquoise) cells transfected in triplicate with pCMV-GFP, pLinker-CMV-GFP, pE4-GFP, pE4S-GFP, pE6ns2-GFP, pE9ns2-GFP and pEgr-1-GFP. * represents a significant fold change in GFP expression between mock-irradiated and irradiated samples ($P < 0.01$ Two-tailed students T test assuming equal variance). N/A data not available.



Except in one unique combination, that of pE4-GFP in LNCaP cells, these results indicate that in MCF-7, PC-3 and LNCaP cells the five radiation responsive enhancers tested; E4, E4S, E6ns2, E9ns2 and Egr-1 were neither consistent in their effects on the expression of GFP compared to a construct containing no enhancer, nor were they responsive to irradiation. The experiments conducted here were analogous to those carried out by Marples *et al.*, (2000; 2002) and Scott *et al.*, (2000; 2002) in which E4, E4S, E6ns2, E9ns2 and Egr-1 radiation responsive enhancers were tested in MCF-7 cells. Their data indicates that synthetic and wild type radiation responsive enhancers could induce between 1.5 and 3 fold increase in the expression of GFP in response to 5 Gy irradiation. In support of this work other groups using the Egr-1 enhancer/promoter in vitro have shown an increase in the expression of downstream genes of between 2.5 and 17 fold in hepatoma (HepG2, Huh7 and PLC/PRF/5), leukaemia (HL525), embryonic kidney (HEK293) glioma (U87, 9L) and glioblastoma (LNZ308) cells exposed to between 3 and 20 Gy irradiation (Weichselbaum *et al.*, 1994b; Joki *et al.*, 1995; Kawashita *et al.*, 1999; Meyer *et al.*, 2002; Hsu *et al.*, 2003; Quinones *et al.*, 2003). In contrast, the data presented here shows that only the E4 enhancer in LNCaP cells is responsive to irradiation leading to a potentially biologically insignificant fold increase in GFP expression of 1.2 after exposure to 5 Gy. GFP expression from MCF-7 and PC-3 cells transfected with synthetic and wild type Egr-1 enhancer constructs was either unchanged or significantly reduced upon irradiation.

The high level of GFP expression from pLinker-CMV-GFP indicates the effectiveness of the CMV promoter alone in inducing GFP expression, in particular in PC-3 and MCF-7 cells. This suggests that there may be transcription factors present in these cells that operate very effectively from the promoter alone, or perhaps bind to unidentified sequences present upstream of the enhancer/promoter region that further enhance the expression of GFP. Interestingly, the same promoter; CMV, was used in conjunction with the radiation responsive promoters in the Scott and Marples series of papers (Marples *et al.*, 2000; Scott *et al.*, 2000; Greco *et al.*, 2002b; Marples *et al.*, 2002; Scott *et al.*, 2002; Greco *et al.*, 2005b). However, in their studies they did not include an enhancer-less promoter as a control, which, in the context of investigating radiation-responsive promoters, would have provided a more appropriate indication of background fluorescence.

It was also observed that GFP expression was enhanced above that seen with pLinker-CMV-GFP background fluorescence under mock-irradiated conditions by all four radiation responsive promoters in LNCaP cells, as well as by the Egr-1 enhancer in MCF-7 cells and the E4S enhancer in PC-3 cells. Taken together with the lack of any consistent effect of

radiation, this suggests that these radiation responsive enhancer/promoters may already be fully active in the absence of irradiation. The most extreme example of this was the pE4S-GFP construct in PC-3 cells which produced levels of GFP expression that were higher than the full-length CMV IE enhancer promoter. However, it is perhaps worth noting that PC-3 cells were the most effective in GFP expression from the promoter-less construct, being about 40% less than CMV. Interestingly, this was also observed to some extent by Scott *et al.,* 2002 in which the radiation responsive enhancers; Egr-1, E4 and E9ns2, induced GFP expression of between 9% and 19% that of the CMV enhancer/promoter in the absence of irradiation. Other publications by the same group do not allude to or reveal the levels of background fluorescence as data is presented as fold increases in GFP expression due to irradiation (Marples *et al.,* 2000; Scott *et al.,* 2000; Greco *et al.,* 2002b; Marples *et al.,* 2002; Scott *et al.,* 2002; Greco *et al.,* 2005b).

After completion of this work two other groups have published data in support of these findings. Schmidt *et al.,* (2004), found that in head and neck carcinoma cell lines (HLaC79 and FaDu), the Egr-1 enhancer induced only weak reporter gene induction upon irradiation and that there was high background expression in non-irradiated control cells. Significant gene expression was also observed from the Egr-1 enhancer and promoter in rat rhabdomyosarcoma (R1H) cells when used to drive GFP expression in the absence of irradiation. While this could be significantly reduced by the insertion of insulating PolyA signals upstream and downstream of the expression cassette, it was not completely ablated, indicating an intrinsic leakiness of the Egr-1 enhancer/promoter (Anton *et al.,* 2005).

It was argued that expression in the absence of radiation may have been caused by cell growth conditions and if this could be reduced, radiation may have resulted in induction. In attempts to reduce the levels of background fluorescence experiments were conducted in which the cells were cultured under hypoxic (section 3.3.2) and/or serum starved (section 3.3.3) conditions. Also, because of the possibility that sequences upstream if the promoter were effecting expression, cells were transfected with a variety of linearised plasmids (section 3.3.4).

3.3.2 Assessment of promoters in MCF-7, PC-3 and LNCaP cells under low oxygen conditions

High oxygen levels are known to induce oxidative stress in cells, leading to the production of reactive oxygen intermediates, which in turn are known to activate CArG elements (Datta *et al.*, 1993; Hallahan 1996). In order to determine whether the relatively high oxygen levels used in the initial experiments were activating the radiation responsive promoters in the absence of irradiation, the above experiments were repeated under low oxygen conditions. MCF-7 cells were routinely cultured in 5% O_2 and, as before, transfected in triplicate with the radiation responsive promoters: pE4-GFP, pE4S-GFP, pE6ns2-GFP, pE9ns2-GFP, and the 2 control vectors: pCMV-GFP and pLinker-CMV-GFP. The experiment was repeated using PC-3 and LNCaP cells.

Figure 3.7 shows the median intensity of GFP expression for mock-irradiated and irradiated MCF-7 (A), PC-3 (B) and LNCaP (C) cells grown under low oxygen conditions and transiently transfected with the various constructs. Interestingly, when compared to culturing under low oxygen versus oxic conditions, median GFP intensity induced by pCMV-GFP in MCF-7 and LNCaP cells reduced from 7000 to 4000 and 2500 to 1500 respectively. In contrast, median GFP expression of pCMV-GFP transfected PC-3 cells increased from 780 to 2250. In the absence of radiation the removal of the enhancer region in pLinker-CMV-GFP led to a decrease in GFP expression of 62%, 58% and 84% in MCF-7, PC-3 and LNCaP cells respectively, when compared to pCMV-mediated GFP expression. Similar to the transfection of LNCaP cells under oxic conditions, the insertion of the radiation responsive enhancers E4S, E6ns2 and E9ns2 into pLinker-CMV-GFP increased GFP expression by 28%, 3% and 4% respectively, however in contrast E4 decreased GFP expression by 3%, shown in figure 3.7 and table 3.3. In addition, in PC-3 cells, the E4S enhancer had a positive effect on GFP expression, increasing it by 25%, while the rest of the radiation responsive enhancers E4, E6ns2 and E9ns2 induced a significant reduction in GFP expression compared to pLinker-CMV-GFP of 16%, 10% and 29% respectively. With the exception of E4S in both PC-3 and LNCaP cells, many of these enhancements in GFP expression, while statistically significant, may not be biologically relevant. None of the radiation responsive enhancers increased GFP expression in MCF-7 cells.

Irradiation of all six constructs in MCF-7, PC-3 and LNCaP cells had no significant effect on GFP expression compared with the corresponding mock-irradiated controls (see figure 3.6 and 3.7). The exceptions to this were MCF-7 cells transfected with pLinker-CMV-GFP and pE4S-GFP and LNCaP cells transfected with pCMV-GFP in which GFP expression was enhanced 1.14, 1.14 and 1.2 fold, respectively, compared to mock irradiated cells (figure 3.7).

Figure 3.7 GFP expression in mock-irradiated (blue) and irradiated (red) A) MCF-7, B) PC-3 and C) LNCaP cells, cultured under low oxygen conditions. Cells were transfected in triplicate with the control plasmid pCMV-GFP and pLinker-CMV-GFP, or the synthetic radiation responsive constructs pE4-GFP, pE4S-GFP, pE6ns2-GFP and pE9ns2-GFP. The vertical bars represent the standard error (SE) between triplicate samples. * represents a significant difference compared to pLinker-CMV-GFP ($P < 0.01$ Two-tailed students T test assuming equal variance).

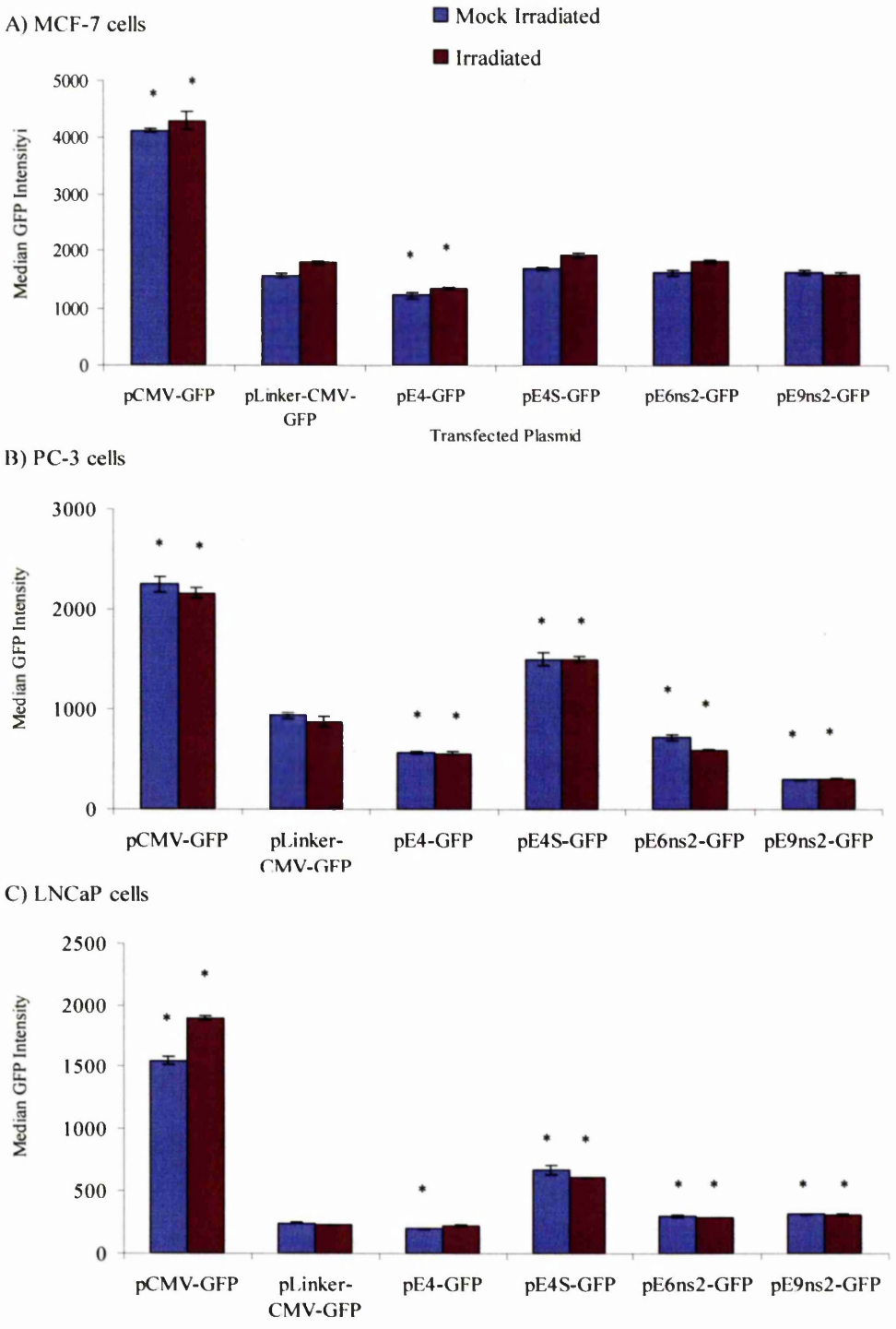
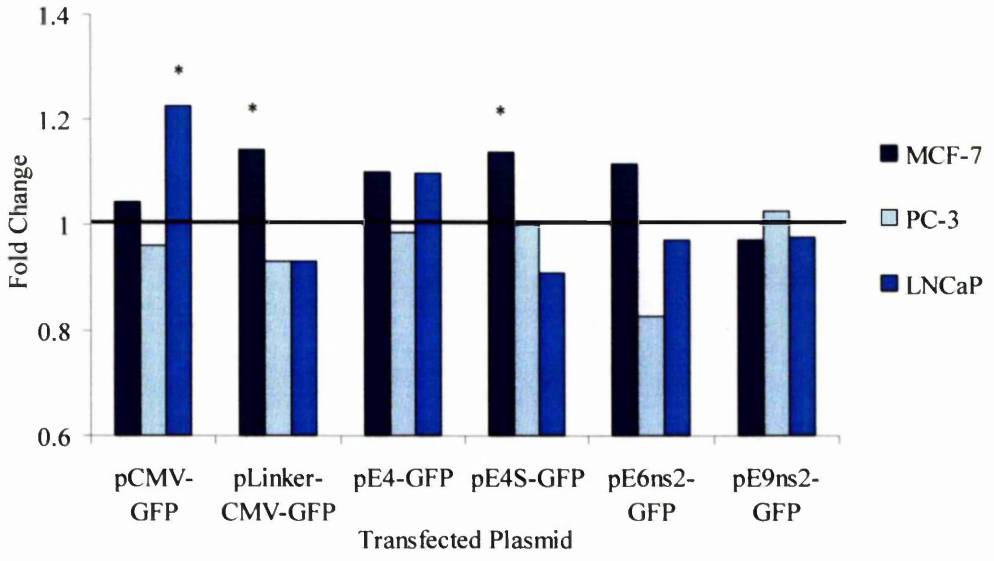


Table 3.3 Changes in the expression of GFP under hypoxic conditions induced by the insertion of the radiation responsive enhancers: E4, E4S, E6ns2 and E9ns2, into the enhancer-less construct pLinker-CMV-GFP ($P < 0.01$). The change in GFP expression is presented as a % of the expression from the CMV enhancer/ promoter which represents 100% activity. Marked increases in GFP expression are highlighted in red. N/S no significant difference.

			Radiation responsive enhancers (versus Linker-CMV)			
Cell line	CMV	Linker-CMV (vs CMV)	E4	E4S	E6ns2	E9ns2
MCF-7	100%	38%	- 8%	N/S	N/S	N/S
PC-3	100%	42%	- 16%	+ 25%	- 10%	- 29%
LNCaP	100%	16%	- 3%.	+ 28%,	+ 3%	+ 4%

Figure 3.8 Fold change in GFP expression induced by irradiation of MCF-7 (dark blue), PC-3 (Pale blue) and LNCaP (turquoise) cells transfected in triplicate under low oxygen conditions with pCMV-GFP, pLinker-CMV-GFP, pE4-GFP, pE4S-GFP, pE6ns2-GFP and pE9ns2-GFP. * represents a significant fold change in GFP expression between mock-irradiated and irradiated samples ($P < 0.01$ Two-tailed students T test assuming equal variance).



Even under low oxygen conditions, there was a high level of background fluorescence from both the radiation responsive promoters and the enhancer-less CMV promoter, pLinker-CMV-GFP. This suggests that reactive oxygen intermediates may not be responsive for activating the CArG elements in the absence of irradiation. If an assumption is made that the putative radiation responsive promoters are already maximally operating, even under low oxygen conditions, then only pE4S-GFP induced levels of GFP expression that were substantially higher than those elucidated by pLinker-CMV-GFP. At 5% oxygen, pE4S-GFP induced the highest levels of GFP expression in LNCaP and PC-3 cells, and this was in both PC-3 and LNCaP cells. In the previous experiment at 20% oxygen pE4S-GFP also produced the highest level of GFP expression but that was only in PC-3 cells, for LNCaP cells it was pE4-GFP. While these maximal 1.6 to 3.4 fold increases in mock-irradiated cells are similar to values reported in the Marples and Scott series of papers for their radiation responsive enhancer constructs in irradiated cells (Marples *et al.*, 2000; Scott *et al.*, 2000; Marples *et al.*, 2002; Scott *et al.*, 2002), the data presented here shows no consistent effects either of the constructs or of the effect of radiation in any of the three cell lines.

The possibility cannot be excluded that even 5% oxygen was sufficient to activate the radiation-responsive elements. However, it is still difficult to rationalise this with the high levels of expression seen with the enhancer-less promoter, unless perhaps there were upstream sequences that could function as occult reactive oxygen species (ROS) or indeed serum response factor (SRF) responsive promoters. Initially, the possibility that SRF, or other factors, present in FCS were activating the CArG elements in the absence of irradiation were explored. After this the effect of vector linearization was examined.

3.3.3 Assessment of promoters in MCF-7 cells starved of serum

SRF, induced by high serum levels, can bind to CArG elements and enhance the transcription of downstream genes (Croissant *et al.,* 1996; Soulez *et al.,* 1996; Spencer and Misra 1996; Arsenian *et al.,* 1998; Spencer and Misra 1999). This could account for the high level of background fluorescence seen in un-irradiated transfected cells. In order to test this hypothesis, the irradiation experiments were repeated only with MCF-7 cells plated, 24 h prior to transfection with pCMV-GFP and pE4-GFP, in RPMI complete media containing 0.5% FCS, rather than 10% FCS.

Figure 3.9 shows GFP expression in MCF-7 cells grown in high (10%) and low (0.5%) serum media and transfected with the control pCMV-GFP and the radioresponsive enhancer construct pE4-GFP. Under mock irradiated conditions replacement of the CMV enhancer with E4 led to a decrease in GFP expression of 79% and 75% when cultured under 10% and 0.5% serum concentrations respectively, when compared to pCMV-GFP. This suggests that even under low serum conditions, the radioresponsive promoter is active in the absence of radiation. Interestingly, irradiation of the transfected MCF-7 cells led to a significant decrease in GFP expression, for both CMV-GFP and E4-GFP, under high serum conditions, but a significant increase of 41% and 66% ($P<0.01$) when cultured under low serum levels for CMV and E4 respectively, when compared to mock irradiated cells. This was the most substantial evidence obtained of a radiation mediated up-regulation of a radiation responsive promoter (1.7 fold); the intact CMV enhancer/promoter also responded to radiation but this was less extensive (1.4 fold). However, there was still a high level of background fluorescence which was considered to be too extensive if the radiation responsive promoters were to be suitable for use in a controlled and targeted gene therapy treatment. In addition, the observations with the enhancer-less promoter construct still remain unexplained.

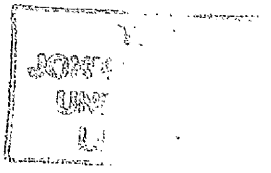
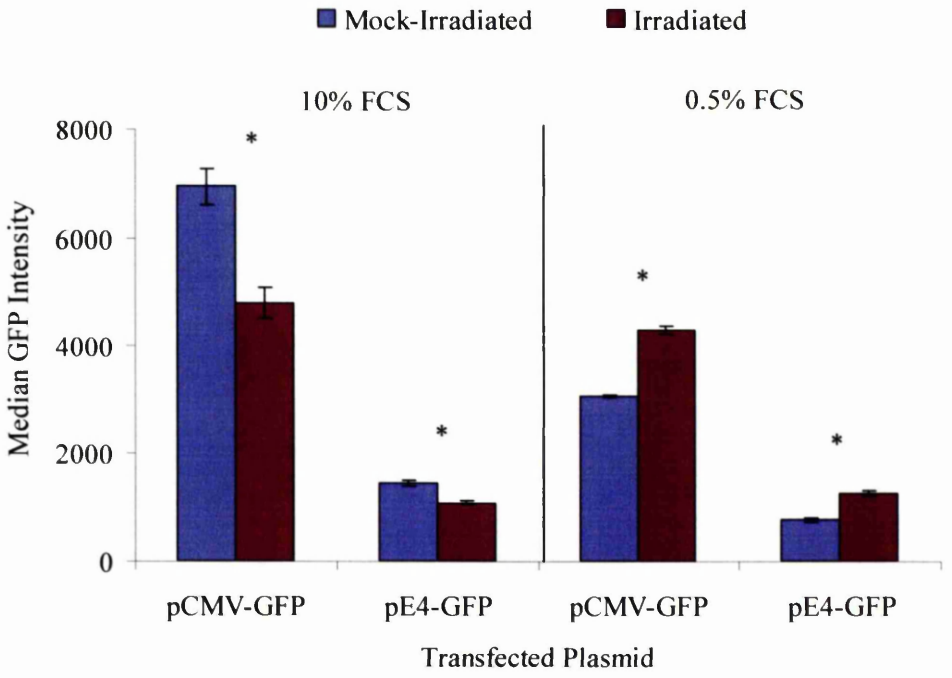


Figure 3.9 GFP expression in mock-irradiated (blue) and irradiated (red) MCF-7 cells cultured under high (10%) and low (0.5%) serum concentrations. The vertical bars represent the standard error (SE) between triplicate samples. * represents a significant difference between mock-irradiated and irradiated samples ($P < 0.01$ Two-tailed students T test assuming equal variance).

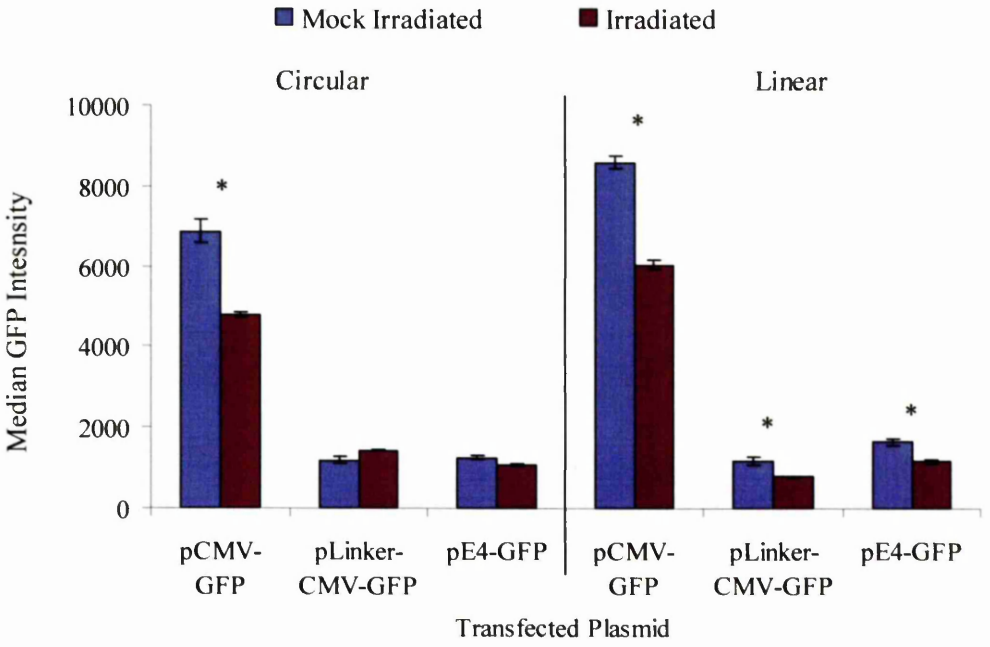


3.3.4 Assessment of linear promoters in MCF-7 cells

Previous experiments have shown that there is a high level of background fluorescence from the vectors containing the radioresponsive enhancer in the absence of irradiation and from the vector containing the enhancer-less CMV promoter. The background fluorescence occurs under both high and low oxygen and serum starved conditions, indicating that activation of the CArG elements by oxidative stress and SRF are not solely responsible. This, and all the previous results, suggests that either the CMV promoter alone is sufficient to drive the expression of GFP or that there are other elements within the pCI-neo vector backbone, such as occult promoters, that are able to elicit the expression of GFP. In order to test this hypothesis the vectors pCMV-GFP, pLinker-CMV-GFP and pE4-GFP were digested with BglII restriction enzyme to linearise the vectors at the 5′ end of the enhancer/promoter regions. This was considered to be an effective way of preventing read-through from the rest of the vector. Digested constructs were purified by phenol/chloroform extraction (section 2.2.4) followed by ethanol precipitation (section 2.2.5), a sample was then analysed by agarose gel electrophoresis (section 2.2.5) to confirm that complete linearisation of the vectors had occurred. MCF-7 cells were transfected with either the circular or linear forms of the three constructs and cultured in 20% O_2 with 10% FCS. GFP expression was then determined in mock irradiated and irradiated cells by FACS analysis.

Following mock-irradiation, linearisation of pCMV-GFP and pE4-GFP led to a significant increase of 25% and 32% respectively, compared to the corresponding circular plasmid (figure 3.10). In contrast, linearisation of the enhancer-less promoter construct, pLinker-CMV-GFP, had no effect on GFP expression resulting in a level of GFP expression that was equivalent to that expressed by pE4-GFP in both circular and linear forms. In addition, irradiation led either to no change or to a further reduction in GFP expression from both circular and linear constructs. This implies that while there may be elements upstream of the enhancer/promoter region of pCI-neo they are not interfering with the radiation inducibility of the radio-responsive promoters nor adding to the high level of background fluorescence seen in un-irradiated cells. However, due to the presence of only a single suitable restriction site at the 5′ end of the enhancer/promoter regions, it is conceivable that the linearised constructs could have been re-ligated enabling read-through from the rest of the vector. On the other hand, this religation must have been extremely efficient since the levels of GFP expression from linear vectors were similar, if not more than, that expressed by circular vectors.

Figure 3.10 GFP expression from mock-irradiated (blue) and irradiated (red) MCF-7 cells transfected with circular and linear forms of the control vectors pCMV-GFP and pLinker-CMV-GFP and the radiation responsive pE4-GFP construct. The vertical bars represent the standard error (SE) between triplicate samples. * represents a significant difference between mock-irradiated and irradiated cells ($P < 0.01$ Two-tailed students T test assuming equal variance).



3.4 Discussion and Conclusions

The experiments conducted were analogous to those carried out by Marples and Scott who reported that synthetic and wild type radiation responsive promoters can induce 1.5 - 3 fold increase in expression of downstream genes in response to irradiation (Marples *et al.*, 2000; Scott *et al.*, 2000; Marples *et al.*, 2002; Scott *et al.*, 2002). However, in contrast to their results, these experiments indicate that the radiation responsive enhancers were not responsive to radiation. In addition, they exhibited a high level of background fluorescence which appeared not to be a consequence either of oxidative stress or of SRF activation of the CArG elements within the radiation responsive enhancers in the absence of irradiation. The disparity between these results and those previously published may stem from the background level of GFP expression from the enhancer-less CMV promoter. This was seen even with a linearised vector and, assuming no extensive re-ligation of the vector had occurred, indicates the universal presence of transcription factors that are effective even on the CMV promoter alone. A likely candidate is the Sp-1 transcription factor which recognises and specifically binds GC-rich sequences (Berg 1992; Kaczynski *et al.*, 2003). It may therefore recognise the oligonucleotide sequence; GGGCGG, present in the CMV promoter. Given this background expression, the development of a truly radiation-controllable and effective system would have needed a considerable investment of time and effort. Rather then continuing to investigate experimental conditions that may have achieved negligible expression of GFP in the absence of radiation, but substantial level of expression after radiation, it was decided to change tactics. Since the aim of this study was to identify a highly controllable or tissue specific enhancer/promoter to drive the molecular switch specifically within prostate cancer cells the radiation responsive enhancers were replaced with prostate specific enhancer/promoters.

Chapter 4

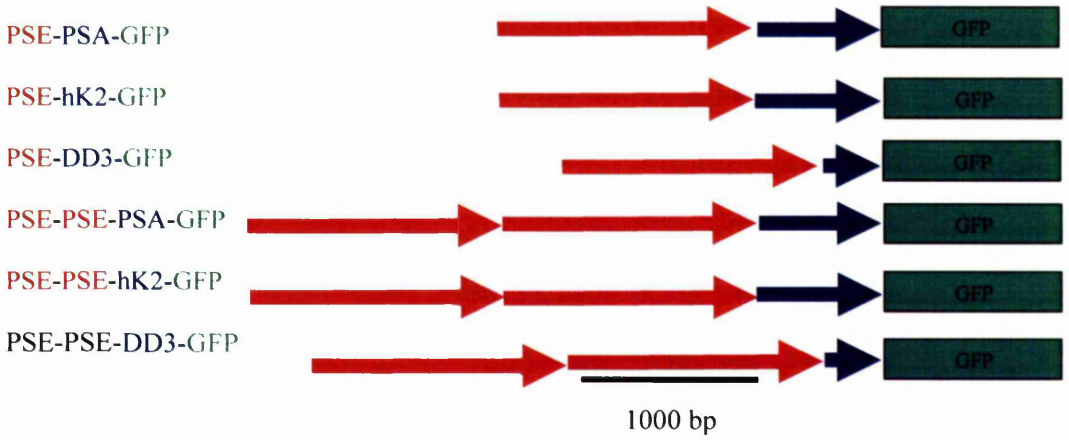
4.0 Results: Prostate specific promoters

4.1 Introduction

As an alternative strategy to drive the molecular switch the CMV enhancer and promoter elements were replaced with prostate specific promoters (PSP) and enhancers (PSE). For an in depth discussion of prostate specific promoters see section 1.4.2. After careful consideration, the promoters selected for driving the molecular switch were PSA and hK2, as they both contain androgen responsive elements and have been used to drive the expression of suicide genes specifically within the prostate. DD3, a newly discovered androgen non-responsive promoter, was also chosen as it is the most prostate cancer specific promoter known to date. In addition, the PSA enhancer (PSE) was used as it has been well documented to enhance prostate specificity and promoter activity.

In order to determine the most suitable prostate specific promoter/enhancer combination to drive the molecular switch, the CMV promoter in pCMV-GFP was replaced with PSA (-630 bp to +12 bp relative to the start of transcription of the PSA gene, NCBI accession number U37672; Brookes *et al.*, 1998; Suzuki *et al.*, 2001), hK2 (-622 bp to +25 bp, AF113169; Latham *et al.*, 2000; van der Poel *et al.*, 2001) or DD3 (-152 bp to +62 bp, AF279290; Verhaegh *et al.*, 2000). In addition, the CMV enhancer upstream of these promoters was replaced with one or two copies of the PSA enhancer (PSE -5322 bp to -3869 bp, U37672; Schuur *et al.*, 1996; Brookes *et al.*, 1998). The different prostate specific promoter/enhancer combinations are shown in figure 4.1.

Figure 4.1 Diagram of the prostate specific promoter and enhancer constructs used to express the GFP reporter gene. Dark blue arrows represent the prostate specific promoters PSA, hK2 and DD3. The red arrows represent one or two copies of the PSA enhancer (PSE) and the green box represent the GFP reporter gene. Scale bar, 1000 bp.



4.2 Creation of vectors

4.2.1 Control vectors pCMV-GFP2 and pLinker-GFP

In order to replace the CMV promoter in pCMV-GFP with prostate specific promoters, see section 4.2.2, it was experimentally advantageous to use the SgfI and XhoI restriction sites, however this meant that the chimeric intron (730-1086 bp) between the CMV promoter and MCS of pCMV-GFP was lost (see figure 4.1). In order to control for any effect this may have on gene expression, a positive control was constructed in which the chimeric intron was replaced with a linker. The synthetic linker (Linker 2) generated from two annealed synthetic oligonucleotides, designed to add 5′ SacI and 3′ NheI restriction sites (see table 4.1 linker 2), was inserted into SacI/NheI digested pCMV-GFP to create pCMV-GFP2 (see figure 4.1). It was subsequently confirmed by FACS analysis of GFP expression from pCMV-GFP and pCMV-GFP2 transfected PC-3 cells (data not shown) that loss of the chimeric intron did not effect gene expression.

Similarly, a negative control vector was constructed in which, based on the findings in chapter 3, the whole of the CMV enhancer and promoter regions and chimeric intron of pCMV-GFP was replaced with a linker to create an enhancer/promoter-less construct (pLinker-GFP). The synthetic linker (linker 3) was generated from two annealed oligonucleotides (see table 4.1 linker 3) and introduced into pCMV-GFP using BglII/SacII restriction sites. Correct insertion of both linkers was initially confirmed by PCR and restriction digest and finally by DNA sequencing.

Figure 4.1 pCMV-GFP vector map and MCS. Linker 2 was inserted into pCMV-GFP using the SacI and NheI restriction sites (blue) removing the chimeric intron between the promoter and MCS to create pCMV-GFP2. Linker 3 was inserted into pCMV-GFP using BglII/SacII restriction sites (blue) thus replacing the CMV enhancer, promoter and Chimeric intron to create pLinker-GFP. The vectors also contain an Amp^r marker for selection in *E. coli* and a neo marker for selection in mammalian cells.

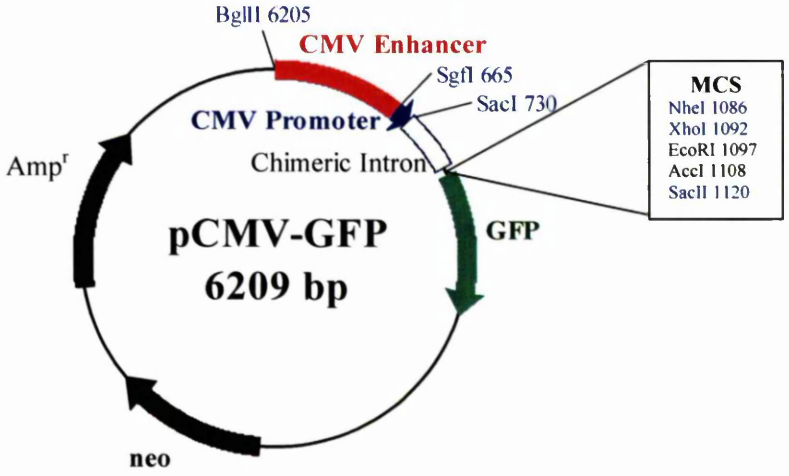


Table 4.1 Synthetic linker oligonucleotides and primers used for PCR amplification of prostate specific enhancer/promoters. The BglII (light blue), XbaI (Red), SgfI (dark blue), SacI (plum), NheI (purple) and XhoI (green) restriction sites, required for cloning into pCMV-GFP are shown.

Oligos	Sequence	
Linker 2	Sense	5′ CGTTTAGTGAACCGTCAGATCG 3′
	Anti-sense	5′ CTAGCGATCTGACGGTTCACTAAACGAGCT 3′
Linker 3	Sense	5′ GATCTGGCCAAAAAGGCCCGC 3′
	Anti-sense	5′ GGGCCTTTTTGGCCA 3′
PSE 1	Sense	5′ GCATAGATCTTCTAGAAATCTAGCTGATATG 3′
	Anti-sense	5′ CGATGCGATCGCAACGTTGAGACTGTCCTGGAGAC 3′
PSE2	Sense	5′ GCATAGATCTTCTAGAAATCTAGCTGATATG 3′
	Anti-sense	5′ CGATTCTAGAAACGTTGAGACTGTCCTGGAGAC 3′
PSA	Sense	5′ CGATGCGATCGCTTCCACATTGTTTGCTGCACG 3′
	Anti-sense	5′ CGCTCGAGAAAGCTTGGGGCTGGGGAGCC 3′
hK2	Sense	5′ GCACGCGATCGCGTGCTCACGCCTGTAATCTC 3′
	Anti-sense	5′ CGCTCGAGGGTGTCCACGGCCAGGTGGTG 3′
DD3	Sense	5′ CGGCGATCGCTGTTCAACATAGTGTGTGAACG 3′
	Anti-sense	5′ CGCTCGAGCCACACAAATCTCCCCTCTG 3′

4.2.2 Prostate specific enhancer/promoter constructs

The prostate specific promoters PSA, hK2 and DD3 were PCR amplified from human male genomic DNA (Promega) using primers designed to add SgfI and XhoI restriction sites to the 5′ and 3′ ends respectively (see table 4.1). A single copy of the PSA enhancer (PSE1) was PCR amplified from pUC18-PSE-2 (a kind gift from G.Verhaegh, University Medical Centre, Nijmegen, Netherlands) using primers designed to add 5′ BglII and XbaI and 3′ SgfI restriction sites. Another copy of PSE (PSE2) was PCR amplified using primers designed to add BglII and XbaI restriction sites to the 5′ and 3′ ends respectively (see table 4.1). The presence of a PCR product was confirmed by agarose gel electrophoresis (see figure 4.2) and it was then purified using the Qiagen PCR purification kit. The PCR products were initially inserted into the pGem-T shuttle vector (Promega; see appendix section 8.1) where sequence fidelity and addition of restriction sites was confirmed before cloning into pCMV-GFP (see figure 4.1). The SgfI/XhoI restriction sites were used for the promoters, the BglII/SgfI restriction sites for the PSE1 and the BglII/XbaI restriction sites for PSE2, thus removing the BglII site between PSE1 and PSE2 (see figure 4.2). Correct insertion and sequence fidelity of the promoters and enhancers was confirmed by PCR, restriction digest and DNA sequencing.

Figure 4.2 Agarose gel electrophoresis of PCR products of the prostate specific promoters and enhancers. Lane 1; PSA (642 bp), Lane 2 hK2 (647 bp), lane 3 DD3 (214 bp) and lane 4 PSE (1453 bp). A 1 kb ladder was used for determination of the size of the PCR products.

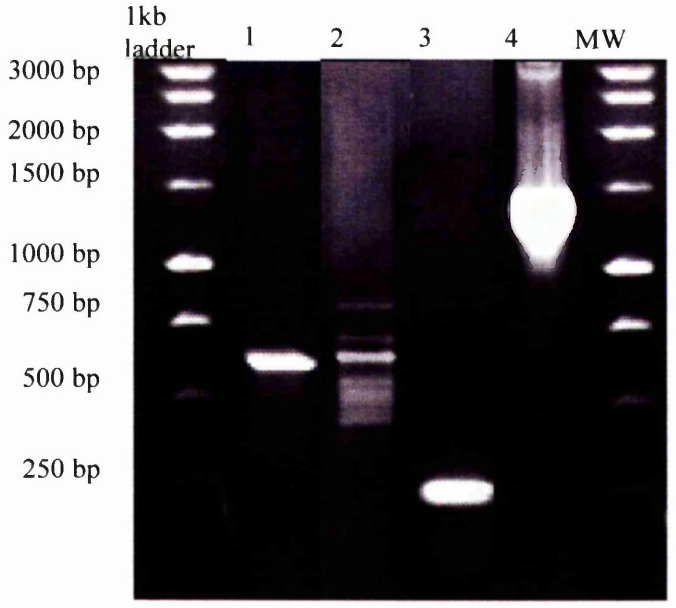
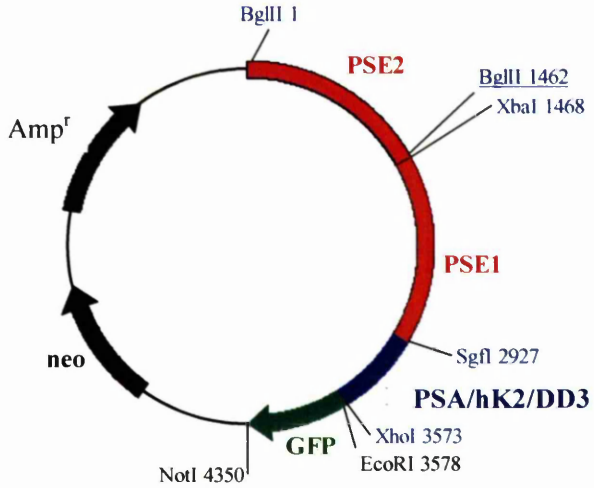


Figure 4.3 Vector map of pPSE-PSE-PSA/hK2/DD3-GFP. The prostate specific promoters: PSA, hK2 and DD3 (dark blue), were all inserted into pCMV-GFP using SgfI and XhoI sites. PSE1 (red) was then inserted using BglII (underlined) and SgfI sites adding the XbaI site. PSE2 (red) was then inserted using BglII and XbaI sites thus removing the underlined BglII site. The vector also contains an Amp^r marker for selection in *E. coli* and a neo marker for selection in mammalian cells.



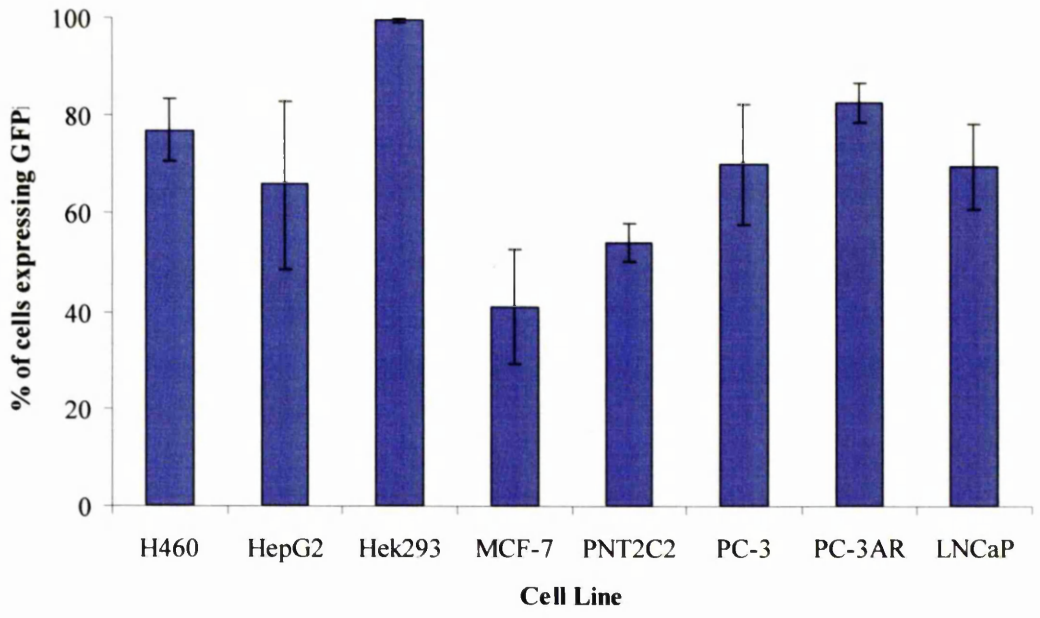
pPSE-PSE-PSA/Hk2/DD3-GFP
8257 - 8685 bp

4.3 Assessment of prostate specific enhancer/promoters

In order to determine the appropriate prostate specific enhancer/promoter combination it was necessary to compare the activity of the different promoters both within the same cell line and between different cells lines of both prostate and non-prostate origin. This was carried out using the following cell lines: three prostate adenocarcinoma cell lines; PC-3, LNCaP, and PC-3AR (PC-3 cells stably transfected with the androgen receptor), the non-cancerous prostate cell line; PNT2C2, and four non-prostate cell lines HepG2 (hepatocellular carcinoma), H460 (lung adenocarcinoma), Hek293 (embryonic kidney) and MCF-7 (breast adenocarcinoma). All experiments were conducted using charcoal stripped FCS which has lipophilic material removed thus reducing the serum concentration of hormones such as testosterone, estradiol and progesterone. This enabled the examination of the effects of added testosterone on the prostate specific enhancer/promoters. To determine the transfection efficiency of the different cell lines, a preliminary experiment was conducted, analogous to the mock-irradiated radiation responsive promoter experiments described in chapter 3. In this, the above cell lines were cultured under oxic conditions, plated in triplicate at a density of 2×10^5 cells and transfected with 1 μg of the positive control vector pCMV-GFP2. As previously described, this vector contains the constitutively activating CMV enhancer/promoter thus giving an indication of the maximum GFP expression a cell line is capable of achieving. GFP reporter gene expression was then measured 48 h later. The experiment was repeated on three separate occasions.

The average percentage of cells expressing GFP (see figure 4.4) is a combined indication of the transfection efficiency in a particular experiment and the ability of the different cell lines to activate gene expression from plasmid DNA. It was found that this was not only inconsistent between the repeat experiments of the same cell line (shown by the SE bars) but also varied considerably between different cell lines. For example, 99% of transfected Hek293 cells were GFP positive, indicating a very high transfection efficiency, whereas only 41% MCF-7 cells were GFP positive. Thus it was not possible to make a direct comparison between the different cell lines. Therefore the % of cells expressing GFP from the promoter in pCMV-GFP2 transfected cells was used as a reference, and defined as a transfection efficiency of 100% to which all the other vectors were compared.

Figure 4.4 GFP expression (as a % of viable FACS sorted cells) of H460, HepG2, Hek293, MCF-7, PNT2C2, PC-3, PC-3AR and LNCaP cells transfected with pCMV-GFP2. The vertical bars represent the SE between 3 separate experiments performed in triplicate.



4.3.1 Assessment of specificity of the prostate specific enhancer/promoters

In order to assess the prostate cell specificity of all the constructs, H460, HepG2, MCF-7, PNT2C2, PC-3, PC-3AR and LNCaP cells were transfected in triplicate with the control vectors, pCMV-GFP2 and pLinker-GFP, and the six different prostate specific enhancer/promoter constructs (see figure 4.1). Hek293 cells were transfected with the two most promising constructs, pPSE-PSE-PSA-GFP and pPSE-PSE-DD3-GFP and the control vectors, pCMV-GFP2 and pLinker-GFP. Experimental conditions were identical to those employed in the transfection efficiency experiment (section 4.3). Each experiment was repeated on three separate occasions.

Remarkably the enhancer/promoter less construct, pLinker-GFP, induced high levels of background GFP expression of between 13% and 77% of pCMV-GFP2 transfected cells for H460, HepG2, Hek293, MCF-7, PNT2C2, PC-3 and PC-3AR cells (see figure 4.5). In LNCaP cells, the background fluorescence was 0.2% compared to the CMV enhancer/promoter, in comparison the prostate specific promoters induced GFP expression of between 1 and 4%, this is the equivalent of an increase above background fluorescence of between 5 and 23 fold. However, these enhancements in GFP expression are not significant due to the low levels of expression and variation between repeat experiments. In all other cell lines, the prostate specific enhancer/promoters either reduced or had no significant effect ($P < 0.01$) on GFP expression compared to pLinker-GFP.

In summary, these data indicate that although the prostate specific enhancer/promoters are not active in all prostate cell lines, they are tissue specific as GFP expression does not exceed background fluorescence in non-prostate cell lines. However, the high level of GFP expression from pLinker-GFP indicates that there may be elements within the pCI-neo backbone that are able to induce GFP expression, thus masking any induction from the relatively weak prostate specific enhancer/promoters. Interestingly, this high background fluorescence is not seen in LNCaP cells suggesting that it is induced by cell-specific transcription factors.

In support of these observations, other groups have shown that the PSA promoter coupled to a PSE or PSE-PSE induced minimal GFP expression in LNCaP cells of 0.9% and 4% respectively compared to the CMV enhancer/promoter, while GFP expression in PC-3 and non-prostate cell lines was less than 0.2% (Latham *et al.*, 2000; Yoshimura *et al.*, 2002). In addition, Pang *et al.*, (1995; 1997) show that an enhancer/promoter less construct, similar to pLinker-GFP, can induce the expression of the reporter gene luciferase, and that luciferase expression driven by CMV enhancer/PSA promoter constructs in non-prostate cell lines was within the range of this negative control. In contrast other groups have shown that PSE-PSA can induce high levels of downstream reporter expression of up to 72 fold compared to the PSA promoter alone (Schuur *et al.*, 1996), and that PSE-PSA, PSE-hK2, and PSE-DD3 showed leaky expression in non-prostate cell lines (van der Poel *et al.*, 2001).

Figure 4.5 GFP expression from H460, HepG2, Hek293, MCF-7, PNT2C2, PC-3, PC-AR and LNCaP cells transfected with plasmids containing the GFP reporter gene controlled by the CMV enhancer/promoter (CMV), no enhancer/promoter (Linker) or the prostate specific enhancer/promoter combinations (PSE-PSA, PSE-hK2, PSE-DD3, PSE-PSE-PSA, PSE-PSE-hK2, and PSE-PSE-DD3). GFP expression is presented as a % of that in pCMV-GFP2 transfected cells. The vertical bars represent the SE of 3 separate experiments performed in triplicate.

CMV
Linker
PSE-PSA
PSE-Hk2
PSE-DD3
PSE-PSE-PSA
PSE-PSE-Hk2
PSE-PSE-DD3
GFP expression (% CMV)
100
80
60
40
20
0
H460
HepG2
Hek293
MCF-7
PNT2C2
PC-3
PC-3AR
LNCaP
Cell Line

4.3.2 Assessment of androgen sensitivity of prostate specific enhancer/promoters

The previous experiment was conducted in the absence of androgen and in charcoal stripped serum. However, the PSE enhancer and the PSA and hK2 promoters all contain androgen responsive elements (ARE) that are up regulated in response to the binding of androgen to androgen receptors (AR) on the cell surface (Riegman *et al.*, 1991a; Riegman *et al.*, 1991b; Murtha *et al.*, 1993; Cleutjens *et al.*, 1997a). It may therefore be possible to enhance gene expression and tissue specificity in LNCaP and PC-3AR cells, the only prostate cell lines available that contain AR, by adding androgen to the culture media. In order to test the androgen responsiveness of the different cell lines the above experiment was repeated in the presence and absence of dihydrotestosterone (DHT) in three separate experiments. Since the physiological range of DHT in the prostate of human males is between 4.5 and 18 nM (Pang *et al.*, 1995), cells were cultured with 10nM DHT (dissolved in ethanol), the same amount of ethanol was added to non-DHT treated cells as a vehicle control.

In the presence of DHT the enhancer/promoter less construct, pLinker-GFP, induced high levels of background GFP expression of between 14% and 85% of pCMV-GFP2 transfected cells for H460, HepG2, Hek293, MCF-7, PNT2C2, PC-3 and PC-3AR cells (see figure 4.6), this is similar to the level of background fluorescence in the absence of DHT seen in figure 4.5. In the AR positive LNCaP cells treated with DHT, the background fluorescence was 2% compared to the CMV enhancer/promoter, however, the expression of GFP from constructs containing prostate specific promoter/enhancer combinations was significantly increased to 26 - 56% of that with the CMV enhancer/promoter (see figure 4.6) compared to 1 - 4% in untreated cells (figure 4.5). This is the equivalent to an increase in GFP expression due to androgen treatment of 34 to 276 fold compared to pCMV-GFP and pLinker-GFP which induced a non significant fold increase of 1.4 and 1.1 respectively (see figure 4.7). Due to large standard error between repeat experiments there was no significant difference between the ability of the six different prostate specific promoters to drive GFP expression. The AR negative cell lines (H460, HepG2, Hek293, MCF-7 and PNT2C2) were unresponsive to androgen showing no significant increase in GFP expression above background fluorescence (figure 4.6) and a fold induction of 0.8 - 2 (figure 4.7) for all the prostate specific enhancer/promoters and the control vectors pCMV-GFP and pLinker-GFP. Surprisingly, and in contrast to previous literature, the PC-3 cell line stably transfected with the AR (PC-3AR) was also unresponsive to androgen even though it is well documented to be androgen responsive (Le Dai *et al.*, 1996; Gkonos *et al.*, 2000; Terouanne *et al.*, 2000; Granchi *et al.*, 2001; Murthy *et al.*, 2003; Pandini *et al.*, 2005). Due to time constraints, it was not possible

to investigate this further, for example by RT-PCR or immunostaining, however one likely possibility is that the transgene had been down regulated.

In support of this work, other groups have shown that the addition of between 1 nM and 10 nM DHT to transfected LNCaP cells, the PSA or hK2 promoter linked to a single PSE enhancer can enhance gene expression by 9 to 30 fold; however two PSE enhancers can further enhance gene expression by as much as 185 fold. This is the equivalent of increasing gene expression from between 0.9% and 4% to between 2.5% and 75% that of the CMV enhancer/promoter. In addition, minimal expression was seen in PC-3 cells and non-prostate cell lines, such as MCF-7, HepG2, R11, HeLa, Hek293 and T24 (Schuur *et al.*, 1996; Brookes *et al.*, 1998; Gotoh *et al.*, 1998; Latham *et al.*, 2000; Wu *et al.*, 2001; Xie *et al.*, 2001; Yoshimura *et al.*, 2002; Tsui *et al.*, 2004). Moreover, Pang *et al.*, (1997) developed a construct similar to pPSE-PSA-GFP that induced a 1000 fold increase in gene expression in response to 10 nM DHT. However, in their construct the final base of the ARE in the PSE (GGAACAtatTGTATC) was mutated from C to T making it more similar to the ARE found in the PSA promoter. Whether this difference is due to a mutation in the ARE sequence or due to variations in the activity of promoters under different experimental conditions, would be worth further investigation. Interestingly, MCF-7 cells, known to contain very low levels of AR, were not responsive to androgen in either my experiments or those conducted by Pang *et al.*, (1995), Brookes *et al.*, (1998), Xie *et al.*, (2001) and Wu *et al.*, (2001). This has also been shown for HeLa cells, which possess functional AR (Pang *et al.*, 1997). Taken together, it suggests that tissue specific factors other than an AR are required for activating the regulatory sequences of PSE, PSA and hK2 in LNCaP cells.

In conclusion, the prostate specific promoters appear to be tissue specific in the presence of androgen, in as much as they induced a very high level of GFP expression above background only in LNCaP cells. However, there was still a very high level of background fluorescence in non-prostate cell lines; attempts to reduce this were therefore undertaken.

Figure 4.6 GFP expression in H460, HepG2, Hek293, MCF-7, PNT2C2, PC-3, PC-AR and LNCaP cells treated with 10 nM DHT and transfected with plasmids containing the GFP reporter gene controlled by the CMV enhancer/promoter (CMV), no enhancer/promoter (Linker) or the prostate specific enhancer/promoter combinations (PSE-PSA, PSE-hK2, PSE-DD3, PSE-PSE-PSA, PSE-PSE-hK2, and PSE-PSE-DD3). GFP expression is presented as a % of that in pCMV-GFP2 transfected cells. The vertical bars represent the SE of 3 separate experiments performed in triplicate. * and ** represent a significant difference compared to pLinker-CMV-GFP ($P < 0.01$ and $P < 0.05$ respectively, Two-tailed students T test assuming equal variance).

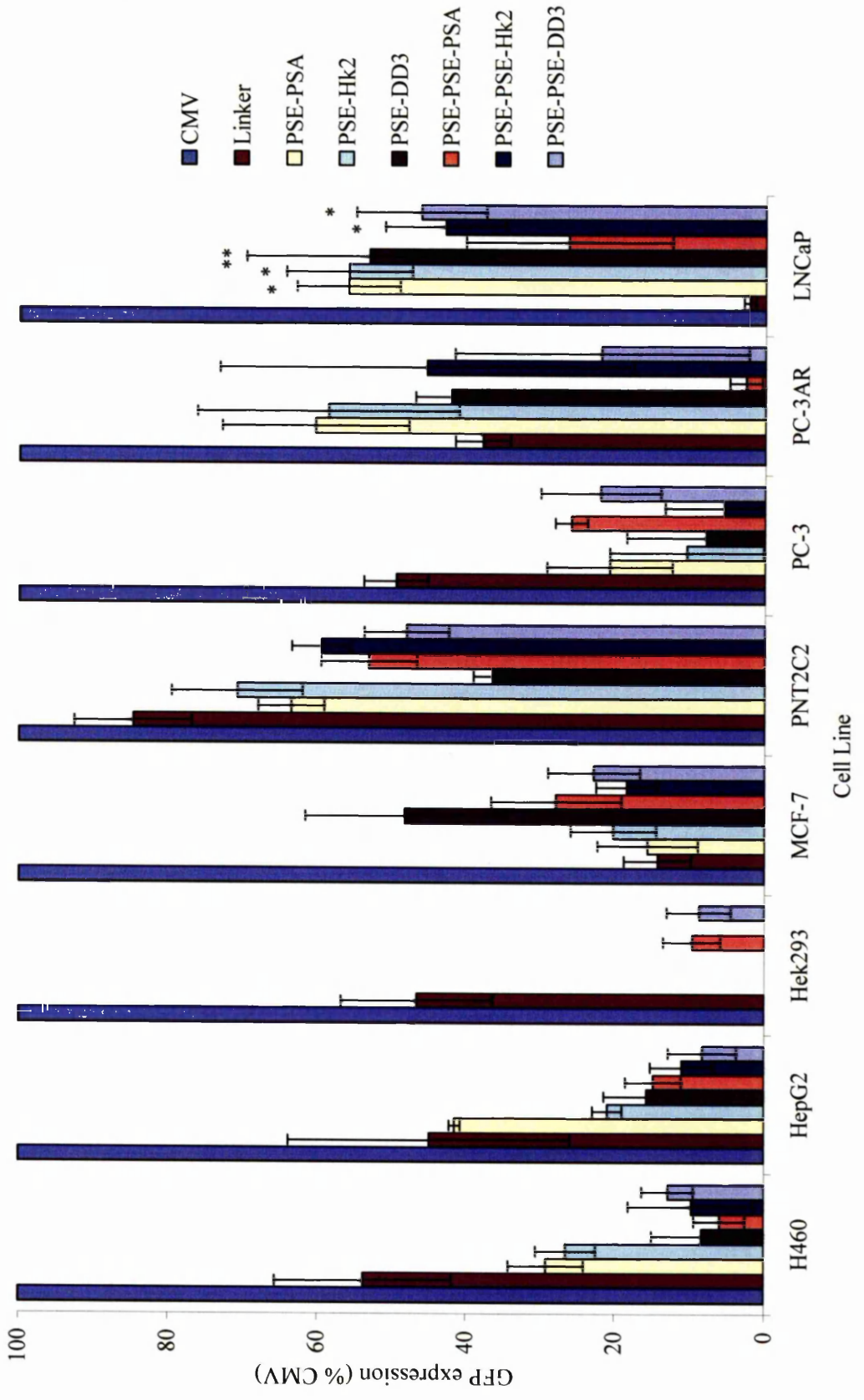
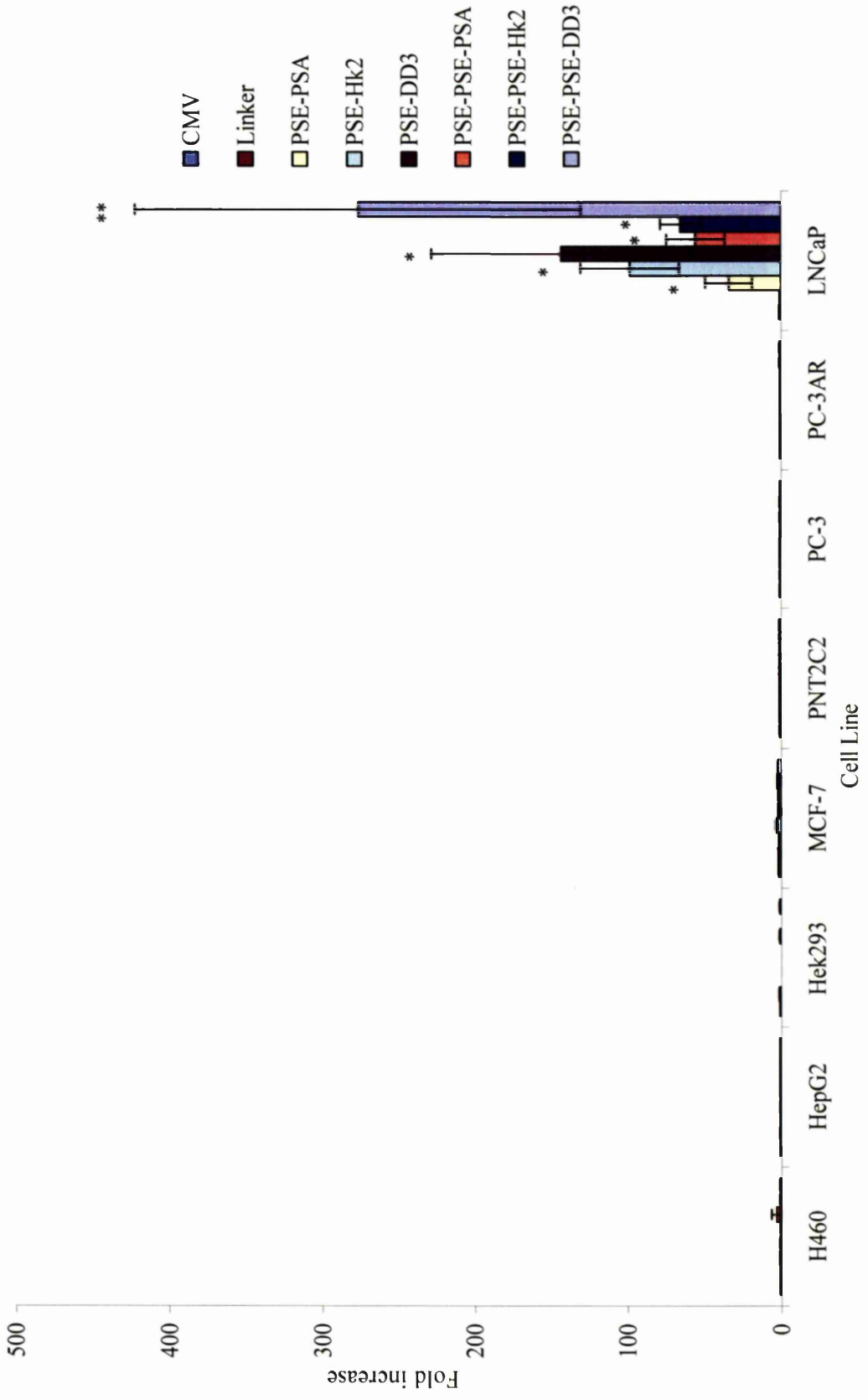

CMV
Linker
PSE-PSA
PSE-Hk2
PSE-DD3
PSE-PSE-PSA
PSE-PSE-Hk2
PSE-PSE-DD3
GFP expression (% CMV)
100
80
60
40
20
0
H460
HepG2
Hek293
MCF-7
PNT2C2
PC-3
PC-3AR
LNCaP
Cell Line

Figure 4.7 Fold increase in GFP expression due to the addition of 10 nM DHT to H460, HepG2, Hek293, MCF-7, PNT2C2, PC-3, PC-AR and LNCaP cells transfected with plasmids containing the GFP reporter gene controlled by the CMV enhancer/promoter (CMV), no enhancer/promoter (Linker) or the prostate specific enhancer/promoter combinations (PSE-PSA, PSE-hK2, PSE-DD3, PSE-PSE-PSA, PSE-PSE-hK2, and PSE-PSE-DD3). The vertical bars represent the SE of 3 separate experiments performed in triplicate. * and ** represents a significant difference compared to pLinker-CMV-GFP ($P < 0.01$ and $P < 0.05$ respectively, Two-tailed students T test assuming equal variance).

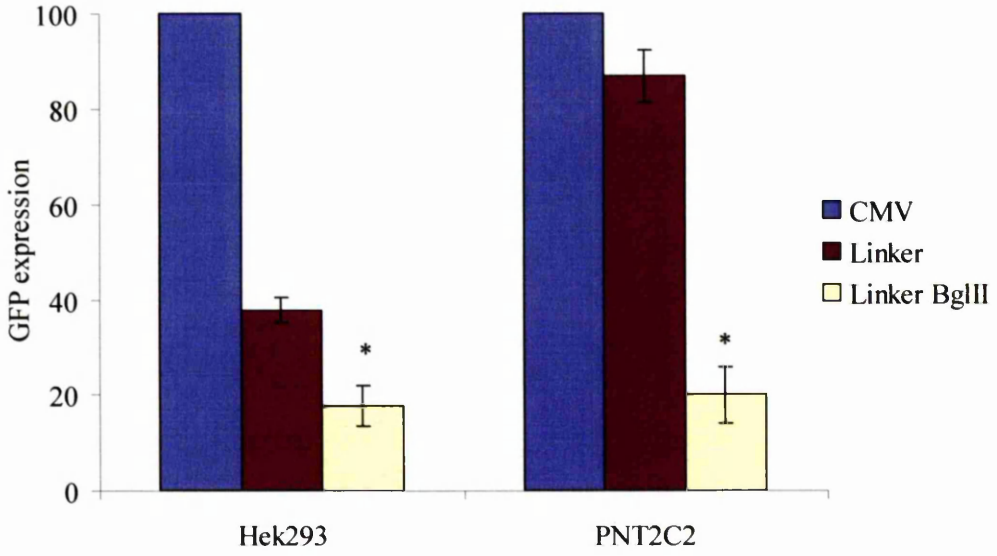

CMV
Linker
PSE-PSA
PSE-Hk2
PSE-DD3
PSE-PSE-PSA
PSE-PSE-Hk2
PSE-PSE-DD3
Fold increase
500
400
300
200
100
0
H460
HepG2
Hek293
MCF-7
PNT2C2
PC-3
PC-3AR
LNCaP
Cell Line

4.3.3 Assessment of GFP expression from linearised prostate specific constructs

Similar to the problems encountered using radiation responsive promoters, a high level of background has also been observed using the prostate enhancer/promoters, indicating a problem with leaky expression from the pCI-neo backbone. In order to test this hypothesis pLinker-GFP was digested with BglII to linearise the vector at the 5′ end of the Linker-GFP region with the intention of preventing read-through from the rest of the vector. Digested constructs were purified by phenol/chloroform extraction (section 2.2.4) followed by ethanol precipitation (section 2.2.5), a sample was then analysed by agarose gel electrophoresis (section 2.2.5) to confirm that complete linearisation of the vectors had occurred. Hek293 and PNT2C2 cells, previously shown to induce high levels of pLinker-GFP mediated background fluorescence, were then plated and transfected with circular pCMV-GFP2, pLinker-GFP and BglII-linearised pLinker-GFP. The experiment was repeated 3 times on separate occasions.

Figure 4.8 shows the % GFP expression compared to pCMV-GFP2 of circular and linear pLinker-GFP constructs in Hek293 and PNT2C2 cells. While the level of GFP expression from circular pLinker-GFP was similar to that seen in the previous experiments (38% and 87% compared to 37% and 77% in figure 4.5 for Hek293 and PNT2C2 cells respectively), linearization significantly reduced GFP expression by 20% and 67%. Assuming that there has been no extensive religation of the vector, these results, combined with those by Pang *et al.,* (1995), support the suggestion that there are additional elements within the vector backbone, in this case pCI-neo, capable of inducing GFP expression and that these elements operate with different efficiencies in different cell lines, presumably as a consequence of differential expression of the appropriate transcription factors.

Figure 4.8 GFP expression from Hek293 and PNT2C2 cells transfected with pCMV-GFP2, pLinker- GFP and BglII-linearised pLinker-GFP. GFP expression is represented as a % of pCMV-GFP2 transfected cells. The vertical bars represent the standard error (SE) between 3 separate experiments performed in triplicate. * represents a significant difference circular and linear pLinker-GFP transfected cells ($P < 0.01$ Two-tailed students T test assuming equal variance).



4.4 Discussion and Conclusions

These results indicate that, while the prostate specific enhancer/promoters are highly sensitive to androgen, at least in the AR expressing cell line LNCaP, there is still a high level of fluorescence in non-prostate cells from both prostate specific constructs and the enhancer/promoter-less construct. This background expression has meant that it has not been possible to determine which construct is the most prostate cell specific. However, the high androgen inducibility of these constructs above background florescence and their previously well documented prostate specificity (Pang *et al.*, 1995; Lee *et al.*, 1996; Schuur *et al.*, 1996; Pang *et al.*, 1997; Brookes *et al.*, 1998; Gotoh *et al.*, 1998; Latham *et al.*, 2000; Verhaegh *et al.*, 2000; Wu *et al.*, 2001; Xie *et al.*, 2001; Lee *et al.*, 2002a; Yoshimura *et al.*, 2002; Tsui *et al.*, 2004) suggest that they are suitable candidates for driving the molecular switch. In addition, PSA based prostate specific promoters have been used successfully *in vivo* and in clinical trials to drive therapeutic gene expression (Rodriguez *et al.*, 1997; Gotoh *et al.*, 1998; Martiniello-Wilks *et al.*, 1998; Latham *et al.*, 2000; Shirakawa *et al.*, 2000; Park *et al.*, 2003; Hsieh *et al.*, 2004). Taken together this indicates that the problem probably lies, not in the prostate specific elements, but in the pCI-neo vector system. Linearisation of all the vectors and repetition of the experiments was one practical approach to this, however, since linearisation of pLinker-GFP did not completely ablate the expression of GFP, probably due to religation (though this hypothesis was not tested), this may not have provided any useful information. To avoid the pCI-neo backbone completely, it was considered feasible and practical to construct the molecular switch in the adenoviral vector pShuttle2 (BD Bioscience, Clontech). pShuttle2 was specifically designed for the incorporation of DNA into an adenovirus by exploiting unique restriction endonuclease sites rather than *LoxP* recombination. This would ensure that only the essential elements of the molecular switch are incorporated into the adenovirus so that there should be no non-specific gene activation. In addition, it was envisaged that the adenovirus would substantially enhance the transfection efficiency of mammalian cells, both *in vitro* and *in vivo*. This approach is considered in chapter 6.

Chapter 5

5.0 Results: GDEPT

5.1 Introduction

The aim of the molecular switch is to drive GDEPT specifically within prostate cells leading to cell kill. It was therefore important to determine which enzyme/prodrug system was optimal under the experimental conditions employed. HSVtk/GCV and NTR/CB1954 systems have both been shown to efficiency kill LNCaP and PC-3 cells (Eastham *et al.*, 1996; Blackburn *et al.*, 1998; Gotoh *et al.*, 1998; Martiniello-Wilks *et al.*, 1998; Blackburn *et al.*, 1999; Latham *et al.*, 2000; Shirakawa *et al.*, 2000; Djeha *et al.*, 2001; Loimas *et al.*, 2001; Pramudji *et al.*, 2001; Yoshimura *et al.*, 2001; Freytag *et al.*, 2002b; Ikegami *et al.*, 2002; Read *et al.*, 2003). However, they have very different mechanisms of action. HSVtk converts the prodrug gancyclovir (GCV) into a nucleoside analogue that is incorporated into DNA during cell division resulting the chain termination and subsequent cell death. It therefore relies on the cells actively dividing during exposure. In contrast NTR activates the prodrug CB1954 and this generates crosslinks in DNA. This system does not require the cells to be actively dividing for apoptosis to be initiated.

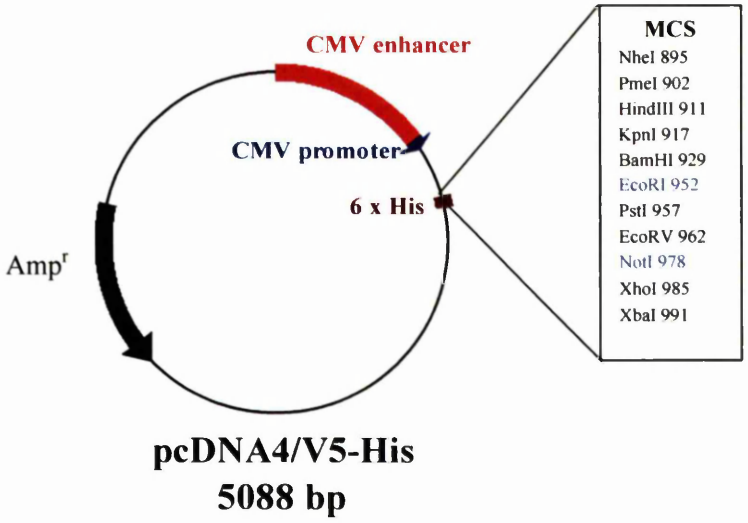
There are no literature reports on a direct comparison of the effectiveness of these two GDEPT systems in prostate cell lines. Therefore, HSVtk/GCV and NTR/CB1954 were tested in both the non-prostate cell line H460, because it is fast growing and highly transfectable, and the prostate cell lines; PC-3 and LNCaP. Vectors were used in which the expression of HSVtk and NTR was controlled by the CMV promoter. Histidine (His)-Tag fusion genes encoding HSVtk and NTR were also generated so that expression of the enzymes in mammalian cells could be confirmed by western blot analysis using anti-His antibodies, and an MTT assay (see section 2.6.4) was used to determine cell growth inhibition after prodrug treatment.

5.2 Creation of vectors

5.2.1 Production of His-tagged HSVtk and NTR fusion proteins

In order to visualise the expression of HSVtk and NTR in mammalian cells, the His-Tag fusion system, using the pcDNA4/V5-His mammalian expression vector (Invitrogen, see figure 5.1), was adopted. The HSVtk and NTR cDNA were inserted upstream of and in frame with a C-terminal His-tag consisting of 6 histidine residues. To achieve this, HSVtk and NTR cDNA were PCR amplified from pORF-HSV1tk (see appendix section 8.5; InvivoGen) and Escherichia *coli* genomic DNA (DH5α, Invitrogen, see section 2.3.3) using primers designed to introduce both 5′ EcoRI and 3′ NotI restriction sites at the ends of the genes. Furthermore, the 3′ primer was designed to eliminate the HSVtk and NTR stop codons, to enable transcription of the gene fused to the His-tag. Additional bases were also added to the 3′ primer so that the ORF of the gene was in frame with the His-Tag (see table 5.1 HSVtk-His and NTR-His). HSVtk and NTR cDNAs were initially inserted into the pGem-T shuttle vector, where sequence fidelity and addition of restriction sites was confirmed, prior to cloning into pcDNA4/V5-His using the EcoRI/NotI restriction sites (see figure 5.1) to create pcDNA4-HSVtk-His and pcDNA4-NTR-His. Correct insertion was confirmed by PCR and DNA sequencing.

Figure 5.1 pcDNA4/V5-His vector map and MCS used to generate the fusion proteins of HSVtk and NTR with the His-tag. The EcoRI/NotI restriction digest sites used to insert HSVtk and NTR cDNA are shown in blue. The 6 x His-tag antibody epitope for the detection of expressed proteins by western blot analysis is shown in dark red and the stop codon (TGA) is highlighted in yellow. The vector also contains an Amp^r marker for selection in *E. coli*.



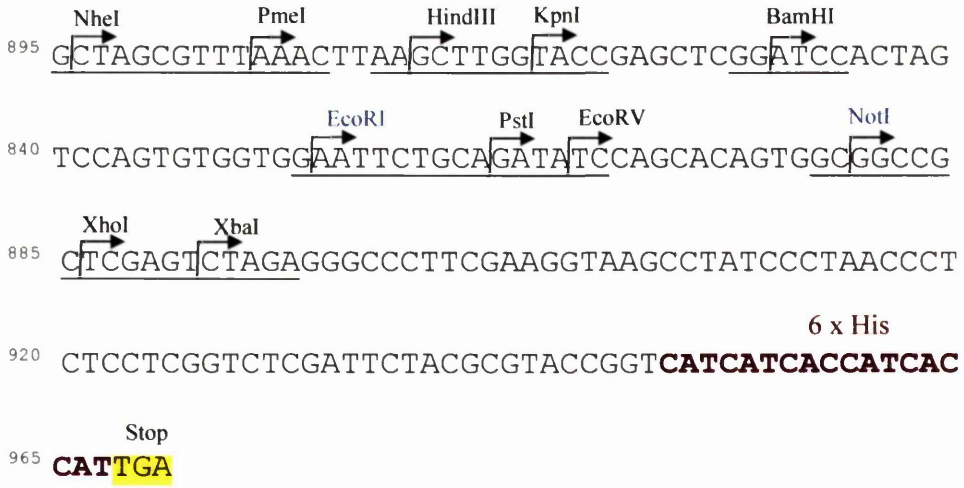


Table 5.1 Oligonucleotides used for PCR amplification of HSVtk and NTR as in frame His-tag fusions in pcDNA4/V5-His (HSVtk-His and NTR-His) and insertion into pCI-neo (HSVtk and NTR). EcoRI and NotI restriction sites are shown in grey and light blue respectively. The initiation Kozac and start codons (ACC ATG) and stop codons (TCA and TTA) are highlighted in yellow. Additional bases required to make in frame HSVtk and NTR His-Tag fusion proteins are underlined.

Oligos	Sequence	
HSVtk-His	Sense	5′ GGGAATTCACC ATG GCT TCG TAC CCC TGC C 3′
	Anti-sense	5′ ATGCGGCCGCAG GTT AGC CTC CCC CAT CTC CCG G 3′
NTR-His	Sense	5′ CGGAATTCACC ATG GAT ATC ATT TCT GTC GCC 3′
	Anti-sense	5′ TAGCGGCCGCAG CAC TTC GGT TAA GGT GAT GTT TTG 3′
HSVtk	Sense	5′ GGGAATTCACC ATG GCT TCG TAC CCC TGC C 3′
	Anti-sense	5′ ATGCGGCCGCTCA GTT AGC CTC CCC CAT CTC C 3′
NTR	Sense	5′ CGGAATTCACC ATG GAT ATC ATT TCT GTC GCC 3′
	Anti-sense	5′ TAGCGGCCGCTTA CAC TTC GGT TAA GGT GAT GTT TTG 3′

5.2.2 Production of vectors for MTT assay

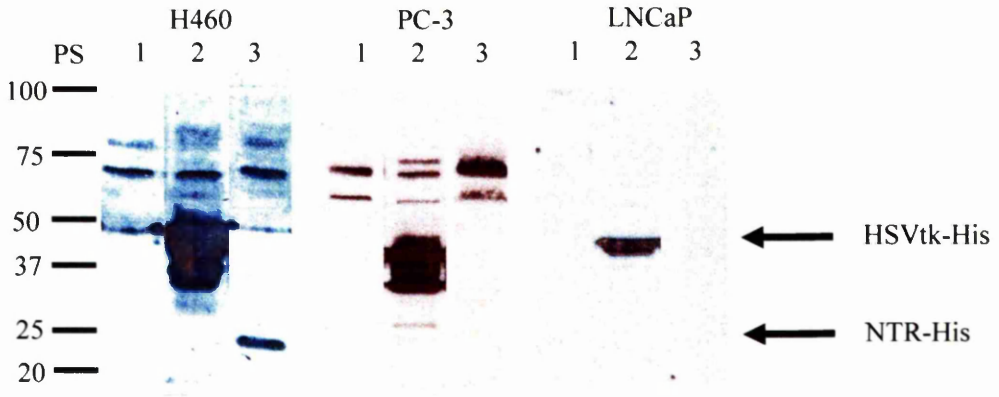
Although the His-tag vectors constructed in section 5.2.1 could, in theory, be used for the MTT assay, in order to ensure that the His-tag did not have any effect on the functional activity of the expressed proteins additional vectors were constructed in pCI-neo without a His-tag. HSVtk and NTR cDNA was PCR amplified as in section 5.2.1 using primers designed to add EcoRI and NotI restriction sites at the 5′ and 3′ ends respectively and a 5' TAA stop codon (see table 5.1 HSVtk and NTR). HSVtk and NTR were initially inserted into the pGem-T shuttle vector where sequence fidelity and addition of restriction sites was confirmed before cloning into pCI-neo using the EcoRI/NotI restriction sites (see figure 3.1) to create pCMV-HSVtk and pCMV-NTR.

5.3 Experiments to assess the expression and functionality of HSVtk and NTR

5.3.1 Western blot analysis of the expression of HSVtk and NTR

In order to confirm that HSVtk and NTR were being expressed in human cells, pcDNA4V5-HSVtk-His and pcDNA4V5-NTR-His (described in section 5.2.1) were transfected into H460, PC-3 and LNCaP cells (as described in section 2.6.1). After incubation at 37°C for 24 h, the cells were harvested, extracts prepared and proteins separated by SDS PAGE (see sections 2.5.4, 2.4.1 and 2.4.2). Expression of the His fusion proteins was then assessed by western blot analysis, using an anti-His antibody (Santa Cruz, as described in section 2.4.3). The estimated molecular weight of HSVtk and NTR are 42 kDa and 25 kDa respectively. As shown in figure 5.2A, although several cross reacting bands were seen, in H460 cells there were bands that corresponded to the correct molecular weights of HSVtk-His and NTR-His confirming that the expression of HSVtk and NTR had occurred. However, in PC-3 and LNCaP cells while there was expression of HSVtk there was no detectable expression of NTR.

Figure 5.2 Western blot analysis of cell extracts (equivalent to 40 µg of total protein per lane as determined by a Bradford assay) from H460, PC-3 and LNCaP cells. Lane 1; control un-transfected cell extract, Lane 2; pcDNA4V5-HSVtk-His transfected and lane 3; pcDNA4V5-NTR-His transfected. To determine protein sizes the precision plus protein dual colour standard was used (PS).



5.3.2 Assessment of the activity of HSVtk and NTR

In order to determine the biological effect of expression of the vector-encoded proteins expressed in H460, PC-3 and LNCaP cells an MTT assay was performed to measure cell growth inhibition after treatment of transfected cells with GCV and CB1954. The cells were plated at a density of $1x10^5$cells/well in 6 well plates and then transfected in duplicate 24 h later with 1 μg pCMV-HSVtk or pCMV-NTR DNA. After an 8 h incubation at 37°C the media was changed and GCV (in 0.1 M HCl, final concentration 0-1000 ng/μl) and CB1954 (in DMSO, final concentration 0-1000 μM) added. Controls involved equivalent volumes of 0.1 M HCl or DMSO. In preliminary experiments with cells transfected with pCMV-HSVtk, the optimum time for the MTT assay was found to be 4 days for H460, 5 days for PC-3 and 7 days for LNCaP cells (data not shown). This variation was due to the differences in rates of cell division of the three cell lines. In contrast, all cells transfected with pCMV-NTR were incubated for 48 h prior to MTT assay as cell division is not necessary for the generation of potentially lethal DNA lesions from CB1954 activation.

pCMV-HSVtk transfected H460 cells were sensitive to GCV; 10 ng/μl GCV led to an 86% reduction in cell growth. In comparison control cells were insensitive to GCV at this concentration. However, control cell growth rapidly dropped by 28% and 73% at 100 ng/μl and 1000 ng/μl GCV respectively (figure 5.3A). In contrast, pCMV-HSVtk transfected PC-3 cells were less sensitive to GCV; 10 ng/μl GCV led to a 48% reduction in cell growth. However, at this concentration control cell growth was reduced by 12% (figure 5.3B). Similarly, LNCaP control cells were also sensitive to GCV with cell growth mimicking that of pCMV-HSVtk transfected cells. However, at a concentration of 100 ng/μl GCV the cell growth in control and pCMV-HSVtk transfected cells was reduced by 28% and 50% respectively (figure 5.3C). In support of these observations other groups have shown that 10 ng/μl GCV reduced cell growth by 80% and 20% to 55% for HSVtk transfected H460 and PC-3 cells respectively (Eastham *et al.*, 1996; Katabi *et al.*, 1999; Loimas *et al.*, 2001; Pramudji *et al.*, 2001). In contrast, 10 ng/μl GCV has been shown to reduce LNCaP cell growth by 25 to 92% (Pramudji *et al.*, 2001; Suzuki *et al.*, 2001; Ikegami *et al.*, 2002), whereas other groups have shown that only 1 ng/μl reduces cell growth by 40% (Yoshimura *et al.*, 2001; Freytag *et al.*, 2002b)

H460 cells were sensitive to increasing doses of CB1954, however, when transfected with pCMV-NTR cell growth rapidly reduced. At a dose of 10 μM CB1954, cell growth reduced by 20% and 62% for control and pCMV-NTR transfected cells respectively (figure 5.4A). In contrast, PC-3 and LNCaP control cells were relatively insensitive to CB1954, even at a concentration of 100 μM CB1954, cell growth was only reduced by 11% and 12% for PC-3 and LNCaP cells respectively. However, while pCMV-NTR transfected PC-3 cells were very sensitive to CB1954; a dose of 10 μM led to a 61% reduction in cell growth, LNCaP cells required a higher dose of 100 μM to reduce cell growth by only 21% (figure 5.4B and C). While no data has been published on the response of H460 cells to NTR/CB1954 therapy, Read *et al.*, (2003) showed that 10 μM CB1954 reduced the cell growth of NTR transfected PC-3 cells by 25-80%. In contrast to my data, Latham *et al.*, (2000) showed that NTR transfected LNCaP cells were more sensitive to CB1954; 10 μM CB1954 induced a reduction in cell survival of 88%, and a dose of 100 μM reduced cell survival to 0%.

Assuming the levels of expression of the normal and His-tagged fusion proteins were similar, the results suggest that westerns might not be as consistently sensitive compared to the MTT assays; H460 cells had the most intense HSVtk-His and NTR-His band and sensitisation to both GCV and CB1945 was evident, however, PC-3 cells expressed no detectable NTR but had the greatest increase in sensitivity to CB1954. LNCaP cells had detectable HSVtk but no detectable NTR expression and no or minimal sensitisation to GCV and CB1954. Taken together, while it has been possible to reduce the cell growth of H460 and PC-3 cells expressing HSVtk and NTR, LNCaP transfected cells were no more sensitive to GCV and CB1954 than the corresponding control cells. Further experiments to improve the killing efficiency of GCV in LNCAP cells were therefore undertaken. In addition, there was some degree of toxicity of control cells to the prodrug and/or its solvent. Interestingly, H460 cells were more sensitive to CB1954, however the prostate cells lines; PC-3 and LNCaP, were more sensitive to increasing concentrations of GCV.

Figure 5.3 The growth of control (blue) and pCMV-HSVtk transfected (red) A) H460, B) PC-3 and C) LNCaP cells in the presence of 0, 1, 10, 100 and 1000 ng/µl GCV. MTT assay was performed on days 4, 5 and 7 for H460, PC-3 and LNCaP cells respectively. Vertical bars represent standard error of duplicate wells.

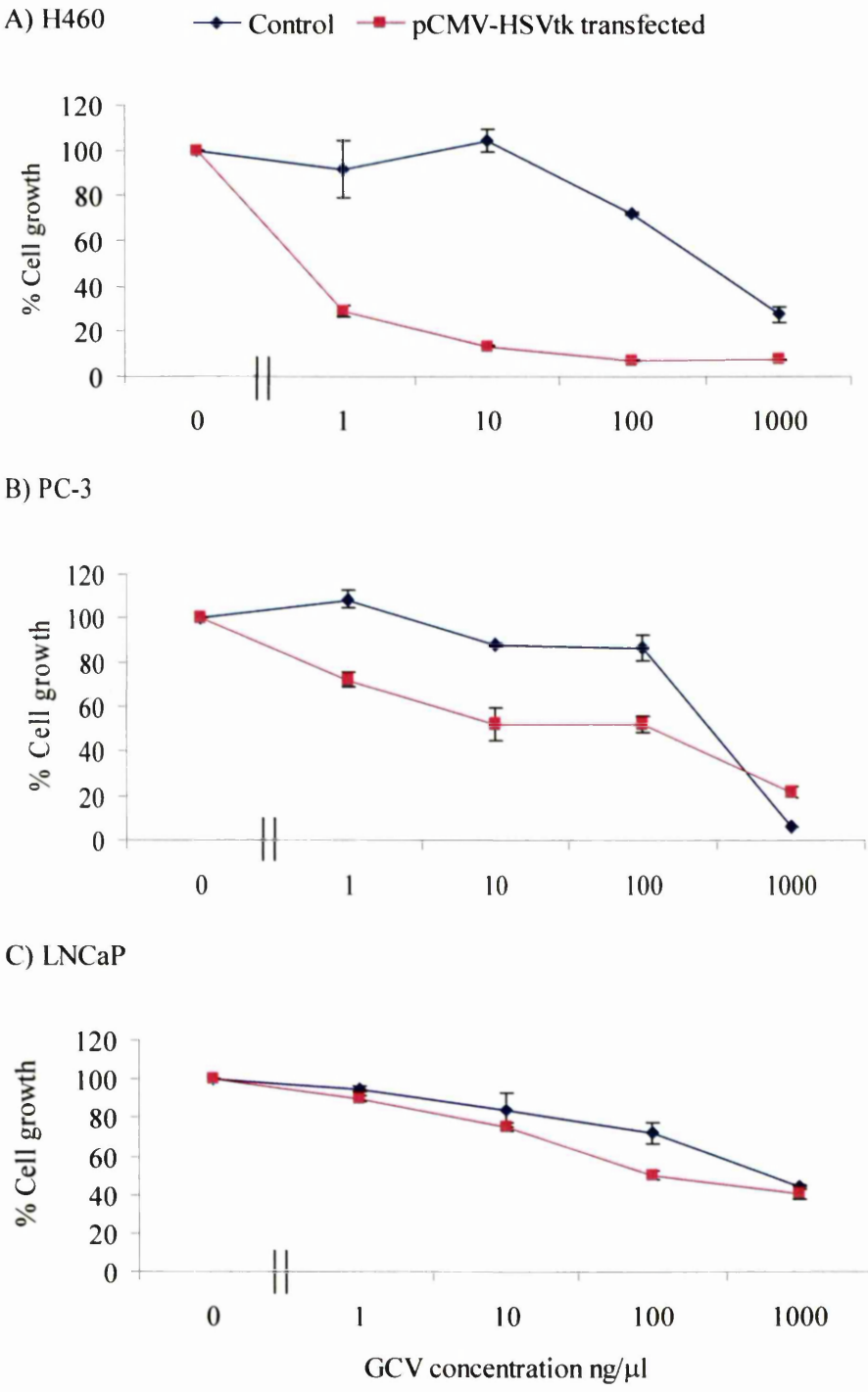
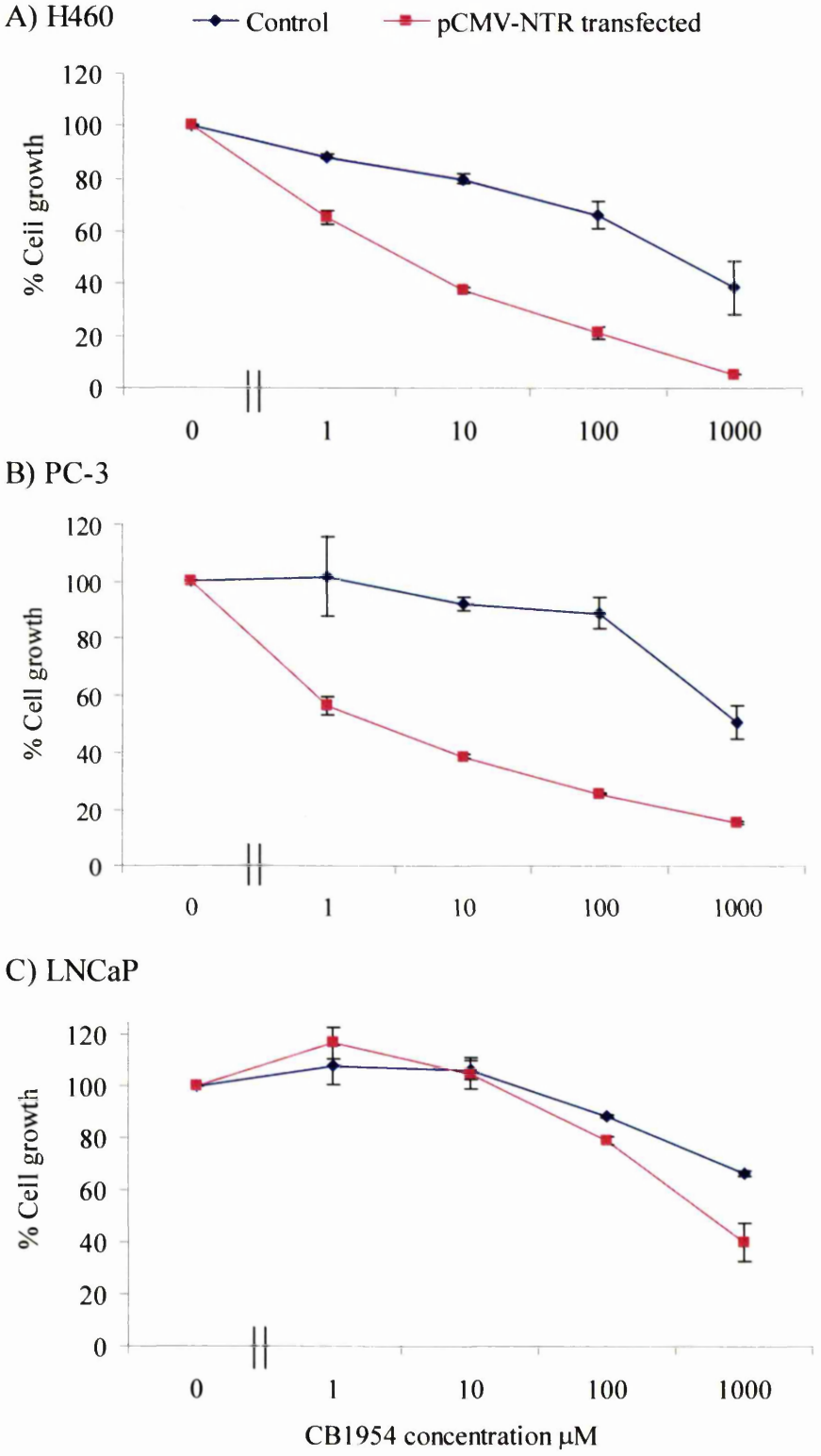


Figure 5.4 The growth of control (blue) and pCMV-NTR transfected (red) A) H460, B) PC-3 and C) LNCaP cells in the presence of 0, 1, 10, 100 and 1000 μM CB1954 cells for 48 h. Vertical bars represent standard error of duplicate wells.

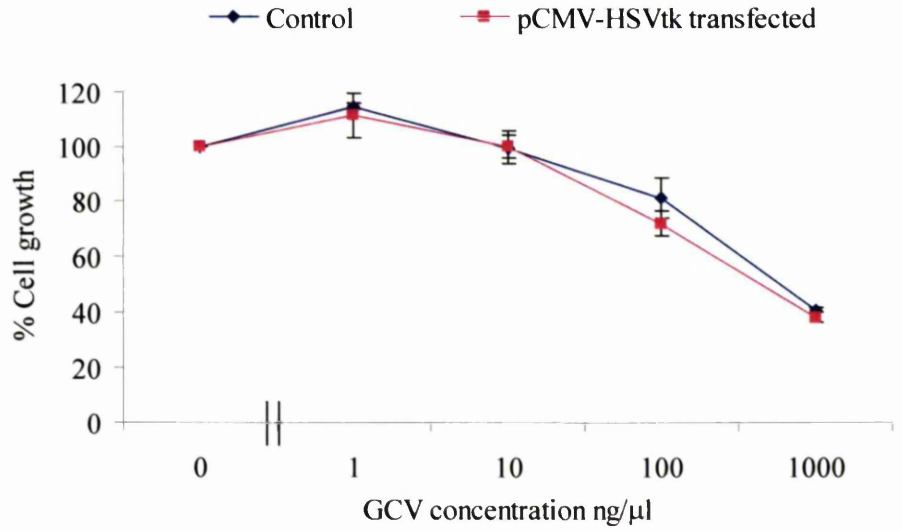


5.3.3 Assessment of the activity of HSVtk in LNCaP cells exposed to repeat doses of GCV

LNCaP cells divide once every 36-96 h and, compared to PC-3 and H460 cells (24-36 h and 23 h respectively), may be too slow for activated GCV to be incorporated into dividing DNA to a sufficient extent to cause chain termination and cell death. In addition, the slow rate of division may provide time for the cells to express or up-regulate mismatch DNA repair mechanisms to counteract the damage of activated GCV (Jiricny 1998a; Prolla 1998; Hoeijmakers 2001; Kaina 2003). To determine if the slow rate of cell division was contributing to the insensitivity of LNCaP cells to active GCV, LNCaP cells were transfected with pCMV-HSVtk, as in the previous experiments, and cultured for 9 days prior to MTT assay with GCV (0-1000 ng/μl in 0.1 M HCl) administered 8 h after transfection (day 0) and on day 3.

Incubating pCMV-HSVtk transfected LNCaP cells for longer (9 days instead of 7) and administering 2 doses of 100 ng/μl GCV instead of one, had the effect of reducing cell growth by 28% (figure 5.5) compared to 50% (figure 5.3). However, this reduction in cell growth is not significantly different to control cells (19%). Taken together, this suggests that the prodrug and/or its solvent are still toxic to the control cells and that repeat doses and longer incubation times are not enhancing the efficiency of pCMV-HSVtk transfected cell killing by active GCV. However, the possibility that no activation of GCV was occurring in LNCaP transfected cells, while it was in H460 and PC-3 cells, could not be excluded.

Figure 5.5 The growth of control (blue) and pCMV-HSVtk transfected (red) LNCaP cells in the presence of 0, 1, 10, 100 and 1000 ng/μl GCV administered on day 0 and day 3. MTT assay was performed on day 9. Vertical bars represent standard error of duplicate wells.



5.4 Discussion and Conclusions

The western blot and MTT analysis indicate that in H460 cells HSVtk and NTR are both expressed and functional, resulting in a reduction in cell growth versus control cells in the presence of either prodrug. In PC-3 cells only the HSVtk protein was detected in the western blot. However, in the MTT assay, CMV-HSVtk and CMV-NTR transfected cells were sensitized to the presence of GCV and CB1954 respectively. This suggests that although active NTR protein is expressed the His-tag is not detectable by the antibody, perhaps because it is expressed at a low level which is nevertheless sufficient to activate CB1954. In LNCaP cells, again NTR was not detected by the antibody, however in this case LNCaP cells showed essentially no increase in sensitivity to CB1954. Perhaps here the levels of expression were too low for this to occur. However, HSVtk was easily detected by the His-antibody, indicating that the LNCaP cells were transfected with this vector and that the protein was expressed, but were nevertheless insensitive to GCV. In addition, it was not possible to increase sensitivity by longer incubation times and repeat doses of GCV suggesting that the relatively slow division of LNCaP cells was not limiting the incorporation of activated GCV into dividing DNA. There are numerous possible reasons for these findings. For example, the LNCaP cells used in these experiments may have expressed a mechanism, such as mismatch DNA repair, not present in PC-3 or H460 cells, that either prevents activation of GCV by HSVtk, or prevents the incorporation of activated GCV into dividing or radiation damaged DNA. (Jiricny 1998a, b; Prolla 1998; Hoeijmakers 2001; Kaina 2003). LNCaP cells may also have translesion synthesis polymerases capable of replicating past the GCV-triphosphate, inserted into elongating DNA in the place of deoxyguanosine triphosphate, thus enabling DNA replication in spite of the presence of a DNA lesion (Lehmann 2003; Friedberg *et al.*, 2005; Lehmann 2005). Alternatively, GCV or the mono- and tri-phosphates, may be expelled from the LNCaP cells by the multi-drug resistance transporters even before they are incorporated into DNA (Doige and Ames 1993; Gottesman *et al.*, 1996). The insensitivity of LNCaP cells to NTR/CB1954 therapy may also be explained by the presence of a DNA repair pathway capable of eliminating the DNA interstrand cross-links, induced by activated CB1954. This is done by a process of novel excision repair reactions that uncouple the cross-link, followed by homologous recombination to provide the genetic information required to complete repair (McHugh *et al.*, 2001).

In conclusion, it has not been possible to induce cell death in LNCaP cells with HSVtk/GCV or NTR/CB1954 GDEPT strategies. However, other groups have shown that the HSVtk/GCV and NTR/CB1954 suicide systems driven by constitutive and/or prostate specific promoters are not only effective in LNCaP and other prostate cell lines *in vitro* and *in vivo*, but HSVtk/GCV and NTR/CB1954 has also been used successfully in phase I/II clinical trials (Eastham *et al.*, 1996; Hall *et al.*, 1997; Blackburn *et al.*, 1998; Martiniello-Wilks *et al.*, 1998; Atkinson and Hall 1999; Blackburn *et al.*, 1999; Hall *et al.*, 1999; Herman *et al.*, 1999; Hassan *et al.*, 2000; Koeneman *et al.*, 2000; Latham *et al.*, 2000; Shirakawa *et al.*, 2000; Chhikara *et al.*, 2001; Djeha *et al.*, 2001; Miles *et al.*, 2001; Nasu *et al.*, 2001; Pramudji *et al.*, 2001; Teh *et al.*, 2001; Yoshimura *et al.*, 2001; Ebara *et al.*, 2002; Freytag *et al.*, 2002a; Lee *et al.*, 2002b; Freytag *et al.*, 2003; Kubo *et al.*, 2003; Park *et al.*, 2003; Corban-Wilhelm *et al.*, 2004; Hsieh *et al.*, 2004; Satoh *et al.*, 2004; Searle *et al.*, 2004; Teh *et al.*, 2004; Tourkova *et al.*, 2004; Hattori and Maitani 2005; Lipinski *et al.*, 2005). Interestingly, in these clinical trials the dose of GCV ranges from approximatly 5 mg/kg to 13 mg/kg twice daily for up to 28 days. Assuming man is mostly water this is the equivalent of 5 – 13 mg/l or 5 – 13 ng/μl. In the experiments presented here, similar doses of GCV were applied to H460, PC-3 and LNCaP cells; 10 ng/μl GCV resulted in a 48% reduction in PC-3 cell growth. However, only a single dose was given and cell growth was measured after 4, 5, and 7 days. Similarly, in clinical trials testing the NTR/CB1954 system, patients recived 24mg/m^2 CB1954 at 3 weekly intervals. This is the equivalent of 0.7 mg/kg or 0.7 mg/l if we assume that an average patient is approximately two m^2 and weighs 75kg. In the above experiments H460, PC-3 and LNCaP cells recived only a single dose of between 1 and 1000 uM CB1954, which is the equivalent of between 0.252 and 252 mg/l. A dose of 2.52 mg/l resulted in a 61% reduction in PC-3 cell growth.

The HSVtk/GCV insensitivity of the LNCaP cells used in these studies remains to be established although it is reasonable to suggest that they may have undergone some form of spontaneous mutation. It was intended to address this at a later stage by obtaining fresh cells from the ATCC. Meanwhile, both HSVtk/GCV and NTR/CB1954 systems clearly were functional in other cell lines. It was therefore considered worthwhile to examine if the molecular switch would work in principle. It was also envisaged that other strategies would be incorporated at a later date to induce cell death in LNCaP cells if this was shown to be necessary.

Chapter 6

6.0 Results: Molecular switch

6.1 Introduction

The basic molecular switch consists of two vectors. One vector contains the prostate specific enhancer/promoter controlling the expression of Cre recombinase. The other vector consists of the strong CMV IE promoter/enhancer controlling the expression of a tumour sensitising gene, the expression of which was silenced by a 'stop' cassette flanked by *loxP* sites. Recombination between the *loxP* sites by Cre recombinase would result in the removal of the 'stop' cassette, activation of transcription and hence, in a therapeutic context, tumour sensitisation.

In chapter 4, different combinations of prostate specific enhancers and promoters were tested for specificity in different prostate and non-prostate cell lines. The experiments were inconclusive as the high level of background fluorescence generated from the pCI-neo backbone made the results difficult to interpret. Nevertheless, in vector-transfected LNCaP cells treated with DHT, GFP expression was statistically significantly higher than in cells transfected with the pLinker-GFP control. The PSE-PSE-DD3 promoter resulted in the highest mean level of increased fluorescence, although this also showed the greatest variation. The PSE-PSE-PSA promoter induced a lower fold increase, but was amongst the least variable in response. Therefore, it was decided that PSE-PSE-PSA and PSE-PSE-DD3 would be compared for their ability to drive Cre recombinase expression from the putative prostate specific construct, vector 1 (see figure 6.1). PSA has been extensively used by other groups in GDEPT strategies thus providing a foundation to which the PSE-PSE-PSA driven molecular switch can be compared (Rodriguez *et al.*, 1997; Gotoh *et al.*, 1998; Martiniello-Wilks *et al.*, 1998; Latham *et al.*, 2000; Nettelbeck *et al.*, 2000; Shirakawa *et al.*, 2000; Yu *et al.*, 2001a; Yoshimura *et al.*, 2002; Park *et al.*, 2003; Foley *et al.*, 2004b; Hsieh *et al.*, 2004; Li *et al.*, 2005; Satoh *et al.*, 2005). DD3 has recently been identified and is proving to be the most prostate cancer specific promoter to date (Rogulski *et al.*, 1997a; Verhaegh *et al.*, 2000; van der Poel *et al.*, 2001; Schalken *et al.*, 2003). In addition, recently published data indicated that duplicate PSE enhancers are more prostate specific and induce higher levels of gene expression than a single enhancer (Latham *et al.*, 2000; Wu *et al.*, 2001).

To prevent transcriptional read through and provide a means of visualising the operation of the molecular switch, the 'stop' cassette was designed to consist of two *LoxP* sites either side of the fluorescent reporter gene, CyFP, which itself had a 3′ PolyA transcription termination

signal. Thus the excision of the stop cassette by Cre mediated *LoxP* recombination would result in the disappearance of CMV driven CyFP fluorescence (see vector 2, figure 6). HSVtk was selected as the tumour sensitising gene as it has been used extensively in prostate cancer GDEPT strategies, and so is ideal to test the 'proof of principle' of the molecular switch system.

The two vectors will be combined into one (see vector 3, figure 6.1) and then inserted into an adenovirus to enhance both transfection efficiency and, based on earlier results using pCI-neo, reduce background gene expression *in vitro* and *in vivo*. In order to generate an adenovirus expressing the molecular switch, without using the standard method of Cre mediated *LoxP* recombination, which may have generated some difficulties, the Adeno-X TM expression system 1 (Clontech Cat No. 631513) was adopted. Initially, the molecular switch was cloned into pShuttle2 and then inserted into the adenovirus using the unique restriction sites I-Ceu I and PI-Sce I (see figure 6.2).

Figure 6.1 Map of the molecular switch in the form of two separate vectors (1 and 2) and combined into a single vector (vector 3). The elements shown are: CMV enhancer (red), CMV promoter (dark blue), HSVtk (turquoise), PSE1 (red), PSE2 (red), PSA/DD3 (dark blue), Cre (light blue) and the 'stop' cassette consisting of; two *LoxP* sites (black), CyFP (lilac) and a PolyA (grey).

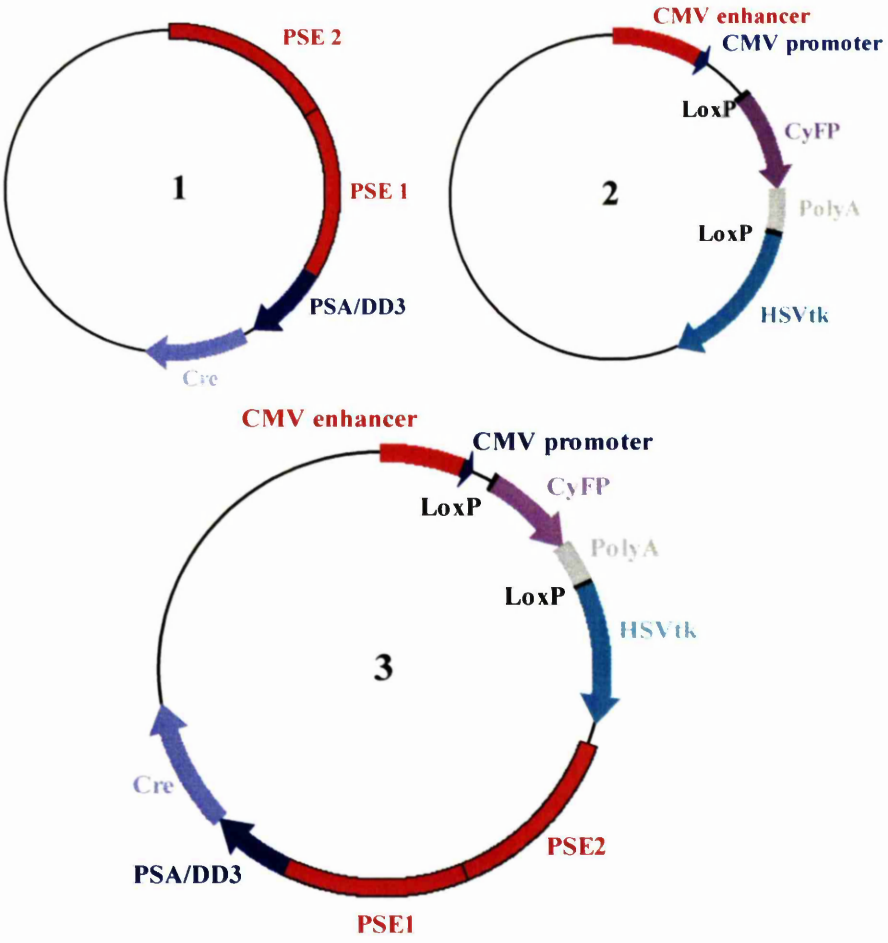
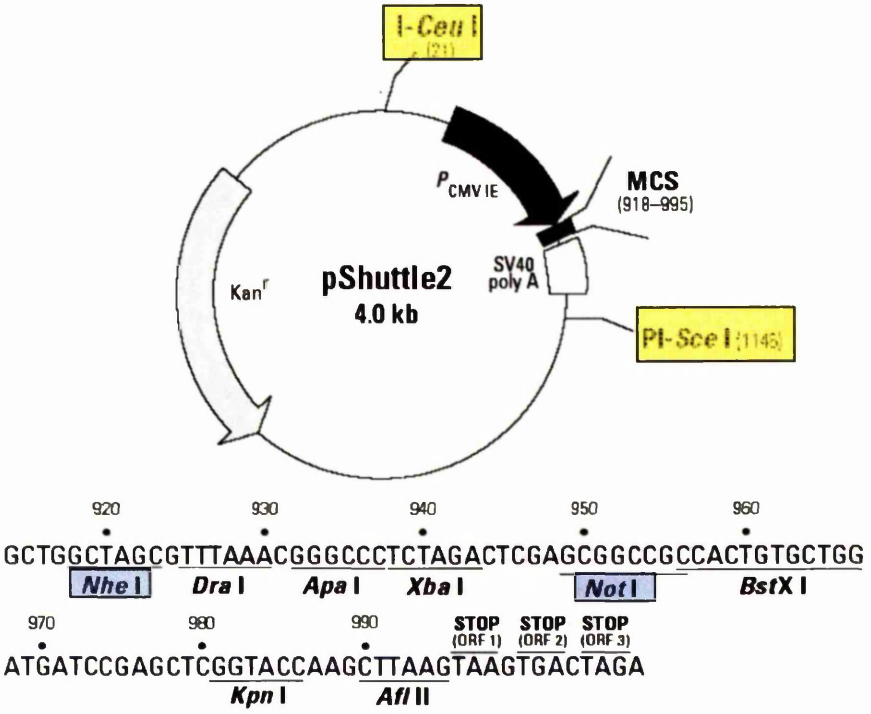
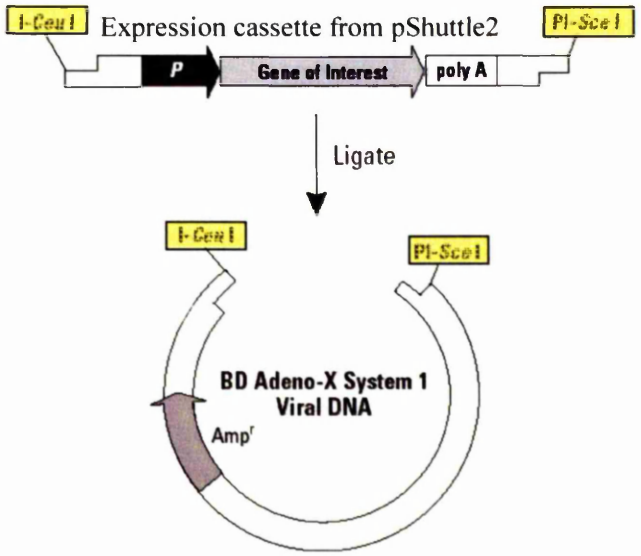


Figure 6.2 A) pShuttle2 vector and MCS sequence map. pShuttle2 was used as the host cloning vector for the construction of the single vector molecular switch system (see figure 6.1). NheI and NotI restriction sites, used for the insertion of the molecular switch elements, are highlighted in light blue and the unique restriction sites, I-Ceu I and PI-Sce I, are highlighted in yellow. B) Strategy for the insertion of the molecular switch into the BD Adeno-X system 1. The expression cassette containing the molecular switch was excised from pShuttle2 using the unique I-Ceu I and PI-Sce I restriction sites and inserted into the adenovirus. pShuttle2 and BD Adeno-X system 1 contain the kanamycin resistance (Kan^r) and ampicillin resistance (Amp^r) genes for selection and propagation in *E. coli*.

A)



B)



6.2 Creation of molecular switch vectors

Prior to insertion into the pShuttle2 vector, the components of the molecular switch were assembled in pCI-neo. This was because many of the elements of the molecular switch had already been cloned into pCI-neo (see chapters 4 and 5) and because it has a more extensive MCS than pShuttle2 providing greater cloning flexibility. At each stage of the cloning procedure, sequence fidelity, correct insertion and orientation of the genes and the presence of the required restriction sites was confirmed by DNA sequencing, PCR and restriction digest. All of the oligonucleotides used in the construction of the molecular switch are shown in table 6.1.

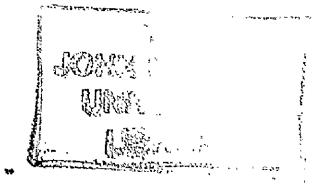


Table 6.1 Oligonucleotides used in the construction and analysis of the molecular switch in pShuttle. Restriction sites shown are: EcoRI (grey), NotI (turquoise), BglII (light blue), NheI (purple), PacI (pink), XbaI (red), PmlI (yellow) and FseI (orange). The initiation Kozac and start codons (ACC ATG) and stop codons (CTA, TTA, TCA) are highlighted in yellow.

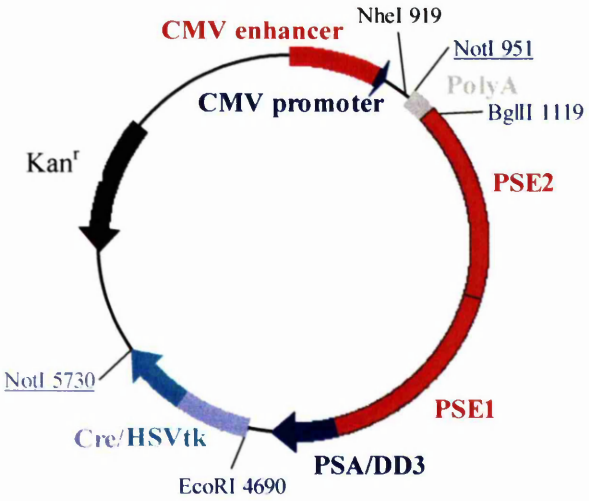
Oligos	Sequence	
Cre1	Sense	5′ GGGAATTCACC ATG TCC AAT TTA CTG ACG GTA C 3′
	Anti-sense	5′ ATGCGGCCGCCTA ATC GCC ATC TTC CAG CAG G 3′
PolyA	Sense	5′ GAAGATCTGCGGCCGCATATCTTTATTTTCATTACATC 3′
	Anti-sense	5′ CTAGATCTGACACAAAAAACCAACACA 3′
Stop Primers	Sense	5′ GCGCTAGCATAACTTCGTATAAT 3′
	Anti-sense	5′ GGGAATTCATATAACTTCCGTATAGCATA 3′
CyFP1	Sense	5′ GCTTAATTAAACC ATG GTG AGC AAG GGC G 3′
	Anti-sense	5′ GCTCTAGATTA CTT GTA CAG CTC GTC CAT GC 3′
GFP	Sense	5′ GCGCTAGCACC ATG GTG AGC AAG GGC GAG G 3′
	Anti-sense	5′ ATGCGGCCGCTTA CTT GTA CAG CTC GTC CAT TGC 3′
HSVtk 2	Sense	5′ GCGCTAGCACC ATG GCT TCG TAC CCC TGC C 3′
	Anti-sense	5′ ATGCGGCCGCTCA GTT AGC CTC CCC CAT CTC C 3′
Cre 2	Sense	5′ GCGCTAGCACC ATG TCC AAT TTA CTG ACG GTA C 3′
	Anti-sense	5′ ATGCGGCCGCCTA ATC GCC ATC TTC CAG CAG G 3′
HSVtk 3	Sense	5′ GGGAATTCACC ATG GCT TCG TAC CCC TGC C 3′
	Anti-sense	5′ ATGCGGCCGCTCA GTT AGC CTC CCC CAT CTC C 3′
Stop	Sense	5′ GCTAGCATAACTTCGTATAATGTATGC TATACGAAGTTATCGTTAATTAAGCTCTAGACGCACGTG AAAGTGGCCGGCCATAACTTCGTATAATGTATGCTATA CGAAGTTATATGAATTC 3′

6.2.1 pShuttle-PolyA-PSE-PSE-PSA/DD3-Cre

The pPSE-PSE-PSA-GFP vector (figure 4.3) was used as the basis for the construction of pShuttle-PolyA-PSE-PSE-PSA-Cre. Cre recombinase cDNA was PCR amplified from pBS185 (see appendix section 8.6; GIBCO Life Technologies) with primers designed to add 5′ EcoRI and 3′ NotI restriction sites (see table 6.1 Cre1). Cre recombinase cDNA was initially inserted into pGem-T and then cloned into pPSE-PSE-PSA-GFP using EcoRI/NotI restriction sites thus replacing GFP to create pPSE-PSE-PSA-Cre.

In addition, a polyadenylation signal (PolyA) was added to the 5′ end of PSE. Specific PCR primers complementary to the 5′ and 3′ ends of the synthetic PolyA signal of the neomycin resistance gene in pCI-neo were used to amplify the PolyA signal and introduce 5′ BglII/NotI and 3′ BglII restriction sites (see table 6.1 PolyA). As before, the PCR product was cloned into pGem-T and then inserted into pPSE-PSE-PSA-Cre using BglII restriction sites to create pPolyA-PSE-PSE-PSA-Cre. PolyA-PSE-PSE-PSA-Cre was then inserted into pShuttle2 on a NotI/NotI restriction digest (see figure 6.2) to create pShuttle-PolyA-PSE-PSE-PSA-Cre, shown in figure 6.3. The same procedure was adopted for the creation of pShuttle-PolyA-PSE-PSE-DD3-Cre from pPSE-PSE-DD3-GFP.

Figure 6.3 Vector map of pShuttle-PolyA-PSE-PSE-PSA/DD3-Cre/HSVtk. The restriction sites EcoRI/NotI and BglII (light blue) were used for the cloning of HSVtk, Cre and PolyA into pPSE-PSE-PSA/DD3-GFP. PolyA-PSE-PSE-PSA/DD3-Cre/HSVtk was then cloned into pShuttle using NotI restriction sites (underlined). This vector also contains a Kan^r marker for selection in *E. coli*.



pShuttle-PolyA-PSE-PSE-PSA/DD3-Cre
8325 - 8753 bp

6.2.2 pShuttle-*LoxP*-CyFPPolyA-*LoxP*-HSVtk

In order to create the 'stop' cassette, an oligonucleotide was designed consisting of two *LoxP* sequences flanking 4 unique restriction sites; PacI, XbaI, PmlI and FseI. In addition, NheI and EcoRI restriction sites were added to the 5′ and 3′ ends respectively (figure 6.4 and table 6.1; Stop). Due to problems directly annealing long fragments with self complementary sections, two primers were designed that were complementary to the 5′ and 3′ ends of the stop oligonucleotide (table 6.1; Stop primers), to PCR amplify the 'stop' cassette using the synthetic stop oligonucleotide as a template. The 'stop' cassette was then cloned into pGem-T before cloning into pCMV-HSVtk (section 5.2.2) using EcoRI/NotI restriction sites to create pCMV-*LoxP*-MCS-*LoxP*-HSVtk.

Figure 6.4 Stop oligonucleotide sequence. Two *LoxP* sequences consisted of two 13 bp inverted repeats (red) with an intervening asymmetric 8 bp core (blue). The restriction sites NheI and EcoRI, for cloning into pCMV-HSVtk, and PacI, XbaI, PmlI and FseI, for the insertion of CyFP and PolyA, are underlined.

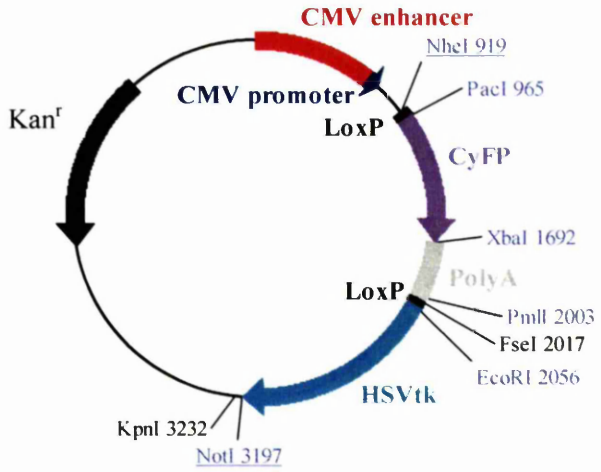
5'GCTAGCATAACTTCGTATAATGTATGCTATACGAAGTTATCGTTAATTAA
NheI PacI

GCTCTAGACGCACGTGAAAGTGGCCGGCCATAACTTCGTATAATGTATGC
XbaI PmlI FseI

TATACGAAGTTATATGAATTC 3'
EcoRI

PCR primers complementary to the 5′ and 3′ ends of the CyFP gene were used to amplify the gene from pCyFP (kind gift from Flavia Moreira-Leite, PICR, Manchester, UK) and introduce 5′ PacI and 3′ XbaI restriction sites (see table 6.1; CyFP1). CyFP was then cloned into pCMV-*LoxP*-MCS-*LoxP*-HSVtk via pGem-T using PacI/XbaI restriction sites. The bovine growth hormone (BGH) PolyA from pcDNA3 (Invitrogen) was then excised using XbaI and PvuII restriction sites and inserted into pCMV-*LoxP*-CyFP-*LoxP*-HSVtk using XbaI and PmlI by blunt end cloning to create pCMV-*LoxP*-CyFPPolyA-*LoxP*-HSVtk. *LoxP*-CyFPPolyA-*LoxP*-HSVtk was then excised from the pCI-neo vector and inserted into pShuttle using NheI/NotI restriction sites to create pShuttle-CMV-*LoxP*-CyFPPolyA-*LoxP*-HSVtk, shown in figure 6.5.

Figure 6.5 Vector map of pShuttle-CMV-*LoxP*-CyFPPolyA-*LoxP*-HSVtk. The restriction sites NheI/EcoRI, used for insertion of 'Stop' cassette into pCMV-HSVtk and PacI, XbaI and PmlI, used for the insertion of CyFP and PolyA into the 'stop' cassette, are shown in light blue. *LoxP*-CyFPPolyA-*LoxP*-HSVtk was then cloned into pShuttle2 using NheI/NotI restriction sites (underlined). This vector also contains a Kan^r marker for selection in *E. coli*.



pShuttle-CMV-LoxP-CyFPPolyA-LoxP-HSVtk
6220 bp

6.2.3 pShuttle-Molecular switch

In order to generate an all-in-one molecular switch for insertion into the adenovirus, PolyA-PSE-PSE-PSA-Cre and PolyA-PSE-PSE-DD3-Cre were excised from pShuttle-PolyA-PSE-PSE-PSA-Cre and pShuttle-PolyA-PSE-PSE-DD3-Cre, respectively, and inserted into pShuttle-CMV-*LoxP*-CyFPPolyA-*LoxP*-HSVtk using NotI/NotI restriction sites. After several attempts, all constructs were found to have lost the 'stop' cassette. It seems reasonable to suggest that the reason for this was that the prostate specific enhancer/promoter combinations chosen to drive Cre expression were active in *E. coli*. The expressed recombinase then recognised the *LoxP* sites leading to excision of the 'stop' cassette creating two separate plasmids: pShuttle-CMV-*LoxP*-HSVtk-PolyA-PSE-PSE-PSA/DD3-Cre and *LoxP*-CyFPPolyA. The all-in-one construct pShuttle-CMV-*LoxP*-CyFPPolyA-*LoxP*-HSVtk-PolyA-PSE-PSE-PSA/DD3-Cre (pShuttle-Molecular switch), could therefore not be propagated in *E. coli* and so vector DNA appropriate for digestion and insertion into the adenovirus could not be produced. I speculate that it may be possible to remedy this by culturing the *E. coli* in minimal media, such as M9 salts, however a more feasible approach would be use another host system altogether, such as yeast, which would be expected to be less promiscuous than *E. coli* in its recognition of sequences.

6.3 Creation of control vectors

6.3.1 pShuttle-GFP, pShuttle-CyFP, pShuttle-HSVtk and pShuttle-Cre

pShuttle-GFP was generated to act as a positive control for FACS analysis since GFP was under the control of the constitutive strong CMV enhancer/promoter, thus providing an indication of the maximum GFP expression a cell line could achieve. Similarly, pShuttle-HSVtk and pShuttle-Cre were controls for the expression of HSVtk and Cre driven by the CMV enhancer/promoter again so that this could be compared to prostate specific promoters. All three constructs were generated by PCR amplification of the genes GFP, Cre and HSVtk (from pEGFP, pBS185 and pORF-HSV1tk respectively) with primers designed to add 5′ NheI and 3′ NotI restriction sites (see table 6.1; GFP2, HSVtk2 and Cre2). The PCR products were cloned into pGem-T and then inserted into pShuttle using NheI/NotI restriction sites (see figure 6.2).

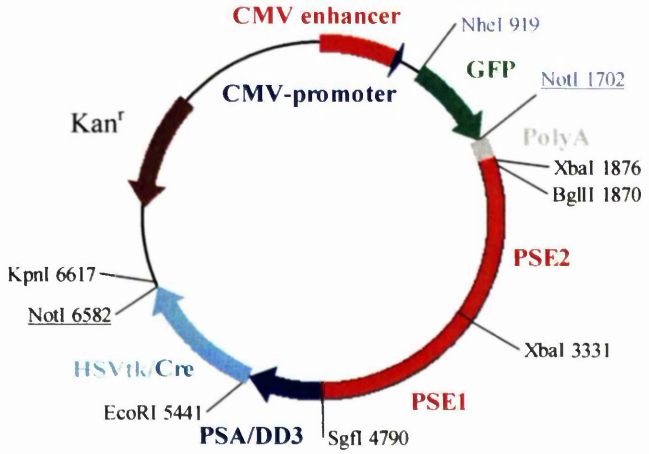
6.3.2 pShuttle-GFP-PolyA-PSE-PSE-PSA/DD3-Cre

Since, for reasons presented above, PolyA-PSE-PSE-PSA/DD3-Cre cannot be inserted into pShuttle-*LoxP*-CyFPPolyA-*LoxP*-HSVtk, it was proposed to test the molecular switch as two separate vectors using lipofectamine to co-transfect prostate and non-prostate cells. To achieve this, GFP was added to the 5′ end of the PolyA signal to ensure that there was no read-through from the CMV promoter in pShuttle2 which could have affected the specificity of the prostate specific enhancer/promoter. In addition, since GFP was expressed from the CMV promoter and thus measurable by FACS analysis, these vectors were used to determine the transfection efficiency of large vectors into prostate cells. PolyA-PSE-PSE-PSA/DD3-Cre was excised from pCMV-PolyA-PSE-PSE-PSA/DD3-Cre (see section 6.2.1) and inserted into pShuttle-GFP using NotI/NotI restriction sites to create pShuttle-GFP-PolyA-PSE-PSE-PSA-Cre and pShuttle-GFP-PolyA-PSE-PSE-DD3-Cre (see figure 6.6)

6.3.3 pShuttle-GFP-PolyA-PSE-PSE-PSA/DD3-HSVtk

pShuttle-GFP-PolyA-PSE-PSE-PSA/DD3-HSVtk vectors were made to enable the comparison of GDEPT driven by prostate specific promoters alone and when incorporated into the molecular switch. The vector was constructed using the same procedure as pShuttle-PolyA-PSE-PSE-PSA/DD3-Cre (see section 6.2.1 and figure 6.3) but using PCR primers designed to amplify HSVtk from pORF-HSV1tk (InvivoGen) adding 5′ EcoRI and 3′ NotI restriction sites (see table 6.1; HSVtk3) instead of Cre. PolyA-PSE-PSE-PSA/DD3-HSVtk was then inserted into pShuttle-GFP using NotI/NotI restriction sites to create pShuttle-GFP-PolyA-PSE-PSE-PSA-HSVtk and pShuttle-GFP-PolyA-PSE-PSE-DD3-HSVtk (see figure 6.6).

Figure 6.6 Vector map of pShuttle-GFP-PolyA-PSE-PSE-PSA/DD3-Cre/HSVtk. GFP was initially cloned into pShuttle using NheI/NotI restriction sites (light blue) and then PolyA-PSE-PSE-PSA/DD3-HSVtk/Cre was inserted into pShuttle-GFP using NotI/NotI restriction sites (underlined). This vector also contains a Kan^r marker for selection in *E. coli*.



pShuttle-GFP-PolyA-PSE-PSE-PSA/DD3-HSVtk/Cre
9177 - 9605 bp

6.4 Experiments to assess the molecular switch in prostate and non-prostate cells.

6.4.1 Assessment of the molecular switch by FACS analysis.

In order to determine the transfection efficiency of large vectors (> 9000 bp) LNCaP, PC-3 and H460 cells were plated in triplicate at a density of 2 x 10^5 cells and transfected with 0.5 μg of each of the control vector pShuttle-GFP and the large test vectors pShuttle-GFP-PolyA-PSE-PSE-PSA-Cre and pShuttle-GFP-PolyA-PSE-PSE-DD3-Cre. GFP expression was then measured 48 h later by FACS analysis. The GFP fluorescence from pShuttle-GFP-PolyA-PSE-PSE-PSA-Cre and pShuttle-GFP-PolyA-PSE-PSE-DD3-Cre was compared to pShuttle-GFP, which was defined as a transfection efficiency of 100%. In LNCaP and PC-3 cells the transfection efficiency of the large vectors varied between 61% and 82% that of pShuttle-GFP, however, in H460 cells the transfection efficiency was reduced to 31% for both pShuttle-GFP-PolyA-PSE-PSE-PSA-Cre and pShuttle-GFP-PolyA-PSE-PSE-DD3-Cre. While the transfection efficiency of large vectors was less than pShuttle-GFP, it was still relatively high and it was considered that it would provide sufficient Cre recombinase to excise the 'stop' cassette upon co-transfections with the molecular switch vectors. Therefore, the possibility of measuring transfection efficiency using transfection media other than lipofectamine (genejuice^{TM}) was not investigated.

To demonstrate that Cre recombinase would recognise the *LoxP* sites and excise the intervening 'stop' cassette in cells transfected with the two vectors, FACS analysis was used to measure the expression of CyFP. Androgen responsive LNCaP cells were plated in media containing 10 nM DHT and transfected with 0.5 μg of pShuttle-CMV-*LoxP*-CyFPPolyA-*LoxP*-HSVtk or co-transfected with 0.5 μg of pShuttle-CMV-*LoxP*-CyFPPolyA-*LoxP*-HSVtk with 0.5 μg of either pShuttle-Cre, pShuttle-PolyA-PSE-PSE-PSA-Cre or pShuttle-PolyA-PSE-PSE-DD3-Cre. In total 1 μg DNA was used per well, of which the expression of 0.5 μg was detected by FACS. The experiment was then repeated six times on different days. The molecular switch was also tested in PC-3 cells (the non-androgen responsive prostate cell line) and H460 cells (the non-prostate control cell line) both without the addition of DHT, and repeated on three separate occasions.

Figure 6.6 shows the % CyFP expression, compared to pShuttle-*LoxP*-CyFPPolyA-*LoxP*-HSVtk, from the co-transfection of the molecular switch vector (pShuttle-*LoxP*-CyFPPolyA-*LoxP*-HSVtk) and the Cre recombinase vector, expression of which was driven by either the CMV enhancer/promoter (pShuttle-Cre) or the prostate specific promoters, PSA and DD3, with two PSE enhancers (pShuttle-PolyA-PSE-PSE-PSA-Cre or pShuttle-PolyA-PSE-PSE-DD3-Cre). In LNCaP cells the molecular switch operates efficiently; CyFP expression was significantly reduced (by 84% $P < 0.01$) when Cre expression was driven by the CMV promoter/enhancer in pShuttle-Cre. However, when Cre expression was driven by the prostate specific promoter/enhancers the CyFP expression reduced by 18% and 28% for pShuttle-PolyA-PSE-PSE-PSA-Cre and pShuttle-PolyA-PSE-PSE-DD3-Cre respectively, but these were not significantly different from the control due to the large variation (show by the SE bars) between replicate experiments. Similarly in PC-3 cells CyFP expression was significantly reduced by 31% ($P < 0.01$) when Cre expression was driven by the CMV enhancer/promoter whereas the prostate specific enhancer/promoters had no significant effect on CyFP expression compared to the control. In contrast, in H460 cells co-transfection of the molecular switch with pShuttle-GFP, pShuttle-PolyA-PSE-PSE-PSA-Cre and pShuttle-PolyA-PSE-PSE-DD3-Cre significantly reduced the expression of CyFP by 79%, 56% and 40% respectively ($P < 0.01$).

Figure 6.6 FACS analysis of CyFP expression in LNCaP, PC-3 and H460 cells transfected in triplicate with pShuttle-CMV-*LoxP*-CyFPPolyA-*LoxP*-HSVtk (column 1: CMV-*LoxP*-CyFPPolyA-*LoxP*-HSVtk) or co-transfected with pShuttle-CMV-*LoxP*-CyFPPolyA-*LoxP*-HSVtk and either pShuttle-Cre (column 2: CMV-*LoxP*-CyFPPolyA-*LoxP*-HSVtk + CMV-Cre), pShuttle-PolyA-PSE-PSE-PSA-Cre (column 3: CMV-*LoxP*-CyFPPolyA-*LoxP*-HSVtk + PSE-PSE-PSA-CRE) or pShuttle-PolyA-PSE-PSE-DD3-Cre (column 4: CMV-*LoxP*-CyFPPolyA-*LoxP*-HSVtk + PSE-PSE-DD3-Cre). CyFP expression was compared to pShuttle-*LoxP*-CyFPPolyA-*LoxP*-HSVtk which was taken as 100% CyFP expression for each cell line. Vertical bars represent SE of 6 different experiments for LNCaP and three for PC-3 and H460 cells. * represents significant difference compared to pShuttle-*LoxP*-CyFPPolyA-*LoxP*-HSVtk ($P < 0.01$, two-tailed students T test assuming equal variance).

Column 1: CMV-*LoxP*-CyFPPolyA-*LoxP*-HSVtk
Column 2: CMV-*LoxP*-CyFPPolyA-*LoxP*-HSVtk + CMV-Cre
Column 3: CMV-*LoxP*-CyFPPolyA-*LoxP*-HSVtk + PSE-PSE-PSA-Cre
Column 4: CMV-*LoxP*-CyFPPolyA-*LoxP*-HSVtk + PSE-PSE-DD3-Cre
% CyFP expression
0
20
40
60
80
100
120
LNCaP
PC-3
H460
1
2
3
4
*

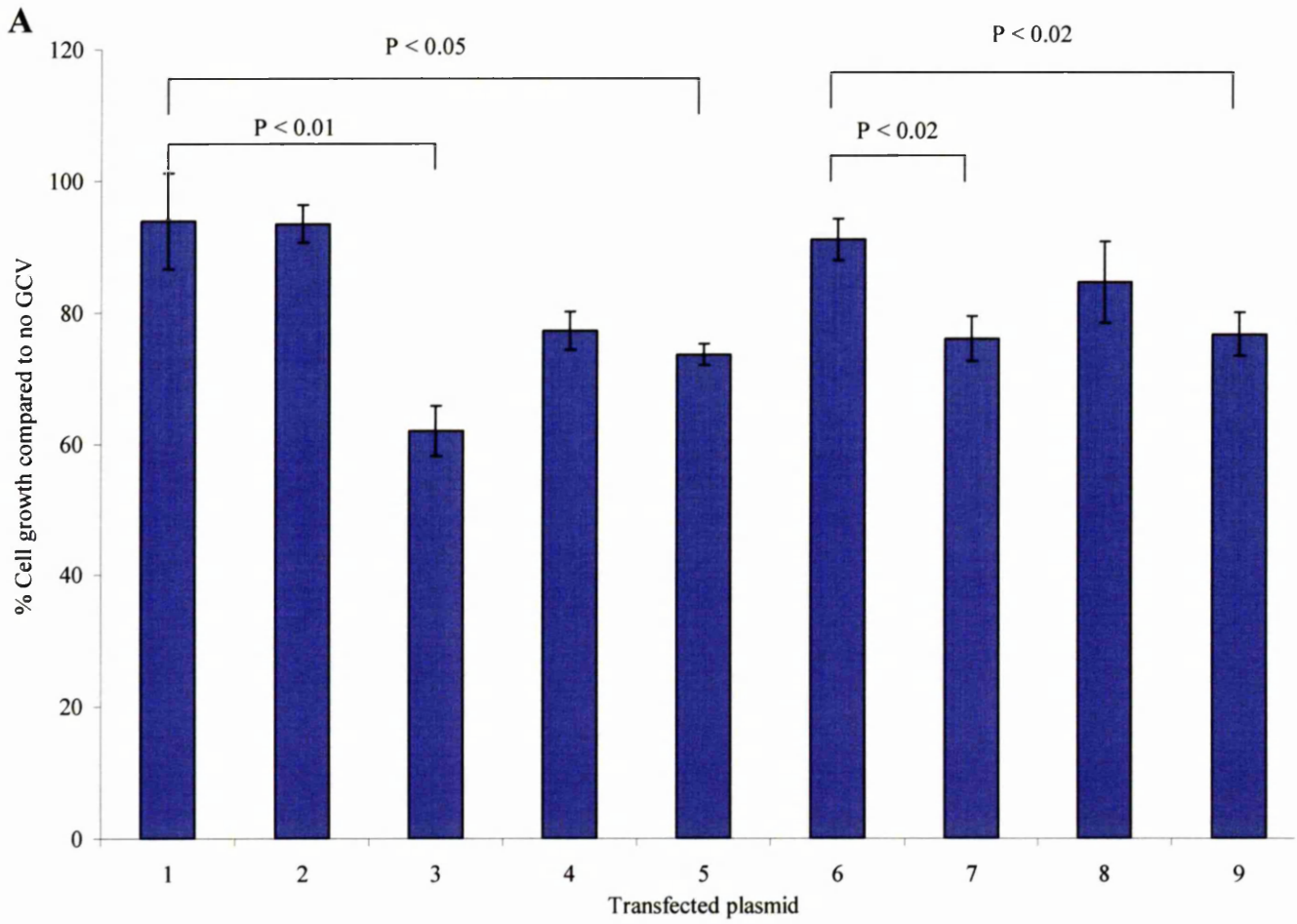
6.4.2 Assessment of the molecular switch by MTT assay

As described above, FACS analysis of CyFP showed that the molecular switch was functional but in order to determine whether, as a consequence of 'stop' cassette removal, HSVtk was being expressed, H460 and PC-3 cells were transfected with 0.5 μg of each of the control vectors pShuttle-GFP, pShuttle-HSVtk, pShuttle-GFP-PolyA-PSE-PSE-PSA-HSVtk or pShuttle-GFP-PolyA-PSE-PSE-DD3-HSVtk or co-transfected with 0.5 μg of pShuttle-CMV-*LoxP*-CyFPPolyA-*LoxP*-HSVtk with either 0.5 μg pShuttle-Cre, pShuttle-GFP-PolyA-PSE-PSE-PSA-Cre or pShuttle-GFP-PolyA-PSE-PSE-DD3-Cre. In total 1 μg DNA was used per well, of which 0.5 μg would encode HSVtk. H460 cells were then exposed to 10 ng/μl of GCV and PC-3 cells to 50 ng/μl of GCV, previously determined to be the optimum drug concentrations to induce cell death in the two cell lines, (see chapter 5). Cell growth was then measured by MTT analysis after 4 and 5 days for H460 and PC-3 cells respectively (see section 2.6.4 and chapter 5). The experiments were repeated on 3 different days. The killing efficiency of HSVtk in the molecular switch was not tested in LNCaP cells as they had previously been shown to be unresponsive to HSVtk/GCV therapy (see chapter 5).

The growth of control cells and transfected cells in the presence and absence of GCV are shown for PC-3 and H460 cells in figure 6.7A and B, respectively. In PC-3 cells there was no significant difference in cell growth between the three controls; un-transfected cells (column 1) and pShuttle-GFP (column 2) or pShuttle-*LoxP*-CyFPPolyA-*LoxP*-HSVtk (column 6) transfected cells. The addition of GCV significantly reduced PC-3 cell growth when transfected with pShuttle-HSVtk (column 3) and pShuttle-GFP-PolyA-PSE-PSE-DD3-HSVtk (column 5) by 32% and 20% respectively compared to un-transfected cells. Cell growth also reduced by 17% when transfected with pShuttle-GFP-PolyA-PSE-PSE-PSA-HSVtk (column 4) however, due to the variation between repeat experiments, this was not significantly different to the control. When pShuttle-*LoxP*-CyFPPolyA-*LoxP*-HSVtk transfected cells were co-transfected with pShuttle-Cre (column 7) and pShuttle-GFP-PolyA-PSE-PSE-DD3-Cre (column 9) there was a significant reduction in cell growth of 15% and 14% respectively compared to pShuttle-*LoxP*-CyFPPolyA-*LoxP*-HSVtk (column 6; $P < 0.02$) but no significant reduction when co-transfected with pShuttle-GFP-PolyA-PSE-PSE-PSA-Cre (column 8). Interestingly, there was no significant difference in cell growth when using CMV or prostate specific enhancer/promoters to drive HSVtk directly or in conjunction with the molecular switch. This indicates that the molecular switch was not a limiting factor of HSVtk expression.

In H460 cells there was also no significant difference in cell growth between the three control wells; un-transfected cells (column 1) and pShuttle-GFP (column 2) or pShuttle-*LoxP*-CyFPPolyA-*LoxP*-HSVtk (column 6) transfected cells. However, there was a significant reduction in cell growth of 46%, 17% and 15% between pShuttle-HSVtk (column 3), pShuttle-GFP-PolyA-PSE-PSE-PSA-HSVtk (column 4) and pShuttle-GFP-PolyA-PSE-PSE-DD3-HSVtk (column 5) respectively compared to un-transfected cells. Similarly, when these promoters were used in the molecular switch to drive Cre expression in co-transfections with pShuttle-*LoxP*-CyFPPolyA-*LoxP*-HSVtk cell growth reduced significantly by 56%, 24% and 40% for CMV (column 7), PSE-PSE-PSA (column 8) and PSE-PSE-DD3 (column 9) respectively, compared to pShuttle-*LoxP*-CyFPPolyA-*LoxP*-HSVtk (column 6). Interestingly, there was also a significant reduction in cell growth when PSE-PSE-DD3 (column 9) was used to drive Cre expression in the molecular switch compared to pShuttle-GFP-PolyA-PSE-PSE-DD3-HSVtk (column 5; $P < 0.01$). This suggests that the molecular switch was effecting the expression of HSVtk when Cre recombinase was driven by the DD3 promoter. These results are surprising as activation of prostate specific promoters in H460 lung carcinoma cells was not expected.

Figure 6.7 Percentage cell growth in A) PC-3 and B) H460 cells treated with GCV compared to non-treated controls. PC-3 and H460 cells were un-transfected (column 1: cells only) or transfected in triplicate with the controls pShuttle-GFP (column 2), pShuttle-HSVtk (column 3), pShuttle-GFP-PolyA-PSE-PSE-PSA-HSVtk (column 4), pShuttle-GFP-PolyA-PSE-PSE-DD3-HSVtk (column 5) and pShuttle-CMV-*LoxP*-CyFPPolyA-*LoxP*-HSVtk (column 6) or co-transfected with pShuttle-CMV-*LoxP*-CyFPPolyA-*LoxP*-HSVtk and pShuttle-Cre (column 7), pShuttle-GFP-PolyA-PSE-PSE-PSA-Cre (column 8), or pShuttle-GFP-PolyA-PSE-PSE-DD3-Cre (column 9). Vertical bars represent standard error of 3 separate experiments. Horizontal bars represent significant difference compared to cells only or pShuttle-*LoxP*-CyFPPolyA-*LoxP*-HSVtk (two-tailed students T test assuming equal variance).



Column 1: cells only

Column 2: CMV-GFP

Column 3: CMV-HSVtk

Column 4: CMV-GFP-PolyA-PSE-PSE-PSA-HSVtk

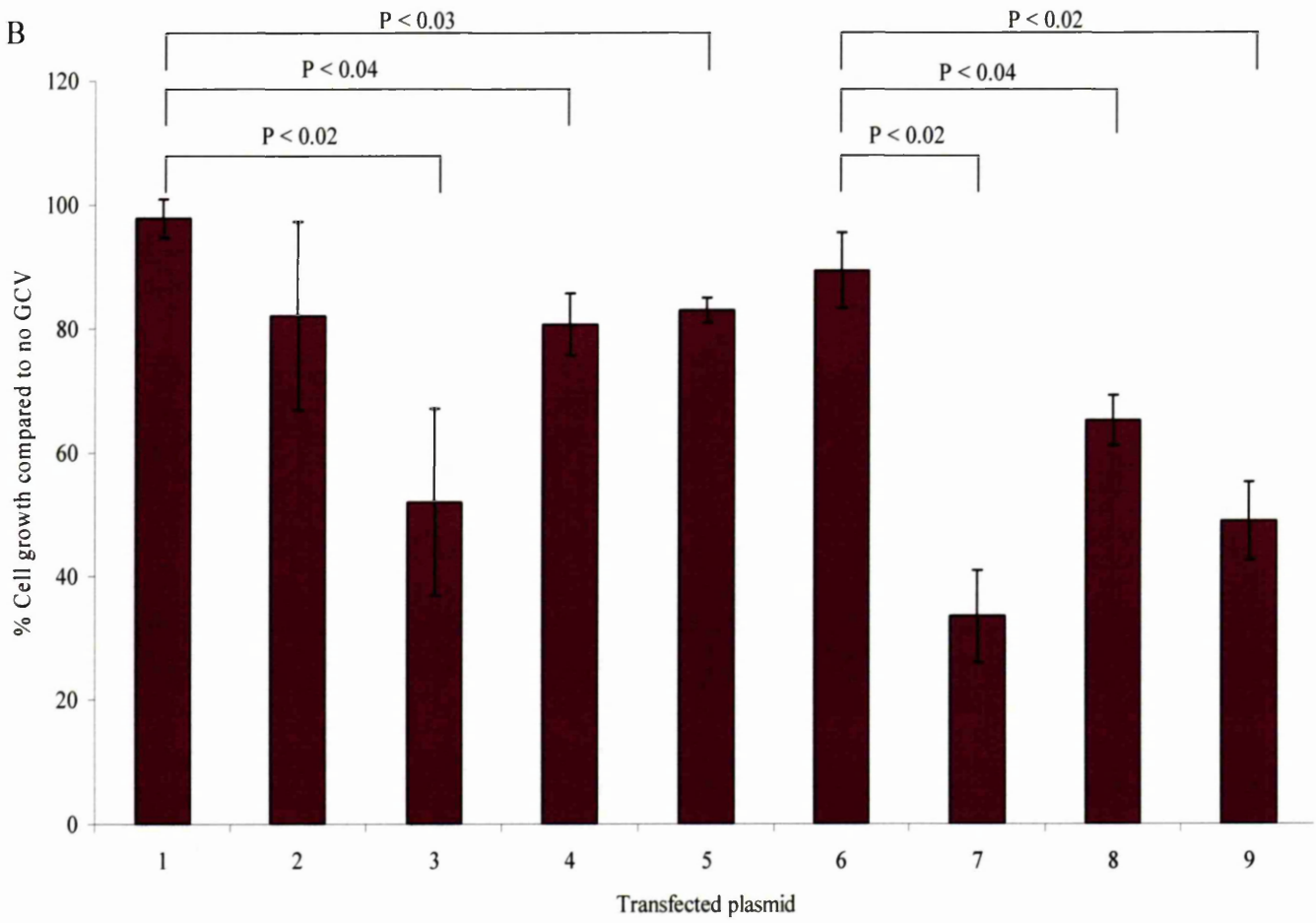
Column 5: CMV-GFP-PolyA-PSE-PSE-DD3-HSVtk

Column 6: CMV-*LoxP*-CyFPPolyA-*LoxP*-HSVtk

Column 7: CMV-*LoxP*-CyFPPolyA-*LoxP*-HSVtk + CMV-Cre

Column 8: CMV-*LoxP*-CyFPPolyA-*LoxP*-HSVtk + PSE-PSE-PSA-Cre

Column 9: CMV-*LoxP*-CyFPPolyA-*LoxP*-HSVtk + PSE-PSE-DD3-Cre



Column 1: cells only

Column 2: CMV-GFP

Column 3: CMV-HSVtk

Column 4: CMV-GFP-PolyA-PSE-PSE-PSA-HSVtk

Column 5: CMV-GFP-PolyA-PSE-PSE-DD3-HSVtk

Column 6: CMV-*LoxP*-CyFPPolyA-*LoxP*-HSVtk

Column 7: CMV-*LoxP*-CyFPPolyA-*LoxP*-HSVtk + CMV-Cre

Column 8: CMV-*LoxP*-CyFPPolyA-*LoxP*-HSVtk + PSE-PSE-PSA-Cre

Column 9: CMV-*LoxP*-CyFPPolyA-*LoxP*-HSVtk + PSE-PSE-DD3-Cre

6.5 Discussion and Conclusions

FACS analysis and MTT assays confirmed that when the CMV enhancer/promoter was used to drive the expression of Cre recombinase the 'Stop' cassette was removed from vector 2 (see figure 6.1) by recombination, resulting in both a reduction in CyFP expression, in LNCaP, PC-3 and H460 cells, and an increase in HSVtk expression, as indicated by the increase in sensitivity to GCV in PC-3 and H460 cells. The activity of the prostate specific enhancer/promoters when used to drive HSVtk expression alone or in conjunction with the molecular switch demonstrated that they could be activated. Indeed in several situations sensitisation to GCV was better or equal to that produced when Cre was expressed from the CMV enhancer/promoter. However, FACS analysis of CyFP expression indicated that PSE-PSE-PSA and PSE-PSE-DD3 were both active, but only in H460 cells. This was confirmed by MTT analysis, which also indicated that PSE-PSE-DD3 was also active in PC-3 cells.

It was originally hypothesised that the prostate specific promoters would only be active in LNCaP cells as these are the only prostate cell line that is androgen responsive. Yoshimura *et al.*, (2002) showed that a lipofectamine transfection of LNCaP cells with a PSE-PSA enhancer/promoter combination in conjunction with a molecular switch induced a 2 fold increase in transcription compared to a single transfection with a PSE-PSA-reporter gene construct. However, it has not been possible to confirm or refute this as LNCaP cells showed a large variation in transfection efficiency and promoter activity over 6 different experiments (shown by the large SE bars in figure 6.6) suggesting that it is not a reliable cell line in which to test the efficiency of a molecular switch. The only other prostate cell line available was the PC-3 cell line. MTT analysis suggested that while PSE-PSE-DD3 was active, there was no significant difference in HSVtk expression between pShuttle-GFP-PolyA-PSE-PSE-DD3-HSVtk and co-transfections of pShuttle-GFP-PolyA-PSE-PSE-DD3-Cre and pShuttle-*LoxP*-CyFPPolyA-*LoxP*-HSVtk. This suggests that the molecular switch operates but it is not enhancing the activity of the relatively weak prostate specific promoters as envisaged. In addition, the prostate specific enhancer/promoters were apparently active in H460, and inconsistently active in PC-3 cells, leading to excision of the 'stop' cassette and a reduction in CyFP expression in H460 cells (figure 6.6) and an increase in HSVtk/GCV mediated cell death of both PC-3 and H460 cells (figure 6.7A and B). This is not surprising: previous experiments indicated that H460 and PC-3 cells were capable of inducing reporter gene expression from both an enhancer/promoter-less construct and prostate specific enhancer/promoters (see chapter 4). These experiments indicated that H460 and PC-3 cells contained transcription factors that were able to recognise sequences within the pCI-neo

vector backbone resulting in reporter gene expression. The same sequences, or similar, may also be present within the pShuttle vector, thus leading to the expression of Cre recombinase and excision of the 'stop' cassette in a non-prostate specific manner.

Taken together these data indicates that it has been possible to co-transfect cells with two relatively large vectors and express sufficient Cre recombinase to excise the 'stop' cassette, leading to the expression of HSVtk. However, due to problems of inconsistently transfecting LNCaP cells using lipofectamine, it has not been possible to compare the efficiency of HSVtk expression from the prostate specific enhancer/promoters when used in conjunction with the molecular switch or on their own. Other groups have demonstrated that cell type specific promoters such as; carcinoembryonic antigen (CEA), α-fetoprotein (AFP) and thyroglobulin, when used in conjunction with a molecular switch enhances gene expression by 5 to 60 fold *in vitro* compared to the promoters alone. The promoters have also been shown to work efficiently *in vivo* following co-transfections of two vectors, very similar to vectors 1 and 2 in figure 6.1 (Sato *et al.*, 1998; Kijima *et al.*, 1999; Nagayama *et al.*, 1999; Ueda *et al.*, 2000; Sakai *et al.*, 2001; Ueda *et al.*, 2001; Leow *et al.*, 2005). However, instead of delivering the constructs directly into the cells using lipofectamine, they were incorporated into an adenovirus delivery vector system. Therefore, incorporation of my molecular switch into two separate adenovirus vectors may improve the transfection efficiency of LNCaP cells. This will enable elucidation of the prostate specificity, functionality and efficiency of the molecular switch compared to using single prostate specific enhancer/promoters vectors *in vitro*. In addition it may eliminate the problem of background gene expression induced by the pShuttle vector backbone, as only the essential elements of the molecular switch would be incorporated. Furthermore, they would enable transfection of *in vivo* prostate cancer models with the molecular switch.

Chapter 7

7.0 Summary

The aim of this project was to combine a radiation controllable molecular switch with a non-specific high activity promoter to achieve high level, persistent and tissue specific expression of a therapeutic gene in a GDEPT context in localised prostate cancer. Once developed this system could then be further manipulated by the incorporation of a number of different prostate specific promoters, to target metastatic cancer and/or hypoxic regions and administered using a number of gene therapy delivery strategies.

To this end four different radiation responsive CArG element configurations and the wild type Egr-1 enhancer were engineered into pCI-neo upstream of the reporter gene GFP. While similar constructs have been tested by a variety of groups and elicited an increase in GFP expression of between 1.5 and 17 fold (Weichselbaum *et al.*, 1994b; Joki *et al.*, 1995; Kawashita *et al.*, 1999; Marples *et al.*, 2000; Scott *et al.*, 2000; Marples *et al.*, 2002; Meyer *et al.*, 2002; Scott *et al.*, 2002; Hsu *et al.*, 2003; Quinones *et al.*, 2003), the data presented here indicated that the radiation responsive enhancers were not responsive to radiation, but, they exhibited a high level of GFP fluorescence in the absence of irradiation. Initially it was hypothesised that this was as a result of either oxidative stress and/or SRFs present in the media, however, further experiments conducted in low oxygen (5% O_2) and in the absence of serum partially discounted this theory. In addition, this would not have accounted for the very high background fluorescence elicited by the enhancer-less CMV promoter. Background fluorescence from this vector was not reduced by linearization of the vector immediately upstream of the promoter. It was therefore concluded that universal transcription factors were present and effective on the CMV promoter alone, thus explaining the overall level of background fluorescence. Interestingly, although the CMV promoter was also used in the Scott and Marples series of papers they appeared not to have experienced any problems with background fluorescence from the CMV promoter (Marples *et al.*, 2000; Scott *et al.*, 2000; Scott *et al.*, 2002; Scott and Greco 2004). It is possible that they used either a different version of the CMV promoter, which, under their culture conditions, did not result in activation of downstream genes in the absence of radiation.

At this point it was decided to change the strategy and replace the radiation responsive CArG enhancers and CMV promoter with prostate specific enhancer/promoters. This was based on the premise that further experiments to reduce the background florescence and achieve

substantial radiation activation would be both time consuming and, in light of more recent publications alluding to the radiation unresponsiveness and non-specific activation of CArG elements (Schmidt *et al.*, 2004; Anton *et al.*, 2005), potentially unproductive.

However, given more time, it would have been interesting to investigate whether the background fluorescence from the enhancer-less CMV promoter could be reduced by mutation of a putative Sp-1 transcription factor binding site present within the CMV promoter. One interesting observation from this work is that the E4S enhancer in PC-3 cells was capable of inducing the equivalent level of GFP expression as the CMV enhancer/promoter. Although the reason for this was not determined, since the E4S enhancer is only 69bp long it could be used as a direct replacement for the CMV enhancer, which is 660bp long, in PC-3 cells if vector size was limiting.

As an alternative strategy to drive the molecular switch, the CMV enhancer elements were replaced with two directly repeating PSEs and the CMV promoter was replaced with the PSA, hK2 or DD3 promoters. In the absence of androgen, the prostate specific enhancer/promoters were not active in all prostate cell lines, and furthermore, high levels of GFP expression were seen in non-prostate cell lines. However, GFP expression from the prostate specific enhancer/promoters in all the cell lines tested was not significantly different from that induced by the enhancer/promoter-less construct, which represented background fluorescence. In contrast, in the presence of androgen, the prostate specific enhancer/promoters were highly prostate specific, inducing levels of GFP expression 35 to 276 fold above background fluorescence in the androgen receptor positive cell line, LNCaP. Moreover, although GFP expression was seen from the prostate specific enhancer/promoters in the other cell lines, it did not exceed background fluorescence and was not androgen inducible. Further experiments, using linearised vectors, indicated that the background fluorescence was partially due to additional elements within the pCI-neo vector backbone capable of inducing GFP expression in the presence of appropriate transcription factors. It was therefore felt that the problem probably lay, not in the prostate specific elements, which have been previously well documented to be prostate specific (Pang *et al.*, 1995; Lee *et al.*, 1996; Schuur *et al.*, 1996; Pang *et al.*, 1997; Rodriguez *et al.*, 1997; Brookes *et al.*, 1998; Gotoh *et al.*, 1998; Martiniello-Wilks *et al.*, 1998; Latham *et al.*, 2000; Shirakawa *et al.*, 2000; Verhaegh *et al.*, 2000; van der Poel *et al.*, 2001; Wu *et al.*, 2001; Xie *et al.*, 2001; Lee *et al.*, 2002a; Yoshimura *et al.*, 2002; Park *et al.*, 2003; Schalken *et al.*, 2003; Hsieh *et al.*, 2004; Tsui *et al.*, 2004), but in the pCI-neo vector system. Since it was envisaged that the all-in-one molecular switch would be constructed in an adenovirus, into which only the essential

elements would be incorporated, the molecular switch driven by the prostate specific enhancer/promoters was still considered a feasible approach to achieving tissue specific expression of a GDEPT strategy within the prostate. The promoters chosen for the molecular switch were PSE-PSE-PSA and PSE-PSE-DD3. PSE-PSE-DD3 was chosen as it resulted in the highest increase in mean fluorescence and, although it has only recently been discovered, is proving to be the most prostate cancer specific promoter to date. In contrast, PSE-PSE-PSA induced a lower fold increase in GFP expression but has been used extensively by other groups in GDEPT strategies thus providing a basis with which the PSE-PSE-PSA driven molecular switch can be compared.

In the future it would be interesting to compare other prostate specific enhancer/promoters combinations, such as osteocalcin and prostate specific membrane antigen, as these do not contain ARE and so are not reliant on androgen for activation. Such an approach would be ideal to drive the molecular switch in patients who have undergone androgen ablation therapies and for whose cancers have progressed into hormone refractory prostate cancer. Moreover, osteocalcin would target the therapy to metastatic cancers for which there is, as yet, no curative option. It would also be interesting to investigate whether it is possible to direct GDEPT expression to regions of hypoxia within localised and metastatic tumours using combinations of HREs and prostate specific promoters. The design of this system means that it is possible to replace any part of the PSE-PSE-PSA-GFP vector system with other enhancers, promoters, and/or downstream reporter genes (see figure 4.3). Therefore, as other specific promoters become available, such as TARP, or new ones are discovered, they can be inserted into the system, along with different effector genes, to further the development of a prostate specific gene therapy strategy.

The next stage was to determine which GDEPT strategy was the most suitable to use in testing the killing efficiency of the molecular switch. Although HSVtk/GCV and NTR/CB1954 have both been used successfully in GDEPT strategies in both PC-3 and LNCaP cells (Eastham *et al.*, 1996; Blackburn *et al.*, 1998; Gotoh *et al.*, 1998; Martiniello-Wilks *et al.*, 1998; Blackburn *et al.*, 1999; Latham *et al.*, 2000; Shirakawa *et al.*, 2000; Djeha *et al.*, 2001; Loimas *et al.*, 2001; Pramudji *et al.*, 2001; Yoshimura *et al.*, 2001; Freytag *et al.*, 2002b; Ikegami *et al.*, 2002; Read *et al.*, 2003), there are as yet no literature reports on a direct comparison of the effectiveness of these two GDEPT systems in prostate cell lines. The prostate cell lines, PC-3 and LNCaP, were therefore transfected with vectors containing HSVtk or NTR, under the control of the CMV enhancer/promoter. In addition, the H460 cell line was also transfected as a non-prostate control. Transfected H460 and PC-3 cells were

both sensitive to GCV and CB1954 induced cell growth inhibition. However, LNCaP cells were insensitive to both HSVtk/GCV and NTR/CB1954 GDEPT systems. It was initially hypothesised that a 7 day activity assay did not provide sufficient time for the HSVtk transfected LNCaP cells to divide and enable activated GCV to be incorporated into the DNA. However, further experiments conducted over a 9 day period led to no increase in cell growth inhibition, indicating that the rate of cell division was not affecting the efficiency of pCMV-HSVtk transfected cell killing by activated GCV. This cell division-based theory also does not explain the insensitivity of LNCaP cells to NTR/CB1954, an enzyme/prodrug combination which does not require cell division to allow incorporation of the toxic metabolite into DNA and hence mediate cell killing. As discussed in section 4.5 there are numerous possible reasons for these findings. LNCaP cells may have up-regulated mechanisms such as mismatch DNA repair, DNA cross-link repair, lesion by-pass and/or multi-drug resistance (Doige and Ames 1993; Gottesman *et al.,* 1996; Jiricny 1998a, b; Prolla 1998; Hoeijmakers 2001; McHugh *et al.,* 2001; Kaina 2003; Lehmann 2003; Friedberg *et al.,* 2005; Lehmann 2005), not present in PC-3 or H460 cells. Alternatively, the LNCaP cells used in my experiments may have undergone some form of spontaneous mutation resulting in insensitivity to both HSVtk/GCV and NTR/CB1954 systems. Future experiments conducted using fresh cells from the ATCC may address this problem. In addition, future collaborations with other prostate cancer research groups may provide expertise in the transfection of LNCaP cells, as well as access to other prostate cancer cells lines not yet commercially available.

Growth inhibition of prostate cells, in particular LNCaP cells, may be achieved using other enzyme/prodrug strategies, such as CD/5-FU or HRP/IAA, the latter of which would also have the advantage that it is effective in against both oxic and hypoxic tumours. In addition, the molecular switch could be designed to enable the expression of two GDEPT enzymes, for example HSVtk/HRP or NTR/CD, either through the generation of fusion proteins or by the use of an internal ribosome entry site (IRES) located between the genes encoding the two separate enzymes. It is hoped that such a strategy would not only improve the effectiveness of cell killing but may even be synergistic. In addition, killing cells via two distinct pathways may also reduce the potential of the tumour cells to develop resistance mechanisms which would reduce the effectiveness of a single therapy.

The HSVtk/GCV and CD/5-FU suicide gene system are well documented in 9L glioma cells and the prostate cell lines; PC-3, RM-1 and LNCaP, to sensitise cells to radiation, it is also known that the cytotoxic effects of these two therapies, when combined, is synergistic (Kim

et al., 1994; Kim *et al.*, 1995; Kim *et al.*, 1997; Rogulski *et al.*, 1997a; Rogulski *et al.*, 1997b; Blackburn *et al.*, 1998; Atkinson and Hall 1999; Chhikara *et al.*, 2001; Freytag *et al.*, 2002b). In addition, combined radiotherapy and in situ gene therapy with an adenovirus containing HSVtk alone, and in combination with CD, has been tested in phase I/II clinical trials. While these results indicate that the treatment was well tolerated with no changes in dose limiting toxicity, further trials are warranted to evaluate long-term toxicity and efficacy (Rogulski *et al.*, 2000; Teh *et al.*, 2001; Freytag *et al.*, 2002a; Freytag *et al.*, 2003; Teh *et al.*, 2004). It would therefore be interesting to combine prostate specific GDEPT with radiation to achieve enhanced cell killing while maintaining prostate specificity. Meanwhile, since both HSVtk/GCV and NTR/CB1954 systems were functional in other cell lines, it was considered worthwhile to examine if the molecular switch would work in principle.

It was initially proposed to assemble the molecular switch as two separate vectors using the pShuttle vector system and then to incorporate it into a single vector for insertion into an adenovirus. As single vectors, one vector contained the prostate specific enhancer/promoters, either PSE-PSE-PSA or PSE-PSE-DD3, controlling the expression of Cre recombinase. The other vector consisted of the strong CMV IE promoter/enhancer controlling the expression of a tumour sensitising gene, HSVtk, the expression of which was silenced by a 'stop' cassette flanked by *loxP* sites. It was envisaged that recombination between the *loxP* sites by Cre recombinase would result in the removal of the 'stop' cassette, activation of HSVtk transcription and, in the presence of GCV, cell sensitisation. Since LNCaP cells were insensitive to the HSVtk/GCV system, the 'stop' cassette was constructed to contain a reporter gene, CyFP, and a polyA signal. This would not only prevent transcriptional read-through, but also enable visualisation of the activation of the molecular switch in LNCaP cells, as the excision of the stop cassette by Cre mediated *LoxP* recombination would result in the disappearance of CMV driven CyFP fluorescence as measured by FACS analysis. During construction of the molecular switch vectors it was found that the method intended to be used to combine the two vectors was not feasible. This was because the prostate specific enhancer/promoter combinations chosen to drive Cre expression were unexpectedly active in *E. coli*, resulting in Cre mediated excision of the 'stop' cassette. The all-in-one construct could therefore not be propagated in *E. coli* and so vector DNA appropriate for digestion and insertion into the adenovirus could not be produced. Thus, in order to test the molecular switch, two separate vectors were introduced simultaneously into cells using lipofectamine.

Control experiments, in which the CMV enhancer/promoter was used to drive the expression of Cre instead of prostate specific enhancer/promoters, indicated that the molecular switch

was functional in LNCaP, PC-3 and H460 cells. When the prostate specific enhancer/promoters, in the presence of DHT, were used to drive the molecular switch in the AR positive LNCaP cells, although there was a reduction in CyFP expression, it was not significant. Thus, over 6 repeat experiments there was a large variation in CyFP expression which was attributed to problems using lipofectamine to consistently transfect LNCaP cells with the two, relatively large, molecular switch vectors. FACS analysis of CyFP expression in the AR negative cell lines, PC-3 and H460, indicated that PSE-PSE-PSA and PSE-PSE-DD3 were both active, but only in H460 cells. This was confirmed by MTT analysis, which also indicated that PSE-PSE-DD3 was active in PC-3 cells. It was hypothesised that the molecular switch driven by the prostate specific enhancer/promoters would only be active in LNCaP cells in the presence of DHT. However, it was not surprising to find that they were also active in PC-3 and H460 cells. Previous experiments had indicated that H460 and PC-3 cells both contained transcription factors that were able to recognise sequences within the pCI-neo vector backbone resulting in reporter gene expression. The same sequences, or similar, may also be present within the pShuttle vector, thus leading to the expression of Cre recombinase and excision of the 'stop' cassette in a manner that was not prostate specific.

A possible solution to these problems would be to use either an adenovirus to deliver the vectors individually or to persevere with attempts to find a method to generate an all-in-one molecular switch vector in an adenovirus. This would improve both the transfection efficiency of the LNCaP cells and their prostate specificity, as only the essential elements of the molecular switch would be incorporated into the adenovirus, thus eliminating the pShuttle vector backbone. Furthermore, it would enable transfection of *in vivo* prostate cancer models with the molecular switch. Approaches that could be tested to achieve an all-in-one adenovirus molecular switch would be to either reduce the activation of the prostate specific elements by *E. coli*, for example by using minimal media, or to investigate the feasibility of using another host, such as yeast. Alternatively, it may be possible to construct an adenovirus containing CMV-*LoxP*-HSVtk-PolyA-PSE-PSE-PSA/DD3-Cre and then to insert the rest of the 'stop' cassette, *LoxP*-CyFPPolyA, using the reverse of the Cre-*loxP* reaction (see section 1.3). However, since the reverse reaction is not as efficient as the forward reaction, the all-in-one adenovirus would be present in relatively small amounts potentially making detection and isolation problematic.

Gene delivery research is still in an experimental phase. However, advancements are constantly being made in the development of non-human viral vectors, such as baculoviruses, which would circumvent the problems of safety and pre-existing immunity related to human

viruses. In addition, research efforts are also focussing on specifically targeting the delivery virus to the desired tissue. In the case of prostate cancer, an ideal target would be one that is over-expressed at all stages of prostate cancer, in particular metastatic cancer, but not expressed in any other tissues. As yet nothing has been identified that completely satisfies these criteria; however, there are several candidate cell surface proteins that are promising. These include STEAP (six-transmembrane epithelial antigen if the prostate), PSMA and PSCA. The potential of such strategies has been comprehensively discussed in a review by Maitland *et al.,* (2004). It is hoped that future collaborations with such groups may lead not only to alternative strategies for incorporating the molecular switch into a viral delivery vector but also increased transfection efficiency and prostate specificity.

The future success of gene therapy strategies such as this one will be remarkably improved by the development of cost effective genetic screens for known biomarkers enabling patient specific treatment. The molecular switch system presents a highly adaptive model that could be modified to incorporate a number of tissue speicifc, cytotoxic or corrective therapies which would be determined by the genetic and expression profile of the patient. For example, a hormone refractory metastatic cancer with regions of hypoxia would be best treated using a gene therapy apporach composed of both metastatic and hypoxia specific enhancer/promoters, such as a hypoxia responsive elements and the osteocalcin promoter with the HRP/IAA GDEPT system which is effective in both oxic and anoxic conditions. In contrast, a localised androgen responsive cancer may be best treated using combinations PSE/PSA/Hk2/PB enhancer/promoters with HSVtk/GCV and CD-5-FU double suicide gene therapy in conjunction with radiation. In addition, the 'stop cassette' could also be utilised to deliver prostate speicifc genes. The reporter gene, CyFP, could be replaced with a therapeutic gene followed by a constitutive promoter both of which are in the reverse orientation compared to the rest of the vector. The gene will therefore only be expressed when the cassette is circularised after prostate specific Cre mediated recombination. The therapeutic gene could either be another cyctoxic GDEPT, alternatively, it could be a gene correcting for a genetic abnormality. The exact gene would be determined by genetic screening, however, possible targets would be the major apoptotic regulators p53 and Bcl-2 both of which are often mutated in prostate cancer and are implicated in cancer progression and hormone resistance. Other possible candidates have been comprehensively reviewed by Quinn *et al.,*(2005), Konishi *et al.,* (2005) and Foley *et al.,* (2004a).

Although it was not possible to test the effectiveness of a prostate specific molecular switch, this project has resulted in a more complete understanding of the limitations of the use of

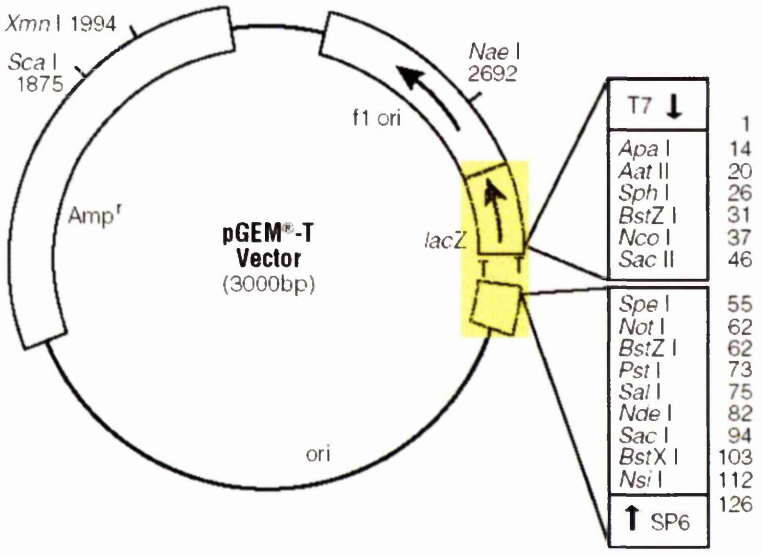
radiation responsive and prostate specific promoters in gene therapy strategies. It has also highlighted the importance of vector design and the need for a reliable *in vitro* prostate model in which such approaches can be tested. There are many hurdles that still need to be overcome in the generation of an effective gene therapy strategy for prostate cancer. However, gene therapy offers us the potential to tailor treatment specifically to the needs of the individual, a direction that will prove to be critical in the challenge to find an effective cure for cancer.

Chapter 8

8.0 Appendix

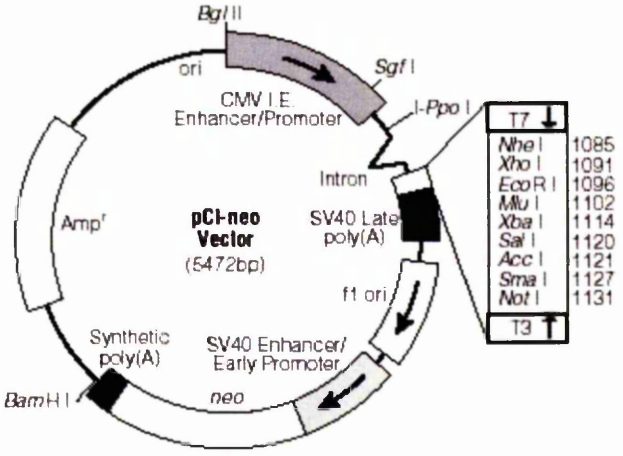
8.1 pGem-T (Promega) vector map

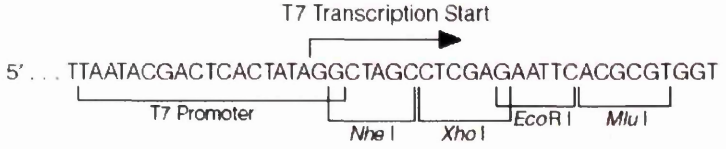
PCR products were initially cloned into the pGem-T T/A cloning vector to facilitate rapid cloning and confirmation of the fidelity of the sequence before excision of the inserts using appropriate restriction sites located either side of the T overhangs. Successful cloning of an insert into pGem-T interrupts the coding sequence of β-galactosidase (lacZ) within the lac operon (highlighted in yellow). β-galactosidase is an enzyme that converts the substrate, X-gal, into an insoluble blue dye, therefore, recombinant clones can be identified as white colonies on X-gal indicator plates. pGem-T also contains T7 and SP6 RNA polymerase promoters to enable the synthesis of RNA from the cloned DNA insert, an origin of replication (ori) for propagation in *E. coli* and an f1 origin (f1 ori) for single-stranded DNA production. In addition, an ampicillin resistance gene (Amp^r), under the control of a bacterial promoter, enables selection in *E. coli* cells.

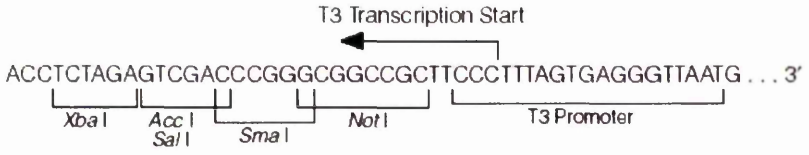


8.2 pCI-neo (Promega) vector map and multiple cloning site (MCS)

pCI-neo was used as the basis for all the radiation responsive promoters (chapter 3), the prostate specific promoters (chapter 4) and pCMV-HSVtk and pCMV-NTR (chapter 5). In addition, pCI-neo was used as an intermediate vector in the construction of the molecular switch in pShuttle (chapter 6). The MCS, flanked by T7 and T3 RNA polymerase promoters for the synthesis of RNA from the cloned DNA insert, enables the insertion of genes under the control of the CMV IE enhancer/promoter. The pCI-neo vector backbone also contains an SV40 and synthetic polyadenylation signals (SV40 Late polyA and Synthetic polyA) to terminate transcription, an SV40 origin of replication (within the SV40 enhancer/early promoter) for transient replication in SV40 T antigen expressing mammalian cells, an origin of replication (ori) for propagation in *E. coli* and an f1 origin (f1 ori) for single-stranded DNA production. In addition, a neomycin phosphotransferase gene (neo), under the control of the SV40 enhancer/early promoter, and an ampicillin resistance gene (Amp^r), under the control of a bacterial promoter, enable selection in mammalian and *E. coli* cells.

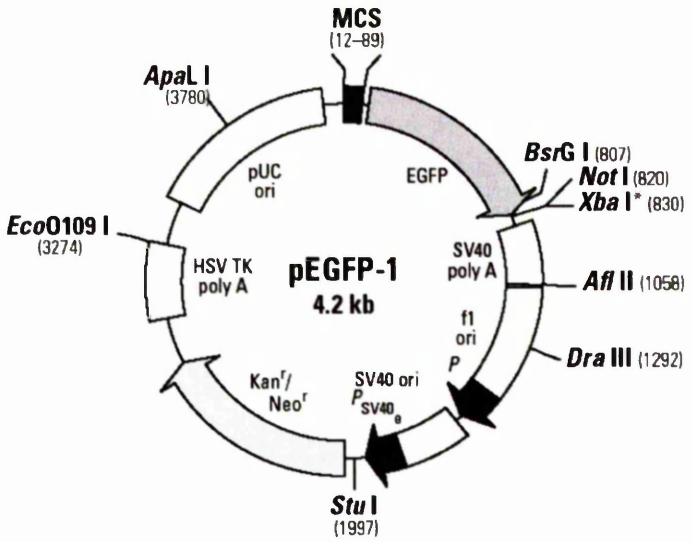


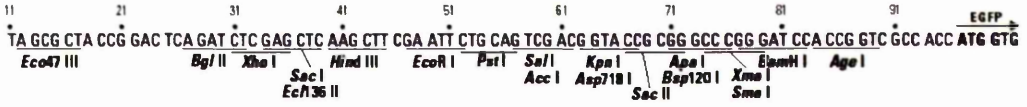




8.3 pEGFP-1 (Clontech) vector map and MCS

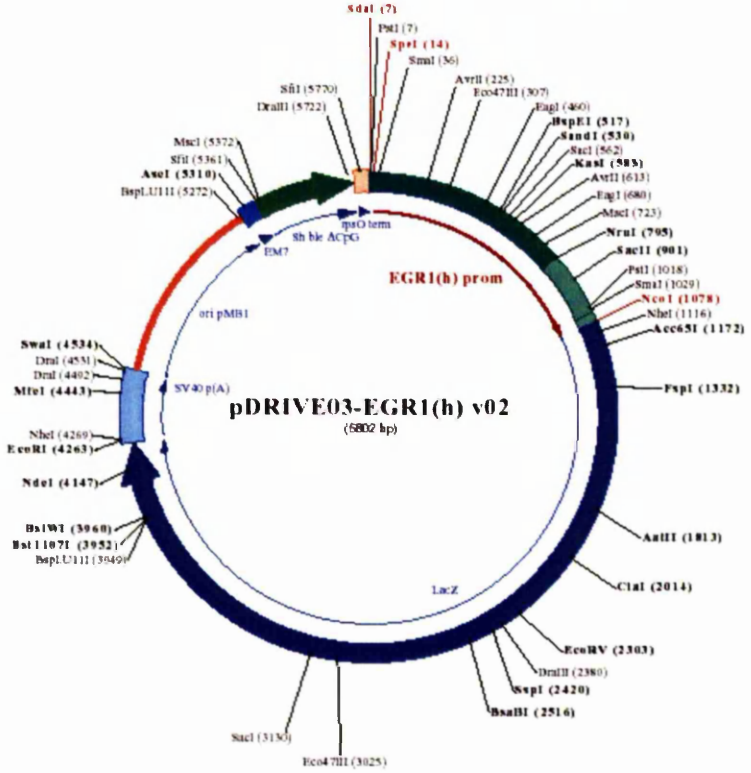
GFP, in section 3.2.1, was excised from pEGFP-1 using EcoR1, in the MCS, and NotI restriction sites (highlighted in yellow). The EGFP-1 vector backbone also contains SV40 and HSVtk polyadenylation signals (SV40 poly A and HSVtk polyA) to terminate transcription, an SV40 origin of replication (SV40 ori)) for transient replication in SV40 T antigen expressing mammalian cells, a pUC origin (pUC ori) of replication for propagation in *E. coli* and an f1 origin (f1 ori) for single-stranded DNA production. In addition, a neomycin phosphotransferase gene (Neo^r), under the control of the SV40 early promoter (P SV40), and a kanamycin resistance cassette (Kan^r), under the control of a bacterial promoter, enable selection in mammalian and *E. coli* cells.





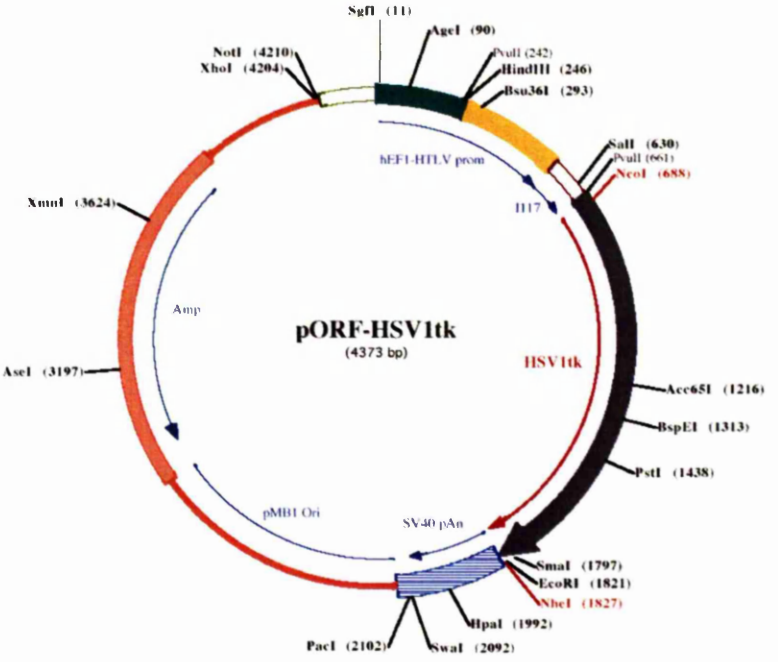
8.4 pDRIVE03-EGR-1(h) v02 (InvivoGen) vector map

pDRIVE03-EGR-1(h) v02 was used to PCR amplify the WT Egr-1 enhancer (section 3.2.2), using the WT Egr-1 primers listed in table 3.1; this corresponded to the sequence between bases 84 and 625 on the map below. In addition, the vector also contained the LacZ gene, encoding β-galactosidase enabling the testing of promoter activity in transient transfection experiments and the SV40 polyadenylation signal (SV40 pA) to terminate LacZ transcription. An *E. coli* origin of replication (ori pMB1) and the Zeocin resistance gene (*Sh ble*), under the control of the EM7 bacterial promoter, enable propagation and selection in *E. coli* cells.



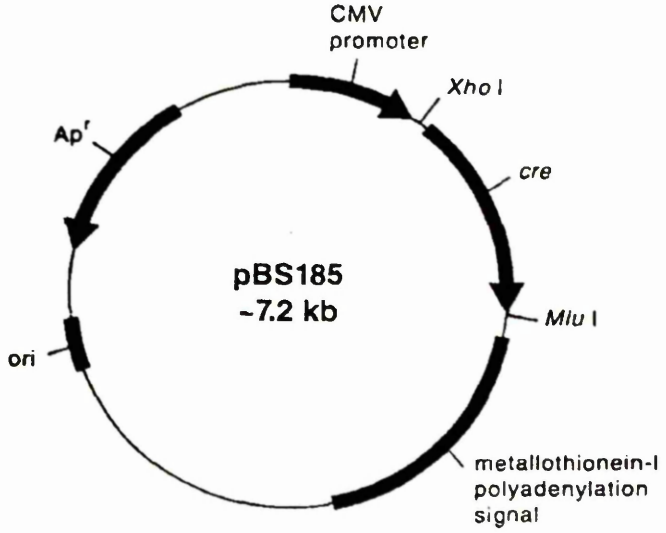
8.5 pORF-HSV1tk (Clontech) vector map

pORF-HSV1tk was used to PCR amplify the HSVtk gene (sections 5.2.1, 6.3.1 and 6.3.3), using HSVtk primers listed in tables 5.1 and 6.1. Expression of HSVtk in this vector is driven by a hybrid promoter (hEF1-HTLV promoter) composed of the Elongation Factor-1α (hEF-1) promoter and the 5′ untranslated region of the Human T-cell Leukaemia Virus (HTLV). In addition, the vector contains an SV40 polyadenylation signal (SV40 pAn) to terminate HSVtk transcription. An *E. coli* origin of replication (pMB1 ori) and the ampicillin resistance gene (Amp), under the control of a bacterial promoter, enable propagation and selection in *E. coli* cells.



8.6 pBS185 (Invitrogen) vector map

pBS185 was used to PCR amplify the Cre recombinase gene (sections 6.2.1 and 6.3.1), using Cre recombinase primers listed in table 6.1. Expression of Cre recombinase (cre) in this vector is driven CMV enhancer/promoter with a metallothionein-I polyadenylation signal to terminate transcription. In addition, an *E. coli* origin of replication (ori) and the ampicillin resistance gene (Ap^r), under the control of a bacterial promoter, enable propagation and selection in *E. coli* cells.



Chapter 9

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