The Effect of Quercetin on the Growth of Primary Bovine Cells and Analysis of its Ability to Modulate the Level of Transcription From the Bovine Papillomavirus Type 4 Long Control Region

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To my parents, James and Teresa Connolly,
and my Nanny, Julia Connolly

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Declaration

The work presented in this thesis is my own work unless otherwise stated.

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Abbreviations

APS	Ammonium persulphate
β-GAL	β-Galactosidase
BPV	Bovine papillomavirus
BSA	Bovine serum albumin
CAT	Chloramphenicol Acetyltransferase
CET	Cholera enterotoxin
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetra-acetic acid
EGF	Epidermal growth factor
FACS	Fluorescence activated cell sorting
hr	Hours
HPV	Human papillomavirus
Luc	Luciferase
LCR	Long control repeat
Neo	Neomycin
min	Minute
MoMuLTR	Moloney murine long terminal repeat
MTT	3-[4,5-Dimethlythiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue
ONPG	*O*-Nitrophenyl-β-D-galactopyranoside
ORF	Open reading frame
PalF	Foetal Bovine Palate Fibroblasts
PalK	Foetal Bovine Palate Keratinocytes
PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline + Tween 20
PCR	Polymerase chain reaction
PE	PBS plus EDTA
PMSF	Phenylmethylsulfonyl Fluoride
PV	Papillomavirus
sec	second
SDS	Sodium dodecyl sulphate
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline + Tween 20
TEMED	N,N,N',N'-tetramethylethylenediamine
TPA	12-*O*-tetradecanoylphorbol-13 acetate
μM	Micromolar
μl	Microlitre
μg	Microgram
bp	Base pair
cm	Centimetres
g	Gram
kb	Kilobase pairs
kD	KiloDalton

kg	Kilogram
l	Litre
M	Molar
mg	Milligram
ml	Millilitre
mM	Millimolar
nm	Nanometres
nu	Nucleotides
OD	Optical density (light absorbance)
pg	picogram
RI	Refractive Index
V	Volts
v/v	Volume per unit volume
w/v	Weight per unit volume

Abstract

It has been demonstrated that the bioflavonoid quercetin can synergise with bovine papillomavirus type 4 (BPV4) DNA plus an activated *ras* oncogene to fully transform primary fibroblasts cells derived from bovine foetal palate (PalF cells). The aim of this thesis has been to identify a mechanism(s) by which quercetin may be contributing to this transformation process.

Quercetin was shown to inhibit the growth of PalF cells in a concentration dependent manner. A quercetin concentration of ~20μM inhibited the growth of PalF cells by approximately 50%. PalF cells were shown to arrest in the G1/G0 and G2/M phases of the cell cycle in response to quercetin treatment. These results indicated that the presence of quercetin in PalF cell culture medium is not conducive to normal cell proliferation.

Investigations were undertaken to determine if quercetin could modulate the level of transcription from the viral long control region (LCR); this region of the virus contains sequences which are involved in regulating expression of viral genes. Results showed that quercetin was capable of increasing the level of viral transcription from the LCR up to 4 fold. In keeping with earlier observations, the timing of exposure to quercetin was critical; the increase in transcription from the viral LCR was only observed when cell were exposed to quercetin *after* cells had been transfected with LCR-containing reporter plasmids. No effect on transcription was observed when cells were treated with quercetin before transfection. The quercetin-mediated effect on LCR transcription activity was also limited to the LCR functioning in a promoter capacity. Quercetin had no effect on the LCR when present as an enhancer.

Removal of nucleotides 311 to 331 from the 3'terminal end of the BPV4 LCR correlated with a drop in the basal level of transcription from the LCR and abrogation of the response of the LCR to quercetin. EMSA experiments identified a cellular factor, designated QX1, as binding to an oligonucleotide corresponding the sequence of the LCR from nucleotides 301 to 331. Although this sequence of the LCR contains a TRE-like binding motif, mutational analysis has confirmed that this motif is not the site to which QX1 specifically binds. Furthermore, AP-1 does not bind to the TRE-

like element. AP1 and QX1 display different mobilities in a polyacrylamide gel showing that QX1 and AP1 are not the same factor. Mutational analysis has thus far not identified specific residues critical for the binding of QX1 to this 3'terminal region of the BPV4 LCR.

Western blot analysis, using a phosphotyrosine antibody, identified several proteins which showed changes in their phosphotyrosine status in response to quercetin exposure. These results confirm that quercetin can alter the phosphorylation status, and possibly also the activity of a number of different proteins, in PalF cells.

The observations made in the course of this thesis indicate that quercetin can induce PalF cells to growth arrest, possibly as a result of DNA damage. Quercetin also causes an increase in the level of viral transcription. Over-expression of viral oncoproteins such as E7, in response to quercetin treatment, would stimulate cells to continue proliferating. The stimulation of damaged cells to proliferate in an adverse environment induced by quercetin may partially explain the ability of quercetin to transform PalF cells in co-operation with BPV4 plus *ras*. Other epigenetic effects, as indicated by the changes induced in several phosphotyrosine-containing proteins, could further contribute to this synergism.

Chapter 1

General Introduction

1.1 Introduction

There are a large number of diseases which fall under the banner of cancer. Nevertheless cancer is often viewed as a single disease because, irrespective of the site or cell type which is initially affected, the nature of disease progression is very often the same. Of course, cancers originating from different cell types are in general very different diseases. Not all cancers progress so rapidly or are as aggressive as others, however many share a similar pattern of evolution. This pattern is described as the "Multistep nature of cancer" (Vogelstein and Kinzler, 1993).

1.2 The Step-by-step Nature of Cancer Progression

In general, cancer incidence increases dramatically with age (Miller, 1980). This relationship led Nordling (1953) and Armitage and Doll (1954) to hypothesise that several successive changes must occur in a cell to elicit cancer. The heterogeneous nature of tumours and their ability to change over time was studied in spontaneous mammary tumours by Foulds (1956) who later concluded that tumour progression is characterised by a series of permanent, irreversible changes that occur individually in each tumour (Foulds, 1975). The multistage hypothesis has since been supported with evidence that has come from several independent sources such as epidemiology, clinical observation and pathology and, more recently, molecular genetic studies.

Cancer is essentially a unique type of genetic disease. Unlike classic genetic diseases like cystic fibrosis, which involves a single germline mutation, cancer develops through the acquisition of several individual somatic mutations (Fearon and Vogelstein, 1990; Solomon *et al.*, 1991).

The process of cancer cell evolution can be broken down into three distinct stages - initiation, promotion and progression. Tumour initiation begins when an induced change occurs in a single normal cell. Transition from one stage to the next, which encompasses the processes of promotion and progression, is marked by the acquisition of additional mutations. Each new mutation can complement and co-operate with those already established to push the tumour towards an increasingly aggressive tumorigenic phenotype.

The concept that cancer originates from a single cell was first outlined in a review by Nowell (1976). He not only concurred with other workers (references cited

within Nowell, 1976) that tumour evolution was the result of sequentially acquired genetic changes, but proposed that all the cells in a malignant tumour derived from the same initial cell. In this model of clonal evolution, a change occurs within a single cell creating a novel cell clone which possess a selective growth advantage over neighbouring normal cells. As a result of genetic instability (Cheng and Loeb, 1993) in the expanding tumour population, mutant cells are produced. Most of the mutant cell variants which arise are not viable and are eliminated by the body. Occasionally a mutation occurs which confers on that cell a further growth advantage with respect to both the normal and the original tumour cells. The cell grows into a small sub-population which possess the ability to outgrow its neighbouring cells. Within this limited population, a cell acquires a third mutation which gives it an even greater growth advantage. This process of mutation followed by selective clonal expansion continues. With each additional mutation the cell becomes increasing abnormal, both genetically and metabolically; the deregulated cells become increasingly refractory to the mechanisms which control proliferation, differentiation and death. Eventually the cells become malignant which enables them to invade and destroy surrounding tissue and ultimately metastasise to numerous sites throughout the body via the circulatory system where they form secondary (metastatic) tumours. Although benign and malignant tumours are implicated in the overall process of carcinogenesis, only malignant tumours are properly referred to as cancer.

The mutation rate for human DNA is very low ($1.4x10^{-10}$) (Cheng and Loeb, 1993). One would expect, therefore, that it would take many years to amass mutations in several independent genes. As mentioned earlier, cancers occur most frequently in older people; the protracted, disease-free lag phase reflects the years required for an individual cell to accumulate the number of separate mutations necessary for a malignant tumour to materialise. However, it has long been debated that the normal rates of mutation are still insufficient to induce cancer (Loeb, 1991) and it has been suggested that tumour cells experience higher rates of mutation and are genetically unstable (Nowell, 1976; Nicolson, 1987). Indeed, some cancers which have a deficiency in mismatch repair mechanisms show mutation rates which are two to three fold higher compared to normal cells (Bhattacharyya *et al.*, 1994; Shibata *et al.*, 1994; Eshleman *et al.*, 1995). Generally though, the multiple genetic alterations which are found in tumour cells appear to be a consequence of gross chromosomal alterations.

These alterations are characterised by extensive regions of chromosomal deletion or duplication which can arise through processes such as mitotic recombination or aberrant mitotic segregation, ultimately giving rise to aneuploidy. Aneuploidy, a condition in which the chromosome number is not an exact multiple of the diploid set, is found in a majority of tumour cells and, although often difficult to prove, it has been suggested that full or partial aneuploidy is a cause rather than the effect of neoplastic progression (Kinzler and Vogelstein, 1996). The mechanism(s) by which these gross chromosomal changes actually arise is unknown (Cheng and Leob, 1993; see Table 1, Nicolson, 1987, for a list of mechanisms which may generate genetic instability) and represents an area requiring extensive further research.

1.3 Evidence for Multistage tumour Progression

Many cancers exhibit a multistep mode of progression. Two cancers in particular, however, have proven to be model systems. Cancers of the colon and cervix lend themselves to detailed analysis at several levels; they have been extensively characterised at the clinical level and, because both are relatively common neoplasias, significant amounts of tumour material are available for analysis. Both forms of cancer develop separately through a series of clinically and histologically distinct lesions. Furthermore, the location of these lesions within the body means biopsy collection is relatively non-invasive. In the next three sections I will consider evidence from independent sources, concentrating mainly on cancer of the colon and cervix, which support cancer progression as a multistage process.

1.3.1 Epidemiology

The identification of potential carcinogens or risk factors which contribute to any stage of cancer development often comes from studies of well defined populations. Association between particular social or occupational behaviour and increased cancer incidence frequently points to individual factors as common denominators in one or more types of cancer.

It was in lung cancer that the first statistical evidence was presented which linked this common form of cancer with tobacco smoke and studies over the past three to four decade have demonstrated that cigarette smoking is the principal cause of lung cancer (for review see Shopland *et al.*, 1991). Exposure to asbestos was identified as

a contributory factor to developing lung cancer and mesothelioma (cancer of the pleura surround the lungs) (for review see Knudson, 1995) and this risk was greatly amplified in individuals who were smokers also (Dawson *et al.*, 1992).

There is strong evidence linking exposure to sources of radiation and a large range of cancers (IARC, 1990, Morgan *et al.*, 1996). Exposure to radiation, whether accidental, natural or iatrogenically administered as a treatment for other medical conditions, has been associated with leukaemias, lymphoma (IARC, 1990) and solid tumours, particularly those of the lung (Preston *et al.*, 1986; IARC, 1988, Morgan and Breit, 1995), bone (Rowland *et al.*, 1978; Mays and Spiess, 1984; Cafiero *et al.*, 1996), thyroid (Prentice *et al.*, 1982; Ron *et al.*, 1987) and breast (Boice and Nonson, 1977; Boice *et al.*, 1979; Elkind, 1996).

A number of viral infections, especially those involving specific types of human papillomaviruses and hepatitis B virus, have been shown to be casually linked to cancer of the cervix (zur Hausen, 1989) and liver respectively (Shafritz and Hadziyannis, 1987).

Finally, cancer of the colon is plainly influenced by the environment. This is reflected in the dramatic geographical variation in incidence. This form of cancer, which occurs most frequently in western urbanised communities, appears to correlate with diets which are deficient in fibre whilst containing high levels of carbohydrate and lipids (Giovannucci and Willett, 1994; Lee and MacDonald, 1992). The peak incidence of colon cancer is still, however, in the over 60 age group (Lee and MacDonald, 1992).

A common feature in all these cancers is that expression of the malignant tumour is generally only evident years or even decades after exposure to a particular carcinogen or infection. The long interval between exposure and malignancy suggests that the damage induced by the identified risk factor is, by itself, insufficient to elicit cancer. The delay has been attributed to the requirement for additional changes to accumulate within the tumour cells before malignancy is achieved; this supports the hypothesis that multiple independent events are necessary for the development of cancer.

1.3.2 Clinical Pathology

A large number of histologically distinct lesions or tumours have been identified within the large intestine. These include small areas of dysplasia identified as aberrant crypt foci (ACF) and adenomas which arise from the colorectal epithelium and appear in the form of elevated polyps (Kinzler and Vogelstein, 1996). Adenomas, which are benign tumours, exhibit varying degrees of dysplasia graded as mild (early), moderate (intermediate) or severe (late); severe dysplasia implies that mucosal invasion is imminent (Wyllie, 1992). The risk of colorectal malignancy is proportional to the number of adenomas which is particularly evident in individuals who are genetically predisposed to adenomatous polyposis (Kinzler and Vogelstein, 1996). In this autosomal dominant condition, individuals typically develop hundreds to thousands of adenomas and, although these tumours are benign, their large numbers virtually guarantee that some will progress to invasive carcinomas. Malignant tumours (carcinomas) can sometimes appear polypoid, possibly reflecting their adenomatous origin, and later they evolve into ulcerating lesions which can lead to rectal bleeding and obstruction. Adenocarcinomas of the colon are usually well or moderately differentiated. Commonly they penetrate the entire thickness of the intestinal wall which can lead to extensive spread throughout the peritoneal cavity (Wyllie, 1992).

Cervical squamous-cell carcinoma, like colon cancer, normally does not appear abruptly. Instead it develops slowly over a period of 2-10 years from normal epithelium which displays progressively severe degrees of abnormality. Premalignant change within the cervix is characterised by the presence of poorly differentiated cells which display loss of polarity, excessive and abnormal mitotic activity and both nuclear and cytoplasmic pleomorphism (Fox, 1992). The term cervical intraepithelial neoplasia (CIN) was introduced to describe and classify the whole range of premalignant lesions which can occur in the cervix (Richart, 1968, 1973). If cells are undifferentiated yet confined to the lower third of the epithelium, the lesion is classed as CIN I. Once these cells extend into the middle third of the epithelium, CIN II is applied. CIN III depicts undifferentiated cells which occupy the upper third or full thickness of the epithelium. Not all cases of CIN I, II or III inevitably develop into invasive carcinomas. Indeed, a parallel system for the cytological diagnosis of cervical lesions which was introduced in America in 1988 (the Bethesda System (NCI

workshop, 1989)), suggests that low grade lesions, originally classified as CIN I, will not progress to carcinoma *in situ* (CIS); only CIN II or III (alternatively classified as high-grade squamous intraepithelial lesion (H-SIL)) have the potential of full neoplastic development (Wright and Kurman, 1996). Despite HPV infection being a relatively frequent event, progression to malignancy is very rare in comparison and there is always a strong possibility that CIN (or SIL) at any stage can either remain stationary or regress. However there is currently no way of distinguishing particular lesions which will continue to evolve neoplastically and therefore all cases of CIN have to be regarded as potentially invasive (Fox, 1992).

Early cytogenetic studies detected aneuploidy in all grades of lesions (Wilbanks *et al.*, 1967). Over the past decade more precise methodologies have been applied to determine ploidy and although some level of aneuploidy is detected in some CIN I lesions, these lesions generally possess normal diploid karyotypes (Fu *et al.*, 1988). CIN II, III and carcinoma *in situ* (malignant lesion) in comparison all displayed aneuploid karyotypes; aneuploidy was detected more frequently as the severity of the lesions increased, i.e., the percentage of carcinomas which were aneuploid was greater than that detected in CIN III, which was greater than that detected in CIN II (Fu *et al.*, 1983; Fujii *et al.*, 1984; Fu *et al.*, 1988).

Analysis of the various lesions which are found in the colon or cervix presents a spectrum of tumours which show progressive levels of dysplasia and neoplasia. The bulk of clinical and histopathological data suggests that malignant tumours arise from pre-existing benign tumours (Sugarbaker *et al.*, 1985; Syrjanen, 1996). Therefore, the concept that more neoplastic tumours originate from less aggressive precursors supports cancer as a stepwise process; the macroscopic changes in tumour morphology reflects the changes which are occurring within the tumour cell at the molecular level.

1.3.3 Molecular Genetics

The epidemiological and clinico-pathological support for a multistep mechanism of carcinogenesis, although very significant, is somewhat circumstantial. The underlying cause for the changes in cellular behaviour lie at a molecular level. The development of molecular genetic technology over the past ten to fifteen years

has provided convincing evidence that cancer progression is a consequence of accumulating permanent alterations to the cell's normal genetic structure.

Two distinct classes of genes have been causally linked to cancer development. These cancer-related genes are called oncogenes and tumour suppressor genes.

1.3.3.1 Oncogenes

The use of certain chemicals, radiation and some viruses has been shown capable of inducing cancer experimentally in animal model systems. Tumour-viruses in particular played a pivotal role in focusing research efforts on the identification of critical genes involved in neoplasia. Compared to most chemical carcinogens, tumour viruses rapidly induce the formation of tumours in animal models and transform cells in culture efficiently and reproducibly (Cooper, 1990). In addition, these viruses comprise small genomes with respect to the cellular genomes of higher eukaryotic cells which makes molecular characterisation of their DNA more amenable.

The discovery of retroviral oncogenes was very significant, providing the first step towards understanding the function of genes specifically involved in carcinogenesis. The oncogenes of acutely transforming retroviruses, such as the Rous sarcoma virus, are a distinct group of genes responsible for viral pathogenesis but which were shown not to be required for viral replication (Weiss *et al.*, 1985). Retroviral oncogenes originated from normal cellular genes called proto-oncogenes. Proto-oncogenes constitute a group of normal cellular genes with potentially transforming activity. An oncogene is an 'activated' proto-oncogene; it is a mutant form of a normal cellular gene which has acquired the ability to induce transformation as a consequence of aberrant gene expression or an alteration in structure and/or function of the protein product. Proto-oncogenes become incorporated into retroviral genomes by virus-cell recombination events (Swanstrom *et al.*, 1983; Neil *et al.*, 1984; Miles and Robinson, 1985). During the recombination event, the cellular gene can become mutated by truncation or point mutation of sequences critical for regulation or biochemical activity of the gene product. Alternatively the gene may be expressed aberrantly under the influence of the powerful viral promoter. The incorporation of a cellular proto-oncogene into a retroviral genome and its transmission into another cell following subsequent viral infection is called

transduction. Even if the transduced cellular gene sustains no mutation in the transfer, its reinsertion into cellular DNA can lead to irregular expression of this gene in the vicinity of retrovirus integration which thus may cause neoplastic growth. Irrespective of the method of activation, retroviral transduction has lead to the identification of many oncogenes including *src* (Duesberg and Vogt, 1970), *ras* (Santos *et al.*, 1982, Parada *et al.*, 1982) and *myc* (Hayward *et al.*, 1981; Payne *et al.*, 1982).

It has long been recognised that many cancer cells, when viewed microscopically, carry damaged chromosomes. The presence of chromosome translocations, inversions, deletions, and amplifications, all involving large genome domains, has provided evidence for the existence of oncogenes for many decades. However, only since the generation of techniques for molecular manipulation and cell culture has it been possible to dissect and test the biological significance of the regions implicated in these gross chromosomal alterations.

The study of cancer in whole animals is difficult to control and quantitate due to the number of uncontrollable variables which are naturally associated with a complex, multicellular organism. The development of techniques for the maintenance and cultivation of cells in culture has proved to be invaluable in the dissection of neoplastic transformation. It has provided a normalised environment in which the growth requirements and behaviour of normal cells can be analysed, properties of normal and cancer cells can be compared under controlled conditions, and quantitative and reproducible assays for cell transformation have been developed. The development of sensitive and reproducible transformation assays in cell culture has been critical in establishing an experimental understanding of the molecular basis of cancer. The assays focus on characteristics such as morphological change, loss of contact inhibition, reduced dependency on growth factors and/or serum for growth, anchorage independent growth and tumorigenicity in nude mice inoculations.

Tumorigenicity studies are particularly important as they provide a link between cells which display transforming characteristics *in vitro* and the ability to form tumours *in vivo*. It is very important to be able to relate results observed *in vitro* with effects *in vivo*: tumorigenicity studies add validity to the *in vitro* transformation assays.

However, these assays are in no way absolute. Many cells display one or more transformed characteristics *in vitro* and yet do not induce tumours in nude mice. Such

partially transformed cells appear only to have acquired some of the alterations needed to convert a normal cell to a tumorigenic phenotype adding support for a multistep mechanism of carcinogenesis

Quantitative *in vitro* assays for neoplastic transformation have defined the parameters that characterise the growth of normal and transformed cells and have led to the identification of factors which control growth and differentiation. However it must be remembered that cell culture is an artificial system. All results must be related to whole animal studies. The *in vitro* growth characteristics of any cell type is only an approximation to the phenotype of the same cells *in vivo*. Nevertheless, cell culture experiments have and continue to play a significant role in the identification and characterisation of potential oncogenes as well as allowing the effects of various chemicals, whether mutagenic, carcinogenic or therapeutic, to be assessed.

DNA from chemically transformed rodent cells was shown to be capable of transforming immortalised but non-transformed mouse recipient NIH 3T3 cells in a series of gene transfer experiments (Shih *et al.*, 1979). Later, DNA extracted from human tumours was found to induce transformation of NIH 3T3 cells with high efficiency in gene transfer assays (Shih *et al.*, 1981; Murray *et al.*, 1981). These results suggested that normal cells contained proto-oncogenes which could be activated experimentally by chemical carcinogens and that human tumours contained biologically active cellular oncogenes. Gene transfer experiments led to the identification of many distinct human oncogenes that were activated by mutation or DNA rearrangements. Some of the cellular oncogenes have been shown to be homologues of retroviral oncogenes, such as the *ras* genes, whereas others are not. In all cases however, the biologically active cellular oncogenes are mutant forms of normal proto-oncogenes. The identification of activated oncogenes in a large number of human tumours clearly implicates cellular genes in the pathogenesis of human cancers.

Further experiments which have confirmed the role of oncogenes in carcinogenesis includes the generation and study of transgenic mice. As mentioned above, cell culture experiments, whilst providing an easily manipulatable system for the analysis of oncogene transforming activity, represents an oversimplified approximation of the development of neoplasias in whole animals. In a transgenic mouse however, an oncogene is introduced into every cell in the animal. This enables

the transforming potential of an activated oncogene to be assessed in an *in vivo* environment.

To generate a transgenic mouse, a construct containing an activated oncogene is microinjected into the pronuclei of fertilised mouse eggs. If the oncogene construct becomes integrated into the germ line DNA, transgenic offspring will be obtained.

The first laboratory to report the function of a cellular oncogene in transgenic animals was that of Philip Leder and colleagues (Stewart *et al.*, 1984). In their experiments the *c-myc* gene was over-expressed by placing it under the transcriptional control of a strong inducible promoter, the mouse mammary tumour virus (MMTV) LTR. Mice that expressed the MMTV/myc transgene developed mammary carcinomas after a latent period of several months. This study provided direct experimental evidence that a cellular oncogene could induce neoplastic disease in a complete, living animal. Important to note, however, was that the MMTV/myc transgene was clearly not the only factor required for cancer development in this system. The long latency period indicated that other changes must have occurred to give rise to the final tumour. Furthermore, only one tumour generally developed in one of the ten mammary glands present in the transgenic mouse despite all the cells carrying the MMTV/myc transgene. These results suggest that one activated oncogene (*c-myc*) was contributory but not sufficient for the formation of mammary carcinomas in these mice.

Many subsequent transgenic mouse models have confirmed the activity of various oncogenes in carcinogenesis (Muller *et al.*, 1988; Tsukamoto *et al.*, 1988). They have also shown that oncogenes can co-operate and further reduce the latency period observed for either oncogene alone (Compere *et al.*, 1989; Thompson, 1989).

The experimental strategies outlined above have been seminal in identifying oncogenes which contribute to the neoplastic process. Ironically, identification of oncogenes and understanding their roles in transformation has lead to a greater understanding of proto-oncogene activity in normal cell function, including the regulation of proliferation, differentiation, development and death.

Oncogenes, however, are not the only, and perhaps not even the most important, genes associated with neoplastic progression. A second class of genes, the tumour suppressor genes, have also been shown to play a crucial role in the genesis of cancers.

1.3.3.2 Tumour Suppressor Genes

As their name suggests, tumour suppressor genes have the ability to suppress neoplastic growth. The identification of tumour suppressor genes, and the elucidation of their role in carcinogenesis, has come from three main areas of interest; somatic cell hybrid analysis, the study of inherited cancer syndromes and the experimental reversion of the neoplastic phenotype *in vitro*.

In 1969 Henry Harris and co-workers fused normal cells with tumour cells (Harris *et al.*, 1969). The resultant normal-malignant cell hybrids were shown not to be tumorigenic. Numerous studies of this type have subsequently confirmed this observation which implies that normal cells contain genetic information that can suppress the neoplastic behaviour of tumour cells (Stanbridge, 1976, Saxon *et al.*, 1986). Many of the non-tumorigenic hybrids obtained after such fusions however continued to display some phenotypic characteristics of transformed cells *in vitro*, including loss of density-dependent inhibition of cell growth and loss of anchorage-dependent growth. Hence the suppression of tumorigenicity in these hybrid cells appears to represent only a partial reversion of cell transformation. This is consistent with the multihit model of carcinogenesis where the activation of dominant oncogenes and loss of tumour suppressor gene functions are independent events, both of which contribute to the fully transformed state. Furthermore, normal-malignant cell hybrids often revert to the tumorigenic phenotype following the loss of particular chromosomes which originated from the normal parent cell line (Stanbridge *et al.*, 1981; Klinger, 1982). These results confirm that loss of a tumour suppressor gene(s) is associated with transformation.

In some human cancer families, tumour susceptibility is inherited in a dominant Mendelian fashion. Retinoblastoma (RB) is a disease that can arise sporadically or, in about 10% of cases, shows a familial pattern of inheritance (Evans, 1993). Familial retinoblastoma affects children at an early age and patients frequently develop multiple tumours in both eyes. Individuals with sporadic retinoblastoma generally develop a single tumour in one eye only and present at an older age compared to those with the inherited form of the disease. Alfred Knudson studied cases of inherited and sporadic retinoblastoma and made statistical comparisons of frequency and age of onset of both forms of the disease. In 1971 he proposed that

retinoblastoma was caused by two mutations, one in each copy of the same gene (Knudson, 1971). In retinoblastoma families, affected individuals inherit one defective copy of the gene which is then present in every cell of their bodies: this kind of inherited mutation is called a germline mutation. Malignant tumours can develop from any cell that sustains a second mutation in the remaining normal gene copy by virtue of a somatic mutation. Sporadic retinoblastoma also involves mutation in both copies of the same gene, however both mutations are derived from separate somatic events. The presence of a germline mutation in hereditary retinoblastoma is what predisposes affected individuals to tumour development, earlier age of onset and the presence of multiple tumours in both eyes. The requirement for mutations in both alleles of a tumour suppressor gene has become known as Knudson's two-hit hypothesis.

Various experimental strategies were applied to identify the chromosome, chromosome region and ultimately the gene implicated in the development of retinoblastoma. The mapping techniques used included karyotype analysis (Knudson *et al.*, 1976; Benedict *et al.*, 1983), single chromosome transfer experiments, genetic linkage studies looking for loss of heterozygosity (LOH) using isozyme markers (Sparkes *et al.*, 1983) and the application of restriction fragment length polymorphisms (RFLP) (Cavenee *et al.*, 1983; Dryja *et al.*, 1986). A gene, named *Rb*, was finally mapped to chromosome 13 band q14. Several genetic studies have shown that functional loss of both copies of the retinoblastoma susceptibility gene is associated with tumour development (Godbout *et al.*, 1983; Friend *et al.*, 1987; Weichselbaum *et al.*, 1988). Therefore, unlike oncogenes which contribute to tumorigenesis through the acquisition of dominant, activating mutations, it is the loss of tumour suppressor gene function which can lead to neoplastic transformation.

The *Rb* gene has been implicated in the genesis of neoplasms other than retinoblastoma including osteosarcomas (Hansen *et al.*, 1985), sarcomas of soft tissue (Weichselbaum *et al.*, 1988) and carcinomas of the breast (Lee *et al.*, 1988; T'Ang *et al.*, 1988), lung (Harbour *et al.*, 1988) and bladder (Weinberg, 1992). The gene encodes a protein (pRb) which has been shown to bind the E2F family of transcription factors that are required for DNA transcription and replication (Adams and Kaelin, 1995). Binding of E2F to the hypophosphorylated form of pRb renders it unavailable

to participate in DNA replication. Therefore by sequestering E2F, pRb effectively inhibits DNA replication.

Since the identification of *Rb*, several other tumour suppressor genes have also been identified including *APC* (Kinzler and Vogelstein, 1996), *DCC* (Cho and Fearon, 1995), *WT-1*(Coppes *et al.*, 1994; Hastie, 1994), *NF-1* (Ponder, 1992; Upadhyaya *et al.*, 1995,1997) and *p53*.

p53 has been mapped to chromosome location 17q13.1 (McBride *et al.*, 1986). This region is frequently deleted in tumours (Baker *et al.*, 1989; Nigro *et al.*, 1989) and mutations in the *p53* gene have been identified in a very large number of disparate cancers (for review see Caron de Fromentel and Soussi, 1992). Indeed, more mutations have been reported in the *p53* gene than in any other gene to date (Hollstein *et al.*, 1991) which highlights the critical role this gene must play in cancer development.

Early experiments identified *p53* as a putative oncogene: it was shown to be capable of transforming early passage rodent cells in conjunction with an activated *c-H-ras* gene (Eliyahu *et al.*, 1984; Jenkins *et al.*, 1984; Parada *et al.*, 1984). In subsequent studies however it was determined that the *p53* clone used in these early studies was in fact a mutant cDNA. Wild type *p53* does not co-operate with *ras* but rather inhibits cellular transformation induced by other oncogenes and *ras* (Eliyahu *et al.*, 1989; Hinds *et al.*, 1989).

A rare familial cancer syndrome called Li-Fraumeni syndrome (LFS) has been identified in which affected individuals inherit an autosomal dominant p53 mutation. As with inherited Retinoblastoma, LFS patients inherit a predisposition to developing an array of diverse tumour types including sarcomas, carcinomas and leukaemia, at an early age (Li *et al*, 1988).

Over-expression of wild type p53 in tumour cell lines is antiproliferative (Baker *et al.*, 1990; Chen *et al.*, 1990; Diller *et al.*, 1990). This ability to reverse the tumorigenic phenotype confirms the tumour suppressor status of p53.

p53 is up-regulated in response to a number of DNA damaging agents, including radiation, which subsequently leads to growth arrest (Maltzman and Cyzyk, 1984; Kastan *et al.*, 1991; Kuerbitz *et al.*, 1992) and activation of DNA repair mechanisms (Kastan *et al.*, 1992). The induction of growth arrest thus enables cells to repair any endogenous DNA damage and is undoubtedly an important regulatory

mechanism which protects cells from accumulating genetic mutations or alterations that may ultimately lead to a malignant phenotype. p53 mediated growth arrest has also been reported where the specific cell culture environment is not conducive to continued cell proliferation, i.e., when serum (Reich *et al.*, 1984) or isoleucine concentration (Steinmeyer *et al.*, 1990) in culture medium is low. The ability of a cell to growth arrest when exposed to cellular stress is again an advantageous response, providing the cell an opportunity to survive in adverse conditions whilst remaining ready to grow when suitable conditions are re-established. In extreme situations where, for example, the level of DNA damage is extensive, p53 has been shown to play a role in inducing programmed cell death (apoptosis) (Shaw *et al.*, 1992; Lowe *et al.*, 1993; Clarke *et al.*, 1993).

The role of p53 in tumorigenesis is, however, quite complex. Particular mutations have been identified which have lead to the production of proteins with novel, aberrant functions. These kinds of mutations, termed gain of function mutations, instead of leading to gene inactivation, result in the acquisition of transforming potential (Wolf *et al.*, 1984; Dittmer *et al.*, 1993).

The inactivation or loss of tumour suppressor genes like *p53* and *Rb* clearly has severe implications in terms of the ability of the cell to regulate vital cellular mechanisms including DNA transcription, DNA repair and replication, and cell death. Loss of tumour suppressor gene function can be achieved not only by genetic mutation and/or deletion but also through the action of particular viral gene products. The role played by viral oncoproteins in the inactivation of p53 and pRb, and the significance of this in virus-associated cellular transformation, is discussed in detail in sections 1.5.3.1 and 1.5.3.2.

As observed for *Rb*, mutations and/or loss of *p53* is never the only event detected in a malignant tumour. Disruption of the normal function of p53 alone, whilst creating an unstable environment in which other genetic changes are more likely to occur, is not sufficient to induce full transformation. p53 dysfunction or loss is one more event which can contribute to neoplastic progression. Nevertheless because tumours classically possess changes in several individual genes, which may or may not include p53, leads us back to the hypothesis that cancer progression is a multistep event that marries the functions of numerous genes in different combinations.

Finally, it would be inaccurate and unrealistic to think that all cancers can be neatly fitted into the multistep concept outlined above. Cancer, as already mentioned, is a collective term for a large number of diseases which display a very varied spectrum of individual characteristics. "*No unitary concept can give a satisfactory explanation of the intimate nature of cancer*" (Vriel, 1979). Cancer is a dynamic, complex process. A successful tumour cell can exploit its immediate, unique environment to gain proliferative dominance, immortality and autonomy. However, a model like the "multistep" scenario, where applicable, provides a framework which can help us understand the progress of cancer and allow certain prognostic predictions to be made. By gaining a better understanding of the mechanism(s) of cancer progression, efficient treatments and strategies can be developed in an attempt to disrupt or prevent the advance of the carcinogenic process.

1.4 Viruses and Cancer

Identifying risk factors is an important step in understanding the aetiology of individual cancers. Indeed, eliminating risk factors can dramatically reduce the cancer incidence rate as exemplified when comparing cohorts of smokers with non-smokers and the frequency of lung cancer. Experimental and epidemiological studies have identified certain viral agents as risk factors in the development of human tumours. It has been estimated that viral infections are directly implicated in 15% of human cancers world-wide (zur Hausen, 1986). Cancer of the cervix and hepatocellular carcinoma both include a viral component in their aetiology and together account for approximately 80% of virus-associated cancers (zur Hausen, 1991b). The mechanisms by which different viruses contribute to tumorigenesis are varied.

Human immunodeficiency virus (HIV) infection induces immunosuppression in affected individuals (Feinberg, 1996). This acquired immunosuppression results in a considerable increased risk of developing Kaposi's sarcoma and non-Hodgkin's lymphoma (Biggar *et al.*, 1994; Schulz *et al.*, 1996). The virus itself has not been shown to be directly transforming however it creates an environment where cells transformed by other physical, chemical or infectious agents can persist: the longer a transformed cell escapes elimination by the body's defence mechanisms the greater the chance that cell will progress to a more neoplastic state. Immunosuppression in

general, whether drug induced, as in the case of transplant patients (London *et al.*, 1995), or genetic (Barnett *et al.*, 1983; Fuchs and Pfister, 1996), is associated with increased risk of tumour formation.

Other viruses have a more direct effect on tumour induction compared to HIV. Human T cell leukaemia-lymphoma virus type I (HTLV-I) is a retrovirus and has been associated with malignant adult T cell leukaemia-lymphoma (ATLL). ATLL is most prevalent in coastal areas of southern Japan, in the Caribbean and in regions of central Africa (zur Hausen, 1991b). The latency period between primary infection and presentation of leukaemia spans several decades indicating that virus infection per se is not sufficient for development of malignant disease. The elucidation of other cellular changes necessary for full neoplastic transformation has not been well characterised. Nevertheless, identification of this retrovirus as a primary element of risk for ATLL development contributed to the isolation of other pathogenic retroviruses including HTLV-II and HIV.

Epstein-Barr virus (EBV) has been linked to several separate human cancers including Burkitt's lymphoma, undifferentiated nasopharyngeal carcinoma, gastric cancer, peripheral T cell lymphoma and Hodgkin's Disease in immunosuppressed individuals (zur Hausen, 1991b). EBV is a widespread viral infection which can be present in up to 100% of a population and persists in the infected host throughout life (zur Hausen, 1991b). The way in which EBV infection contributes to the development of malignant tumours is obscure and not well understood however it remains the first human tumour virus to be identified as a cofactor in cancer genesis (Epstein *et al.*, 1964).

The link between chronic hepatitis B virus (HBV) and hepatocellular carcinoma was initially suspected from epidemiological observations. Areas of the world which show endemic HBV infection, including south-east Asia and China, coincidentally report the highest incidence of primary liver cancer (Bosch and Munoz, 1989). Although current evidence suggests that primary liver cancer is multifactorial in origin, HBV infection and chemical agents remain the major risk factors for this type of disease (Saracco, 1995). Aflatoxins are believed to be particularly important cofactors as they are a common food contaminant and potent liver carcinogens as demonstrated in a variety of animal species. The risk of developing primary liver cancer following HBV infection and aflatoxin exposure is increased (Saracco, 1995).

The appearance of primary liver cancer in humans is generally only observed after several decades of chronic liver disease and persistent HBV infection. Several other reports indicate that HBV genome persistence is critical for progression to the tumorigenic state (for review see zur Hausen, 1991b); HBV genome sequences have been detected in malignant tumours originating in individuals from high-risk areas (Nagaya *et al.*, 1987).

Papillomaviruses are another group of viruses which possess transforming characteristics and have been isolated from a variety of animal species including humans. In the great majority of cases these viruses induce benign lesions of the skin and mucosa, however infection with selective variants has been link to the development of malignancies (Syverton, 1952; zur Hausen, 1991a; Campo *et al.*, 1994). In humans, infection with 'high-risk' HPV types 16 and 18 has been associated with anogenital cancers, in particular cancer of the cervix. More than 90% of cervical cancer tumours contain viral DNA (Munoz and Bosch, 1996); in the majority of cases the DNA has integrated into the host cell genome (Durst *et al.*, 1985; Schwarz *et al.*, 1985). The transforming potential of the high-risk HPVs has been attributed to specific transcripts derived from the viral genes E6 and E7 (Vousden, 1994).

The role of HPV infection in the formation of neoplastic lesions is of direct relevance to the work in this thesis. In subsequent sections, I will review all aspects of papillomavirus biology concentrating particularly on bovine papillomavirus type 4, the specific papillomavirus which has been the focus of the experimental work presented in later chapters.

1.5 Papillomaviruses: Historical Overview

Although the first papillomavirus was identified more than 60 years ago, progress in trying to understand the molecular biology of these viruses has been slow.

Early work on animal papillomas was limited mainly to transmission studies. Transmission of the canine oral papilloma was performed back in 1898 (McFadyean and Hobday, 1898); the experimental transfer of a skin papilloma from horse to horse was successfully achieved in 1901 (Cadeac, 1901). The fact that papillomas could exhibit such contagious behaviour suggested the involvement of an infectious agent. A bacterial or protozoan aetiology was ruled out when Ciuffo demonstrated in 1907 that transmission of common human warts was possible using cell-free filtrates

(Ciuffo, 1907). Then in 1933 the first papillomavirus, the cottontail rabbit (Shope) papillomavirus (CRPV), was isolated and characterised (Shope and Hurst, 1933). This was the first virus to be identified which was known to cause tumours.

Since that time very many more papillomaviruses have been identified and isolated from a wide species range. They have been shown to be transmissible not only between animals of the same species, but the naturally occurring papillomaviruses from cattle, sheep, deer and cottontail rabbit have been shown to cause tumours in other species also (Olson, 1987).

Even though an extensive spectrum of investigations was carried out on papillomaviruses since the early 1930s up until the mid to late 1970s, little insight was gained about how these viruses were actually producing papillomas. Much of this was due to the limited amount of viral material/DNA available for study; initially papillomavirus particles had to be purified from warts or papillomavirus lesions. The amount of virus present in a lesion is very variable and large amounts of virus infected tissue was required to purify significant amounts of virus for analysis. Up until the early 1970s it was believed that there was in fact only one type of human papillomavirus and that the morphological and behavioural differences seen between papillomavirus-induced lesions was influenced purely by the location at which the lesion occurred on the body (Rowson and Mahy, 1967). Development of an efficient *in vitro* culture system for the propagation of papillomaviruses has proved elusive even to the present day. However, the advent of molecular cloning technology provided the first real opportunity to begin experimental analysis of the various molecular events involved in papillomavirus-associated tumour induction and progression to malignancy.

1.5.1 Papillomavirus Structure and Classification

The papillomaviruses (PVs) are distinct from other members of the papovaviridae family, such as SV40 and polyoma viruses, in that they have larger virions, their closed circular double-stranded superhelical DNA genome is larger and transcription of the large, overlapping open reading frames (ORF) occurs from only one DNA strand (Danos *et al.*, 1982; Chen *et al.*, 1982). PV genomes are on average ~7.9kb in size: virus sizes can range from ~7.2kb, as observed for BPV4 (Campo *et*

al., 1980), HPV9, 15, 17, 22 and 23 (Kremsdorf *et al.*, 1982, 1984), up to ~8.4kb for the deer papillomavirus (DPV)(Groff and Lancaster, 1985).

Genes encoded by PVs are subdivided into two groups, namely the 'early' and 'late' genes. Early genes are expressed prior to DNA replication. They encode proteins which control and modulate vegetative viral DNA replication and transcription. The early genes are also responsible for cell transformation. The late genes encode structural proteins, L1 (major protein) and L2 (minor protein), which aggregate to form the viral capsid. There is also a non-coding region (NCR), alternatively termed the LCR (long control region) or URR (upstream regulatory region), which contains several *cis*-acting regulatory elements (Ward *et al.*, 1989). The LCR is defined as the region that extends from the stop codon of the L1 open reading frame (ORF) to the start codon of E6; this non-coding region is the least conserved between papillomavirus types.

Papillomaviruses infect epithelial cells of the skin and mucosa. A few papillomaviruses, such as BPV1, 2 and 5, DPV, EEPV, RPV and sheep papillomavirus, are capable of inducing fibroblast tumours as well as being tropic for epithelial cells. Infection by human papillomaviruses (HPVs) however is purely epitheliotropic in nature.

Papillomavirus replication and virion assembly occurs in keratinocytes and is closely coupled to keratinocyte differentiation. Virus is undetectable in basal stem cells although the basal layer is believed to be a site of latent viral infection. Viral DNA has been detected in suprabasal cells and in successive layers by *in situ* hybridisation (McDougall *et al.*, 1986). Mature virions are only found in the most superficial and highly differentiated epithelial layers. The vegetative life cycle of papillomaviruses therefore apparently requires specific host cellular factors that are sequentially produced in a predefined order according to the differentiation program of keratinisation. The synchronisation of papillomavirus gene expression and particle maturation with keratinocyte differentiation has consequences for normal host cell function. Papillomavirus infection causes modifications of host gene expression and interferes with normal cellular control mechanisms. In normal tissue, DNA replication is restricted to basal and parabasal cells. In papillomavirus infected cells, however, DNA synthesis has been detected in keratinising cells (Rashad, 1969). Hence papillomavirus infection must be capable of altering the tolerance of

differentiating cells to DNA replication. Differentiation markers, such as keratins, desmosome-related antigens and cell surface glycoproteins, all show changed expression in the presence of a papillomavirus infection (Steinberg, 1986). At a gross level, changes in the normal pattern of differentiation and maturation gives rise to an anomalous morphology in the form of a papilloma.

Individual PV types are generally classified according to criteria such as host range and by comparing levels of nucleic acid homology. A nomenclature for the classification and organisation of papillomaviruses was originally proposed in 1979 and was determined by heterologous DNA reassociation kinetics (Coggin and zur Hausen, 1979). The definition for classifying new virus isolates has been amended several times since then and today a papillomavirus is recognised as a novel type if the sequence of its entire L1 ORF shares less than 90% identity with any other L1 sequence. There have been more than 75 HPV types cloned over the past 15 years, approximately 63 of these HPVs have been completely sequenced (van Ranst *et al.*, 1996). The final tally, if ever realised, relies very much on the continued enthusiasm in the search for new types; at present perhaps the sky's the limit.

In terms of evolution, however, host specificity does not always reflect the existence of a natural relationship. For example, BPV1, 2 and 5 (Group A) infect cattle as do BPV3, 4 and 6 (Group B). There is however little DNA homology between the group A and B bovine papillomaviruses (Lancaster and Olsen, 1978; Jarrett *et al.*, 1984). BPV1 in fact shows a greater degree of homology with viruses that infect other animal species, namely the deer (DPV), European Elk (EEPV) and reindeer (RPV). Likewise HPV1 is more closely related to BPV1(cattle) and CRPV(cottontail rabbit) than to some other HPVs, e.g. HPV2 or 4.

Interestingly, PVs that are related at the molecular level exhibit similar biological properties such as type of lesion produced and site of infection. Homologies which exist both at the molecular and biological level between viruses from quite divergent species cannot be wholly attributed to tissue tropism. A better understanding of the various individual viral genomes' organisation and biological properties must be attained before evolutionary relationships can be more accurately assessed.

1.5.2 Papillomavirus Disease and Cancer

Infection by a papillomavirus results in hyperproliferation of epithelial (and in a few cases fibroblast) cells. This leads to the formation of papillomas (condylomas or warts) which can exhibit many different morphological and histological patterns depending primarily on the identity of the infecting PV type. Individual virus types are remarkably species and tissue specific. The majority of lesions produced are benign. These benign tumours can either persist in this inert form or can spontaneously regress. However, infection by a few specific PV types is associated with the formation of papillomas which have the potential to progress to carcinomas providing additional cofactors, such as genetic background, environment and/or other viral or microbial infections, exist in parallel.

1.5.2.1 Cottontail Rabbit Papillomavirus (CRPV)

Papillomatosis is endemic in rabbits in particular states in America, specifically those states which lie in the Mississippi valley (Kreider and Bartlett, 1981). The aetiological agent, identified by Shope, was shown to be a virus (Shope and Hurst, 1933). The Shope or cottontail rabbit papillomavirus (CRPV) was later identified as the first DNA virus capable of inducing tumours.

CRPV naturally infects the hairy epithelium of cottontail rabbits (Kidd and Parsons, 1936). It will induce papillomas in both cottontail and domestic rabbits (Shope, 1935). Interestingly it has been observed that, whereas 25% of papilloma-carrying cottontail rabbits eventually developed malignant lesions, 75% of domestic rabbits also with benign papillomas ultimately developed carcinomas (Syverton, 1952). This suggests therefore that papillomas in domestic rabbits are more likely to progress to fully malignant and even metastatic tumours than papillomas in the natural host, the cottontail rabbit. The genetic background of the domestic rabbit apparently renders it considerably more susceptible to CRPV-associated carcinogenesis compared to the virus' natural host, the cottontail rabbit (Kreider, 1980).

Carcinomas in either domestic or cottontail rabbits only appeared several months after viral infection or inoculation (Syverton *et al.*,1950a, b; Syverton, 1952). The delay implicates cofactors as necessary elements in the development of malignant disease. CPRV infections were later shown to synergise with the actions of known carcinogenic chemicals, especially tar (Rous and Kidd, 1936; Kidd and Rous, 1937).

The interaction between virus and chemical carcinogen(s) greatly reduced the latency period between infection and cancer (*ibid.*).

Finally, viral particles were not detected in cancerous lesions (Syverton, 1952) suggesting that production of mature CRPV virions is not permitted in malignant cells possibly as a result of disruption to cellular differentiation.

1.5.2.2 Human Papillomaviruses

Genetic background was shown to influence the susceptibility of domestic rabbits in developing cancer when infected with the cottontail rabbit papillomavirus (CRPV). Genetic background is also a fundamental contributory factor to cancer development in Epidermodysplasia verruciformis (EV) patients. EV is a rare, lifelong, multifactorial disease which involves genetic and extrinsic factors in addition to HPV infections. This condition can arise sporadically or is inherited; generally inheritance of this disease follows an autosomal dominant pattern however there is one report of a pedigree where inheritance is X-linked (Androphy *et al.*, 1985). Lesions, induced by a range of specific (Group D) HPVs, are widespread and some can undergo malignant transformation. The development of multiple skin carcinomas arises 10 to 30 years after the onset of the disease and is observed in 30-50% of EV individuals (Iftner *et al.*, 1990); these cancers occur most frequently in sun exposed areas of the skin. The risk of malignant conversion very much depends on the HPV type present in the lesion and also on skin type; malignant conversion is very rare in black-skin patients (Jacyk and de Villiers, 1993). More than 20 HPV types have been isolated from EV-associated skin lesions however, HPV5 and 8 are the predominant types, being found in 90% of squamous cell carcinomas from EV patients (Arends *et al.*, 1990).

Many EV patients have been shown to have impaired cell-mediated immunity (Jablonska and Orth, 1985). Immunosuppression in general, whether it be genetic, acquired through infection (HIV)(Vernon *et al.*, 1995; Adams *et al.*, 1995) or iatrogenically induced (renal allograft recipients)(Trenfield *et al.*, 1993; Petry *et al.*, 1994), is often accompanied by an increased incidence of warts and skin cancers (Benton and Arends, 1996). Dysfunctional immune responses to PV in these situations, as in EV, appears also to be cell-mediated in nature.

A number of other HPVs have been implicated in the genesis of anogenital (zur Hausen, 1991a; Arends *et al.*, 1990 and refs. therein) and oral/upper respiratory tract cancers (Kahn *et al.*, 1986; Brandsma *et al.*, 1986; Zarod, 1988; Watts *et al.*, 1991). In particular, HPV16 and 18 have been classified as 'High-risk' types for genital cancer. These are related to HPV6 and 11 which are representative of the 'Low risk' group of HPV types. Why high and low risk HPVs have different malignant potentials is not well understood. zur Hausen (zur Hausen, 1991a) suggested that it may be partially due to differential reliance on exogenous cofactors for carcinogenic advance. As will be subsequently discussed (section 1.5.3), the ability of some viral proteins to interact with important cellular, regulatory proteins must also be an important determinant of HPV oncogenic potential.

Cervical cancer is the second most common cancer in women world -wide exceeded only by breast cancer (Parkin *et al.*, 1993). Around 2,000 women die of cervical carcinoma in the United Kingdom each year although this number is slowly decreasing due to improvements in screening and treatment of identified premalignant lesions. World wide, there are an estimated 500,000 new cases of cervical cancer each year (Scheffner *et al.*, 1994). In sub-Saharan Africa alone, an estimated 53,000-130,000 women develop this malignancy each year (Feldmeier, *et al.*, 1996). Analysis of invasive squamous cervical carcinomas, cervical adenocarcinomas and cell lines derived from these tumour have identified HPV specific sequences in more than 90% of cases (Munoz and Bosch, 1996 for review); HPV16 was the predominant type isolated from carcinomas whereas HPV18 was the most common isolate in adenocarcinomas . The high level of HPV detection in cancers of the cervix strongly supports a role for this virus in initiation, progression to and maintenance of the malignant phenotype, although HPV infection is considered to have its greatest effect early in the transformation programme.

The long latency period seen between HPV infection and carcinoma *in situ* (CIS) presentation combined with the low frequency of lesions progressing to full malignancy implicates additional agents or cofactors in the genesis of cervical cancer. Apart from the clear association between cervical cancer and certain HPV infections, epidemiological studies have identified other contributory factors which increase the risk of malignant conversion; these include age (20-29 years being the age group at highest risk), multiple sexual partners/increased sexual activity per week, a history of

genital warts in the proband or any sexual partner and cigarette smoking (Kataja *et al.*, 1993; Syrjanen, 1996).

1.5.2.3 Bovine Papillomaviruses

Six separate bovine papillomaviruses have been identified to date. These have been divided into two groups, groups A and B (Campo *et al.*, 1980, 1981; Jarrett *et al.*, 1984). The group A BPVs include BPV1, 2 and 5 while the group B BPVs include BPV3, 4 and 6. Group A BPVs can infect both fibroblast and epithelial cells and induce fibropapillomas; Group B BPVs, like HPVs, are purely epitheliotropic in nature. Of the six BPVs so far identified, BPV1 has been most extensively studied and much of the early work on papillomavirus biology was performed using BPV1.

BPV1 infections induce only benign fibropapillomas in cattle. Infection with BPV4 and 2 however is associated with the development of alimentary canal cancer (Campo *et al.*, 1994) and urinary bladder cancer (Campo *et al.*, 1992) respectively in the natural host. In both cases, cattle's consumption of bracken fern has been identified as a cofactor. Bracken fern is known to contain a selection of biologically potent chemicals including mutagens, carcinogens (Evans, I. A. *et al.*, 1982) and immunosuppressants (Evans, W. C. *et al.*, 1982) however the exact mechanism of synergy between BPV4 or 2 and bracken has not been elucidated. The role of BPV4 in upper gastrointestinal (GI) tract cancer in cattle appears to differ from that of the high-risk genital HPVs 16 and 18. Whereas high-risk HPV DNA has been detected in the large majority of cervical tumours and derived cells lines, BPV4 DNA has not been detected in naturally occurring cancers in cattle (Campo *et al.*, 1985). Similarly, when bovine palatine tissue was infected with BPV4 *in vitro* and subsequently placed into the renal capsule of nude mice, malignant cells developed. Nevertheless no BPV4 DNA was detected in these malignant cells (Gaukroger *et al.*, 1991). Therefore, unlike high-risk HPVs, such as types 16 and 18, which persist in cervical tumours to malignancy, a 'hit and run' mechanism has been proposed for the contribution of BPV4 in upper GI cancer in cattle (Smith and Campo, 1988). BPV4 is implicated only in the early stages of transformation and viral DNA is lost as subsequent genetic alterations are acquired. BPV4 is apparently not necessary for progression to, or maintenance of, the malignant phenotype.

Papillomavirus infections, apart from having the potential in some cases to cause cancer, also produce benign lesions which in their own right can be life threatening, as in the case of laryngeal papillomatosis (Steinberg, 1987; Kashima and Mounts, 1987; Kashima *et al.*, 1996), disfiguring and, in individuals with anogenital warts, can severely reduce quality of life (Oriel, 1971). There is also increasing interest in establishing the role of HPV infection in nonmelanoma skin cancers from both normal and immunosuppressed patients (Shamanin *et al.*, 1996). Infection of cattle and horses have agricultural and financial implications (Jarrett, 1985). Therefore, there are several very serious conditions, including cancer, that justify the need for developing methods of controlling and/or preventing papillomavirus infections. Strategies for the treatment and control of papillomavirus infections are areas which presently attract active research (Kinbauer *et al.*, 1996; Campo, 1997). Nevertheless, in order for such strategies to be optimally successful and efficient, researchers require an in-depth understanding of the natural biology of the viruses implicated, putative cofactors which synergise with the viruses in the disease process, the location of pools of latent virus infection, the pathology of the diseases induced and the nature of host immune responses to these infections. The identification of key viral transforming genes and analysis of their functions and interactions are areas of prime importance.

1.5.3 Papillomavirus Genes and Transformation

Transcription of papillomavirus genes is dependent on both viral and cellular transcription factors which can interact with the viral regulatory sequences present in the LCR. Both viral E1 and E2 gene products are necessary for viral replication (Lambert, 1991; Yang *et al.*, 1991). E1 of BPV1 is important for maintenance of the viral genome as extrachromosomal episomes (Arends *et al.*, 1990). The BPV1 E2 protein can function as a transcriptional transactivator when present as a full length molecule or as a repressor in its truncated form (Lambert *et al.*, 1987). The E2 proteins from high risk HPVs, such as HPV18 and 16, in general function as repressor proteins (Romanczuk *et al.*, 1990;). In a large majority of cervical carcinomas and in all cell lines derived from cervical cancers, viral DNA has integrated into the host genome (Yee *et al.*, 1985; Durst *et al.*, 1987a; Popescu *et al.*, 1987). Integration appears to be a random event with viral DNA located at different genomic sites in

different cancers and cells lines. However the site at which the viral genome is disrupted for integration shows remarkable specificity. Integration regularly involves cleavage within the E1/E2 region. As a consequence the E2 gene product(s) is either lost or altered. As mentioned above, E2 acts primarily as a repressor of transcription from the E6/E7 promoter of HPV16 and 18 which results in the downregulation or even 'switching-off' of genital HPV expression (Romanczuk *et al.*, 1990). Loss of E2 repressor function, as a consequence of integration, may lead to high levels of viral gene expression; irregular expression of viral oncongenes E6 and E7 is associated with cellular transformation (Vousden, 1994).

Often one of the consequences of viral genome integration is that synthesis of the capsid proteins L1 and L2 is lost. These structural proteins almost certainly contain important epitopes which, if displayed on the outside of the virion, may allow host immune systems to recognise virus and virus infected cells. Integration of the viral genome therefore may provide a mechanism whereby a virus-infected cell could evade recognition by host immune surveillance which previously prevented any further progression of a premalignant lesions to carcinoma.

The mechanism for achieving cell transformation is not the same for all papillomaviruses. Only the E6 gene product of EV specific HPV8 has transforming ability (Iftner *et al.*, 1988). Generally papillomaviruses express two oncogenes which co-operatively achieve transformation. Table 1.1 highlights the oncogenes which have been identified in several papillomaviruses.

Table 1.1 Summary of the oncogenes which have been identified in several Papillomaviruses

	E5	E6	E7	E8
BPV1	+	+	-	-
BPV4	-	-	+	+
CRPV	-	+	+	-
HPV16	+	+	+	-
HPV8	-	+	-	-

Symbols: + : indicates recognised oncogenes
- : indicates either non-transforming or absent ORF

(Taken from Campo, 1992)

1.5.3.1 E7

E7 has been identified as one of the major oncogenes present in high-risk HPVs including HPV16 and 18 (Bedell *et al*, 1987; Vousden *et al.*, 1988; Dyson *et al*, 1989b; Munger *et al.*, 1989a; Hudson *et al.*, 1990). Indeed, in BPV4, E7 is the major *in vitro* transforming gene (Pennie *et al.*, 1993). HPV16 E7 contains a pRb binding domain (Munger *et al.*, 1989b), two zinc binding Cys-x-x-Cys motifs (Barbosa *et al.*, 1989) and casein kinase II (CK II) phosphorylation sites (Barbosa *et al.*, 1990). The zinc finger regions of E7 are essential for the transactivating activity of this viral oncoprotein (Phelps *et al.*, 1988). The CKII sites are important for transformation by HPV16 E7 but to a lesser extent than the pRb and zinc finger binding domains (Barbosa *et al.*, 1990; Storey *et al.*, 1990). The E7 proteins of BPV1 and HPV8 contain neither Rb-binding or CKII sites and are consequently non-transforming (Iftner *et al.*, 1990); BPV4 E7 is transforming however it does not possess a CKII phosphorylation site (Jackson *et al.*, 1996).

pRb, an important tumour suppressor gene product, when bound to E7 cannot interact with its natural target, namely E2F (Chellappan *et al.*, 1992; Morris *et al.*, 1993). The ability of HPV E7 to interact with pRb, which can lead to inappropriate DNA synthesis, RNA expression and cell proliferation, is proposed as the major mechanism by which E7 contributes to cellular transformation. This is supported by the observation that the E7 proteins encoded by low risk genital HPV, like HPV6 and 11, bind pRb with a much lower affinity than high-risk HPV types 16 or 18 and this activity correlates with transforming potential (Munger *et al.*, 1991). BPV4 E7 also contains the pRb binding region (Jackson *et al.*, 1991) however it has not yet been determined if it can bind the pRb protein. Experiments are currently underway to address this question.

Work by Jewers *et al.* (1992) has shown that, in the context of the whole HPV16 genome, binding of pRb by E7 is not necessary for the immortalisation of primary human keratinocytes. Instead, immortalisation has been linked to one of the Cys-x-x-Cys motifs in the C-terminus of E7. These results suggest that the ability of E7 to induce immortalisation in human primary keratinocytes is independent of its ability to bind pRb.

It is very likely that E7's interaction with pRb is not the only function of this viral protein in transformation. Indeed, E7 has been shown to associate with several other proteins including the Rb-related protein, p107 (Davis *et al.*, 1993), cyclin A and the protein kinase $p33^{CDK2}$ (Tommasino *et al.*, 1993) and members of the AP1 family including c-Jun, JunB JunD and c-Fos (Antinore *et al.*, 1996). The significance of these interactions with E7 in relation to cellular transformation is at present unclear. The induction of chromosomal abnormalities, whether dependent on the disruption of normal pRb function or not, is another interesting feature of E7 from high-risk genital HPVs (Hashida and Yasumoto, 1991; White *et al.*, 1994).

1.5.3.2 E6

Mutation in p53, whilst commonly detected in a wide range of cancers, is rarely detected in cancers of the cervix (Fujita *et al.*, 1992; Busby-Earle *et al.*, 1994). This is attributed to the ability of a viral gene, E6, to bind p53 and effectively disrupt its function, negating the need for further inactivation by mutation. The E6 open reading frame (ORF) is present in all PVs isolated to date except the group B BPVs (BPV3, 4 and 6)(Jackson *et al.*, 1991). E6 has been shown not only to bind p53 but also to target the cell cycle regulator and tumour suppressor protein for ubiquitin-mediated degradation (Scheffner *et al.*, 1990; Lechner and Laimins, 1994). E6 proteins from high-risk HPVs have a greater affinity for p53 compared to low-risk HPVs (Lechner and Laimins, 1994); low risk HPV E6 proteins also show no clear degradative activity compared to high-risk HPV E6 proteins (Scheffner *et al.*, 1990). This variation in affinity and degradative potential of different E6 proteins for p53 may partially explain the differences in oncogenic potential seen between HPV types. Other viral proteins, including SV40 large T and adenovirus E1B, have been shown to form stable complexes with p53 yet neither protein results in the ubiquitin-linked degradation of p53; SV40 large T and adenovirus E1B proteins however increase the half-life of the p53 protein. Unlike papillomaviruses, adenovirus or SV40 have not been linked to any form of cancer in their natural hosts.

The ability of E6 to lead to the degradation of p53 appears to be a crucial extra function of this viral oncoprotein in transformation. While binding of p53 can abrogate its activities of DNA binding and transcriptional transactivation/ transrepression (Lechner *et al.*, 1992; Lechner *et al.*, 1994; Crook *et al.*, 1994),

degradation of the protein has been shown necessary to abrogate p53 induced G1 arrest (Foster *et al.*, 1994; Slebos *et al.*, 1995) and establish resistance to p53 mediated apoptosis (Haupt *et al.*, 1995). The E6 domains involved in binding p53 and targeting p53 for degradation are separate suggesting that the two functions of E6 are independent (Crook *et al.*, 1991; Thomas *et al.*, 1995; Mansur *et al.*, 1995).

The interaction of E6 with p53 is undoubtedly central to its role in transformation however other roles for E6 in transformation have been suggested. In work by Linda Scobie (unpublished results), introduction of HPV16 E6 into p53 null mouse fibroblasts was shown to confer immortality. Work by Pim and colleagues (Pim *et al.*, 1994) also demonstrated that the ability of E6 to immortalise primary mouse fibroblasts was independent of its interaction with p53. The ability of E6 to act as a repressor (Etscheid *et al.*, 1994) and/or activator of transcription (Akutsu *et al.*, 1996) has also been shown to be independent of p53. Several other proteins have been shown to interact with E6, in particular E6BP, a calcium binding protein which appears to associate only with high-risk E6 proteins (Chen *et al.*, 1995), however the significance of any of these interactions in relation to cellular transformation has not as yet been determined. An interesting observation is that while E6 is the main oncogene in EV-associated HPV8, no complex formation has been detected between the HPV8 E6 protein and cellular p53 (Steger and Pfister, 1992). This suggests that E6 proteins from separate HPV types employ different strategies of achieving cellular transformation.

The disruption of p53 activities by high-risk HPV E6 appears to be important in the early stages of tumorigenesis only (Crook and Vousden, 1996). This correlates well with the model that HPV infection contributes most significantly to the initiation of the carcinogenic process while playing a less critical role in the progression to or maintenance of the malignant phenotype. Like E7, expression of E6 has been shown to generate genomic instability, presumably through its disruption of p53 functions (White *et al.*, 1994). Perhaps the generation of genomic instability, through the deregulation of proliferation, is the single most critical contribution of papillomavirus infection to neoplastic transformation.

Other functions of E6 which have been reported and can be associated with cellular transformation include the inhibition of differentiation in human keratinocytes (Sherman and Schlegel, 1996) and the activation of telomerase (Klingelhutz *et al.*,

1996); telomerase activation is associated with cell immortalisation and can be detected in a majority of cell lines and tumours.

As mentioned above, the group B BPVs including BPV4 do not possess an E6 ORF. Despite the contributions of E6 to transformation observed for other papillomaviruses, it is evident that cellular transformation can be achieved by BPV4 without the actions of E6. It has not been determined if other viral proteins encoded by BPV4 can substitute for the absence of E6 or indeed if E6 functions are not necessary for transformation by BPV. It has been shown however that E6 provides additional functions not supplied by the other BPV4 viral oncoproteins, E7 and E8, as HPV16 E6 can confer immortality on primary bovine palate fibroblasts (PalF cells) in the presence of BPV4 E7 and *ras* with or without BPV4 E8 (Pennie *et al.*, 1993).

1.5.3.3 E5

E5 from BPV1 (DiMaio *et al.*, 1986; Schiller *et al.*, 1986; Schlegel *et al.*, 1986) and HPV16 (Bouvard *et al.*, 1994; Valle and Banks, 1995) among others have been shown to possess transforming potential, although BPV1 E5 is more strikingly transforming compared to HPV16 E5.

BPV1 E5 can activate membrane receptors such as EGF and PDGF (Martin *et al.*, 1989; Petti *et al.*, 1991); this has been attributed to the ability of BPV1 E5 to bind and stimulate phosphorylation of the PDGF (Petti DiMaio, 1992; Cohen *et al.*, 1993) and EGF receptors (Cohen *et al.*, 1993). BPV1 E5 also binds the cellular protein 16K ductin which is a constituent of gap junctions and vacuolar ATPases (Goldstein and Schlegel, 1990; Goldstein *et al.*, 1991). Complex formation between E5 and 16K of vacuolar ATPases may result in altered intercellular pH which favours prolonged ligand-receptor interaction. Thus a receptor would be actively signalling for longer which may contribute to cellular transformation. Furthermore, by interfering with 16K ductin of gap junctions, effective cell-cell communication may be reduced sufficiently for the E5 expressing cell to escape proliferative control signals provided by neighbouring cells (Campo, 1992).

The transforming functions of E5 appear to be more complex than its direct activation of particular growth factor receptors and inhibition of receptor downregulation via its interaction with the ductin component of vacuolar H^+-ATPase. Recent experiments have shown that mutations within the transmembrane region of

E5, while maintaining the ability of E5 to complex ductin, renders the protein non-transforming (Sparkowski *et al.*, 1996). In addition, BPV1 E5 retained in the endoplasmic reticulum can still induce autophosphorylation of the PDGF receptor but is no longer able to transform cells (Sparkowski *et al.*, 1995).

The HPV16 E5 gene, unlike HPV16 E6 and E7, is generally lost in advanced carcinomas often as a consequence of viral integration. Hence E5 appears not to play a role in the conversion of HPV transformed cells to a fully malignant phenotype. Nevertheless, E5 may play a significant role in the early stages of carcinogenesis perhaps principally by increasing the cell's responsiveness to particular growth factors and/or making cells less responsive to the regulatory effects of neighbouring cells through the downregulation of intercellular gap junctional communication.

1.5.3.4 BPV4 E8

BPV4 does not possess an E6 ORF. There is however a small ORF upstream of the E7 ORF designated E8. The E8 peptide is homologous to BPV1 E5 in that it is very hydrophobic with a high leucine content (Jackson *et al.*, 1991). It encodes a polypeptide 42 residues in length and is found in Golgi and endoplasmic reticulum (ER) membranes of transformed cells; occasionally E8 has been detected in the plasma membrane (Campo, 1992; Pennie *et al.*, 1993). E8 is a true early protein being expressed in the basal and suprabasal layers of a papilloma and at early stages of tumour development (Anderson *et al.*, 1997); E8 is not detected in areas of vegetative viral DNA replication and expression levels are decreased in late stage papillomas (*ibid.*). Over-expression of E8 plus activated *ras* alone is lethal to PalF cells *in vitro* (Campo, 1992) but when co-expressed with E7 or expressed within the context of the whole BPV4 genome induces anchorage-independent growth (Pennie *et al.*, 1993). E8, like BPV1 E5, can complex with 16K ductin *in vitro*. Furthermore E8 expressing cells show significant loss of gap junction intercellular communication (Faccini *et al.*, 1996). It is still unclear if E8 binds gap junctions or H^+-ATPases or both *in vivo*. The precise mechanism by which E8 contributes to cell transformation is not fully understood.

1.5.4 Transcriptional regulation of viral genes

The long control region (LCR) is the least conserved region between different papillomavirus genomes, however different LCRs share a general level of organisation in that each possess a promoter and tissue specific enhancer. Analysis of several papillomavirus LCRs has identified binding sites for numerous regulatory factors, both viral and cellular in origin, which are arranged in a complex modular structure. Cellular factors which bind and modulate papillomavirus gene expression include AP-1 (Offord *et al.*, 1990, 1993; Mack and Laimins *et al.*, 1991; Thierry *et al.*, 1992), Sp1 (Dong *et al.*, 1994; Tan *et al*, 1992, 1994), NF-1/CTF (Gloss *et al.*, 1989a, 1989b; Apt *et al.*, 1993), Oct-1 (Chong *et al.*, 1991; Sibbet *et al.*, 1995), PVF and NFA (Chong *et al.*, 1990), TEF (Ishiji *et al.*, 1992), KRF-1 (Mack and Laimins, 1991), glucocorticoid and progesterone response elements (Chan *et al.*, 1989), PEF-1 (Cuthill *et al.*, 1993; Sibbet *et al.*, 1995), PEBP2 (Jackson and Campo, 1995) and C/EBP (McCaffery and Jackson, 1994; Auborn *et al.*, 1991). Not all the cellular factors identified above bind each LCR from every papillomavirus.

In addition to the number and range of transcription factor binding sites, their arrangement is such that many of the elements are closely spaced, and in some cases they even overlap. The location of the various binding sites in relation to one another suggests that the regulation of viral transcription may involve competition and/or co-operation between the different transcription factors.

Papillomaviruses exhibit an extremely limited host cell range. One way in which gene expression can be limited to a particular cell or tissue type is by virtue of a factor or factors which are present exclusively in the target tissue, such as MyoD in striated muscle (Weintruab *et al.*, 1991). While papillomavirus gene expression is limited to epithelial cells, no such epithelial-specific factor has, as yet, been identified. The nuclear factors which have been shown to bind to the various papillomavirus LCRs, including AP-1, NF-1 and Oct-1, are generally ubiquitous proteins and thus cannot explain the tissue specific nature of papillomavirus gene expression.

Tissue specificity may be achieved by regulatory factors of partial or low cell specificity, depending on the combination of factors binding to the LCR at any one particular time. It is known that the range of genes expressed can change as a cell differentiates. The vegetative life cycle of papillomaviruses is closely coupled to the differentiation program of keratinocytes therefore the temporal expression of

particular transcription factors may be an important aspect of papillomavirus transcriptional regulation. Alternatively, the stability of a particular protein may be variable in different cell types. Indeed, AP1 proteins levels have been shown to be higher and more stable in keratinocytes compared to fibroblast cells (Offord *et al.*, 1993).

Another way in which gene transcription can be limited to a specific cell type is through the action of co-activator proteins. In a series of experiments by Ishiji *et al.* (1992), it was demonstrated that the HPV16 E6 and E7 promoter can be activated by TEF-1 only in the presence of a co-activator protein. However, the cell specificity displayed by HPV16 is more restricted than that of TEF-1 and its associated co-activator indicating that this interaction only partially contributes to the complex mechanism which regulates viral transcription.

In low grade cervical lesions, HPV DNA is generally present as episomes and a wide spectrum of viral early genes are expressed (Durst *et al.*, 1985). In high grade CIN and cervical carcinomas, and in cell lines derived from cervical cancers, the viral DNA is frequently found integrated into the host cell genome (Durst *et al.*, 1985; Schwarz *et al.*, 1985) and only the E6 and E7 ORFs are expressed at high levels (Schwarz *et al.*, 1985; Pater and Pater, 1985; Baker *et al.*, 1987). While the site of integration within the host genome is random, disruption of the HPV genome within the E1 or E2 ORF is common.

Papillomavirus LCRs contain multiple binding sites for the viral DNA-binding protein E2. The E2 ORF can encode a full length or truncated form of the protein. The full length E2 protein has been shown to possess transcriptional transactivating activity (Spalholz *et al.*, 1985; Cripe *et al.*, 1987; Lees *et al.*, 1990) whereas the truncated protein, which lacks the N-terminal transactivation domain, functions as a repressor of transcription (Cripe *et al.*, 1987; Chin *et al.*, 1988; Thierry and Yaniv, 1987; Romanczuk *et al.*, 1990; Dong *et al.*, 1994; Jackson and Campo, 1995). Integration into the cellular genome is often accompanied by the loss, disruption or inactivation of the E2 ORF (Schneider-Gadicke and Schwarz, 1986; Smotkin and Wettstein, 1986; Baker *et al.*, 1987; Cullen *et al.*, 1991). Loss of E2 repressor activity has been linked to the unregulated expression of the transforming viral early genes, E6 and E7. Therefore loss of E2 functions could represent an important event in the progression to invasive carcinoma.

1.5.5 Co-operation with Cellular Genes to Achieve Transformation

The expression of the E6 and E7 ORFs from high-risk HPV types has been shown necessary and sufficient to transform and immortalise human keratinocytes and fibroblast cells *in vitro* (Durst *et al.*, 1987b; Pirisi *et al.*, 1987; Munger *et al.*, 1989a; Kaur and McDougall, 1989). Nevertheless, such cells are not tumorigenic when transplanted into nude mice (*ibid.*). Tumorigenicity is only achieved when these cells are grown for long periods in culture (Kaur and McDougall, 1989; Hurlin *et al.*, 1991) or additional activated oncogenes, such as *ras*, are cotransfected (Durst *et al.*, 1989; DiPaolo *et al.*, 1989; Schneider *et al.*, 1991). Co-operation between virus and cellular genes in the attainment of a fully transformed phenotype is consistent with the observation that papillomavirus infection alone in insufficient to induce a malignant state (zur Hausen, 1991a) and indeed that carcinogenesis is a multistep process.

Cytogenetic analysis of HPV-associated lesions have identified a correlation between grade of lesion and the severity of genetic change. Low grade, preinvasive cervical lesions (CIN I and/or II) in general possess apparently normal, diploid genomes compared to high grade (CIN III) and cancerous lesions which more often display chromosomal anomalies including aneuploidy (Kirkland *et al.*, 1967, 1970). No one specific chromosomal abnormality or rearrangement has been consistently or exclusively identified in cervical cancers in association with HPV infection, however several chromosomes have been implicated including chromosomes 1, 3, 4, 6, 11, 13, 17, 18 and 21 (for reviews see DiPaolo *et al.*, 1993; Stanley and Sarkar, 1996). Of these, aberrations involving chromosome 1 have been reported most frequently (Atkin and Baker, 1977, 1979, 1982; Sreekantaiah *et al.*, 1988) however genetic changes in chromosome 1 are also very common in other solid epithelial tumours (Rodriguez *et al.*, 1994).

Loss of a whole chromosome or chromosome region in cancer progression generally indicates the presence of a putative tumour suppressor gene. Allelic deletions involving regions of chromosomes 3 and 11, among others, have been implicated in cervical carcinogenesis. The transfer of a single chromosome 11 into the immortal cervical cancer cell line HeLa lead to a loss of tumorigenic potential as determined in nude mouse assays (Saxon *et al.*, 1986). This result, in combination with several other studies (Koi *et al.*, 1989; Hampton *et al.*, 1994), not only support the existence of a tumour suppressor gene on the long arm of chromosome 11 but

further suggest that loss of this chromosome region is a late event in cervical cancer and is associated with the acquisition of a tumorigenic phenotype.

A deletion of 11p induced anchorage independence in embryonic human fibroblasts (Smits *et al.*, 1988). The transcriptional activity of HPV16 was subsequently shown to be greater in cells which carry a deletion of loci on chromosome 11p (Smits *et al.*, 1993) which has been associated with the loss of transcriptional repressor proteins that bind the TATAA motif in the viral promoter. At present however there is no strong evidence supporting the existence of a tumour suppressor gene on 11p.

Loss of heterozygosity in cervical carcinomas has also been detected at loci on chromosome 3 at relatively high frequency (Kohno *et al.*, 1993). Alterations of chromosome 3 including deletions and rearrangements are often found in primary cervical cancers and HPV transformed cell lines indicating that genes relevant to the development of cervical cancer are located on the short arm of chromosome 3. Loci on chromosome 3p however appear not to be specifically associated with cervical cancer development as similar deletions have been reported in other epithelial cancers (Rodriguez *et al.*, 1994).

The activation of specific oncogenes in the genesis of cervical cancer has not been extensively characterised. c-Ha-*ras* mutations have been detected in naturally occurring alimentary canal cancer in cattle which are believed to have arisen from BPV4 induced lesions (Campo and Jarrett, 1987). Mutations in c-H-*ras* have been reported in association with late stage cervical cancers (Riou *et al.*, 1985, 1988). Furthermore, in some cervical cancers carrying mutations in *ras*, high levels of c-*myc* expression were concomitantly detected (Riou *et al.*, 1985). The amplification and over-expression of c-*myc* has been associated with recurrent disease and progression to malignancy *in vivo* (Ocadiz *et al.*, 1987; Riou *et al.*, 1987). Integration of HPV near c-*myc* has also been reported in cervical cancers (Couturier *et al.*, 1991) and amplification of cellular sequences adjacent to the site of HPV integration has been observed in several cases (Lazo *et al.*, 1989; Wagatsuma *et al.*, 1990).

Amplification and overexpression of c-myc has been detected in HPV16 transformed rodent cells (Crook *et al.*, 1989) and a HPV16 immortalised human keratinocyte cell line (Crook *et al.*, 1990) *in vitro*. In certain cell lines, integration of HPV DNA has been identified at a locus close to c-*myc*; the deregulation of c-*myc* in

these cells lines may be a consequence of local HPV integration (Durst *et al.*, 1987a; Popescu *et al.*, 1987). *In vitro*, an activated *ras* gene is necessary for BPV4 to induce morphological changes and anchorage independent growth in primary foetal bovine palate fibroblast cells (PalF cells)(Jaggar *et al.*, 1990; Pennie and Campo, 1992). HPV16 was shown to co-operate with a v-*fos* gene in the transformation of primary mouse epithelial cells however these cells were dependent on glucocorticoid hormones for proliferation (Crook *et al.*, 1989).

As mentioned earlier, analysis of cervical cancer tissue, cell lines derived from cervical cancers or HPV transformed cells lines has not revealed highly consistent or characteristic genetic aberrations. This is in contrast to colorectal cancer in which a number of defined mutations in both proto-oncogenes and tumour suppressor genes have been characterised. Karyotype analysis of HPV transformed cell lines, for example, as outlined above has revealed complex and variable profiles regardless of the HPV type used (Durst *et al.*, 1987b; Smith *et al.*, 1989; Popescu *et al.*, 1990; Gilles *et al.*, 1993). This may be a reflection of the overall genetic instability generated by the expression of the viral oncoproteins E6 and E7. Papillomavirus infection is considered to be an initiation event occurring early in cervical carcinogenesis. The establishment of genetic instability as a consequence of viral infection thus generates an environment in which a range of genetic changes are more likely to occur (White *et al.*, 1994), ultimately leading to continued cellular transformation and progression to malignancy. It is clear however that an accumulation of genetic changes, which can involve a wide range of different gene combinations, is required for papillomavirus transformed cells to progress to malignancy.

1.6 Bovine Papilloma Virus type-4 (BPV4)

Animal model systems can be used to increase our understanding of similar processes occurring in human diseases, such as cervical cancer. There are many aspects of a BPV4 infection which make it an ideal animal model system for studying papillomavirus-associated carcinogenesis. BPV4 infects the mucous epithelium of the upper gastrointestinal tract in cattle and induces papillomatosis (Jarrett *et al.*, 1978a; Campo *et al.*, 1980). Like HPV16 and 18 infections in humans, BPV4 infection is associated with the development of cancer in its natural host. Also like humans, cattle

are large, long lived animals. The major advantage of the BPV4-cattle system is that, unlike humans, cattle are amenable to experimental manipulations. This provides an opportunity to identify putative cofactors which can synergise with the virus to induce cancer. Furthermore, it facilitates the efficacy of novel therapeutic and/or prophylactic vaccines to be assessed (Kinbauer *et al.*, 1996; Campo 1997).

There are however two major difference between BPV4 and the high-risk human papillomaviruses, such as HPV16 and 18; in HPV induced cervical carcinomas the viral DNA is generally retained whereas in BPV4 associated cancers the viral DNA is not detected (Campo *et al.*, 1985; Gaukroger *et al.*, 1991). Also, the E6 ORF, which is one of the two transforming genes in high-risk HPV, is not present in the BPV4 genome (Jackson *et al.*, 1991) suggesting that the mechanisms of transformation by these viruses are quite different.

Despite the differences between BPV4 and high-risk HPVs , BPV4 remains one of only a few papillomaviruses which is associated with cancer in its natural host. Indeed, the unique ability of BPV4 to induce cancer in the absence of the powerful transforming E6 gene is perhaps one of the most intriguing features of this particular papillomavirus. BPV4 alone is a poorly transforming virus and the carcinogenic potential of BPV4 is only realised in the presence of potent cofactors, such as bracken fern (Campo *et al.*, 1994) which will be discussed further in section 1.7.1.

1.6.1 Genome Structure

BPV4 is one of the smallest papillomaviruses isolated to date possessing a genome only 7265 nucleotides in length. The general structure of the BPV4 genome however is very similar to that of other papillomaviruses, as is illustrated in Figure 1.1. It comprises a series of early and late ORFs in addition to a non-coding LCR. Some of the properties of the various ORF in BPV4 have been discussed earlier in section 1.5.3. T

The BPV4 genome possess eleven separate ORFs, however two of the eleven ORFs, E3 and E5, contain no ATG and are therefore suspected of having no function. The ORFs are divided into the early and late genes, accounting for the 'E' or 'L' prefix. There are three polyadenylation sites within the BPV4 genome located at nucleotides 4004, 7155 and 7191.

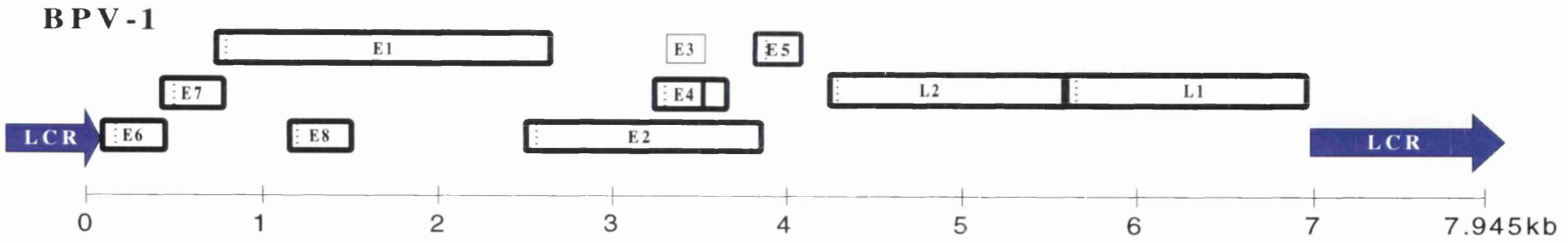
Figure 1.1

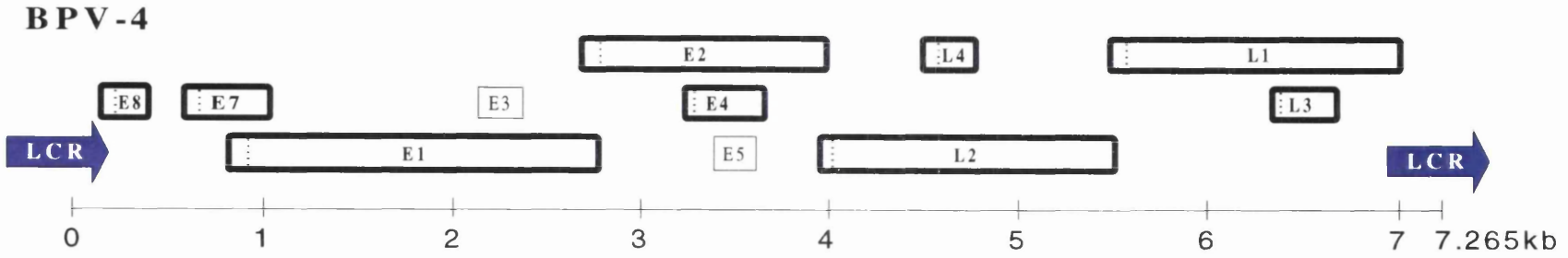
Figure 1.1 Genetic Maps of three papillomaviruses

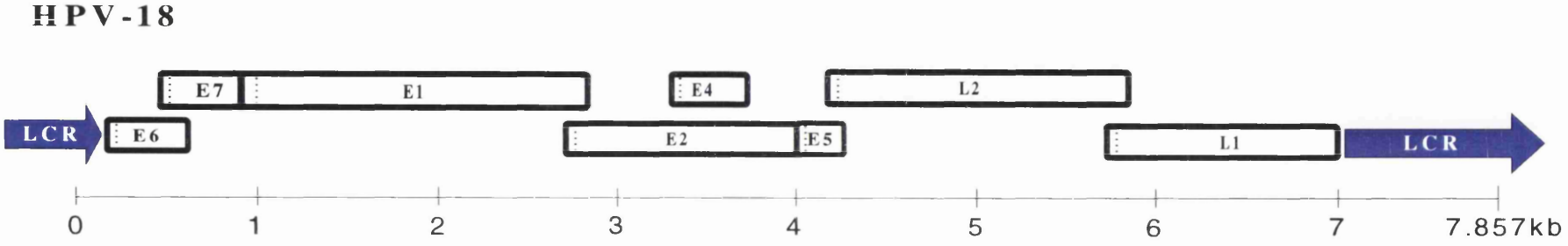
The figure compares the genetic maps of three papillomaviruses - BPV1, BPV4 and HPV18. The genomic organisation of each virus was deduced from their primary DNA sequence.

Each genome is displayed in a linear fashion. The various open reading frames (ORFs) of the early (E) and late (L) regions are indicated. The dotted vertical line in each ORF represents the first ATG codon. ORFs without an ATG codon are shown as thin-lined boxes

The LCR extends continuously from the end of the L1 ORF through to the beginning of the E6 ORF in each viral genome.


BPV-1
E1
E3
E5
E7
E4
L2
L1
LCR
E6
E8
E2
LCR
0
1
2
3
4
5
6
7
7.945kb


BPV-4
E2
L4
L1
E8
E7
E3
E4
L3
LCR
E1
E5
L2
LCR
0
1
2
3
4
5
6
7
7.265kb


HPV-18
E7
E1
E4
L2
LCR
E6
E2
E5
L1
LCR
0
1
2
3
4
5
6
7
7.857kb

Transcription of the early genes terminates at the 4004 polyadenylation (polyA) site whereas the late gene transcripts terminate at either of the two remaining polyA sites. In common with other papillomaviruses, mRNA molecules are generated by a series of complex splicing events between ORFs (Smith *et al.*, 1986; Stamps and Campo, 1988).

The E1 ORF is transcribed into a number of separate mRNAs however the function(s) of any of these transcripts has not yet been determined. The BPV4 E1 ORF shows a high degree of homology with E1 ORFs from other papillomaviruses, such as BPV1 E1, suggesting that this protein plays a role in the replication of viral DNA (Lambert, 1991).

Similar to the E1 ORF, the E4 ORF gives rise to several mRNA molecules. Two of the transcripts, 7E11 and a 1.6Kb transcript, encode E1-E4 fusion proteins, although the two proteins utilise different regions of E1. The exact function of E4 has not been elucidated however its expression is greatest in the differentiating layers of papillomas (Anderson *et al.*, 1997). This coincides with productive viral DNA replication (Campo *et al.*, 1994). A similar pattern of expression has been demonstrated for HPV1 E4 (Breitburd *et al.*, 1987) and this E1-E4 fusion protein has been shown to alter cytokeratin assembly (Doorbar *et al.*, 1990). The BPV4 7E11 transcript may fulfil a similar function and by interfering with normal epithelial differentiation may in some way contribute to BPV4 virion production.

Transcripts corresponding to the full length BPV4 E2 ORF have so far not been identified. A smaller unspliced transcript, that relates only to the 3' portion of the E2 and E4 ORFs, possibly represents an N-terminal truncated form of E2; N-terminal truncated E2 proteins have been shown to function primarily as repressors of transcription in other systems (Cripe *et al.*, 1987; Lambert *et al.*, 1987; Chin *et al.*, 1988; Thierry and Yaniv, 1987; Romanczuk *et al.*, 1990; Dong *et al.*, 1994; Jackson and Campo, 1995). The role of E2 in the autoregulation of BPV4 transcription is discussed fully in section 1.6.2.

E7 and E8 have been identified as the primary transforming genes of BPV4 (see sections 1.5.3.1 and 1.5.3.4). BPV4 E7 is homologous to other E7 proteins from high-risk HPVs, such as HPV16 E7 (Jaggar *et al.*, 1990; Jackson *et al.*, 1991). It contains two Cys-x-x-Cys zinc-binding motifs and a putative Rb-binding domain; the Rb-binding domain has not yet been shown to functionally bind pRb. Deletion of the

3' terminal third of the BPV4 E7 ORF, which includes one of the two Cys-x-x-Cys domains (Jaggar *et al.*, 1990), or mutations in the Cys-x-x-Cys or Rb-binding motif, have all been shown to abolish the protein's transforming potential *in vitro* (Jackson *et al.*, 1996). The structural and functional characteristics of BPV4 E8, as detailed in section 1.5.3.4, suggest that E8 may transform cells in a way analogous to that of BPV1 E5. The effects of E7 and E8 on the transformation of established and primary cells is outlined in sections 1.6.3 and 1.6.4.

There are four late ORFs, numbered L1 to L4. L1 and L2 are the main structural proteins of the BPV4 capsid; L1 is the major capsid protein and L2 is the minor protein. Two related transcripts, 2.8kb and 4.2kb, have been identified. The 2.8kb transcript encodes the L1 protein and the 4.2kb transcript has the capacity to encode the L1 and L2 proteins. The two remaining late ORFs, L3 and L4, each possess an ATG start codon, however their function in relation to BPV4 replication or virion assembly has not been determined.

1.6.2 Long Control Region (LCR)

DNaseI footprinting of the BPV4 LCR revealed 16 sites where nuclease digestion was prevented by the binding of nuclear factors (Jackson and Campo, 1991). Figure 1.2 is a schematic illustration of the putative nuclear factor binding sites and various control elements which have been identified within the BPV4 LCR. Among these sites are three consensus E2 binding sites - E2(1), E2(2) and E2(4). In addition to the three consensus sites, a fourth degenerative E2 site, called E2(3), which differs from the consensus E2 binding mofit at one nucleotide only, has been identified (Jackson and Campo, 1991). E2(1) has been associated with E2 mediated transactivation. E2(4) is the main binding site for E2 functioning as a repressor. This E2 site is located immediately upstream of the TATA box in the viral promoter. Binding of E2 to the E2(4) site may prevent binding of proteins involved in the initiation of transcription thus preventing or repressing viral transcription.

E2(2) is an important cis-regulatory element in the BPV4 LCR which may bind several cellular nuclear factors, including PEBP2, in addition to viral E2 (Jackson and Campo, 1995). Indeed, PEBP2 was shown to bind within the E2(2) site in EMSA (*ibid.*). In view of the PEBP2 binding motif being located within the E2(2), PEBP2 and E2 would be expected to compete for binding to the E2(2) site.

Figure 1.2

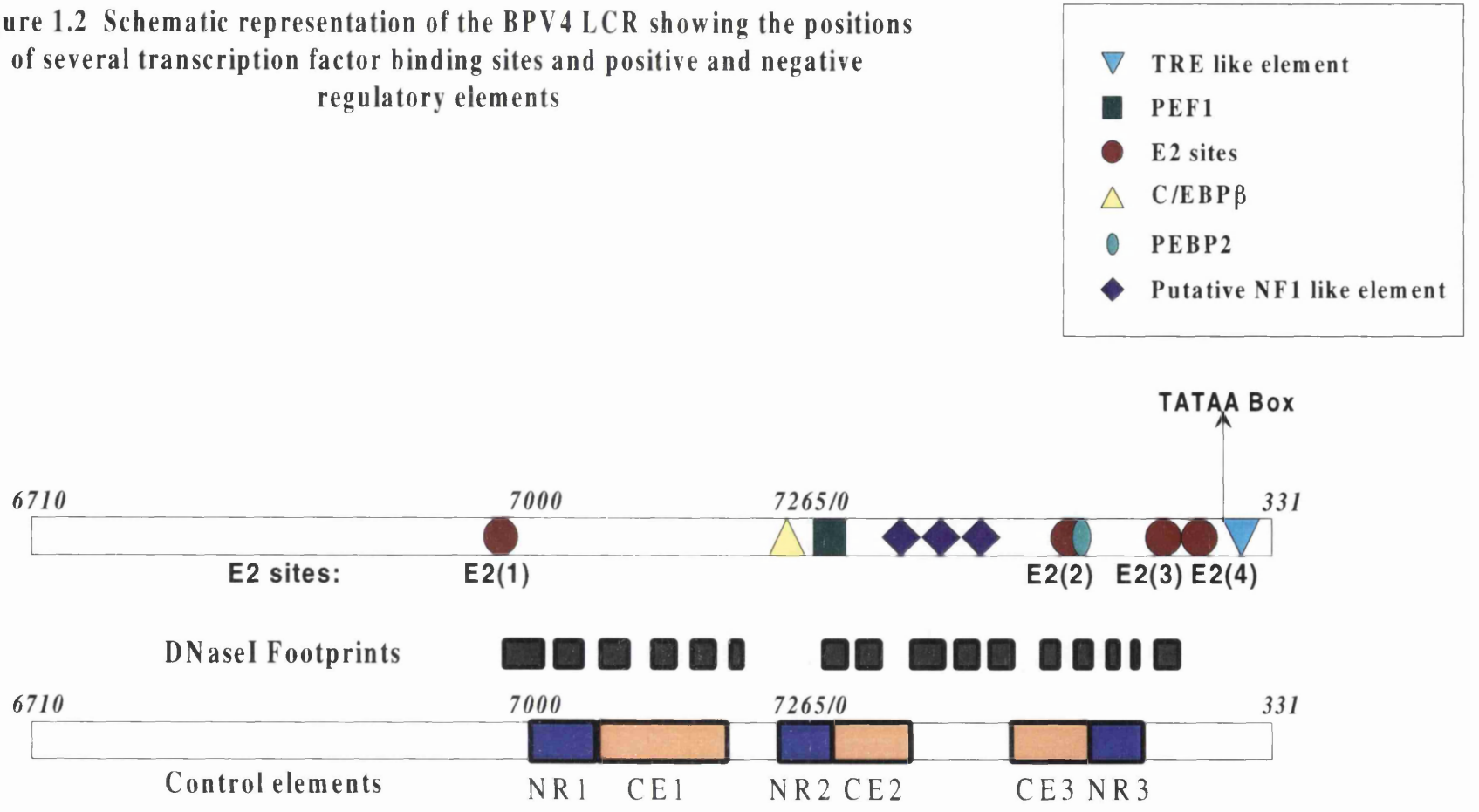
Figure 1.2 Schematic representation of the BPV4 LCR showing the positions of several transcription factor binding sites and positive and negative regulatory elements

The BPV4 LCR (nucleotides 6710-331) maps between the 3' end of the L1 ORF and the 5' end of the E8 ORF.

CE1-3 are positive transcription regulatory elements and NR1-3 are the negative elements.

The *in vitro* footprints, identifying binding sites for regulatory factors, are indicated in relation to the negative and positive *cis*-elements (for details, see Jackson and Campo, 1991).

Figure 1.2 Schematic representation of the BPV4 LCR showing the positions of several transcription factor binding sites and positive and negative regulatory elements



Other cellular factors in addition to E2 and PEBP2 are suspected of binding to this region of the LCR. Deletion or mutation of sequences at this site have resulted in a 60 to 85% drop in LCR activity (Jackson and Campo, 1991, 1995).

The four E2 binding sites within the BPV4 LCR display differential affinity for E2; E2(1) and E2(2) formed more stable complexes with oligonucleotides containing the E2 binding motif compared to E2(3) and E2(4). The arrangement of the E2 sites along the BPV4 LCR is comparable with the location of E2 sites of other mucosal papillomaviruses, particularly the positioning of the two E2 site adjacent to the TATA box (Tan *et al.*, 1992, 1994; Dong *et al.*, 1994). Depending on the cellular concentrations of the E2 protein, the activity of the LCR can be stimulated or inhibited; at low concentrations of E2, the protein occupies the E2(1) site primarily and the LCR is transactivated. As the level of E2 protein increases the other E2 sites become occupied, positive transcription factors, such as PEBP2 or the transcription initiation machinery which bind the TATA box, become displaced or are prevented from binding and the activity of the LCR is repressed (Jackson and Campo, 1995).

Another interesting observation was that when all four E2 binding sites were mutated and E2 binding within the LCR was apparently abolished, BPV4 E2 was still capable of inducing a twofold increase in LCR activity (Jackson and Campo, 1995). Such transcriptional transactivation, independent of DNA binding, has also been reported for BPV1 E2 (Haugen *et al.*, 1988) however the mechanism of how E2 achieves this is unknown.

In addition to the E2 binding sites, several E2-independent regulatory elements, both positive and negative, have been mapped along the LCR. These cis-acting elements are arranged in such a way that each positive element is paired closely with a negative element (see figure 1.2). The pairing of positive and negative elements along the LCR reflects the complex yet highly ordered and regulated nature of BPV4 transcription.

1.6.3 Work in Established Cells

BPV4 has been identified as the aetiological agent of papillomatosis in the upper alimentary canal of cattle (Jarrett *et al.*, 1978a; Campo *et al.*, 1980). BPV4 induced lesions are, in general, benign papillomas which eventually regress spontaneously (Jarrett, 1985). In bracken-grazing cattle however, the level and

persistence of BPV4 induced papillomatosis is more widespread when compared to animals which have not been exposed to bracken. Furthermore, animals infected with BPV4 and grazing on a diet which contains bracken fern are at high risk of developing squamous cell carcinomas within the areas of papillomatosis (Jarrett *et al.*, 1978b). The synergism between BPV4 and bracken in the induction of upper GI tract cancer in cattle has been reproduced experimentally (Campo *et al.*, 1994) and is discussed fully in section 1.7.1.

In an attempt to characterise the transforming properties of BPV4, established mouse fibroblast cell lines, in particular NIH3T3 cells and a sub-line of C127 cells (C127sc), were transfected with the whole BPV4 genome *in vitro*. BPV4 was demonstrated to transform both cell lines *in vitro* and several of the subclones generated were further capable of inducing tumours when implanted into nude mice (Campo and Spandidos, 1983; Smith and Campo, 1988).

Transformation of C127 cells was however dependent on a number of experimental parameters. For example, cells had to be grown either at low density or in the presence of the tumour promoting agent, TPA, for cells to be morphologically transformed by BPV4. The dependence on low cell density or TPA, which is known to disrupt intercellular communication (Murray and Fitzgerald, 1979; Yotti *et al.*, 1979), suggests transformation may be prevented by inhibitory effects of neighbouring cells (Smith and Campo, 1988). High serum levels in the culture medium also contributed to a higher focus frequency as compared to low serum levels (*ibid.*); growth factors in the serum have been postulated as allowing expression of the transformed phenotype.

Finally, the physical state of the viral DNA transfected into C127 cells significantly affected the level of transformation achieved; a circular BPV4 genome was unable to fully transform C127 cells unlike a linear or fragmented genome. This suggests that repressor functions of the BPV4 genome are lost or disrupted when the genome is linearised or fragmented. Indeed, integration of HPV DNA into the host cell genome, as observed in many cervical cancers, is often accompanied by loss of the E2 repressor protein (Cripe *et al.*, 1987; Dong *et al.*, 1994; Jackson and Campo, 1995). Alternatively, deletion of negative elements, several of which have been identified within the BPV4 LCR (Jackson and Campo, 1991; McCaffery and Jackson,

1994) may lead to enhanced transformation potential by the remaining BPV4 sequences.

A small viral fragment which contained the E7 and E8 ORFs was shown to retain the ability to transform C127 cells (Smith and Campo, 1988). This was the first indication that E7 and E8 were the main transforming proteins of BPV4. Recent work on the specific role of E8 in cellular transformation has shown that, while E8 is only weakly transforming when expressed alone in established rodent fibroblasts, cells were capable of anchorage independent growth in semi-solid medium (O'Brien, V., unpublished results). Confirmation of E7 and E8's role in cellular transformation was obtained from subsequent transfection experiments performed in primary cells (see section 1.6.4).

An important observation from these early *in vitro* experiments was that viral DNA was generally absent in fully transformed C127 cell lines. Out of 60 cell lines analysed, only nine had retained BPV4 DNA (Smith and Campo, 1988); it was apparent that BPV4 DNA was progressively lost the longer cells were cultured *in vitro*. Even in the minority of cell lines which retained BPV4, viral sequences were not expressed (Smith and Campo, unpublished data). These results reflect the mechanism of transformation by BPV4 *in vivo* where viral DNA is only rarely detected in carcinomas (Campo *et al.*, 1985). Therefore, not only is the *in vitro* transformation of established mouse fibroblasts consistent with BPV4 transformation *in vivo* (Campo *et al.*, 1985), these results indicate that BPV4 gene expression is not required for maintenance of the malignant phenotype. The absence of viral DNA from cancerous lesions has so far only been reported for BPV4.

1.6.4 Work in Primary Cells

BPV4 is a weakly transforming papillomavirus. Unlike high-risk HPVs such as HPV16 or 18, which individually and alone can immortalise primary epithelial cells (Woodworth *et al.*, 1989), BPV4 can only morphologically transform primary fibroblast in association with another activated oncogene, such as *ras* (Jaggar *et al.*, 1990). This is in keeping with transformation by BPV4 *in vivo* where cofactors, in addition to virus infection, are necessary for the generation of a malignant phenotype (Jarrett *et al.*, 1978b).

In an attempt to establish an experimental system which provides a more accurate representation of the papillomavirus-host cell interaction *in vivo*, BPV4 was transfected, *in vitro*, into primary fibroblasts cells which had been explanted from the soft palate of a bovine foetus (PalF cells) (Jaggar *et al.*, 1990). Unlike established cells which empirically possess certain characteristics of transformation, primary PalF cells are behaviourally and genetically more similar to the virus' target cells *in vivo*. Furthermore PalF cells are derived from the species and anatomical site which BPV4 infects naturally. PalF cells however are not the natural host cell of BPV4. Nevertheless, transfection experiments using PalF cells, as detailed below, have supplied very interesting and informative results about the transforming potentials of the complete BPV4 genome and individual BPV4 ORFs.

PalF cells transfected with BPV4 alone are non-transformed (Jaggar *et al.*, 1990). When PalF cells are transfected with BPV4 in the presence of an activated *ras* gene, the cells are morphologically transformed, have an extended life span and can grow in methocel, independent of anchorage (Pennie *et al.*, 1993). These cells however are not immortal nor are they tumorigenic when transferred into nude mice (Jaggar *et al.*, 1990; Pennie *et al.*, 1993). Various subgenomic fragments of BPV4 have been transfected into PalF cells in an attempt to assign individual transforming characteristics to particular viral ORFs. Table 1.2 summarises the results of a series experiments in which combinations of BPV4 subgenomic fragments where transfected into PalF cells. Several characteristics of transformation were assessed. These included, morphological transformation, anchorage independent growth, immortality and tumorigenicity in nude mice. To reiterate, all the BPV4-induced transformation results discussed here were dependent on the cotransfection of an activated *ras* gene.

Transfection of PalF cells with the E7 ORF resulted in morphological transformation (Pennie *et al.*, 1993). Introduction of a subgenomic fragment containing E7 and E8 produced cells which were morphologically transformed and capable of anchorage independent growth (*ibid.*). When the E8 ORF and *ras* were transfected into PalF cells in the absence of any other BPV4 sequences, it was found to be lethal to recipient cells. The mechanism of how or why E8 expression is lethal to PalF cells is not fully understood, however work by V. O'Brien in our laboratory is currently addressing these questions.

Table 1.2 Summary of transforming potential of BPV genes in PalF cells either alone or in combination with the HPV16 E6 gene or quercetin

Transfected DNA (all included *ras*)	Morphological Transformation	Anchorage Independence	Immortality	Tumorigenic in nude mice
BPV4 (whole genome)	+	+	-	-
E7	+	-	-	-
E7 + E8	+	+	-	-
E7 + E8 + HPV16 E6	+	+	+	-
BPV4 + Quercetin	+	+	+	+
E7 + Quercetin	+	+	+	+*
E7 + E8 + Quercetin	+	-	N/D	-

+ : Phenotype detected
- : Phenotype not detected
+* : Only seen when quercetin was administered soon after transfection
N/D : Not determined

Stable PalF transfectants containing BPV4 E7, E8 and HPV16 E6 (plus *ras*) have been generated by H. Ashrafi. These cells were examined for their ability to communicate via gap junctions. Results showed that PalF cells expressing E8 showed a loss of gap junction intercellular communication (GJIC) whereas the same cells without E8 did not (Faccini *et al.*, 1996). Similar effects on gap junction communication have been observed with HPV16 E5 (Oelze *et al.*, 1995). BPV1 E5 shows a high degree of homology with BPV4 E8 (see section 1.5.3.3) and it has been suggested that these two proteins interfere with GJIC through an interaction with a component of gap junctions, namely ductin (Faccini *et al.*, 1996).

BPV4 does not possess an E6 ORF (Jackson *et al.*, 1991). This gene is crucial to the transforming ability of other papillomaviruses, such as HPV16 and 18, as discussed earlier in section 1.5.3.2. It is currently unknown if other BPV4 encoded proteins can substitute for the actions of E6 or if the mechanism of BPV4 transformation is independent of E6-related functions. Transformation experiments have demonstrated however that introduction of an exogenous E6 can provide additional characteristics of transformation which could not be achieved by BPV4

alone (see Table 1.2). When HPV16 E6 was cotransfected with BPV4 E7 (and *ras*) into PalF cells in the presence or absence of E8, colonies formed were immortal (Pennie *et al.*, 1993). The same cells could only grow in methocel, a measure of anchorage independent growth, when E8 was present. Hence E6 possess transforming activity, particularly the ability to induce cellular immortality, which has not been achieved by any of the BPV4 encoded proteins.

The effect of E6 on cellular p53 (see section 1.5.3.2) has been proposed as a very significant interaction in terms of cellular transformation. Nevertheless, experiments by Linda Scobie (Scobie *et al.*, submitted) have demonstrated that E6 can transform cells independent of its interaction with p53. A mouse fibroblast cells line which contains no endogenous p53 gene (p53-null mouse fibroblasts) was transfected with the entire BPV4 genome in the presence or absence of an exogenous HPV16 E6. The results obtained demonstrated that an immortal phenotype was only achieved when the HPV16 E6 ORF was present.

An important observation in all these experiments has been that BPV4 transformed PalF cells, even in the presence of HPV16 E6, are not tumorigenic in nude mouse assays (Pennie *et al.*, 1993). This indicates that other cofactors are necessary for BPV4 transfected PalF cells to attain a fully transformed phenotype. The dependence on additional cofactors to achieve a malignant state is supported by BPV4 transformation *in vivo* (Jarrett *et al.*, 1978b; Campo *et al.*, 1994) as well as other papillomavirus-associated cancers (Jackson *et al.*, 1993).

1.7 Cofactors in Papillomavirus-Associated Cancer

CRPV is unique among papillomavirus in its ability to induce malignant tumours in domestic and cottontail rabbits in the absence of any obvious cofactors (Rous and Beard, 1935; Syverton *et al.*, 1950a, 1950b; Syverton, 1952). Nevertheless, the time taken for a benign papilloma to progress to a malignant tumour was dramatically reduced when the skin of rabbits was treated with a carcinogen, such as tar (Rous and Kidd, 1936; Kidd and Rous, 1937). When the rabbits' skin was exposed to the carcinogen in the absence of virus, no malignant tumours developed (Rous and Beard, 1935). These results indicate that the carcinogen alone was insufficient to cause cancer however, in the presence of virus, the two could co-operate in the rapid induction of malignancy.

Cigarette smoking and tobacco have been implicated as risk factors in a large number of cancers (Shopland *et al.*, 1991). Several epidemiological studies have likewise identified cigarette smoking as a risk factor in developing oral (Spitz *et al.*, 1992) and cervical cancer (Trevathan *et al.*, 1983; Reeves *et al.*, 1987; Cuzick *et al.*, 1990; Basu *et al.*, 1991; Darling *et al.*, 1992). More specifically, the cervical mucous from smokers was shown to be mutagenic in the *S. typhimurium* microsomal test (Holly *et al.*, 1986), low grade cervical lesions were found to be larger in women who smoke compared to non-smokers (Szarewski *et al.*, 1996), and cigarette smoke condensates were shown to transform HPV18-immortalised exocervical cells to tumorigenicity (Nakao *et al.*, 1996). Furthermore, the level of nicotine in cervical flushes was shown to be proportional with smoking frequency (Schiffman *et al.*, 1987), and a larger number of DNA adducts were detected in cervical scrapes from smokers (Phillips *et al.*, 1990; Phillips and Nishe, 1993).

In addition to the toxic by-products of cigarettes and tobacco, smoking has also been shown to compromise the immune system (reviewed by Holt, 1987). A reduced immune response has been implicated in a number of HPV-associated cancers (see below).

Radiotherapy has been associated with the progression of laryngeal papillomas to carcinoma (Lindeberg *et al.*, 1986; Lindeberg and Elbrond, 1991). Radiation exposure, in the form of UV light, is thought to contribute to the carcinogenic progression of HPV-induced skin lesions in patients suffering from EV (see section 1.5.2.2) (Ifner *et al.*, 1990). Radiation, apart from being capable of inducing DNA damage, has also been shown to be immunosuppressive (Boyle *et al.*, 1984).

EV individuals are reported as having genetically impaired cell-mediated immunity (Jablonska and Orth, 1985). When the immune system is compromised, as in the case of EV, in allograft transplant patients (Trenfield *et al.*, 1993; Petry *et al.*, 1994), or in individuals infected with HIV (Boccalon *et al.*, 1996), an increased incidence of warts and skin cancer is often observed (Benton and Arends, 1996). So far HIV infection has not been shown to significantly increase the risk of developing cervical cancer however it is proposed as a cofactor in other HPV-linked cutaneous or mucocutaneous cancers (Valle, 1987). The immune system, therefore, appears to be an important regulator, restricting the progression of benign HPV-initiated lesions to a cancerous state.

Secondary viral infections apart from HIV, including Herpes simplex virus type 2 (HSV2) (Merino, 1991; Yamakawa *et al.*, 1994), cytomegalovirus (CMV) (Shen *et al.*, 1993), and human herpes virus (HHV) (Yamakawa *et al.*, 1994), have all been suggested as cofactors in anogenital carcinogenesis. Bacterial and parasitic infections, including *Mycoplasma hominis* infection (Guijon *et al.*, 1992) and schistosomiasis (Feldmeier *et al.*, 1996) respectively, have also been suggested as possible cofactors.

The LCR of HPV16, the most abundant papillomavirus found in cervical cancers, has demonstrated the presence of a progesterone and glucocorticoid response element (Chan *et al.*, 1989; *Chong et al.*, 1990; Mittal *et al.*, 1993). Changes in hormones levels during pregnancy or early adolescence (Shen *et al.*, 1993), as a result of altered metabolism (Auborn *et al.*, 1991), or as a consequence of taking an oral contraceptive (Beral *et al.*, 1988; Hildesheim *et al.*, 1990), may each contribute to changes in viral gene expression and promote, even in part, neoplastic transformation.

In vitro, several studies have shown a co-operation between papillomaviruses and the classical tumour promoting agent, TPA (Cuzin *et al.*, 1985; Tsang and Stich, 1988; Gaukroger *et al.*, 1993). Apart from the ability of TPA to increase the expression of cellular oncogenes (Dotta *et al.*, 1985) and papillomavirus genes (Amtmann and Sauer, 1982; Smith *et al.*, 1987; Gius and Laimins, 1989), it has also been shown to stimulate viral replication *in vitro* (Amtmann and Sauer, 1982; Smith *et al.*, 1987; Smith and Campo, 1988) and *in vivo* (Amtmann *et al.*, 1984; Gaukroger *et al.*, 1993). An increase in viral gene expression/replication has been linked to more frequent and efficient *in vitro* transformation (Smith and Campo, 1988).

BPV4 can co-operate with a tumour promoter (TPA) or initiator (DMBA) to induce carcinomas arising from BPV4-infected bovine palatine tissue when implanted into the renal capsule of nude mice in the presence of slow releasing pellets of TPA and/or DMBA (Gaukroger *et al.*, 1993). The ability of BPV4 to co-operate with both chemicals in neoplastic transformation suggests that this virus can achieve transformation by interacting with a range of activated cellular genes.

A strong association between naturally occurring cancers in cattle and their consumption of bracken fern has been identified in animals in the West Highlands of Scotland. The effects of bracken consumption on cattle with concomitant papillomavirus infections are discussed in more detail below.

1.7.1 Bracken Fern as a Cofactor in Cattle Cancer

Bracken fern, whether fresh, dried or cooked, as well as bracken fern extracts have been shown to be both toxic and carcinogenic in a range of domestic and experimental animals (Rosenberger *et al.*, 1960; Pamukcu, 1963, 1969; Evans and Widdop, 1966; Hirono *et al.*, 1970; Evans, 1984; Santos *et al.*, 1986, 1990; Santos, 1987; Campo *et al.*, 1992, 1994). It has been observed that cattle often suffer from chronic enzootic haematuria and bladder tumours when bracken is a constituent of their diet (Pamukcu *et al.*, 1967, 1976). When cattle were fed bracken fern experimentally, they were seen to develop haematuria and bladder tumours which were histopathologically identical to naturally occurring bladder tumours (Price and Pamukcu, 1968; Pamukcu *et al.*, 1976; Campo *et al.* 1992). Bracken fern, which is widespread in particular areas of the world, is known to contain mutagens, carcinogens (Evan, I. A. *et al.*, 1982) and immunosuppressants (Evans, W. C. *et al.*, 1982). Apart from the toxic nature of bracken fern, infection with a papillomavirus has also been implicated in the development of urinary bladder tumours in cattle.

A series of experiments by Olson and co-workers showed that extracts from bovine cutaneous warts, when injected into the bladder of cows, induced malignant tumours (Olson *et al.*, 1959); alternatively, suspensions of naturally occurring, bracken-associated bovine bladder tumours gave rise to skin and vaginal warts, as well as bladder polyps and fibromas in test calves (Olson *et al.*, 1965). These early experiments were a strong indication that a transmissible agent was involved in the production of naturally occurring bladder tumours in cattle.

Subsequently, experiments by Campo and colleagues have shown a synergism between bovine papillomavirus type 2 (BPV2) and bracken (Campo and Jarrett, 1986; Campo *et al.*, 1992). Results from a number of long term experiments showed that cattle inoculated on the skin with BPV2 and fed on a diet of hay developed cutaneous warts at the site of injection. Likewise, animals inoculated with BPV2 and fed on bracken developed warts at the site of injection as expected. All animals which were fed bracken became severely immunosuppressed and bladder cancers frequently developed. These bladder tumours were histopathologically indistinguishable from bladder tumours which arise spontaneously. BPV2 DNA was detected in a large proportion of these cancers independent of whether the animal had been injected with

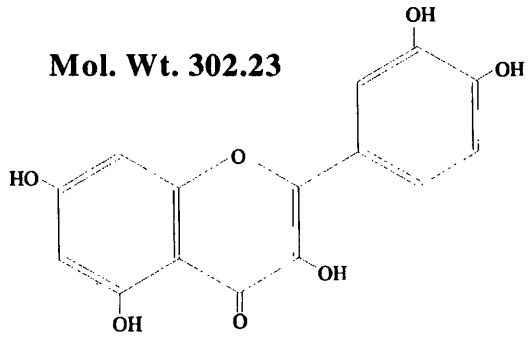
BPV2 or not. This observation suggests that chemicals in the bracken, most likely immunosuppressants such as sesquiterpene pterosins and pterosides (Evans, W. C. *et al.*, 1982), were responsible for the activation of a latent viral infection and together they contributed to bladder cancer in these animals. Immunosuppression has been identified as a cofactor in several other papillomavirus-associated cancers (sections 1.5.2.1, 1.5.2.2 and 1.7)

Synergism between a second bovine papillomavirus, BPV4, and bracken has also been established. As mentioned earlier, BPV4 infects and induces papillomatosis of the upper gastrointestinal (GI) tract in cattle (Campo *et al.*, 1980). These lesions have been shown to progress to squamous cell carcinomas at high frequency in animal grazing on bracken fern (Jarrett *et al.*, 1978b; Campo *et al.*, 1994). In the absence of bracken, BPV4 gave rise to benign papillomas only at the site of injection. As mentioned above, animals fed on bracken fern become chronically immunosuppressed however immunosuppression alone, induced either by bracken or azothioprene, was insufficient to induce cancer of the upper gastrointestinal tract (Campo *et al*, 1992, 1994). Only animals inoculated with BPV4 and fed bracken developed cancer of the upper GI tract and lower bowel (Campo *et al.*, 1994). These results confirm that BPV4 and bracken fern can co-operate in the induction of cancer in this natural system. Furthermore, immunosuppression while enhancing the level of papillomatosis, is not sufficient to synergise with BPV4 to produce cancer and other components of the bracken fern must therefore play a role in this carcinogenic process.

1.7.2 Quercetin

In an attempt to identify individual compounds of bracken fern which have the potential to synergise with BPV4, one of the more potent and extensively studied mutagens present in bracken fern, quercetin, was selected and used in a series of *in vitro* experiments.

Figure 1.3 The Chemical Structure of Quercetin



Quercetin (3,3',4',5,7- pentahydroxyflavone) (see Figure 1.3) is a ubiquitous, naturally occurring bioflavonoid which is found in a wide range of edible plant products including fruits, vegetables, wine and tea (Herrmann, 1976). It has been shown to exhibit a variety of biological effects some of which are beneficial including the inhibition of tumour induction by chemical carcinogens (Kato *et al.*, 1983), antiproliferative action on several tumour cells lines (Avila *et al.*, 1994 and references therein) and free-radical scavenging antioxidant properties (Bors *et al.*, 1987; Negre-Salvayre *et al.*, 1992). However, many other reports identify other, less salutary effects of quercetin. These include quercetin's ability to form adducts with DNA (Rahman *et al.*, 1990), to induce mutations in both prokaryotic and eukaryotic cells (Bjieldanes and Chang, 1977; Amacher *et al.*, 1979; Maruta *et al.*, 1979; Nakayasu *et al.*, 1986; Ishikawa *et al.*, 1987), to cause clastogenic damage (Ishidate *et al.*, 1988) and to initiate mammalian cells in a two-stage transformation assay *in vitro* with TPA as a promoter (Sakai *et al.*, 1990). In addition to its genotoxic effects, quercetin has been shown to inhibit and activate a range of enzyme activities (Avila *et al.*, 1994 and ref. therein; Elliott *et al.*, 1992) and to induce protein fragmentation (Ahmed *et al.*, 1994).

Synergism between BPV4 and quercetin has been demonstrated in a series of *in vitro* experiments (Pennie and Campo, 1992). As discussed in section 1.6.4, primary bovine palate fibroblasts (PalF cells) when transfected with BPV4 alone exhibit no morphological change. BPV4 can achieve morphological transformation and anchorage independent growth of PalF cells only when cotransfected with an

additional oncogene, such as an activated *ras* gene (Jaggar *et al.*, 1990). These partially transformed cells however are not immortal nor are they tumorigenic as determined in a nude mouse assay. *ras* alone, like BPV4 alone, is not sufficient to transform PalF cells.

The oncogenes of BPV4 have been identified as E7 and E8 (see sections 1.6.3 and 1.6.4). PalF cells transfected with E7 and *ras* are morphologically transformed but remain anchorage dependent, are not immortal and are non-tumorigenic in nude mice (Pennie *et al.*, 1992). Transfection of E7 + E8 + *ras* into PalF cells leads to morphologically transformation and anchorage independent growth, however these same cells do not achieve immortality or tumorigenicity (*ibid.*). Hence, it is now accepted that E7 can morphologically transform PalF cells while E8 can confer anchorage independence. Immortality of PalF cells is only observed when an exogenous E6 gene from HPV16 is transfected into PalF cells in combination with the whole BPV4 genome (or E7 + E8) plus *ras*. Nevertheless, when PalF cells are transfected with E7 and *ras* only and then treated with a single dose of 20μM quercetin, they become fully transformed attaining not only anchorage independence and immortality but also tumorigenicity in nude mice assays (Cairney and Campo, 1995). Therefore quercetin can confer anchorage independent growth in the absence of E8 and immortality in the absence of an exogenous E6. These observation highlight that, not only can quercetin substitute for the activities of several viral oncoproteins, it can provide additional functions in transformation. Synergism between virus and quercetin is emphasised in this transformation system as the extent of transformation observed cannot singly be achieved by either the virus or chemical treatment alone (Pennie and Campo, 1992).

In another set of experiments, the timing of exposure to quercetin was also shown to be important with regard to its ability to synergise with BPV4. Quercetin was demonstrated to synergise with BPV4 (whole genome or various subgenomic fragments) when cells were exposed to 20μM quercetin shortly before or soon after transfection with BPV4 (Cairney and Campo, 1995). The longer the time interval between exposure to quercetin and transfection, or vice versa, the less evident was the co-operation between chemical and virus in PalF cells. This temporal synergism would suggest that, at this concentration, quercetin was not inducing any permanent genetic change within the PalF cells. Furthermore, the results indicate that the effect

of quercetin on the PalF cells was transient supporting a role for quercetin inducing epigenetic change rather than genetic change.

Another observation from these experiments was that expression of E8 and exposure to quercetin were apparently antagonistic (Cairney and Campo, 1995). When PalF cells were transfected with E7/E8 plus *ras* and exposed to quercetin shortly before or after transfection, cells were not immortal, colonies grew poorly or not at all in an anchorage independent environment and cells were not tumorigenic in nude mice. Antagonism between E8 and quercetin was not evident however when E8 was expressed in the context of the full BPV4 genome. This would suggest that expression of E8 is more tightly regulated in the context of the whole BPV4 genome and that E8 expression and exposure to quercetin are antagonistic only, perhaps, when E8 is aberrantly expressed.

While quercetin has been shown to substitute for the ability of E8 to induce anchorage independent growth (Pennie and Campo, 1992), treatment of cells with quercetin had no apparent effect on gap junction intercellular communication (Margaret Cairney, unpublished data). The down regulation of GJIC has been ascribed to the ability of E8 to physically interact with the 16k ductin component of gap junctions (Faccini *et al.*, 1996), an effect which is not achieved by exposure of cells to the chemical quercetin.

These results suggest that quercetin is one component of bracken fern which has the potential to co-operate with BPV4 *in vivo* to achieve neoplastic transformation. Nevertheless, quercetin and BPV4 alone are insufficient to induce cancer in cattle *in vivo* (Campo *et al.*, 1992). When quercetin was administered to animals infected with BPV4, the papillomas remained benign. This indicates that other constituents of bracken fern are additionally required for the initiation of cancer *in vivo*. Immunosuppresion has been identified as a cofactor in other papillomavirus-associated cancers. Therefore, it may be hypothesised that immunosuppressants in bracken fern, in combination with quercetin, may be sufficient to induce cancer *in vivo*. Alternatively, because bracken fern is a rich source of toxic chemicals, it may be that a cocktail of several bracken chemicals co-operate to synergise with BPV4 to induce cancer in cattle.

Chapter 2

Materials and Methods

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James Burrough (FAD) Ltd
70 Eastways Industrial Park
Witham
Essex
CM8 3YE
UK

Johnson and Johnson Medical Limited
Coronation Road
Ascot
Berks.
UK

Labsystems (UK) Ltd
Unit 5
The Ringway Centre
Edison Road
Basingstoke
Hampshire
RG21 27H
UK

National Diagnostics
Unit 3
Chamberlain Road
Aylesbury
Buckinghamshire
HP19 3DY
UK

Northumbria Biologicals Ltd.(NBL) Gene Sciences
South Nelson Industrial Estate
Cramlington
Northumberland
NE23 9BL
UK

Nunc A/S
PO Box 280
Kampstrup
DK 4000
Roskilde
Denmark

Perkin Elmer Corporation
Roche Molecular Systems, Inc.
Branchburg
New Jersey
U.S.A

Pharmacia Biotech Ltd
23 Grosvenor Road
St Albans
Hertfordshire
AL1 3AW
UK

Premier Beverages
Knighton
Adbaston
Stafford
ST20 0QJ
UK

Promega Ltd
Delta House
Enterprise Road
Chilworth Research Centre
Southhampton
SO16 7NS
UK

Qiagen Ltd
Unit 1
Tillingbourne Court
Dorking Business Park
Station Road
Dorking
Surrey
RH4 1HJ
UK

Santa Cruz Biotechnology, Inc
2161 Delaware Avenuw
Santa Cruz
California 95060
USA

Sartorius AG
37070 Goettingen
Germany

Severn Biotech Ltd
Unit 2
Park Lane
Kidderminster
Worchester
DY11 6TJ
UK

Sigma Chemical Co Ltd
Fancy Road
Poole
Dorset
BH12 4XA
UK

Technical Photo Systems
55 Napier Road
Ward Park North
Cumbernauld
G68 0EF
Scotland

Unipath
Wade Road
Basingstoke
Hants RG24 0PN
UK

Wallac Oy
PO Box 10
SF-20101
Turku
Finland

Whatman International Ltd
Whatman Labsales
St Leonards Road
20/20 Maidstone
Kent
ME16 0LS
UK

Worthington Biochemical Company
Halls Mill Road
Freehold
New Jersey
07728
USA

2.2 MATERIALS

2.2.1 Antibodies

Sigma Immuno Chemical Co.
Anti-Mouse IgG (whole molecule) alkaline phosphatase conjugate (raised in Goat)

Amersham International plc.
Anti-Mouse IgG horseradish peroxidase linked whole antibody (raised in Sheep)

Affiniti Research Products Limited
Anti-Phosphotyrosine (PY20) IgG2b Monoclonal antibody (Mouse Host)

Santa Cruz Biotechnology, Inc
Anti-cFos (K-25)-G rabbit polyclonal IgG antibody

Anti-cJun (730-5), a polyclonal antibody raised in rabbit, and rabbit pre-immune serum (943-0), were both a kind gift from Dr. Elizabeth Black, Beatson Institute for Cancer Research, Glasgow.

Anti-JunB (725/5) and Anti-JunD (783/2) antibodies were a kind gift from Dr. David Gillespie, Beatson Institute for Cancer Research, Glasgow. The JunB and JunD antibodies were raised in rabbit against bacterially expressed fusion proteins. The cDNA clones used to construct the fusion proteins were of mouse origin (Kovary and Bravo, 1991).

2.2.2 Bacteriology

Beatson Institute Central Services
L-Broth

Becton Dickinson Labware
Falcon 1059 polypropylene tubes

Beta Laboratories
Yeast-extract

Bibby Sterilin Ltd
90mm bacteriological petri dishes

Difco Laboratories
Bacto-Agar
Bactotryptone

Fisons Scientific Equipment
Glycerol

Gibco BRL Europe Life Technologies Ltd
E.Coli DH5α competent cells

Nunc
Sterile Disposable Inoculating Loops

Sigma Chemical Co. Ltd
Ampicillin
Lysozyme

2.2.3 Chemicals and Reagents

Chemicals and reagents used were of analytical grade (AnalaR) when possible.

Amersham International plc
ECL Western detection agent

BDH Analar Laboratory Supplies
Ammonium persulfate (APS)
D-glucose
Ethyl Acetate
Napthalene black
Repelcote silicone treatment

Boehringer Mannheim Ltd
Caesium chloride
RNase A

Fisons Scientific Equipment
Acetic acid
Betaplate Scint
Butan-2-ol
Chloroform
di-Potassium hydrogen orthophosphate (anhydrous)
Diaminothanetetra-acetic acid (EDTA) disodium salt
DMSO (dimethyl sulfoxide)
Hydrochloric acid
Magnesium chloride
Magnesium sulphate
Methanol
Potassium chloride
Potassium dihydrogen orthophosphate
Propan-2-ol
Sodium acetate
Sodium carbonate
Sodium chloride
Sodium dodecyl sulphate (SDS)

Gibco BRL Europe Life Technologies Ltd
Agarose (Ultrapure electrophoresis grade)
Tris base

James Burrough Ltd
Ethanol

National Diagnostics
Ecoscint A (Biodegradable Scintillation Solution)

Pharmacia Biotech Ltd
poly(dI-dC)

Severn Biotech Ltd
30% (w/v) acrylamide:0.8% (w/v) bis-acrylamide
40% (w/v) acrylamide:2.1% (w/v) bis-acrylamide

Sigma Chemical Co. Ltd
β-mercaptoethanol
Acetly Coenzyme A
Aprotinin
Benzamidine
Bicinchoninic Acid solution
Bovine Serum Albumin
Bromophenol Blue
Coomassie Brilliant Blue R
Copper(II) sulphate (pentahydrate 4% (w/v) solution)
Dithiothreitol (DTT)
Ethidium Bromide
Ficoll (type 4000
HEPES
Leupeptin
Nonidet P-40 (NP40)
ONPG
Phenol:Chloroform:Isoamyl Alcohol (25:24:1 (v/v))
PMSF
Ponceau S solution
Salmon testes DNA (sodium salt)
TEMED (N,N,N',N'-tetramethylethylenediamine)
Tween-20 (Polyoxyethylene sorbitan nonolaurate)

Unipath
PBS tablets

2.2.4 Enzymes and Kits

Boehringer Mannheim Ltd
Random primed DNA labelling kit

Gibco BRL Europe Life Technologies Ltd
All restriction enzymes and stock reaction buffer solutions used were obtained from Gibco BRL unless other otherwise stated.

NBL Gene Sciences
Alkaline phosphatase
T4 DNA ligase

Perkin Elmer Corporation
GeneAmp PCR Core Reagents

Qiagen Ltd
QIAprep Spin plasmid miniprep kit
QIAquick gel extraction kit

2.2.5 General Laboratory Supplies and Miscellaneous

Aldrich Chemical Company, Inc
Pre-coated TLC sheets (Silca gel on polyester without fluorescent indicator)

Alpha Laboratories Ltd
Microcentrifuge tubes
Pastettes

Cadisch and Sons
70μm filter nylon gauze

Canberra Packard Ltd
Superpolyethylene scintillation vials

Cruachem Ltd
Cruachem oligonucleotide purification (COP) cartridges

Du Pont (UK) Ltd
Polyallomer ultracentrifuge tubes

Gelman Sciences Ltd
Sterile 0.2μm Acrodisc filter units

Premier Beverages
Marvel (Dried Skimmed Milk)

Sartorius AG
Collodion Bags

Wallac Oy
Printed filtermat A (glass fibre)
Sample bag (for filtermat A)

Whatman International Ltd
Whatman 3MM filter paper
Whatman 1 filter paper

2.2.6 Mammalian Cells

PalF cells are primary fibroblasts explanted from bovine foetal palate.

PalK cells are primary keratinocytes explanted from bovine foetal palate.

Swiss 3T3 feeders (ATCC CCL92), originally established in 1962 by G. Todaro and H. Green (Todaro and Green, 1963), are an immortalised mouse fibroblast cell line which were a kind gift from Roselyn McCaffery, Beatson Institute for Cancer Research, Glasgow.

2.2.7 Membranes, Radiochemicals and X-ray Film

Amersham International plc
[methyl-^{3}H]Thymidine
D-threo[dichloroacetyl-1-^{14}C]Chloramphenicol
Hybond-C extra
Redivue [α^{32}P]dTTP

Decon Laboratories Limited
Decon 75

Eastman Kodak Company
X-OMAT AR X-ray film
X-OMAT S X-ray film

Technical Photo Systems
Fuji RX medical X-ray film

2.2.8 Molecular Weight Markers

Amersham International plc
Rainbow^{TM} coloured protein molecular weight markers (14,300 - 200,000 Da)

Gibco BRL Europe Life Technologies Ltd
φX174 RF DNA/ *Hae* III fragments
1Kb DNA ladder
λ DNA/*Hind* III fragments

2.2.9 Plasmids Vectors

pOLuc is a promoter assay vector containing the firefly luciferase coding sequence cloned into a modified pGEM3 vector (Brasier *et al*, 1989). This plasmid was a gift from Dr. Maria Jackson (Beatson Institute, Glasgow).

pLCRLuc contains nucleotides 6710-331 of the BPV-4 genome cloned into the *BamH* I site of the vector pOLuc (Jackson and Campo, 1995). This vector was a gift from Dr Maria Jackson (Beatson Institute, Glasgow).

psLCRLuc contains nucleotides 6710-310 of the BPV-4 genome, cut from p41X-PIN using *BamH* I, and cloned into the *BamH* 1 site of the vector pOLuc.

p41X is an enhancer test plasmid which contains the herpes simplex virus thymidine kinase (TK) promoter upstream of the coding sequence for the bacterial CAT protein. This vector, which was originally made by Jas Lang, was a gift from Dr Maria Jackson (Beatson Institute, Glasgow).

p41X-PINT contains nucleotides 6710-331 of the BPV-4 genome cloned into the *Xho*I site of p41X upstream of the TK promoter and CAT coding sequence. This vector was a gift from Dr Maria Jackson (Beatson Institute, Glasgow).

p41X-PIN contains nucleotides 6710-310 of the BPV-4 genome cloned into the *Xho*I site of p41X (Jackson and Campo, 1991). This vector was a gift from Dr Maria Jackson (Beatson Institute, Glasgow).

pGL3 is a luciferase reporter vector obtained commercially from Promega.

pGL3-PINT contains nucleotides 6710-331 of the BPV-4 genome cloned into the *Bgl* II site of pGL3. This plasmid was a gift from Dr. Iain Morgan.

pGL3-PIN contains nucleotides 6710-310 of the BPV-4 genome cloned into the *Bgl* II site of pGL3. This plasmid was a gift from Dr. Iain Morgan.

pGL3-Δ57.1 contains nucleotides 7058-310 of the BPV-4 genome cloned into the *Bgl* II site of pGL3. This plasmid was a gift from Dr. Iain Morgan.

pGL3-Δ10 contains nucleotides 7131-310 of the BPV-4 genome cloned into the *Bgl* II site of pGL3. This plasmid was a gift from Dr. Iain Morgan.

pGL3-Δ36 contains nucleotides 7212-310 of the BPV-4 genome cloned into the *Bgl* II site of pGL3. This plasmid was a gift from Dr. Iain Morgan.

pGL3-Δ170 contains nucleotides 44-310 of the BPV-4 genome cloned into the *Bgl* II site of pGL3. This plasmid was a gift from Dr. Iain Morgan.

pGL3-Δ151 contains nucleotides 149-310 of the BPV-4 genome cloned into the *Bgl* II site of pGL3. This plasmid was a gift from Dr. Iain Morgan.

pGL3-Δ114 contains nucleotides 184-310 of the BPV-4 genome cloned into the *Bgl* II site of pGL3. This plasmid was a gift from Dr. Iain Morgan.

The LCR deletion mutants, Δ57.1, Δ10, Δ36, Δ170, Δ151 and Δ114 were originally generated and cloned into the *BamH* I site of p41X by Dr. Maria Jackson (Jackson and Campo, 1991) and subsequently subcloned into pGL3 by Dr. Iain Morgan and Joan Grindlay.

pGL3-wtLCR contains nucleotides 6710-331 of the BPV-4 genome cloned between the Hind III and Kpn I sites of pGL3.

pGL3-sLCR contains nucleotides 6710-310 of the BPV-4 genome cloned between the Hind III and Kpn I sites of pGL3.

pGL3-AP1LCR contains nucleotides 6710-331 of the BPV-4 genome cloned between the Hind III and Kpn I sites of pGL3. Nucleotide 316 has been mutated from G to T, using PCR-based mutagenesis, thus generating a consensus AP-1 binding motif (TGATGCA) - nucleotides 313-319.

pGL3-randLCR contains nucleotides 6710-310 of the BPV-4 genome followed by a stretch 21 random nucleotides cloned between the Hind III and Kpn I sites of pGL3.

pCH110 is a control eukaryotic assay vector which contains a functional *lacZ* gene which is expressed either from the SV40 early promoter or from the *E.coli gpt* promoter (Hall *et al*, 1983). This vector was commercially obtained from Pharmacia.

pTKCAT contains the herpes simplex virus TK promoter upstream of the coding sequence for the bacterial CAT protein. This plasmid was a gift from Dr. Elizabeth Black (Beatson Institute, Glasgow).

p5xTRE-CAT contains five copies of nucleotides -73 to -65 of the human collagenase gene ligated head to head, flanked by *Hind* III/*BamH* I linker sequences and cloned into the *Hind* III/*BamH* I cut vector pBL-CAT2. pBL-CAT2 was derived from pUC18 and contains the herpes simplex virus TK promoter upstream of the coding sequence for the bacterial CAT protein. This plasmid was a gift from Dr. Elizabeth Black (Beatson Institute, Glasgow).

pMoMuLTR-CAT contains the Moloney murine long terminal repeat element upstream and driving transcription of the chloramphenicol acetyl transferase (CAT) gene. This plasmid, originally constructed by Jack Lenz (Albert Einstein College of

Medicine, Bronx, New York, USA), was a kind gift from Dr Jim Neil (Glasgow University Veterinary School).

2.2.10 Tissue Culture

Alpha Laboratories Ltd
Plastic disposable cuvettes

Beatson Institute Central Services
Kanamycin
Penicillin
Sterile distilled water
Sterile glycerol
Sterile phosphate-buffered saline (PBS)
Sterile phosphate-buffered saline + EDTA (PE)
Streptomycin

Becton Dickinson Labware
18 gauge sterile syringe needles
60 and 90mm tissue culture dishes
Falcon 2054 polystyrene round bottomed tubes
Falcon 2097 polypropylene tubes
Falcon 2098 polypropylene tubes
Serological plastic pipets
Sterile Plastipak syringes

Bibby Sterilin Ltd
Sterile plastic universal containers

Costar Corporation
96 well culture plates
Disposable cell scrapers

Gibco BRL Europe Life Technologies Ltd
100mM sodium pyruvate
10x Dulbecco's Modified Eagles Medium
10x Nutrient Mixture F-10(HAM)
2.5% Trypsin solution
200mM L-glutamine
7.5% sodium bicarbonate
Special Liquid Medium

Globepharm
Foetal Calf Serum

ICN Biomedicals Inc
Mycoplasma removal agent

Johnson and Johnson Medical Limited
PRESEPT* effervescent disfectant tablets

Labsystems (UK) Ltd
Clinicon disposable measuring cuvettes

Promega Ltd
Luciferase Assay System
Reporter Lysis 5x Buffer

Nunc
Cryotubes
T25, T75 and T175 cm^2 culture flasks

Sigma Chemical Co. Ltd
Adenine
Cholera enterotoxin
EGF
Hydrocortisone
Insulin
MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue)
Polybrene (Hexadimethrine bromide)
Quercetin (3,3',4',5,7-pentahydroxyflavone)
TPA (12-O-tetradecanolyphorbol 13-acetate)

Worthington Biochemical Company
Trypsin

2.3 METHODS

2.3.1 Tissue Culture

Bovine foetal samples were obtained from the veterinary post-mortem room at the Glasgow University School of Veterinary Medicine. All cell culture work was performed following strict aseptic protocols inside a laminar flow hood (Class II Microbiological Safety Cabinets; Medical Air Technology Ltd., Manchester, UK).

2.3.1.1 Swiss 3T3 Feeder Cells

Swiss 3T3 mouse fibroblasts were routinely grown in Special Liquid Medium containing 10% (v/v) FCS, 2mM L-glutamine, 37.5μg/ml penicillin and 10μg/ml streptomycin. Cells were seeded at 10^6 cells/T175 tissue culture flask and incubated at 37°C in a humid atmosphere containing 5% (v/v) CO_2 (Heraeus, Essex, UK) until completely confluent. Medium was changed twice weekly.

After confluency was reached, the cells were trypsinised (section 2.3.14) using a solution of 0.25% (w/v) trypsin in 1xPE buffer (PBS containing 1mM EDTA) and resuspended in fresh growth medium. Cells were lethally irradiated by exposure to 60Gy of γ-irradiation using a ^{60}Co source. Following irradiation the feeder cells, now incapable of further cell division, were plated out immediately or stored for up to 48 hours at 4°C without loss of feeding capacity. Feeder cells were plated at a density of $1x10^6$ cell/90mm culture dish before addition of keratinocytes.

Feeder cells were removed from keratinocyte cultures by a process of selective trypsinisation; typsin solution was added to the mixed culture and after a 2 to 3 minute incubation at 37°C all feeders were detached from the culture plastic. Keratinocytes remained attached, requiring longer incubations in trypsin to be dislodged. Unwanted feeders were washed away using phosphate buffered saline (PBS:0.14M NaCl, 27mM KCl, 10mM Na_2HPO_4, 15mM K_2HPO_4)

2.3.1.2 Isolation of Primary Bovine Keratinocytes

A small section of palate tissue was taken from a bovine foetus which had completed no more than five months gestation. The palate tissue was immediately placed in a solution of PBS containing 100μg/ml penicillin, 400μg/ml streptomycin,

100μg/ml kanamycin and 2.5μg/ml amphotericin. After washing a further two times in the PBS/antibiotic solution, the tissue was incubated overnight at 4°C in PBS containing 0.01% EDTA and 0.25% Worthington trypsin. Following a further 30 minute incubation at 37°C, the trypsin was neutralised by the addition of foetal calf serum (FCS) to a final concentration of 10% (v/v). The tissue was transferred to a 90mm tissue culture dish and keratinocyte (PalK) cells were separated from underlying mesenchyme by scraping along the surface of the tissue using a scalpel blade. The remaining solid tissue was placed in a second culture dish for further isolation of fibroblast cells (see section 2.3.1.3).

The freshly extracted PalK cells were suspended in 10ml keratinocyte medium, transferred to a 25ml universal tube and centrifuged at 1000rpm for 5 minutes at room temperature. Primary Bovine Keratinocyte (PalK) growth medium comprises Special Liquid Medium supplemented with 4mM glutamine, 0.31% (w/v) sodium bicarbonate, 2% (v/v) 10x Nutrient Mixture F-10, 10% (v/v) FCS, 10ng/ml cholera enterotoxin, 0.5μg/ml hydrocortisone, 5μg/ml insulin, 180μM adenine, 10ng/ml EGF, 37.5μg/ml penicillin, 10μg/ml streptomycin and 100μg/ml kanamycin.

The PalK cell pellet was gently resuspended as a single cell suspension in culture medium and counted (section 2.3.1.4). $5x10^5$ viable cells were transferred onto a layer of irradiated Swiss 3T3 feeder cells (section 2.3.1.1) in a T175 tissue culture flask. The presence of the 3T3 feeder layer is necessary for keratinocytes to initiate colony formation (Rheinwald and Green, 1975a). Hydrocortisone in keratinocyte culture medium promotes orderly colony morphology and helps maintain an increased growth rate (Rheinwald and Green, 1975b). The addition of CET, by raising cAMP levels, appears to restrict keratinocyte enlargement (Green, 1978) and may thus prevent the onset of terminal differentiation (Sun and Green, 1976).

2.3.1.3 Isolation of Primary Bovine Fibroblasts

The isolation of fibroblasts from foetal bovine palate was described previously by Jaggar *et al.* (1990). Briefly, following removal of the keratinocyte layer (section 2.3.1.2), the remaining tissue mass was cut into pieces approximately 2-4mm^2 in size and placed into a 90mm dish with individual fragments being well spaced apart. Incubating the dish for five minutes at 37°C facilitated adhesion of the tissue pieces to

the dish surface. Culture medium (Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% foetal calf serum, 2mM glutamine, 1mM pyruvate, 0.375% sodium bicarbonate, 37.5μg/ml penicillin and 10μg/ml streptomycin) was added slowly to the dish so as not to disturb the adherent samples. Medium was subsequently changed twice weekly. Following a two week period sufficient outgrowth of fibroblasts had occurred that the original tissue chips could be removed. The newly extracted fibroblasts were trypsinised and reseeded into a larger tissue culture flask. Cells were expanded until sufficient numbers were obtained for stocks to be made and stored in liquid nitrogen.

2.3.1.4 Maintenance, Counting and Passage of Cells in Culture

Cells were fed every three to four days with the appropriate culture medium and incubated at 37°C in a humid atmosphere containing 5% (v/v) CO_2 (Heraeus, Essex, UK).

PalF cells were grown to approximately 90% confluence and then passaged.

PalK cells were grown to 80 to 90% confluence and then passaged (1 in 4 split) or seeded into smaller culture dishes at a specified density. The background feeder layer was first removed by trypsinising for 3-4 minutes. After washing with PBS twice to remove the 3T3 cells, a second volume of trypsin was added which eventually dislodged the PalK cells. The feeder cells can be separated from PalK cells by this method of selective trypsinisation because they are less firmly attached to the culture plastic compared to the epithelial PalK cells.

Swiss 3T3 feeders were grown to 90% confluency before passaging or 100% confluency before irradiation.

To passage cells, spent medium was aspirated off and the cell monolayer washed two times with PBS pre-warmed to 37°C. After removing the second volume of PBS, trypsin solution (0.25% trypsin in 1x PE; PBS containing 1mM EDTA), also at 37°C, was added to the cells. The tissue culture flasks or plates were incubated at 37°C until the cells detached from the culture plastic. Cells were resuspended in the appropriate growth medium, transferred to a sterile universal tube and centrifuged at 1000rpm for 5 minutes at room temperature; serum present in the growth medium neutralised the trypsin. The cell pellet was resuspended in medium and the concentration determined.

The concentration of a cell suspension was determined after cells were mixed with the dye napthalene black to enable the number of viable cells in the suspension to be evaluated. Living cells are impermeable to napthalene black whereas dead cells cannot exclude the dye and stain black (Kaltenbach *et al*, 1958). 20µl of a cell suspension was mixed with 80µl of staining solution (1% (w/v) napthalene black in PBS). Stained cells were applied to the counting chamber of a covered haemocytometer slide (Improved Neubaer) and examined under a light microscope using a 10x objective lens. The number of living cells was counted and from the average of two separate counts the actual concentration of the cell suspension was calculated taking into account the sample area ($1mm^2$), depth of chamber and dilution factor. Cell concentrations were expressed as viable cells per millilitre. Cells were finally seeded at the required density.

2.3.1.5 Mycoplasma Screening

A 60mm dish of NRK (Newborn Rat Kidney) cells was provided by Marian Lacey (Beatson Institute, Glasgow). 2ml of growth medium, taken from cells being tested for the presence of mycoplasma, was added to the dish of NRK cells containing 2ml fresh medium (SLM supplemented with 10% (v/v) FCS and mM glutamine) and incubated for 3-4 days in a humidified incubator at 37°C in an atmosphere of 5% CO_2. The growth medium from cells being screened must have been in contact with the cells for a minimum of 2 days. At the end of the incubation period all medium was removed and cells washed twice with PBS. 2.5ml of PBS followed by 2.5ml of fixative (75% (v/v) methanol, 25% (v/v) glacial acetic acid) was added to the dish. This was removed and 5ml fixative only added. Fixative was aspirated off and a second 5ml volume of fixative added and then incuated at room temperature for 10 minutes. Fixative was poured off and the dish placed at 37°C for 15 minutes to dry completely. 5ml of Heochst 33458 stain (0.05µg/ml in PBS) was added and the cells allowed to stain for 5 minutes at room temperature. When the staining solution was removed the plate was rinsed twice with distilled water and left to air dry. A drop of distilled water was added to the centre of the dish and covered with a clean glass cover slip. Cells were finally examined using a water immersion lens on a fluorescence microscope (Leitz Wetzlar) with a Mercury vapour lamp light source. Mycoplasma, if

present, can be identified as extranuclear fluorescent specs against the dark background.

2.3.1.6 Long Term Cell Storage

To freeze cells stocks for storage, cells were trypsinised and pelleted as outlined above (section 2.3.1.4). The pellet was resuspended at a concentration of approximately 10^6 cell/ml in ice cold growth medium containing 10% (v/v) DMSO. The DMSO in the medium acts as a cryoprotectant but all solutions must be chilled as DMSO is toxic to cells at room temperature. The cells were transferred in 1ml aliquots into chilled Nunc cryotubes on ice. The cryotubes were transferred into a polystyrene rack, wrapped in cotton wool and frozen by placing in a -70°C freezer overnight. The insulation around of the cell ampoules ensures gradual cooling and eventual freezing of the cells which increases the viability of the cells. All cells were finally transferred to a storage rack and placed in liquid nitrogen (-195.8°C).

Frozen cells were recovered by placing an ampoule of cells directly from liquid nitrogen into a small, covered bucket of water at 37°C. Once thawed, the cells were added to 10ml of the appropriate pre-warmed growth medium, centrifuged, resuspended in fresh medium and seeded into a tissue culture flask. PalK cells were plated out onto a layer of irradiated feeders (section 2.3.1.1).

2.3.1.7 Transient Transfection of Primary Bovine Fibroblasts

PalF cells were transiently transfected with a range of plasmid DNAs (see section 2.2.9) using the standard protocol for calcium phosphate-mediated transfection. Exponentially growing cells were trypsinised and 1-2x10^5 viable cells replated into each 60mm tissue culture dish containing 5ml of appropriate growth medium. Cell were replaced in a humidified incubator at 37°C containing 5% (v/v) CO_2 for 24 hours.

For each 60mm monolayer of PalF cells to be transfected the following mixture was set up. These volumes can be multiplied according to the number of cells being transfected.

To 250µl of 2x HEPES buffered saline (280mM NaCl, 10mM KCl, 1.5mM $Na_2HPO_4.2H_2O$, 12mM D-glucose, 50mM HEPES), 250µl of a DNA solution (up to

20μg of total plasmid DNA(s) in 0.1xTE pH8 containing 250mM $CaCl_2$) was added. The addition of the DNA solution to the 2xHBS must done very gradually with constant but gentle mixing. The mixture was left to stand at room temperature for 20 to 30 minutes during which time a fine precipitate formed giving a slight blue/grey tinge to the transfection mix. At the end of the incubation, the mixture was gently pipetted up and down to resuspend the precipitate.

The calcium phosphate-DNA suspension was added directly into the medium above the cell monolayer. The dish was rocked slowly to disperse the transfection solution throughout the culture medium. All dishes of cell were incubated for 18-24 hours in the presence of the transfection mixture.

Following this incubation, medium and precipitate were completely removed by aspiration. The monolayer was washed twice with pre-warmed PBS and fresh medium was added to each dish. Cells were returned to the incubator for a further 24 hour after which time 1-100μM quercetin or an equivalent volume of its diluent, ethanol, was added to the growth medium of the PalF cells. Incubation continued for a further 24 to 48 hours after which time the cells were harvested.

2.3.1.8 Transient Transfection of Primary Bovine Keratinocytes

PalK cells cannot be transfected using the calcium phosphate method of transfection because high concentrations of calcium in the cellular environment triggers terminal differentiation of keratinocytes (Boyce and Ham, 1983). Instead a procedure employing the use of the polycation Polybrene was found to give satisfactory transient transfection efficiency in PalK cells.

$5x10^5$ PalK cells were seeded in 60mm dishes in the presence of the appropriate culture medium (section 2.3.1.2) but in the absence of an irradiated feeder layer. 18 hours later the medium was removed and 2ml of transfection mix was added to each 60mm dish of cells. The transfection mix contained up to 10μg/ml of total plasmid DNA and 10μg/ml Polybrene in PalK culture medium. The cells were incubated for 6 hours in the presence of the transfection medium with occasional rocking to enhance adsorption and prevent any area of the monolayer from drying out.

The transfection mix was aspirated off and 5ml of special liquid medium containing 35% (v/v) DMSO was added to each dish of cells and incubated for

3 minutes at room temperature. After removal of the DMSO-shock volume, the cells were washed twice with warm PBS and refed with a fresh volume of growth medium. Cells were incubated overnight (16 to 24 hours) after removal of the transfection mix before quercetin at various concentrations or an equivalent volume of its solvent, ethanol, was added to the medium. Cells were incubated in the presence of quercetin or ethanol for a further 24 hours before being harvested.

2.3.1.9 Harvesting Cells

(a) **For Reporter Gene Assays**

At the end of the required incubation in the presence of quercetin or ethanol the culture medium was removed. Medium containing quercetin at any concentration was pipetted into a conical flask and detoxified by the addition of a PRESEPT* tablet; one PRESEPT* table is sufficient to disinfect 500µl of medium containing any COSHH 3 chemical.

Cell monolayers were washed twice with PBS, the PBS was completely removed by aspiration and 300µl of 1x Reporter Lysis Buffer (Promega) added to each 60mm dish. Following a 15 minute incubation at room temperature, cells were scrapped off the culture plastic and each lysate transferred to a 1.5ml eppendorf. Cell debris was pelleted by spinning lysates at 4°C in a microcentrifuge at 14,000rpm for 5 minutes. The supernatant was transferred to a second eppendorf taking care not to disturb the cell pellet. The lysates were either assayed for reporter enzyme activity immediately or stored at -20°C.

(b) **For Phosphotyrosine Analysis**

PalF cells were grown in 90mm culture dishes in the presence of culture medium containing 20µM quercetin or an equivalent volume of ethanol. The duration of quercetin exposure and specific experimental conditions are given in detail in section 5.2.1.

Cells were harvested for total cellular protein under conditions designed to maintain the phosphorylation status of tyrosine-containing proteins at the time of lysis. Medium was removed from the cells and disposed of appropriately. Monolayers were

washed twice with ice-cold PBS before the addition of 500µl of boiling lysis solution (1% SDS (w/v), 10mM Tris pH7.4) to each 90mm dish. Cells were scrapped from the culture dish, transferred to a microcentrifuge tube and boiled for 5 minutes. Samples were centrifuged for 5 minutes at 4°C at 14,000rpm in a microcentrifuge and the supernatant transferred to a chilled 1.5ml microfuge tube. Protease inhibitors PMSF and aprotinin were added to final concentrations of 2mM and 1µg/ml respectively. Cell lysates were stored at -20°C.

(c) Preparation of Nuclear Extract from Cells in Culture

PalF cells were treated with quercetin or ethanol according to the schedule detailed in section 4.10.1. Following the appropriate treatment, medium was removed from the cells and the monolayer washed twice with ice-cold PBS. 5ml of PBS was added to the culture flask and the cells removed from the plastic surface using a cell scraper. The cell solution was transferred to a 50ml falcon tube and the flask rinsed with another 10ml PBS. The cells were pelleted by centrifugation at 2,000rpm for 5 minutes at 4°C. The pellet was resuspended in 5ml of PBS and re-centrifuged as before. The PBS was removed and the pellet suspended in 160µl of freshly prepared hypotonic buffer (10mM Tris-Cl (pH7.3), 10mM KCl, 1.5mM $MgCl_2$, 2mM PMSF, 4mM β-mercaptoethanol, 0.5mM benzamidine, 1µg/ml leupeptin, 1µg/ml aprotinin) and transferred to a clean eppendorf tube. The solution was spun in a microcentrifuge for 30sec at 14,000rpm at 4°C. The pellet was resuspended in 260µl fresh lysis buffer (0.4% (v/v) Nonidet P-40,10mM Tris-Cl (pH7.3), 10mM KCl, 1.5mM $MgCl_2$, 2mM PMSF, 4mM β-mercaptoethanol, 0.5mM benzamidine, 1µg/ml leupeptin, 1µg/ml aprotinin) and left on ice for 10 minutes before spinning in a microcentrifuge (14,000rpm) for 5min at 4°C. The supernatant was removed and the pellet resuspended in 25µl of 0.02M KCl buffer (20mM Tris-Cl (pH7.3), 25% (v/v) glycerol, 1.5mM $MgCl_2$, 0.2mM EDTA, 20mM KCl, 2mM PMSF, 4mM β-mercaptoethanol, 0.5mM benzamidine, 1µg/ml leupeptin, 1µg/ml aprotinin) followed by the drop-wise addition of 100µl 0.6M KCl buffer (20mM Tris-Cl (pH7.3), 25% (v/v) glycerol, 1.5mM $MgCl_2$, 0.2mM EDTA, 0.6M KCl, 2mM PMSF, 4mM β-mercaptoethanol, 0.5mM benzamidine, 1µg/ml leupeptin, 1µg/ml aprotinin). The solution was incubated on ice for 30min then centrifuged in a microcentrifuge (14,000rpm) for

15min at 4°C. The supernatant was aliquoted into clean cryotubes tubes, frozen on dry ice and stored at -70°C.

2.3.1.10 Growth Rate Analysis

Three separate methods were used to assess the effect of different concentrations of quercetin on the growth characteristics of PalF cells.

(a) Direct Cell Counting

Cells were trypsinised and replated at a density of $5x10^5$ or 10^6 cells per 90mm dish in the appropriate growth medium (section 2.3.1.3). After leaving the cells overnight to adhere to the culture plastic, old medium was aspirated off and fresh medium containing 1-100μM quercetin or control medium containing ethanol was added to the appropriate dishes of cells. The volume of ethanol in control medium was equivalent (v/v) to the highest concentration of quercetin; quercetin or ethanol never exceeded 0.5% (v/v) in medium. Dishes of cells were set up in triplicate for each concentration of quercetin (1, 20, 50 and 100 μM), ethanol or medium alone.

Medium was changed every 24 hours to ensure that the active concentration of quercetin in the medium was maintained as constant as possible. Quercetin-containing medium must be properly disposed of (section 2.3.1.9a). Cell cultures were examined daily using a 4x and 10x objective lens on a light microscope. When monolayers reached approximately 90% confluency cells were trypsinised, removed from the culture dish and counted using a haemocytometer (section 2.3.1.4) before 10^6 cells were reseeded. This procedure was continued over a period of nine days. At the end of the nine days all dishes of cells were trypsinised and the total number of cells in each counted. The cumulative number of cells was individually calculated for each dish of cells grown in the presence of a specific concentration of quercetin, ethanol or medium alone. The average number of cells from the three plates for each growth condition was plotted against time in days and graphically reproduced using Excel 5.0 spreadsheet software.

(b) MTT assay

MTT is a water soluble tetrazolium salt yielding a yellow coloured solution when dissolved in water or PBS. Soluble MTT can be converted to an insoluble

formazan which is deep purple in colour. Active mitochondrial dehydrogenase enzymes will cause this conversion whereas dead cells will not. The water insoluble formazan can be solubilized in DMSO or other such solvents and measured spectrophotometrically (Carmichael *et al*, 1987). The absorbance read at 590nm is a function of concentration of dye converted.

1000-2500 PalF cells were added to each well in a 96 well tissue culture plate. One 96 well plate of cells was set up for each day in the growth curve duration including a plate for Day 0. The outer wells along the edge of the plate did not receive cells. Instead, 200µl of sterile distilled water was added to each of these wells to reduce evaporation from the inner cell-containing wells.

After an overnight incubation, during which time the cells attached to the bottom of each well, medium was changed. The fresh medium was supplemented with various concentrations of quercetin or ethanol present at the same dilution as the highest concentration of quercetin, i.e. 1% v/v. Five wells of cells received normal PalF medium only. The first plate, corresponding to Day 0, was incubated for four hours only after the addition of fresh medium then treated with MTT and assayed. Each day thereafter one plate of cells was treated with MTT to determine the number of living cells in each well.

Treatment of cells with MTT was performed as follows; Medium from all cells in one 96 well plate was aspirated off using an 18 gauge needle. Any medium containing quercetin was disposed of in the appropriate manner (section 2.3.1.9a). Each well of cells was washed once with pre-warmed PBS before addition of 200µl medium containing 400µg/ml MTT. MTT was made from powder as a 5mg/ml stock solution in PBS. The plate was replaced in a humid incubator (37°C and 5% (v/v) CO_2) for 3 hours. MTT-medium was removed from each well into a COSHH 3 conical flask using a 18 gauge needle and detoxified using PRESEPT* tablets as for quercetin medium. Following a single wash with PBS, 100µl DMSO was added to each well and the plate placed on a horizontal shaking plateform for 10 minutes at room temperature. The absorbance in each well was read immediately at 590nm using a multiwell plate reader (Dynatech MR7000).

(c) **Thymidine incorporation**

The uptake of tritiated thymidine applied to determining the number of viable cells in a culture population was first described by Elkind *et al*, (1963).

1000 or 2500 PalF cells were added to the inner wells of a 96 well plate. The wells along the edge of the plate contained sterile distilled water only which reduced the level of evaporation from the inner wells. After an overnight incubation, the medium was removed and replaced with medium containing increasing concentrations of quercetin ranging from 1-100μM. Control wells of cells received either PalF medium alone or medium supplemented with ethanol, the solvent in which quercetin was dissolved.

The plate was incubated for three days in a humid 37°C incubator with an atmosphere of 5% (v/v) CO_2. The medium containing quercetin or ethanol was changed in the wells each day. On the fourth day medium was removed using an 18 gauge needle and cells washed with PBS. 20μl of PalF medium containing 0.5μCi of ^{3}H-thymidine was added to each well of cells and incubated for a further 6 hours. The radioactive medium was removed into a conical flask containing DECON and disposed of down a designated radiochemical sink. 50μl of trypsin solution (0.25% trypsin in 1x PE) was added to each well, the plate incubated at 37°C for 15 minutes and the cells harvested onto printed Filtermat A glass fibre filters using a multiwell harvester. Filters were left overnight at room temperature to dry completely before being sealed in sample bags with 15ml Betaplate Scint scintillant fluid and counted in a Beckman LS6000IC scintillation counter.

2.3.1.11 Luciferase Reporter Assay

Cells were lysed using 1x Reporter Lysis Buffer (Promega) as detailed in section 2.3.1.9a. 50μl of cell lysate was aliquoted carefully into the bottom of a Clinicon disposable measuring cuvette. Luciferase activity in each sample was assayed using the Luciferase Assay System (Promega) according to the manufacturer's instructions. The amount of luminescence produced was measured in a BioOrbit LKB 1251 Luminometer for 30 seconds and readings expressed as millivolts per second.

2.3.1.12 β-Galactosidase Reporter Assay

The plasmid pCH110 was used in all transient transfections as an internal control against which the efficiency of transfection could be normalised. β-galactosidase can catalytically convert colourless o-nitrophenyl-β-D-galactopyranoside (ONPG) to yellow o-nitrophenol. The level of activity of this enzyme can be assayed by measuring changes in light absorbance at 420nm.

Cells were lysed as detailed in section 2.3.1.9a. To 50µl of each cell lysate, 1ml of solution I (60mM Na_2HPO_4, 40mM NaH_2PO_4, 10mM KCl, 1mM $MgCl_2$, 50mM β-mercaptoethanol) and 0.2ml of solution II (60mM Na_2HPO_4, 40mM NaH_2PO_4, 2mg/ml) was added. After mixing, all samples were incubated at 37°C for 30-60 minutes or until a yellow colour change could be seen. Samples were transferred to plastic disposable cuvettes and the reactions stopped by the addition of 0.5ml 1M sodium carbonate. The absorbance was read at 420nm using a Beckman DU 650 spectrophotometer.

2.3.1.13 Chloramphenicol Acetly Transferase (CAT) Reporter Assay

40µl of CAT assay master mix (250mM Tris pH7.5, 0.2µCi ^{14}C- chloramphenicol, 0.5mg/ml acetyl CoA) was added to 5-50µl of each cell lysate sample and incubated at 37°C for 1 hour. After the addition of 400µl ethyl acetate, samples were mixed thoroughly by inverting the tubes several times and spun at room temperature for 30 seconds in a microcentrifuge to separate the aqueous and organic phases. The top solvent layer was transferred to a clean eppendorf taking care not to disturb the interface. All solvent was evaporated off by drying samples under vacuum in a speedivac. Each sample residue was resuspended in 10µl ethyl acetate, spotted onto a thin layer chromatography (TLC) plate and placed in a closed glass tank containing 95% (v/v) chloroform and 5% (v/v) methanol. As the solvent front moved up the TLC plate the various acetylated products of each sample were separated out. After 35 minutes, the plate was removed from the tank and placed on a sheet of 3MM Whatman paper to air dry. The dried plates were placed in a light tight lead cassette and exposed at room temperature to X-OMAT AR X-ray film overnight.

The film was automatically developed by passing it through a Kodak

X-OMAT 480 RA Processor and placed on a light box. The TLC plates were placed on top of the film, aligned, and the position of the non-acetylated substrate and mono-acetylated products marked lightly with a pencil outline. These areas were cut out and placed in individual scintillation vials; the non-acetylated substrate was placed in a separate vial from its acetylated products. The vials were loaded into racks in a Beckman LS 6000 IC scintillation counter and readings for each sample obtained in Counts per minute (CPM). The percentage of substrate converted into acetylated product was calculated using the following formula;

$$\% \text{ Conversion} = \frac{\text{CPM monoacetylated product}}{\text{CPM non-acetylated product} + \text{CPM monoacetylated product}} \times 100$$

2.3.1.14 Flourescence Activated Cell Sorting (FACS) Analysis

Flourescence Activated Cell Sorting was used to analyse the cell cycling status of PalF cells in a particular population. Cells were grown in the presence or absence of quercetin according to the experimental conditions given in detail in section 3.3.1.

Cells were harvested by trypsinisation and pelleted at 4°C in growth medium as descibed previously in section 2.3.1.4. Each cell pellet was washed twice with ice cold PBS spinning cells, as before, between each wash. The second volume of PBS was aspirated off and the pellet was resuspended in a small residual volume of PBS (~ 200µl) before cells were fixed by the slow, drop by drop addition of 5ml ice cold 70% ethanol. Samples were left on ice for 1 hour. Cells were either stored at -20°C under 70% ethanol for no longer than 1 month or stained immediately.

To stain cells for analysis, each cell suspension in 70% ethanol was pelleted at 4°C for 5 minutes at 2000rpm. Ethanol was aspirated off and cells resuspended in 500µl of staining solution (PBS containing 250µg/ml RNAse A, propidium iodide (10µg/ml), 0.2% (v/v) Tween 20). Samples were left at room temperature for 30 minutes to 1 hours before analysis.

Stained cell samples were filtered through 70μm nylon gauze into Falcon 2054 polystyrene round bottom tubes. Samples were assayed using a Becton Dickinson FACScan machine and analysed using the 'Cell Quest' software package.

2.3.2 Molecular Biology

2.3.2.1 Oligonucleotide synthesis

Single stranded oligonucleotides were synthesised on an Applied Biosystems 392 DNA/RNA synthesiser using the manufacturers protocols and Cruachem reagents by Beatson Institute technical services staff. The final oligonucleotides were made with or without trityl group protection. All primers were deprotected after synthesis by incubating in a 55°C water bath overnight.

Oligonucleotides carrying the trityl moiety were detritylated using a Cruachem oligonucleotide purification (COP) cartridge according to manufacturers instructions. Each oligonucleotide was ultimately eluted from the COP cartridge minus the tritly group using 1 to 2ml of 20% (v/v) acetonitrile. The acetonitrile was evaporated off and the purified oligonucleotide was resuspended in 0.5 to 1ml sterile distilled water or TE pH8.0. The absorbance of each oligonucleotide solution was measuring at 260nm and the concentration of the sample determined. An O.D. reading of 1 at 260nm corresponds to an oligonucleotide concentration of ~33μg/ml. Oligonucleotide solutions were stored at -20°C.

"Trityl off" oligonucleotides were provided in ammonia. The oligonucleotides were deprotected by heating to 55°C overnight then purified by precipitation with butan-1-ol. 1ml butan-1-ol was added to 150μl oligonucleotide solution and microcentrifuged at 13,000g for 20 minutes at room temperature. The butanol solution was carefully removed and the pellet dried by centrifugation under vacuum. The oligonucleotide was finally dissolved in an appropriate volume of sterile distilled water or TE pH8.0 and the concentration determined as described above.

2.3.2.2 PCR-based Mutagenesis

Primers were designed to contain the appropriately altered base(s) plus a restriction enzyme site, e.g. *Hind* III. Single stranded oligonucleotide primers were generated and purified as detailed in section 2.3.2.1.

Appropriate primers were used to set up PCR reactions using a GeneAmp PCR Kit from Perkin Elmer according to the manufacturers instructions; the total volume of each reaction mix was 100µl containing 1ng of template DNA plus 40pmol of each primer. Each reaction contained 1X PCR buffer, 200mM each dNTP and 2.5 units AmpliTaq DNA polymerase. The final concentration of $MgCl_2$ in each reaction was optimised in the range of 1-4mM.

The PCR reaction volumes were contained in 0.5ml sterile eppendorf tubes. One drop of sterile mineral oil was added to each tube to cover the top of the reaction volume to prevent sample evaporation. The tubes were placed securely in a Perkin Elmer Cetus DNA thermal cycler 480. The samples were heated to 95°C for 1min (denaturing step), 55°C for 1min (annealing of primers step) and 72°C for 2min (elongation step). This cycle of denaturing, annealing and elongation was repeated a total of 30 times. The PCR products were phenol:chloroform extracted as detailed in section 2.3.2.4 before being digested with the appropriate restriction enzyme(s) (sections 2.3.2.3). After digestion, the PCR products were further extracted with phenol:chloroform to remove any enzyme which may interfere with the ligation reaction of product into vector cut with compatible enzyme(s). Inserts were ligated into the appropriate vector as detailed in section 2.3.2.8.

2.3.2.3 Restriction Enzyme Digests

Digestion of DNA was performed in small reaction volumes using enzymes and their appropriate concentrated buffer solutions according to the manufacturers instructions. 5-10 units of restriction enzyme were added per µg of DNA ensuring that the total volume of enzyme added did not exceed one tenth of the final reaction volume. Small quantities of plasmid DNA (<5µg) were routinely digested in a 20µl reaction volume for 1 to 2 hours at 37°C. Larger digests were carried out in proportionally larger reaction volumes. Digestion of DNA with more than one enzyme (double digestion) was performed either sequentially or in a single reaction

providing the buffering conditions for the individual enzymes, as specified by the manufacturer, were compatible. When DNA was digested with different enzymes separately, the DNA was ethanol precipitated after the first round of digestion and resuspended in an appropriate volume of TE before the second reaction mix was set up. Reactions were terminated by the addition of 0.5M EDTA (pH 8.0) to a final concentration of 10mM. The digested DNA was finally ethanol precipitated and resuspended in an appropriate volume of TE.

2.3.2.4 DNA Extraction and Precipitation with Organic Solvents

DNA samples were purified by extraction with a mixture of phenol and/or chloroform to remove contaminants such as residual enzyme activities from a restriction reaction or detergents which may interfere with subsequent manipulations. In the first round of extraction, an equal volume of phenol:chloroform:isoamyl alcohol (24:24:1 v/v/v) was added to the DNA solution. The aqueous DNA and organic phases were mixed thoroughly by vortexing, then separated by centrifugation in a bench top microcentrifuge at 14,000rpm for 5min at room temperature. The upper aqueous phase was carefully removed, transferred to a clean microcentrifuge tube and the process repeated. Extraction with phenol:choloform:isoamyl alcohol was repeated until the interphase between the upper and lower phases was clear. The aqueous phase was then extracted with an equal volume of choloform:isoamyl alcohol (24:1 v/v) to remove any traces of phenol from the aqueous phase. After vortexing and centrifugation to separate the two phases, the upper aqueous DNA phase was removed to a fresh tube for ethanol precipitation.

Ethanol precipitation concentrated DNA samples and removed solute contaminants such as salt. To the aqueous solution of DNA, one tenth of the volume of 3M sodium acetate (pH 5.2) and 2-2.5 volumes of ice cold ethanol was added. The solution was mixed by inversion several times and then placed at -20°C for at least 30 minutes to facilitate DNA precipitation. Precipitated DNA was recovered by centrifugation in a microcentrifuge at 14,000rpm for 15min at 4°C. The DNA pellet was washed with 70% ethanol to remove any traces of salt, dried under vacuum and resuspended in an appropriate volume of TE buffer (pH8.0). The concentration of the DNA solution was determined as detailed in section 2.3.2.5.

2.3.2.5 DNA Quantification

The concentration of DNA in aqueous solutions was measured spectrophotometrically in a Beckman DU 650 spectrophotometer. Samples were diluted in TE buffer and transferred to a quartz cuvette with a path length of 1cm. The spectrophotometer was initially calibrated using TE buffer only as a blank. The optical density readings were obtained at 260nm and 280nm; an O.D. reading of 1 at 260nm (A_{260} = 1) corresponds approximately to a concentration of 50μg/ml of double stranded DNA. The ratio between readings at 260nm and 280nm (A_{260}:A_{280}) provided an estimate of the DNA sample purity; a ratio of ~1.8 indicated that preparations contained essentially no protein or phenol contamination.

2.3.2.6 Agarose Gel Electrophoresis

Horizontal gel cast apparatus from Pharmacia was used. Agarose gels were generally 0.8% (w/v) in 1xTAE buffer unless otherwise stated. The appropriate percentage (w/v) of agarose was dissolved in 1xTAE buffer (40mM Tris base, 16mM acetic acid, 1mM EDTA pH8.0) by heating the solution in a glass conical flask in a microwave until all the particles of agarose had dissolved. Ethidium bromide was added to the molten agarose to a final concentration of 0.5μg/ml just before the gel was poured. The gel was poured when the molten agarose was hand hot and a comb with the required number and size of teeth placed immediately into the gel to form the sample wells. The solidified gel was placed in the gel tank and submerged in 1xTAE buffer containing 0.5μg/ml ethidium bromide. Samples containing 1x loading buffer (10x loading buffer; 0.45% (w/v) bromphenol blue, 1% (w/v) SDS, 100mM EDTA, 2.5% (w/v) Ficoll 400 in TE) were loaded into individual wells. An appropriate sized DNA marker was generally loaded into the first and/or last well in the gel. DNA markers used were;

- 1Kb DNA ladder (Suitable for sizing linear double-stranded DNA from 500bp to 12kb)
- λ *Hind* III (Suitable for sizing linear double-stranded DNA from 125bp to 23kb)
- φX174 *Hae* III (Suitable for sizing linear double-stranded DNA from 72 to 1,353 bp)

DNA was separated on the gel by running at 70-100 constant voltage usually until the samples' blue dye front was 1-3cm from the end of the gel. The separated DNA was

visualised by illimination on a short wave (312nm) UV light box and photographed onto videoprint paper using an Appligene Imager.

2.3.2.7 Isolation and Purification of DNA Restriction Fragments from Agarose Gels

DNA fragments to be used for cloning were separated from unwanted products of the restriction digest reaction by electrophoresis on 0.6% non-denaturing agarose gels and visualised as described in section 2.3.2.6. The fragment band was cut out of the gel using a clean scalpel blade and the gel slice placed in a sterile microcentrifuge tube. Extraction of the DNA fragment from the agarose was achieved using a QIAquick gel extraction kit (Qiagen) following the manufacturer's instructions. Alternatively, DNA fragments were purified from the gel slices using extraction with phenol:chloroform as detailed in section 2.3.2.4. First the gel slice was weighed then three volumes of TE buffer were added to the tube containing the gel fragment. The tube was place at 50°C until the gel had dissolved completely in the TE. Extraction with phenol:chloroform was performed as described earlier.

2.3.2.8 Cloning DNA Fragments into Plasmid Vectors

Vector DNA and the DNA fragment to be inserted into the vector were separately digested according to conditions detailed in section 2.3.2.3. The DNA fragment to be cloned into the vector was prepared as described in section 2.3.2.7.

The cut ends of the vector DNA were dephosphorylated to prevent the vector from religating to itself. After the vector DNA had been linearized by digesting with the required enzymes in a 20µl reaction volume, 2.2µl of 10x alkaline phosphatase buffer and 1µl (1 unit) of alkaline phosphatase were added to the reaction mixture and incubated at 37°C for a further 35 minutes. All enzyme activity in the reaction was finally stopped by heating the reaction volume to 65°C for 5 minutes. Sterile distilled water was added to the inactivated reaction to a final volume of 60µl before undergoing phenol chloroform extraction (section 2.3.2.4).

The DNA fragment was inserted into dephosphorylated vector at a ratio of 3:1 respectively. The vector (100ng) and insert DNA were incubated together in a reaction containing 1x ligase buffer and 1µl (4 units) of T4 DNA ligase and incubated

overnight at 11°C. Dilutions of this reaction volume were used to transform competent bacterial cells (section 2.3.2.9).

2.3.2.9 Transformation of Competent Bacterial Cells

E.Coli DH5α competent cells obtained commercially were used for the propagation of plasmid DNAs. Stocks of competent cells were stored at -70°C. Bacteria were transformed according to the manufacturer's instructions.

Briefly, DH5α cells were thawed on wet ice and after gentle mixing, 20µl of the cell suspension was aliquoted into a chilled polypropylene tube (Falcon 2059). Any unused bacterial cells were re-frozen rapidly in a dry-ice/ethanol bath and replaced in a -70°C freezer. Any one batch of cells should not be freeze-thawed more than once. 1-2ng of plasmid DNA was gradually added to the cells while mixing gently. The cells were left on ice for 30 minutes. Cells were heat shocked by placing in a 42°C water bath for 40 seconds then immediately returned to ice for 2 minutes. 80µl of sterile SOC (2% bactotryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose) was slowly added to the cells and the tube then placed securely in a shaking (225rpm) incubator at 37°C for 1 hour. Finally, the total transformation mix was spread onto a pre-warmed L-agar plate containing antibiotic at the appropriate concentration (eg. 100µg/ml ampicillin), inverted and incubated at 37°C overnight. Colonies obtained were either picked immediately or the plates stored at 4°C up-side-down and wrapped in parafilm for up to two weeks.

2.3.2.10 Glycerol Stocks

Stocks of transformed bacterial cells were made by first picking a single colony of newly transformed cells with a sterile yellow pipette tip. This was used to inoculate a 5ml volume of warm L-broth containing the appropriate antibiotic. The 5ml culture was incubated overnight at 37°C whilst shaken at 225rpm. 850µl of the overnight culture, which contained exponentially growing cells, was added to 150µl of sterile glycerol in a 1.5ml Nunc cryotube. The mixture was vortexed to ensure that the glycerol was mixed throughout the bacterial cell suspension and frozen rapidly by placing the tube into a dry-ice/ethanol bath for 5 minutes. Glycerol stocks were stored at -70°C.

2.3.2.11 Small Scale Preparation of Plasmid DNA (Miniprep)

Small amounts of plasmid DNA were simultaneously extracted from several individual, transformed bacterial colonies using either a QIAprep Spin plasmid miniprep kit following the manufacturer's instructions or the alkaline lysis method as described by Maniatis *et al*, 1989.

Briefly, a single colony of bacteria was picked from an agar+antibiotic plate using a sterile yellow pipette tip. This was used to inoculate a 5ml culture of L-Broth (1% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl). 20 to 40 separate colonies were generally picked for screening at any one time. The 5ml cultures were incubated at 37°C in a shaking incubator (225rpm) overnight. 1.5ml of each confluent bacterial culture was pelleted by spinning in a microcentrifuge (14,000rpm) for 30 seconds at room temperature.

Each bacterial pellet was resuspended in 100µl lysozyme solution (50mM glucose, 25mM Tris-HCl (pH8.0), 10mM EDTA, 2mg/ml lysozyme) and let stand at room temperature for 5 minutes. 200µl of freshly prepared solution 2 (0.2M NaOH, 1% (w/v) SDS) was then added, mixed in gently and left at room temperature for 5 minutes. 150µl of ice-cold 3M potassium acetate (pH4.8) was added, mixed thoroughly and the solution placed on ice for 5 minutes. The precipitate formed was pelleted by spinning the samples in a microcentrifuge (14,000rpm) for 3 minutes at room temperature and the supernatant removed to a clean eppendorf tube. The nucleic acids were precipitated by adding 2 volumes of ice-cold ethanol to the supernatant and standing the tube at room temperature for 5 minutes followed by centrifugation in a microcentrifuge (14,000rpm) for a further 10 minutes at 4°C. The pellet was washed with 70% ethanol before drying under vacuum for 5 minutes to remove all traces of ethanol. The pellet was dissolved in 20µl TE and stored at -20°C. Contaminating RNA in these samples can interfere with the detection of DNA fragments on an agarose gel. Hence RNA in these samples was destroyed by the addition of DNase-free RNase A (0.5mg/ml) to the digestion mixture.

2.3.2.12 Large Scale Preparation of Plasmid DNA (Maxiprep)

1ml of a 5ml overnight culture was used to inoculate 500ml of Super Broth (1.2% (w/v) bactotryptone, 2.4% (w/v) yeast extract, 0.8% (v/v) glycerol, 0.72M K_2HPO_4, 0.17M KH_2PO_4) containing antibiotic (100μg/ml ampicillin) in a 2 litre glass conical flask. The culture was incubated at 37°C for 48 hours shaking at 225rpm.

Bacterial cells were collected by centrifuging for 10 minutes at 6000g at 4°C. The bacterial pellet was weighted; 5g of bacterial pellet was resuspended in 20 ml GTE solution (50mM glucose, 25mM Tris-Cl (pH8.0), 10mM EDTA). When the pellet was completely resuspended, 5ml of 25mg/ml lysozyme in GTE solution was added, mixed thoroughly and the whole solution allowed to stand at room temperature for 10 minutes. 50ml of freshly prepared 0.2M NaOH / 1%(w/v) SDS was added and stirred in gently with a pipette until the solution became homogenous and clear; this viscous solution was incubated on ice for 10 minutes. 37.5ml of 3M potassium acetate solution was added and the whole solution inverted sharply five times. The viscosity of the solution was reduced and a large, white precipitate formed. The solution was placed on ice for a further 10 minutes followed by centrifugation at 20,000 x g at 4°C.

The supernatant was decanted through several layers of filter tissue and 0.6 volumes of propan-2-ol added. The whole solution was mixed by inversion several times and left to stand at room temperature for 10 minutes. Nucleic acid was recovered by centrifuging for 10 minutes at 15,000 x g at room temperature. The pellet was washed with 70% ethanol, centrifuged briefly to collect the pellet, excess ethanol drained off and the pellet dried under vacuum.

The pellet was resuspended in 8ml TE buffer. When completely dissolved, 8.8g CsCl and 0.4ml 10mg/ml ethidium bromide were added. The refractive index of the solution was adjusted to 1.395. The solution was transferred to a 10ml polyallomer ultracentrifuge tube and the tube filled (if necessary) using CsCl/TE solution (with R.I.=1.395). The tube was sealed and placed in a T1270 rotor with a protective metal cap over the top of each tube. The plasmid DNA was banded by centrifuging at 55,000rpm for 24 hours at 20°C in a Sorvall OTD Combi 80 ultracentrifuge.

The tube was carefully removed from the centrifuge rotor and placed securely in a clamp on a retort stand. An 18 gauge needle was first inserted into the top of the tube to act as an air inlet and then a second needle attached to a 2ml syringe was inserted into the side of the centrifuge tube ~1cm below the lower plasmid band. The plasmid DNA band was then carefully extracted and transferred to a clean ultracentrifuge tube. The tube was filled with CsCl/TE (RI=1.395) as before and a second round of ultracentrifugation at 50,000rpm (20°C) was performed for 48 hours.

The plasmid DNA band was removed from the tube as described above and the ethidium bromide in the solution removed by extracting with an equal volume of TE saturated butan-2-ol. The solution was mixed and the ethidium bromide separated with the upper organic phase which was carefully removed and discarded into appropriate waste bottles. The extraction with butanol was repeated until the lower aqueous phase was clear and colourless. The plasmid DNA was ethanol precipitated directly (section 2.3.2.2) or dialysed.

To dialyse, the plasmid DNA solution was transferred into a dialysis tube (Collodion Bag) and placed in a large beaker containing 2.5 litres of TE. Plasmid DNA was dialysed against the TE on a magnetic stirrer for 4 hours at room temperature. The TE buffer was changed and dialysis continued for a further 4 hours at room temperature or overnight at 4°C. After dialysis, plasmid DNA was ethanol precipitated as detailed in section 2.3.2.4.

2.3.2.13 DNA Sequencing

The sequence of all new plasmids was checked using Taq terminator sequencing on an Applied Biosystems 373A automated DNA sequencer which was performed by Beatson Institute technical servives staff.

The region to be sequenced first underwent PCR amplification. 0.5μg of template DNA was added to 12μl RQ grade H_20 plus 3.2pmoles the appropriate primer. 8μl of dye terminator cycle sequencing ready reaction premix was then added to each reaction volume contained in 250μl thin walled eppendorf tubes. The sample were placed in a PTC-100 programmable thermal controller (Genetic Research Instrumentation Ltd) and exposed to 25 cycles of 95°C for 30secs, 50°C for 15secs and 60°C for 4min. The PCR products were ethanol precipitated as detailed in section

2.3.2.4, washed with 70% ethanol and finally dried under vacuum before being given to a member of technical services for loading onto the sequencing gel.

2.3.2.14 End Labelling Double Stranded Oligonucleotide

Single stranded oligonucleotides were synthesised using Cruachem chemicals and an Applied Biosystems 392 DNA/RNA automated oligonucleotide synthesising machine by Beatson Institute technical services. Oligonucleotides were deprotected by incubating at 55°C overnight, ethanol precipitated and resuspended in TE buffer. The concentration of oligonucleotide solutions was measured spectophotometrically at described in section 2.3.2.3. However, an optical density reading of 1 at 260nm (Abs_{260nm}) for a single stranded oligonucleotide is roughly equivalent to a solution of 33μg/ml compared to 50μg/ml for double stranded DNA.

Complementary single stranded oligonucleotides were mixed together in equimolar amounts, heated to 95°C for 5 minutes and then allowed to cool slowly to room temperature which enabled the two strands to anneal together into double stranded molecules. The concentration of the double stranded oligonucleotide solution was measured spectrophotometrically and diluted with TE buffer to give a final concentration of 0.1mg/ml. Oligonucleotide solutions were stored at -20°C.

Double stranded oligonucleotides with sticky ends were end labelled as follows. Using a random primed labelling kit (Boehringer Mannheim), 1μl (0.5nmol) dCTP, 1μl (0.5nmol) dATP and 1μl (0.5nmol) dGTP solutions were added to 200ng of double stranded oligonucleotide. The reaction volume was made up to 12μl with distilled water. 2μl of 10x Klenow buffer (0.5M Tris-Cl (pH7.2), 0.1M $MgSO_4$, 1mM DTT, 0.5mg/ml bovine serum albumin (BSA - fraction V)) was added followed by 5μl [α-^{32}P]dTTP and 1μl (2 units) Klenow enzyme (also taken from the random primed labelling kit). The contents of the reaction were mixed and incubated at 37°C for 30 to 60 minutes. The radiolabelled oligonucleotide was separated from unincorporated nucleotides by polyacrylamide gel electrophoresis as described in section 2.3.2.15.

2.3.2.15 Purifying Radioactive Probe using Polyacrylamide Gel Electrophoresis

A 6% non-denaturing polyacrylamide gel was made by adding 15% (v/v) 40% (w/v) acrylamide:2.1% (w/v) bisacrylamide solution to 0.5x TBE buffer (89mM Tris

base, 89mM orthoboric acid, 2mM EDTA (pH8.0)). Polymerization of the gel was catalysied by the addition of 0.07% APS and 0.08% TEMED. After mixing thoroughly, the solution was poured between two glass plates which had been siliconized with repelcote, washed with detergent and cleaned with 70% ethanol prior to use. A comb forming sample wells was placed in the top of the gel immediately after pouring. The gel, 2mm thick, was left to polymerize in a verticle position for 1 hour before use.

The gel spacer was removed and the gel, formed between the two glass sheets, was placed vertically into the electrophoresis tank (ATTO) containing 0.5x TBE and secured in place. 0.5x TBE buffer was added to the top reservoir of the tank to cover the top of the gel. The comb was removed carefully from the gel and each well rinsed out with 0.5x TBE buffer using a 20ml syringe. The gel was run for 15 minutes at 150 volts to equilibrate the gel before any samples were loaded.

The radioactively labelled sample(s) was loaded into a single gel well. No loading dye was added to the sample, however 10µl of bromophenol blue loading buffer was added to the extreme wells to monitor the gels progress. The sample(s) was electrophoresed at 150 volts at room temperature until the blue dye front was approximately 5cm from the end of the gel. The position of each radiolabelled oligonucleotide was determined by exposing the gel, covered with cling film, to a sheet of Fuji RX medical X-ray film for 1-2 minutes. Using the X-ray film as a guide, the band containing the labelled probe was excised from the gel using a clean scalpel blade. The gel slice was transferred to a clean eppendorf tube and TE buffer was added to the tube sufficient to cover the gel slice. The tube was placed in a lead pot, sealed and left at room temperature overnight during which time the oligonucleotide eluted from the gel slice into the TE buffer. The polyacrylamide gel slice was removed from the solution and the radioactive oligonucleotide solution stored at -20°C.

2.3.2.16 Electrophoretic mobility Shift Assay (EMSA)

PalF cells were harvested for their nuclear extract as described previously in section 2.3.1.9c. Samples of nuclear extract were removed from storage at -70°C and thawed on wet ice. To 5µl (10-15µg) of nuclear protein extract, still on ice, 5µl 10x binding buffer (100mM HEPES (pH7.9), 2mM EDTA, 1M NaCl, 1mg/ml BSA, 40%

glycerol), 60μg/ml polydIdC and 4.4mM DTT was added followed by 1μl (~1ng) of radioactively labelled double stranded oligonucleotide DNA (section 2.3.2.14). 100ng of various unlabelled (cold) double stranded oligonucleotide was added to selected samples for competition analysis. The volume of each reaction mix was made up to 50μl with distilled water, mixed and incubated on ice for 30 minutes.

Each sample was loaded into a separate well on a 5% non-denaturing polyacrylamide gel prepared as described in section 2.3.2.17. 5μl loading dye mixed with 45μl 1x binding buffer was added to the end wells on the gel to allow migration of the samples down the gel to be monitored. Samples were electrophoresed at 150 volts at 4°C until the blue dye front in the tracking wells was ~3cm from the end of the gel. The gel was transferred onto Whatman 3MM filter paper and dried down on a Biorad 583 gel drier at 80°C for 2 hours using a slow rise in temperature cycle. The dried gel was placed in a lead, light tight casette with intensifying screens, covered with a sheet of Fuji RX medical X-ray film and placed at -70°C overnight.

2.3.2.17 Non-denaturing Polyacrylamide Gel electrophoresis

A pair of 2mm ATTO glass plates were siliconized with repelcote, washed with detergent and cleaned with 70% ethanol prior to use. A 5% polyacrylamide gel was made by adding 12.6% (v/v) of 40% (w/v) acrylamide:2.1% (w/v) bisacrylamide solution to 0.5x TBE buffer (89mM Tris base, 89mM orthoboric acid, 2mM EDTA (pH8.0)). Polymerization of the gel was catalysied by the addition of 0.07% APS and 0.08% TEMED. After mixing thoroughly, the solution was poured between two glass plates held securely together in the gel forming apparatus (ATTO) . A comb to make the samples wells was placed in the top of the gel immediately after pouring. The gel was left to polymerize in a verticle position for 1 hour before use.

The gel spacer was removed and the gel, formed between the two glass sheets, was placed vertically into the electrophoresis tank (ATTO) containing 0.5x TBE and secured in place. The upper reservoir was filled with 0.5x TBE buffer, the comb was removed carefully from the gel and each well rinsed out with 0.5x TBE buffer using a 20ml syringe. The gel was run for 15 minutes at 150 volts prior to use to equilibrate the gel.

2.3.2.18 Electrophoretic mobility SuperShift Assay

Supershift assays provide a means of identifying a protein which shows binding affinity to a short sequence of DNA. The protocol employed in supershift assays was essentially identical to that detailed in section 2.3.2.16 except that antibody was added to the binding reaction before the addition of radiolabelled oligonucleotide. The antibody used is specific for a protein, or family of related proteins, which is suspected of binding to the oligonucleotide used in the EMSA assay.

2μl (0.2ng) of antibody was added to a reaction mix containing 5μl (10-15ug) nuclear extract, 1x binding buffer (10mM HEPES (pH7.9), 0.2mM EDTA, 0.1M NaCl, 0.1mg/ml BSA, 4% glycerol), 60μg/ml polydIdC and 4.4mM DTT. This reaction mix was incubated on ice for 30 minutes after which time 1μl (~1ng) of radiolabelled double stranded oligonucleotide was added to the mix. The whole reaction was incubated on ice for a further 30 minutes. The samples were electrophoresed on a 5% non-denaturing polyacylamide gel (section 2.3.2.17) and after the gel was dried it was exposed to X-ray film as detailed in section 2.3.2.16.

2.3.3 Biochemistry

2.3.3.1 Determination of Protein Concentration in Cell Lysis Samples

Cells were harvested for total cellular protein as described in section 2.3.1.9b. The concentration of protein in a sample was determined by application of the BCA/$CuSO_4$ protein assay.

Proteins reduce alkaline Cu(II) to Cu(I) in a concentration-dependent manner. Bicinchoninic acid is a highly specific chromogenic reagent for Cu(I), forming a purple complex with an absorbance maximum at 562nm.

A small volume of each protein sample was diluted 1/10 and then 10μl of undiluted and diluted protein solution were placed in separate wells in a 96 well plate. 200μl of developing solution (5ml BCA (Bicinchoninic acid) solution, 100μl of 4% (w/v) $CuSO_4$ (copper II sulphate pentahydrate solution) was added to the diluted and undiluted protein samples and the plate incubated at 37°C for 1 hour.

The absorbance of each sample was read at 590nm using a Dynatech MR7000 automatic plate reader. The absorbance reading was converted to concentration in μg/μl for each sample using a standard curve generated from a series of control BSA solutions of known concentration. The actual concentration of each protein sample was calculated after multiply by the relevant dilution factor.

2.3.3.2 SDS-Polyacrylamide Gel Electrophoresis and Western Blotting

The resolving gel, containing 8-11% polyacrylamide in 1x resolving gel buffer-pH8.8 (4x RGB: 1.5M Tris, 0.4% SDS - pH to 8.8 with HCl) was poured between two glass plates siliconised and cleaned as described in section 2.3.2.17. 0.08% TEMED and 0.07% APS were also added to the liquid resolving gel mixture, before the gel was poured, to catalyse polymerisation. The top of the gel was covered with water saturated butan-2-ol and allowed to polymerise for 1 hour. The butanol was poured off and the top of the gel washed with water and blotted dry. The stacking gel consisting of 5-7% polyacrylamide in 1 x stacking gel buffer-pH6.8 (4x SGB: 0.5M Tris, 0.4% SDS - pH to 6.8) was poured on top of the resolving gel, a comb inserted and left to polymerise for at least 30 minutes before use. Again TEMED (0.08%) and APS (0.07%) were added to the gel mix to catalyse polymerisation. Due to the difference in pH between the stacking and resolving gels, the stacking gel should only be poured up to 1 hour before use to minimise merging of the pH's.

Equivalent amounts of each protein sample (20-40μg) was mixed with an equal volume of 2x SDS gel loading buffer (100mM Tris-Cl (pH6.8), 2% (v/v) β-mercaptoethanol, 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol), boiled for 3 minutes then placed immediately on ice. Samples were loaded into consecutive wells and 5μl Rainbow™ protein molecular weight marker mix (molecular weight range 14.3KD-200KD) added to the first or last well on the gel. The gel was run at 80mA (constant current) for several hours or at 25mA overnight in 1x Tris-glycine buffer (25mM Tris, 250mM glycine, 0.1% (w/v) SDS) at room temperature until the sample dye front was ~1-2cm from the end of the gel.

The separated protein bands were visualised by placing the gel in 20-30ml staining solution (0.25% Coomassie Brilliant Blue (R250) dissolved in 45% (v/v) methanol, 45% (v/v) H_2O, 10% (v/v) glacial acetic acid) at room temperature on a slowly shaking platform for 20min followed by destaining in the same solution

without coomassie blue. Several changes of destain solution must be made for background staining to be minimised. The stained gel was dried down onto Whatman 3MM paper using a Biorad 583 gel drier.

The gel **was not stained** with Coomassie blue if the proteins were to be Western blotted onto a nitrocellulose filter. Instead, one of the glass plates was removed and unused portions of the gel, including the stacking gel, were cut away and disgarded. The gel was rinsed twice in distilled water and placed on top of two sheets of Whatman 3MM paper, cut to the same size as the gel, which had been soaked in Towbin buffer (0.3% (w/v) Tris, 0.19M glycine, 10% (v/v) methanol, 0.03% (v/v) conc. HCl) (Towbin *et al*, 1979). A piece of nitrocellulose or Hybond C^{extra} membrane, cut to the same size as the gel, was soaked in Towbin buffer and placed carefully on top of the gel. Finally two more pieces of Towbin soaked Whatman 3MM paper cut to gel size were placed on top of the membrane and the whole assembly placed in a Bio-Rad cassette between two sponges which had been presoaked in Towbin. It is very important that no air bubbles exist between any of the paper, gel or membrane layers as these will interfer with transfer. The whole sandwich was placed in the Bio-rad wet transfer tank, the nitrocellulose side of the sandwich being positioned closest to the cathode. The tank was filled with Towbin buffer and the transfer performed overnight at 4°C at 30 volts.

After transfer the gel was stained with Coomassie blue staining solution as described above and the filter stained for 5 minutes in a solution of Ponseau S which allowed the fidelity of transfer and loading consistency to be checked. Ponseau S stain is a temporary stain which can be washed off the membrane with water. Proteins on the filter were detected using antibodies as described in section 2.3.3.3.

2.3.3.3 Phosphotyrosine Protein Detection on Western Blot

The filter was placed in 100ml blocking buffer (5% (w/v) Marvel (dried milk) in PBS-T (0.1% (v/v) in PBS)) and incubated at room temperature for 2 hours or at 4°C overnight on a gently shaking platform. If the filter was blocked overnight, the blocking buffer was changed and the filter blocked at room temperature for 1 hour before addition of the primary antibody solution. The filter was placed in 25ml blocking buffer containing PY20 antiphosphotyrosine mouse monoclonal antibody at a 1/5000 dilution and incubated at room temperature for 1 hour with gentle agitation.

The primary antibody solution was removed and filter rinsed in blocking buffer then washed 3 times, each for 15 minutes, in 100ml volumes of fresh blocking buffer. The filter was then incubated in 25ml blocking buffer containing a 1/1000 dilution of anti-mouse IgG horse-radish peroxidase linked whole antibody for 1 hour at room temperature with gentle agitation.

The filter was rinsed, washed twice for 15 minutes in blocking buffer and finally washed for 15 minutes a further three times in PBS-T buffer only. Excess surface liquid was removed from the filter by briefly blotting with a piece of Whatman 3MM paper. Antibody was detected by immersing the filter in ECL detection reagent for 1 minute, the filter wrapped in cling film and exposed to Kodak X-OMAT S (fast) x-ray film at room temperature over a range of time intervals.

Chapter 3

The Effect of Quercetin on the Growth Characteristics of PalF cells

Chapter 3 The effect of quercetin on the growth characteristics of PalF cells

3.1 Introduction

Quercetin (3,3',4',5,7-pentahydroxy flavone) is a member of a large class of natural substances called flavonoids which display a wide variety of biological actions (Gabor, 1988). Quercetin is one of the more extensively studied flavonoids and is present in most edible fruits and vegetables (Kuhnau,1976); humans are estimated to consume approximately 1 gram of mixed flavonoids each day as part of a normal diet, and of this amount quercetin is estimated to constitute 50 to 500mg (Jones and Hughes, 1982). Quercetin is classified as a weakly toxic drug and doses of 1g per day per adult has been shown to have no serious side effects (Havsteen, 1983). It has been previously used therapeutically for the treatment of inflammation, allergy, bee sting and ulcer (Hofmann *et al.*, 1990).

Quercetin in the diet has been proposed to have beneficial qualities, namely as a chemopreventive agent (Verma *et al.*, 1988; Deschner *et al.*, 1991; Kato *et al.*, 1983) and as an inhibitor of tumour growth and invasion (Bracke *et al.*, 1988; Okada *et al.*, 1990; Scambia *et al.*, 1990c, 1992; Ranelletti *et al.*, 1992). These positive qualities of quercetin have been related to its antioxidant and growth inhibitory properties. As an antioxidant quercetin displays free radical scavenging activity (Bors and Saran, 1987; Negre-Salvayre and Salvayre, 1992; Morel *et al.*, 1993). However, quercetin can also function as a prooxidant; auto-oxidation of quercetin has been shown to lead to the concomitant production of semiquinone radicals, superoxide anion radicals (O_2^{+}), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\cdot}OH$) (Elliott *et al.*, 1992). The prooxidant actions of quercetin may contribute to the compound's genotoxicity (Fazal *et al.*, 1990; Rahman *et al.*, 1990; Elliott *et al.*, 1992). Furthermore, the ability of quercetin to behave as a pro- or antioxidant appears to be dependent on the redox state of the cellular background (Plaumann *et al.*, 1996).

Quercetin has been shown to be a genotoxic agent (Bjeldanes and Chang, 1977; Brown, 1980; Nagao *et al.*, 1981); it can induce mutations in both prokaryotic and eukaryotic cells (Bjieldanes and Chang, 1977; MacGregor and Jurd, 1978; Amacher *et al.*, 1979; Maruta *et al.*, 1979; Nakayasu *et al.*, 1986; Ishikawa *et al.*, 1987) and cause clastogenic damage (Yoshida *et al.*, 1980; Ishidate *et al.*, 1988). Work by Suzuki *et al.* (1991) revealed that quercetin could induce recombination of

minisatellite sequences in a mouse fibrosarcoma cell line and in a mouse mammary tumour cell line in culture. The genotoxicity of quercetin correlates with its ability to complex with DNA and induce strand breaks in the presence of Cu(II) ions and oxygen (Rahman *et al.*, 1990; Fazal *et al.*, 1990). The free radicals generated by quercetin in the presence of Cu(II) can also lead to the fragmentation of proteins such as albumin (Said Ahmed *et al.*, 1994).

Compounds which are demonstrated to possess mutagenic properties are often shown to concurrently function as carcinogens. However, while the studies outlined above demonstrate that quercetin can act as a mutagen, few reports have identified quercetin to be carcinogenic. A study by Pamukcu and co-workers found that albino non-inbred male and female rats fed on a diet containing 0.1% quercetin for approximately one year developed multiple tumours of the ileal intestine and bladder (Pamukcu *et al*, 1980). In other long term animal feeding experiments, where quercetin was given at various concentrations in the diet to a number of different rodent species, quercetin was not found to be carcinogenic (Hirono, 1981; Hirono *et al.*, 1981; Morino *et al.*, 1982). The carcinogenicity of quercetin observed by Pamuku *et al.* (1980) may be partially attributable to the genetic background and/or microflora in the gut of the experimental animals used; Norwegian rats may be particularly sensitive to the carcinogenic effects of quercetin. The purity of quercetin use by Pamukcu *et al.* (1980) was reported as >99% compared to >95% in other studies. It is possible, although unlikely, that the less pure quercetin preparations contained a potent inhibitor of quercetin-induced carcinogenesis.

An interesting report by Ishikawa *et al.* (1987) showed that a mouse fibrosarcoma cell line (BMT-11 cl-9) treated with quercetin *in vitro* and then transplanted into C57Bl/6 female mice, lead to the appearance of both regressing and progressing tumours. Thus, in these experiments, quercetin was shown to possess both a tumour promoting and anti-tumourigenic properties. The authors suggest that the fate of cells exposed to quercetin is determined by the specific spectrum of genes altered genetically, epigenetically or both. A later study by the same group suggested that tumour regression is associated with a decrease in prostaglandin E_2 production (Okada *et al.*, 1990).

The carcinogenicity of quercetin in the presence of other carcinogenic compounds has also been studied. The cooperative effects of 7,12-

dimethylbenz[a]antracene (DMBA) and 12-O-tetradecanoylphorbol-13-acetate (TPA) in the production of skin tumours in mice has been extensively studied and well characterised (Balmain and Pragnell, 1983; Balmain *et al.*, 1984). From such experiments TPA has been classified as a tumour promoter while DMBA has been shown to function as a tumour initiating agent. Quercetin has been observed to inhibit the promotive effects of TPA on skin tumours initiated by DMBA in a mouse skin model (Kato *et al.*, 1983). The ability of quercetin to inhibit TPA-induced tumour promotion has been associated with the concomitant inhibition of epidermal lipoxygenase activity (*ibid.*). Deschner and co-workers found that quercetin in the diet could inhibit azoxymethanol (AOM)-induced colonic tumours in CF1 female mice (Deschner *et al.*, 1991); Verma *et al.* (1988) observed that DMBA and N-nitrosomethylurea-induce rat mammary cancers were similarly inhibited by dietary quercetin.

Finally, quercetin has further been shown to synergise with known anti-proliferative drugs such as cisplatin (C-DDP), busulphan and adriamycin to inhibit tumour growth both *in vitro* (Hoffman *et al.*, 1988; Hofmann *et al.*, 1989; Scambia *et al.*, 1990a, 1994b) and *in vivo* (Hofmann *et al.*, 1990; Scambia *et al.*, 1992).

In direct contrast to the results described above, quercetin given orally was shown to cause a dose-dependent enhancement of tumours induced by azoxymethane (AOM) in a rat colon model system (Pereira *et al.*, 1996). In this setting, quercetin exhibits co-carcinogenic activity, functioning as a tumour promoting agent, analogous to the actions of TPA in the skin mouse model (Kato *et al.*, 1983). It is particularly interesting to compare the work of Deschner *et al.* 1991 and Pereira *et al.* 1996; in both studies, the duration of the experiment was very similar, as was the purity and concentration of quercetin in the diet. The inducer (AOM) and amount given was identical, however while both groups gave their animals 30mg AOM per kg body weight (30mg/kg) in total, Deschner *et al.* (1991) administered 3 separate sub-cutaneous injections of 10mg AOM per kg body weight (10mg/kg) compared to a single sub-cutaneous injection by Pereira *et al.* (1996). Perhaps the most notable difference between the two studies was that Deschner *et al.* (1991) used CF1 female mice whereas Pereira *et al.* (1996) used male Discher 344 rats. The age of the animals in both studies was very similar. In both experiments quercetin was shown not to inhibit the formation of aberrant crypt foci or nuclear aberrations, two alterations

attributable to the actions of AOM. Hence, it would appear that quercetin does not inhibit the initiating effects of AOM.

In support of the results obtained by Pereira *et al.* (1996), quercetin has further been demonstrated to modulate the mutagenic properties of aromatic amines and acetamides (Ogawa *et al.*, 1987a, 1987b), to enhance tumour induction by methylcholanthrene (Ishikawa *et al.*, 1985) and act as a tumour initiator in co-operation with TPA in a two-stage transformation assay in mammalian cells *in vitro* (Sakai *et al.*, 1990). Finally, quercetin was shown to transform normal primary PalF cells to a tumourigenic phenotype in nude mice in cooperation with BPV4 sequences and an activated *ras* gene (see section 1.7.2 for details; Pennie and Campo, 1992).

In keeping with the *in vivo* observations outlined above, quercetin has also been reported to have contradictory effects on various cell lines *in vitro*. The vast majority of work published however describes a growth inhibitory role of quercetin in a wide range of tumour cell lines derived from numerous cell types and anatomical sites. The growth of several leukaemic cell lines (Scambia *et al.*, 1990b; Yoshida *et al.*, 1992; Post and Varma, 1992) as well as acute myeloid leukaemia (AML) and acute lymphoid leukaemia (ALL) primary leukaemic blasts (Larocca *et al.*, 1990) has each been inhibited by quercetin. Furthermore quercetin was shown to inhibit AML and ALL progenitors in colony forming assays (Larocca *et al.*, 1991,1995). In addition to leukaemic cells, quercetin exerts growth inhibitory action on human mammary and breast cancer cell lines (Markaverich *et al.*, 1988; Scambia *et al.*, 1991, 1993), human ovarian tumour and cultured cancer cells (Scambia *et al.*, 1990c, 1992, 1994a), human meningiomas (Piantelli *et al.*, 1993), human melanoma cells (Piantelli *et al.*, 1995), and colon-cancer cell lines and primary colorectal tumours (Piantelli *et al.*, 1990; Hosokawa *et al.*, 1990a; Ranelletti *et al.*, 1992). In most of these studies, quercetin was seen to affect growth inhibition in a concentration-dependent manner. In addition to assessing the general growth inhibitory action of quercetin, the effect of quercetin on cell cycle status in some cell lines has also been analysed.

Quercetin was seen to elicit a G_1/S phase arrest in human gastric cancer cells (IC50 of 32-55μM) (Yoshida *et al.*, 1990) and human colon cancer cells (Hosokawa *et al.*, 1990a). A late G_1 arrest was observed when human leukaemic T-cells (70μM) (Yoshida *et al.*, 1992) and human peripheral blood lymphocytes (Gong *et al.*, 1994) were exposed to quercetin. In the latter experiments, cell cycle arrest followed the

onset of cyclin E expression (Gong *et al.*, 1994). OVCA 433 cells (ovarian cancer cells)(Scambia *et al.*, 1990c) and colon cancer cell lines (Ranelletti *et al.*, 1992) were all blocked in the G_0/G_1 phase of the cell cycle by quercetin at concentrations ranging from 10nM to 10μM.

However not all cells were induced to stop in the G_0/G_1 to late G_1/S phase of the cell cycle. MDA-MB468 cells (human breast cancer cell line) arrested at the G_2/M step in the cell cycle following a six day exposure to 30μg/ml (~89μM) quercetin (Avila *et al.*, 1994); arrest of the MDA-MB468 cells in G_2/M correlated with the inhibition of translation of endogenous mutant p53. p53 mediated G_2/M arrest was similarly observed in a non-tumour cell line C3H10T1/2CL8 (Plaumann *et al.*, 1996). In contrast to the results of Avila *et al.* (1994), this G_2/M arrest was not observed in association with mutant p53 inhibition. Rather, quercetin was found to induce the accumulation of wild type p53. In addition to the G_2/M arrest, quercetin further induced apoptosis in C3H10T1/2CL8 cells which occurred out of the G_2/M phase. The G_2/M arrest and apoptosis observed in these cells following a 72hr exposure to 120μM quercetin was determined to be p53 dependent as the same effects were not observed in p53 knockout fibroblasts (Plaumann *et al.*, 1996). Although p53 is generally associated with blocking the cells at the G_1/S transition point in the cell cycle, several reports have also identified a role for p53 in the establishment of a G_2/M checkpoint later in the cell cycle (Agarwal *et al.*, 1995; Siegel *et al.*, 1995; Stewart *et al.*, 1995).

In relation to the cell cycle/growth inhibitory effects of quercetin, a number of cellular target molecules, in addition to p53 discussed above, have been implicated in mediating the actions of quercetin. Many reports identify the ability of quercetin to interact with estrogen type II binding sites (type-II EBS). Type-II EBS, originally described by Clark *et al.*, (1978) in the rat uterus, while displaying the same steroid and tissue specificity seen for the 'true' estrogen receptor (ER), occur at higher concentrations and exhibit a lower affinity for its ligand, estradiol, compared to ERs (Markaverich *et al.*, 1979; Syne *et al.*, 1982). Indeed, the binding affinity of type-II EBS for estradiol is so low, it is unlikely that these sites would be occupied by estrogens *in vivo*. Markaverich *et al.* (1990) demonstrated that type-II EBS in normal and malignant cells are occupied by a flavonoid-like ligand which displays growth inhibitory activity. Furthermore, type-II EBS but not ERs in several human cancer

cells lines bind flavonoids (Markaverich *et al.*, 1983) and inhibit cell growth possibly by mimicking the endogenous ligand (Markaverich *et al.*, 1984).

Quercetin has subsequently been shown to induce type-II EBS. Levels of type-II EBS is human breast-cancer cell lines were seen to increase following exposure to quercetin (Scambia *et al.*, 1993). The ability of quercetin to induce type-II EBS was well correlated with the relative binding affinity of quercetin for type-II EBS. More importantly, quercetin-induced enhancement of type-II EBS was accompanied by increased sensitivity of the breast cancer cell lines to the inhibitory effects of quercetin (Scambia *et al.*, 1993). Other reports further support a role for type-II EBS in mediating the actions of quercetin. The growth inhibitory potential and sensitivity of colon-cancer cell lines and primary tumours to quercetin correlated with the affinity for and number of type-II EBS per cell respectively (Ranelletti *et al.*, 1992). Type-II EBS have likewise been associated with the growth inhibiting actions of quercetin in primary and cultured ovarian cells (Scambia *et al.*, 1990b, 1990c), human menigiomas (Piantelli *et al.*, 1993) and melanoma (Piantelli *et al.*, 1995) as well as several myeloid and lymphoid leukaemic cells (Larocca *et al.*, 1990; Teofili *et al.*, 1992) and normal bone marrow (Larocca *et al.*, 1991).

Although the reports described above implicate type-II EBS in at least partially mediating the growth inhibitory effects of quercetin, the complete mechanism of action of quercetin is still poorly understood. TGFβ1 (transforming growth factor-β) has been presented as another potential candidate involved in quercetin-induced growth inhibition (Scambia *et al.*, 1994a; Larocca *et al.*, 1995). TGFβ1 is one member of a family of structurally related proteins (Keller *et al.*, 1992; Jacobsen *et al.*, 1993). It has been isolated from normal and neoplastic cells (Roberts and Sporn, 1985) and can significantly influence the growth and differentiation of numerous cell types. It can stimulate and/or inhibit the growth of myeloid progenitor cells depending on their differentiation status (Aglietta *et al.*, 1989; Hatzfeld *et al.*, 1991; Jacobsen *et al.*, 1991; Bursuker *et al.*, 1992; Keller *et al.*, 1992) and has further been shown to inhibit the growth of several human cancer cell lines (Knabbe *et al.*, 1987; Zugmaier *et al*, 1989; Berchuck *et al.*, 1990).

Quercetin has been shown to enhance TGFβ1 secretion from human ovarian cancer cells (Scambia *et al.*, 1994a). In this study, quercetin-induced growth inhibition was reversed by addition of a TGFβ1 neutralising antibody. Furthermore

the induction of TGFβ1 following exposure to quercetin appeared to be specific as other anti-proliferative compounds were seen to have no effect on TGFβ1 secretion (*ibid.*). In a second study, quercetin was found to induce TGFβ1 expression in leukaemic progenitor cells (Larocca *et al.*, 1995). Analogous to the work by Scambia *et al.* (1994a), the use of TGFβ1 antisense oligonucleotides and neutralising monoclonal antibodies was shown to block the growth-inhibitory action of quercetin (*ibid.*).

It has been proposed that TGFβ1 expression is a consequence of type-II EBS induction. This is supported by experiments using other anti-proliferative drugs, such as tamoxifen, which like quercetin is known to bind type-II EBS. Tamoxifen, an anti-estrogen used in the treatment of breast cancer, has been demonstrated to bind type-II EBS and subsequently induce TGFβ1 production (Knabbe *et al.*, 1987; Colletta *et al.*, 1990).

Type-II EBS and TGFβ however are not the only possible mechanism(s) through which quercetin may be achieving growth inhibition. Quercetin is also reported to deregulate and/or inhibit the actions of a large number of enzymes including ATP-utilizing enzymes, such as protein kinase C (PKC) (Gschwendt *et al.*, 1983), phosphatidylinositol 3-kinase (PI3-kinase) (Matter *et al.*, 1992), the Na,K-ATPase (Lang and Racker, 1974; Kuriki and Racker, 1976), the *src* encoded tyrosine kinase (Graziani *et al.*, 1983) as well as several other tyrosine kinases (Srivastava and Chiasson *et al.*, 1986) by competitive inhibition of ATP binding. Inhibition of enzyme activity, particularly kinases and phosphatases which mediate signal transduction signals, represents another way in which quercetin interferes with cellular proliferation. Quercetin can inhibit several other enzymes including cyclo-oxygenase and lipoxygenase (Kato *et al.*, 1983; Elia and Santoro, 1994). It has been suggested that lipoxygenase products are essential for tumour promotion (Fischer *et al.*, 1982), therefore inhibition of lipoxygenase by quercetin may constitute another putative mechanism by which quercetin prevents tumour promotion (Kato *et al.*, 1983; Nakadate *et al.*, 1984). In addition to the inhibition of specific enzyme activities, quercetin has also been shown to increase cyclic AMP levels (Graziani *et al.*, 1979), inhibit aerobic glycolysis, DNA, RNA and protein synthesis, and the transport of lactate, D-glucose and Ca^{2+} (Hosokawa *et al.*, 1990b; Yoshida *et al.*, 1992 and

references therein; Graziani *et al.*, 1983 and references therein; Uddin and Choudhry, 1995).

The concentration of quercetin capable of inhibiting tumour cell growth *in vitro* is in the range of 1nM to 1μM (Scambia *et al.*, 1993; Piantelli *et al.*, 1995). A quercetin concentration at this level has been shown to affect type-II EBS but is lower than the concentrations reported to inhibit many enzyme activities (≥50μM) (Elliott *et al.*, 1992; Lang and Racker, 1974; Monaham *et al.*, 1975; Kuriki and Racker, 1976; Shoshan and MacLennam, 1981). This would suggest that the growth inhibitory effects of quercetin at concentrations ≤1μM involves a mechanism mediated by type-II EBS. However not all cells will express type-II EBS and therefore higher local concentrations of quercetin may potentially be needed for significant growth inhibition to be realised. Notwithstanding, some enzymes, including lipoxygenase and PI3-kinase, and also certain cellular processes such as lactate transport and glycolysis, are more sensitive to quercetin and have been shown to be inhibited at quercetin concentrations similar to those sufficient to induced type-II EBS (Kato *et al.*, 1983; Matter *et al.*, 1992; Belt *et al.*, 1979). Therefore, whatever the mechanism, it is quite probable that quercetin achieves growth inhibition by targeting several cellular mechanisms either individually or simultaneously. The specific cell type and local concentration of active quercetin will also dictate how quercetin will ultimately affect the cells' normal activity.

Analysis of the anti-proliferative action of quercetin described above has been performed using, almost exclusively, tumourigenic cell lines or tumour material. Even when non-oncogenic cell lines were used, the cells were nonetheless immortal. The effect of quercetin on primary, non-transformed cells of any origin has not been assessed in any detail. It is therefore not known if there is a differential response to quercetin by normal, primary cells compared to immortal or tumourigenic cells. Determining the sensitivity of various cell types at different stages of transformation would be very relevant and beneficial when considering the use of quercetin as an anticancer agent; it would naturally be very convenient to have an agent which exclusively or more specifically inhibited the growth of tumour cells in comparison to normal cells. Therefore, in light of the growth inhibitory activity of quercetin observed for a large number of tumour cell lines by several independent groups, we

wished to evaluate the effect of various concentrations of quercetin on the growth of normal, primary fibroblasts (PalF cells) originally derived from the soft palate of a bovine foetus. Furthermore, and more importantly, with respect to earlier results where quercetin was shown to act as a co-carcinogen in the BPV4 transformation system (Pennie and Campo, 1992; Cairney and Campo, 1995), we wished to determine if PalF cells continue to grow in the presence of quercetin particularly in concentrations which were effective at inducing cellular transformation in cooperation with BPV4 E7 and an activated *ras*.

3.2 Analysis of Growth Rate of PalF cells in the presence of quercetin

Earlier experiments demonstrated that quercetin, given at a concentration of 20μM, was able to synergise with BPV4 (or subgenomic fragments of BPV4) and *ras* in the transformation of PalF cells to a fully tumourigenic phenotype (Pennie and Campo, 1992; Cairney and Campo, 1995). In an attempt to evaluate the growth of normal PalF cells in the presence of quercetin, PalF cells were grown in medium containing increasing concentrations of quercetin. Three separate assays were used to characterise the growth response of PalF to a range of quercetin concentrations: these assays were MTT, tritiated-thymidine uptake and direct counting of viable cells (see section 2.3.1.10 for experimental details).

Why use three assays to analyse essentially the same characteristic of PalF cells' behaviour in the presence of quercetin? By using three separate assays to look at the growth response of PalF cells quercetin, we would expect to find that the results from one assay generally confirmed or supported the results from the other assays, thus reinforcing that any effect of quercetin on PalF cell growth was genuine.

Each of the three assays used in the course of the work presented here have their own advantages. The manual counting of cells using a haemocytometer and visualising viable cells using dye exclusion and a light microscope (see section 2.3.1.10a) has previously been the most direct and frequently used method for determining cellular growth rates. However, direct cell counting is slow and relies on personal assessment and accuracy in counting the number of viable cells. Also, larger culture dishes/flasks requiring greater volumes of medium are needed. Therefore it

would be beneficial to find an alternative method which was accurate and consistent with direct cell counting but which may be performed more rapidly and easily.

The MTT assay features several advantages in that it can be performed in 96-well plates which means fewer cells and less medium is needed in each well, multiple replicates can be set up for each quercetin concentration being analysed, a wide range of quercetin concentrations can be assessed using a single 96-well plate, and all 96 wells can be read rapidly and simultaneously using a scanning multiwell spectrometer. The MTT assay is easy to do, convenient, quick and less labour intensive than the direct cell counting method. However, one disadvantage and important consideration when using the MTT assay with application to quercetin is that quercetin itself has been shown to directly reduce MTT (Habtemariam, 1995). Because this assay is dependent on the cellular reduction of MTT by the mitochondrial dehydrogenase of viable cells to the blue formazan product (Carmichael *et al.*, 1987), care had to be taken when quercetin was used in association with MTT. It was important, therefore, that all quercetin containing medium was removed from each well and that cell monolayers were washed with PBS before fresh medium containing MTT reagent was replaced (see section 2.3.1.10b for details of method employed). In this way the reduction of MTT could be attributed to the viable cells present per well and not to residual quercetin.

Like the MTT assay, the tritiated (H^3)-thymidine uptake assay was performed in 96-well plates. However, thymidine-uptake was not used as a means of assessing the number of viable cells on a daily basis. Rather, this assay provided information relating to the specific concentration of quercetin which produced a 50% reduction in the uptake of H^3-thymidine relative to control cells, that is, cells which were not exposed to quercetin. The concentration of quercetin (or any compound) which inhibits the uptake of H^3-thymidine by 50% is called the I.C.50 value. Indirectly, the I.C.50 reading can be related to the proliferation rate of cells as the uptake of thymidine is generally proportional to cell number.

By employing these three separate assays for assessing the proliferative response of PalF cells to quercetin in culture medium, it was hoped that comparisons could be made whereby the results of one assay would be supported by the results of the other two. Furthermore, by comparing the results of the MTT assay with those

obtained from direct cell counting, the accuracy and applicability of the MTT assay as a more convenient and rapid method of assessing the growth rate of PalF cells may be considered.

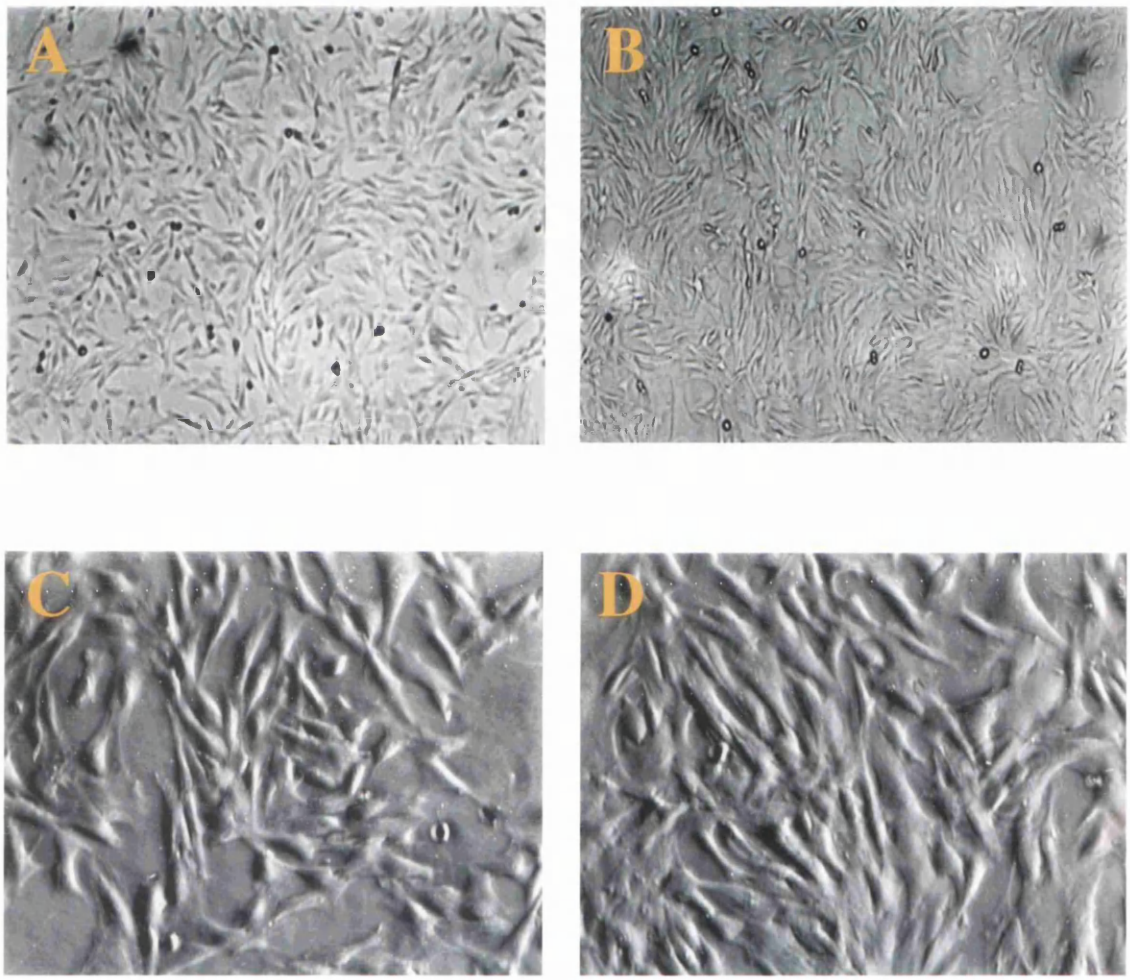
3.2.1 Experimental Procedure

Normal, low passage PalF cells were used in all three assays detailed below. Figure 3.1 shows photographs of normal, sub confluent PalF cells as viewed using a light microscope.

For direct cell counting, cells were seeded at $5x10^5$ or 10^6 cells per 90mm tissue culture dish as detailed in section 2.3.1.10a. Triplicate dishes were set up for each concentration of quercetin (1, 20, 50 and 100μM). Control dishes were also set up in triplicate where cells were incubated in medium alone or medium supplemented with 0.5% ethanol (0.5% v/v ethanol was equivalent to the volume of quercetin dissolved in ethanol which was added to medium to give final quercetin concentration of 100μM). Fresh medium, with or without quercetin/ethanol, was replenished in each dish every 24 hours in an attempt to keep the effective concentration of quercetin in the culture medium constant. Cell monolayers were trypsinised and counted, according to the procedure detailed in section 2.3.1.10a, when cells reached approximately 90% confluence. The growth of PalF cells was assayed according to this method over a 9 day time interval.

In the MTT assay, 1000 or 2500 cells were plated into each well in 96-well plates as indicated in section 2.3.1.10b. Following an overnight incubation during which time the cells adhered to the bottom of each well, the medium was changed. Where appropriate the medium was supplemented with quercetin to a final concentration of 0.1, 1, 10, 15, 25, 35, 50, 75, 100 or 200μM. Five wells of cells were exposed to each of the quercetin concentrations (quintuplicate wells). In addition to the specific quercetin concentrations, two control groups of five wells were each incubated in either medium alone or medium supplemented with 1% v/v ethanol (1% v/v ethanol was equivalent to the volume of quercetin in ethanol added to wells to give a final quercetin concentration of 200μM). Medium was replenished in all wells

Figure 3.1 Photographs of Normal Primary PalF cells



Panels A and B: Bright field images: final magnification x20

Panels C and D: Phase contrast images: final magnification x50

PalF cell monolayers in each photograph were approximately 90% confluent at the time pictures were taken.

every 24 hours. PalF cell proliferation rates were assayed using MTT for a period of 7 days.

Similar to the conditions set for the MTT assay, for the H^3-thymidine uptake assay PalF cells were seeded into separate wells in 96-well plates at a density of 1000 or 2500 cells per well (see section 2.3.1.10c) . Plates were incubated overnight to allow cells to adhere to the culture plate's surface. The following day, old medium was removed and replaced with either fresh medium alone, medium + 1% v/v ethanol, or medium containing quercetin at the appropriate concentration (0.1, 1, 10, 15, 25, 35, 50, 75, 100 or 200μM). Quintuplicate wells were set up for each culture medium condition. Medium (+/- quercetin or ethanol) was changed every 24 hours for the next three days. On the fourth day, H^3-thymidine in PalF medium was added to each well and the plates incubated in the appropriate atmosphere for a further 6 hours, as detail in materials and methods (section 2.3.1.10c).

3.2.2 Results

(a) Direct cell counting

Figure 3.2 shows the growth curves for PalF cells which were cultured in medium containing quercetin over a nine day period. The cell doubling time for untreated PalF cells was shown to be 36 to 48 hours. Quercetin present at 1μM has no significant effect on the growth of PalF cells. However, when quercetin was present at concentrations of 20μM and higher, the doubling time for PalF cells was increased. The higher the concentration of quercetin in the culture medium, the greater the inhibitory effect on PalF cell growth rate. Cell proliferation was almost totally inhibited when quercetin was present at concentrations ≥ 50μM. From the graphs, 20μM was the concentration of quercetin which was seen to inhibit the growth rate of PalF cells by more than 50%.

(b) MTT assay

Figure 3.3 corresponds to the second set of growth curves for PalF cells cultured in the presence of quercetin over a six day period. The absorbance readings are proportional to cell number. Consistent with the results shown in figure 3.2,

quercetin was seen in inhibit the growth rate of PalF cells in a concentration dependent manner. A quercetin concentration of 10 to 15μM was seen to inhibit the growth rate of PalF cells by approximately 50%.

(c) Tritiated thymidine uptake

Figure 3.4 illustrates the effect of increasing concentrations of quercetin on the uptake of H^3-Thymidine by PalF cells. A quercetin concentration of 25 to 35μM was effective at inhibiting the uptake of thymidine by PalF cells by approximately 50% (I.C.50 = 25-35μM). This is consistent with results obtained by other workers (Dr. Judith Brown, personal communication). The I.C.50 is slightly greater than the concentrations of quercetin which were found to inhibit the growth rate of PalF cells as indicated in the results shown in figure 3.2 and 3.3. One explaination for this is that the thymidine uptake assay involved treating PalF cells with quercetin for only three days compared to nine and six days in the growth rate assays described above.

Nevertheless, the results from each of the three independent assays showed that quercetin inhibited the growth of, and uptake of thymidine by, normal PalF cells in a concentration-dependent manner. The concentration of quercetin which inhibited the growth of PalF cells by approximately 50%, as determined by direct cell counting (~20μM) (Figure 3.2) and MTT (10-15μM) (Figure 3.3), correlated relatively closely with the I.C.50 value obtained from the H^3-thymidine uptake assay (25-35μM) (Figure 3.4). The results indicate that all three assays yield similar results. Furthermore, the results support the use of the MTT assay as a attractive alternative to direct cell counting as a rapid and convenient method of analysing the growth rate of PalF cells. Hence the MTT assay may be applied in future studies to analyse the growth response of PalF, PalF transfected cells or other similar cell types to quercetin and/or other test compounds. Finally, it must be stressed that care must always be taken when using quercetin in conjunction with MTT due to the potential of quercetin to independently reduce MTT.

Figure 3.2 Growth curves of PalF cells cultured in medium containing increasing concentrations of quercetin
Cell number was determined using the method of Direct Cell Counting

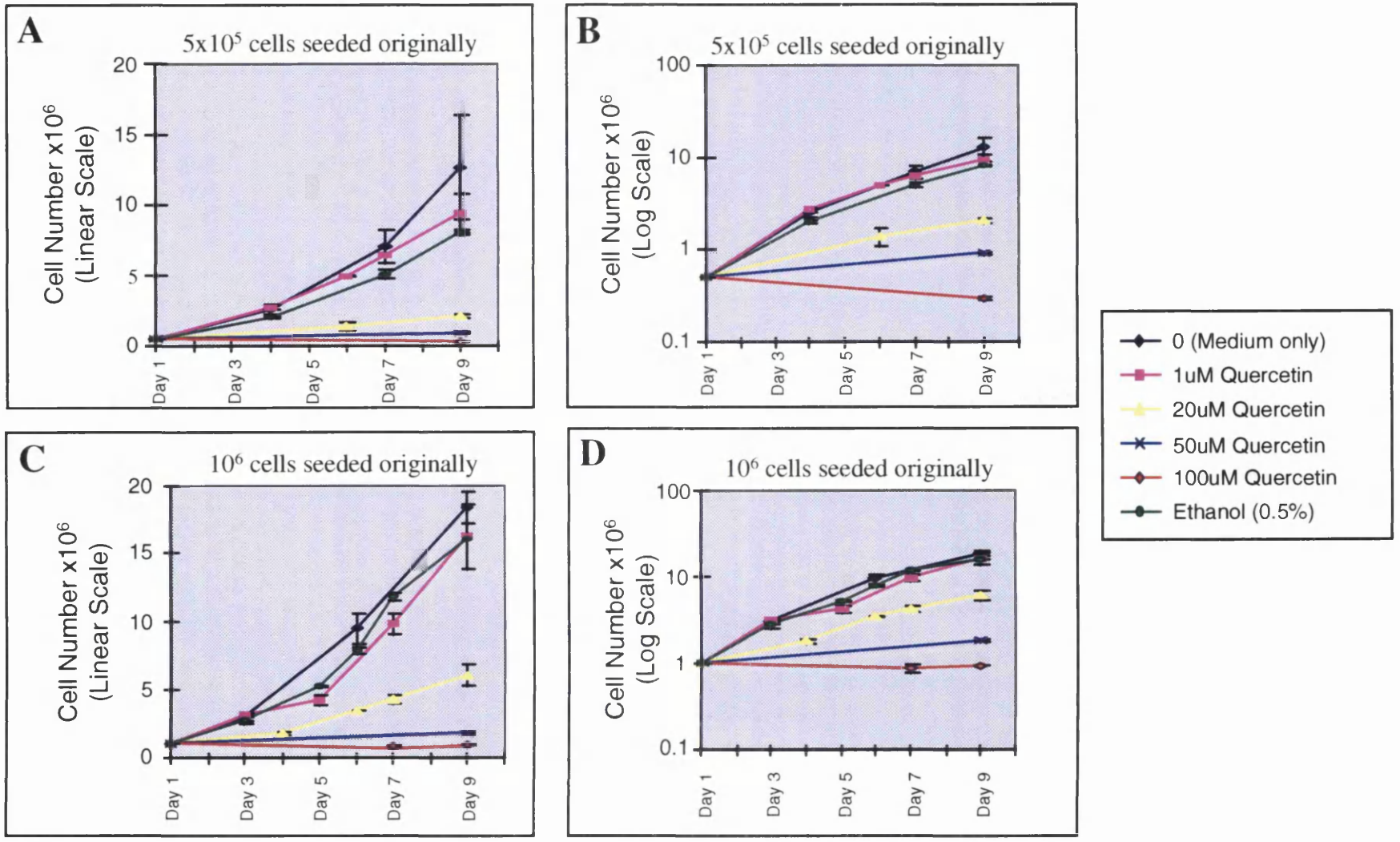
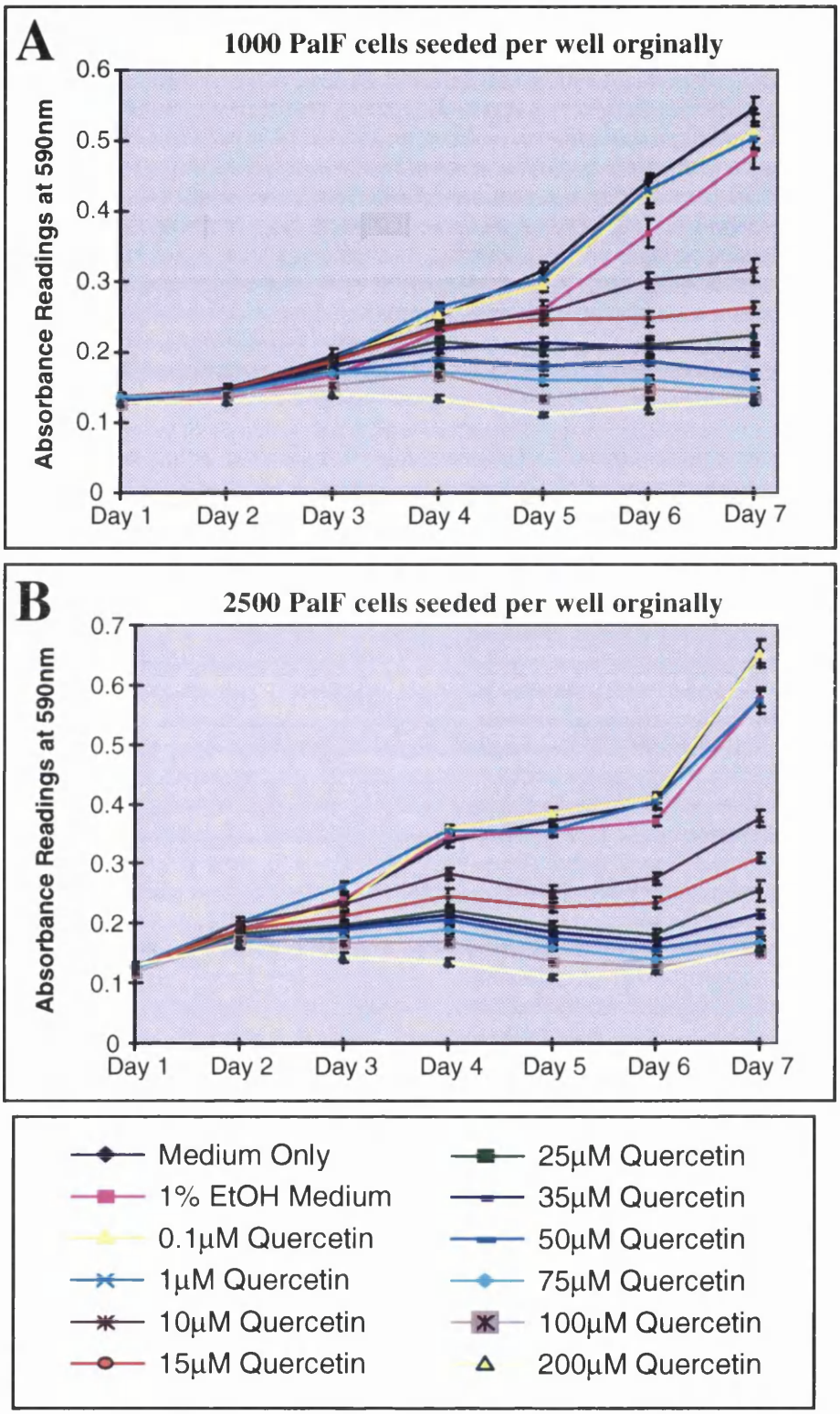
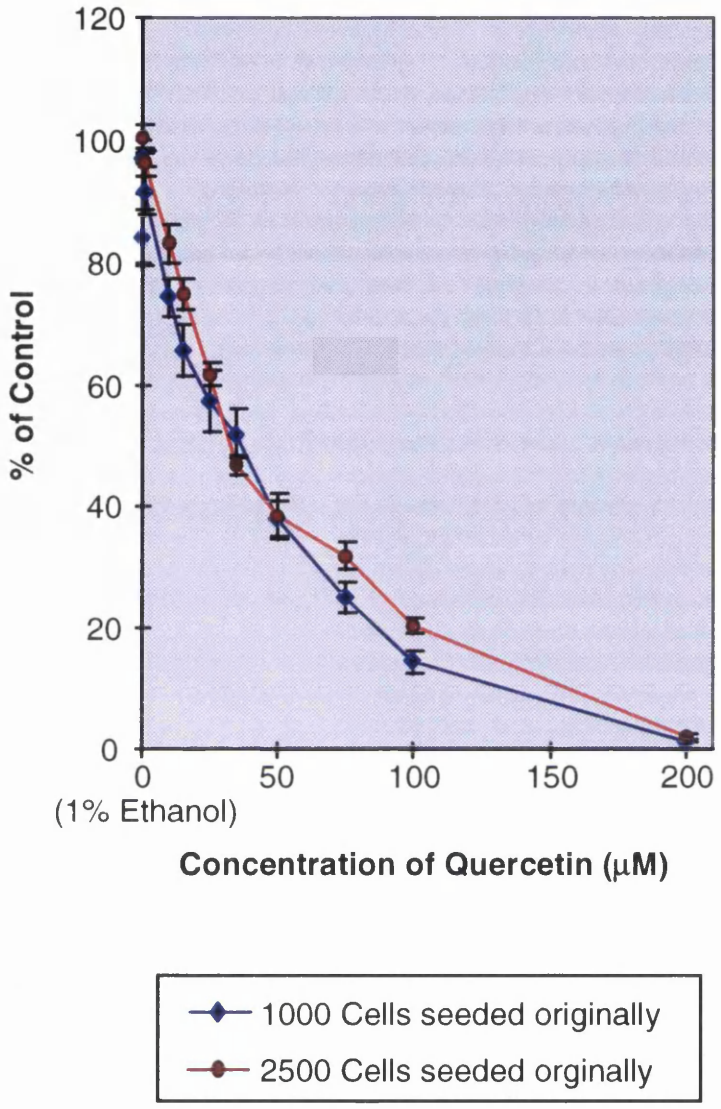


Figure 3.3 MTT assay results illustrating the growth rates of PalF cells cultured in increasing concentrations of quercetin.



Data in panels A and B represents the mean of two separate experiments with quintuplicate samples in each. Vertical bars correspond to the standard error.

Figure 3.4 H^3-Thymidine uptake by PalF cells exposed to increasing concentrations of quercetin.



All results are expressed as a percetage of control cells which were cultured in 1xDMEM medium only, in the absence of quercetin. Points on the plot corresponding to 0% quercetin represent cells which were actually cultured in medium containing 1% ethanol (1% ethanol is equivalent to the volume of quercetin in ethanol which was added to cells to give a final quercetin concentration of 200μM).

The data points in each series represent the mean from 2 separate experiments with quintuplicate samples in each experiment. The vertical bars indicate standard error.

3.3 Cell cycle analysis of PalF cells exposed to quercetin

As outlined above in section 3.1, quercetin has been shown to induce cell cycle arrest in several tumour cell lines and tumour material. The results from the growth analysis (section 3.2) showed that quercetin inhibited the growth of PalF cells in a concentration dependent manner. However, the growth curves gave no indication as to how quercetin was affecting proliferation; it was not clear whether the inhibition of cell growth was due to an increase in the rate of apoptosis, such that the rate of cell death was equal to the rate of proliferation, or if quercetin was inducing growth arrest at a specific or random stage in the cell cycle. As a means of addressing these questions, cell cycle analysis was performed on PalF cells cultured in medium containing increasing concentrations of quercetin.

3.3.1 Experimental Procedure

$5x10^5$ low passage PalF cells were seeded into 90mm tissue culture dishes. After an overnight incubation during which time the cells adhered to the culture plastic, the medium was changed. Cells were cultured in either medium alone, medium + 0.5% ethanol, or medium supplemented with 1, 20, 50 or 100μM quercetin. Medium was replenished in all dishes every 12 hours. Duplicate dishes were set up for each culture condition. Cells were grown in the appropriate medium for 12, 24, 36 or 48 hours. Following treatment cells were harvested and the percentage of cells in each phase of the cell cycle was determined using fluorescence activated cell sorting (FACS) as detailed in section 2.3.1.14.

The effect of quercetin on the cell cycle was not studied for a period longer than 48 hours. Within the context of earlier experiments, PalF cells treated with quercetin for 48 hours was shown to be sufficient to induce transformation to tumourigenicity in co-operation with BPV4 and an activated *ras* oncogene (Pennie and Campo, 1992; Cariney and Campo, 1995). We therefore only wished to analyse the cell cycle status of PalF cells exposed to quercetin within a 48 hour time interval.

3.3.2 Results

The photographs of PalF cells exposed to a variety of quercetin concentrations for 48 hours (figure 3.5) show that quercetin can induce certain morphological

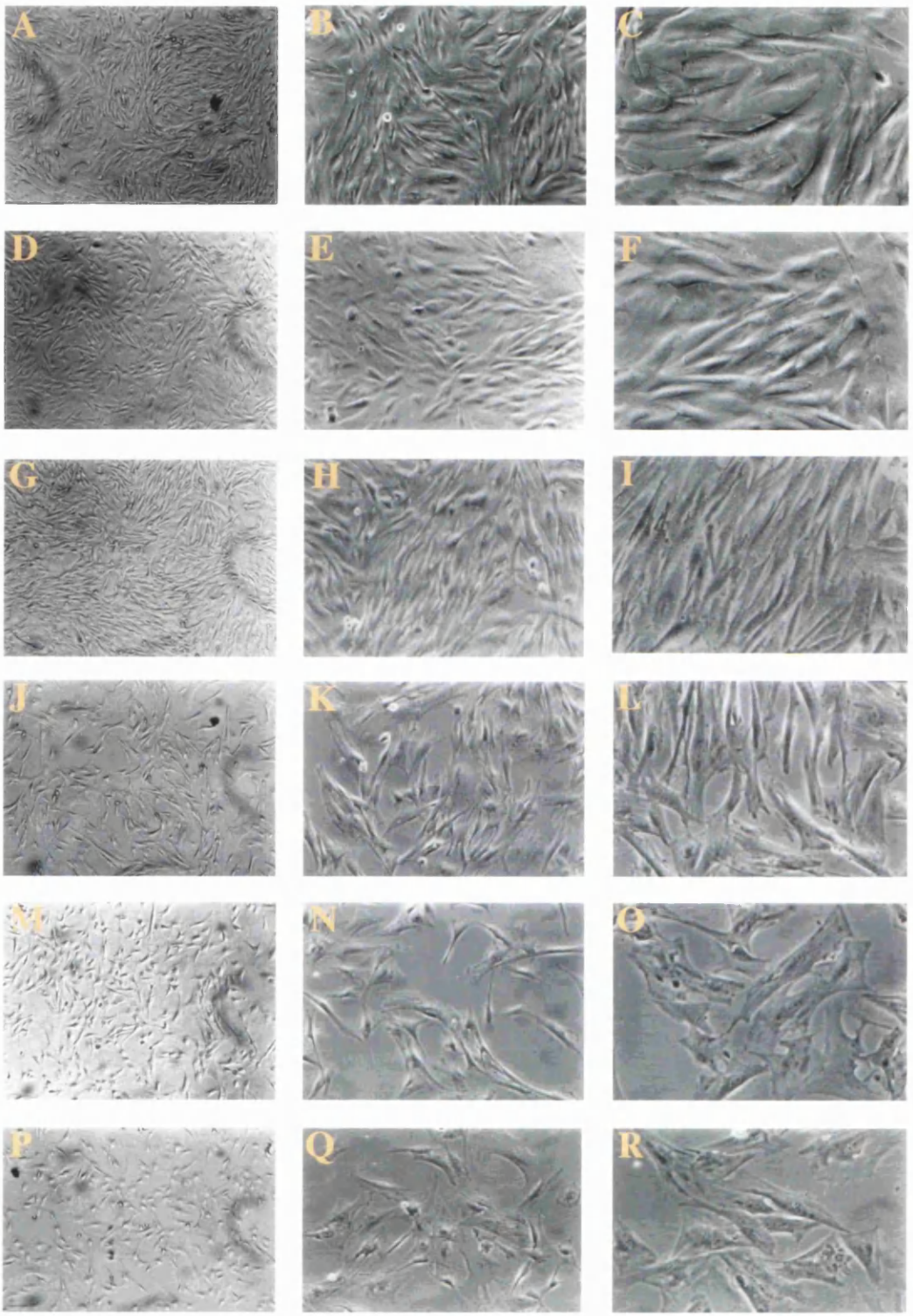
Figure 3.5

Figure 3.5 Photographs of PalF cells taken after exposure to various concentrations of quercetin for 48 hours

Panel A, B and C: Cells exposed to 1xDMEM medium only
Panel D, E and F: Cells exposed to 1xDMEM supplemented with 0.5% ethanol
Panel G,H and I: Cells exposed to medium supplemented with 1μM quercetin
Panel J, K and L: Cells exposed to medium supplemented with 20μM quercetin
Panel M, N and O: Cells exposed to medium supplemented with 50μM quercetin
Panel P, Q and R: Cells exposed to medium supplemented with 100μM quercetin

Panels A, D, G, J, M and P: Bright field images; final magnification x20
Panels B, E, H, K, N and q: Phase contrast images: final magnification x50
Panels C, F, I, L, O, and R: Phase contrast images: final magnification x100

Figure 3.5



changes particularly when present at concentrations as high as 100μM (Figure 3.5). In addition to an obvious reduction in cell density, cells were flatter and the cytoplasm was more vacuolar in appearance when grown in medium containing higher levels of quercetin. Nevertheless, the number of floating cells in the medium did not appear to be dramatically increased when cells were cultured in higher quercetin concentrations (personal observation). This would suggest that the concentrations of quercetin used were not sufficiently cytotoxic to induce significant cell death within 48 hours after first addition. However, no specific assay to accurately assess the level of apoptosis was performed. Therefore, the ability of quercetin to induce apoptosis in PalF cells has not yet been determined.

As the line plots in figure 3.7 illustrate, 12 hours after the addition of 50 or 100μM quercetin to culture medium, the proportion of cells in the G_0/G_1 phase of the cell cycle was seen to fall dramatically. Simultaneously, the proportion of cells in S phase was seen to increase accordingly. A similar drop in the percentage of cells in G_2/M phase was also observed 12 hours after quercetin was first added to the culture medium; the fall in the proportion of cells in G_2/M was again only observed for cells treated with 50 or 100μM quercetin. These observations suggest that quercetin, at concentrations of 50 and 100μM, can stimulate cells in G_0/G_1 to enter S phase and cells in G_2/M to progress through mitosis (M phase) and on into G_1. An alternative explanation is that cells' exit from S phase was delayed in the presence of quercetin. Whether quercetin is stimulating cells to enter S and M phases of the cell cycle or delaying their exit into successive phases, this effect of quercetin appears to be an early and transient event only. By 24 hours the accumlation of cells in S was no longer evident.

As the growth rate studies indicate (figures 3.2, 3.3 and 3.4), quercetin inhibits the growth of PalF cells when constantly present in the culture medium. Therefore, the effects of quercetin on PalF cells appears to be incompatible with normal cell proliferation. Indeed, cell cycle analysis of PalF cells treated with quercetin for longer time periods suggests quercetin can induce a proportion of cells to arrest.

A partial arrest in the G_2/M phase of the cell cycle was observed in cells exposed to quercetin for 48 hours (Figure 3.6 and 3.7). The percentage of cells arrested in G_2/M was proportional to the concentration of quercetin in the

Figure 3.6 DNA profiles of PalF cells exposed to various concentrations of quercetin over a two day period

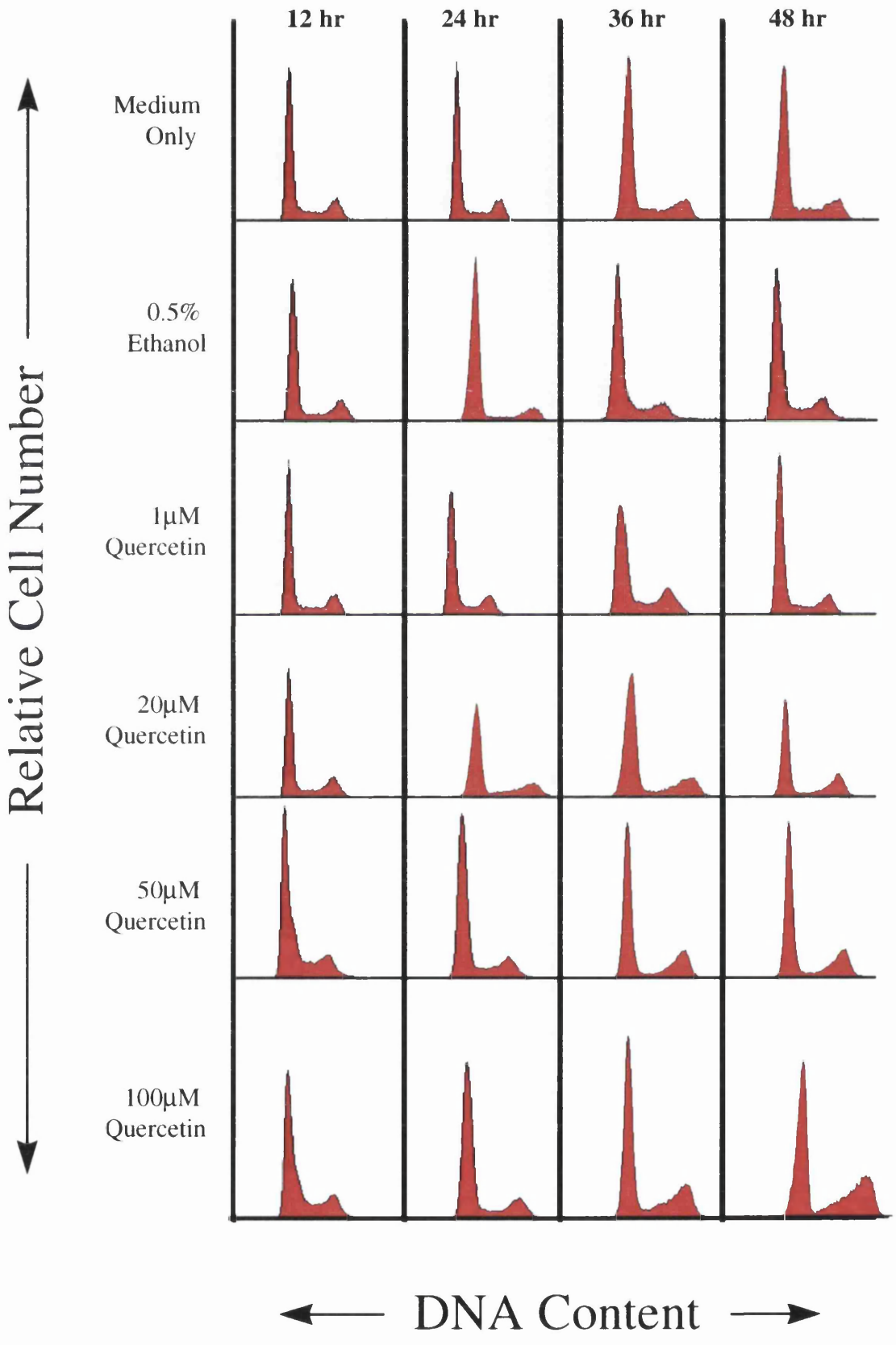
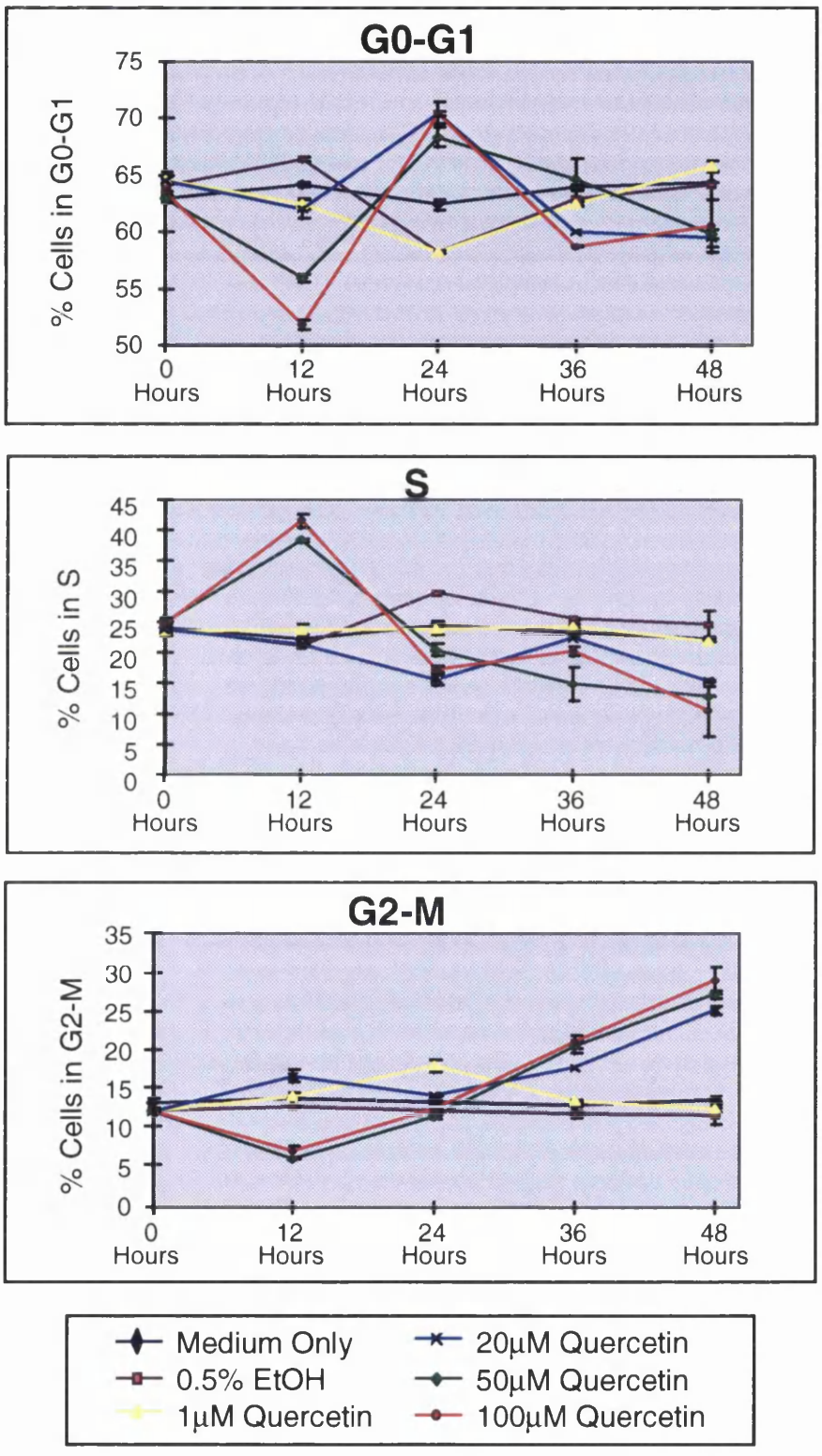


Figure 3.7 Plots showing the percentage of PalF cells in each phase of the cell cycle following exposure to increasing concentrations of quercetin over a 48 hour period



All data shown above represents the mean of two separate experiments with duplicate samples in each. The vertical bars correspond to the standard error of the mean.

culture medium. Furthermore, the percentage of cells arresting in G_2/M in response to quercetin increased steadily over the 48 hours of the experiment (Figure 3.6 and 3.7).

It was observed in early, preliminary cell cycle experiments that if quercetin was added only once to each culture dish, that a similar G_2/M arrest began to appear within 24 hours after quercetin was first added to the medium. Consistent with the results above, the percentage of cells arresting in G_2/M was dependent on and proportional to the concentration of quercetin in the medium. However, over the next 24 hours, the percentage of cells in G_2/M steadily declined rather than continuing to increase as expected. The apparent recovery of PalF cells from this quercetin-induced arrest suggested that the effect of quercetin on the cell cycle was merely transient. Nevertheless, results from the later experiments where quercetin was replenished in the medium every 12 hours (Figures 3.6 and 3.7), showed that the proportion of cells arresting in the G_2/M phase of the cell cycle increased steadily over the 48 hour assay period. Quercetin in culture medium appears to undergo a process of gradual inactivation or degradation: hence the effective concentration of active quercetin in medium seeems to decline over time. Thus in all subsequent experiments, quercetin was replenished every 12 to 24 hours in an attempt to maintain the active concentration of quercetin as constant as possible.

As mentioned above, the cell cycle arrest of PalF cells in response to quercetin exposure was concentration dependent. The extent of the growth arrest observed was greatest for the highest concentrations of quercetin ($\geq$ 50μM). However, it is evident from the DNA histogram plots (figure 3.6) and from the line plots illustrating the percentage of cells in each phase of the cell cycle (figure 3.7) that quercetin, even when present at a concentration 100μM, did not induce a complete G_2/M arrest in PalF cells. This is consistent with observations made by Hosokawa *et al.* (1990a) and Plaumann *et al.* (1996).

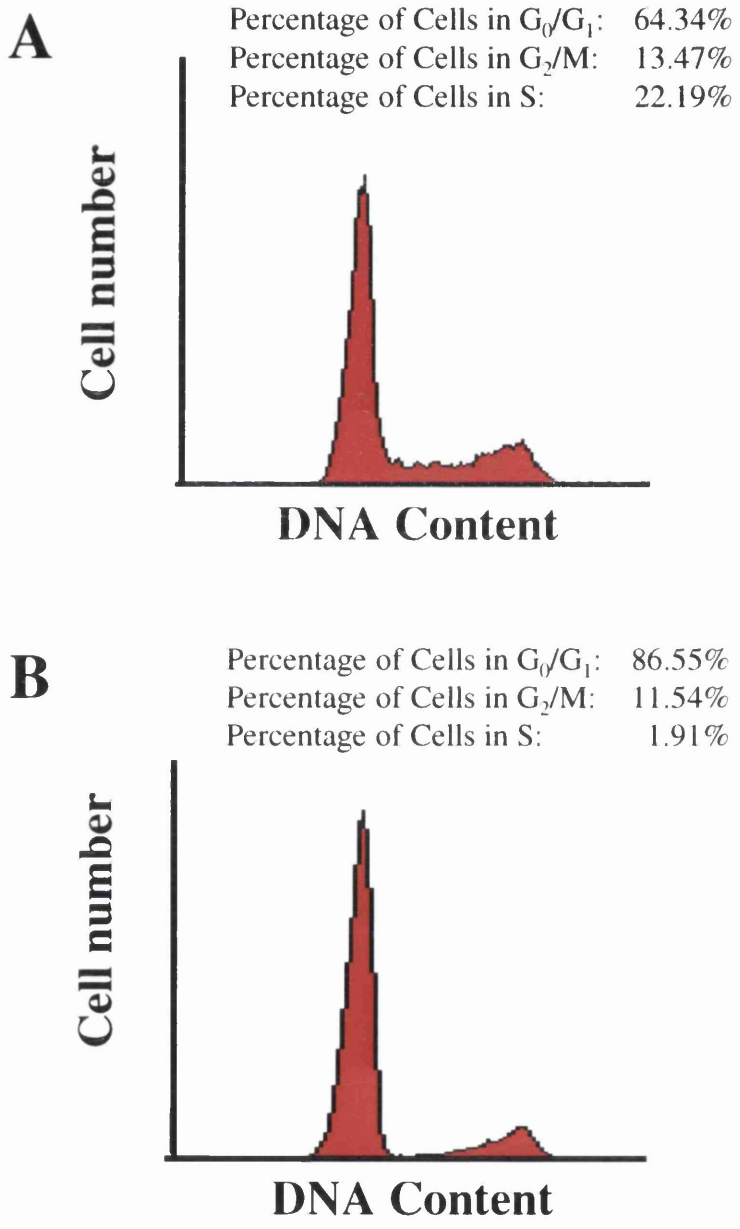
One explanation for this may be that concentrations of quercetin greater than 100μM are necessary to induce a complete G_2/M phase arrest in PalF cells. Indeed, a quercetin concentration of 120μM was required to induce a significant growth arrest in C3H10T1/2CL8 cells (Plaumann *et al.*, 1996).

A further explanation is that the experiment was not continued long enough to allow all cells to arrest in G_2/M. From the growth rate analyses (section 3.2), PalF cells in these experiments have been shown to have a cell doubling time of 36 to 48 hours. Because the cell cycle analysis was only observed over a 48 hour period, it is possible that not enough time had elapsed for all susceptible cells to arrest in any particular stage of the cell cycle.

A third possibility may be that quercetin can induce cells to arrest in more than one phase of the cell cycle. Indeed, as the results in figure 3.7 indicate, the level of cells in the G_0/G_1 phase of the cell cycle peaked 24 hours after quercetin was first added to the culture medium and dispite a drop by 36 hours, the proportion of cells in the G_0/G_1 phase of the cell cycle remained relatively high. Thus the results suggests that quercetin can simultaneously induce a G_2/M and a G_1 arrest in PalF cells

PalF cells can be induced to arrest largely in G_0/G_1. This was confirmed when PalF cells were cultured in medium containing low serum concentrations (0.5% serum compared to a normal concentration of 10%). After 48 hours, 86.56% of cells were in G_0/G_1, 11.56% of cells were in G_2/M and 1.91% of cells were in S phase (Figure 3.8). For normal, untreated PalF cells, 64.34%, 13.47%, and 22.19% of cells were in G_0/G_1, G_2/M and S phase respectively. This suggests that the ability of quercetin to induce a G_2/M arrest is a specific effect of quercetin and not merely a consequence of PalF cells' inability to arrest in G_0/G_1.

Figure 3.8 DNA profiles of PalF cells grown in (A) high (10%) and (B) low serum (0.5%)1xDMEM medium for 48 hours



Pencentages given correspond to the mean of two separate experiments with duplicate samples in each experiment.

Chapter 4

Analysis of the Effects of Quercetin on BPV4 transcription

Chapter 4 Analysis of the effects of quercetin on BPV4 transcriptional activity

4.1 Introduction

As discussed in detail in earlier chapters, the bioflavonoid quercetin can synergise with BPV4 DNA sequences, in the presence of an exogenous activated *ras* gene, and induce several characteristics of transformation in bovine foetal palate fibroblast (PalF) cells including tumorigenicity (Pennie and Campo, 1992; Cairney and Campo, 1995) (see section 1.7.2). The mechanism(s) involved in this transformation system is not known. The principle aim of this thesis was to attempt to identify possible mechanisms by which quercetin contributes to the cellular transformation of PalF cells in association with BPV4.

4.2 The effect of quercetin on the transcriptional activity of the wild type, full length BPV4 LCR acting in a promoter configuration

We hypothesised that quercetin may contribute to the transformation of PalF cells, in co-operation with BPV4, by altering the level of viral transcription; an increase in viral transcription may give rise to higher levels of viral proteins which could contribute to the transformation observed. To tests this hypothesis, PalF cells were treated with 20μM quercetin for 48 hours both before and after transfection with a plasmid carrying the entire BPV4 LCR cloned upstream and driving expression of a luciferase reporter gene. Cells were exposed to 20μM quercetin as this was the concentration of quercetin which was sufficient to transform PalF cells, in co-operation with BPV4 viral sequences and an activated *ras* gene, as detailed in earlier experiments (Pennie and Campo, 1992; Cairney and Campo, 1995).

4.2.1 Experimental procedure

Quercetin solid (powder) was dissolved in ethanol to give a stock solution of 20mM. A fresh stock solution of quercetin was made every week and stored at -20°C.

10^5 low passage PalF cells were seeded into 60mm tissue culture dishes as specified in section 2.3.1.7. Following an overnight incubation, during which time the cells adhered to the tissue culture dish, the culture medium was removed and replaced with either fresh medium supplemented with 20μM quercetin or medium

supplemented with an equivalent volume of ethanol. Cells were incubated in the appropriate medium for 48 hours. At the end of 48 hours, the cells were co-transfected with either 7.5μg pLCRLuc plus 2.5μg pCH110 vectors or 7.5μg pOLuc plus 2.5μg pCH110 vectors according to the protocol detailed in section 2.3.1.7. The plasmid vector pCH110 contains the *lacZ* gene; this second reporter vector was included in all transfections as an internal control providing a means of determining and correcting for the efficiency of each transfection.

After transfection, cells which had initially been exposed to quercetin before transfection received medium supplemented with ethanol, while cells which were previously cultured in ethanol containing medium were given medium plus quercetin; quercetin was present at a final concentration of 20μM. Each dish of cells was incubated in the appropriate medium for a further 48 hours. At the end of the 48 hours, cells were harvested (see section 2.3.1.9a) and assayed separately for luciferase and β-galactosidase enzyme activities, as detailed in sections 2.3.1.11 and 2.3.1.12.

Table 4.1 summarises the various incubation and transfection conditions which were used in this experiment. Conditions #1 and #2 correspond to control sets in which cells were not exposed to quercetin. In each experiment, duplicate cell samples were set up for each of the 6 conditions.

The luciferase reading for each sample was corrected for efficiency of transfection using the β-galactosidase readings according to the following equation;

(Sample's luciferase reading - background luciferase reading (lysis buffer only)) x (average of all β-gal. readings ÷ sample's β-gal reading)

Background transcription, attributable to the empty vector (pOLuc) alone, was corrected for by subtracting the average luciferase readings from condition #1, #3 and #5 from the average luciferase readings from condition #2, #4 and #6 respectively. The 'No quercetin' reading was then set =1 and the 'Quercetin before' and 'Quercetin after' readings were subsequently normalised to the 'No quercetin' reading. Once normalised, the results from all the separate experiments were amalgamated; the average luciferase reading, and the standard error of the mean (S.E.M), were calculated for each condition (quercetin before transfection, quercetin after

transfection and no quercetin) using the results from all experiments combined. The Student's t-Test was used to calculate **p**; **p** provides a measure of how statistically significant is the difference between two values, or how likely it may be that the difference between two values may have occurred by chance. A **p** value less than or equal to (≤) 0.05 is taken as being statistically significant.

Table 4.1 Summary of the experimental conditions used when PalF cells were transfected with the BPV4 LCR present in a promoter configuration, and treated with or without quercetin.

	Condition #1	Condition #2	Condition #3	Condition #4	Condition #5	Condition #6
Before transfection	Med + EtOH	Med + EtOH	Med + Quer.	Med + Quer.	Med + EtOH	Med + EtOH
Transfected with	pOLuc + pCH110	pLCRLuc + pCH110	pOLuc + pCH110	pLCRLuc + pCH110	pOLuc + pCH110	pLCRLuc + pCH110
After transfection	Med + EtOH	Med + EtOH	Med + EtOH	Med + EtOH	Med + Quer.	Med + Quer.

Abbreviations used: Med = medium (1X DMEM)
Quer. = quercetin (to final concentration of 20μM)
EtOH = ethanol (a volume equivalent to the volume of quercetin added in other conditions, ie. 0.1% v/v)

4.2.2 Results

Figure 4.1 shows the results obtained from 8 separate experiments. Quercetin was shown to increase the transcriptional activity of the BPV4 LCR by an average of 2.67 fold over untreated control cells. Exposure of cells to quercetin before transfection had no apparent effect on the activity of the LCR. The p values obtained were as follows;

- Quercetin before transfection compared to no quercetin; p=0.48
- Quercetin after transfection compared to no quercetin; p=0.0036**
- Quercetin after transfection compared to quercetin before; p=0.0024**

* significant p value, ** very significant p value

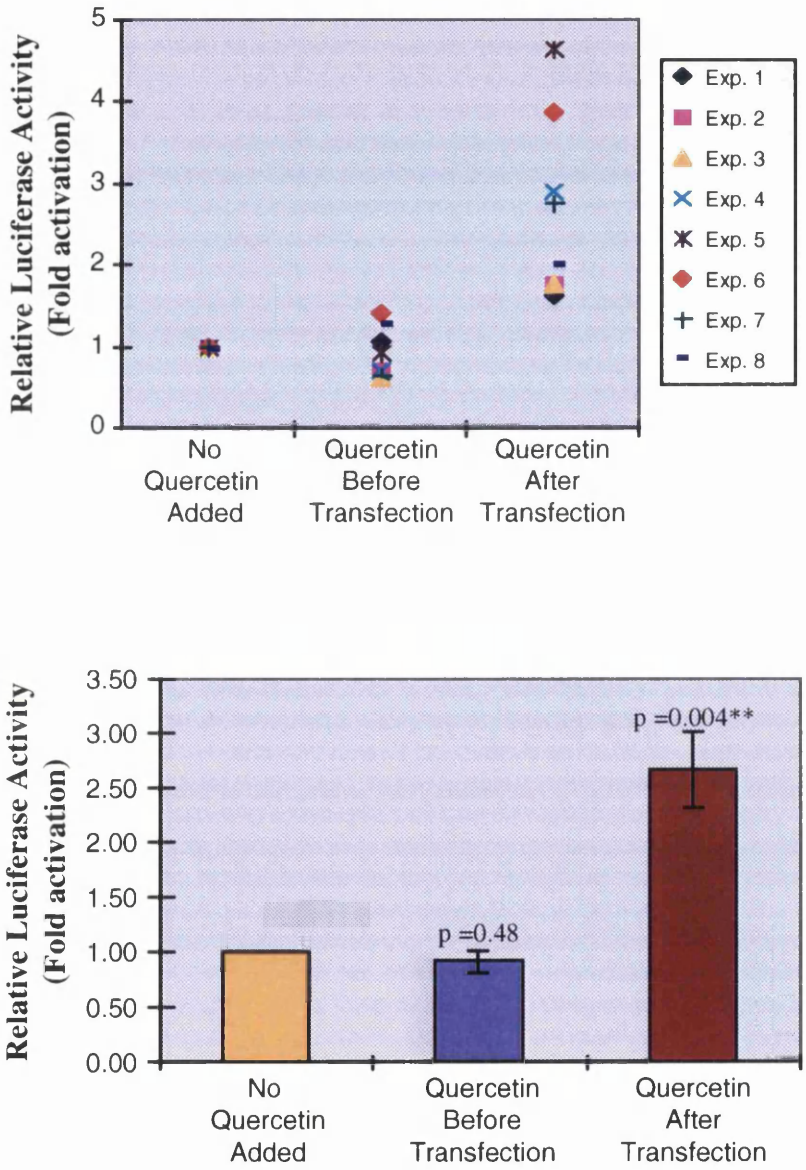
Figure 4.1

Figure 4.1 Full length BPV4 LCR acting as a promoter driving a luciferase reporter gene in PalF cells

Top panel shows a scatter plot which represents the results from each of 8 separate experiments.

Bottom panel shows the results from all 8 experiments combined. The vertical bars correspond to the standard error of the mean (S.E.M).

Figure 4.1 Full length BPV4 LCR acting as a promoter driving a luciferase reporter gene in PalF cells



As the p values indicate, the increase in transcription of the BPV4 LCR in response to quercetin treatment after transfection was significant compared to cells either not treated with quercetin or treated with the same concentration of quercetin (20μM) before transfection.

The observation that the activity of the LCR is only altered if quercetin is added to culture medium after cells have been transfected with the LCR suggests quercetin and the LCR must be present simultaneously for an alteration in LCR activity to be achieved. It further suggests that any epigenetic changes which may occur as a consequence of quercetin treatment are relatively short lived.

4.3 The effect of quercetin on the transcriptional activity of the full length, wild type BPV4 LCR functioning in an enhancer configuration

As the results in figure 4.1 demonstrated, quercetin was seen to increase the transcriptional activity of the BPV4 LCR approximately 2.5-3 fold when the LCR was present in a promoter orientation. We wished to determine if quercetin could induce a similar effect on the activity of the BPV4 LCR if functioning as an enhancer.

PalF cells were transfected as described in section 4.2.1. A different set of plasmid vectors was used in place of those described in the previous experiment. The plasmids used were p41X-PINT and p41X, again in conjunction with the pCH110 vector. p41X-PINT carries the entire BPV4 LCR cloned upstream of the herpes simplex virus thymidine kinase (TK) promoter and together these control the expression of the bacterial chloramphenicol acetyltransferase (CAT) reporter gene . p41X is the same vector as p41X-PINT but without the BPV4 LCR sequences.

4.3.1 Experimental procedure

10^5 PalF cells were treated as described in section 4.2.1. Briefly, PalF cells were exposed to either quercetin at 20μM or an equivalent volume of ethanol for 48 hours before transfection. Cells were then co-transfected with either 7.5μg p41X plus 2.5μg pCH110 plasmids or 7.5μg p41X-PINT plus 2.5μg pCH110 plasmids as detailed in section 2.3.1.7.

After transfection, cells which were previously exposed to medium containing ethanol were given medium plus 20μM quercetin, and cells which were originally treated with quercetin were given medium supplemented with an equivalent volume of ethanol. Cells were cultured in the appropriate medium for a further 48 hours after which time all cells were harvested as described and section 2.3.1.9a and assayed for β-galactosidase and CAT enzyme activities (see sections 2.3.1.12 and 2.3.1.13). Table 4.2 summarises the experimental rationale used.

Table 4.2 Summary of the experimental conditions used when PalF cells were transfected with the BPV4 LCR present in an enhancer configuration, and treated with or without quercetin.

	Condition #1	Condition #2	Condition #3	Condition #4	Condition #5	Condition #6
Before transfection	Med + EtOH	Med + EtOH	Med + Quer.	Med + Quer.	Med + EtOH	Med + EtOH
Transfected with	p41X + pCH110	p41X-PINT + pCH110	p41X + pCH110	p41X-PINT + pCH110	p41X + pCH110	p41X-PINT + pCH110
After transfection	Med + EtOH	Med + EtOH	Med + EtOH	Med + EtOH	Med + Quer.	Med + Quer.

Abbreviations used: Med = medium (1X DMEM)
Quer. = quercetin (to final concentration of 20μM)
EtOH = ethanol (a volume equivalent to the volume of quercetin added in other conditions, ie. 0.1% v/v)

Each CAT reading was corrected for the efficiency of transfection using the corresponding β-galactosidase reading and applying the equation given in section 4.2.1. The effect of the empty vector (p41X) on transcription was further corrected for as described in section 4.2.1. Within each experiment, the results were normalised to the 'No quercetin' condition which was set =1. Using the combined results from all experiments, the average CAT reading and the S.E.M for each condition was calculated and the Student's t-Test was applied to determine statistical significance between the various conditions.

4.3.2 Results

Figure 4.2 illustrates the results obtained from 5 separate experiments. Quercetin, given before or after transfection, had no significant effect on the transcriptional activity of the BPV4 LCR when acting as an enhancer. The p values obtained were as follows;

- Quercetin before transfection compared to no quercetin; p=0.06
- Quercetin after transfection compared to no quercetin; p=0.33
- Quercetin after transfection compared to quercetin before; p=0.25

This result suggests that the effect of quercetin on the activity of the full length BPV4 LCR in PalF cells is promoter specific and is not observed if the LCR is present in enhancer mode.

4.4 Comparison of the effect of quercetin and/or TPA on the transcriptional activity of the Collagenase TRE and the BPV4 LCR in high and low serum culture conditions

Sequence analysis of the BPV4 LCR identified binding motifs for several known transcription factors both viral and cellular in origin. These include sites for BPV4 E2, C/EBPβ, PEBP2, putative NF1-like binding sites and a TRE-like element. Figure 4.3 presents a schematic illustration of these various binding motifs as they appear within the BPV4 LCR. Although three separate NF1-like motifs have been identified, binding of NF1 to these sites has not yet been investigated.

TRE (TPA responsive elements) or AP1-binding motifs are known to bind the well characterised transcription factor AP1(Angel *et al.*, 1987; Lee *et al.*, 1987). AP1 is a general term to describe a transcription factor which can comprise a selection of different protein subunits coming together to form either homo- or heterodimers. Purified preparations of AP1 have shown that AP1 can constitute a homodimer of Jun or Jun-related proteins; alternatively AP1 can incorporate a heterodimer of Jun and cFos as well as several Fos-related proteins called Fras (Fos-related antigens) (Halazonetis *et al.*, 1988; Latchman, 1991). Homodimers of the Fos protein are unable to bind AP1-binding sites (Latchman, 1991). Increased levels of Jun and Fos have been detected in cells after treatment with phorbol esters such as TPA

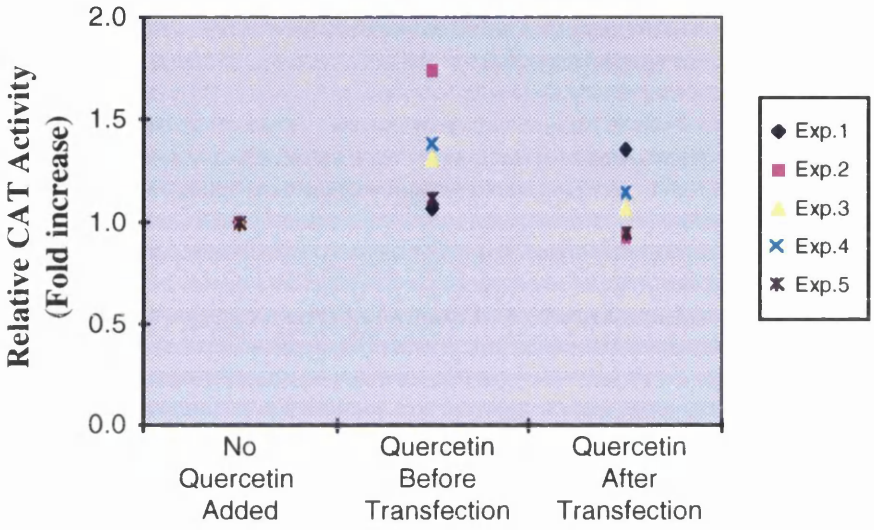
Figure 4.2

Figure 4.2 Full length BPV4 in enhancer configuration in PalF cells

Top panel shows a scatter plot which represents the results from each of 5 separate experiments.

Bottom panel shows the results from all 5 experiments combined. The vertical bars correspond to the standard error of the mean (S.E.M).

Figure 4.2 Full Length BPV4 LCR in enhancer configuration in PalF cells



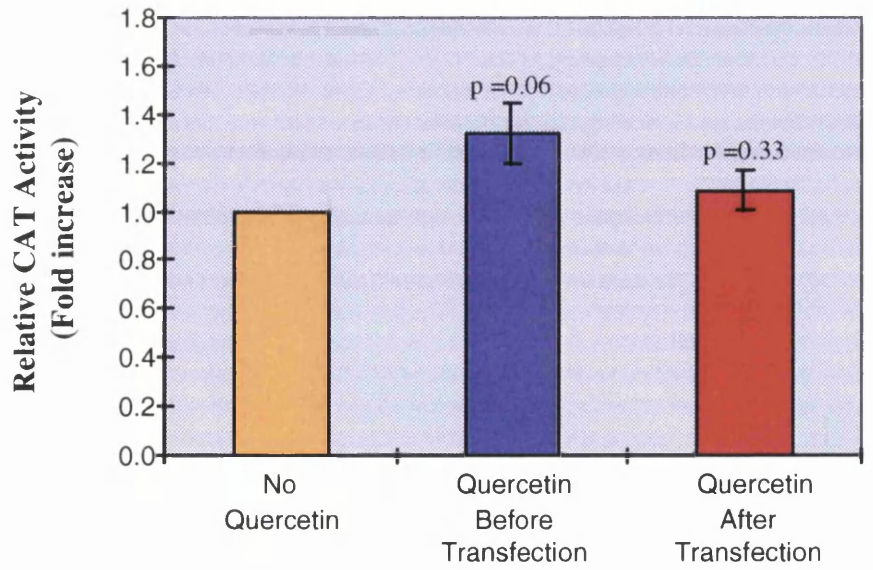
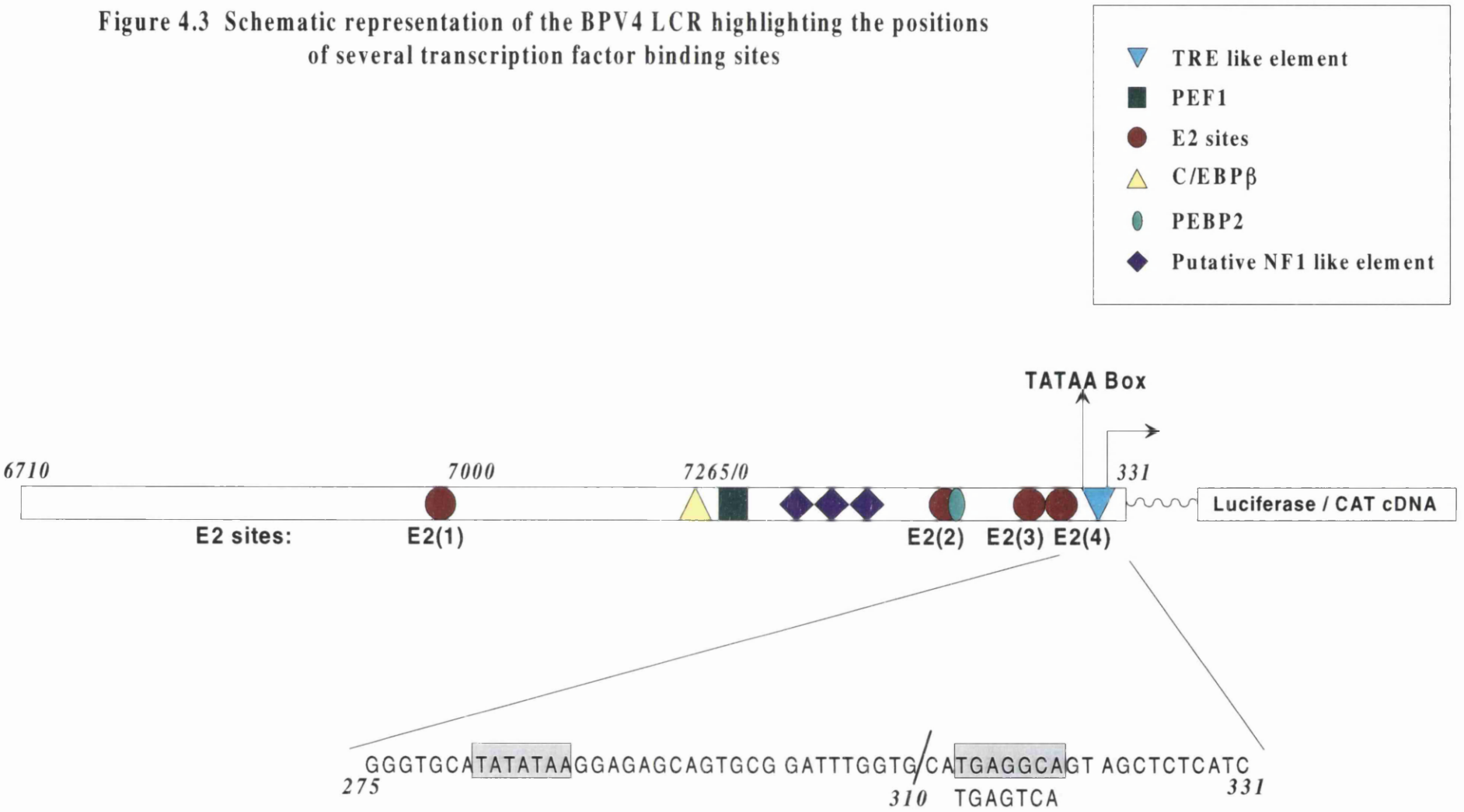


Figure 4.3

Figure 4.3 Schematic representation of the BPV4 LCR highlighting the positions of several transcription factor binding sites

The sequence along the bottom of the figure corresponds to nucleotides 275 to 331 of the LCR. The TATAA box and the TRE-like motifs are highlighted in grey boxes. The sequence below the TRE-like motif corresponds to the consensus sequence which binds the transcription factor AP1.

Figure 4.3 Schematic representation of the BPV4 LCR highlighting the positions of several transcription factor binding sites



(Lamph *et al.*, 1988). Likewise, Jun and Fos have also been induced in quiescent cells after treatment with serum or growth factors (Lamph *et al*, 1988; Rauscher *et al.*, 1988). Jun and Fos have both been identified as potential oncoprotein as uncontrolled expression of these proteins leads to cellular transformation (Cooper, 1990).

As mentioned above, the BPV4 LCR contains a TRE-like element. The nucleotide sequence at this site is identical to an AP1-consensus binding motif except for a single base difference.

- TRE-like element sequence: TGAGGCAG
- AP1 consensus binding site sequence: TGAGTCAG

AP1-binding sites are generally located in enhancers or upstream to promoter elements. In this respect the location of the TRE-like element within the BPV4 LCR is rather unusual, being located within the 3' extreme end of the promoter region and lying downstream of the TATAA box.

Despite the single base change and its location with the LCR, the TRE-like element remains a potential target site through which quercetin may be mediating a increase in transcription. We therefore decided to test whether quercetin was perhaps altering the transcriptional activity of the BPV4 LCR through AP-1 activation.

Firstly, as a control, a plamid containing five copies of the collagenase TRE was transfected into PalF cells in both high and low serum culture conditions. Cells were treated with TPA which is known to induce AP1.

AP1 in normal, cycling cells is constitutively expressed. In order to best visualise an induction of AP1, background AP1 levels must first be reduced. This can be achieved by driving cells into a quiescent state. Removal or marked reduction of serum from the cells' culture medium can force cells into quiescence. Against a backdrop of depleted AP1 levels, the effect of a treatment(s) which may induce AP1 expression can then be observed to maximum effect. In light of this, cells were precultured in a low serum environment before treating with TPA

Cells transfected with the collagenase TRE plasmid were also treated with quercetin in high and low serum containing medium to see if quercetin could induced a similar effect on AP1 expression.

The same experimental rationale was also applied using a plasmid carrying the BPV4 LCR. The transcriptional activity of the LCR in PalF cells in response to

treatment with quercetin, TPA or both was determined as for the collagenase TRE-containing plasmid.

4.4.1a Experimental procedure

TPA (12-O-tetradeconolyphorbol 13-acetate) was dissolved in ethanol to give a stock solution of 100μg/ml. This stock solution was stored at -20°C when not in use.

10^5 PalF cells were seeded into the appropriate tissue culture dish as specified in section 2.3.1.7. The following day cells were transfected with either 7.5μg p5xTRECAT plus 2.5 μg pCH110 or 7.5μg pTKCAT plus 2.5μg pCH110. After transfection, cells were fed with a fresh volume of medium containing either high (10% v/v) or low (0.5% v/v) serum. All cells were cultured in the appropriate serum-containing medium for a period of 48 hours after which time the medium was replenished. The fresh medium contained the same concentration of serum as before but was now supplemented with either ethanol, quercetin (20μM), TPA (100ng/ml) or quercetin plus TPA; fresh medium was added to the appropriate dishes of cells according to the protocol summarised in table 4.3. The volume of ethanol added to cells in conditions #1 and #2 was equivalent to the total volume of quercetin plus TPA which was added to cells in conditions #7 and #8. Cells were cultured in the new supplemented medium for a further 24 hours. At the end of this time, all cells were harvested (section 2.3.1.9a) and the resultant cell lysates assayed for both CAT and β-galactosidase enzyme activities (section 2.3.1.12 and 2.3.1.13).

Each CAT assay reading was corrected for efficiency of transfection and the contribution of empty vector (pTKCAT) on transcription was removed. The final readings were normalised to the 'No quercetin' reading set = 1, as described in detail in section 4.3.1.

Table 4.3 Summary of the experimental conditions used when PalF cells were transfected with the Collagenase TRE and treated with or without quercetin and/or TPA

	Cond. #1		Cond. #2		Cond. #3		Cond. #4		Cond. #5		Cond. #6		Cond. #7		Cond. #8	
Transfected with	pTKCAT + pCH110		p5xTRE + pCH110		pTKCAT + pCH110		p5xTRE + pCH110		pTKCAT + pCH110		p5xTRE + pCH110		pTKCAT + pCH110		p5xTRE + pCH110	
Serum content (%)	H (10)	L (0.5)	H (10)	L (0.5)	H (10)	L (0.5)	H (10)	L (0.5)	H (10)	L (0.5)	H (10)	L (0.5)	H (10)	L (0.5)	H (10)	L (0.5)
Treated with	E		E		Q		Q		T		T		Q+T		Q+T	

Abbreviations: E = ethanol
Q = quercetin (20μM)
T = TPA - 12-O-tetradecanoylphorbol 13-acetate (100ng/ml)
H = high serum (10%)
L = low serum (0.5%)

*In each of the conditions given in the table 4.3, cells were incubated in either high (10%) or low (0.5%) serum containing medium for a period of 48 hours after transfection and before the addition of ethanol, quercetin and/or TPA. For all conditions, duplicate dishes of cells were used.

4.4.1b Experimental procedure

The experimental procedure described in section 4.4.1a was simultaneously repeated with PalF cells but using a different set of plasmid vectors. PalF cells were alternatively transfected with either 7.5μg pLCRLuc plus 2.5μg pCH110 or 7.5μg pOLuc plus 2.5μg pCH110. After transfection, cells were cultured for 48 hours in 1xDMEM containing either a high (10%) or low (0.5%) serum content. At the end of the 48 hour incubation a fresh volume of medium containing the same concentration of serum as before, but freshly supplemented with either ethanol alone, quercetin (20μ M), TPA (100ng/ml), or quercetin plus TPA, was added to the appropriate dishes in each experimental conditions, as indicated in table 4.4. All cells were cultured in supplemented medium for a further 24 hours. At the end of the incubation, all dishes

of cells were harvested and assayed for luciferase and β-galactosidase enzyme activities (section 2.3.1.11 and 2.3.1.12). Table 4.4 summarises the protocol followed in this particular experiment.

Each luciferase assay reading was corrected for efficiency of transfection, the contribution of empty vector (pOLuc) on transcription was removed, and the final readings were normalised to the 'No quercetin' reading set = 1, as described in detail in section 4.3.1.

Table 4.4 Summary of the experimental conditions used when PalF cells were transfected with the BPV4 LCR functioning as a promoter and treated with or without quercetin and/or TPA.

	Cond. #1		**Cond. #2**		**Cond. #3**		**Cond. #4**		**Cond. #5**		**Cond. #6**		**Cond. #7**		**Cond. #8**	
Transfected with	pOLuc + pCH110		pLCRLuc + pCH110		pOLuc + pCH110		pLCRLuc + pCH110		pOLuc + pCH110		pLCRLuc + pCH110		pOLuc + pCH110		pLCRLuc + pCH110	
Serum content (%)	H (10)	L (0.5)	H (10)	L (0.5)	H (10)	L (0.5)	H (10)	L (0.5)	H (10)	L (0.5)	H (10)	L (0.5)	H (10)	L (0.5)	H (10)	L (0.5)
Treated with	E		E		Q		Q		T		T		Q+T		Q+T	

Abbreviations: E = ethanol
Q = quercetin (20μM)
T = TPA - 12-O-tetradecanoylphorbol 13-acetate (100ng/ml)

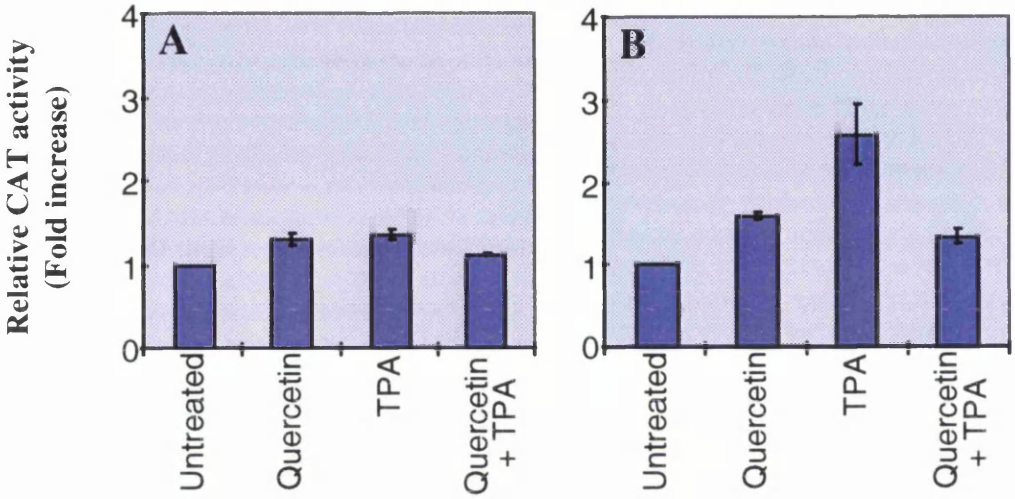
*All cells were incubated in either high (10%) or low (0.5%) serum containing medium for a period of 48 hours after transfection and before the addition of ethanol, quercetin and/or TPA. For each condition given in table 4.4, duplicate dishes of cells were used.

4.4.2 Results

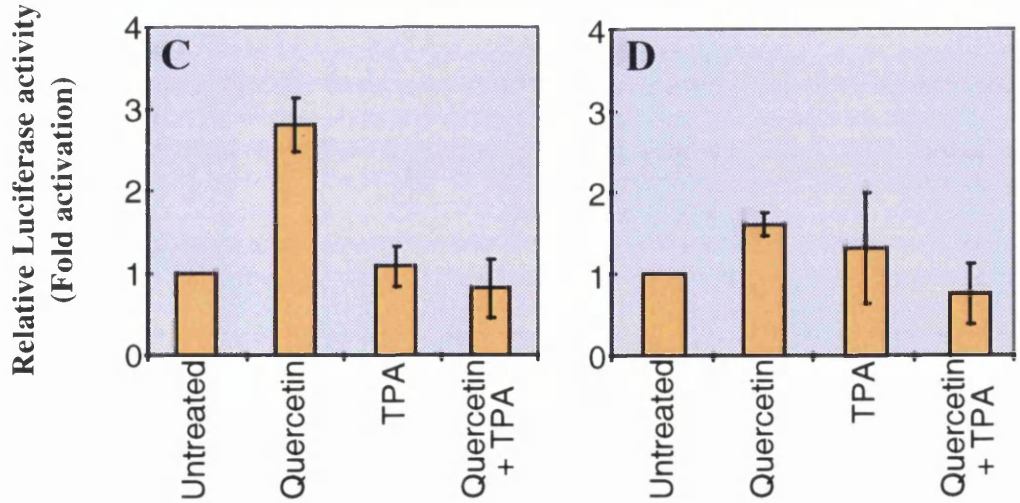
Figure 4.4 illustrates the results obtained from 3 separate experiments. TPA increased the activity of the collagenase TRE. As expected, this was more clearly observed when cells were preincubated in low serum containing medium

Figure 4.4

Effect of quercetin and/or TPA on the Collagenase TRE in PalF cells grown in (A) high (10%) and (B) low (0.5%) serum containing medium



Effect of quercetin and/or TPA on the BPV4 LCR in PalF cells grown in (C) high (10%) and (D) low (0.5%) serum containing medium



(see figure 4.4, panels A and B). Quercetin had a small effect on the transcriptional activity of the collagenase TRE and, like TPA, the increase in transcriptional activity was more obvious in cells cultured in a low serum environment. Quercetin and TPA, when present in the culture medium simultaneously, appeared to have antagonistic effects on the transcriptional activity of the collagenase TRE; the activity of the collagenase TRE was reduced to a level below that observed for either quercetin or TPA alone. This would suggest that the pathway(s) which is activated by TPA is inhibited by quercetin and *vice versa.*

TPA was unable to induce an increase in the activity of the BPV4 LCR when added to cells incubated in either high or low serum containing medium (figure 4.4, panels C and D). The effect of quercetin on the upregulation of the BPV4 LCR's transcriptional activity was most strikingly observed for cells cultured in medium containing 10% serum (panel C). This suggests that quercetin requires certain serum factors, perhaps serum growth factors, to achieve the increase in LCR activity. Alternatively, the observed increase in transcription as a result of quercetin exposure may reflect the need for carrier proteins present in serum, such as albumin, for quercetin to gain access to the cell. Notwithstanding, effects of quercetin are not abolished in a low serum environment. This is exemplified by the observed reduction in response of the collagenase TRE to TPA in low serum when quercetin is also present. The mechanism of interference or antagonism between TPA and quercetin is not known.

Similar to the results described for the collagenase TRE, the effect of quercetin on the BPV4 LCR was not observed when TPA was simultaneously present in the culture medium. This supports the earlier suggestion that quercetin and TPA have a competitive or antagonistic effect on particular cellular function(s).

4.5 Analysis of the effect of a 21 base pair 3' truncation of the BPV4 LCR on transcriptional activity of the LCR acting as a promoter

Results from the previous experiments suggested that quercetin did not induce an increase in the transcriptional activity of the BPV4 LCR via activation of an AP1 transcription factor. Nevertheless, it was not clear if the TRE-like element, or another as yet unidentified site within the 3' 21 base pair sequence of the BPV4 LCR (nucleotides 311-331), was mediating the effect of quercetin on the LCR's

transcriptional activity. Therefore, a plamid carrying a 21 base pair deletion from the 3' end of the BPV4 LCR, a region which included the TRE-like element, was introduced into PalF cells and the transcriptional activity of this LCR deletion mutant was assayed.

4.5.1 Experimental procedure

A 21 base pair, 3' truncated form of the BPV4 LCR was contained in the p41X-PIN plasmid vector. However in p41X-PIN, the LCR was present as an enhancer element lying upstream of a TK promoter. This truncated form of the LCR was cut out of p41X-PIN using an appropriate restriction enzyme and sub-cloned into the *BamH* I site of the pOLuc vector as a promoter element, thus generating the psLCRLuc vector (see sections 2.2.9 and 2.3.2 for details).

10^5 low passage PalF cells were seeded in the appropriate tissue culture dish and incubated overnight as indicated in section 2.3.1.7. The culture medium was removed and replaced with a fresh volume of medium supplemented with either 20μM quercetin or an equivalent volume of its diluent, ethanol. Cells were cultured for 48 hours in this fresh medium. At the end of the 48 hours, the medium was changed to a fresh volume of 1xDMEM and the cells transfected with a combination of either 7.5μg psLCRLuc plus 2.5μg pCH110 or 7.5μg pOLuc plus 2.5μg pCH110. After an overnight incubation (section 2.3.1.7) the cells were re-fed with fresh medium supplemented with 20μM quercetin or an equivalent volume of ethanol; cells which previously were cultured in quercetin received ethanol containing medium, whereas cells which were previously grown in the presence of ethanol received quercetin medium. Control cells which were cultured in ethanol-containing medium before transfection were again given a fresh volume of medium containing ethanol.

Cells were incubated in the appropriate conditions for a further 48 hours after which time all cells were harvested. The cell lysates obtained were assayed for luciferase and β-galactosidase enzyme activities (section 2.3.1.11 and 2.3.1.12). All results were corrected for efficiency of transfection and normalised to the 'No quercetin' condition as described in section 4.2.1. The results from each of the experiments were combined and used to calculate the average fold increase in reporter gene expression, also detailed in section 4.2.1.

Table 4.5 Summary of the experimental conditions used when PalF cells were transfected with a 3' truncated BPV4 LCR present as a promoter and treated with or without quercetin.

	Condition #1	Condition #2	Condition #3	Condition #4	Condition #5	Condition #6
Before transfection	Med + EtOH	Med + EtOH	Med + Quer.	Med + Quer.	Med + EtOH	Med + EtOH
Transfected with	pOLuc + pCH110	psLCRLuc + pCH110	pOLuc + pCH110	psLCRLuc + pCH110	pOLuc + pCH110	psLCRLuc + pCH110
After transfection	Med + EtOH	Med + EtOH	Med + EtOH	Med + EtOH	Med + Quer.	Med + Quer.

Abbreviations used: Med = medium (1X DMEM)
Quer. = quercetin (to final concentration of 20μM)
EtOH = ethanol (a volume equivalent to the volume of quercetin added in other conditions, ie. 0.1% v/v)

4.5.2 Results

Quercetin produced a smaller increase in the level of transcription from the 3'truncated BPV4 LCR compared to the response of the full length LCR. This reduced effect of quercetin on the transcriptional activity of the 3' truncated LCR was only seen in cells which were treated with 20μM quercetin for 48 hours after transfection. This is similar to the observation described for the full length LCR (section 4.2.2). Also in keeping with observations described in section 4.2.2, the same concentration of quercetin had no effect on short-LCR activity when added to the culture medium before transfection.

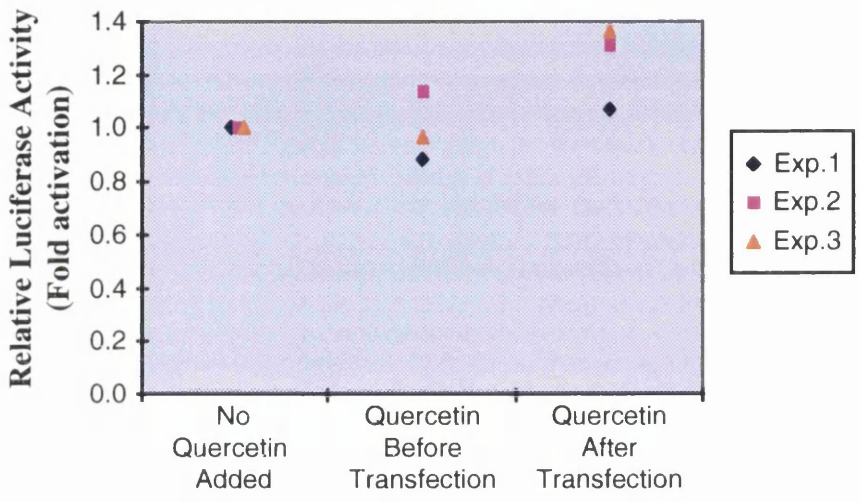
Despite the removal of nucleotides 311 to 331 from the BPV4 LCR, a slight increase the transcriptional activity of the LCR in response to quercetin exposure was observed (see figure 4.5). The response of this truncated form of the BPV4 LCR to quercetin however was less than that seen for the full length LCR (see section 4.2.2). Furthermore, the increase in transcription observed for the shortened LCR in cells treated with quercetin after transfection was found not to be statistically significant, according to Student t-Test analysis. p values obtained indicate no significant difference in the relative luciferase activity for cells treated with quercetin

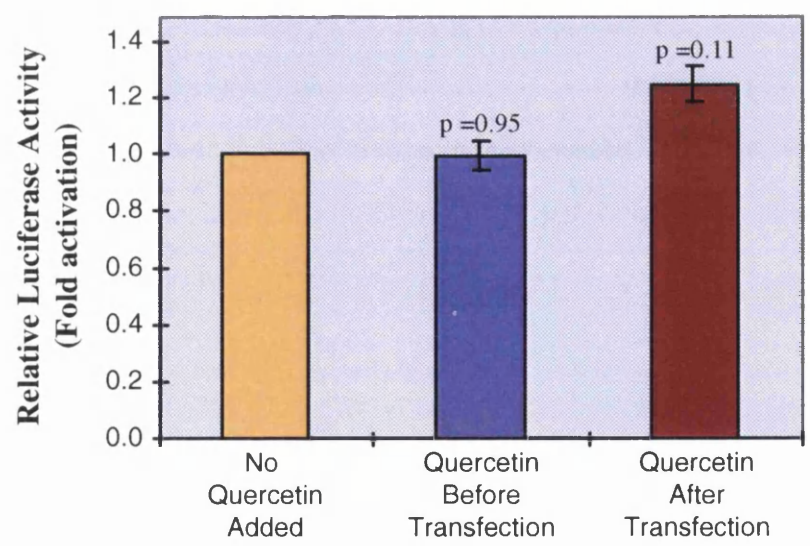
Figure 4.5 Activity of the 3'truncated BPV4 LCR acting as a promoter driving a luciferase reporter gene in PalF cells

Top panel shows a scatter plot which represents the results from each of 3 separate experiments.

Bottom panel shows the results from all 3 experiments combined. The vertical bars correspond to the standard error of the mean (S.E.M).

Figure 4.5 Activity of the 3' truncated BPV4 LCR acting as a promoter driving a luciferase reporter gene in PalF cells





after transfection compared to cells either not exposed to quercetin or exposed to quercetin before transfection.

- Quercetin before transfection compared to no quercetin; p=0.95
- Quercetin after transfection compared to no quercetin; p=0.11
- Quercetin after transfection compared to quercetin before; p=0.08

4.6 Analysis of the effect of a 21 base pair 3' truncation of the BPV4 LCR on the transcriptional activity of the LCR acting as an enhancer

Results from the experiment described in section 4.5.2 indicated that removal of 21 base pairs from the 3' end of the BPV4 LCR acting as a promoter correlated with a reduction in the response of the LCR to quercetin. Similar to the procedure presented in section 4.3.1, and as an extension of the results in section 4.5.2, we wished to determine if this shortened LCR showed similar activity, with relation to quercetin treatment, when the LCR was present in an enhancer orientation.

4.6.1 Experimental procedure

The experimental procedure employed was identical to that described in section 4.5.1. Briefly, 10^5 low passage PalF cells were seeded in 60mm tissue culture dish and incubated overnight as indicated in section 2.3.1.7. Cells were grown for the next 48 hours in medium supplemented with either quercetin (20μM) or an equivalent volume of ethanol. Following this the cells were transfected with a combination of plasmid vectors comprising either 7.5μg p41X-PIN plus 2.5μg pCH110 or 7.5μg p41X plus 2.5μg pCH110. After transfection (section 2.3.1.7), the medium was changed. Cells which were initially cultured in the presence of quercetin were given medium supplemented with ethanol, while cells which were previously treated with ethanol before transfection received medium supplemented with 20μM quercetin. Control cells were cultured in ethanol-containing medium for 48 hours both before and after transfection. At the end of the 48 hours, all dishes of cells were harvested and their cell lysates assayed for CAT and β-galactosidase enzyme activities (section 2.3.1.12 and 2.3.1.13).

Each CAT assay reading was corrected for efficiency of transfection, the contribution of empty vector (p41X) on transcription was removed, and the final

readings were normalised to the 'No quercetin' reading set = 1, as described in detail in section 4.3.1.

Table 4.6 Summary of the experimental conditions used when PalF cells were transfected with the 3' truncated BPV4 LCR present as an enhancer and treated with or without quercetin.

	Condition #1	Condition #2	Condition #3	Condition #4	Condition #5	Condition #6
Before transfection	Med + EtOH	Med + EtOH	Med + Quer.	Med + Quer.	Med + EtOH	Med + EtOH
Transfected with	p41X + pCH110	p41X-PIN + pCH110	p41X + pCH110	p41X-PIN + pCH110	p41X + pCH110	p41X-PIN + pCH110
After transfection	Med + EtOH	Med + EtOH	Med + EtOH	Med + EtOH	Med + Quer.	Med + Quer.

Abbreviations used: Med = medium (1X DMEM)
Quer. = quercetin (to final concentration of 20μM)
EtOH = ethanol (a volume equivalent to the volume of quercetin added in other conditions, ie. 0.1% v/v)

4.6.2 Results

The p values obtained using the Student's t-Test are given below;

- Quercetin before transfection compared to no quercetin; p=0.23
- Quercetin after transfection compared to no quercetin; p=0.15
- Quercetin after transfection compared to quercetin before; p=0.02*

The enhancer activity of 21 base pair 3' truncated form of the BPV4 LCR was not significantly altered by exposure to 20μM quercetin (see figure 4.6). The relatively poor response of the LCR as an enhancer to quercetin was independent of whether quercetin was added to the culture medium before or after transfection with p41X-PIN. The p-value obtained when comparing cells treated with quercetin before and after transfection appears significant. Nevertheless, results from the other experiments detailed in earlier sections indicate that the effect of quercetin on the activity of the BPV4 LCR is generally limited to cells treated with quercetin after

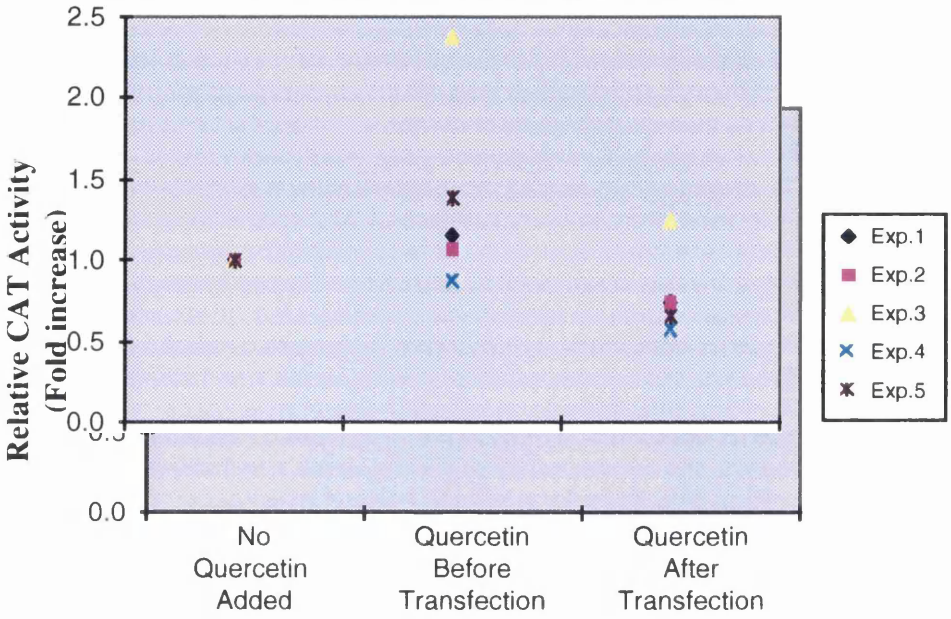
Figure 4.6

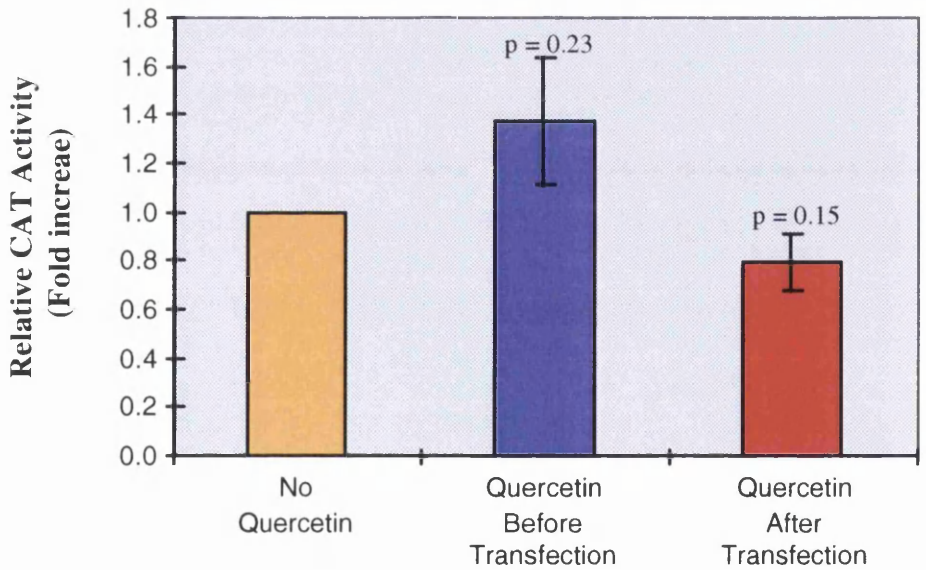
Figure 4.6 Activity of the 3'truncated BPV4 LCR in enhancer configuration in PalF cells

Top panel shows a scatter plot which represents the results from each of 5 separate experiments.

Bottom panel shows the results from all 5 experiments combined. The vertical bars correspond to the standard error of the mean (S.E.M).

Figure 4.6 Activity of the 3' truncated BPV4 LCR in enhancer configuration in PalF cells





transfection. The p-value obtained when comparing untreated cells with cells treated with quercetin after transfection is not significant therefore it is more likely that quercetin has relatively little to no effect on the activity of the LCR when present as an enhancer. This result in very similar to that described in section 4.3.2.

Hence, it would appear that the BPV4 LCR, either full length or 3' truncated, does not respond to quercetin when present as an enhancer in PalF cells.

A review of the results described in sections 4.2.2, 4.3.2, 4.5.2 and 4.6.2 suggest that the response of the BPV4 LCR to quercetin exposure is only observed if the LCR is present as a promoter and quercetin is added to the culture medium after cells have been transfected with the BPV4 LCR.

4.7 Time course experiment to determine the optimum length of quercetin treatment before cells are harvested, to detect the maximum increase in LCR activity

The results presented in sections 4.2.2 and 4.5.2 showed that the transcriptional activity of the BPV4 LCR in PalF cells was increased only when cells were treated with 20μM quercetin for 48 hours after transfection with a reporter vector carrying the BPV4 LCR. Cells were treated with quercetin for 48 hours as this was in keeping with the experimental protocol described by Cairney and Campo (1995); treatment of PalF cells with 20μM quercetin for 48 hours after transfection with BPV4 DNA (and *ras*) was sufficient to induce full cellular transformation.

However, it had not been established if treating cells with quercetin for 48 hours was the optimum incubation interval to achieve or observe the maximum increase in the LCR's transcriptional activity. For this reason, a time course experiment was performed in order to determine the duration of quercetin treatment which would produce in the greatest fold activation, as determined by reporter protein levels.

4.7.1 Experimental procedure

Approximately $2x10^5$ PalF cells were transfected with either 7.5μg pLCRLuc plus 2.5μg pCH110 or 7.5μg pOLuc plus 2.5μg pCH110. After transfection cells were treated with 20μM quercetin for either 1.5, 3, 6, 12, 24, 32, 40 or 48 hours. Control cells were treated with an equivalent volume of ethanol for 48 hours. After cells had been exposed to quercetin for the required time interval, cells were harvested (section 2.3.1.9a) and assayed for luciferase and β-galactosidase activities (sections 2.3.1.11 and 2.3.1.12).

Each luciferase assay reading was corrected for efficiency of transfection as described in section 4.3.1. The contribution of empty vector (pOLuc) on transcription was removed by subtracting the corrected luciferase reading for the pOLuc transfected cells treated with ethanol from the corresponding reading for cells transfected with pLCRLuc and treated with ethanol; the luciferase reading for pOLuc transfected cells treated with quercetin was likewise subtracted from the luciferase readings obtained for cells transfected with pLCRLuc and treated with quercetin. The average luciferase reading for the duplicate plates from each culture condition was calculated and the readings normalised to the 'No quercetin' reading set = 1, as described in detail in section 4.3.1.

4.7.2 Results

Results from the time course experiment are given in figure 4.7. The biggest increase in LCR transcriptional activity was observed when PalF cells were incubated in 20μM quercetin for 24 to 32 hours after transfection. In keeping with this observation, and in an attempt to optimise any potential response to quercetin treatment, exposure of cells to quercetin in all subsequent experiments was performed for a duration 24 hours.

4.8 Comparing the difference in response to quercetin treatment between full length and 21 base pair 3' truncated BPV4 LCRs in PalF cells

The activity of both the full length and truncated BPV4 LCRs cloned into the pOLuc vector is not very high in PalF cells. The reasons for this may be two fold. Firstly, the pOLuc vector is not a particularly strong expression vector in PalF cells. Secondly, PalF cells are not the natural host cell for a BPV4 infection; BPV4 is

Figure 4.7 Time course of full length LCR promoter activity in 20μM quercetin

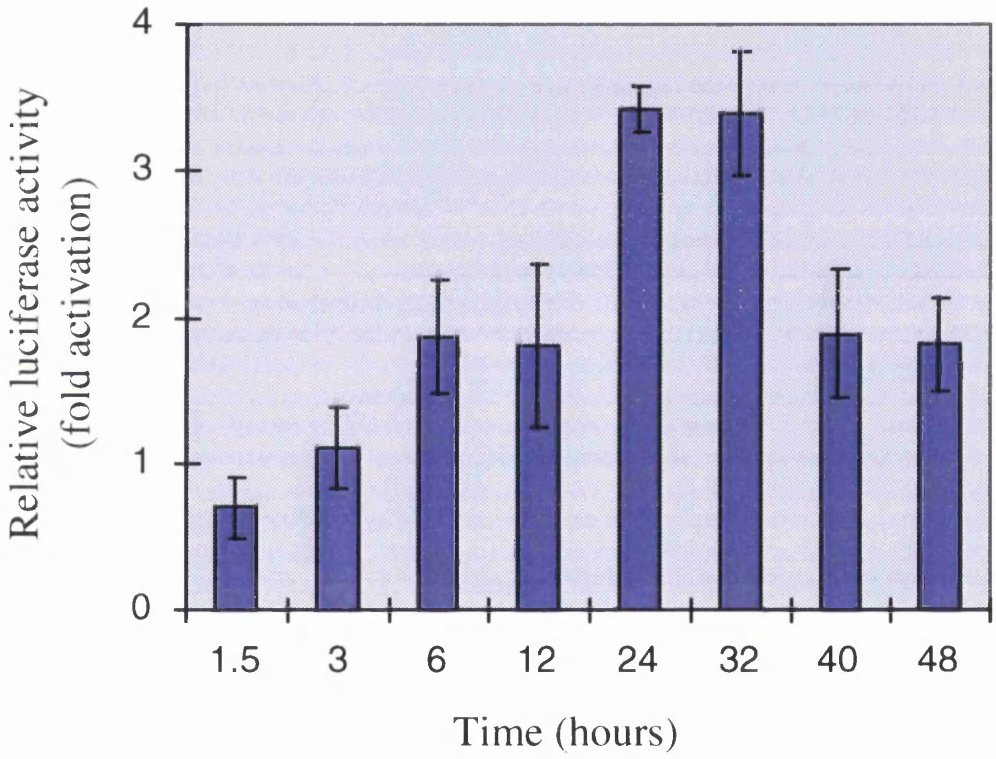


Chart above represent the average results from two separate experiments.
Duplicates plates were used for each time point in both experiments.
Vertical bars correspond to standard error of the mean value (S.E.M)

naturally tropic for keratinocytes, therefore it is reasonable to expect the activity of the LCR to be less than optimum in fibroblasts.

As a means of improving the level of reporter gene expression, the full length and 21 base pair truncated BPV4 LCRs were cloned into the *Bgl* II site of the pGL3 expression vector (Promega) by Dr Iain Morgan (Beatson Institute, Glasgow). In addition to sub-cloning the BPV4 LCRs into a more efficient expression vector, quercetin treatment of cells was observed for 24 hours, rather than 48 hours as in previous experiments, in keeping with the results of the time course experiment (section 4.7.2).

The following experiments compared the expression of the full length and short LCRs, present as promoter elements in the pOLuc and pGL3 vectors, in response to quercetin exposure in PalF cells.

4.8.1 Experimental procedure

Quercetin was only given after transfection as no appreciable alteration to the level of transcription was detected when cells were treated with quercetin before transfection, as observed from the experimental results detailed above (section 4.2.2 and 4.5.2).

$2x10^5$ PalF cells were seeded into 60mm tissue culture dishes. The following day, cells were transfected with either 7.5μg of pOLuc/pGL3 plus 2.5μg pCH110 (condition #1), 7.5μg pLCRLuc/pGL3-PINT plus 2.5μg pCH110 (condition #2), or 7.5μg psLCRLuc/pGL3-PIN plus 2.5μg pCH110 (condition #3) according to the schedule detailed in section 2.3.1.7. After transfection, two dishes of cells from each condition (duplicate dishes) were given medium supplemented with either 20μM quercetin or an equivalent volume of ethanol. All cells were cultured for a further 24 hours and then harvested as described in section 2.3.1.9a. The resultant cell lysates were assayed for luciferase and β-galactosidase enzyme activities (sections 2.3.1.11 and 2.3.1.12).

Each luciferase assay reading was corrected for efficiency of transfection (see section 4.3.1). The contribution of empty vector (pOLuc or pGL3) on transcription was removed by subtracting the luciferase reading for pOLuc/pGL3 transfected cells treated with ethanol or quercetin from the luciferase reading for cells transfected with pLCRLuc/pGL3-PINT or psLCRLuc/pGL3-PIN similarly treated with ethanol or

quercetin respectively (ethanol readings are subtracted from ethanol readings and quercetin readings are subtracted from quercetin readings). The final readings were normalised to the 'No quercetin' reading set = 1, as described in detail in section 4.3.1.

Table 4.7a Summary of the experimental conditions used when PalF cells were transfected with the pOLuc vectors, carrying the full length and truncated BPV4 LCRs present as a promoter, and treated with or without quercetin.

	Condition # 1		Condition # 2		Condition # 3	
Transfected with	pOLuc + pCH110		pLCRLuc + pCH110		psLCRLuc + pCH110	
Treated with	E	Q	E	Q	E	Q

Table 4.7b Summary of the experimental conditions used when PalF cells were transfected with the pGL3 vectors, carrying the full length and truncated BPV4 LCRs present as a promoter, and treated with or without quercetin

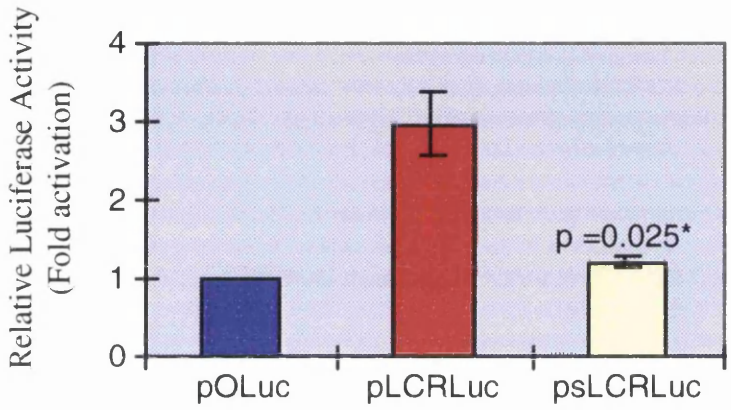
	Condition # 1		Condition # 2		Condition # 3	
Transfected with	pGL3 + pCH110		pGL3-PINT + pCH110		pGL3-PIN + pCH110	
Treated with	E	Q	E	Q	E	Q

4.8.2 Results

Figure 4.8 illustrates the response of the full length and 3' truncated BPV4 LCRs in pOLuc (panel A) and pGL3 (panel B) vectors to quercetin. Removal of 21 base pairs from the 3' end of the BPV4 LCR correlated with a significant decrease in response to quercetin treatment. A significant decrease in response to quercetin treatment was observed independent of whether the truncated LCR was cloned in the pOLuc ($p=0.025$) or pGL3 based vector ($p=0.037$). Nevertheless, the response of the truncated LCR to quercetin treatment in either vector was not totally abolished

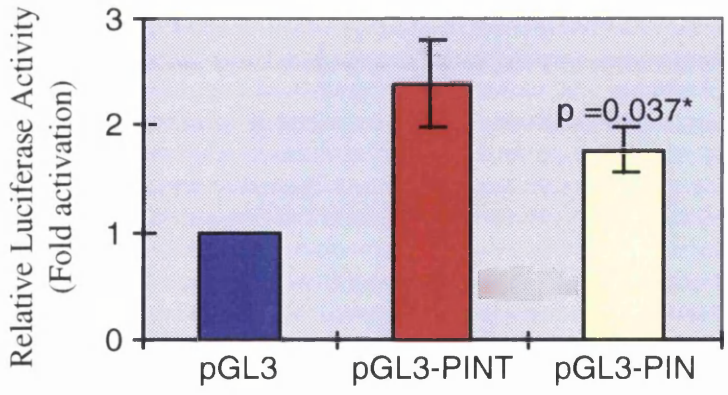
Figure 4.8 The transcriptional response of the full length and 3' truncated BPV4 LCRs in (A) pOLuc and (B) pGL3 vectors in PalF cells treated with 20μM quercetin

A



p value shown reflects the difference between pLCRLuc and psLCRLuc
The chart represents results from 4 separate experiments

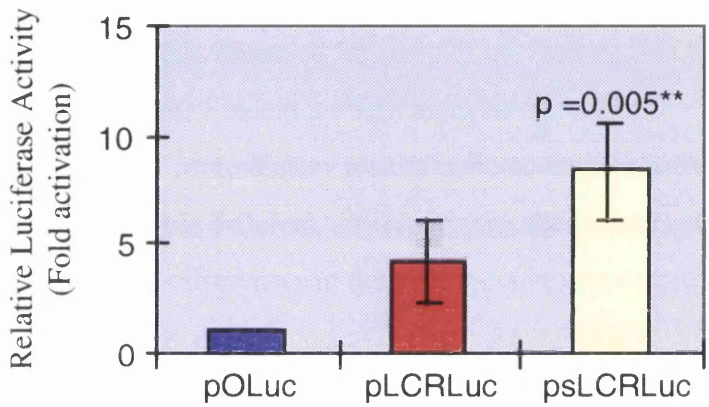
B



p value shown reflects the difference between pGL3-PINT and pGL3-PIN
The chart represents results from 5 separate experiments

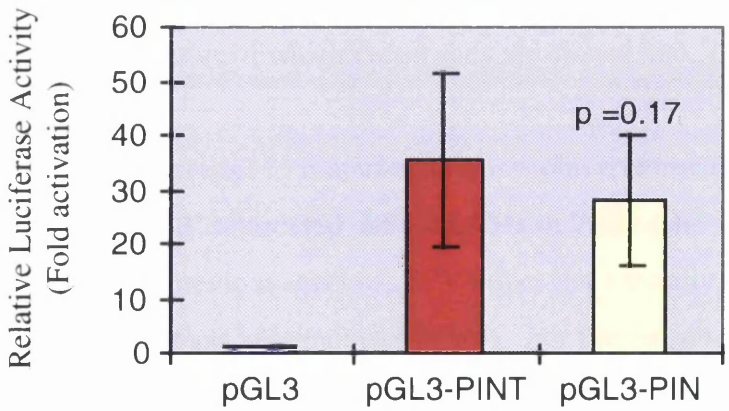
Figure 4.9 The basal transcriptional activity of the full length and 3' truncated BPV4 LCRs in (A) pOLuc and (B) pGL3 vectors in PalF cells

A



p value shown reflects the difference between pLCRLuc and psLCRLuc
The chart represents results from 4 separate experiments

B



p value shown reflects the difference between pGL3-PINT and pGL3-PIN
The chart represents results from 5 separate experiments

suggesting that other regions of the LCR are involved in mediating the transcriptional effects of quercetin.

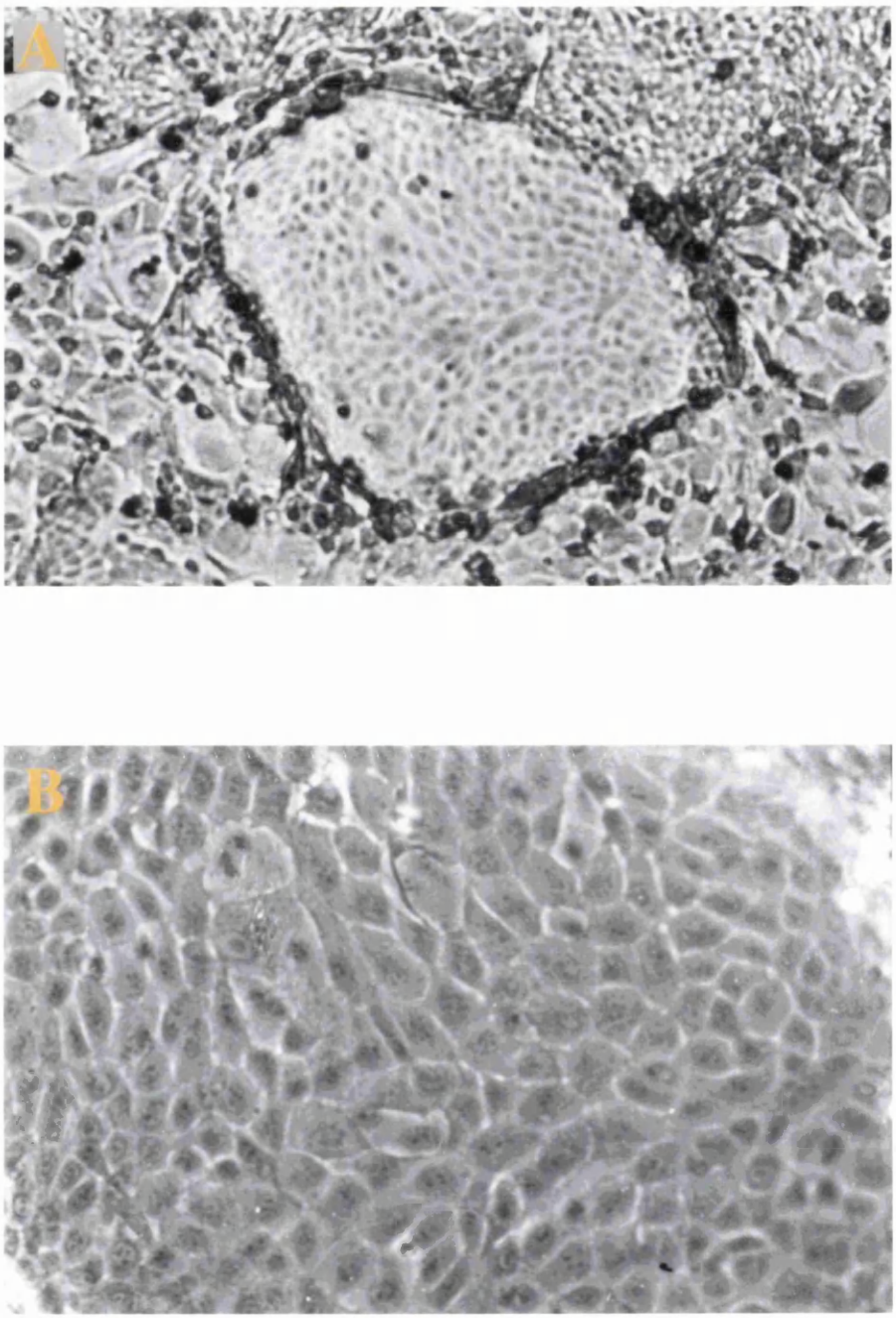
Figure 4.9 shows the basal activity of the full length and 3' truncated forms of the BPV4 LCR in pOLuc (panel A) and pGL3 (panel B) vectors. The truncated BPV4 LCR appears more active in PalF cells compared to the full length LCR, when cloned in pOLuc based vectors (panel A). This difference appears to be significant according to a Student's t-Test analysis ($p=0.005$). In the pGL3 based vectors however, the short LCR appears less active compared to full length LCR (figure 4.9 - panel B). The difference in basal activities of the full length and truncated forms of the BPV4 LCR in pGL3 vectors, unlike that observed for the pOLuc vectors, was found not to be significant according to the Student's t-Test analysis ($p=0.17$).

These apparently contradictory results indicate that the activity of the truncated LCR in these two vectors is different. Alternatively, the variant activities observed may reflect fundamental differences in the vector constructs directly, independent of the BPV4-specific sequences also present. There are noticeable differences in the basal activities of the LCRs in these two vectors (figure 4.9) which is reflected in their respective relative luciferase activities; the activity of the full length and truncated LCRs in greater in the pGL3 based vectors compared to corresponding activities in the pOLuc base vectors. Nevertheless, the fold increase in transcriptional activity in response to quercetin for the full length and truncated forms of the BPV4 LCRs is roughly the same irrespective of which vector they are cloned into.

4.9 Comparing the difference in response to quercetin treatment between full length and 21 base pair 3' truncated BPV4 LCRs in PalK cells

As mentioned in previous sections, BPV4 does not naturally infect fibroblast cells. Rather the virus infects keratinocytes *in vivo*. For this reason we wished to determine the activity of the BPV4 LCR, both full length and truncated, in a more physiologically relevant cell type and to this end primary bovine keratinocyte cells explanted from the soft palate of a bovine foetus (PalK cells) were used in a series of transient transfection experiments. Figure 4.10 contains photographs of PalK cells viewed at two magnifications as seen with a light microscope. Similar to the protocol described in section 4.8, both the pOLuc and pGL3 vectors, containing the full length

Figure 4.10 Photographs of PalK cells



Panel A: Bright field image of PalK cells (centre area) grown on a layer of irradiated Swiss 3T3 feeder cells.
Final magnification x20.

Panel B: Phase contrast image of PalK cells.
Final magnification x50.

and 21base pair truncated LCRs, were transfected into bovine keratinocytes. The cells were treated with quercetin or ethanol for 24 hours *after* transfection only.

4.9.1 Experimental procedure

The procedure followed in these experiments was essentially identical to that detailed in section 4.8.1. However, in place of PalF cells , $5x10^5$ primary bovine foetal palate keratinocyte (PalK) cells were seeded in the appropriate tissue culture dishes, in the absence of a feeder layer, and transfected as described in section 2.3.1.8. All cells were transfected with a total of 10μg of DNA as indicated in tables 4.8a and 4.8b; cells in condition #1 received 7.5μg pOLuc/pGL3 plus 2.5μg pCH110, condition #2 received 7.5μg pLCRLuc/pGL3-PINT plus 2.5μg pCH110 and condition #3 received 7.5μg psLCRLuc/pGL3-PIN plus 2.5μg of pCH110. The appropriate dishes of cells were subsequently treated with either 20μM quercetin or an equivalent volume of ethanol for 24 hours after transfection according to the schedules summarised in table 4.8a and 4.8b. All dishes of cells were harvested at the end of the 24 hour incubation period (2.3.1.9a). Each cells lysate was then assayed for luciferase and β-galactosidase enzyme activities (sections 2.3.1.11 and 2.3.1.12).

Each luciferase assay reading was corrected for efficiency of transfection using the corresponding β-galactosidase reading as described in section 4.3.1. The contribution of empty vector (pOLuc/pGL3) on transcription was removed by subtraction, as detailed in section 4.8.1. Each reading was finally normalised to the 'No quercetin' reading set = 1 (section 4.3.1).

Table 4.8a Summary of the experimental conditions used when PalK cells were transfected with the pOLuc vectors, carrying the full length and truncated BPV4 LCRs present in a promoter configuration, and treated with or without quercetin.

	Condition # 1		**Condition # 2**		**Condition # 3**	
Transfected with	pOLuc + pCH110		pLCRLuc + pCH110		psLCRLuc + pCH110	
Treated with	E	Q	E	Q	E	Q

Table 4.8b Summary of the experimental conditions used when PalK cells were transfected with the pGL3 vectors, carrying the full length and truncated BPV4 LCRs present in a promoter configuration, and treated with or without quercetin

	Condition # 1		Condition # 2		Condition # 3	
Transfected with	pGL3 + pCH110		pGL3-PINT + pCH110		pGL3-PIN + pCH110	
Treated with	E	Q	E	Q	E	Q

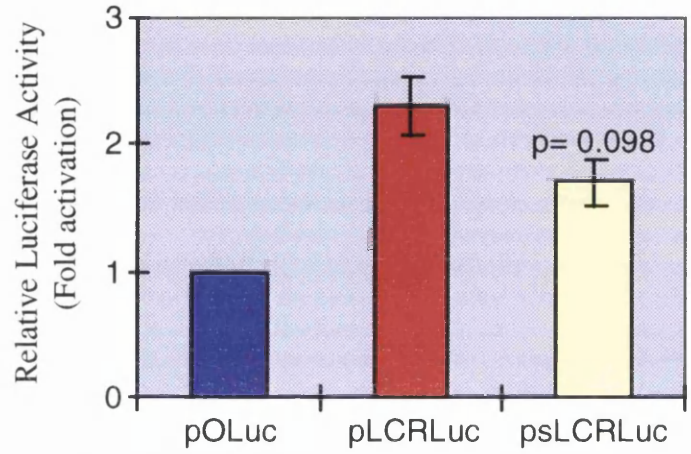
4.9.2 Results

Figure 4.11 shows the response to quercetin treatment of the full length and 3' truncated BPV4 LCRs, cloned in both pOLuc (panel A) and pGL3 (panel B) vectors, following transfection into PalK cells. Removal of 21 base pairs from the 3' end of the BPV4 LCR correlated with a decrease in the transcriptional response to quercetin for the psLCRLuc vector only. A similar decrease was not observed for the truncated LCR cloned in the pGL3 vector (pGL3-PIN). For both vectors, the response of the truncated LCR to quercetin treatment was not significantly different from the response of the full length LCR; p values comparing the quercetin response of the truncated versus the full length LCR in the pOLuc- and pGL3-based vectors were 0.098 and 0.78 respectively. As was observed for the same vectors transfected into PalF cells (section 4.8.2), the response of the truncated LCR to quercetin treatment in either vector was not abolished. This further supports the suggestion that other regions of the LCR are involved in mediating the transcriptional effects of quercetin.

Figure 4.12 represents the basal activity of the full length and 3' truncated forms of the BPV4 LCR, in pOLuc (panel A) and pGL3 (panel B) vectors, in PalK cells. In contrast to the results obtained with the pOLuc vectors in PalF cells (section 4.8.2), the 3' truncated BPV4 LCR was less active in PalK cells compared to the full length LCR (panel A). The fall in the basal level of transcriptional activity observed for the psLCRLuc (truncated LCR) vector as compared to the pLCRLuc (full length LCR) was determined not to be significant according to a Student's t-Test analysis ($p=0.2$). The basal activities for psLCRLuc described in this section and in section 4.8.2 highlight that the same vector can display contrasting activity in different cell types.

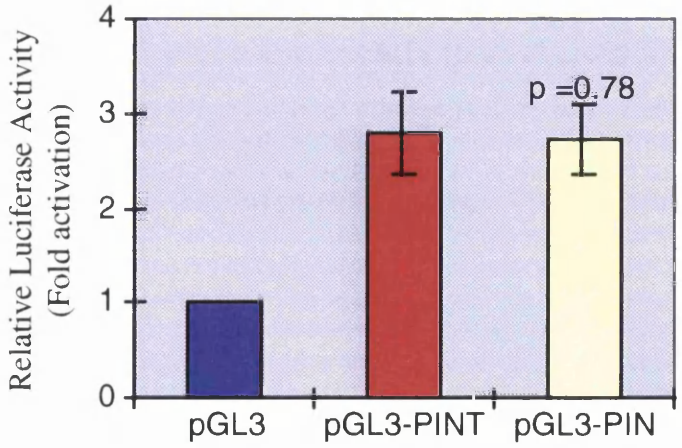
Figure 4.11 The transcriptional response of the full length and 3' truncated BPV4 LCRs in (A) pOLuc and (B) pGL3 vectors in PalK cells treated with 20μM quercetin

A



p value shown reflects the difference between pLCRLuc and psLCRLuc
The chart represents results from 5 separate experiments

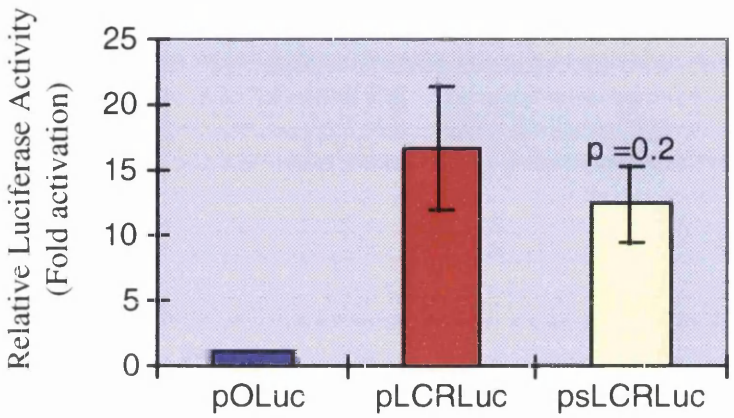
B



p value shown reflects the difference between pGL3-PINT and pGL3-PIN
The chart represents results from 8 separate experiments

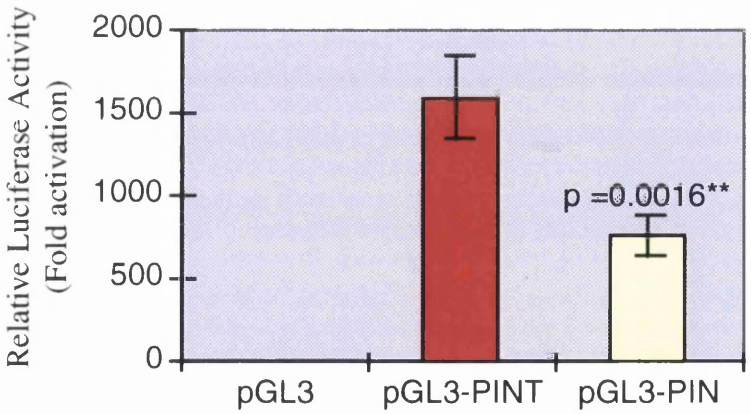
Figure 4.12 The basal transcriptional activity of the full length and 3' truncated BPV4 LCRs in (A) pOLuc and (B) pGL3 vectors in PalK cells

A



p value shown reflects the difference between pLCRLuc and psLCRLuc
The chart represents results from 5 separate experiments

B



p value shown reflects the difference between pGL3-PINT and pGL3-PIN
The chart represents results from 8 separate experiments

The truncated LCR in the pGL3 based vector in PalK cells also showed a decrease in the basal level of transcription with respect to the full length LCR, however unlike the pOLuc-based vector, the observed drop in response to quercetin was found to be very significant (p=0.0016) (figure 4.12 - panel B).

Results from the experiments described in sections 4.8 and 4.9 show that a sequence of the BPV4 LCR can display varying activities, both at a basal level and in response to quercetin treatment, depending on the vector into which the sequence is cloned. Furthermore, the cell type into which the vector is transfected can also affect the levels of activity observed. Notwithstanding, results from sections 4.8 and 4.9 indicate that nucleotides 311-331 of the BPV4 LCR are at least partially involved in mediating the increase in transcriptional activity of the BPV4 LCR in response to quercetin treatment. In addition, the drop in basal reporter gene activity upon removal of these nucleotides from the 3' end of the BPV4 LCR suggests that this stretch of the LCR is also important in terms of the basal activity and regulation of viral transcription.

4.10 Identification of a factor, QX1, which binds within the 3' terminal 21 base pair sequence of the BPV4 LCR

Results from the transfection experiments detailed above have shown that the increase in transcription, in response to quercetin treatment, was less for the truncated form of the LCR when compared to the response of the full length LCR. This suggested that a site(s) within or adjacent to nucleotides 311-331 of the BPV4 LCR was mediating, at least in part, the effect of quercetin on the LCR's transcriptional upregulation. We therefore decided to use an oligonucleotide corresponding to the last 25 base pair sequence from the BPV4 LCR in a series of electrophoretic mobility shift assays (EMSA), in an attempt to identify any factor(s) which has the potential to bind within this region of the LCR.

4.10.1 Experimental procedure

The experimental protocol which was used is given in detailed in section 2.3.2.14-2.3.2.17. Briefly, 10^6 low passage PalF cells were seeded in each of 8xT175 tissue culture flasks. The cells were left overnight to adhere to the tissue culture plastic. The following day, cells were re-fed with a fresh volume of 1xDMEM

medium. Cells were incubated for 24 hours under normal conditions. After 24 hours, the medium in 4 of the T175 flasks was supplemented with 20μM quercetin; medium in the 4 remaining T175 flasks was supplemented with an equivalent volume of ethanol (0.1% v/v). Cells were cultured in the presence of 20μM quercetin or ethanol for 24 hours. At the end of the incubation period, the cells were harvested for nuclear extract as detailed in section 2.3.1.9c.

Approximately 10μg of nuclear extract from the quercetin and ethanol treated PalF cells was mixed with the appropriate radioactively labelled oligonucleotide containing the binding site of interest, plus non-radioactively labelled competitor oligonucleotide where specified. Details of the oligonucleotides used as labelled probes and unlabelled competitors in this and other EMSAs is given in figure 4.13. The reaction mixtures were incubated as described in section 2.3.2.16 and then separated on a polyacrylamide gel as detailed in section 2.3.2.17.

4.10.2 Results

As illustrated in figure 4.14, two complexes were detected binding to the labelled 3'wtLCR oligonucleotide. Both complexes were detected in nuclear extract from PalF cells independent of whether the cells were exposed to quercetin or its diluent ethanol prior to harvesting.

The lower band corresponds to a complex which binds non-specifically to the labelled probe, as indicated by the lack of competition with any of the unlabelled oligonucleotides which were used.

The second complex, indicated by the arrows, was designated QX1. Exposure of PalF cells to 20μM quercetin for 24 hours prior to harvesting had no apparent effect on the binding of QX1 (figure 4.14 - lane 5); the EMSA performed with an identical amount of nuclear extract from cells similarly treated with ethanol before harvesting showed no change in the binding of QX1 (figure 4.14 - lane 2). The QX1 band was competed away with unlabelled 3'wtLCR oligonucleotide or with an unlabelled oligonucleotide corresponding to the sequence of the collagenase TRE which contained a consensus AP1-binding site. The competition observed indicates that the binding of QX1 to a region of the LCR between nucleotides 311 and 331 is specific. Binding probably involves the nucleotides in or adjacent to the TRE-like element; this

Figure 4.13 Oligonucleotides used in Electrophoretic Mobility Shift Assays (EMSA)

3'wt LCR: 5' - GGATTTGGTGCATGAGGCAGTAGCT - 3'
Mutant #1: 5' - TCGAGGATTTGGTGCATGAGTCAGTAGCTTCCATC - 3'
Mutant #2: 5' - TCGAGGATTTGGTGCAAAAGGCAGTAGCTTCCATC - 3'
Mutant #3: 5' - TCGAGGATTTGGAACATGAGGCAGTAGCTTCCATC - 3'

Col. TRE: 5' - AAGCATGAGTCAGACACCTC - 3'
Myc: 5' - CCCCCACCACGTGGTGCCTGA - 3'

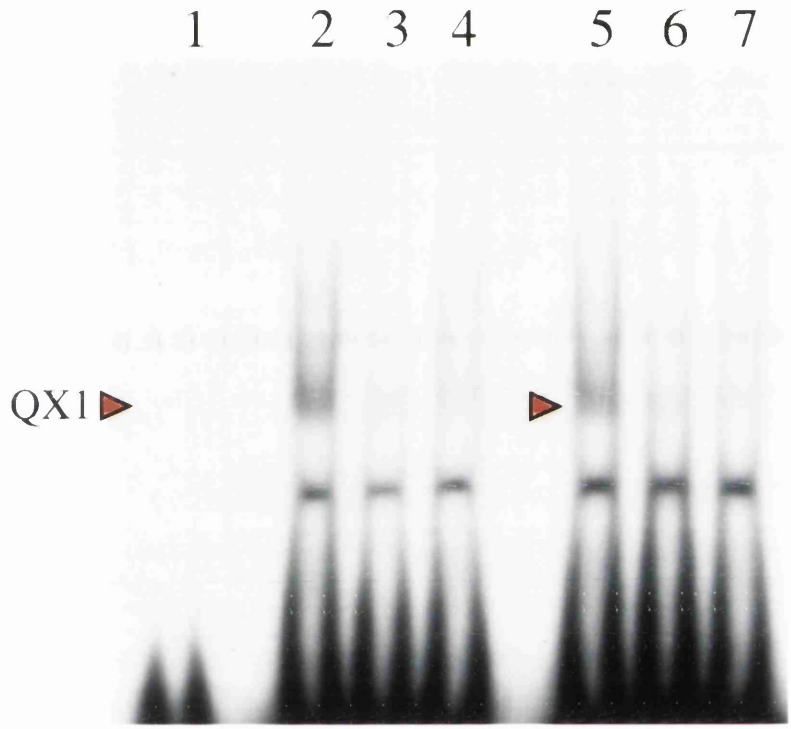
- Shaded region in 3'wt (wild type) LCR sequence highlights the TRE-like element
- Shaded bases in mutants #1, #2 and #3 indicate nucleotide changes compared to 3'wt LCR
- Shaded region in Col. TRE (collagenase TRE) sequence highlights the AP1-binding motif
- The sequence for the Myc oligonucleotide was taken from Blackwood and Eisenman, 1991

All oligonucleotide sequences used in the binding assays were double stranded and end labelled with ^{32}P, as detailed in sections 2.3.2.1 and 2.3.2.14. Only the sequence of the leading strand of each double stranded oligonucleotide is shown above.

The 3' wild type (wt) LCR, mutant # 1, mutant # 2 and mutant # 3 oligonucleotides were synthesised by Beatson Institute technical services. Each strand of the oligonucleotide was generated separately and then annealed in equimolar quantities after purification (see section 2.3.2.1 for details).

The collagenase TRE and Myc double stranded oligonucleotides were kindly provided by Dr. David Gillespie and Dr. Kevin Ryan (Beatson Institute) respectively.

Figure 4.14 EMSA showing the binding of factor QX1 to a BPV4 LCR oligonucleotide (nucleotides 308-331) using nuclear extract from PalF cells treated with or without quercetin

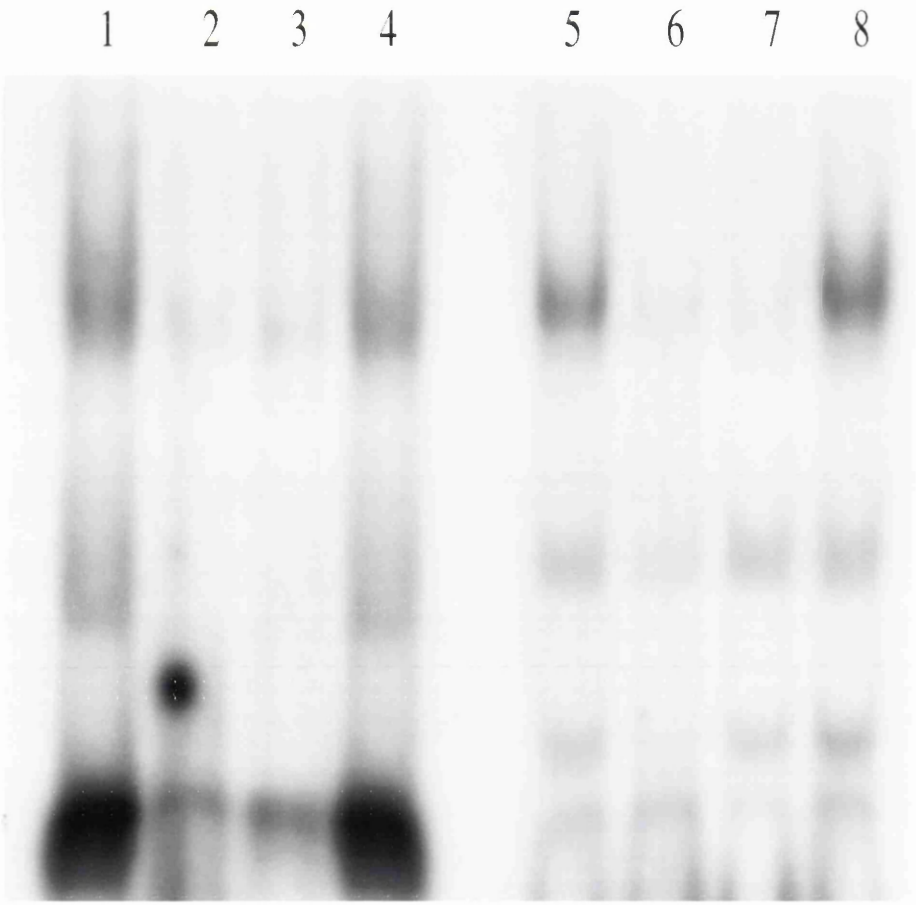


Lane 1 contain no nuclear extract
Lanes 2-4 each contain ~10µg nuclear extract from PalF cells exposed to 0.1% v/v ethanol for 24 hours before cells were harvested
Lane 5-7 each contains ~10µg nuclear extract from PalF cells exposed to 20µM quercetin for 24 hours before cells were harvested

Lane 1: 3'wtLCR probe alone (no extract)
Lane 2: 3'wtLCR
Lane 3: 3'wtLCR probe + cold 3'wtLCR competitor (self)
Lane 4: 3'wtLCR probe alone + cold Col.TRE competitor
Lane 5: 3'wtLCR probe
Lane 6: 3'wtLCR probe + cold 3'wtLCR competitor (self)
Lane 7: 3'wtLCR probe alone + cold Col.TRE competitor

Each competitor was added to the lanes as indicated in a 100 fold excess with respect to labelled probe

Figure 4.15 EMSA showing a factor, QX1, binds within the 3'terminal 21bp sequence of the BPV4 LCR and that QX1 is present in both chick embryo fibroblasts and bovine foetal palate fibroblasts



Lanes 1-4 contain whole cell extract from chick embryo fibroblasts (CEF)
Lanes 5-8 contain nuclear extract from bovine foetal palate fibroblasts (PalF)

Lane 1: 3'wtLCR probe
Lane 2: 3'wtLCR probe + Cold 3'wtLCR competitor (Self)
Lane 3: 3'wtLCR probe + Cold Col.TRE competitor
Lane 4: 3'wtLCR probe + Cold Myc competitor

Lane 5: 3'wtLCR probe
Lane 6: 3'wtLCR probe + Cold 3'wtLCR competitor (Self)
Lane 7: 3'wtLCR probe + Cold Col.TRE competitor
Lane 8: 3'wtLCR probe + Cold Myc competitor

◀ QX1: Factor which is seen to bind between nucleotides 310 and 331 of BPV4 LCR

is supported by the similar degree of competition observed with the collagenase TRE and 3'wtLCR oligonucleotides alike.

Figure 4.15 shows that QX1 is not specific to PalF cell nuclear extract. A band, apparently identical to QX1, was similarly detected in nuclear extract from chick embryo fibroblasts (CEF) (see QX1 band in lane 1). The QX1 band detected in CEF extract was competed away with unlabelled 3'wtLCR and unlabelled collagenase TRE oligonucleotides (lanes 2 and 3 respectively). An unlabelled oligonucleotide carrying the binding site for the c-myc protein was unable to compete for the binding of QX1. The pattern of binding and competition of QX1 observed with CEF extract was the same as that obtained when using PalF nuclear extract. This supports the conclusion that the two bands correspond to the same factor, namely QX1, and that QX1 is present in fibroblast cells both bovine and chick in origin.

4.11 Factor QX1 is not classical AP-1

It was not clear if the QX1 band, identified in figures 4.14 and 4.15, was a novel factor or if this band corresponded to an AP1 factor. AP1 was a potential candidate factor for binding to the BPV4 LCR oligonucleotide considering the high degree of homology between the TRE-like element and the AP1-binding site (see section 4.4 for sequence comparison).

To address this question it was first necessary to determine the level of AP1 in PalF cells. This was done in an EMSA using an oligonucleotide containing the canonical AP1-binding site from the collagenase gene. Secondly, we wish to determine if QX1 and AP1 were the same factor; if QX1 and AP1 were indeed the same, then the bands for QX1 and AP1 should display similar mobility in an EMSA.

4.11.1 Experimental procedure

Approximately 10μg of chick embryo fibroblast (CEF) or PalF nuclear extract was incubated with a radiolabelled oligonucleotide probe containing either the collagenase AP1-binding site or the TRE-like element found in the BPV4 LCR. Competitor oligonucleotides were included in reaction mixes where specified (see figure 4.16 and 4.17 for details of competitors used). Binding to the radiolabelled oligonucleotides was determined by running the reaction samples in individual lanes

on a non-denaturing polyacrylamide gel and exposing the dried gel to X-ray film at -70°C, as described in sections 2.3.2.16 and 2.3.2.17.

4.11.2 Results

PalF cells contain readily detectable levels of AP1 (see figure 4.16). The level of AP1 detected in PalF cells was roughly identical to that observed for CEF extracts (figure 4.16). The AP1 band was completely competed away using the same unlabelled TRE oligonucleotide (self competition) for both CEF and PalF extracts (lanes 3 and 8). Addition of unlabelled 3'wtLCR oligonucleotides to the reaction mixes resulted in only a small degree of competition. This indicates that the interaction between AP1 and the 3'wtLCR sequence is not as specific as AP1 for the collagenase TRE (lanes 4 and 9). The unrelated c-myc oligonucleotide had no affect on the binding of AP1 to the collagenase TRE (lane 5 and 10).

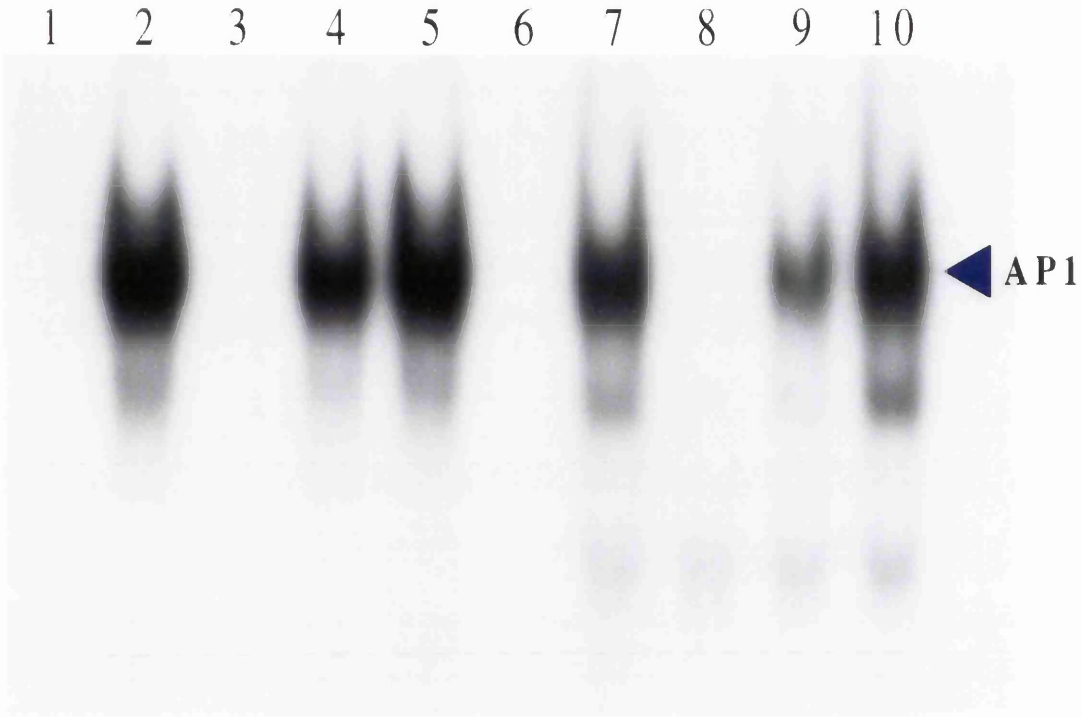
As is illustrated in figure 4.17, the QX1 and AP1 bands appear at very different position on the same gel. This indicates that the two factors are not the same. An unlabelled collagenase TRE oligonucleotide was able to compete well with the 3'wtLCR for the binding of QX1 (figure 4.17 - lane 4). Conversely, the 3'wtLCR competed poorly with the collagenase TRE for the binding of AP1.

Other complexes in addition to AP1 and QX1 were detected in the EMSAs presented in figures 4.16 and 4.17. These other complexes however displayed a non-specific pattern of binding or were not consistently detected in every assay and are thus not discussed further here or in following sections.

4.12 Analysis to determine if QX1 and AP1 share any common structural components

Although it would appear from the results in section 4.11.2, that QX1 and AP1 are not the same, this does not rule out the possibility that the two factors each contain a common structural component(s), particularly when considering the similarity between their individual binding motifs. As mentioned in section 4.4, AP1 may exist as a homo- or heterodimer; Jun proteins may combine to form homodimers, or Jun may combine with Fos (or Fras) to produce the heterodimer form of AP1. We therefore used antibodies to the cJun and cFos to test if QX1 contained either of these two proteins as structural subunits.

Figure 4.16 EMSA to detect AP1 in nuclear extracts from Chick embryo fibroblasts and Bovine foetal palate fibroblasts

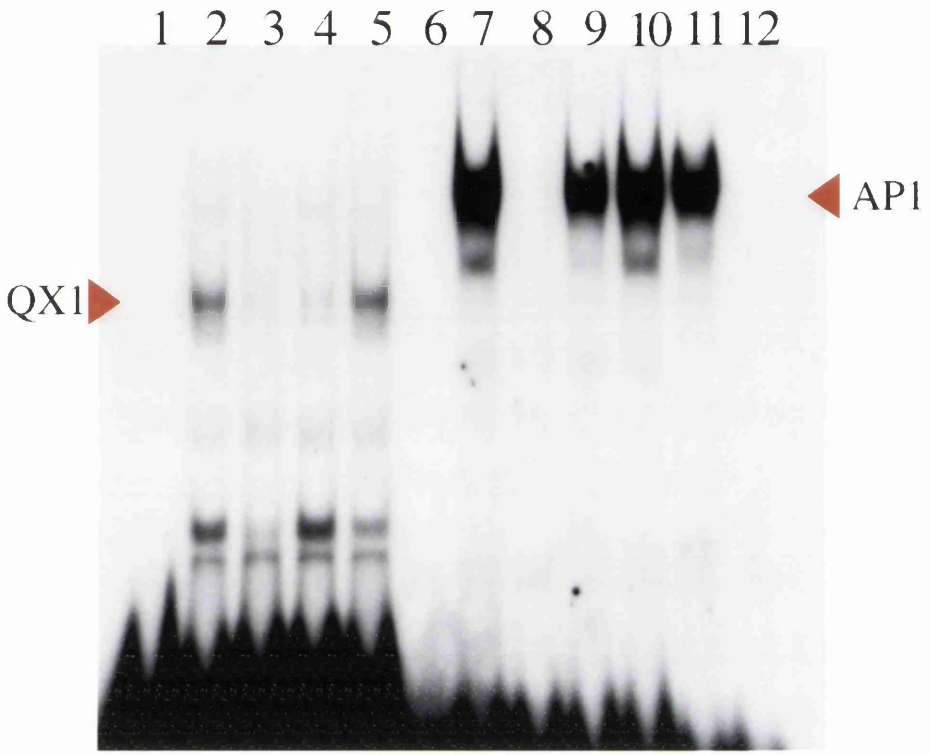


Lanes 2-5 contain whole cell extract from chick embryo fibroblasts (CEF)
Lanes 7-10 contain nuclear extract from bovine palate fibroblasts (PalF)

Lane 1: Col.TRE probe alone (no extract)
Lane 2: Col.TRE probe
Lane 3: Col.TRE probe + Cold Col.TRE competitor (Self)
Lane 4: Col.TRE probe + Cold 3'wtLCR competitor
Lane 5: Col.TRE probe + Cold Myc competitor
Lane 6: Col.TRE probe alone (no extract)
Lane 7: Col.TRE probe
Lane 8: Col.TRE probe + Cold Col.TRE competitor (Self)
Lane 9: Col.TRE probe + Cold 3'wtLCR competitor
Lane 10: Col.TRE probe + Cold Myc competitor

Competitor oligonucleotides were present in 100 fold excess with respect to labelled probe

Figure 4.17 EMSA with nuclear extract from Bovine Foetal Palate Fibroblasts to determine if QX1 and AP1 are the same factor



Lane 1 and 6 contain no cellular extract
Lanes 2-5, 7-10 contain extract from PalF cells
Lane 11 and 12 contain whole extract from chick embryo fibroblasts (CEF)

Lane 1: 3'wtLCR probe alone (no extract)
Lane 2: 3'wtLCR probe
Lane 3: 3'wtLCR probe + Cold 3'wtLCR competitor (Self)
Lane 4: 3'wtLCR probe + Cold Col.TRE competitor
Lane 5: 3'wtLCR probe + Cold Myc competitor
Lane 6: Col.TRE probe alone (no extract)
Lane 7: Col.TRE probe
Lane 8: Col.TRE probe + Cold Col.TRE competitor (Self)
Lane 9: Col.TRE probe + Cold 3'wtLCR competitor
Lane 10: Col.TRE probe + Cold Myc competitor

Lane 11: Col.TRE probe alone
Lane 12: Col.TRE probe + Cold Col.TRE competitor

Each competitor oligonucleotide used was present in 100 fold excess with respect to labelled probe

4.12.1 Experimental procedure

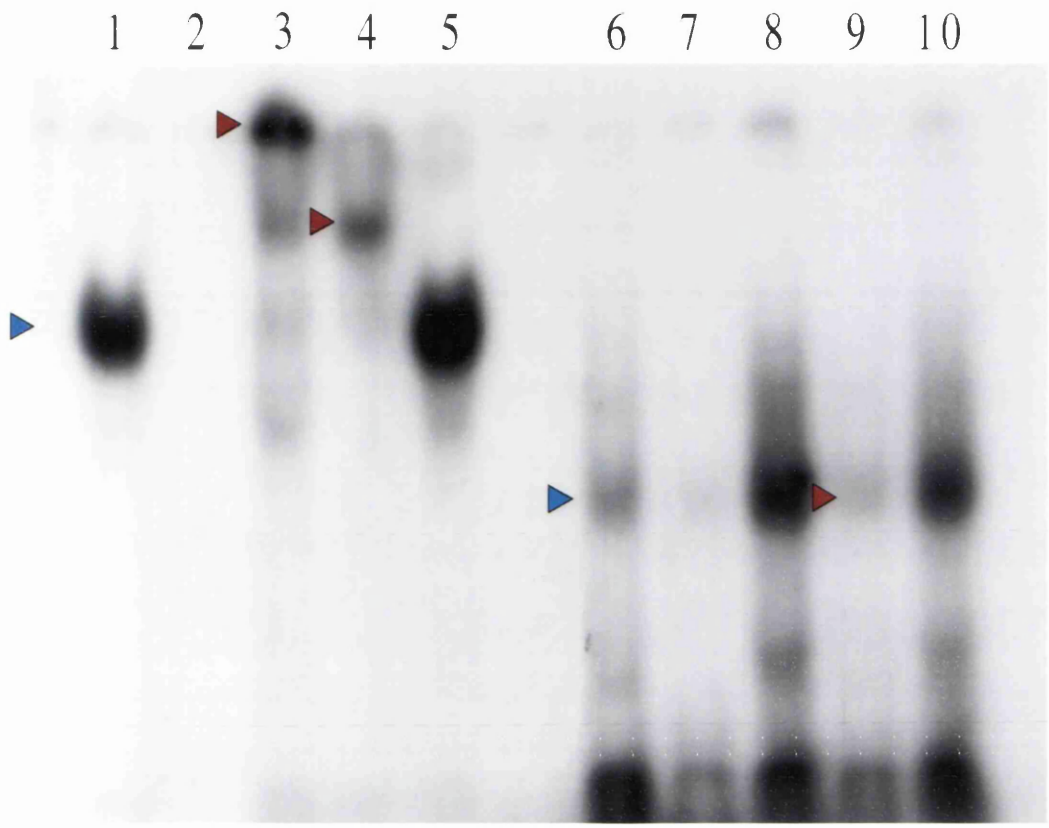
For details of the basic experimental procedure followed, see section 2.3.2.18. The supershift assay was performed first with chick embryo fibroblast (CEF) nuclear extract and subsequently with PalF nuclear extract. The CEF extract was used to determine if the antibodies were producing a supershift of the AP1 band as expected. The CEF extract was also used to assess if similar disruption or shift could be obtained for the QX1 band. The antibodies used were known to shift the CEF AP1 band (personal communication by Dr. David Gillespie), however it was not known if the same antibodies would bind to and shift the bovine proteins with the same efficacy. Therefore the supershift experiment using the CEF extract functioned primarily a positive control, to confirm that the antibodies were able to produce the desired supershift of AP1. Furthermore, if the antibodies did induce change in the QX1 band, this may be more evident with CEF extract, particularly if the antibodies recognise the CEF proteins more specifically than the bovine proteins.

4.12.2 Results

Figure 4.18 shows the supershift EMSA result obtained using CEF extract. The complex in lane 1 indicated by the first blue arrow corresponds to AP1. The AP1 complex was competed away with an unlabelled collagenase TRE oligonucleotide (lane 2). Addition of either the cJun or cFos antibodies resulted in a shift of the AP1 band up the gel (lanes 3 and 4 respectively). Addition of pre-immune serum to the reaction mix had no effect on the mobility of the AP1 band.

The complex in lane 6, indicated by the second blue arrow, corresponds to QX1. QX1 was successfully competed away by addition of a 100 fold excess of unlabelled 3'wtLCR oligonucleotide to the reaction mix (lane 7). Inclusion of the cJun antibody to the reaction mix had no effect on the position of the QX1 band (lane 8). Although a weak complex was detected at the top of the gel (see arrow at the top of lane 8), a similar complex was also detected with pre-immune serum (lane 10). The cFos antibody, while not producing a clear band shift as in lane 4, disrupted the binding of QX1 to the 3'wtLCR oligonucleotide, which is reflected in the reduced band intensity (lane 9). As observed in lane 5, addition of pre-immune serum had no effect on the binding of QX1 to the 3'wtLCR oligonucleotide (lane 10). These results indicate that QX1 in CEF may comprise AP1 components. The absence of a clear

Figure 4.18 Electrophoretic mobility Super Shift assay using nuclear extract from Chick embryo fibroblast cells (CEF) and antibodies to components of the AP1 complex (cJun and cFos)



All lanes contain ~10ug of nuclear extract from chick embryo fibroblast cells (CEF)

Lane 1: Col.TRE probe
Lane 2: Col.TRE probe + Cold Col.TRE competitor (Self)
Lane 3: Col.TRE probe + Anti-cJun antibody
Lane 4: Col.TRE probe + Anti-cFos antibody
Lane 5: Col.TRE probe + pre-immune serum
Lane 6: 3'wtLCR probe
Lane 7: 3'wtLCR probe + Cold 3'wtLCR competitor (Self)
Lane 8: 3'wtLCR probe + Anti-cJun antibody
Lane 9: 3'wtLCR probe + Anti-cFos antibody
Lane 10: 3'wtLCR probe + pre-immune serum

Blue arrows indicate position of AP1 (lane 1) and QX1 (lane 6) complexes

Red arrows indicate bands which have been shifted up the gel (or disrupted) as a consequence of antibody binding

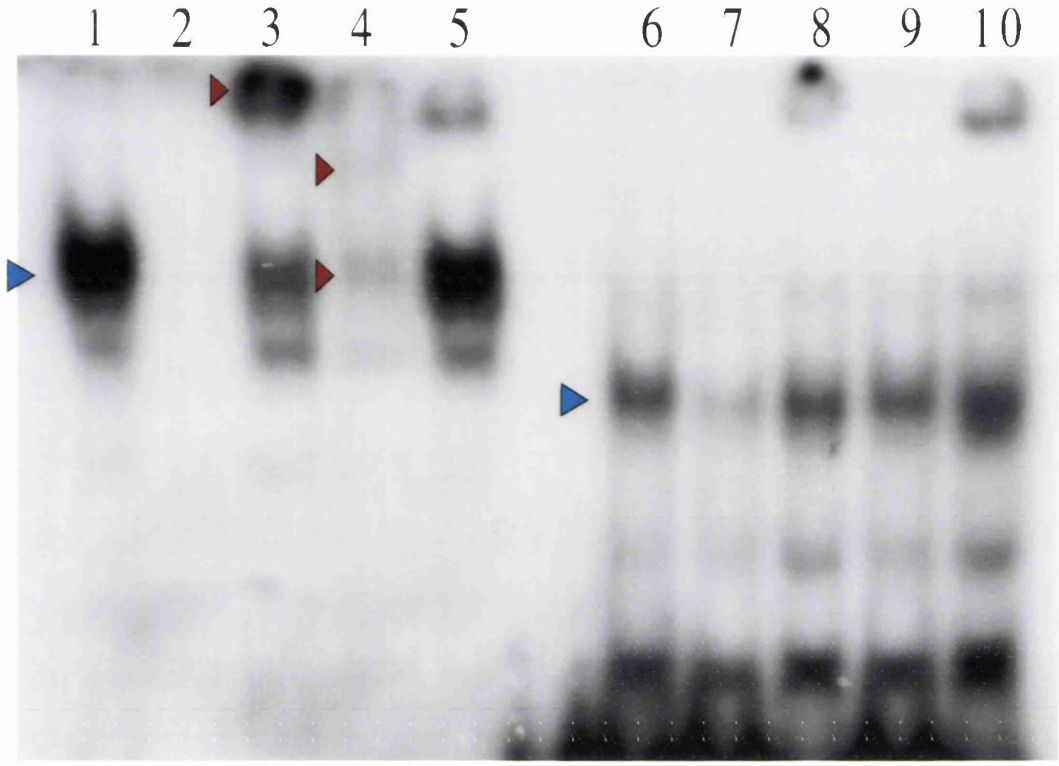
shift with cJun suggest that cJun is probably not a component of QX1. Notwithstanding, a different cJun-related protein, which is perhaps not well recognised by the specific cJun antibody which was used in this experiment, may be involved. Likewise, cFos or one of the other fos related proteins (Fras) may be a constituent of the QX1 factor.

Figure 4.19 illustrates the Supershift EMSA results obtained using PalF extract. The results obtained with the PalF extract are very similar to those observed with CEF extract. The cJun and cFos antibodies shifted or disrupted the AP1 band (lanes 3 and 4 respectively). The supershift obtained upon addition of the cJun antibody to the reaction mix with PalF nuclear extract was not as efficient as that observed with CEF extract. The incompleteness of the shift may simply reflect a drop in specificity of the cJun antibody for the bovine cJun protein. The same argument could apply to the banding pattern observed in lane 4; the cFos antibody had less of an effect on the bovine AP1 band compared to the supershift profile observed with the CEF extract (figure 4.18 - lane 4).

The cJun and cFos antibodies had little or no effect on the binding of QX1 to the 3'wtLCR with PalF extract. The cJun antibody appeared to have no effect on the position of QX1. A weak band was detected at the top of lane 8 but, as was observed with CEF extracted, a similar band was produced when pre-immune serum was added to the reaction mix. (see lane 10). Addition of cFos to the reaction mix also appeared to have no effect on the position or intensity of the QX1 band (lane 9). As was suggested earlier, the antibodies which were used in the respective reaction mixes are probably more specific for chicken proteins. The structure of the bovine proteins may be sufficiently diverse in structure from those of chicken origin so as not to be efficiently recognised.

As the results from figure 4.18 indicate, it is probable that QX1 is not composed of either cJun or cFos. This however does not exclude the involvement of proteins related to either cJun, cFos or both. Failure therefore to achieve a clear supershift of the QX1 band with either the cJun or cFos antibodies when using PalF extract may be partially attributable to potential diversity in the constituent proteins between species. In addition to this, the level of relatedness of the QX1 proteins to cJun and/or cFos even within each cell species probably further influenced the sensitivity the assay.

Figure 4.19 Electrophoretic mobility Super Shift assay using nuclear extract from Bovine foetal palate fibroblast cells (PalF) and antibodies to components of the AP1 complex (cJun and cFos)



All lanes contain ~10ug of nuclear extract from Bovine foetal palate fibroblasts (PalF)

Lane 1: Col.TRE probe
Lane 2: Col.TRE probe + Cold Col.TRE competitor (Self)
Lane 3: Col.TRE probe + Anti-cJun antibody
Lane 4: Col.TRE probe + Anti-cFos antibody
Lane 5: Col.TRE probe + pre-immune serum
Lane 6: 3'wtLCR probe
Lane 7: 3'wtLCR probe + Cold LCR competitor (Self)
Lane 8: 3'wtLCR probe + Anti-cJun antibody
Lane 9: 3'wtLCR probe + Anti-cFos antibody
Lane 10: 3'wtLCR probe + pre-immune serum

Blue arrows indicate position of AP1 (lane 1) and QX1 (lane 6) complexes

Red arrows indicate bands which have been shifted up the gel (or disrupted) as a consequence of antibody binding

4.13 Analysis of the effect of mutations within or adjacent to the TRE-like element in the BPV4 LCR on the binding of QX1

Sequence comparison between the TRE-like element and the AP1-binding site revealed a single base difference between the two sites. We were interested to know if mutating the TRE-like element to a consensus AP1-binding site would alter the binding profile of this 3' terminal stretch of the BPV4 LCR. We were also interested in trying to abolish QX1 binding by means of mutating nucleotides adjacent to the TRE-like element; by altering or disrupting QX1 binding, it may be possible to identify critical residues within the QX1 binding motif.

4.13.1 Experimental procedure

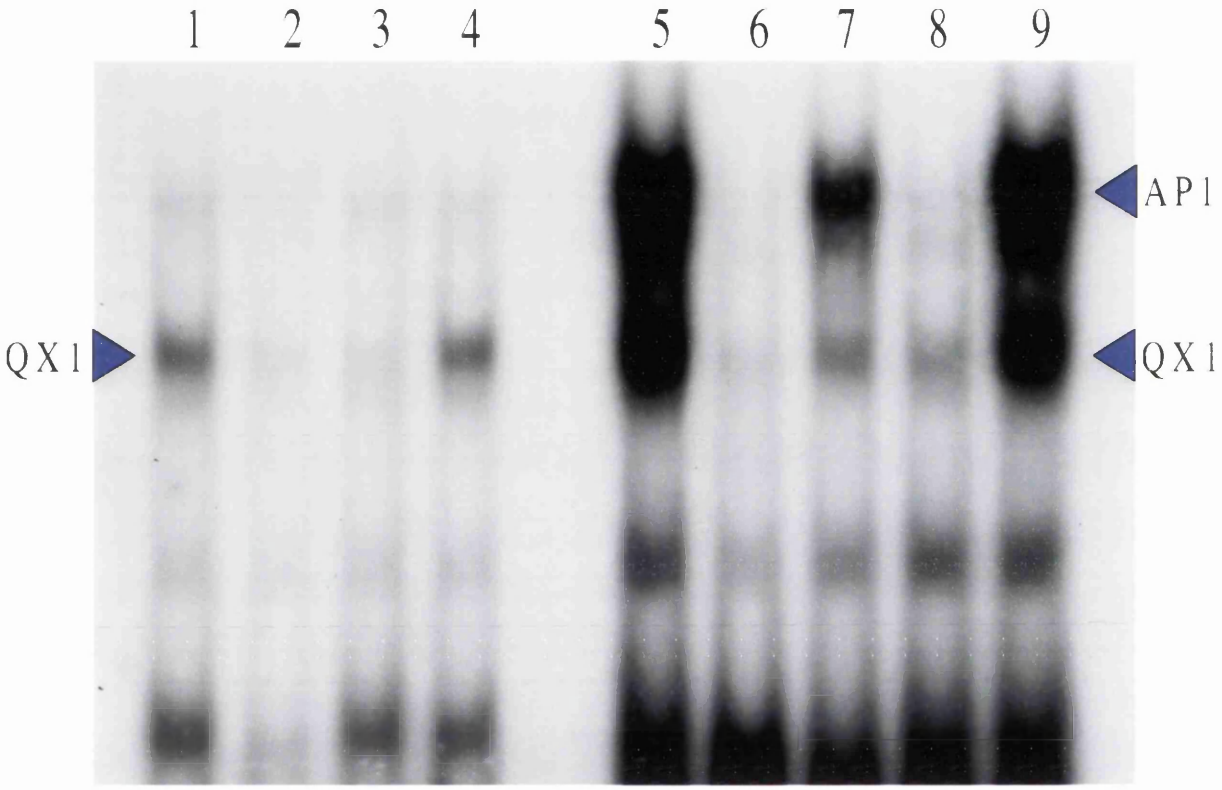
Three oligonucleotides were synthesised which contained a single or double base pair change with respect to the wild type sequence of the LCR (nucleotides 311 to 331) - see figure 4.13 for nucleotide sequences of the mutant LCR oligonucleotides used. Mutant #1 harbours a G-T transversion which changes the TRE-like element to a consensus AP1-binding site. Mutants #2 and #3 contain changes in nucleotides which are at the 5'end of or are immediately adjacent to the TRE-like element.

The mutant oligonucleotides were mixed with ~10μg of PalF nuclear extract as described in section 2.3.2.16. Unlabelled oligonucleotides were added as competitors, as specified in figure 4.20 and 4.21.

4.13.2 Results

Figure 4.20 illustrates the effect of a single base change within the BPV4 TRE-like element on the binding of QX1 and AP1. The G-T mutation introduced at nucleotide 317 of the BPV4 LCR, which converted the TRE-like element to a consensus AP1-binding motif, facilitated the binding of AP1 to this mutated oligonucleotide. In addition to the de novo binding of AP1, this mutant oligonucleotide retained the ability to bind QX1. Furthermore, the level of QX1 binding to mutant oligonucleotide #1 appeared to be enhanced. It would appear however that AP1 and QX1 can not bind simultaneously to the 3'wtLCR sequence as a third complex corresponding to both complexes bound was not detected above the AP1 band on the gel. Both the AP1 and QX1 complexes were competed away by addition of unlabelled mutant#1 oligonucleotide. Addition of unlabelled 3'wtLCR

Figure 4.20 EMSA showing the effect of a single base change (G-T) within the BPV4 LCR TRE-like element



Lane 1: 3'wtLCR probe
Lane 2: 3'wtLCR probe + Cold 3'wtLCR competitor (Self)
Lane 3: 3'wtLCR probe + Cold Col.TRE competitor
Lane 4: 3'wtLCR probe + Cold Myc competitor
Lane 5: Mutant LCR #1 probe
Lane 6: Mutant LCR #1 probe + Cold Mutant #1 competitor (Self)
Lane 7: Mutant LCR #1 probe + Cold 3'wtLCR competitor
Lane 8: Mutant LCR #1 probe + Cold Col.TRE competitor
Lane 9: Mutant LCR #1 probe + Cold Myc competitor

Each competitor is present in a 100 fold excess with repect to labelled probe
All lanes contain ~10ug of PalF cell nuclear extract

oligonucleotide competed away QX1 more efficiently than AP1. Conversely, unlabelled collagenase TRE oligonucleotide competed away AP1 more specifically than QX1. Addition of an unlabelled myc-binding oligonucleotide had no effect on the binding of AP1 or QX1 to the mutant#1 oligonucleotide sequence. These observations highlight the significance of this single nucleotide base with respect to both QX1 and AP1 binding.

The effect of the two other mutant LCR oligonucleotides on the binding of QX1 was less dramatic. As the results in figure 4.21 demonstrate, QX1 was seen to bind to mutant #2 and mutant #3 oligonucleotides with an efficacy similar to that observed for the wild type LCR. Because the binding of QX1 to mutant oligonucleotides #2 and #3 was apparently unaffected by the respective nucleotide changes, it may be concluded that the residues which were mutated are not critical for the binding of QX1 to this region of the BPV4 LCR.

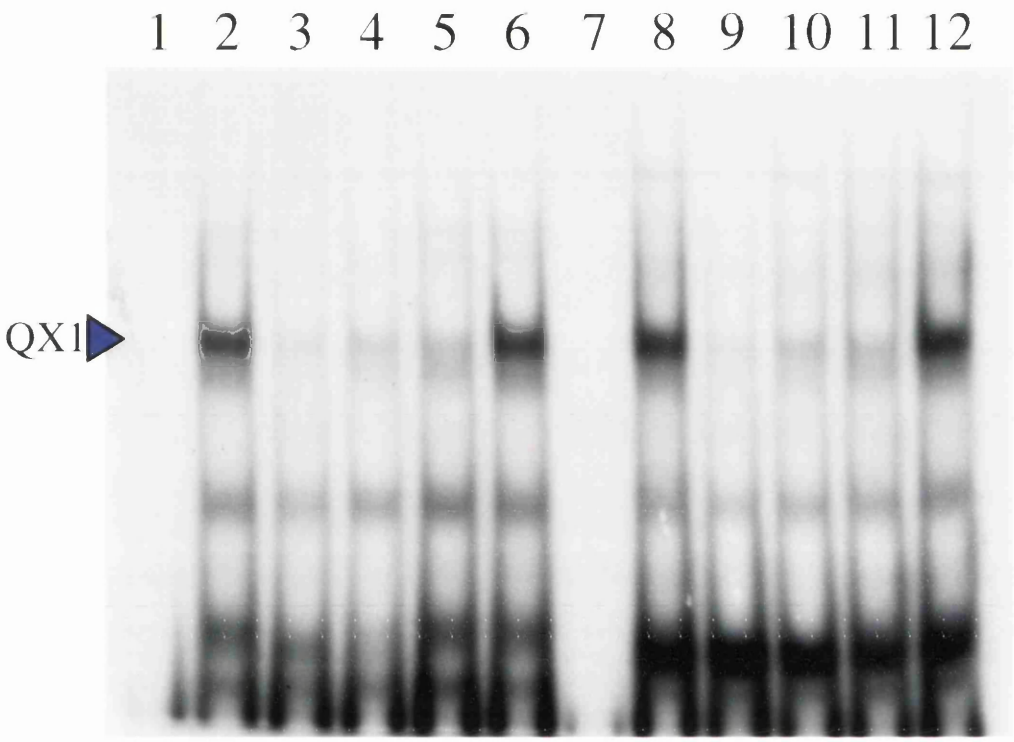
4.14 The effect of mutations within the 3' terminal 21 base pairs of the BPV4 LCR on the transcriptional activity of the LCR

In figure 4.20, a dramatic change in the binding profile of the EMSA was observed when a single nucleotide within the 3'wtLCR oligonucleotide was mutated to generate a consensus AP1-binding site (figure 4.13). This single base pair change within the TRE-like motif was sufficient to allow AP1 to bind a sequence within nucleotides 311-331 of the BPV4 LCR. As an extension of the experiment and results described in section 4.13, mutant variants of BPV4 LCR were created and cloned into the pGL3 expression vector. The basal transcriptional activity and response to quercetin treatment of these newly generated mutant LCR-containing plasmids was then assessed.

4.14.1 Experimental procedure

Four new plasmid vectors were generated using a PCR based method (see section 2.3.2.2 for details). Mutations were introduced into the LCR by designing specific PCR primers which contained the desired mutation. On the end of each of the PCR primers, a separate recognition site for a restriction enzyme was included. This facilitated subsequent cloning of the new LCR inserts into the appropriate expression vector and in the correct orientation. The sequences of the oligonucleotide primers

Figure 4.21 EMSA showing the effect of BPV4 LCR mutants #2 and #3 on the binding of factor QX1



All lanes, except lanes 1 and 7, contain ~10ug nuclear extract from bovine foetal palate fibroblasts (PalF)

Lane 1: BPV4 LCR mutant #2 probe alone (no extract)
Lane 2: Mutant #2 probe
Lane 3: Mutant #2 probe + Cold mutant #2 competitor (self)
Lane 4: Mutant #2 probe + Cold 3'wtLCR competitor
Lane 5: Mutant #2 probe + Cold Col.TRE competitor
Lane 6: Mutant #2 probe + Cold Myc competitor
Lane 7: BPV4 LCR Mutant #3 probe alone (no extract)
Lane 8: Mutant #3 probe
Lane 9: Mutant #3 probe + Cold mutant #3 competitor (self)
Lane 10: Mutant #3 probe + Cold 3'wtLCR competitor
Lane 11: Mutant #3 probe + Cold Col.TRE competitor
Lane 12: Mutant #3 probe + Cold Myc competitor

used to generate the LCR mutants are given in figure 4.22. The forward primer annealed to the bottom strand at the 5' end of BPV4 LCR (at nucleotide 6710); the reverse primers, which carried the particular point mutations, annealed to the top strand at the 3' end of the BPV4 LCR (at nucleotide 310 (sLCR) or nucleotide 331 (wt, AP1 and random). The same forward primer was used in all PCR reactions.

The AP1 primer was designed to change the TRE-like element in the LCR to a classic AP1-binding site. This involved a single base change at nucleotide 317; the remainder of the LCR sequence was unchanged. The random primer was designed to replace nucleotides 311-331 with a string of 21 random nucleotides. This was to address the question of whether the specific sequence of nucleotides 311-331 is important with respect to the basal activity and response to quercetin of the BPV4 LCR.

The PCR products, corresponding to the various mutant LCR inserts, were separated from unwanted by products of the PCR reaction by electrophoresis through an agarose gel. The mutant LCR inserts were purified from the gel and their ends cut with the appropriate restriction enzymes. The pGL3 expression vector was linearised with the same enzymes used to cut the ends of the mutant LCR PCR products; the enzymes chosen allowed the mutant LCRs to be inserted into the vector in the correct orientation. After being linearised, the vector was incubated with alkaline phosphatase to prevent the vector from religating to itself. The mutant LCR PCR products were ligated into the pGL3 vector and subsequently transformed into competent DH5α bacterial cells. Transformed colonies were screened for vectors which contained the BPV4 LCR inserts. Positive colonies were picked and cultured in 500ml or 1 litre volumes of LB broth containing the appropriate selection antibiotic. The mutant LCR vectors were purified from the bacterial cultures and the sequence of each of the mutant LCRs checked using an automated sequencing machine. Details of all the methods used in the process described above can be found in section 2.3.2

$2x10^5$ low passage PalF cells or $5x10^5$ PalK cells were seeded into 60mm tissue culture dishes. The following day, cells were transfected with either 7.5μg pGL3 plus 2.5μg pCH110, or 7.5μg pGL3-wtLCR plus 2.5μg pCH110, or 7.5μg pGL3-sLCR plus 2.5μg pCH110, or 7.5μg pGL3-randLCR plus 2.5μg pCH110,

Figure 4.22 PCR primers used to introduce mutations into BPV4 LCR

LCR 6719: 5' - TGCAGGTACCTGGAAGAATGGGATTTGT - 3' *(Forward primer)*

wt LCR: 5' - TGCCAAGCTTGATGAGAGCTACTGCCTCATG - 3' (*Reverse primer*)

AP1 LCR: 5' - TGCCAAGCTTGATGAGAGCTACTGACTCATG - 3' (*Reverse primer*)

sLCR: 5' - TGCCAAGCTT * * * * * * * * * * * CACCAAATCCGCACTGCTCTC - 3' (*Reverse Primer*)

Random LCR: 5' - TGCCAAGCTTACCTGAGCCACCTGCATCGATCACCAAATCCGCACTGCTC - 3' (*Reverse Primer*)

NB Random LCR sequence corresponds to a noncoding region from the mouse x chromosome

or 7.5μg pGL3-AP1LCR plus 2.5μg pCH110 as described in section 2.3.1.7 (for PalF cells) or section 2.3.1.8 (for PalK cells). After transfection, a fresh volume of medium, supplemented with 20μM quercetin or an equivalent volume of ethanol, was added to the appropriate dishes of cells, as summarised in table 4.9. Cells were cultured in this medium for 24 hours and finally harvested as described in section 2.3.1.9a. The cell lysates were assayed for β-galactosidase and luciferase enzyme activities (section 2.3.1.11 and 2.3.1.12).

Each luciferase assay reading was corrected for efficiency of transfection using the corresponding β-galactosidase reading, as described in section 4.3.1. The contribution of empty vector (pGL3) on transcription was removed by subtraction, as detailed in section 4.8.1, and the final readings were normalised to the 'No quercetin' reading set = 1 (section 4.3.1).

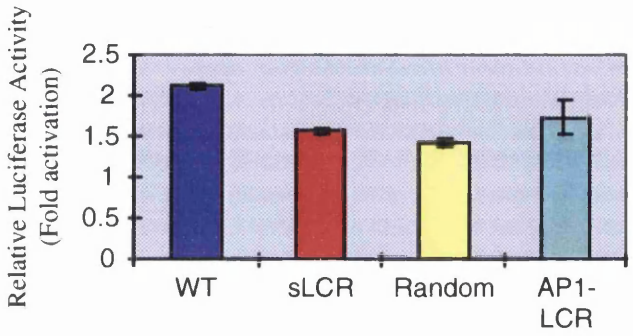
Table 4.9 Summary of the experimental conditions used when PalF and PalK cells were transfected with pGL3 vectors, carrying mutant BPV4 LCRs present in a promoter configuration, and treated with or without quercetin

	Condition #1		**Condition #2**		**Condition #3**		**Condition #4**		**Condition #5**	
Transfected with	pGL3 + pCH110		pGL3-wtLCR + pCH110		pGL3-sLCR + pCH110		pGL3-randLCR + pCH110		pGL3-AP1LCR + pCH110	
Treated with	E	Q	E	Q	E	Q	E	Q	E	Q

4.14.2 Results

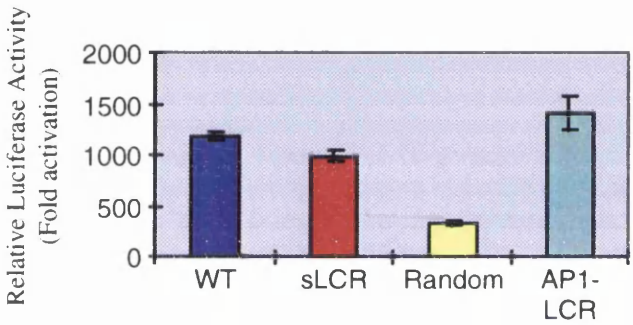
Figure 4.23 illustrates the response to quercetin exposure and the basal transcriptional activity of four newly generated BPV4 LCR-containing plasmids. Similar to observations detailed in earlier results sections, the transcriptional activity the wild type BPV4 LCR was increased approximately 2 fold when PalF cells were treated with 20μM quercetin for 24 hours after transfection (figure 4.23 - panel A). Also in keeping with earlier observations, the activity of the 21 base pair truncated form of the BPV4 LCR was increased in response to quercetin treatment

Figure 4.23 Bar charts showing the basal activities and response to quercetin treatment for a range of BPV4 LCR mutants



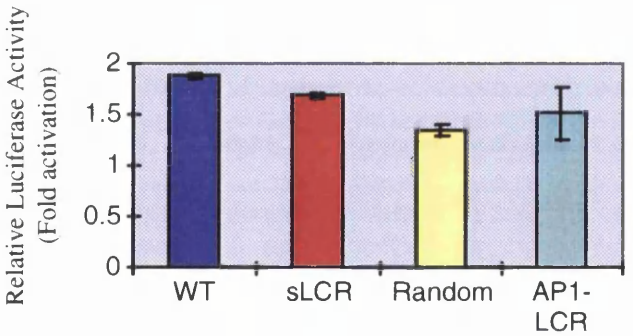
A

PalF cells
Response to Quercetin



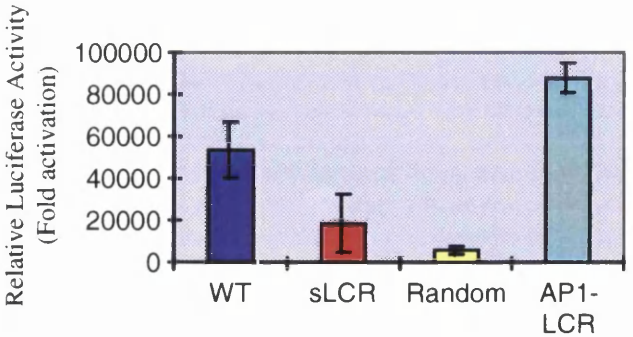
B

PalF cells
Basal activity



C

PalK cells
Response to Quercetin



D

PalK cells
Basal activity

(~1.5 fold increase), but the level of the response was less in comparison to the wild type LCR. The random-LCR plasmid showed a response to quercetin similar to that observed for the truncated LCR. The response to quercetin treatment of the LCR plasmid which carried an AP1-binding site was similar to that seen for the truncated and random LCRs but less than the response of the wild type LCR sequence.

The response of the same four vectors to quercetin exposure after transfection into PalK cells was essentially the same as that seen with PalF cells (see figure 4.23 - panel C). The largest response to quercetin was observed with the wild type LCR, although the level of the response was slightly less than the response seen for the same vector in PalF cells. The truncated LCR again showed a slightly reduced response with respect to the wild type LCR however the level of the response was very similar to that achieved by the same vector in PalF cells. The random LCR, as in PalF cells, showed the smallest response to quercetin treatment. It would appear that replacing nucleotides 311 to 331 of the BPV4 LCR with a stretch of random nucleotides has a greater effect on response to quercetin than simply deleting the same bases.

The activity of the AP1-LCR was increased in response to quercetin. The fold increase observed was similar to that seen for the truncated LCR but less than response of the wild type LCR. This result indicates that a single base substitution, which changes the TRE-like motif to a canonical AP1-binding site, directly affects the response of the LCR to quercetin treatment.

The basal transcriptional activities of the four vectors was more varied than their individual responses to quercetin treatment (figure 4.23 - panels B and C). In both PalF and PalK cells, the AP1-containing LCR displayed the strongest transcriptional activity. Thus, by replacing the TRE-like element in the BPV4 LCR with a canonical AP1-binding site, by virtue of a single base pair change, the basal level of transcription was increased. Hence the TRE-like element must contribute to the normal activity of the BPV4 LCR.

The wild type LCR showed good activity in both PalF and PalK cells. The activity of the truncated LCR was less than that seen for either the wild type or AP1-containing LCR. The difference in activity between the wild type and truncated LCR was more evident in PalK cells; the activity of the truncated LCR was only slightly less than the activity of the wild type LCR in PalF cells.

The basal transcriptional activity of the random LCR was dramatically less than the wild type LCR in both PalF and PalK cells. In the random-LCR vector, nucleotides 311-331 of the BPV4 LCR were replaced with a stretch of random nucleotides; these nucleotides are situated down stream of and did not directly involve the TATAA box. Nevertheless, it seems clear that the specific sequence of nucleotides 311 to 331 of the BPV4 LCR is important for maintaining the basal level of transcription from the viral LCR and this sequence cannot simply be replaced by any sequence of random nucleotides.

One very clear observation was that the activity of all four vectors was considerable higher in PalK cells in comparison to their corresponding activities in PalF cells. Each of the vectors was approximately 40 to 50 times more active in PalK than in PalF cells. This difference in the basal level of transcriptional activity strongly reflects the natural tropism of BPV4 for epithelial cells.

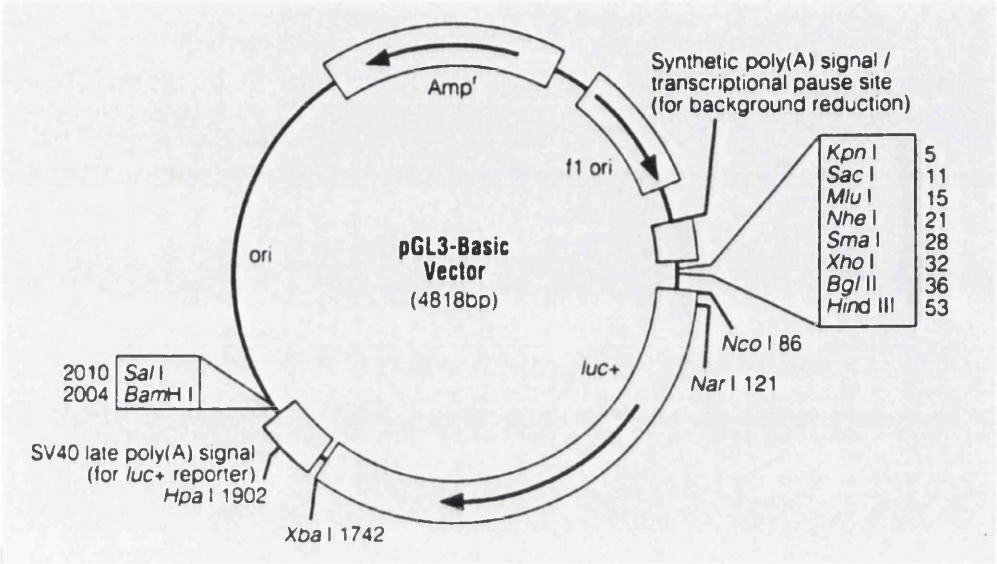
4.15 Identification of other potential regions in the BPV4 LCR which may mediate the effects of quercetin on the LCR's transcriptional activity

The results from experiments with the full length and 3' truncated forms of the BPV4 LCR indicated that, although the sequence between nucleotides 311 and 331 may be partially mediating the effect of quercetin on levels of transcription, other regions of the LCR also appear to be involved. A panel of 5' truncated BPV4 LCR mutants was generated by Dr. Iain Morgan (Beatson Institute). Figure 4.24 is a schematic representation of the BPV4 LCR sequences which are included in each of the mutant LCR plasmids. By transfecting these mutants into PalF cells, it was possible to screen for other regions of the BPV4 LCR which may be contributing to the upregulation of transcription in response to quercetin exposure.

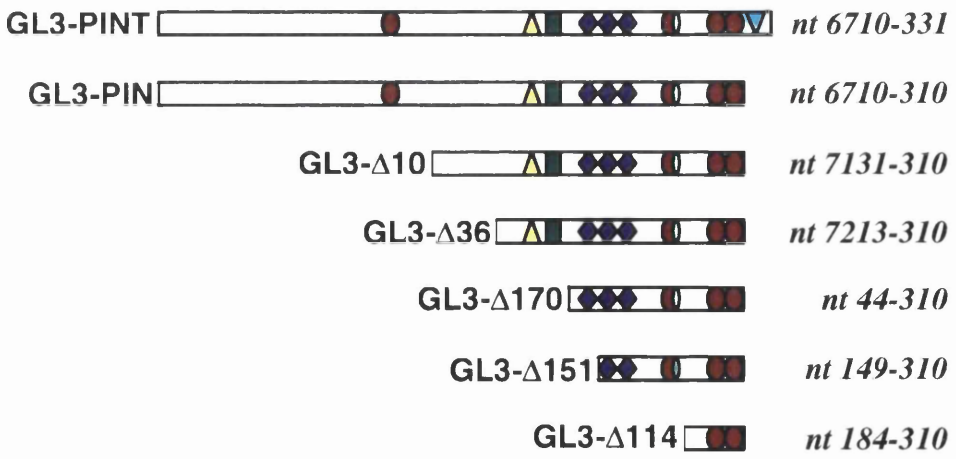
4.15.1 Experimental procedure

$2x10^5$ low passage PalF cells were plated into 60mm tissue culture dishes. After an overnight incubation, the cells were transfected with one of the following plasmid DNA combination;

Figure 4.24 pGL-3 basic vector map
(taken from the Promega biological research products catalogue, 1997)



The following BPV4 LCR deletion mutants were cloned into the *Bgl*II site of pGL-3 basic vector (see section 2.2.9 for details)



- 7.5μg pGL3 + 2.5 μg pCH110
- 7.5μg pGL3-PIN + 2.5 μg pCH110
- 7.5μg pGL3-Δ10 + 2.5 μg pCH110
- 7.5μg pGL3-Δ170 + 2.5 μg pCH110
- 7.5μg pGL3-Δ114 + 2.5 μg pCH110
- 7.5μg pGL3-PINT + 2.5 μg pCH110
- 7.5μg pGL3-Δ57.1 + 2.5 μg pCH110
- 7.5μg pGL3-Δ36 + 2.5μg pCH110
- 7.5μg pGL3-Δ151 + 2.5 μg pCH110

Cells were transfected according the protocol detailed in section 2.3.1.7. After transfection, the cells were treated with a fresh volume of medium supplemented with either 20μM quercetin or an equivalent volume of ethanol (see table 4.10 for details). Cells were cultured in the appropriate supplemented medium for 24 hours. At the end of the incubation period, all cells were harvested as described in section 2.3.1.9a and the cell lysates obtained were assayed for β-galactosidase and luciferase enzyme activities (see sections 2.3.1.11 and 2.3.1.12).

Each luciferase assay reading was corrected for efficiency of transfection using the corresponding β-galactosidase reading, as described in section 4.3.1. The contribution of empty vector (pGL3) on transcription was removed by subtraction, as detailed in section 4.8.1, and the final readings were normalised to the 'No quercetin' reading set = 1 (section 4.3.1).

4.15.2 Results

Figure 4.25 -panel A illustrates the response of each the 5' deletion mutants to quercetin exposure. As expected the full length, wild type LCR displayed the greatest response to quercetin treatment. Removal of nucleotides 311-331 from the LCR correlated with a drop in response to quercetin treatment to approximately 1.5 fold. Deletion of 421 nucleotides from the 5' end of the LCR had no effect on the response of the vector to quercetin exposure. Deletion of a further 82 nucleotides from the 5'end of the LCR however resulted in a drop in the response to quercetin back to a level comparable with the empty vector. This would indicate that nucleotides in this region of the LCR may be involved in mediating part of the response of the BPV4 LCR to quercetin. More extensive 5' deletions of the BPV4 LCR had little further effect on response of the LCR to quercetin.

Figure 4.25 Charts showing the (A) the response to quercetin and (B) the basal transcriptional activity for a series of 3'terminal deleted BPV4 LCR mutants in PalF cells

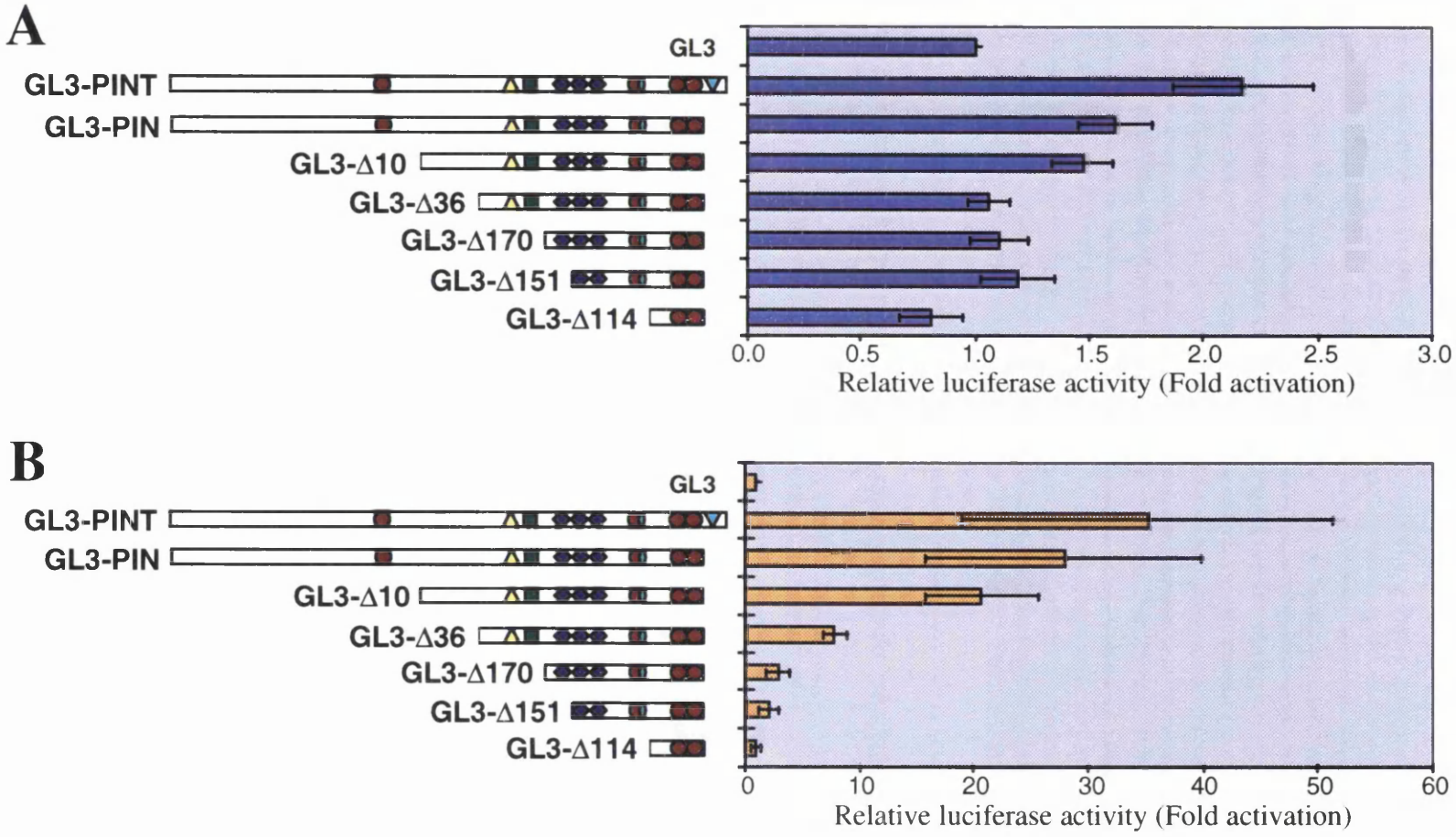


Table 4.10 Summary of the experimental conditions used when PalF cells were transfected with pGL3 vectors carrying various 5' and 3'terminal truncated mutants of the BPV4 LCRs present in a promoter configuration, and treated with or without quercetin

	Transfected with	Treated with
Condition #1	7.5μg pGL3 + 2.5μg pCH110	E (2 dishes) Q (2 dishes)
Condition #2	7.5μg pGL3PINT + 2.5μg pCH110	E (2 dishes) Q (2 dishes)
Condition #3	7.5μg pGL3-PIN + 2.5μg pCH110	E (2 dishes) Q (2 dishes)
Condition #4	7.5μg pGL3-Δ57.1 + 2.5μg pCH110	E (2 dishes) Q (2 dishes)
Condition #5	7.5μg pGL3-Δ10 + 2.5μg pCH110	E (2 dishes) Q (2 dishes)
Condition #6	7.5μg pGL3-Δ36 + 2.5μg pCH110	E (2 dishes) Q (2 dishes)
Condition #7	7.5μg pGL3-Δ170 + 2.5μg pCH110	E (2 dishes) Q (2 dishes)
Condition #8	7.5μg pGL3-Δ151 + 2.5μg pCH110	E (2 dishes) Q (2 dishes)
Condition #9	7.5μg pGL3-Δ114 + 2.5μg pCH110	E (2 dishes) Q (2 dishes)

Figure 4.25 - panel B illustrates the basal transcriptional activities of each of the LCR deletion mutant plasmids. The activity of the full length BPV4 LCR (pGL3-PINT) is approximately 35 fold greater than the empty vector. Removal of nucleotides 311-331 from the 3'end of the LCR resulted in a decrease in the basal level of activity. This is in keeping with observations from earlier experiments (sections 4.8.2 and 4.9.2). Sequential deletion of nucleotides from the 5' end of the LCR correlated with a steady decline in the basal transcriptional activity of the LCR. This suggests that a number of regulatory elements which influence the activity of the

LCR are located at different sites along the length of the LCR. A significant drop in activity was observed with pGL3-Δ36 when nucleotides 7131 to 7212 were deleted (figure 4.24). Deletion of these residues was also associated with a drop in response to quercetin treatment (figure 4.25 - panel A). Therefore, the region of the BPV4 LCR encompassing nucleotides 7131-7212 appears to contains sequences which are important in terms of both basic levels of transcriptional activity and response to quercetin treatment.

4.16 Analysis of the effect of quercetin treatment on the transcriptional activity of the Moloney Murine Long Terminal Repeat (MoMuLTR) acting as a promoter element

Experiments described by Cairney and Campo (1995) demonstrated that transfection of PalF cells with the BPV4 E7 gene and an activated *ras* gene, followed by a 48 hour exposure to 20μM quercetin, was sufficient to induce cellular transformation of PalF cells. The same degree of transformation was not observed however when the same cells were similarly transfected but not treated with quercetin. In these experiments, expression of the BPV4 E7 coding sequence was not controlled by the viral LCR but rather the Moloney Murine long terminal repeat (MoMuLTR). The MoMuLTR is a strong viral promoter. It had not been determined if the activity of the MoMuLTR could be altered by exposure to quercetin as has been observed for the BPV4 LCR. We therefore transfected PalF cells with a plasmid carrying the CAT reporter gene under the transcriptional control of the MoMuLTR. The cells were treated with 20μM quercetin for 24 hours after transfection and ultimately screened for any changes in reporter protein levels.

4.16.1 Experimental procedure

$2x10^5$ low passage PalF cells were plated into the appropriate tissue culture dishes and incubated overnight as described in section 2.3.1.7. The following day the cells were transfected with one of the following plasmid DNA combinations;

- 7.5μg pCAT + 2.5μg pCH110
- 7.5μg pMoMuLTR-CAT + 2.5μg pCH110
- 2μg pMoMuLTR-CAT + 2.5μg pCH110 + 5.5μg salmon sperm DNA

- 1μg pMoMuLTR-CAT + 2.5μg pCH110 + 6.5μg salmon sperm DNA
- 0.2μg pMoMuLTR-CAT + 2.5μg pCH110 + 7.3μg salmon sperm DNA

Salmon sperm DNA was added to transfection reactions as indicated above to act as carrier DNA and to bring the total amount of DNA in each reaction to a total of 10μg.

After transfection, a fresh volume of medium, supplemented with either 20μM quercetin or an equivalent volume of ethanol, was added to the appropriate dishes; two dishes in each transfection condition were treated with quercetin while the other two were treated with ethanol. The cells were incubated in this medium for 48 hours and then harvested as detailed in section 2.3.1.9a. The cell lysates were assayed for CAT and β-galactosidase enzyme activities as described in sections 2.3.1.12 and 2.3.1.13.

Each CAT assay reading was corrected for efficiency of transfection using the corresponding β-galactosidase reading, as described in section 4.3.1. The contribution of empty vector (pCAT) on transcription was removed by subtraction, as detailed in section 4.8.1, and the final readings were normalised to the 'No quercetin' reading set = 1 (section 4.3.1). The results from 3 separate experiments are presented in figure 4.26a and b.

4.16.2 Results

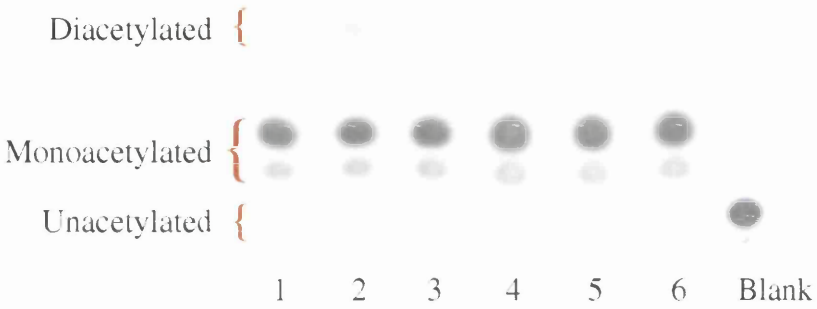
The Molony Murine LTR was found to be a very strong promoter element. The results in figure 4.26a show that when 7.5μg of plasmid vector containing the MoMuLTR was transfected into PalF cells, expression of the CAT reporter gene was high. This was reflected in the level of conversion of chloramphenicol substrate to mono-and even diacetylated products; 2μl of each cell lysate, from a total lysis volume of 350μl, was sufficient to convert almost 100% of the chloramphenicol substrate. Usually 50μl of a 350μl cell lysate is used in each CAT assay reaction and even then such a high conversion level is often not observed. No difference was seen in the percentage conversion for cells which had been exposed to 20μM quercetin for 48 hours after transfection. From the results shown in figure 4.26a, no conclusion as to the response of the MoMuLTR to quercetin can be made as

any effect of quercetin on the activity of the MoMuLTR has probably been obscured by the high basal level of expression for this promoter element.

Figure 4.26b shows the results from two separate experiments in which the amount of MoMuLTR reporter vector had been sequentially reduced in an attempt to identify any change in activity of the MoMuLTR in response to quercetin treatment. As the average percentage conversion values given in each table show, quercetin appeared to actually inhibit the activity of the MoMuLTR. This is in contrast to the response of the BPV4 LCR to quercetin treatment. The effect of quercetin on the activity of the BPV4 LCR has not been assessed using nanogram amounts of a BPV4 LCR-containing vector, as was done with the MoMuLTR (as detailed in section 4.16.1). Nevertheless, it would appear from the results with the MoMuLTR and the BPV4 LCR that the response of different promoters to quercetin treatment is not the same.

Although the results in figure 5.26b suggest that quercetin treatment can partially inhibit the activity of the MoMuLTR, this effect was only observed when small amounts of the reporter vector were transfected into the host cells and less than usual amounts of the cell lysate samples were used in the assay reactions. It is very clear that the MoMuLTR is a very powerful promoter even in the presence of quercetin. In relation to the experiments described by Cairney and Campo (1995), it is unlikely that a crucial role of quercetin was to alter the level of transcription of viral genes which were under the transcriptional control of the MoMuLTR. Nevertheless, quercetin is known to affect a wide range of cellular functions. It is very possible that the effect of quercetin on one or more other cellular targets synergised with the high level of viral gene expression from the MoMuLTR and thus contributed to cellular transformation in PalF cells.

Figure 4.26a Transcriptional activity of the Moloney Murine Long Terminal Repeat (LTR) in PalF cells



Lane	1, 2, and 3	4, 5, and 6
Transfected with	7.5µg pMoMuLTR-CAT + 2.5µg pCH110	7.5µg pMoMuLTR-CAT + 2.5µg pCH110
Treated with	Ethanol	Quercetin
Average % conversion	97.92%	97.69%

Figure 4.26b Transcriptional activity of the MoMu-LTR in PalF cells

A

1 2 3 4 5 6 7 8 9 10 11 12 Blank

Lane	1 and 2	3 and 4	5 and 6	7 and 8	9 and 10	11 and 12
Transfected with	2µg pMoMuLTR-CAT + 2.5µg pCH110		1µg pMoMuLTR-CAT + 2.5µg pCH110		0.1µg pMoMuLTR-CAT + 2.5µg pCH110	
Treated with	Ethanol	Quercetin	Ethanol	Quercetin	Ethanol	Quercetin
Average % conversion	26.35	15.54	7.18	4.55	1.42	1.12

B

1 2 3 4 5 6 7 8 9 10 11 12 Blank

Lane	1 and 2	3 and 4	5 and 6	7 and 8	9 and 10	11 and 12
Transfected with	2µg pMoMuLTR-CAT + 2.5µg pCH110		1µg pMoMuLTR-CAT + 2.5µg pCH110		0.1µg pMoMuLTR-CAT + 2.5µg pCH110	
Treated with	Ethanol	Quercetin	Ethanol	Quercetin	Ethanol	Quercetin
Average % conversion	60.89	49.96	26.59	12.13	13.26	7.53

Chapter 5

The Effect of Quercetin on Protein Phosphotyrosine Levels in PalF cells

Chapter 5 The effect of quercetin on Protein Phosphotyrosine levels

5.1 Introduction

Quercetin is known to affect a number of different cellular functions. Many of the quercetin-induced effects have been attributed to the ability of quercetin to inhibit or alter the activity of a large number of cellular enzymes. The inhibition of enzymes such as mitochondrial succinoxidase, NADH-oxidase, enzymes involved in arachidonic acid metabolism, aldose reductase and several other oxido-reductases (Elliott *et al.*, 1992 and references therein) account for the ability of quercetin to inhibit various metabolic processes including lactate transport and glycolysis (Belt *et al.*, 1979). The inhibition of several kinases including pp60src (Graziani *et al.*, 1983), casein kinase II (Cochet *et al.*, 1982), protein kinase C (Gshwendt *et al.*, 1983), phosphorylase kinase (Srivastava, 1985), phosphatidylinositol-3-kinase (Matter *et al.*, 1992) and several tyrosine kinases associated with mammary tumours (Levy *et al.*, 1984), suggest a role for quercetin in the inhibition and/or de-regulation of a number of different signal transduction pathways.

The extensive repertoire of enzymes which can be inhibited by quercetin is undoubtedly a critical aspect of quercetin's ability to induce such a large number of varied and sometimes contradictory effects.

5.2 Determining the phosphotyrosine status of proteins in PalF cells after quercetin treatment

In chapter 4, the ability of quercetin to modulate the level of transcription from the BPV4 LCR was analysed. In spite of the observed increase in transcription from the LCR, it was clear that this was not the only effect of quercetin which would explain the ability of quercetin to synergise with BPV4 and activated *ras* to induce the malignant transformation of PalF cells. A previous study showed that quercetin, BPV4 and *ras* expression were synergistic and necessary for the complete transformation of PalF cells even when the BPV4 genes were under the transcriptional control of the Molony Murine LTR promoter (Cairney and Campo, 1995). The results presented in figure 4.26b showed that, unlike the BPV4 LCR, the activity of the Molony Murine LTR was not increased in response to quercetin. Thus other effects of

quercetin must have been responsible for the significant enhancement to transformation. The nature of these other effects had not as yet been studied.

In an attempt to identify other cellular changes induced in PalF cells as a result of exposure to quercetin, bearing in mind the observations which have been made in other studies (refer to section 5.1), we decided to test if quercetin was having an effect on protein phosphotyrosine levels.

5.2.1 Experimental procedure

10^6 PalF cells were seeded into 90mm tissue culture dishes. After an overnight incubation, the medium was changed. The fresh medium was supplemented with quercetin to a final concentration of 20μM. Two extra dishes, in which medium was supplemented with an equivalent volume of absolute ethanol (0.1% v/v), were set up in parallel to act as controls.

Cells were cultured in the appropriate environment (37°C and 5% CO_2) for 48 hours. At the end of the incubation period, medium containing quercetin (or ethanol) was removed and all cell monolayers were washed twice with PBS to remove any traces of quercetin. Two (duplicate) dishes of cells were harvested for total cellular protein according to the method detailed in section 2.3.1.9b. These protein samples corresponded to cells being harvested at time = 0 after removal of quercetin. The control (ethanol treated) cells were also harvested at this time.

To all other dishes of cells, a fresh volume of 1xDMEM medium was added. Cells were replaced in the appropriate incubator and harvested at various time points after removal of quercetin. The rationale for harvesting cells at various times after removal of quercetin was to determine how long a phosphotyrosine change, if detected, would persist. Earlier experiments showed that the timing of exposure of PalF cells to quercetin was critical with regard to quercetin's ability to synergise with BPV4 and *ras* and induce malignant transformation of PalF cells (Cairney and Campo, 1995). Only cells treated with quercetin for 48 hours either immediately before or immediately after transfection with BPV4 and *ras* became fully transformed.

The concentration of each protein sample was determined according to the method described in section 2.3.3.1. Approximately 20μg of each protein sample was loaded into separate wells in an SDS-polyacrylamide gel. The protein samples were electrophoresed through the SDS-polyacrylamide gel and subsequently transferred

onto Hybond C^{extra} or a nitrocellulose filter as detailed in section 2.3.3.2. Phosphotyrosine proteins were detected using the appropriate primary and secondary antibodies as described in section 2.3.3.3.

5.2.2 Results

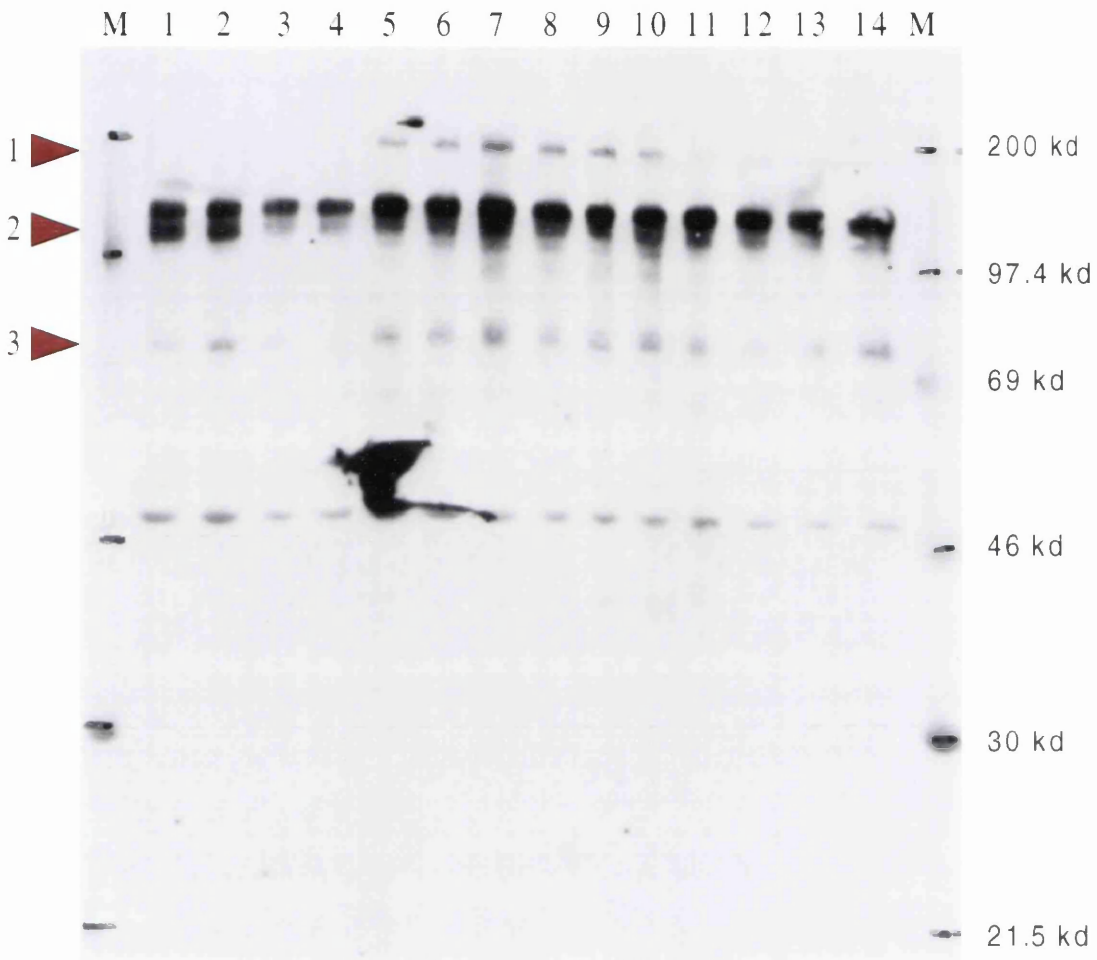
Figure 5.1 shows a number of proteins phosphorylated on tyrosine residues which were detected in extracts from PalF cells.

Compare first the pattern of protein bands in lanes 1 and 2 with those in lanes 3 and 4. The protein extracts run in lanes 1 and 2 came from cells which were treated with 0.1% ethanol for 48 hours before harvesting and should represent normal phosphotyrosine-protein levels. The extracts run in lanes 3 and 4 came from cells which were similarly treated but with 20μM quercetin before harvesting. The arrows numbered 2 and 3 indicate two bands which are less intense in lanes 3 and 4 compared to the bands at the same position in lanes 1 and 2. This observation suggests that the levels of phosphotyrosine in these proteins were reduced as a result of exposure to quercetin. Removal of quercetin permitted these proteins to regain a normal level of phosphotyrosine. The recovery of the phosphotyrosine levels was already apparent 30 minutes after quercetin had been removed from the culture medium (lanes 5 and 6).

The band at the top of the gel (indicated by arrow number 1) shows a different phosphotyrosine pattern. This protein was not readily detected in ethanol treated cells nor was it detected in cells harvested immediately after a 48 hour exposure to quercetin. Its expression was initially detected 30 minutes after quercetin had been removed from the culture medium. It was detected up to four hours after quercetin had been removed however was undetectable again by 8 hours. The transient increase in this one particular protein may be an artefact or a consequence of changing the medium. Alternatively it may be cell cycle related.

Certain proteins, such as cyclins, are only expressed in particular phases of the cell cycle. The transient expression of such proteins can be clearly detected in populations of synchronised cells. A synchronised cell population is one in which all the cells are progressing through the same phase of the cell cycle at the same time. Results in chapter 3 highlighted the ability of quercetin to induce growth inhibition and cell cycle arrest in PalF cells. Removal of quercetin releases cells from its inhibitory effects permitting them to grow normally. The sudden and synchronised

Figure 5.1 Western blot using protein extract from PalF cells and an antibody to detect phosphotyrosine



Approximately 20µg of protein extract was added to each lane in the gel

M: Size markers
Lane 1 and 2: Control lanes (PalF cell treated with ethanol (0.1% v/v) for 48 hours
Lane 3 and 4: PalF cells treated with 20µM quercetin for 48 hours before harvesting

In all other lanes, cells were treted with 20µM quercetin for 48 hours. At the end of the 48 hours, the medium was changed and cells were harvested at various times after removal of quercetin

Lane 5 and 6: Cells harvested 30 minutes after removal of quercetin
Lane 7 and 8: Cells harvested 1 hour after removal of quercetin
Lane 9 and 10: Cells harvested 2 hours after removal of quercetin
Lane 11 and 12: Cells harvested 4 hours after removal of quercetin
Lane 13 and 14: Cells harvested 8 hours after removal of quercetin

release of cells back into a normal cycle would result in the synchronised expression of cell cycle regulated proteins. Thus protein band number 1 (figure 5.1) may correspond either to a protein which is transiently phosphorylated on tyrosine or a phosphotyrosine protein which is transiently expressed in the cell cycle.

The observations made from figure 5.1 suggest that quercetin can alter the phosphotyrosine status of several proteins in PalF cells.

Many enzymes, including kinases, are phosphorylated on tyrosine residues (Levy *et al.*, 1984). The activity of such enzymes often correlates with their phosphorylation status. Thus the ability of quercetin to alter the phosphorylation and subsequent activity of several proteins in a number of cell lines (Levy *et al.*, 1984; Srivastava and Chiasson, 1986; Matter *et al.*, 1992) and now in PalF cells, may largely explain why quercetin can have such a profound effect on the normal functioning of so many different cell types.

5.3 Analysis to determine if the change in phosphotyrosine was due to a change in medium

It is possible that the changes which were induced in several of the phosphotyrosine proteins were independent of quercetin. They may have occurred as a result of simply changing the medium at the end of the 48 hours. To control for this possibility, the experiment described in the previous section was repeated in the absence of quercetin. If the changes in phosphotyrosine were genuinely the result of the medium being changed, the pattern of phosphotyrosine should again been seen to change, as illustrated in figure 5.1.

5.3.1 Experimental procedure

The experimental procedure employed was essentially identical to that described in section 5.3.1. The only difference was that cells were not treated with quercetin. Instead cells were cultured for 48 hours in 1xDMEM supplemented with 0.1% v/v absolute ethanol. Control dishes of cells were cultured in 1xDMEM only. As detailed in section 5.2.1, the medium in all dishes was changed at the end of the 48 hour incubation. Control cells and cells corresponding to the 0 time point were harvested for total cellular protein immediately after the 48 hours. All other dishes of cells received a fresh volume of 1xDMEM and were replaced in an appropriate

incubator. Cells were sequentially harvested at various times after the medium change.

5.3.2 Results

As the protein blot in figure 5.2 illustrates, treating cells with ethanol for 48 hours, followed by a medium change, did not elicit the same changes to the phosphotyrosine proteins that were produced by quercetin. This result confirms that quercetin can specifically induce changes to several phosphotyrosine proteins in PalF cells and that simply changing the medium has no detectable effect on phosphotyrosine levels.

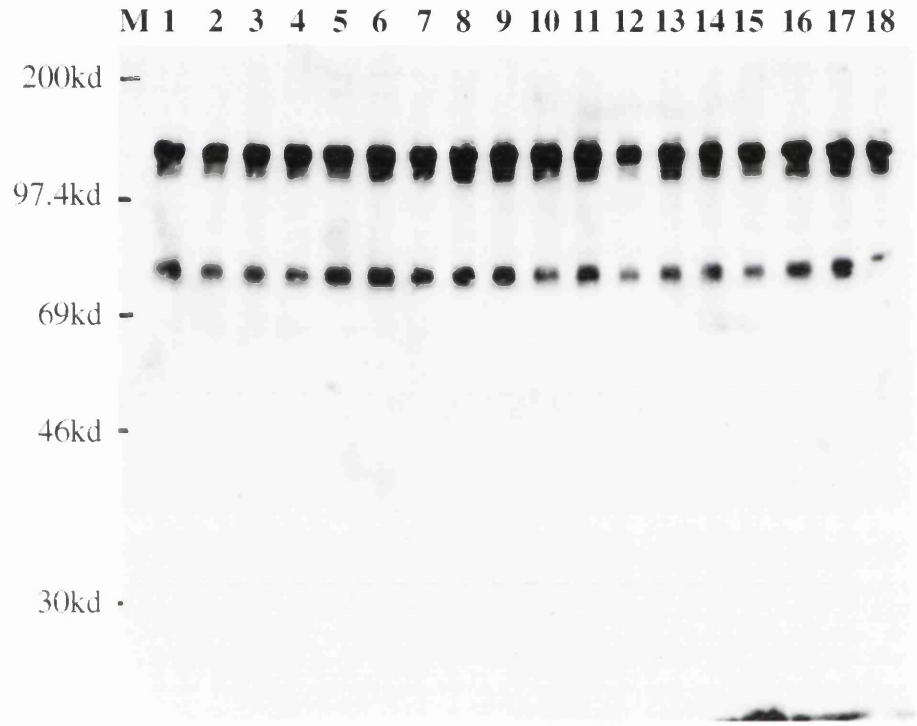
5.4 The effect of quercetin on other PalF cell proteins

It is unlikely that the effects of quercetin would be limited to those proteins which can be phosphorylated on tyrosine residues. With the total cellular protein extracts already available, another SDS-polyacrylamide gel was run. The total protein profile was visualised by staining the gel with Coomassie blue and subsequently analysed for differences.

5.4.1 Experimental procedure

Approximately 20-30μg of total cellular protein extract, made from cells exposed to either quercetin or an equivalent volume of ethanol, was loaded into separate wells on an SDS-polyacrylamide gel. The protein extracts used were similar to those described in section 5.2.1 except that an extra time point (24 hours after removal of quercetin) was included. After electrophoresis, the gel was stained in a solution of 0.25% Coomassie Brilliant Blue (R250) for 20 minutes at room temperature followed by several rounds of destaining. The stained gel was then placed on a clean light box and photographed before being dried down onto Whatman 3MM paper using a Biorad 583 gel drier. The methods of SDS-polyacrylamide gel electrophoresis and gel staining are described in detail in section 2.3.3.2.

Figure 5.2 Western blot showing that changing the medium does not alter the levels of phosphotyrosine in PalF cells



Each lane contains approximately 20µg of protein extract. M = Size markers

Lane 1 and 2: Control lanes (untreated PalF cells)

Cells were treated with 0.1% v/v ethanol for 48 hours. At the end of this time, the medium was changed. Cells were harvested at various times after the medium change

Lane 3 and 4: Cells harvested immediately after 48 hour incubation in 0.1% ethanol
Lane 5 and 6: Cells harvested 15 minutes after medium change
Lane 7 and 8: Cells harvested 30 minutes after medium change
Lane 9 and 10: Cells harvested 1 hours after medium change
Lane 11 and 12: Cells harvested 2 hours after medium change
Lane 13 and 14: Cells harvested 4 hours after medium change
Lane 15 and 16: Cells harvested 8 hours after medium change
Lane 17 and 18: Cells harvested 24 hours after medium change

5.4.2 Results

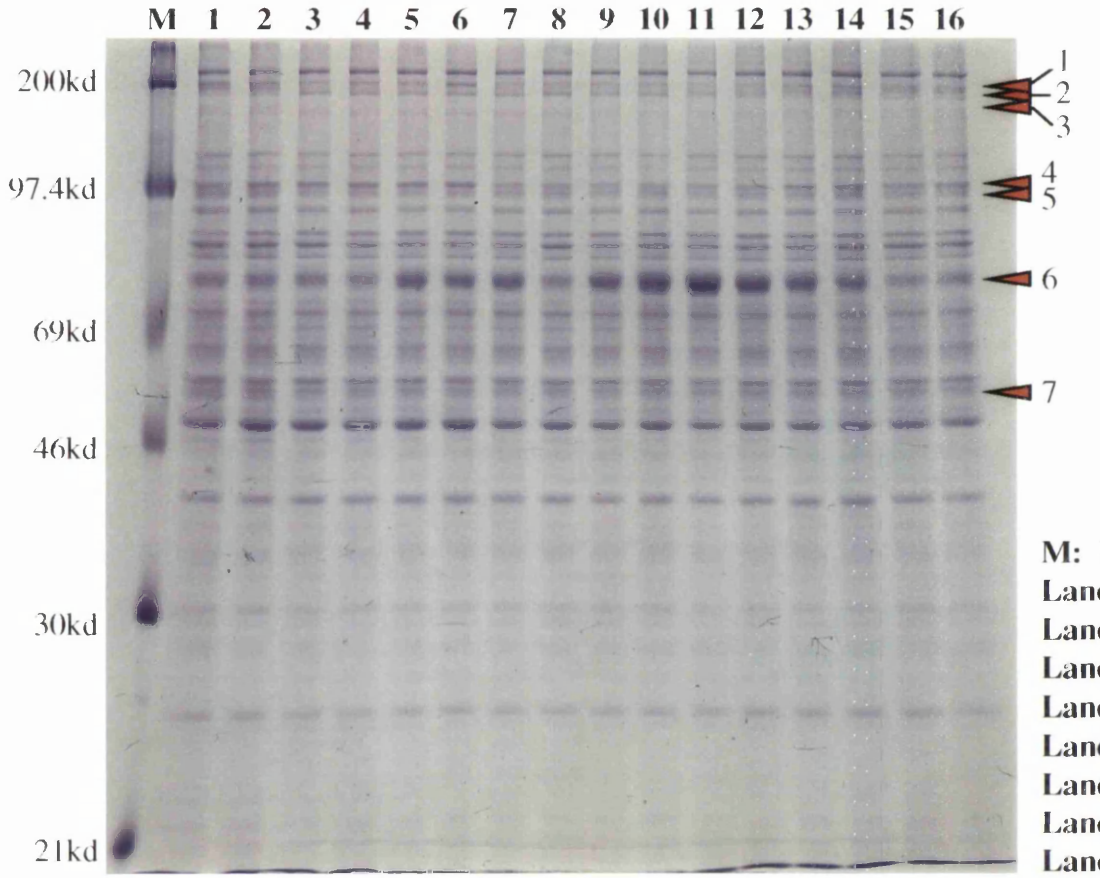
The Coomassie blue stained gel, shown in figure 5.3, only shows up the most abundant proteins in the total PalF protein extracts. Nevertheless, as the arrows to the right of the picture indicate, several protein bands show alterations in either their intensity or position within the gel.

Comparing primarily the banding patterns in lanes 1 and 2 with lanes 3 and 4; these lanes correspond to cells treated with either ethanol alone or 20μM quercetin respectively for 48 hours before harvesting. Close examination of the gel picture (figure 5.3) shows banding differences between the protein extracts (see bands indicated by the red arrows). Following the bands across the gel it can be seen that by 24 hours after removal of quercetin, the protein profile of quercetin treated cells has reverted back to that of cells treated with ethanol. Some of the proteins recovered in less that 24 hours.

One of the protein bands, indicated by arrow #6, however only begins to change 30 minutes after quercetin has been removed. A similar effect was seen in figure 5.1, where the uppermost band (see band #1 - figure 5.1) appeared 30 minutes after quercetin was removed, remained for up to 4 hours and then disappeared down to background levels again. As was suggested in section 5.2.2, the band indicated in figure 5.3 (approximately 70kd in size) may correspond to a protein which is cell cycle regulated. As shown in chapter 3, a quercetin concentration of 20μM can inhibit the growth of PalF cells by approximately 50% and induce at least a partial growth arrest. Removal of quercetin would release cells from this growth inhibition and allow cells to enter into a normal cycle of cell proliferation. This may explain the cyclic nature of the change in certain proteins.

Although the identity of any of the proteins shown to be altered in response to quercetin has not been determined, these results confirm that quercetin can directly affect a number of different proteins, and perhaps also their respective activities, in PalF cells.

Figure 5.3 Coomassie Blue stained gel representing the total protein profile from PalF cells



Red arrows indicate protein bands in which differences can be observed

M: Molecular weight markers
Lane 1 and 2: Normal untreated PalF cells
Lane 3 and 4: Time 0 after quercetin removed
Lane 5 and 6: 30 minutes after quercetin removed
Lane 7 and 8: 1 hour after quercetin removed
Lane 9 and 10: 2 hours after quercetin removed
Lane 11 and 12: 4 hours after quercetin removed
Lane 13 and 14: 8 hours after quercetin removed
Lane 15 and 16: 24 hours after quercetin removed

Chapter 6

Discussion

Chapter 6 Discussion

6.1 Introduction

BPV4 naturally infects the mucous epithelium of the upper gastrointestinal tract in cattle and induces the formation of benign papillomas (Jarrett *et al.*, 1978a; Campo *et al.*, 1980). These benign papillomas however have been shown to progress to squamous cell carcinomas at high frequency in animals which consume bracken fern in their diet (Jarrett *et al.*, 1978b; Campo *et al.*, 1984). Bracken fern, which covers extensive areas of the West Highlands of Scotland, is known to contain a number of compounds which possess mutagenic, carcinogenic and/or immunosuppressive properties (Evans,I.A. *et al.*, 1982; Evans,W.C. *et al.*, 1982). One of the more extensively studied mutagens found abundantly in bracken fern is quercetin. Quercetin is not exclusive to bracken fern; it is present in a wide range of edible plant products including vegetable, fruits, tea and wines (Herrmann, 1976; Kuhnau, 1976).

BPV4 alone, when transfected into cells *in vitro*, is non-transforming. The transforming potential of BPV4 is only realised when BPV4 DNA is cotransfected into PalF cells in co-operation with an exogenous oncogene such as activated *ras* (Jaggar *et al.*, 1990). BPV4 plus *ras*, while inducing several cellular changes consistent with the transformed state, only partially transform PalF cells; the cells retain a finite life span and are unable to induce tumours in nude mice (Pennie *et al.*, 1993). It was observed however that PalF cells exposed to 20μM quercetin for a period of 48 hours after transfection with BPV4 DNA (or selected BPV4 sequences) plus *ras*, displayed additional characteristics of transformation; quercetin treated PalF cells transfected with BPV4 DNA plus activated *ras* were immortal and tumorigenic in the nude mouse assay system (Pennie and Campo, 1992; Cairney and Campo, 1995). These observations suggested that quercetin was capable of synergising with BPV4 (plus *ras*) to achieve full transformation of PalF cells. Furthermore, quercetin provided additional functions necessary for the complete transformation of PalF cells which were not provided by either BPV4 or *ras*.

The aim of this thesis has been to assess the effect of quercetin on PalF cells and attempt to identify a mechanism(s) by which quercetin may synergise with BPV4 (and *ras*) to induce full cellular transformation.

6.2 The effects of quercetin on PalF cell growth

The effect of quercetin on the growth of PalF cells was analysed using three separate assay methods. Each of the three assays produced similar results and indicated that a quercetin concentration in the range of 10-35μM inhibited the growth rate and uptake of thymidine by approximately 50% (see section 3.2.2 and figures 3.2, 3.3 and 3.4). The level of growth inhibition was seen to be proportional to the concentration of quercetin in the culture medium showing that quercetin inhibits the growth of PalF cells in a concentration dependent manner.

As was discussed in detail in section 3.1, quercetin has been shown to inhibit the growth of a large number of cancer cell lines and primary cancer cells derived from a variety of anatomical sites (Scambia *et al.*, 1990a, b, and c, 1993, 1994b; Piantelli *et al.*, 1993, 1995; Ranelletti *et al.*, 1992; Avila *et al.*, 1994; Larocca *et al.*, 1995; Plaumann *et al.*, 1996). In keeping with the observations for PalF cells, quercetin-induced growth inhibition was shown to be concentration dependent. Unlike the cells and cell lines which have been used in previous studies, PalF cells are normal, primary bovine fibroblasts. The results from the experiments described in section 3.2.2 therefore confirm that quercetin can inhibit the growth of normal, untransformed cells in addition to partially or fully transformed tumour cells or cell lines.

The concentration of quercetin capable of inhibiting the growth of particular cells or cell lines is variable. Some studies report a quercetin IC 50% (the concentration of quercetin inhibiting growth of cells by 50%) ranging from 1nM to 1μM (Scambia *et al.*, 1990b, 1991; Ranelletti *et al.*, 1992; Larocca *et al.*, 1995; Piantelli *et al.*, 1995). Other cells however were only inhibited when quercetin was present at concentrations of ~ 20μM and higher (Hosokawa *et al.*, 1990; Avila *et al.*, 1994). Although the mechanism underlying the antiproliferative activity of quercetin remains to be clarified, there is evidence suggesting a role for type-II estrogen binding sites (type-II EBS) in many instances. Several studies have shown that quercetin can regulate the levels of type-II EBS and that the degree of quercetin-induced growth inhibition if often proportional to the number of type-II EBS expressed by a particular

cell type. Cell which express type-II EBS are generally inhibited by quercetin present at nM concentrations. Furthermore, the relative type-II EBS binding affinities are in good correlation with the capacity of quercetin to induce these sites (Ranelletti *et al.*, 1992; Scambia *et al.*, 1991, 1993). Cells which have fewer or are negative for type-II EBS appear only to be inhibited by higher concentrations of quercetin (Hosokawa *et al.*, 1990; Avila *et al.*, 1994). In such cases, growth inhibition is more probably a consequence of quercetin's ability to inhibit the activity of a number of cellular enzymes (Lang and Racker, 1974; Monaham *et al.*, 1975; Kuriki and Racker, 1976; Graziani *et al.*, 1983; Shosham and MacLennam, 1981; Kato *et al.*, 1983; Nishino *et al.*, 1984; Nakadate *et al.*, 1984; Verma *et al.*, 1988; Matter *et al.*, 1992).

The concentration of quercetin which was effective at inhibiting the growth of PalF cells was in the range of 10-35μM (section 3.2.2). This would indicate that PalF cells do not express or poorly express type-II EBS and that growth inhibition is more likely to be related to cellular enzyme inhibition.

Growth inhibition induced by quercetin is generally reversible (Scambia *et al.*, 1990b; Ranelletti *et al.*, 1992). Cells can continue to grow normally after quercetin is removed from the culture medium. The same recovery from quercetin treatment has also been observed for PalF cells (personal observation). However a point is reached when the concentration of quercetin, rather than being simply growth inhibitory, becomes cytotoxic. Quercetin has been shown to induce apoptosis in cells which have been treated with higher quercetin concentrations (generally >50μM) particularly for prolonged periods of time (Yoshida *et al.*, 1992; Avila *et al.*, 1994; Plaumann *et al.*, 1996). The non-tumour cell line C3H10T1/2CL8 underwent apoptosis but only after cells had been treated with 120μM quercetin for 72 hours (Plaumann *et al.*, 1996). The ability of quercetin to induce growth arrest and/or cell death under certain conditions is hardly surprising. It is quite usual for cells exposed to cellular stress or adverse culture conditions to growth arrest while retaining the potential to resume cycling when the cellular environment is again favourable. Yet in situations where adverse conditions persist, cells are generally induced to undergo apoptosis (Plaumann *et al.*, 1996; Iwata *et al.*, 1997; Zhan *et al.*, 1997).

Addition of quercetin to PalF cell culture medium, even at concentrations as high as 100μM, appeared not to induce significant levels of cell death as judged by the

small number of floating cells in the medium (personal observation). Nevertheless, no specific assay was performed to accurately assess the level of apoptosis which may have been occurring as a result of quercetin treatment.

In summary, the results from the growth and cell cycle experiments described in chapter 3, and from other studies with quercetin (Scambia *et al.*, 1994a; Avila *et al.*, 1994; Larocca *et al.*, 1995), suggest that the presence of quercetin is not compatible with normal cell growth of primary PalF cells and a large number transformed cell lines alike.

The ability of quercetin to inhibit the growth of a large number of tumour cell lines *in vitro* has lead to it being proposed as a potential therapeutic antitumour agent (Scambia *et al.*, 1990a; Avila *et al.*, 1994; Shimoi *et al.*, 1994; Piantelli *et al.*, 1995). The maximum tolerated dose for quercetin in humans has not yet been determined, however plasma concentrations of 12μM were achieved following an intravenous infusion of 100mg with no apparent side effects (Gugler *et al.*, 1975). A quercetin concentration of 12μM is greater than that which has been shown effective in inhibiting several tumour cell lines and primary tumour cells *in vitro* (Scambia *et al.*, 1990b; Larocca *et al.*, 1995). As has been discussed in detail in section 3.1, the effects of quercetin are numerous and diverse. Because quercetin can affect a large number of cellular targets, it is possible that its actions may cause the inhibition of one or more steps of the carcinogenic process. This may be viewed as a beneficial quality for an anticancer agent. With the potential to target a large number of cellular activities, quercetin may thus be more effective at inhibiting the growth of a larger number of diverse tumour types which possess a variety of molecular alterations when compared to an agent which inhibits one specific cellular function.

Alternatively, the fact that quercetin has such a large number of potential cellular effects suggests that the final outcome may be rather unpredictable. The unpredictable nature of quercetin treatment is clearly reflected in the results from a number of studies in which quercetin was shown to actually contributed to carcinogenesis (Pamukcu *et al.*, 1980; Pereira *et al.*, 1996). The mechanism(s) underlying the ability of quercetin to produce such contradictory effects is not understood. There is evidence to suggest that the actions of quercetin may be influenced by a number of factors including specific cell type, by what other cellular

changes have occurred in those cells as well as the physical, redox state of the cellular background (Okada *et al.*, 1990; Perona and Serrano, 1988; Dreher and Junod, 1996; Plaumann *et al.*, 1996). The ability of quercetin to contribute to the carcinogenic process highlights the need for additional study of the action of quercetin and its further evaluation as an anticancer agent should proceed with caution.

In addition to quercetin's potential as an antitumour agent, the consumption of quercetin and other flavonoids as part of a normal diet has been related to a lower incidence of cancers of the stomach, colon (Haenszel *et al.*, 1980; Ranelletti *et al.*, 1992), and breast (Adlercreutz, 1984) in humans. The chemopreventive properties of quercetin have been related to its antioxidant properties. However, quercetin has also been demonstrated to possess pro-oxidant properties. Quercetin has been shown to inhibit glutathione reductase (Elliott *et al.*,1992) a key antioxidant enzyme which protects a cell against oxidative stress. Combining this with quercetin's additional ability to produce reactive oxygen species (O_2^+, H_2O_2, $^{\bullet}OH$) (Canada *et al.*, 1990; Elliott *et al.*, 1992) suggests that quercetin could be promoting extensive oxidative stress *in vivo*. Furthermore, quercetin inhibits O-methylation of catecholestrogens (Zhu and Leibr, 1996). Catecholestrogens have been postulated to mediate the induction of kidney tumours by estradiol in male Syrian hamsters. Hence quercetin has the potential to increase the production of potentially mutagenic free radicals and thereby potentiate estrogen-induced renal tumorigenesis.

Similar claims of chemopreventive activity have also been proposed for another common dietary antioxidant, β-carotene (Hennekens, 1986). β-carotene was suspected of lowering the risk of developing cancer, specifically lung cancer, and cardiovascular disease. Results from a large, long-term Scandinavian trial (ATBC trial) (Heinonen *et al.*, 1994) however failed to detect any significant protective effect with regard to lung cancer. In fact, there were significantly more new cases of lung cancer in the group given β-carotene dietary supplements. Although the finding of ATBC trial does not necessarily exclude potential benefits of β-carotene in the diet, it suggests that predicted benefits, regarding cancer prevention, may have been overestimated and highlights the potential of antioxidants such as β-carotene to have some harmful effects. Thus the chemopreventive activity of *in vivo* antioxidants has not yet been proven (Hennekens *et al.*, 1994).

6.3 The effect of quercetin on cell cycle status and morphology of PalF cells

Preliminary analyses showed that the effects of quercetin on the cell cycle were only transient unless quercetin was replenished in the culture medium every 12 hours. Quercetin appears to undergo a process of inactivation in culture medium although the specific mechanism of how quercetin is inactivated is not understood. One possibility is that quercetin is gradually oxidised. Because of the problems with quercetin stability, it was necessary to replenish the medium with a fresh volume of active quercetin every 12 hours to obtain a measurable cell cycle arrest.

Analysis of the effect of quercetin on the cell cycle status of PalF cells was performed for a range of quercetin concentrations over a 48 hour time period. The results, illustrated in figure 3.6 and 3.7 and described in section 3.3.2, show that quercetin can induce PalF cells to undergo cell cycle arrest. This observation complements the PalF cell growth assays discussed in sections 3.2.2 and 6.2 and shows that the growth inhibitory action of quercetin is not simply a dynamic balance between continued cell proliferation and cell death.

PalF cells treated with 20, 50 and 100μM quercetin were seen to steadily accumulate in the G2/M phase of the cells cycle. An interesting observation however was that PalF cells were observed to accumulate in S-phase within 12 hours after quercetin was first added to the culture medium (see figure 3.7, panel 3). This accumulation of cells in S-phase was only clearly evident for PalF cells which were treated with 50 or 100μM quercetin and had disappeared by 24 hours.

A complete G2/M arrest was not observed for PalF cells even when exposed to the highest concentration of quercetin. This observation suggests that a quercetin concentration of 100μM was still insufficient to induce a complete cell cycle arrest. Alternatively, the experiment may have been terminated before a complete cell cycle arrest could be achieved. This is a plausible suggestion considering PalF cells were shown to take 36 to 48 hours to complete a single cell cycle (see section 3.2.2).

The results presented in figure 3.6 and 3.7 indicate that quercetin can also induce cells to arrest in G1/G0. Thus exposure of PalF cells to quercetin is associated with the establishment of two separate restriction points in the cell cycle, one in G1, the other in G2/M.

The observations from the PalF cell cycle analysis have lead to the following model. After the addition of quercetin to the culture medium, cells begin to accumulate in S-phase. Either cells in mid to late G1 are being stimulated to enter S-phase or their ability to exit S is delayed or inhibited. Whatever the mechanism, the accumulation of cells in S-phase is transient. All cells that have already passed the quercetin-induced restriction point in G1 continue through the cell cycle until they reach the second restriction point in G2. Likewise, cells which have travelled beyond the G2 block before quercetin has been added to the medium would enter and complete mitosis (M phase). Cell would then stop cycling once the G1 block was reached.

Quercetin is known to induce different types of cell cycle arrest (Yoshida *et al.*, 1990; Hosokawa *et al.*, 1990; Gong *et al.*, 1994; Sato *et al.*, 1994; Avila *et al.*, 1994; Plaumann *et al.*, 1996). This may be related to the cell type involved, the cellular processes affected and/or the specific concentration of quercetin used. The specific mechanism(s) relating to a quercetin-induced cell cycle arrest has not been fully clarified. Nevertheless, some of the potential candidate proteins which have been shown to be modulated by quercetin and which may have a direct impact on normal cell proliferation include kinase enzymes such as phosphatidylinositol-3 kinase (PI3-kinase) and viral *src* (Matter *et al.*, 1992; Graziani *et al.*, 1983), TGFβ (Scambia *et al.*, 1994a), heat shock proteins (Elia and Santoro, 1994), and both wild type and mutant p53 (Plaumann *et al.*, 1996; Avila *et al.*, 1994).

In addition to growth inhibition and cell cycle arrest, quercetin was also shown to induce certain morphological changes in PalF cells (Figure 3.5). Cell density was reduced however this was most likely related to the decrease proliferation rate which was seen to be a consequence of quercetin in the culture medium. Other morphological changes induced by exposure to quercetin included cells having a flatter appearance and a more vacuolar cytoplasm. The morphological changes induced were more pronounced in PalF cells which were exposed to the highest concentrations of quercetin (50 and 100μM). Thus, similar to the observations for PalF growth inhibition, the level of morphological change induced by quercetin was found to be dependent on and proportional to the concentration of quercetin present in the culture medium.

6.4 Quercetin can increase the level of transcription from the BPV4 LCR when present as a promoter

As was mentioned in section 6.1, one of the primary aims of this thesis was to attempt to identify ways in which quercetin may be synergising with BPV4 DNA (plus activated *ras*) to achieve malignant transformation of PalF cells. One hypothesis was that quercetin was able to increase the level of viral transcription and consequently the levels of viral oncogenes. As a means of testing this hypothesis, a series of transient expression assays were performed to analyse the effect of quercetin treatment on the transcriptional activity of the BPV4 LCR.

Quercetin was shown to increase the level of transcription from the BPV4 LCR by two to four fold in PalF (figure 4.1, 4.8) and PalK cells (figure 4.11). This effect however was only observed when the LCR was functioning in a promoter capacity (figure 4.1); the LCR when present as an enhancer was unaffected by exposure to quercetin (figure 4.2). Thus the effect of quercetin on the BPV4 LCR is promoter specific.

The increase in transcription was also only realised when cells were treated with quercetin immediately after transfection (figure 4.1). This suggests that the effect(s) of quercetin is relatively short lived and that a cell will recover from exposure to quercetin quite quickly. The transient nature of quercetin treatment generally rules out the possibility that quercetin was inducing changes at the genetic level; a genetic change, such as a mutation, constitutes a permanent change and would thus persist in a cell in the long term.

The experiments described in section 4.2 showed that quercetin could consistently increase the level of viral transcription up to four fold over untreated cells. Although this may be regarded as a small to moderate increase in viral transcription, it may have a profound effect on cells *in vivo* and be sufficient to push cells into a more transformed state. Indeed, the degree of phenotypic transformation detected in various cell lines has been shown to correlate with the level of viral oncogene expression (von Knebel Deoberitz *et al.*, 1994; Liu *et al.*, 1995; Trujillo and Mounts, 1996).

The 3'terminal 21 base pair sequence of the LCR, encompassing nucleotides 311-331, was proposed as a potentially interesting site with respect to quercetin

responsiveness because of an identified TRE-like binding motif. Deletion of this 21 base pair stretch corresponded with a general drop in basal transcriptional activity (figure 4.9 and 4.12) and a drop in the response of the LCR to quercetin in both PalF and PalK cells (figure 4.8, 4.11). Replacement of this 21 base pair sequence with a stretch of 21 random nucleotides again resulted in a drop in response to quercetin in both PalF and PalK cells (figure 4.23). The drop in response to quercetin with the 'random' LCR was similar to that observed with the 21 base pair truncated form of the LCR (figure 4.23). A significant drop in the basal level of transcription was observed for the LCR containing the 21 random nucleotides in place of the normal 3'sequence (figure 5.23). These results indicate that nucleotides 311 to 331 of the BPV4 LCR are important with respect to basal transcriptional activity and in mediating the transcriptional effects of quercetin.

TPA and quercetin were shown to have antagonistic effects on the activity of the collagenase TRE and on the BPV4 LCR (figure 4.4). This may be related to the known ability of quercetin to inhibit the action of enzymes which are activated by TPA treatment, such as protein kinase C (Gschwendt *et al.*, 1983; Angel *et al.*, 1987). Alternatively, quercetin and TPA may induce separate signal transduction pathways which are themselves incompatible and antagonistic.

An increase in the activity of the BPV4 LCR was only observed when cells were cultured in medium contain 10% serum (figure 4.4). It would appear that quercetin is dependent on serum growth factors, or anther constituent of serum, to modulate the activity of the BPV4 LCR. It is possible that the action of quercetin is in some way associated with a particular signal transduction pathway which is only activated by particular factors present in serum. Disruption of signal transduction as a consequence of low serum levels in the culture medium may thus inhibit the ability of quercetin to transduce its affects to various cellular targets. At present however the exact nature of quercetin's dependence on serum is not known and represents an area for further study.

6.5 A cellular factor QX1 mediates the transcriptional effects of quercetin within the BPV4 LCR

Results from the transient expression assays indicated that the 21 base pair 3'terminal sequence of the BPV4 LCR, encompassing nucleotides 311-331, was

important in mediating the transcriptional changes induced by exposure of PalF and PalK cells to quercetin. A series of electromobility shift assays (EMSA) were performed in an attempt to ascertain if any protein was capable of binding to this region of the BPV4 LCR. As the results in figure 4.14 and 4.15 illustrate, a factor designated QX1 was identified as binding to an oligonucleotide which corresponded to the sequence of nucleotides from 301 to 331 of the BPV4 LCR. The addition of several unlabelled oligonucleotides as competitors confirmed that the binding of QX1 to this oligonucleotide was specific. QX1 was also detected in chick embryo fibroblast cells (CEFs) (figure 4.15). Thus QX1 is not a PalF specific factor but is expressed in at least one other primary fibroblast cell from a different species.

The presence of a TRE-like element lead to the initial hypothesis that QX1 may in fact be AP1. Further EMSA experiments however showed that QX1 and AP1 displayed very different mobility in a polyacrylamide gel and confirmed that QX1 and AP1 are not the same factors (figure 4.17). Supershift EMSAs, which employed antibodies to various subunits of classic AP1, indicated that QX1 was not composed of cJun or cFos subunits. As the results in figure 4.18 suggest however, QX1 may contain a subunit(s) which is related to cFos although the exact identity of any QX1 composite protein has not yet been confirmed. The exclusion of AP1 as a candidate for QX1 is in agreement with expression assay results in which TPA, which is known to increase the levels of AP1, had no affect on the transcriptional activity of the BPV4 LCR. Furthermore, quercetin and TPA together displayed antagonistic effects with respect to the transcriptional activity of the BPV4 LCR and the collagenase TRE (figure 4.4).

A series of point mutations were introduced into the wtLCR oligonucleotide in an attempt to abolish binding of QX1 and thus potentially identify residues critical for the binding of QX1 to a region of the LCR. Mutating the TRE-like element to a canonical AP1-binding site, by virtue of a G to T transversion, did not exclude the binding of QX1 (figure 4.20). Indeed the binding of QX1 appeared to be strengthened. Furthermore, the conversion of the TRE-like element to a consensus AP1 binding motif enabled AP1 to bind strongly to the LCR mutant oligonucleotide (figure 4.20). Although both AP1 and QX1 were shown to bind to this mutant LCR oligonucleotide, binding of each individual factor was mutually exclusive. This would suggest that while AP1 and QX1 bind separate sites within the LCR

oligonucleotide, their respective binding sites are actually located very close to each other and may in fact be overlapping.

Two more mutant LCR oligonucleotides were generated in which nucleotides 5' to the TRE-like element were changed (see figure 4.13 for details of mutants oligonucleotide sequences). Neither mutant #2 nor mutant #3 abolished binding of QX1 to the LCR. These observations exclude any of the mutated nucleotides as being important in the binding of QX1 to the LCR and further suggest that the binding site of QX1 lies downstream (3'), albeit marginally, of the TRE-like motif.

The only other binding site which has been identified within nucleotides 311 to 331 of the BPV4 LCR is putative INR (initiator) element (Novina and Roy, 1996) . INRs have been identified downstream of the TATA box in other viral and cellular genes. They have a general consensus sequence of PyA(+1)NT/APyPy (Javaheri *et al.*, 1994), where Py corresponds to a pyrimidine base. A similar sequence, TAGCTCT, exists at nucleotide 321-327 of the BPV4 LCR. The putative INR is consistent with the suspected position of the QX1 binding site. INRs bind a host of cellular proteins (IBP) many of which have been identified as either TAF, GTF or USF family members. Binding of an IBP to the INR assists the formation of the transcription pre-initiation complex and enhances promoter strength (Kaufmann and Smale, 1994). Deletion of nucleotides 311 to331 corresponded with a drop in the level of basal transcription and response of the LCR to quercetin, effects which are consistent with the removal of an INR. Thus QX1 may be an IBP which is regulated by quercetin.

There was no difference in the binding of QX1 from quercetin and ethanol treated control cells (figure 4.14). This shows that QX1 is also present in cells which have not been treated with quercetin. It further suggests that the effect of quercetin on QX1 is probably not to induce the de novo expression of QX1. It is possible however that quercetin may be affecting the activity of QX1 at the post-transcriptional level. In fact a similar effect of quercetin has been reported with regard to TGFβ (Scambia *et al.*, 1994a). Alternatively, quercetin treatment may induce phosphorylation changes on QX1 thus altering its activity.

6.6 Epigenetic effects of quercetin

The effects of quercetin are numerous, diverse and complex. In light of this it is reasonable to suggest that alterations to the level of LCR transcription is not the only effect of quercetin which may explain its synergistic association with BPV4 DNA and *ras*. Indeed, quercetin was seen to synergise with BPV4 plus *ras* to achieve full cellular transformation even when the BPV4 genes were being expressed from the Moloney murine LTR (figure 4.26a and 4.26b). This powerful viral promoter was found not to be up-regulated in response to quercetin exposure; rather the activity of the Moloney murine LTR was slightly reduced following quercetin treatment (figure 4.26b).

As a means of identifying other cellular targets affected by quercetin in PalF cells, the phosphotyrosine status of proteins was analyses. Quercetin was shown to alter the phosphorylation status of a number of cellular proteins. Although the identity of the proteins affected has not been determined the results are strongly suggestive of the potential of quercetin to induce change at an epigenetic level.

Epigenetic changes are generally non-permanent, reversible changes to proteins or DNA. Proteins may be modified in a number of ways including phosphorylation. A phosphate moiety can be transferred on and off tyrosine, serine or threonine residues in many proteins. The presence or absence of protein phosphorylation is often related to the functional activity of a protein. Results from the phosphotyrosine analysis described in section 5.2.2, in combination with observations made by other workers (Graziani *et al.*, 1983; End *et al.*, 1987; Van Wart-Hood *et al.*, 1989; Matter *et al.*, 1992) confirm the potential of quercetin to affect the activity of various cellular proteins possibly by altering their phosphorylation status.

DNA can also be epigenetically modified by a process of methylation. DNA methylyation involves the enzymatic transfer of a methyl group from s-adenosyl-L-methionine to the 5' position on a cytosine ring. Depending on the species, between 2 to 7 percent of cytosine residues in animal cell DNA are methylated (Lewin, 1990). Most methyl moieties are found in CpG 'doublets'. Between 60 to 90 percent of CpGs are methylated which accounts for most, if not all, of the methylcytosine in vertebrate genomes.

Many cellular genes which are constitutively expressed, such as 'housekeeping' genes, contain clusters of CpG sequences located upstream of the site of transcription initiation (Bird, 1986). These CpG-rich islands are generally unmethylated. Hypomethylation is generally associated with a gene which is undergoing transcription. Hypermethylation of CpG islands is often observed when gene expression has been silenced.

Quercetin has been shown to induce DNA hypermethylation (Ishikawa *et al.*, 1987). This suggests that quercetin treatment, through DNA hypermethylation, may lead to the transcriptional silencing of particular cellular genes, such as tumour suppressor genes. Thus by reducing the expression of cellular genes which can protect the cell against uncontrolled proliferation, quercetin may be promoting cellular transformation.

The ability of quercetin to synergise with BPV4 and *ras* to achieve full transformation has been shown to be limited to a time period shortly after transfection of PalF cells with BPV4 and *ras* DNA (Cairney and Campo, 1995). This suggests that the effects induced by quercetin which contribute to this synergistic process are relatively short lived. This supports the hypothesis that certain epigenetic events, induced by quercetin, are critical for the full transformation of PalF cells in association with BPV4 and *ras* expression.

6.7 Potential model for the role of quercetin in BPV4-associated cellular transformation

In light of the experimental observations which have been described in this thesis, a model to explain the contribution of quercetin to BPV4-associated cellular transformation is proposed.

Quercetin induces cell cycle arrest, possibly as a direct result of quercetin-induced DNA damage or cellular stress. The expression of BPV4 genes has been shown to be modulated by quercetin treatment. Increased expression of a viral gene like E7, which can bind and affect the normal activity of pRb, would stimulate cells to continue through the cell cycle. The stimulation of cells to undergo DNA synthesis under adverse conditions may give rise to cellular mutation. Topoisomerase II, an enzyme involved in the repair of DNA damage, has also been shown to be inhibited by quercetin and may result in a mutation going uncorrected (Austin *et al.*, 1992).

Other epigenetic effects of quercetin, as indicated by the ability of quercetin to alter the phosphorylation status of several cellular proteins, suggests other cellular targets of quercetin, which when altered, may further contribute to the process of transformation.

The synergism between BPV4 (plus *ras*) expression and quercetin treatment appears only to occur in a limited time window, that is the period immediately following cellular transfection. Viral gene (and *ras*) expression may have to be at a maximum before quercetin can have a synergistic effect. Coupled with this is the observation that the effects of quercetin are short lived; quercetin appears to undergo a process of inactivation upon addition to cell culture medium.

The combined effects of quercetin treatment, oncogenic *ras* and increased BPV4 expression could potentially lead to the attainment of a DNA mutation(s) which ultimately leads to the establishment of a fully transformed cellular phenotype.

The model proposed above may be extended to the situation *in vivo*. However, certain physical restraints argue against quercetin having a role in BPV4-associated carcinogenesis. Quercetin is poorly absorbed in the gastrointestinal tract and is degraded by intestinal microflora (Ueno *et al.*, 1982). Furthermore, quercetin which is absorbed is rapidly inactivated by O-methylation catalysed by catechol-O-methyltransferase (Zhu *et al.*, 1994). These facts however are less important when considering BPV4-associated carcinogenesis *in vivo*. Quercetin (in bracken fern) would be ground directly into oral lesions via the mechanical action of chewing and would be in direct contact with lower GI lesions as the food was ingested. Thus the concentrations of quercetin, along with other chemicals in bracken necessary for carcinogenesis, are highest at the site of immediate interest.

Quercetin may be a contributory factor in similar cancers, namely of the oral cavity and gastrointestinal track, in humans. There has been a recent surge in interest in establishing the role of papillomaviruses in the aetiology of oral cancers (Yeudall, 1992; Woods *et al.*, 1993; Chen *et al.*, 1997).

6.8 Future prospects

Results in chapter 3 showed that quercetin could inhibit the growth of PalF cells in a concentration dependent manner. It would be interesting to determine if

PalF cells stably transfected with BPV4 DNA (plus *ras*) were more or less sensitive to the growth inhibitory effects of quercetin. Similarly, could BPV4 plus *ras* transformed cells be induced to undergo the same kind of growth arrest induced in normal PalF cells following continued quercetin treatment.

It is currently unknown if quercetin can induce or suppress the expression of wild type p53 in PalF cells. A study by Plaumann *et al.* (1996) showed that quercetin could induce p53 expression in a non-tumorigenic cell line, C3H10T1/2CL8. The ability of quercetin to induce the expression of p53 indicates that quercetin may be inducing a level of DNA damage. The presence of DNA damage has been shown to result in cell cycle arrest (Plaumann *et al.*, 1996). Therefore by determining the p53 status in quercetin treated PalF cells it may be possible to identify a potential mediator in the quercetin induced cell cycle arrest.

The ability of quercetin to induce apoptosis in PalF cells has not yet been determined. If apoptosis is induced it would be interesting to determine if this was p53 dependent or not.

Results in this thesis have shown that quercetin can increase the level of transcription from the BPV4 LCR. The next step would be to determine if quercetin treatment can actually give rise to a detectable increase in the level of viral proteins. It is appreciated though that this may be a technically very difficult experiment to perform.

The use of mutant oligonucleotides has thus far been unsuccessful in positively identifying bases critical for the binding of QX1 within the 3'terminal 21 base pair region of the BPV4 LCR. The initial screening was rather limited with only 3 different mutant oligonucleotides used. It is reasonable to suggest that by generating several more mutant LCR oligonucleotides, carrying base substitutions 3' to those previously changed, a QX1 binding site may be identified.

In the event that a specific binding motif was identified, its response to quercetin could be assess by cloning the element upstream of a suitable promoter driving a reporter gene and thus confirm that quercetin can induce an increase in transcription. It would also be interesting to determine if other quercetin response elements (QRE) were located elsewhere in the LCR or in the regulatory regions of other viruses or cellular genes.

As yet it is not known if QX1 is a novel transcription factor. With the identification of a QRE, the next step would therefore be to try to purify and characterise QX1.

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