The Transformation Biology of Bovine Papillomavirus Type 4 in Established and Primary Cells.

Margaret Cairney

This thesis is submitted in part fulfilment of the Degree of Doctor of Philosophy in the University of Glasgow.

Beatson Institute for Cancer Research,
CRC Beatson Laboratories,
Bearsden, Glasgow.

Faculty of Medicine,
University of Glasgow,
Glasgow.

April 1996

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

For my parents

ABSTRACT

Bovine papillomavirus type 4 (BPV-4) induces papillomas in the upper alimentary canal of cattle which can progress to carcinomas in animals feeding on bracken fern. Viral DNA is rarely detected in either naturally occurring or experimentally induced cancers. Similarly, BPV-4 DNA is seldom found in *in vitro* transformed established cells. These results suggest that presence of BPV-4 is not required for progression to or maintenance of the transformed state. The frequency with which BPV-4 DNA is lost both *in vivo* and *in vitro* also suggests that there may be active selection against whole or part of the BPV-4 genome. Several BPV-4-transfected established lines were analysed to examine whether this proposed negative selection was observable at the DNA level.

In common with previous *in vitro* studies, BPV-4 DNA was found to be progressively lost on continued sub-culture. Analysis of cell lines containing BPV-4 DNA showed that there was no overt mutation or rearrangement of viral DNA sequences. The apparent wildtype organisation of viral genes included the E8 open reading frame (ORF). It was originally hypothesised that this viral ORF, which is the second major transforming gene of BPV-4, was an attractive target for negative selection due to the detrimental effect observed on transfecting primary cells with BPV-4 E8 DNA.

Previous work also reported amplification and rearrangement of specific host sequences in BPV-4-transformed lines. It was therefore proposed that induction of cellular DNA amplification may be an important aspect of BPV-4 transformation activity. This was investigated in virally-transformed established cells. No BPV-4-mediated DNA amplification was apparent in any of the lines, although involvement of cellular regions of repetitive sequences in manifestation of such amplification may be indicated.

The co-factors involved in BPV-4-associated carcinogenesis have been identified as including the mutagens, carcinogens and immunosuppressants present in bracken fern. One of major mutagens present is the flavonoid quercetin. Quercetin has discernible effect on BPV-4 transformation *in vitro* as it synergises with the virus to fully transform primary bovine fibroblasts (PalF cells). The work described in this thesis confirmed these initial results and extended these findings. Quercetin-treated cells showed a more aggressive transformed morphology than untreated transfectants, whether they had been transfected with whole genome BPV-4 or sub-genomic fragments. However, whereas in non-treated cells the E8 gene was required for anchorage independence, quercetin-treated cells containing the BPV-4 E7 gene alone were found to be capable of anchorage-independent growth. Conversely, and contrary to expectation, quercetin-treated cells transfected with the E8/E7 genes grew very poorly or not at all in semi-solid media. The reasons for the antagonistic action of E8 and quercetin are not yet understood. Independent of quercetin action, results also provided circumstantial evidence that the E8 oncoprotein is responsible for downregulation of gap junctional intercellular communication in BPV-4-transformed cells. BPV-4 does not have an E6 gene and immortalisation of BPV-4-transformed cells is achieved only in the presence of an exogenous E6 gene. Quercetin treatment however conferred immortality on cells transformed by whole genome BPV-4 or by the E7 gene alone.

The observed synergism between BPV-4 and quercetin was also found to be dependent on time of treatment. Treatment of cells with quercetin either immediately before or after DNA transfection resulted in an increased degree of cellular transformation compared to that seen on lengthening the interval between treatment with the chemical and transfection with BPV-4 genes. PalF cells treated with quercetin immediately after transfection with the E7 gene were tumourigenic. The tumours grew far more aggressively than when cells were treated with quercetin before transfection with whole

genome BPV-4. This indicated that the timing of exposure of viral products and quercetin is crucial and that in certain circumstances only the E7 gene is required for full malignant transformation.

No discernible mutation was found either as a result of treatment with quercetin and / or transfection with BPV-4 genes. However, quercetin was found to cause epigenetic changes in PalF cells as measured by transient alteration in phosphotyrosine levels of, as yet, unidentified proteins.

These results strongly support the hypothesis that quercetin acts as a co-carcinogen in BPV-4-associated carcinogenesis *in vivo* and suggest that this *in vitro* experimental system provides a useful model for analysis of viral / chemical co-operation in papillomavirus-associated carcinogenesis.

ACKNOWLEDGEMENTS

First and foremost I would like to thank my supervisor Dr. Saveria Campo for all her help, advice and enthusiasm. In addition my thanks to all members of R6 (past and present) for their generous practical help and engaging banter. My thanks also to my advisor, Dr. Ruth Fulton, for fruitful discussion and continual interest. In addition I appreciate and gratefully acknowledge the contribution of the following people involved in various aspects of this work: Drs. John Pitts, Ken Smith, Ruedi Fries, Sabina Solinas, Jim Russell, Alex Lewis, Andrew Povey and Charlie Walker. Thanks also to the Beatson animal house staff, particularly Tom Hamilton, Central Services staff (the Institute's unsung heroes), David Tallach for the photography and Liz Gordon for many a literature search. I would also like to acknowledge the financial support of the Medical Research Council and the Cancer Research Campaign.

Last but not least, heartfelt thanks to John, my family and friends for their encouragement, love and support.

Declaration

The work described in this thesis was performed personally unless otherwise acknowledged.

'It was the best of times, it was the worst of times...'

Charles Dickens *'A Tale of Two Cities.'*

TABLE OF CONTENTS

LIST OF FIGURES

Chapter 1

Chapter 3

Chapter 4

Chapter 5

LIST OF TABLES

Chapter 3

Chapter 4

Chapter 5

ABBREVIATIONS

A_X	Absorbance $_{\text{wavelength (nm)}}$
ALV	Avian leukosis virus
ARS	Autonomously replicating sequences
ATP	Adenosine triphosphate
β-gal	β-galactosidase
bp	Base pair
BSA	Bovine serum albumin
BPV	Bovine papillomavirus
C	Cytosine
c-	Cellular
°C	Degrees centigrade
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
cf.	Compare
Ci	Curie
CIN	Cervical intraepithelial neoplasia
cm	Centimetre
cpm	Counts per minute
CRPV	Cottontail rabbit papillomavirus
DMBA	7,12-dimethylbenz(a)anthracene
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	3' deoxyribonucleoside 5' triphosphate
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetra-acetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
g	Gram
g.e.	Genome equivalent
G418	Geneticin, G418-sulphate
GJIC	Gap junctional intercellular communication
Gy	Gray
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma

HIV	Human immunodeficiency virus
HPV	Human papillomavirus
hr	Hour
Hz	Hertz
IgG	Immunoglobulin G
kb	Kilobase pairs
kD	KiloDalton
λ	Bacteriophage lambda
l	litre
LOH	Loss of heterozygosity
LTR	Long terminal repeat
M	Molar
mA	Milliamps
μg	Microgram
μl	Microlitre
μm	Micrometre
μM	Micromolar
mg	Milligram
ml	Millilitre
mm	Millimetre
mM	Millimolar
min	Minute
MoLV LTR	Moloney murine leukaemia virus 5' long terminal repeat
mRNA	Messenger ribonucleic acid
nA	Nanoamp
neo^R	Neomycin resistance
ng	Nanogram
NP-40	Nonidet P40 (Octylphenoxy polyethoxy ethanol)
nts	Nucleotides
8OHdG	8-hydroxy-deoxyguanosine
ONPG	*o*-Nitrophenyl-β-D-galactopyranoside
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PalF	Primary bovine fibroblasts
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
pg	picogram

PKC	Protein kinase C
PMSF	Phenylmethylsulfonyl fluoride
PV	Papillomavirus
RFLP	Restriction fragment length polymorphism
RhPV	Rhesus monkey papillomavirus
RNA	Ribonucleic acid
RNAse	Ribonuclease
ROS	Reactive oxygen species
rpm	Revolutions per minute
SCC	Squamous cell carcinoma
SDS	Sodium dodecyl sulphate
sec	Second
SSCP	Single strand conformation polymorphism
SV40	Simian virus 40
TAE	Tris-acetate / EDTA electrophoresis buffer
TBE	Tris-borate / EDTA electrophoresis buffer
TE	Tris / EDTA
TEMED	N,N,N',N'-Tetramethylethylenediamine
TPA	12-*O*-tetradecanoylphorbol-13-acetate
Tris	Tris(hydroxymethyl)aminomethane
Tween 20	Polyoxyethylene sorbitan monolaurate
U	Unit
UV	Ultraviolet
v-	Viral
V	Volts
v/v	Volume for volume
w/v	Weight for volume

CHAPTER 1

INTRODUCTION

Chapter 1

INTRODUCTION

1.1 The multifactorial nature of cancer

Carcinogenesis is a multistep process arising from complex interactions between both environmental and genetic factors (Pike & Forman, 1991; Henderson *et al.*, 1991 for reviews). This results in disruption of normal cellular control and, over time, ultimately leads to malignancy. This disease (perhaps more accurately described as collection of diseases as there are many different forms of cancer and type of tissue affected) is not confined to humans as many multicellular organisms can develop malignancies. However as at least one in five humans will develop cancer (Franks & Teich, 1991) it is perhaps not surprising that there is great impetus to extrapolate findings from cancer studies in other multicellular organisms to that of the human condition - the ultimate goal.

Evidence for the multistep nature of carcinogenesis has been gathered from pathological / clinical observation of human tumours, direct *in vivo* experimentation using animal model systems and more recently from molecular analysis. Epidemiological evidence includes the observation that incidence of the most common human cancers increases dramatically with age, analysis of this relationship suggesting that 3-7 sequential hits are required (Miller, 1980). The apparent clonal origin of malignant growth suggests that cancer arises through the mutation of a single progenitor cell followed by clonal expansion and, over time, acquisition of further mutations, fixed in turn by further clonal expansion (Nowell, 1976). Eventually the cell accumulates enough mutations to make it malignant and in some cases may mutate further allowing the malignant cells to invade and colonise distant organs i.e. to metastasise.

Evidence supporting a multi-hit hypothesis includes data generated from study of human cancers of the colon and the cervix. In colon cancers there is clear morphological evolution from benign tumours (adenomas) which are small and appear almost normal histologically, to malignant tumours which show a high level of cellular disorganisation (Sugarbaker *et al.*, 1985). These clinical observations have been supported by molecular analyses (Fearon & Vogelstein, 1990). A morphological evolution is also seen in cervical carcinomas where lesions are classed according to the degree of cellular disruption (Richart, 1968). Similar to findings in colorectal tumours, molecular analyses of cervical tumours has indicated activation of cellular genes during malignant progression (Riou *et al.*, 1985).

Evidence such as the above and work on animal models points to there being at least three discernible stages in the process of carcinogenesis - initiation, promotion and progression. Study of the mouse skin model (Hecker *et al.*, 1982) has been of particular use as the aetiology of tumour development in this system is well documented and can be controlled. This is in contrast to human tumour studies where, in most cases, causative agents have yet to be identified. In this classical mouse system initiation requires only a single exposure of skin to a carcinogen, such as 7,12-dimethylbenz(a)anthracene (DMBA). It appears to involve DNA damage, in this case carcinogenesis-specific point mutations in the c-Ha-*ras* gene (Quintanilla *et al.*, 1986). Promotion, which results in the appearance of benign tumours (papillomas), involves multiple exposure to chemical agents which do not damage DNA directly e.g. 12-*O*-tetradecanoylphorbol-13-acetate (TPA). This stage is now thought to consist of several steps and has been largely associated with epigenetic mechanisms such as the activation of protein kinase C (PKC), the direct membrane target protein receptor of phorbol esters (Castagna *et al.*, 1982). PKC represents a large gene family of isoenzymes which act as Ser / Thr-specific kinases and play a role in signal

transduction. This is a process by which cells respond to extracellular signals and although there are a number of different ways in which these signals are transduced, all pathways result in the regulation or modulation of gene expression (Hug & Sarre, 1993). It would be expected that disruption of signal transduction pathways would be of great consequence to cells, affecting normal cellular proliferation and differentiation.

A proportion of the mouse skin papillomas induced by initiating and promoting agents progress to malignant carcinomas. This third stage, progression, can be regarded as an open-ended process as malignant tumours may gain further mutations and become more aggressive. Data from the mouse skin model has suggested that tumour progression may be due to genetic events, as while genotoxic carcinogens are found to enhance conversion of benign papillomas to carcinomas, non-genotoxic tumour promoters do not (Hennings *et al.*, 1983).

1.1.1 Risk factors

These studies have successfully shown that in most cases cancer results from a complex interplay between environmental and genetic factors. Epidemiology has identified broad categories of risk factors. These include physical agents (such as UV light or X-rays), chemical agents (both carcinogens and mutagens) and infectious agents. Bacteria, fungi, parasitic animals and viruses have all been considered in this last category. Of these, viruses have been studied in greatest detail, although there are notable exceptions such as recent work implicating infection with the bacterium *Helicobacter pylori* as a co-factor in the development of stomach cancer (Forman *et al.*, 1990; Nomura *et al.*, 1991; Parsonnet *et al.*, 1991).

Other influences to be considered include the host immune status (Beverley, 1991). For example, deliberately immunosuppressed transplant patients and virally-immunosuppressed HIV (human immunodeficiency virus) patients have a substantially

increased risk of developing B cell lymphomas compared to immunocompetent patients (e.g. zur Hausen, 1991). HIV patients are also likely to develop otherwise rare tumours such as Kaposi's sarcoma at multiple sites (Fauci, 1988). Similarly, genetic predisposition to particular forms of cancer is also a risk factor (Ponder, 1990). Predisposition is most easily recognised in inherited cancer syndromes which include childhood tumours of the eye (retinoblastoma) and of the kidney (Wilms' tumours); these examples will be discussed more fully in the next section. Inherited cancer syndromes are thought to account for 1-2 % of cancer incidence (Ponder, 1990).

1.1.2 Oncogenes and tumour suppressor genes

Whereas many environmental factors have long been recognised to contribute to carcinogenesis it is only over the last 20 years, since the emergence of molecular biological techniques, that the identification and preliminary dissection of some of the genetic elements involved in cancer has been possible. Study of both *in vivo* and *in vitro* systems has implicated two classes of genes in cancer - oncogenes and tumour suppressor genes. Disruption of the normal activities of such genes appears to be a common and important event in the genesis of cancer (Bishop, 1991 for review).

Oncogenes are activated forms of cellular genes (proto-oncogenes) and were first described in rapidly transforming retroviruses (Cooper, 1982) where they were presumed to be viral in origin. However, further work showed that these oncogenic sequences were derived from normal non-transforming cellular DNA picked up by the virus sometime during its evolution (Bishop & Varmus, 1982). Study of retroviruses has allowed identification and subsequent characterisation of many cellular proto-oncogenes. Such genes encode products which are fundamental to normal cell growth and development. These include growth factors, growth factor receptors and proteins involved in signal transduction, DNA replication and transcription factors (Teich, 1991).

As such it is not difficult to imagine that disruption of the balanced regulation of such complex processes might result in cell transformation.

Proto-oncogenes can be converted to oncogenes by a variety of events including point mutation (Capon *et al.*, 1983), chromosomal translocation (Erikson *et al.*, 1983) and amplification (Alitalo *et al.*, 1983). Other events which can lead to activation include viral transduction (Neil *et al.*, 1984) and insertion of viral sequences near to proto-oncogenes. For example, examination of chicken bursal lymphomas induced by the retrovirus avian leukosis virus (ALV), showed that the proto-oncogene c-*myc* was activated in over 80 % of these neoplasms and that the most frequent site of ALV DNA integration in these lymphomas was between exons 1 and 2 of the c-*myc* gene (Hayward *et al.*, 1981). Activation of proto-oncogenes such as c-*ras* and c-*myc,* whether through direct mutation of their DNA or aberrant expression, has been found in a variety of human tumours (Alitalo & Schwab, 1986; Rodenhuis, 1992) indicating the critical role of these genes in normal cellular growth and proliferation.

The second class of cancer genes are the tumour suppressor genes (Stanbridge, 1985). These, in common with proto-oncogenes, are normal cellular genes although unlike the latter, tumour suppressor genes are largely thought to restrain cell proliferation. However, whereas oncogenes are thought to contribute to carcinogenesis by being activated by dominant mutations, tumour suppressors are thought to contribute to the oncogenic process mainly through loss of function. Early evidence for their existence came from somatic cell hybridisation studies which showed that fusion of tumour cells with normal cells invariably resulted in non-tumourigenic hybrids (Harris *et al.*, 1969; Harris, 1985). The observation that somatic cell hybrids had unstable karyotypes, frequently lost chromosomes and reverted to tumourigenicity when specific normal chromosomes were lost, led to the conclusion that tumour development may arise through loss of critical growth-regulating genes. As a result, tumour suppressor

research focused on the study of these key chromosomes for identification of candidate genes. This was achieved by transferring in single chromosomes using the technique of microcell transfer (Fournier & Ruddle, 1977). Initial studies showed that genes present on chromosome 11 were important in suppressing tumours in both HeLa cells, an established human cervical carcinoma line containing papillomavirus DNA, (Saxon *et al.*, 1986) and Wilms' tumour cells (Weissman *et al.*, 1987). Similar experiments have shown that other chromosomes are also associated with tumour suppression. However, as a chromosome contains many genes it was important to be able to 'home in' on intrachromosomal regions. Molecular biological techniques have been of particular use in this regard. Using a combination of methodologies including cytogenetic analyses and restriction fragment length polymorphisms (RFLPs) to implicate chromosomal regions of interest, probes can be identified which map to particular regions. Such probes are of use as the mutational events which lead to inactivation of tumour suppressor genes often involve large scale chromosomal rearrangements and deletions encompassing both the tumour suppressor gene and flanking chromosomal regions. If these flanking regions are heterozygous in untransformed cells but are consistently reduced to the homozygous state in transformed cells, this loss of heterozygosity (LOH) suggests the presence of a tumour suppressor gene nearby, the loss of which is important in that tumour biology (Hansen & Cavenee, 1987).

Retinoblastoma was the first cancer in which a tumour suppressor gene was identified using this combination of cytogenetic and molecular analyses. This disease, which results in the formation of ocular tumours, affects young children (Knudson, 1971). There are both sporadic and hereditary forms of this cancer which has a world-wide incidence of about 1 : 20,000. In approximately ten percent of cases tumours are heritable (Evans, 1993). It was postulated that development of this rare eye cancer, whether familial or sporadic in origin, was due to two successive lesions in the cell genome (Knudson, 1971). In sporadic retinoblastoma, i.e. in children with no family

history of the disease, Knudson argued that both mutations occur somatically in the same precursor retinal cell, whereas in familial retinoblastoma one of the mutations is acquired from a parent or results during gametogenesis, the second mutation occurring somatically. Karyotypic analyses of retinoblastoma tumours showed that a proportion of these tumours had a deletion of chromosome 13q14, suggesting that deletion or inactivation of a gene at this site was important in the aetiology of this cancer (Godbout *et al.*, 1983; Sparkes *et al.*, 1983). These findings were instrumental in the ultimate identification and isolation of the retinoblastoma (RB) gene (Friend *et al.*, 1986; Lee *et al.*, 1987). Further studies have shown that the protein encoded by this gene (p105Rb) acts as a negative regulator of cellular proliferation by inhibiting the activity of the E2F family of transcription factors. E2F binding sites are present in a variety of genes involved in initiating DNA synthesis (Lam & La Thangue, 1994 for review). It has also been proposed that p105Rb may play a role in differentiation (Chen *et al.*, 1989). Apart from its central involvement in development of retinoblastomas, mutation of the RB gene has been observed in osteosarcomas, soft tissue sarcomas and carcinomas of the breast, lung and bladder (Weinberg, 1992).

Further evidence for the existence of tumour suppressor genes was provided by analysis of Wilms' tumour, another childhood cancer. This malignancy has an incidence of approximately 1 : 10,000 and accounts for 85% of all childhood kidney cancer (Matsungaga, 1981). As found for retinoblastoma, there are both sporadic and hereditary forms of this cancer although hereditary cases are not as frequent. Studies indicated that the tumour suppressor gene locus involved in Wilms' tumour mapped to chromosome 11p13 (Koufos *et al.*, 1984). However results from other studies suggest that other loci are involved in the pathogenesis of this disease. This includes evidence for loss of heterozygosity at 11p15 without involvement of 11p13 (Mannens *et al.*, 1988; Reeve *et al.*, 1989). The genetics of Wilms' tumour would therefore appear to be

far more complex than those of retinoblastoma although both involve loss or inactivation of tumour suppressor genes.

The importance of tumour suppressor genes is perhaps best exemplified by the tumour suppressor gene p53. The importance of this gene's functional activity is indicated by the observation that p53 is mutated at high frequency in the development of many sporadic human cancers (Harris & Hollstein, 1993). Similarly, germline mutations in the p53 gene are associated with Li-Fraumeni syndrome, a disorder that predisposes individuals to developing multiple forms of cancer at a young age including breast carcinoma, soft tissue sarcoma, osteosarcoma and leukaemia (Malkin *et al.*, 1990; Malkin, 1993). Like the product of the RB gene, the p53 protein appears to play an important role in negatively regulating progress through the cell cycle. p53 is a sequence-specific DNA-binding protein that functions as a transcription factor. The transcriptional activity of p53 is essential to its role as a tumour suppressor gene (Pietenpol *et al.*, 1994) although it must be considered that novel biological properties of the p53 protein may also be involved (Picksley & Lane, 1994).

It has been suggested that in some cases coupled inactivation of p53 and p105Rb proteins may be important for transformation. For example, and of particular significance to this thesis, several DNA tumour viruses including certain papillomaviruses, encode proteins that bind to and inactivate both p53 and p105Rb. Such inactivation is thought to be critical for transformation by these viral proteins both *in vivo* and *in vitro* (Van Dyke, 1994 for review). This will be discussed more fully later (Chapter 1.2.2).

1.1.3 Viruses

It is estimated that viruses have an aetiological role in approximately 15 percent of human cancers world-wide (zur Hausen, 1991). Although essential, viral infection

alone is not sufficient for development of these cancers and other factors are required. Viral contribution can either be direct or indirect. Indirect mechanisms include induction of immunosuppression which leaves the host prone to developing diseases, including cancers, not directly related to the suppressing virus. A classic example of this is seen in patients infected with human immunodeficiency virus (HIV) who show a greatly increased risk of developing specific tumours, particularly Kaposi sarcomas and B cell lymphomas (Fauci, 1988). As regards direct mechanisms of viral contribution to carcinogenesis the most common examples are cancers of the liver and cervix which together account for about 80 % of known virus-induced tumours (zur Hausen, 1991). Of these, both epidemiological (Trichopoulos *et al.*, 1976) and DNA analyses of liver tumours (Nagaya *et al.*, 1987) have linked hepatitis B virus (HBV) infection to the development of hepatocellular carcinoma (HCC). Hepatitis C virus (HCV) infection may also play a role in the development of this disease (Zavitsanos *et al.*, 1992). Similar studies have shown an association between certain types of human papillomavirus (HPV) and cervical cancer (zur Hausen, 1976; 1989a). It is certain members of the latter family of viruses - the papillomaviruses - which are of direct relevance to this thesis and which are therefore considered in greater detail below.

1.2 Papillomaviruses

Papillomaviruses are small DNA viruses which induce tumours in the skin and mucosa of a wide variety of animals including man. Although such tumours are predominantly benign and often regress spontaneously, some can progress to malignancy. The general widespread presence of papillomaviruses within human populations and the observed malignant conversion of some of these lesions has accorded these viruses great clinical importance. As such this has generated much interest in the study of their genetic make-up and transformation potential.

Although papillomaviruses show great specificity with regard not only to host species but also anatomical site, many types show similar genomic organisation (Figure 1.1). In all cases the viral genome is closed circular double-stranded DNA of approximately 8 kb (kilobase) pairs in size, with all the major open reading frames (ORFs) overlapping to a large extent and located on one strand only. These ORFs show notable conservation of position and sequence homology between different viral types. Viral transcription is unidirectional with viral mRNAs being generated by complex splicing mechanisms, some of which encode fusion polypeptides. Functions have been assigned to several ORFs by means of deletion-insertion mutagenesis of sub-genomic DNA fragments and cDNAs (Campo & Jarrett, 1987 for review). As such the viral genome can be roughly divided into three functionally distinct regions. These consist of an upstream regulatory region, the long control region (LCR), an early region which encodes proteins that control replication and transcription of viral DNA and proteins involved in cellular transformation, and a late region that codes for viral structural proteins.

1.2.1 Cottontail rabbit papillomavirus

Cottontail rabbit (Shope) papillomavirus (CRPV) was the first model for studying viral oncogenesis in mammals (Shope, 1933; Rous & Beard, 1935). *In vivo* this virus induces benign skin papillomas which usually regress, however these papillomas can progress to squamous skin carcinomas in up to 25 % of rabbits (Kidd & Rous, 1940). Study of this virus has proved useful for, and pertinent to, analysis of the multifactorial nature of papillomavirus-associated carcinogenesis as co-factors are involved in malignant progression. The important role played by environmental co-factors is demonstrated by the limited geographical incidence of CRPV infection, as although these rabbits are found throughout the United States it is only animals found in regions bordering the Mississippi river which develop CRPV-induced papillomatosis (Shope, 1933).

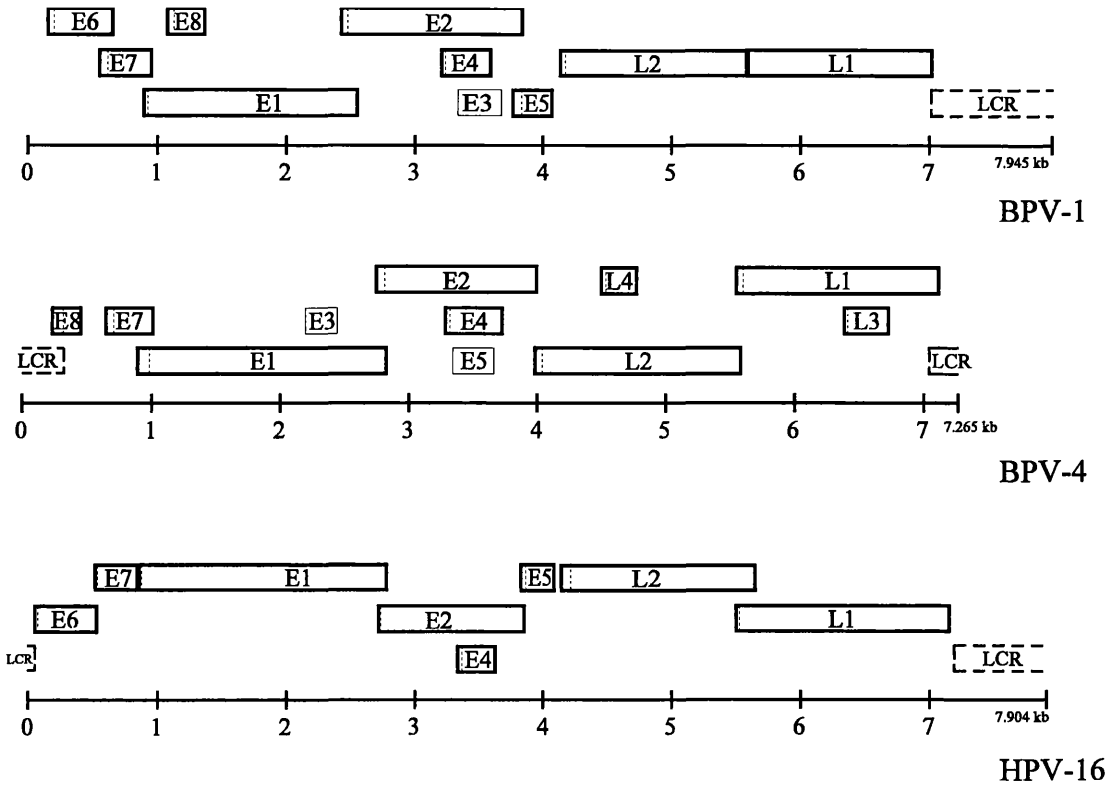


Figure 1.1 Genomic organisation of BPV-1, BPV-4 and HPV-16. In all cases the viral genome is represented as linear and the boxes represent ORFs. The early ORFS are designated E, and the late ORFs L. Dashed lines represent the long control region (LCR). The first ATG codon in an ORF is indicated by a vertical dashed line; ORFs without an ATG codon are represented as thin lined boxes.

The ability of CRPV to induce tumours in experimental animals has allowed examination of papillomavirus-induced cellular transformation. Furthermore this was the first papillomavirus shown to synergise with chemical carcinogens resulting in malignant transformation of CRPV-induced lesions (Rous & Beard, 1935; Rous & Friedewald, 1941; 1944).

Examination of CRPV transforming functions *in vitro* has demonstrated that this virus encodes at least three transforming proteins; two encoded by the E6 ORF and one by the E7 ORF. Although the functions of these proteins have yet to be elucidated (Meyers *et al.*, 1992) the CRPV E7 protein has been shown to bind to the retinoblastoma product p105Rb (Haskell *et al.*, 1993). Binding of the HPV-16 E7 oncoprotein to this tumour suppressor protein is thought to be important to HPV-16-mediated cellular transformation (Dyson *et al.*, 1989 and Chapter 1.2.2 below). Similarly, binding of the CRPV E7 protein to p105Rb may be involved in CRPV-mediated cellular transformation.

1.2.2 Human papillomaviruses

To date molecular cloning techniques have allowed the identification of over 65 different types of human papillomaviruses (HPVs) (Van Ranst *et al.*, 1992). These can be naturally divided into 2 major sub-groups initially classified by tissue type infected-namely cutaneous and mucosal. Approximately equal numbers have been assigned to each class. Human papillomaviruses cause a variety of lesions in distinct anatomical sites. These range from benign common warts (verrucae vulgares), to the progression of some genital flat warts to malignancy. However the degree of site specificity varies among types. For example HPV-13 is associated solely with oral lesions (Pfister *et al.*, 1983) whereas HPV-11 has been found in both laryngeal and ano-genital infections (Gissmann *et al.*, 1983).

Many of the cutaneous HPVs have been isolated almost exclusively from patients suffering from epidermodysplasia verruciformis (EV). EV is a rare life-long disease in which the patient suffers continuous multiple-type papillomavirus infection (Orth, 1987). Some of these virally-induced lesions can progress to malignancy over time, this long 'incubation' being indicative of the requirement of co-factors for full transformation. The exact nature of this disease has yet to be elucidated. Lutzner (1978) suggested that the high frequency of parental consanguinity in EV patients and the observed familial occurrence of the disease pointed to the importance of genetic factors in development of this disease. Pedigree analyses of such families suggested that EV is an autosomal recessive disorder with both sexes being equally affected. However, another mode of EV transmission has also been suggested by the study of a family in which only males were affected (Androphy *et al.*, 1985). Pedigree analysis of this family suggested an X-linked, recessive inheritance of the disease (Androphy *et al.*, 1985). EV patients have normal immunoglobulin levels (Lutzner, 1978) however most have impaired cell-mediated immunity (Jablonska & Orth, 1985). It is thought that this immunologic defect prevents patients mounting a full response to papillomavirus infection (Jablonska & Orth, 1985).

However, with regard to percentage of general population affected and thereafter progressing to malignant lesions, the mucosal HPVs, particularly those infecting the lower genital tract, pose a greater threat than cutaneous HPVs. As a consequence mucosal HPVs have attracted more medical and scientific interest.

HPV infections of the genital tract are now recognised to be among the most prevalent sexually transmitted diseases (U.S. Dept. of Health and Human Services as cited by Shah & Howley, 1990). Infection often results in production of genital warts. These can be either exophytic warts (condylomata acuminata) or the more recently recognised but related flat wart (condyloma planum). In males, exophytic condylomas occur on the

penis, around the anus, on the perineum and, rarely, on the scrotum. In females, this type of wart is found at the entrance of the vagina, on the vulva, around the anus, on the perineum and, very occasionally, on the uterine cervix where flat warts are found. Lesions from both types of warts can be identified cytologically and histologically. Although most regress spontaneously or respond to treatment, some recur. Recurrence has been correlated to presence of virus in normal epithelium adjacent to the lesion (Ferenczy *et al.*, 1985). The role of the immune system in such regression / recurrence is not well understood. Evidence to date suggests that human papillomavirus infection is controlled by the host's cellular immune response and that the degree of this control differs between viral types (Frazer & Tindle, 1992).

Some genital warts, most often flat warts, progress to malignancy. An association between papillomaviruses and genital cancer was suggested in the 1970s (zur Hausen, 1976). The most common, and thus best studied, genital cancer is squamous cell carcinoma (SCC) of the uterine cervix. Molecular studies examining the presence and expression of HPV genomes in invasive cancers have provided data supporting a viral contribution to the development of this disease. These studies have been complemented by findings from epidemiological, clinical and pathological examination of cervical neoplasms.

Epidemiological studies have demonstrated that infection by genital HPVs and cancer of the cervix both share characteristics of sexually transmitted diseases. For example cervical cancer is not seen in nuns and other virgins whereas there is a strong link between the cancer and early onset of sexual activity, multiple sexual partners and multiple kinds of infection (Brinton, 1992). Lesions of the cervical epithelium are termed cervical intraepithelial neoplasia (CIN) and are classified as follows:

CIN-1 - mild dysplasia

CIN-2 - moderate dysplasia

CIN-3 - severe dysplasia.

Clinical and pathological evidence suggesting an association between HPVs and cervical cancer included the fact that the flat cervical warts seen in CIN-1 are often indistinguishable from those resulting from papillomavirus infection. This argument was given further credence when analyses of many primary carcinomas, metastatic tumours and cell lines derived from cervical tumours were shown to contain HPVs 16 and 18 or related types of HPV DNA (Lancaster *et al.*, 1986; Ostrow *et al.*, 1982). As estimates for the annual world-wide incidence of cervical carcinoma currently stand at 500,000 with about 45% mortality, even with medical intervention (Broker & Botchan, 1986), elucidation of papillomavirus / host interaction is of prime concern for both therapeutic and ultimately prophylactic treatments.

Although almost all types of genital tract HPVs are found in mild lesions and sub-clinical infections, it is only types 16 and 18 which predominate in invasive cancers. In most malignant tumours the viral DNA is integrated and actively transcribed. The pattern of viral transcription is less uniform in severe dysplasia than mild lesions. In the former, viral transcripts originate from two specific open reading frames, E6 and E7 (Schwarz *et al.*, 1985), whereas all ORFs are expressed in mild lesions (Shah & Howley, 1990). *In vitro* experiments (Phelps *et al.*, 1988; Storey *et al.*, 1988; Watanabe *et al.*, 1989) have demonstrated that the transforming proteins of HPVs are encoded by the E6 / E7 ORFs. These viral proteins have also been shown to inactivate cellular tumour suppressor gene proteins. The HPV-16 E7 protein binds to the retinoblastoma protein p105Rb (Dyson *et al.*, 1989) and the E6 oncoprotein has been shown to bind to and promote degradation of the p53 protein (Werness *et al.*, 1990; Scheffner *et al.*, 1990). As both p53 and p105Rb are thought to act as cellular 'policemen', normally preventing uncontrolled cellular proliferation, disruption of tumour suppressor protein

functions may well be critical to the transforming activity of the E6 and E7 ORFs and to transformation in general.

In malignant tumours containing viral DNA, integration is specific with regard to the virus but not to intrachromosomal locations. Linearisation of the viral genome often occurs within the E1 / E2 region, thus preventing expression of the E2 ORF. In HPVs-16 and 18, E2 proteins are known to repress expression from the promoter regulating expression of the E6 / E7 ORFs (Cripe *et al.*, 1987; Thierry & Yaniv, 1987). Thus inactivation of E2 may allow unregulated expression of the E6 / E7 ORFs. Constitutive expression of these genes is seen in practically all HPV-positive cancers and HPV-containing cell lines. The E6 / E7 genes of the high-risk, but not low-risk HPVs ('risk' refers to an association with malignant conversion) are capable of immortalising keratinocytes *in vitro* (Dürst *et al.*, 1987; Kaur & McDougall, 1988; Schlegel *et al.*, 1988). Although immortalised, these cells are not malignant suggesting that co-factors are required for progression to carcinomas. That addition of activated *ras* to HPV-16-immortalised human cervical cells results in full transformation, as assayed in nude mice, is further evidence that progression to cervical cancer is multistep and multifactorial (DiPaolo *et al.*, 1989).

1.2.3 Bovine papillomaviruses

The clinical importance of HPV-induced lesions has ensured a healthy interest in the study of papillomaviruses. Due to difficulties in finding a suitable *in vitro* system for study of human papillomaviruses, early work involved analyses of their animal counterparts. The bovine papillomaviruses (BPVs) have been of great experimental use although recent work (Ostrow *et al.*, 1990; Schneider *et al.*, 1991) suggests that rhesus monkey papillomavirus (RhPV) may provide a better model for human genital virus types as RhPV infects the same type of tissue and shows similar *in vitro* transformation characteristics to HPV-16.

Bovine papillomaviruses share a number of factors in common with human papillomaviruses;

(a) they show great specificity with regard to site of infection

(b) certain viral types are associated with malignant progression

(c) such progression requires interaction between viral, cellular and environmental co-factors

An additional advantage in studying animal papillomaviruses is that, unlike the human viruses, direct *in vivo* experimentation is feasible.

To date six bovine papillomaviruses have been identified. These can be divided into two sub-groups using such criteria as degree of genome homology, site specificity and clinical manifestation (Campo *et al.*, 1980; 1981; Jarrett *et al.*, 1984). Sub-group A viruses include BPVs-1, 2 and 5, which cause fibropapillomas. Sub-group B includes types 3, 4 and 6 which are purely epitheliotropic. Data from both immunological and Southern hybridisation studies show that there is no cross-reactivity / cross-homology between the two sub-groups (Campo & Jarrett, 1987).

The ability of the BPVs causing fibropapillomas to transform somatic cells *in vitro*, and also induce tumours in experimental animals has been of great use in assigning functions to particular viral ORFs. In this regard the BPV-1 genome has been studied in greatest detail. Transforming activity has been localised to a region covering 69% of the genome. Further genetic dissection has identified the relevant genes as E5 and E6 (Yang *et al.*, 1985; Groff & Lancaster, 1986; Schiller *et al.*, 1986). The E5 oncoprotein is the major transforming protein and has been shown to induce cellular DNA synthesis and activate the receptors for epidermal growth factor (EGF), colony stimulating factor (CSF-1) (Martin *et al.*, 1989) and platelet derived growth factor (PDGF) (Petti *et al.*, 1991). BPV-1 E5 protein has also been shown to bind to a 16 kD (kiloDalton) cellular

protein called ductin. Ductin is a component of gap junctions and also of the vacuolar proton channel-forming ATPase (Finbow *et al.*, 1991; Goldstein *et al.*, 1992). The second oncoprotein of BPV-1, the E6 protein, contains cys-x-x-cys zinc-binding domains (Barbosa *et al.*, 1989) and has transcriptional transactivator activity (Lamberti *et al.*, 1990). This gene's contribution to the transformation process could be via deregulation of cellular genes.

Although the BPV-1 E7 protein contains a zinc-binding domain and such binding has been shown to be important for the transforming activities of HPVs 16 and 18 E7 proteins (Watanabe *et al.*, 1990; McIntyre *et al.*, 1993), BPV-1 E7 protein does not appear to have a major role in cell transformation. Rather, this protein is involved in the control of viral DNA replication and viral genome copy number (Lusky & Botchan, 1985).

The function of the BPV-1 E8 gene has yet to be established. However, as found for all papillomaviruses, the structural proteins of BPV-1 are encoded by the L1 and L2 ORFs. The major capsid protein is encoded by L1 and the minor capsid protein by L2 (Favre *et al.*, 1975).

1.3 Bovine papillomavirus associated cancers *in vivo*

Although BPV-1 has been widely used to study the *in vitro* biology of papillomaviruses, this particular virus is not associated with the development of cancer in its natural host *in vivo*. Of the two BPVs which are, BPV-2 is associated with bladder cancer (Campo *et al.*, 1992) and BPV-4 with cancer of the upper alimentary canal (Jarrett *et al.*, 1978; Campo *et al.*, 1980). In both diseases ingestion of bracken fern plays a pivotal role in malignant progression. The fern is documented as containing mutagens, carcinogens and immunosuppressants (Evans, I.A. *et al.*, 1982; Evans, W.C. *et al.*, 1982). In the case of BPV-2, an epidemiological role for bracken was established as cattle fed

bracken under experimental conditions developed bladder tumours histologically indistinguishable from those found naturally (Price & Pamukcu, 1968; Pamukcu *et al.*, 1976). The viral factor was confirmed by reciprocal experiments inducing bladder cancer by injecting extracts of bovine warts into the organ (Olson *et al.*, 1959); conversely production of skin and vaginal lesions were seen in animals treated with bladder tumour extracts (Olson *et al.*, 1965). However it was not until more recently that the identity of the viral type was confirmed in a series of experiments which also examined the synergism between bracken fern and the virus. In these studies bracken-fed cattle developed cutaneous warts at the site of BPV-2 injection. They were also found to be immunosuppressed and developed bladder cancers indistinguishable from those found naturally (Campo & Jarrett, 1986; Campo *et al.*, 1992). 'Bracken-free' animals developed skin warts but not bladder cancers. Interestingly some of the bracken-fed control animals developed bladder cancers even though they had not been injected with BPV-2. Further analysis showed that BPV DNA was present in these cancers, suggesting that bracken-induced immunosuppression of the host had allowed reactivation of latent virus (Campo *et al.*, 1994a). Sixty-nine percent of the experimentally induced bladder cancers analysed were found to contain BPV-2 DNA as compared to 46% of field cases which were positive for BPV-2 DNA. The above results support roles for both virus and chemical factors in urinary bladder carcinogenesis.

Papillomavirus and bracken fern have also been identified as co-factors for cancer of the upper alimentary canal of cattle (Jarrett *et al.*, 1978). BPV-4, the viral type associated with this disease, induces papillomas in the upper alimentary canal (Jarrett *et al.*, 1978; Campo *et al.*, 1980). Although most of these lesions are benign and regress spontaneously, some can progress to malignant carcinomas in cattle feeding on bracken fern. These cancers are often accompanied by adenomas and adenocarcinomas of the lower bowel and urinary bladder cancer. The observation that bracken-grazing cattle

show more widespread and persistent papillomatosis than animals who have a bracken-free diet has been reproduced in controlled *in vivo* experiments (Campo & Jarrett, 1987; Campo *et al.*, 1994b). Of six animals injected in the palate with BPV-4 and kept on a diet of bracken, three developed cancer of the lower bowel and two of these developed cancer of the upper alimentary canal (Campo, 1987; Campo *et al.*, 1994b). Cancer was not observed in either 'virus-only' or 'bracken-only' control animals thus indicating pivotal roles for both virus and fern in malignant progression.

1.4 Bovine papillomavirus type 4

Of the bovine papillomaviruses, BPV-4 has proved a useful model for neoplasia of the uterine cervix in humans. It is both associated with a naturally occurring cancer, providing *in vivo* and *in vitro* systems for experimentation, and is also purely epitheliotropic (as seen for the cervical cancer associated HPVs). One major difference between BPV-4 and HPVs-16 and 18 is that, unlike the majority of human cervical cancers, BPV-4 DNA is rarely found in carcinomas. Whilst not negating its interest to cervical cancer studies this fact does raise the question as to whether BPV and HPV transform cells by different mechanisms. Ongoing studies of viral / cellular interactions in both systems may well clarify this point.

Apart from its obvious usefulness as an *in vivo* model system for papillomavirus-associated carcinogenesis, the transformation biology of BPV-4 *in vitro* is also of interest.

1.4.1 Transformation biology of BPV-4 in established cells

The earliest attempts to study the transforming properties of BPV-4 were carried out in established mouse fibroblast cells. The virus was found to transform both NIH3T3 and a C127 sub-line (C127sc) *in vitro*. Such cells were found to be tumourigenic in nude mice (Campo & Spandidos, 1983; Smith & Campo, 1988). In the C127sc study a

number of experimental parameters were examined. Results showed that, as *in vivo*, several factors contributed to viral DNA-dependent morphological transformation. The optimal *in vitro* conditions included high serum concentrations, presence of the tumour promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA), and linearisation of the viral DNA within the E1 ORF, with the additional removal of nucleotides from the 3' end of this ORF (Smith & Campo, 1988). It was proposed that the higher frequency of focus induction seen with high serum levels was due to the presence of stimulatory growth factors allowing expression of the transformed phenotype. The role the phorbol ester TPA plays in encouraging progression to the fully transformed state in the above experimental system is more difficult to assess as this chemical causes many cellular changes including activation of PKC and disruption of intercellular communication (Murray & Fitzgerald, 1979; Castagna *et al.*, 1982). It was proposed that linearisation or fragmentation of the BPV-4 DNA may have allowed full transformation of cells by disrupting viral repressor functions. The E2 ORF, which overlaps the 3' end of the E1 ORF, encodes a protein that acts as a transcriptional regulator and several positive and negative control elements have now been identified in the BPV-4 LCR (Jackson & Campo, 1991; 1995; McCaffery & Jackson, 1994).

This early work on established mouse lines defined the BPV-4 E7 and E8 ORFs as the transforming genes, as a viral fragment containing this region induced focus formation (Smith & Campo, 1988). Amplification and rearrangement of specific host sequences were also observed subsequent to transfection with BPV-4 DNA (Smith & Campo, 1988; 1989). It was also noted that viral DNA was absent in the majority of fully transformed cell lines. This mirrored findings *in vivo* where presence of the virus, although found in benign papillomas, is only rarely detected in carcinomas (Campo *et al.*, 1985). This suggested that although required for induction of the papillomas, presence of BPV-4 is not necessary for progression to or maintenance of the transformed state.

1.4.2 BPV-4 gene expression

Numerous BPV-4 transcripts have been identified in both papillomas and virally-transformed established cells (Smith *et al.*, 1986; Stamps & Campo, 1988; Campo *et al.*, 1994c). BPV-4 mRNAs, in common with other papillomavirus transcripts, are generated by complex splicing mechanisms. The viral transcripts fall into two classes. Those encoded by the early ORFs terminate at the polyadenylation site at nt 4004 of the BPV-4 genome and are detectable both in productive papillomas and in transformed cells *in vitro*. Transcripts representing the late ORFs, the second class, terminate at either of the polyadenylation sites at nts 7155 and 7191 and are only found in papillomas.

The BPV-4 E1 ORF is transcribed into a series of mRNAs. The functions of this ORF are not yet characterised and it is not yet known which, if any, of the mRNAs encodes the functional E1 protein.

The E2 ORF is transcribed into a mRNA identified as Q. Q represents only the 3' half of the E2 ORF and transcripts corresponding to the full ORF have not yet been identified. As found for BPV-1, the BPV-4 E2 protein is involved in both positive and negative regulation of viral transcription through interaction with regulatory elements in the LCR (Jackson & Campo, 1991; 1995).

The E4 ORF is transcribed into several RNA species. Two of these, the 7E11 and 1.6 kb transcripts, encode a potential E1-E4 fusion peptide, although the region of E1 involved differs between these two transcripts. The 7E11 transcript is most likely to encode the E1-E4 fusion peptide described for HPV-1 (Doorbar *et al.*, 1988). The HPV-1 E1-E4 fusion protein interferes with cytokeratin assembly (Doorbar *et al.*, 1991); this may upset normal cellular differentiation and favour production of virion

progeny (Doorbar, 1991). Although functions of BPV-4 E4 have yet to be elucidated they may be similar to those of HPV E4. As found for HPV-1 E4 (Breitburd *et al.*, 1987) the majority of BPV-4 E4 protein is found in the differentiating layers of papillomas, coincident with the vegetative replication of viral DNA (Campo *et al.*, 1994c).

The E7 and E8 ORFs are transcribed into a 3.0 kb RNA. The proteins encoded by these genes are instrumental in cellular transformation and their proposed mechanisms of action will be discussed in greater detail later. Both the E3 and E5 ORFs lack an initiation codon and these ORFs may well be non-functional.

The structural proteins of BPV-4 are encoded by the L1 and L2 ORFs. Two related transcripts have been identified; one of 2.8 kb which encodes L1 and the second of 4.2 kb which has the capacity to encode both L1 and L2. These RNAs are found in papillomas but not in transformed cells, a finding consistent with the lack of productive vegetative replication of viral DNA *in vitro*. Although the L3 and L4 ORFs present contain ATG start codons their function, if any, is not as yet known.

1.4.3 Transformation biology of BPV-4 in primary cells

More recent work has looked at the biology of the virus in primary bovine fibroblasts (PalF cells) derived from foetal palate (Jaggar *et al.*, 1990; Pennie *et al.*, 1993). This approach has a number of obvious advantages; i.e. such cells are non-established, come from the natural host and although fibroblastic (keratinocytes are the natural target cell), are derived from the natural site of infection (Jarrett, 1985).

BPV-4 can morphologically transform primary fibroblasts only in co-operation with an activated *ras* gene (Jaggar *et al.*, 1990). Such cells have an extended life span but are not immortal and although capable of anchorage-independent growth are not

tumourigenic in nude mice (Jaggar *et al.*, 1990). This indicates that additional factors are required for full transformation. Thus this system appears to mimic the *in vivo* situation where the virus requires co-factors for development of the upper alimentary canal carcinomas (Jarrett *et al.*, 1978).

As suggested by earlier work in established cells, the transforming functions of BPV-4 in PalF cells have been mapped to the E7, E8 ORFs (Jaggar *et al.*, 1990). BPV-4 E7 protein is homologous to the HPV-16 E7 protein (Jaggar *et al.*, 1990; Jackson *et al.*, 1991). It has two cys-x-x-cys zinc-binding motifs and a potential p105Rb binding domain (Jaggar *et al.*, 1990; G.J. Grindlay, personal communication). These domains are important for the transforming activities of the HPV-16 E7 protein (Edmonds & Vousden, 1989; Chesters *et al.*, 1990). Although yet to be studied in detail, BPV-4 E7 may well have similar functions to the HPV-16 protein. As deletion of the 3' terminal third of the E7 ORF abolishes *in vitro* transformation (Jaggar *et al.*, 1990) evidence points to this ORF being the major transforming gene of BPV-4.

A number of observations suggest that the BPV-4 E8 ORF may also contribute to transforming activity although its exact role has yet to be determined. Immunohistochemistry of alimentary canal papillomas has shown E8 to be expressed in the basal and suprabasal layers where little or no vegetative viral DNA replication takes place (R. Anderson & M.S. Campo, unpublished results). In conjunction with the observation that BPV-4 E8 protein is only detected in young papillomas, this marks E8 as an early protein. BPV-4 E8 protein encodes a small hydrophobic polypeptide similar in length (42 residues) and hydrophobicity to E5, the major oncoprotein of BPV-1 (Jackson *et al.*, 1991). However, although BPV-1 E5 protein transforms cells *in vitro* (Schiller *et al.*, 1986) BPV-4 E8 does not, and over-expression of this ORF in the absence of other BPV-4 genes is lethal in primary cells (Jaggar *et al.*, 1990; Pennie *et al.*, 1993). Although not recognised as a transforming gene *per se*, E8 contributes to

the process by conferring anchorage independence (Pennie *et al.*, 1993). In addition, both BPV-1 and BPV-4 transformed cells lack gap junctional intercellular communication (W.D. Pennie, 1992). BPV-1 E5 oncoprotein has been shown to bind the 16 kD ductin component of gap junctions (Goldstein & Schlegel, 1990). Recent work, using a cell-free *in vitro* assay system, has shown that BPV-4 E8 protein can also bind ductin (A. Faccini, personal communication).

BPV-4 and the other sub-group B viruses BPV-3 and BPV-6, lack a recognisable E6 ORF (Jackson *et al.*, 1991). This gene has been identified as the second major oncogene of many papillomaviruses including both BPV-1 and HPV-16 (Schiller *et al.*, 1984; Yang *et al.*, 1985; Münger *et al.*, 1989; Watanabe *et al.*, 1989). It binds zinc through cys-x-x-cys repeats (Barbosa *et al.*, 1989), has been shown to be a transcriptional activator (Lamberti *et al.*, 1990), and also to bind to and degrade one of the cell's negative regulators, p53 (Werness *et al.*, 1990). This degradation is through the ubiquitin pathway (Scheffner *et al.*, 1990). That BPV-4 does not possess an E6 ORF raises the question whether E6-like functions are necessary for BPV-4 transformation. Recent work has shown that exogenous HPV-16 E6 can co-operate with BPV-4 to immortalise BPV-4-transformed cells (Pennie *et al.*, 1993). However such cells remain non-tumourigenic. Current studies are addressing whether other BPV-4 proteins complex with p53 protein, or alternatively whether BPV-4 transforming activity can bypass the negative regulatory effects of this protein.

1.5 Environmental co-factors in BPV-4-associated carcinogenesis

Elucidation of the interplay among viral, host and environmental co-factors in carcinogenesis is of paramount importance for both treatment and prevention of the disease. The co-factors in BPV-4-associated carcinogenesis have been identified as including the mutagens, carcinogens and immunosuppressants present in bracken fern. One of the major mutagens present in bracken is the flavonoid quercetin (Evans W.C.

et al., 1982). *In vitro* studies have shown this compound can bind DNA and induces a variety of genetic lesions in both bacterial and mammalian cells (Jackson *et al.*, 1993 for review). Quercetin can also induce clastogenic damage (Ishidate, 1988). This last observation is of particular significance as bracken-grazing cattle show a wide variety of cytogenetic abnormalities (Moura *et al.*, 1988). Although not a carcinogen itself (Morino *et al.*, 1982; Hirono *et al.*, 1987), quercetin can act as an initiator in a two stage transformation assay in mammalian cells *in vitro* (Sakai *et al.*, 1990). In addition, quercetin has been shown to interfere with phosphorylation / dephosphorylation mechanisms (Van Wart-Hood *et al.*, 1989; Matter *et al.*, 1992). In light of these early studies the possible role of quercetin as an initiating agent in full transformation of primary bovine fibroblasts has been undertaken in this laboratory.

BPV-4 transforms PalF cells only in co-operation with activated *ras*. Although morphologically transformed these cells are not immortal and are non-tumourigenic in nude mice. Addition of exogenous HPV-16 E6 - one of the major transforming genes of HPV-16 but lacking in BPV-4 - to this transfection cocktail confers immortality to BPV-4-transformed cells. However they remain non-tumourigenic indicating additional events are needed for full transformation. Pre-treatment of cells with quercetin before subsequent transfection with BPV-4 and *ras* resulted in a much more aggressive transformed morphology and such cells were found to be tumourigenic in nude mice (Pennie & Campo, 1992). These initial results strongly support the hypothesis that quercetin acts as a co-carcinogen in the naturally occurring alimentary canal cancers of bracken-grazing cattle.

1.6 Aims

Previous work from this laboratory has provided a continuous source of information on the transformation biology of BPV-4 in both established and primary cells. Building on this body of information, the specific aims of the work presented in this thesis were:

(a) to investigate whether the routine loss of viral DNA from BPV-4-transfected established cells was due to mutation and / or rearrangement of the BPV-4 genome

(b) to determine whether transfection with BPV-4 DNA resulted in amplification of host sequences

and finally

(c) to examine the synergistic relationship between BPV-4 and the flavonoid quercetin in primary bovine fibroblasts and to elucidate possible mechanisms of quercetin action, whether these be genetic, epigenetic or indeed a combination of the two.

CHAPTER 2

MATERIALS AND METHODS

Chapter 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Antibodies

Sigma Chemical Co., Ltd., Poole, Dorset, England.

Anti-mouse IgG alkaline phosphatase conjugate

Upstate Biotechnology Inc., New York, USA

Mouse monoclonal anti-phosphotyrosine (monoclonal IgG2bk)

2.1.2 Bacterial Hosts

Gibco Europe Life Technologies Ltd., Paisley, Scotland.

E. coli DH5α competent cells

2.1.3 Cells

Established Cells

C127 cells. This established mouse fibroblast line was maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal calf serum, 2 mM glutamine, 1 mM pyruvate, 0.375% sodium bicarbonate, 37.5 μg / ml penicillin, 50 μg / ml streptomycin and 2.5 μg / ml amphotericin B.

C127sc. This is a sub-line of C127 and contains 5.2 kb of the BPV-1 genome (Smith *et al.*, 1993). This line was maintained in DMEM supplemented with 10% foetal calf serum, 2 mM glutamine, 1 mM pyruvate, 0.375% sodium bicarbonate, 37.5 μg / ml penicillin, 50 μg / ml streptomycin and 2.5 μg / ml amphotericin B.

C4Ta2a. This origin of this line is detailed in Smith & Campo (1988) and is also described in Chapter 3.2.1.1. In brief, this line is a secondary transfectant line which resulted from transfection of C127sc cells with BPV-4 DNA in the presence of the tumour promoter TPA. This line was maintained in DMEM supplemented with 10% foetal calf serum, 2 mM glutamine, 1 mM pyruvate, 0.375% sodium bicarbonate, 37.5 μg / ml penicillin, 50 μg / ml streptomycin and 2.5 μg / ml amphotericin B.

ID14 cells originated from transformation of C127 cells by BPV-1 virions (Law *et al.*, 1981). This line was a gift from Dr. P.M. Howley (Dept. of Pathology, Harvard Medical School, Boston) and was maintained in DMEM supplemented with 5% foetal calf serum, 2 mM glutamine, 1 mM pyruvate, 0.375% sodium bicarbonate, 37.5 μg / ml penicillin, 50 μg / ml streptomycin and 2.5 μg / ml amphotericin B.

ID14 transfectants. Derivation of these BPV-4-transfected lines is outlined in Chapter 3.2.1.1. Transformed cells were selected by maintaining cultures in the same medium as the parental ID14 cells with the addition of 800 μg / ml of the neomycin analogue G418.

ΔM9 cells were derived from transformation of C127 cells with BPV-1 DNA (Burnett *et al.*, 1988). This line was a gift from Dr. S. Burnett (Dept. of Medical Genetics, University of Uppsala) and was maintained in DMEM supplemented with 10% foetal calf serum, 2 mM glutamine, 1 mM pyruvate, 0.375% sodium bicarbonate, 37.5 μg / ml penicillin, 50 μg / ml streptomycin and 2.5 μg / ml amphotericin B.

ΔM9 transfectants. These lines were derived from transfection of ΔM9 cells with either whole or sub-genomic fragments of BPV-4 as detailed in Chapter 3.2.2.1. These lines were selected by maintaining cultures in DMEM 2% foetal calf serum,

2 mM glutamine, 1 mM pyruvate, 0.375% sodium bicarbonate, 37.5 μg / ml penicillin, 50 μg / ml streptomycin and 2.5 μg / ml amphotericin B, with the addition of 800 μg / ml G418.

Primary Cells

PalF cells are primary bovine fibroblasts and were routinely grown in DMEM supplemented with 10% foetal calf serum, 2 mM glutamine, 1 mM pyruvate, 0.375% sodium bicarbonate, 37.5 μg / ml penicillin, 50 μg / ml streptomycin and 2.5 μg / ml amphotericin B.

PalF transfectants. These lines were derived from transfection of PalF cells with either whole or sub-genomic fragments of BPV-4 as detailed in Chapter 4.2.2.2. Transformed cells were selected by maintaining cultures in the same medium as the parental PalF cells with the addition of 500 μg / ml of the neomycin analogue G418.

2.1.4 Chemicals

Amersham International plc, Amersham, Bucks., England.

α-^{32}P dCTP 3000 Ci / mmol

BDH Chemicals Ltd., Poole, Dorset, England.

Where possible, chemicals were of analytical grade (AnalaR).

Acrylamide

bis-acrylamide

n-butanol

Calcium chloride

D-glucose

Ethylene diamine tetraacetate (EDTA) disodium salt

Glycerol

Hydrochloric acid

Magnesium chloride

Magnesium sulphate

Napthalene Black

di-Potassium hydrogen orthophosphate anhydrous

Potassium dihydrogen orthophosphate

Sodium acetate

Sodium chloride

Sodium deoxycholate

Sodium dodecyl sulphate (SDS)

Sodium hydroxide

Tris

Beta Lab., East Mosley, Surrey, England.

Yeast Extract

BioRad Laboratories Ltd., Hemel Hempstead, Herts., England.

TEMED (N,N,N',N'-tetramethylethylenediamine)

Boehringer Mannheim UK Ltd., Lewes, East Sussex, England.

Caesium chloride

DOTAP (N-[1-(2, 3-Dioleoyloxy) propyl]-N, N, N-trimethylammoniummethylsulfate)

Protease K

RNase A

James Burrough Ltd., Witham, Essex, England.

Ethanol

Difco Laboratories, Detroit, Michigan, USA.

Bacto-Agar

Bactotryptone

Fisons Scientific Equipment, Loughborough, England.

Dimethyl sulfoxide (DMSO)

Isopropanol

Fluka AG, Chemische Fabrik CH-9470 Buchs.

Methocel MC 4000

Gibco Europe Life Technologies Ltd., Paisley, Scotland.

All DNA restriction enzymes and appropriate buffer concentrates were obtained from Gibco Life Technologies (BRL) unless otherwise stated. The following reagents were also obtained from Gibco:

Agarose (ultrapure grade)

Low melting point agarose (ultrapure grade)

10x Dulbecco's Modified Eagles Medium

Foetal calf serum

10x F10 (Ham) Medium

200 mM glutamine

Geneticin. G418 sulphate

MEM amino acids solution (50x)

7.5% sodium bicarbonate

100 mM sodium pyruvate

Trypsin

Rathburn Chemicals Ltd., Walkerburn, Scotland.

Phenol (water saturated)

Sigma Chemical Co., Ltd., Poole, Dorset, England.

Ampicillin

Aprotinin

Coomassie Brilliant Blue R

Ethidium bromide

Ficoll

Formamide

Heparin (Type II)

IPTG

Lysozyme

ONPG

Quercetin (3,3',4',5,7-pentahydroxyflavonel dihydrate)

Salmon testes DNA (sodium salt)

Sodium azide

2.1.5 Kits

BioRad Laboratories Ltd., Hemel Hempstead, Herts., England.

Prep-A-Gene DNA Purification Kit

Boehringer Mannheim UK Ltd., Lewes, East Sussex, England.

Random Primed DNA Labelling Kit

2.1.6 Columns, Membranes, Paper and X-ray Film

Amersham International plc, Amersham, Bucks., England.

Hybond-C Extra

Hybond ECL nitrocellulose

Hybond N^+

Anderman Co. Ltd., Kingston upon Thames

Schleicher & Schuell Elutip-d columns

Eastman Kodak Co., Rochester, New York, USA

X-ray film (XAR-5)

Whatman International Ltd., Maidstone, Kent, England.

3MM chromatography paper

Whatman 1 filter paper

2.1.7 Plasmids

pBV4 contains the whole BPV-4 genome (7.265 kb) cloned into the BamH I site of pAT153 (Campo & Coggins, 1982). See Figure 3.5.

pJ4Ω16.E6 was a gift from Dr. L. Crawford (Dept. of Pathology, University of Cambridge). This plasmid construct is a pBR322 derivative. It contains the HPV-16 E6 open reading frame (ORF) cloned into the BamH I / EcoR I sites of pJ4Ω downstream of a MoLV LTR promoter (Storey *et al.*, 1988). The plasmid codes for the entire E6 ORF, except for the C-terminal amino acid leucine which is replaced by His-Gly followed by a stop codon.

pSVE8E7 contains the Xho II fragment of BPV-4 (nts 6487-1274) cloned into the BamH I site of pSV2neo (Jaggar *et al.*, 1990). pSV2neo contains the bacterial Tn5 transposon (which encodes neomycin resistance) under the control of the simian virus 40 (SV40) early promoter thereby allowing selection of transfected cells in medium containing the neomycin analogue G418 (Southern & Berg, 1982). In pSVE8E7 the BPV-4 genes are under the transcriptional control of the BPV-4 long control region (LCR) although the enhancer in the (SV40) early promoter region may be expected to influence their expression.

pT24 is a pUC13 derived plasmid containing the 6.6 kb activated human c-Ha-*ras* oncogene from the T24 human bladder carcinoma line originally cloned in pBR322 (Santos *et al.*, 1982). This plasmid construct was a gift from M. O'Prey (Beatson Institute, Glasgow).

pZipE8E7 contains the Xho II fragment of BPV-4 (nts 6487-1274) cloned into the BamH I site of pZipneoSV(XI) (Jaggar *et al.*, 1990). In pZipE8E7 the BPV-4 genes are under the transcriptional control of the Moloney murine leukaemia virus 5' long terminal repeat (MoLV LTR). See Figure 3.5.

pZipE7 contains nts 652-1250 of the BPV-4 genome cloned into the BamH I site of pZipneoSV(XI) (Pennie *et al.*, 1993). In pZipE7 the BPV-4 E7 gene is under the transcriptional control of the Moloney murine leukaemia virus 5' long terminal repeat (MoLV LTR). See Figure 3.5.

pZipneoSV(XI) (referred to as **pZipneo** throughout the text) consists of a Moloney murine leukaemia virus (MoLV) transcriptional unit, including the long terminal repeats (LTRs), and pBR322 sequences. This construct has a unique BamH I cloning site and also contains DNA sequences derived from the transposon Tn5, which encodes G418-resistance (neomycin resistance) in mammalian cells (Cepko *et al.*, 1984).

p41XLCR+ contains nts 6710-310 of the BPV-4 genome cloned into the Xho I site of p41X (Jackson & Campo, 1991).

pALTER-E8 contains nts 236-590 of the BPV-4 genome cloned into the BamH I site of the vector pALTER^{TM}-1 (Promega). This plasmid construct was a gift from R. Anderson (Beatson Institute, Glasgow).

pA-Sma 1.5 contains 1.5 kb of the bovine c-Ha-*ras* gene cloned into pUC8. The bovine sequences present included intron 1a and exon 1 of the c-Ha-*ras* 1 gene and also some satellite-like sequences (McCaffery *et al.*, 1989).

pBov.16K contains the complete ductin cDNA sequence (465 bp) cloned into the Xho I / Xba I sites of pBluescript II SK+. This plasmid construct was a gift from A. Faccini (Beatson Institute, Glasgow).

pBS*ras* contains a 576 bp fragment of the murine c-Ha-*ras* gene cloned into the Sma I site of pBluescript II SK+. This plasmid construct was a gift from W. Lambie (Beatson Institute, Glasgow).

pBV1a contains the whole BPV-1 genome (7.945 kb) cloned into the Hind III site of pAT153 (Campo & Coggins, 1982).

pHSV-βgal contains the *E. coli* β-galactosidase gene (*lacZ*) under the control of the herpes simplex virus immediate early 4 promoter. This plasmid construct, used as an internal control for transfection efficiency in PalF cell transient expression studies, is derived from the *lacZ*-containing plasmid pCH110 (Hall *et al.*, 1983). Transcription of the *lacZ* gene is driven by the HSV2 IE-5 promoter obtained from pLW2 (Gaffney *et al.*, 1985).

pIC-ARS consists of a 719 bp fragment containing ARS-like sequences from the amplified cellular fragment HL-10 (Smith *et al.*, 1993), cloned into the Pst I / BamH I site of pIC20H.

pJ33.6 consists of a 720 bp fragment containing the core minisatellite from λ33.6 (Jeffreys *et al.*, 1985) cloned into the EcoR I / Hind III site of pUC18.

pJ33.15 consists of a 600 bp fragment containing the core minisatellite from λ33.15 (Jeffreys *et al.*, 1985) cloned into the EcoR I / Hind III site of pUC19.

pLCRluc contains nts 6710-331 of the BPV-4 genome cloned into the BamH I site of the vector p0luc upstream of the luciferase coding sequence (Jackson & Campo, 1995).

pME2 contains 415 bp of the human c-*myc* gene (nts 4604-5018) cloned into the Pst I site of pBR322. This plasmid construct was a gift from Dr. G. Birnie (Beatson Institute, Glasgow).

pURE2 contains nts 2979-3814 of the BPV-4 genome cloned into the BamH I site of pUR278.

pURE7 contains nts 647-1250 of the BPV-4 genome cloned into the BamH I site of pUR278.

pURL1a contains nts 5737-6524 of the BPV-4 genome cloned into the BamH I site of pUR278.

pURL1b contains nts 5627-5737 of the BPV-4 genome cloned into the BamH I site of pUR278.

pURL1c contains nts 6524-0082 of the BPV-4 genome cloned into the BamH I site of pUR278.

pURL2a contains nts 4610-4989 of the BPV-4 genome cloned into the Hind III site of pUR278.

pURL2b contains nts 4042-4610 of the BPV-4 genome cloned into the BamH I site of pUR278.

pURL2c contains nts 4989-5629 of the BPV-4 genome cloned into the BamH I site of pUR278.

Plasmid constructs pURE2 through to pURL2c were gifts from G.J. Grindlay (Beatson Institute, Glasgow).

2.1.8 Molecular Weight Markers

Amersham International plc, Amersham, Bucks., England.

Rainbow™ coloured protein molecular weight markers

Gibco Europe Life Technologies Ltd., Paisley, Scotland.

Bacteriophage λ DNA (EcoR I digested)

Bacteriophage λ DNA (Hind III digested)

2.1.9 Research Supplies and Miscellaneous

Beatson Institute Central Services

Amphotericin B

L-broth

Kanamycin

Penicillin

Sterile distilled water

Sterile glycerol

Sterile phosphate-buffered saline (PBS)

Sterile phosphate-buffered saline + EDTA (PE)

Streptomycin

Merck Ltd., Poole, England

Silicone grease

2.1.10 Tissue Culture and Bacteriological Plasticware

Alpha Laboratories Ltd., Eastleigh, Hampshire, England.

Microfuge tubes

Becton Dickinson Labware, Plymouth, England.

Falcon 1059 polypropylene tubes

Falcon 2059 polypropylene tubes

Falcon 2098 polypropylene tubes

Sterile Plastipak syringes

Becton Dickinson Labware, Plymouth, England. (continued)

18G sterile syringe needles

60 and 90 mm tissue culture dishes

Bibby Sterilin Ltd., Stone, Staffs., England.

60 and 90 mm bacteriological petri dishes

Sterile plastic universal containers

Costar Corporation, High Wycombe, Bucks., England

24 well tissue culture plates

DuPont UK Ltd., Stevenage, Herts., England

Polyallomer ultracentrifuge tubes

Gelman Sciences, Northampton, England.

Sterile 0.2 μm Acrodisc filters

Nunc, Roskilde, Denmark.

T25, 80 and 175 cm^2 tissue culture flasks

Cryotubes

2.1.11 Water

Distilled water for the preparation of buffer stocks was obtained from a Millipore MilliRO 15 system. Water for protein, enzyme, RNA or recombinant DNA procedures was further purified on a Millipore MilliQ system to 18MΩ / cm.

2.2 Methods

2.2.1 Cell Culture and Transfection

2.2.1.1 Cell Culture

All cell culture work was carried out in laminar flow hoods (Class II Microbiological Safety Cabinets; Medical Air Technology Ltd., Manchester, England.) using aseptic techniques. Cells were grown in humidified 37°C incubators containing 5% (v/v) CO_2 (Heraeus, Essex, England) and were routinely screened for mycoplasma infection using a fluorescent dye technique (M. Freshney, Beatson Institute, Glasgow).

2.2.1.2 Isolation of Primary Bovine Fibroblasts

PalF cells were isolated as described in Jaggar *et al.* (1990). Briefly, soft palate tissue was removed from bovine foetuses of less than 5 months gestation obtained from Glasgow University Veterinary School. The palate tissue was washed in 70% ethanol and then dissected into small cubes using crossed scalpels. These cubes (approximately 2 mm in size) were then placed in 90 mm tissue culture dishes and tissue was allowed to adhere to the plastic by placing the dishes in a dry 37°C incubator containing 5% (v/v) CO_2 for 5 minutes. Medium was then added to each dish, care being taken not to dislodge the tissue explants. The explants were fed twice weekly over a period of two weeks in which time fibroblasts and keratinocytes grew out of the tissue mass. The medium used for both isolation and subsequent routine growth of PalF cells was DMEM supplemented with 10% foetal calf serum, 2 mM glutamine, 1 mM pyruvate, 0.375% sodium bicarbonate, 37.5 μg / ml penicillin, 50 μg / ml streptomycin and 2.5 μg / ml amphotericin B. This medium selectively favoured the outgrowth of fibroblasts and, as expected, keratinocytes died. After this time the fibroblasts were trypsinised and reseeded into large (T175 cm^2) flasks. Cultures were expanded and stocks of PalF cells were frozen down and stored in liquid nitrogen.

2.2.1.3 Maintenance of Primary Bovine Fibroblasts in Culture

Cells were fed twice weekly. Old medium was aspirated from sub-confluent flasks and fresh medium added. PalF cells were grown until just sub-confluent whereupon they were passaged approximately 1 in 4. Replating was performed as follows: for a T80 cm^2 tissue culture flask medium was aspirated off and the cells washed twice with 5 ml phosphate-buffered saline (PBS; 137 mM NaCl, 44 mM KCl, 1.4 mM KH_2PO_4, 8.4 mM Na_2HPO_4). The PBS was removed and 1 ml of trypsin solution (0.25% trypsin in 1x PE buffer; PBS with the addition of EDTA to 1 mM), which had been pre-warmed to 37°C, was added to cells. Tissue culture flasks were transferred to the 37°C hot room until the monolayer could be detached by gentle agitation. The cells were resuspended in growth medium and reseeded at an appropriate density.

2.2.1.4 Viability Staining and Cell Counting

The concentration of cell suspensions was determined using a haemocytometer (Improved Neubauer) examined under low-power microscopy. Cells were mixed with the dye napthalene black to allow simultaneous appraisal and quantification of cell viability. Viable cells are impermeable to this dye whereas dead cells will stain black due to dye uptake (Kaltenbach *et al.*, 1958). Trypsinised cells were resuspended in medium and 20 µl of this suspension added to a microfuge tube containing 80 µl of viability stain (1% napthalene black in PBS). The contents of the tube were gently pipetted up and down and 20 µl of the mix added to the counting chamber of a covered haemocytometer slide. The slide was placed on a microscope under a 10 x objective. The number of stained cells and the total number of cells were counted within a 1 mm^2 area. The average of two counts was calculated per cell line and the sample concentration determined, taking into account the area counted, depth of chamber and dilution factor of cells loaded. Results were expressed as the number of viable cells per ml.

2.2.1.5 Cell Storage

Stocks of cells were stored long term in liquid nitrogen. Confluent cultures were trypsinised, complete medium was added and the cell suspension transferred to a sterile universal. Cells were pelleted by centrifugation at 1000 rpm for 5 min at room temperature. The pellet was then resuspended at a concentration of approximately 10^6 cells / ml in growth medium containing 10% (v/v) DMSO which acts as a cryoprotectant. Suspensions were divided into 1 ml aliquots in 1-2 ml Nunc cryotubes and frozen, well insulated, at -70°C overnight to ensure a slow rate of cooling. The ampoules were then transferred to a liquid nitrogen bank containing labelled storage racks until required. Frozen stocks were recovered by removing the ampoules from liquid nitrogen and placing them in a beaker containing water heated to 37°C. Once thawed, the cells were pelleted by centrifugation at 1000 rpm for 5 min at room temperature, resuspended in fresh growth medium and transferred to 80 cm^2 flasks.

2.2.1.6 Quercetin Treatment of Primary Bovine Fibroblasts

Four different protocols, QA-QD, were used for the transfection studies in which the order and time interval between quercetin-treatment and viral DNA transfection varied (summarised in Table 4.8) although the concentration (20 μM) and duration (48 hr) of the single dose quercetin treatment was constant. The effect(s) of quercetin on PalF cells was also examined in the absence of viral genes. These analyses included experiments, described in Chapter 4.5.2.2, 4.5.3.2 and 4.5.4.2, in which actively growing PalF cells were treated with five different concentrations of quercetin, namely 20, 45, 100, 200 and 300 μM. For the 20 μM and 45 μM concentrations the quercetin solvent used was ethanol (stock solution: 10 mM stored at -20°C) while for the higher concentration experiments (100-300 μM) dissolution in DMSO was necessary (stock solution: 100 mM made fresh).

2.2.1.7 Growth Curves

The growth characteristics of PalF cells grown in medium containing 20 μM quercetin were assessed. Control classes consisted of PalF cells grown in complete medium with or without ethanol (the quercetin solvent). 2 x 10^4 cells were seeded per 25 cm^2 tissue culture flask and cells allowed to settle overnight. After this time cells were counted daily over a ten day period with duplicate flasks set up for each time point. For counting, medium was aspirated from the flasks and the cells washed twice in pre-warmed PBS to remove any non-adherent cells. The adherent cells were then trypsinised, resuspended in an appropriate volume of complete medium and counted using a haemocytometer. Four counts were performed per class per time point. Data are presented as the average count from duplicate flasks.

2.2.1.8 DNA Transient Transfection of Primary Bovine Fibroblasts

DNA transfection was performed using the cationic lipid N-[1-(2, 3-Dioleoyloxy) propyl]-N, N, N-trimethylammoniummethylsulfate (DOTAP; Boehringer Mannheim BCL) following the manufacturer's recommendations. Each reaction contained 5 μg of each relevant plasmid DNA (Chapter 2.1.7) plus 2 μg of a plasmid construct containing the selectable marker gene for neomycin resistance (pZipneo; Chapter 2.1.7). Transfection classes are described in Chapter 4.2.2.2. Reactions were made up to 20 μg with sonicated salmon sperm DNA (Sigma). 5 x 10^5 PalF cells were seeded into an 80 cm^2 flask 24 hr prior to transfection. The transfection mix was as follows; 80 μl of DOTAP was diluted up to 250 μl with complete medium in a polystyrene reaction vial. DNA (20 μg) was also diluted up to 250 μl with complete medium in a separate reaction vial. Both solutions were mixed together and incubated for 10 min at room temperature. This mixture was then slowly added to the flask of cells and incubated at 37°C overnight. Following withdrawal of the medium, cells were washed twice in PBS and fresh complete medium added to the flask. The next day cells were split at a dilution of 1:2 and allowed to settle for 24 hr prior to selection.

2.2.1.9 Selection of Transfected Cells

Cells were selected in medium containing 500 μg / ml G418 for 21-28 days, being refed twice weekly. After this time, G418-resistant colonies greater than 5 mm in diameter and showing a piled up, transformed morphology were scored (Chapter 4.2.2.3). Where appropriate, several colonies were picked from each transfection class.

2.2.1.10 Isolation of Clonal Populations

Well separated G418-resistant colonies were identified and their position highlighted by marking flasks using a permanent marker pen. The cells were then washed twice in PBS and, leaving cells covered in 10 ml PBS, the top of each flask was removed using a red hot scalpel. The PBS was aspirated off and colonies were ringed using sterile 6 mm stainless steel cloning rings. The base of each cloning ring had been dipped in sterile silicone grease (Merck, England) prior to placing over colonies thus providing a waterproof seal round each isolated colony. A total volume of 100 μl trypsin solution, which had been pre-warmed to 37°C, was pipetted within each cloning ring. After 1-2 min an equal volume of complete medium was added and the cell suspension transferred to multiwell plates (Costar) for expansion into cell lines.

2.2.1.11 Growth in Methocel

The ability of a cell line to form colonies in semi-solid media is taken as a phenotypic measure of its degree of transformation. Anchorage independence was assayed by plating cells in a Methocel based medium. Three grams of Methocel MC 4000 (Fluka) was added to 200 ml of sterile water and autoclaved. The Methocel was left to dissolve with continual stirring for 2-3 days at 4°C. Following dissolution, 22 ml of 10 x F10 (Ham) medium (Gibco), 4 ml of 50 x minimum essential amino acids solution (Gibco), 4 ml of 0.1 M sodium pyruvate, 5 ml of 7.5% sodium bicarbonate and 100 ml of foetal calf serum were added to the solution. Cells (2.5×10^5) were added to 10 ml of this solution and the mixture added to a bacteriological grade petri dish. Bacteriological

petri dishes were used to discourage cells from adhering to the bottom of the dishes. Cells, tested in duplicate, were left at 37°C for 7-10 days before being scored. The efficiency of Methocel colony formation was determined as described in Chapter 4.2.2.3.

2.2.1.12 Tumourigenicity Assay in Nude Mice

The ability to form tumours is an indicator of full cellular transformation. The malignant potential of transformed cells was assayed in nude mice. Four-week old female athymic nude mice, strain MF1 nu/nu (Harlan-Olac, Bicester, England), were injected subcutaneously with 10^7 cells suspended in 0.1 ml of complete medium. Three mice were injected per assay and examined for tumour growth at 1 week intervals up to 15-20 weeks post-injection. If no tumour had developed by then the cells were considered to be non-tumourigenic.

2.2.1.13 DNA Transfection and Luciferase Expression Assay

Possible quercetin-induced effects on the transcriptional activity of the BPV-4 LCR (long control region) were assayed as measured by the enzyme activity of the reporter gene firefly luciferase. A luciferase expression plasmid was constructed by cloning nucleotides 6710-331 of the BPV-4 genome, a region containing the LCR, into the BamH I site of the vector p0luc upstream of the promoterless luciferase coding sequence. This construct, pLCRluc (fully detailed in Chapter 2.1.7), was a gift from Dr. M.E. Jackson (Beatson Institute, Glasgow). PalF cells were treated with 20 μM quercetin for 48 hr either immediately before or after transfection with 15 μg pLCRluc + 5 μg of the control plasmid, pHSV-βgal. Control classes included transfection of PalF cells treated for 48 hr with ethanol (the quercetin solvent) and vector alone controls (p0luc + pHSV-βgal). After incubation with the transfection solution at 37°C overnight, the medium was removed and the cells washed twice with PBS and refed. The cells were harvested 48 hr later in Reporter Lysis Buffer (Promega). 300 μl of Reporter

Lysis Buffer were used per 80 cm^2 flask. Luciferase assays were performed on 50 µl aliquots of the cell lysates using the Luciferase Assay System (Promega) according to the manufacturer's instructions. Light production was measured for 1 minute using a BioOrbit LKB 1251 luminometer. Luminescence values were expressed as millivolts per second. β-galactosidase assays (Chapter 2.2.1.14) were used to standardise luciferase activities.

2.2.1.14 β-*Galactosidase Assay*

The plasmid pHSV-βgal (described in Chapter 2.1.7) was used as an internal control to standardise for transfection efficiency in PalF cells. The levels of β-galactosidase activity were quantified by the catalytic conversion of colourless *o*-nitrophenyl-β-D-galactopyranoside (ONPG) to yellow *o*-nitrophenol. Typically, 100 µl of cell lysate was incubated with 0.5 ml Solution I (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgCl_2$, 50 mM β-mercaptoethanol) and 0.1 ml Solution II (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 2 mg / ml ONPG) at 37°C for 30-60 min, or until a yellow colour change was apparent. The reaction was stopped with 0.25 ml of 1 M sodium carbonate and an absorbance reading taken at 420 nm in a plastic cuvette with a path length of 1 cm.

2.2.2 Recombinant DNA Techniques

2.2.2.1 Transformation of Bacterial Hosts

Commercially available *E. coli* DH5α competent cells (Gibco, Life Technologies) were routinely used to propagate plasmids. Stocks of competent cells were kept at -70°C until use. Bacteria were transformed following manufacturer's instructions. After thawing on ice, 20 µl of competent cells were aliquoted into a chilled polypropylene tube (Falcon 2059) and 1-2 ng of the appropriate plasmid DNA added with gentle mixing. The cells were then incubated on ice for 30 min before being heat shocked at 42°C for 45 seconds. The tube was then returned to ice for 2 min. 80 µl of room

temperature SOC buffer (2% bactotryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgS0_4$, 20 mM glucose) was then added and the tube transferred to a shaking 37°C incubator for 1 hr to allow expression of the antibiotic resistance marker. After this time cells were spread on an L-agar plate containing the appropriate antibiotic. The plate was inverted and incubated overnight at 37°C to allow colony formation.

2.2.2.2 Glycerol Stocks

E. coli transformants bearing plasmids were stored as glycerol stocks for future retrieval. 850 µl of an overnight culture was mixed gently with 150 µl glycerol in a sterile plastic 1-2 ml Nunc cryotube and stored at -70°C. A sterile plastic loop was used to retrieve an aliquot of cells as and when required.

2.2.2.3 Large Scale Preparation of Plasmid DNA

Large quantities of plasmid DNA were prepared using a modification of the method described by Birnboim and Doly (1979). Bacteria containing the plasmid of interest were streaked onto an L-agar plate containing the appropriate antibiotic and the plate inverted and incubated overnight at 37°C to allow colony formation. A single colony, picked using a sterile toothpick, was used to inoculate a sterile universal tube containing 10 ml of Superbroth medium and the appropriate antibiotic. Superbroth medium is composed of two solutions-A and B. Solution A consists of 12 g of tryptone, 24 g of yeast extract and 5 ml of glycerol made up to a final volume of 900 ml with distilled water. Solution B consists of 12.5 g of di-potassium hydrogen orthophosphate (K_2HPO_4) and 3.8 g of potassium dihydrogen orthophosphate (HK_2PO_4), made up to a final volume of 100 ml. Both solutions were autoclaved separately and combined just prior to use. The universal was transferred to a 37°C incubator shaking at 225 rpm for approximately 8 hr. This culture was then added to two 1 litre flasks each containing

500 ml of Superbroth (to allow good aeration), and the appropriate antibiotic, and returned to the shaking incubator for 36 hr.

Bacterial cultures were decanted into 250 ml polypropylene centrifuge bottles (Sorvall Instruments, DuPont) and cells were pelleted by centrifugation in a Sorvall RC-5B centrifuge (Sorvall GS-3 rotor) at 3,000 rpm for 5 min at 4°C. The supernatant was removed and the pellets resuspended in a total of 36 ml of Solution I (50 mM glucose, 10 mM Tris-HCl, 10 mM EDTA (pH 8.0)). Resuspended pellets were pooled into one centrifuge bottle and 4 ml of Solution I containing 5 mg / ml lysozyme were added. The suspension was mixed gently and left at room temperature for 10 min. 80 ml of freshly prepared Solution II were added (0.2 M NaOH, 1% SDS) and the suspension mixed gently before being placed on ice for 5 min. 40 ml of cold Solution III (294.4 g KAc and 115 ml glacial acetic acid made up to 1 litre in water) were added , the bottle shaken rapidly and then returned to ice for 20 min. The flocculate was then centrifuged at 8,000 rpm for 5 min at 4°C in a Sorvall SS34 rotor and the supernatant filtered through Whatman 1 paper. The nucleic acid in this cleared lysate was precipitated in 0.6 volumes of isopropanol and pelleted by centrifugation at 8,000 rpm for 5 min at 4°C. The supernatant was removed and the nucleic acid pellet washed with 70% ethanol to remove any salt. The solution was centrifuged for a further 5 min at 8,000 rpm at 4°C. After discarding the supernatant, the pellet was allowed to dry at room temperature for 10 min before being resuspended in 8.8 ml of TE (10 mM Tris-HCl, 1 mM EDTA pH 8.0).

Ultracentrifugation through a caesium chloride (CsCl) density gradient was then carried out to further purify the plasmid DNA. 8.3 g of caesium chloride was added to the solution and allowed to dissolve at room temperature. 0.4 ml of a 10 mg / ml ethidium bromide solution was added and the refractive index of this solution adjusted to 1.4. The solution was transferred to a sealable centrifuge tube and the gradient run in a

balanced Beckman ultracentrifuge at 55,000 rpm for 16 hr at 20°C, followed by a slower run at 40,000 rpm for 1 hr at 20°C. After centrifugation, any contaminating RNA was found to have pelleted to the bottom of the tube. Two bands were discernible; the upper band contains sheared linear plasmid DNA and residual bacterial chromosomal DNA while the lower band contains closed circular plasmid DNA. The top of the tube was pierced with an 18-gauge needle and the lower band was gently withdrawn by similarly piercing the side of the tube 5 mm below the lower band with an 18-gauge needle connected to a syringe. Plasmid DNAs to be used in transfection studies underwent further centrifugation through CsCl prior to plasmid extraction.

The removed band was transferred to a 50 ml Falcon tube. 3 ml of TE (pH 8.0) were added for each ml of CsCl solution collected. 4 ml of *n*-butanol were added to this and the tube briefly centrifuged at 1,000 rpm for 5 min at 4°C to separate phases. The upper butanol layer was aspirated off and discarded. This extraction process was repeated three more times and the lower, aqueous phase was transferred to a fresh 50 ml Falcon tube. 8 ml of ethanol were added for each ml of original CsCl band solution and the tube was placed on ice for 20 min as lower temperatures cause the CsCl salt to precipitate. The tube was subsequently spun at 1,000 rpm for 20 min at 4°C and the supernatant discarded. Excess liquid was drained from the tube and the pellet was resuspended in 500 µl TE (pH 8.0). This DNA solution was then transferred to a 1.5 ml microfuge tube and extracted once with phenol / chloroform prior to ethanol precipitation. The DNA pellet was finally resuspended in 0.5-1 ml TE (pH 8.0), the final volume chosen depending on the size of the pellet. The DNA concentration was quantified by spectrophotometry (Chapter 2.2.2.6).

2.2.2.4 Eukaryotic DNA Analysis: Preparation of Genomic DNA from Cell Lines

Genomic DNA was isolated from all cell lines using a high salt method of DNA extraction (Miller *et al.*, 1988). Cells, grown to approximately 80% confluency in a

80 cm^2 tissue culture flask, were washed twice with PBS, trypsinised and pelleted at 1,000 rpm at room temperature for 5 min in a 15 ml polypropylene tube. The cell pellet was resuspended in 3 ml of nuclei lysis buffer (10 mM Tris-HCl, 400 mM NaCl and 2 mM Na_2EDTA, pH 8.2) and digested overnight at 37°C with 0.2 ml of 10% SDS and 0.5 ml of a protease K solution (1 mg protease K in 1% SDS and 2 mM Na_2EDTA). After digestion, 1 ml of saturated NaCl (approximately 6 M) was added to the tube which was shaken vigorously for 15 seconds, followed by centrifugation at 2,500 rpm at room temperature for 15 min. The supernatant, containing the DNA, was transferred to a fresh 15 ml polypropylene tube.

2.2.2.5 Extraction with Organic Solvents and Ethanol Precipitation

DNA samples were further purified by extraction with chloroform and / or phenol to remove contaminants that might interfere with subsequent manipulations e.g. residual enzyme activities (restriction, nuclease) and detergents. Typically, the initial extraction was performed in an equal volume of phenol / chloroform. Phenol / chloroform was freshly prepared by mixing equal volumes of 1 M Tris-HCl (pH 8.0)-equilibrated phenol and chloroform / isoamyl alcohol (24:1 v/v). The aqueous and organic phases were mixed thoroughly by vortexing and then separated by centrifugation in a bench top microcentrifuge at 14,000 rpm for 5 min at room temperature. The aqueous phase was carefully removed, transferred to a fresh tube and the extraction process repeated. Following centrifugation, the aqueous phase was carefully removed, transferred to a fresh tube and extracted with an equal volume of chloroform (chloroform : isoamyl alcohol, 24 : 1) by vortexing and centrifugation as described above. This was repeated to remove any traces of phenol from the upper aqueous phase. After the final extraction the aqueous phase was collected for ethanol precipitation.

Ethanol precipitation was used to collect and concentrate samples of DNA, and also to remove solute contaminants such as salt. The aqueous solution of DNA was mixed with

one tenth volume of 3 M sodium acetate, pH 5.2 and 2-2.5 volumes of ice cold ethanol. The sample was mixed by inversion and stored at -20°C overnight or, alternatively, placed on dry ice for 15-30 min to precipitate the DNA. Precipitated DNA was recovered by centrifugation in a bench top microcentrifuge at 14,000 rpm for 30 min at 4°C. The DNA pellet was washed with 70% ice cold ethanol to remove any traces of salt, air dried, and resuspended in an appropriate volume of TE buffer (pH 8.0) before quantification.

2.2.2.6 Quantification of DNA by Spectrophotometric Analysis

The concentration of DNA in aqueous solutions was measured spectrophotometrically. Samples were diluted as appropriate in TE buffer and absorbance readings taken at 260 nm and 280 nm in a quartz cuvette with a path length of 1 cm. The spectrophotometer was calibrated using a TE blank. An A_{260} value of 1 was taken as being equivalent to a concentration of 50 μg / ml double stranded DNA. The ratio between the readings at 260 nm and 280 nm (A_{260} : A_{280}) provided an estimate for the purity of DNA samples. A ratio of 1.8-2.0 indicated that preparations were essentially pure.

2.2.2.7 Restriction Digests

Restriction digests were performed in small volumes using commercially obtained buffer concentrates supplied with the enzymes. 1-10 units of restriction enzyme were added per μg of DNA, depending on the enzyme used and the number of restriction sites present. The total volume of enzyme added did not exceed one tenth of the final reaction volume. Small quantities of plasmid DNA (< 5 μg) were digested in a volume of 20 μl for 1-2 hr at 37°C. Larger digests were carried out in proportionally larger volumes. Double digests were performed sequentially or as a single reaction depending on the buffering conditions specified by the manufacturer. Digestion of genomic DNA (typically 20 μg) was carried out essentially as described, except that digestion was

continued overnight. Reactions were terminated by the addition of 0.5 M EDTA (pH 8.0) to a final concentration of 10 mM. The digested DNA was then phenol / chloroform extracted, ethanol precipitated and resuspended in an appropriate volume of TE.

2.2.2.8 Agarose Gel Electrophoresis

DNA restriction fragments were resolved on non-denaturing agarose gels and visualised by ethidium staining. Unless otherwise stated, gels were 0.8% agarose made up in 0.5 x TBE buffer (5 x TBE is 54 g Tris base, 27.5 g boric acid and 20 ml 0.5 M EDTA (pH 8.0) made up to 1 litre in water). Gel mixes were prepared in glass flasks by microwaving the agarose in the appropriate volume of 0.5 x TBE until all the particles had dissolved. The solution was cooled to approximately 50°C and the contents of the flask were poured into a gel cast and allowed to set at room temperature. The gel comb (well former) was removed and the gel installed in a horizontal sub-marine gel electrophoresis tank (Pharmacia) containing 0.5 x TBE. Samples for electrophoresis were mixed with one sixth volume of gel loading dye (6 x stock is 0.25% bromophenol blue, 15% Ficoll (Type 400)) and pipetted through the TBE buffer into the wells. Molecular weight standards (Hind III digested bacteriophage λ DNA or Hind III / EcoR I digested bacteriophage λ DNA (Gibco Life Technologies) were treated in the same manner. The samples were resolved by electrophoresis towards the anode. Running conditions for 20 cm gels (Pharmacia GNA 200) were 80V for 4 hr and 25V for 16 hr. Once run, the DNA fragments were visualised by staining the gel in running buffer containing 0.5 μg /ml ethidium bromide with gentle agitation for 30 min at room temperature. The gel was placed on a UV transilluminator and a permanent record made using a Polaroid camera.

2.2.2.9 Southern Transfer

5-10 μg of genomic DNA was digested with an appropriate enzyme and resolved by gel electrophoresis. After electrophoresis each gel was stained in running buffer containing 0.5 μg / ml ethidium bromide and photographed. Gels were subsequently destained in running buffer without ethidium bromide. The separated DNAs were then transferred to a charged nylon membrane, Hybond-N^+ (Amersham International plc), following the manufacturer's recommended protocol for alkali blotting of DNA. This included gentle agitation of the gels in 0.25 M HCl at room temperature before blotting. This depurination step improved transfer of DNA greater than 10 kb in size. After blotting gels overnight in 0.4 M NaOH the resulting membranes were air-dried and then baked at 80°C for 1 hour. Blotted gels were subsequently stained with ethidium bromide to check efficient transfer of DNA had taken place.

2.2.2.10 Elution of DNA Fragments from Agarose Gels

DNA fragments for isotopic labelling (Chapter 2.2.2.11) were recovered from low melting point agarose gels. Conditions for digestion of the relevant plasmid construct were designed such that the band of interest contained at least 250 ng of DNA. Agarose gel concentrations varied according to the size of the fragment of interest. Effective resolution was also dependent upon the difference in size between insert and vector bands. For most purposes a gel concentration of 0.8% agarose was used. The gel slice containing the fragment of interest was excised from the gel using a sterile scalpel and was trimmed of excess agarose over a UV source. The gel slice was transferred to a microfuge tube which was then placed in a 65°C water bath until the agarose had melted. An equal volume of TE (pH 8.0) was added and the solution extracted twice with phenol / chloroform and once with chloroform. DNA was concentrated by ethanol precipitation and the DNA pellet resuspended in an appropriate volume of TE.

2.2.2.11 Isotopic Labelling

DNA probes used for hybridisation of DNA (Chapter 2.2.2.9) and RNA (Chapter 2.2.3.2) membranes, isolated as described in Chapter 2.2.2.10, were isotopically labelled with 20 μCi α-^{32}P using a 'Random Primed DNA Labelling Kit' (Boehringer Mannheim) following the manufacturer's instructions. Each labelling reaction contained 25-40 ng of DNA. Unincorporated nucleotides were removed by passing the labelled reaction through an Elutip-d column (Schleicher & Schuell) as described by the manufacturer. Labelled probes were denatured at 95-100°C for 10 min and cooled on ice prior to addition to the appropriate hybridisation mix.

2.2.2.12 Southern Prehybridisation and Hybridisation Conditions

For prehybridisation, the baked membranes were put into individual plastic bags and 25 ml buffer mix was added. Membranes were prehybridised for 1.5 hr in a 65°C water bath with constant shaking. The prehybridisation buffer mixture consisted of 5 x SSPE (20 x SSPE is 3 M NaCl, 0.2 M NaH_2PO_4, 20 mM EDTA pH 7.4), 5 x Denhardt's solution (100 x Denhardt's solution is 2% (w/v) bovine serum albumin, 2% (w/v) Ficoll, 2% (w/v) polyvinylpyrolidone), 0.5% SDS and 20 μg / ml of denatured salmon sperm DNA. After this time 25-40 ng of labelled DNA probe was denatured by heating to 100°C for 10 minutes and was added to the prehybridisation solution. Membranes were hybridised overnight at 65°C with constant shaking.

2.2.2.13 Washing of Hybridised Membranes and Autoradiography

DNA membranes were washed to the following stringency: 2 x SSC, 0.1% SDS for 10 min at room temperature to remove unbound probe and then at 0.1 x SSC, 0.1% SDS for 30 min (2 x 15 min) at 65°C. Membranes were then bagged in 2 x SSC to keep them moist and exposed to X-ray film with two intensifying screens at -70°C.

DNA membranes were stripped for reprobing by immersing the membranes in a solution of boiling 0.5% SDS and allowing the solution to cool to room temperature. Efficiency of stripping was checked by exposing the membranes to X-ray film overnight at -70°C.

2.2.2.14 DNA Fingerprinting

Multilocus DNA fingerprinting was carried out using both human minisatellite and M13 probes.

Electrophoresis and Southern Blotting

This procedure was identical for both DNA fingerprinting methodologies. Restricted DNA samples (5 μg per track) were separated by electrophoresis through 20 cm long 0.8% agarose gels in 1 x TAE buffer (50 x TAE is 242 g Tris, 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA made up to 1 litre in water). Running conditions were 40V for 26 hr. DNA fragments were then Southern blotted onto Hybond N nylon membranes (Amersham International plc) following the manufacturer's instructions; i.e. depurination (in 0.25 M HCl for 15 min), denaturation (in 0.5 M NaOH, 1.5 M NaCl for 45 min) and neutralisation (in 1.5 M NaCl, 0.5 M Tris-HCl, pH 7.2 for 45 min) followed by overnight capillary blotting in 20 x SSC (3 M sodium chloride, 0.3 M sodium citrate). Membranes were then briefly rinsed in 2 x SSC, baked at 80°C for two hours and finally stored dry until required.

Probes and Isotopic Labelling

Human minisatellite probes:

Two human minisatellite polycore probes J33.6 & J33.15 (Jeffreys *et al.*, 1985) were used. Both probes were isolated from commercially available plasmid constructs (Cellmark Diagnostics, Abingdon, Oxon, England). The J33.6 insert (720 bp) was cloned into pUC18 and released by digestion with EcoR I + Hind III. The J33.15 insert (600 bp) was cloned into pUC19 and was also released by digestion with EcoR I +

Hind III. Inserts were separated from vector fragments by electrophoresis and isolated from the agarose gel using a 'glassmilk' isolation procedure (Prep-A-Gene; BioRad Ltd). The probes (20 ng) were isotopically labelled with 20 μCi α–^{32}P dCTP by random priming as described earlier (Chapter 2.2.2.11) and denatured (100°C for 10 min) prior to addition to hybridisation mix.

Bacteriophage M13 probe

The M13 probe was isotopically labelled by a partial primer extension reaction using commercially available bacteriophage M13 DNA. The method described by Wells (1988) was followed:

1. 400 ng of single stranded M13 vector (Pharmacia), 4 ng of 17-mer universal sequencing primer (Pharmacia) and 1 μl of 10 x Klenow buffer were added to a microfuge tube. The reaction was made up to a final volume of 10 μl and incubated at 58°C for one hour.
2. The tube was spun briefly in a microcentrifuge prior to addition of 10 μl of AGT mix (i.e. equal volumes of TE buffer and 0.5 mM dATP, dGTP, dTTP), 6 μl TE buffer, 30 μCi α–^{32}P dCTP (3000 Ci /mmol); and 2 U of DNA polymerase I (Klenow fragment). The reaction was mixed by gentle pipetting up and down.
3. After incubation at 37°C for 15 min the reaction was stopped by addition of 70 μl 3 x SSC.

The polymerisation reaction is stopped before it crosses the tandem repeat regions of the M13 genome (positions 1700-1900 and 2300-2500). The new strand (of up to 4.5 kb) contains the radiolabelled nucleotides while the proximal 2.5 kb of the M13 genome, including the tandem repeats, remains single-stranded (Wells, 1988). This allows the tandem repeats to hybridise to their membrane-bound complements when the labelled phage is used as a hybridisation probe. The labelled M13 DNA was added directly to

the hybridisation solution, i.e. without denaturing the DNA. It was not necessary to remove free nucleotides before use.

Hybridisation and Autoradiography

The same hybridisation / detection protocols were used for the human minisatellite and bacteriophage M13 probes. Hybridisations were carried out in a rotisserie style hybridisation oven (Hybaid, England). Heparin-based hybridisation solutions (Wells, 1988) were used; i.e. prehybridisation solution, 20 ml, pH 7.2 - 50% formamide (deionised), 3 x SSC, 0.2% SDS, and 50 μg / ml heparin (Type II); hybridisation solution, 15 ml, pH 7.2 - 50% formamide (deionised), 3 x SSC, 5% dextran sulphate, 0.2% SDS, and 200 μg / ml heparin (Type II). Membranes were prehybridised for 6 hr at 42°C and hybridisation was carried out overnight (16 hours) at 42°C. Following hybridisation the membranes were washed at low stringency, i.e. 2 washes in 1.5 x SSC, 0.1% SDS, 40°C for 30 min each. Autoradiography was generally carried out at -70°C overnight with one intensifying screen or at room temperature for 1-4 days without an intensifying screen. Membranes were later stripped by immersion in 0.1% SDS at 85°C for 30 minutes.

2.2.3 RNA Analysis

2.2.3.1 Total RNA Extraction and Quantification

Total RNA was isolated from cells grown to approximately 80% confluency following the RNAzol B method of extraction (Biogenesis Ltd., England). Briefly, cells in a T175 flask were washed twice with PBS and 5 ml RNAzol B solution added directly to the flask. The resulting mixture was then transferred to a Falcon 1059 polypropylene centrifugation tube and 1 ml of chloroform was added with vigorous pipetting. The top of the tube was then covered with Parafilm (American National Can, USA) and the tube was left on ice for 15 min to allow phase separation to take place. After this time the tube was spun at 10,000 rpm for 15 min at 4°C in an HB-4 rotor in a Sorvall RC-5B

centrifuge. The aqueous phase was transferred to a fresh tube and an equal volume of isopropanol added. The tube was briefly vortexed before being stored overnight at -20°C to allow precipitation of RNA. After precipitation, the tube was centrifuged as before and the pellet washed with 6 ml ice cold ethanol (75% in diethylpyrocarbonate (DEPC)-treated RNase-free water) with further centrifugation. The RNA pellet was resuspended in DEPC-treated RNase-free water and the concentration of RNA was measured spectrophotometrically as described in Chapter 2.2.2.6. The spectrophotometer was calibrated using a blank of DEPC-treated water. An A_{260} value of 1 was taken as being equivalent to a concentration of 40 µg / ml RNA. RNA samples were stored at -70°C.

2.2.3.2 RNA Dot Blots

20 µg aliquots of total RNA from test and control cell lines were dotted onto the supported nitrocellulose membrane Hybond-C Extra (Amersham International plc) using a Bio-Dot Microfiltration Apparatus (Bio-Rad Ltd.) according to the manufacturer's instructions. Once blotted, membranes were baked for 2 hours at 80°C.

2.2.3.3 Prehybridisation and Hybridisation Conditions - RNA

Membranes were prehybridised using the same buffer as for DNA membranes (Chapter 2.2.2.12) but in the presence of 50% formamide. Labelled DNA probes were denatured as above. Prehybridisation and hybridisation were carried out at 42°C.

2.2.3.4 Washing of Hybridised Membranes and Autoradiography - RNA

RNA membranes were washed to the following stringency: 2 x SSC, 0.1% SDS for 10 min at room temperature to remove unbound probe and then at 0.1 x SSC, 0.1% SDS for 30 min (2 x 15 min) at 65°C. Membranes were then bagged in 2 x SSC to keep them moist and exposed to X-ray film with two intensifying screens at -70°C.

RNA membranes were stripped for reprobing by immersing the membranes in a solution of boiling 0.1% SDS and allowing the solution to cool to room temperature. Efficiency of stripping was checked by exposing the membranes to X-ray film overnight at -70°C.

2.2.4 Protein Analysis

2.2.4.1 Whole cell protein extraction

Cells, grown to approximately 80% confluence in 90 mm tissue culture dishes, were washed twice with pre-chilled sterile PBS. The last traces of PBS were removed by aspiration. Dishes were placed on ice and 0.5 ml of pre-chilled lysis buffer (100 mM NaCl, 50 mM Tris-HCl (pH 7.4), 1% Triton X-100, 0.1% SDS, 0.5% Na deoxycholate, 10 mM $CaCl_2$, 2 mM phenylmethylsulfonyl fluoride (PMSF) and 1% aprotinin) was added per dish. Cells were incubated on ice for 20 min and then scraped, using a disposable cell scraper (Costar, England), into pre-chilled screw cap microfuge tubes. Tubes were centrifuged in a bench top microcentrifuge at 14,000 rpm for 2 min at 4°C. The supernatant from each tube was aliquoted into fresh microfuge tubes (to minimise sample deterioration due to repeat freeze-thawing) and stored at -70°C until required.

2.2.4.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was employed to resolve proteins according to their apparent molecular weight. Gels were subject to electrophoresis using an Atto Dual Slab E.P. Chamber apparatus. Resolving gels were typically 11% with respect to acrylamide and stacking gels were 7% acrylamide. A stock solution containing 44% (w/v) acrylamide, 0.8% *bis*-acrylamide was used for both types of gel. Acrylamide solutions of the required concentration were mixed with the appropriate gel buffer to a final concentration of 1x with distilled water (dH_20).

4x Resolving Gel Buffer (RGB): 1.5 M Tris (pH 8.9), 0.4% SDS

4x Stacking Gel Buffer (SGB): 0.5 M Tris (pH 6.7), 0.4% SDS

Resolving gels were prepared by mixing the following:

stock acrylamide/ *bis*-acrylamide	13.6 ml
dH_20	24.0 ml
4x RGB	12.5 ml
10% ammonium persulphate (APS)	0.40 ml
TEMED	0.15 ml

The gel mix was pipetted between the gel plates and overlaid with water-saturated butan-2-ol. After polymerisation (30-60 min) the overlay was removed and the top of the resolving gel washed extensively with distilled water. Excess water was removed using Whatman 3MM paper and the appropriate comb inserted before the stacking gel was poured.

The stacking gel consisted of:

stock acrylamide/ *bis*-acrylamide	3.30 ml
dH_20	11.0 ml
4x SGB	5.00 ml
10% APS	0.40 ml
TEMED	0.04 ml

The stacking gel was allowed to polymerise for approximately 30 min. After polymerisation the comb was carefully removed and the gel transferred to a gel tank. Both reservoirs were filled with 1x SDS-PAGE running buffer (10x SDS-PAGE running buffer, pH 8.3: 522 mM Tris, 4% glycine, 1% SDS). Wells were flushed with running buffer and bubbles removed from the lower surface of the gel plates before the samples were loaded.

Protein samples (20 µl aliquots of lysates from 80% confluent T80 flasks) were denatured at 100°C for 3-5 min in an equal volume of sample buffer (100 mM Tris-HCl (pH 6.8), 20% glycerol, 4% SDS, 2% β-mercaptoethanol and 0.2% (w/v) bromophenol blue), chilled on ice and microcentrifuged at 14,000 rpm for 20 seconds prior to loading. Gels were run at 100 mA, maximum voltage for 6-7 hours with pre-stained protein molecular weight markers (Amersham International plc). Gels were run in duplicate.

Following electrophoresis, the front gel was used for Western blotting (Chapter 2.2.4.3 below) and the back gel was stained, as follows, with Coomassie Brilliant Blue stain to check for equal loading of samples. The back gel was carefully removed from the plates and incubated at room temperature in staining solution (0.1% (w/v) Coomassie Brilliant Blue in 45 : 45 : 10 (v/v) methanol: dH_20: acetic acid) for 30-40 min with gentle shaking. Subsequently, the gel was destained in 45 : 45 : 10 (v/v) methanol: dH_20: acetic acid for 3-4 hr with several changes of destaining solution.

2.2.4.3 Western Blotting

Proteins extracts were resolved on SDS-PAGE gels together with pre-stained molecular weight markers. Proteins were blotted onto Hybond ECL nitrocellulose membranes (Amersham International plc) using a Sartorius semi-dry blotting apparatus according to the manufacturer's instructions. Briefly, twelve pieces of Whatman 3MM paper and a single piece of nitrocellulose membrane were cut to the size of the gel and equilibrated in 1 x transfer buffer (60 mM Tris, 500 mM glycine, 16 mM SDS, 20% (v/v) methanol). Six pieces of Whatman 3MM paper were placed on the blotter and the gel, which had also been rinsed in transfer buffer, was placed on top. The nitrocellulose membrane was placed on the gel and covered with the remaining six pieces of Whatman paper. Air bubbles were carefully removed and transfer effected at full power (200 mA) for approximately 2 hr. The membrane was removed after transfer of pre-stained molecular weight markers was complete and marker sizes were highlighted with a soft-lead pencil.

The nitrocellulose membrane could either be used immediately or alternatively, air-dried and stored at 4°C until required.

The nitrocellulose membrane was blocked for 30 minutes at room temperature in blocking buffer (3% bovine serum albumin (BSA, fraction V) in PBS, 0.1% NP-40 and 0.01% sodium azide) with continuous shaking. Primary antibody (mouse monoclonal anti-phosphotyrosine antibody; UBI, New York) was diluted 1:1000 in PBS as recommended by the suppliers and incubated with the membrane for 4 hr at 4°C with continuous shaking. The membrane was subsequently rinsed with PBS, blocked with blocking buffer for 10 min and rinsed again in PBS before incubation with anti-mouse IgG secondary antibody conjugated to alkaline phosphatase (1:5000) in blocking buffer containing 1.5% BSA for 2 hr at room temperature. The membrane was rinsed once in PBS, three times in blocking buffer and once in alkaline phosphatase buffer (100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 5 mM $MgCl_2$) before development with chromogenic alkaline phosphatase substrate. This substrate was prepared by dissolving 16 mg nitroblue tetrazolium and 8 mg of 5-bromo-4-chloro-3-indolyl phosphate in 50 ml of alkaline phosphatase buffer. Once sufficient intensity of colour had developed the reaction was stopped by rinsing the membrane in water.

2.2.5 Statistical Analyses

Statistical analyses were carried out using the 'Analysis ToolPak' within Microsoft Excel Version 4.0.

CHAPTER 3

TRANSFECTION OF ESTABLISHED MURINE CELL LINES WITH BPV-4 DNA

Chapter 3

TRANSFECTION OF ESTABLISHED MURINE CELL LINES WITH BPV-4 DNA

3.1 Introduction

BPV-4 is the causative agent of papillomatosis of the upper alimentary canal in cattle (Jarrett *et al.*, 1978; Campo *et al.*, 1980). While this virus is necessary in the initial stages of tumour formation, and BPV-4 DNA is present in benign papillomas, viral DNA is rarely detected in carcinomas (Campo *et al.*, 1985). Similar results have been observed in experiments reproducing the malignant conversion of a BPV-4-induced papilloma in the renal capsule of a nude mouse (Gaukroger *et al.*, 1991). Although BPV-4 DNA was present in the papilloma fronds it could not be detected in the carcinoma or in a splenic metastasis derived from the primary malignancy. Analysis of cell lines derived from transfection of a sub-line of the established mouse fibroblast line C127 with BPV-4 genes (detailed below), showed that the majority of transformed lines did not contain viral DNA (Smith & Campo, 1988). All the above data indicate that the virus is required for the early stages of transformation but presence of BPV-4 is not necessary for, or may even interfere with, the progression to and maintenance of the fully transformed state. A similar situation has been described for transformation of C127 cells by human papillomavirus type 6b (HPV-6b) (Morgan *et al.*, 1990). The BPV-4 and HPV-6b results are examples of viral transformation by means of a 'hit and run' mechanism in which the viral genome although involved in initiating cellular events that can lead to the malignant phenotype, may not be consistently maintained or transcribed in the transformed cells. Such a mechanism is also postulated for transformation by herpes simplex virus and cytomegalovirus (Macnab, 1987).

In a previous study, transfection of whole genome BPV-4 into C127 cells gave rise to a number of transformed lines, several of which proved to be tumourigenic in nude mice (Smith & Campo, 1988). Only nine out of 60 cell lines examined contained BPV-4 DNA. Comparison of cell line DNAs from early versus late passages showed that BPV-4 DNA was progressively lost on continued sub-culture. Viral DNA was not detected in the majority of fully transformed cell lines indicating that there was no apparent relationship between the continued presence of BPV-4 DNA and the transformed phenotype. It was proposed that the persistence of BPV-4 DNA in the minority of cell lines was fortuitous and was due to integration into the cell genome. As expression of the viral DNA was not detected in these transformed cells the presence of BPV-4 sequences was considered to be irrelevant to the maintenance of the transformed phenotype (Smith & Campo, 1988).

Further characterisation of the BPV-4-containing lines revealed amplification and rearrangement of specific host sequences (Smith & Campo, 1988; 1989). A rearranged BPV-4 DNA fragment from one of these lines (a secondary transfectant line C4Ta2a) was cloned and analysed (Smith & Campo, 1989). This fragment was found to consist of BPV-4 DNA flanked by 'cellular' sequences. These non-BPV-4 sequences (designated Am sequences) were sub-cloned and radiolabelled Am sequences were used to probe Southern blots of digested DNAs from the various transformed cell lines. Sequences homologous to the Am probe were found to be amplified and rearranged in all the BPV-4-transformed cells analysed (and also in tumour DNAs derived from injection of these lines into nude mice), irrespective of the presence of BPV-4 DNA. These amplified sequences were found to be transferred and maintained in other second and third round transformants in the absence of BPV-4 DNA (Smith & Campo, 1989). In fact C4Ta2a was the only cell line which stably maintained BPV-4 DNA. Interpretation of these results led to the proposal that the amplified DNA found in all these transfected C127 cells was activated in response to BPV-4 transformation and that

this contributed to the maintenance of the transformed phenotype (Smith & Campo, 1989). Amplification of cellular genes is a frequent event in the progression of pre-malignant cells to malignancy (Stark & Wahl, 1984; Alitalo *et al.*, 1986) and is commonly found after treatment with carcinogens or viruses (Kleinberger *et al.*, 1986).

However, cloning and sequencing of a DNA fragment detected by the Am probe and amplified in a tumour induced by a BPV-4-transformed line, unexpectedly revealed that this fragment consisted of 5.2 kb of the BPV-1 genome (nts 7154-4450) integrated into a cellular region which showed homology to murine autonomously replicating sequences (ARS) (Smith *et al.*, 1993). Studies in yeast have identified ARS elements as putative chromosomal origins of DNA replication by virtue of their ability to undergo autonomous replication when cloned into plasmids (Struhl *et al.*, 1979; Stinchcomb *et al.*, 1979; Chan & Tye, 1980). Similar DNA sequences from other eukaryotes have also been shown to function as ARS elements in yeast (Struhl *et al.*, 1979; Botchan & Dayton, 1982; Roth *et al.*, 1983; Montiel *et al.*, 1984). Smith *et al.* (1993) found on subsequent DNA sequencing of the Am DNA probe that it too contained BPV-1 DNA and mouse sequences. Retrospective analysis showed that the sequences amplified in transformed cells were present at single copy level in the parental C127 line (Smith *et al.*, 1993). The parental cells (subsequently termed C127sc) showed none of the properties of transformed cells (Smith & Campo, 1988), indicating that the resident BPV-1 sequences were 'silent'. Northern analysis of mRNAs from both transformed and parental cells showed that while the BPV-1 sequences in the transformed lines were actively transcribed, BPV-1 RNA was not detected in the parental line, although as the BPV-1 sequences present in these cells were at single copy level this negative result could have been due to lack of sensitivity of detection (K. Smith personal communication). The origin of the BPV-1 component of these cells is unknown.

In the above work the possibility that the observed phenotypes were due, in part or whole, to the BPV-1 sequences could not be ruled out, although manifestation of these effects was dependent on transfection with BPV-4 genes. Although BPV-1 and BPV-4 belong to different subgroups (A & B respectively) and data from both immunological and Southern hybridisation studies show there is no cross-reactivity / homology between the two sub-groups (Campo & Jarrett, 1987), it must be considered that the phenotypes and behaviour of BPV-4 DNA was due to interaction (whether direct or indirect) between the two viral genomes. In the above studies BPV-4 DNA was either absent or rearranged and eventually lost (with one exception, C4Ta2a) on continued sub-culture (Smith & Campo, 1988). Amplification of integrated BPV-1 sequences and flanking cellular sequences was also reported. In light of these findings two separate studies were undertaken to investigate the relationship between rearrangement and loss of the BPV-4 genome and also BPV-4 dependent DNA amplification in BPV-1-containing cell lines.

3.2 Experimental Rationale / Methods

The following experimental work was carried out on DNA extracted from transfected cell lines which had previously been derived by a former worker in the laboratory, Dr. K. Smith.

3.2.1 Status of BPV-4 DNA in transfected cell lines

The BPV-1 sequences appeared 'silent' in the parental C127sc cells. However, their activation post-transfection could well play a role as to whether BPV-4 DNA is lost or retained. That such contribution might depend on the level of BPV-1 expression was investigated by DNA analysis of various BPV-4-transfected lines derived from transfection of a cell line (ID14) containing episomal, actively expressing BPV-1 DNA. Southern blot membranes of DNAs from the above cell lines were screened with whole and sub-genomic BPV-4 DNA probes in order to establish whether the frequently

observed 'hit and run' mechanism of BPV-4-induced cell transformation was due to active selection against all or part of the viral genome (and whether the presence of actively transcribed BPV-1 affected maintenance / loss of BPV-4 DNA). If selection processes are involved, an attractive candidate for negative selection is the BPV-4 E8 ORF as previous studies have shown that overexpression of this ORF is detrimental to primary cells (Jaggar *et al.*, 1990; Pennie *et al.*, 1993).

3.2.1.1 Cell lines

The parental line, called ID14, originated from transformation of C127 cells by BPV-1 virions. The BPV-1 DNA in these cells is actively transcribed, stably maintained at approximately 40 genome equivalents per diploid cell and is present exclusively as supercoiled or relaxed circular extrachromosomal DNA molecules (Law *et al.*, 1981). Five BPV-4 clonal lines (ID1 - ID5) were derived by transfection of ID14 cells with 10 μg BamH I linearised pBV4 DNA using a calcium phosphate precipitation technique (K. Smith, personal communication; pBV4 is detailed in Chapter 2.1.7). A plasmid encoding a neomycin resistance gene (pSV2neo) was co-transfected (1 μg) to allow for subsequent selection of transfected cells in medium containing the neomycin analogue G418 (Gibco, BRL). Transformed cells were selected by maintaining cultures in DMEM supplemented with 5% foetal calf serum and 800 μg / ml G418. An initial analysis of these lines confirmed the presence of episomal BPV-1 DNA and also showed that they contained BPV-4 DNA (K. Smith, personal communication). For two of the clonal lines, ID4 and ID5, DNA from early and later passages were compared. The BPV-4 status of the late passage cells had not previously been determined.

DNA from the secondary transfectant line C4Ta2a was also analysed. The origin of the C4Ta2a line is detailed in Smith & Campo (1988). In brief, C4Ta2a is a secondary transfectant line which resulted from an original transfection of C127sc cells with BPV-4 DNA in the presence of the tumour promoter TPA. This gave rise to the cell

line C4Ta. The secondary transfection was carried out using DNA from these C4Ta cells, one of the resulting lines being C4Ta2a. This line is anchorage independent, shows no contact inhibition and is tumourigenic in nude mice. Unlike the majority of BPV-4-transfected cells C4Ta2a was found to maintain rearranged BPV-4 DNA even on prolonged sub-culture.

3.2.1.2 DNA Analysis

Initial probing with whole genome BPV-4 DNA was undertaken to establish the presence and status of this viral DNA in transfected cell lines. Subsequent Southern blot / RFLP mapping of BPV-4 sequences present in the various cell line DNAs was undertaken using a combination of five restriction enzymes (BamH I, EcoR I, Hinc II, Hind III and Pst I) and seven sub-genomic BPV-4 probes (Table 3.1). The restriction enzyme sites in wildtype (i.e. episomal) BPV-4 DNA are detailed diagramatically in Figures 3.1-3.4 together with the sequence specificity of each sub-genomic BPV-4 probe used. The expected detectable fragment sizes for each restriction enzyme / probe combination are detailed in Table 3.2.

Total genomic DNA was extracted from the cell lines as described in Chapter 2.2.2.4. Generally 10 μg of each restriction enzyme digested cell line DNA and appropriate controls were run on 0.8% agarose gels (14 tracks) under standard electrophoretic conditions (described in Chapter 2.2.2.8). Each gel contained a λ Hind III (500 ng) marker lane and a lane containing 1 μg bovine oesophageal papilloma DNA (which contains episomal BPV-4 DNA) digested with one of the five restriction enzymes used for the RFLP mapping analysis. Two BPV-4-transfected cell line DNAs were analysed per gel, with each of the five different restriction enzyme digests being loaded in consecutive tracks. At least one track of an appropriate control (either ID14 DNA for ID1-5 or C127 for C4Ta2a analyses) digested with one of the five restriction enzymes was also included. Due to the large number of separate probings required (i.e. 8-9)

Table 3.1 List of BPV-4 genomic DNA probes used in the study of C4Ta2a and BPV-4 transfected ID14 cell lines.

Probe	Size, region and construct origin[a]	
BPV-4	7.265 kb (whole genome) isolated from pBV4	
BPV-4 LCR	866 bp (nts 6710-310) isolated from p41XLCR+	
BPV-4 E8	355 bp (nts 236-590) isolated from pALTER-E8	
BPV-4 E7	238 bp (nts 669-906) isolated from pURE7	
BPV-4 E1	2009 bp (nts 1139-3147) isolated from EcoR I digested circular BPV-4 DNA	
BPV-4 E2	836 bp (nts 2979-3814) isolated from pURE2	
BPV-4 L2a	380 bp (nts 4610-4989) isolated from pURL2a	Equal quantities of L2a, b and c used for L2 probe
BPV-4 L2b	569 bp (nts 4042-4610) isolated from pURL2b	
BPV-4 L2c	641 bp (nts 4989-5629) isolated from pURL2c	
BPV-4 L1a	788 bp (nts 5737-6524) isolated from pURL1a	Equal quantities of L1a, b and c used for L1 probe
BPV-4 L1b	111 bp (nts 5627-5737) isolated from pURL1b	
BPV-4 L1c	824 bp (nts 6524-0082) isolated from pURL1c	

[a] All probe constructs are detailed in Chapter 2.1.7

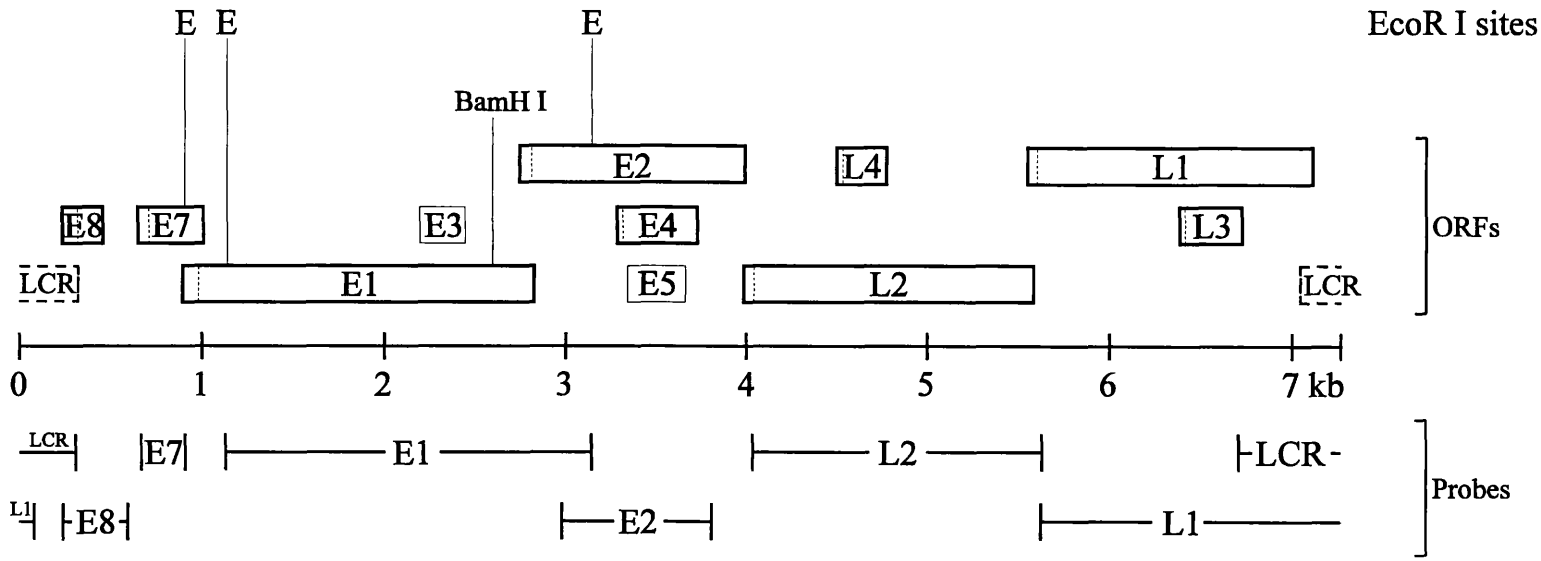


Figure 3.1 Genomic organisation of BPV-4 together with EcoR I restriction sites (above scale) and specificity of sub-genomic BPV-4 DNA probes (below scale). The viral genome (7.265 kb) is represented as linear. Closed boxes represent ORFs and the dashed box the long control region (LCR). The early ORFs are designated E, and the late ORFs L. The first ATG codon in an ORF is indicated by a vertical dashed line; ORFs without an ATG codon are represented as thin lined boxes. The position of the single BamH I site is also indicated.

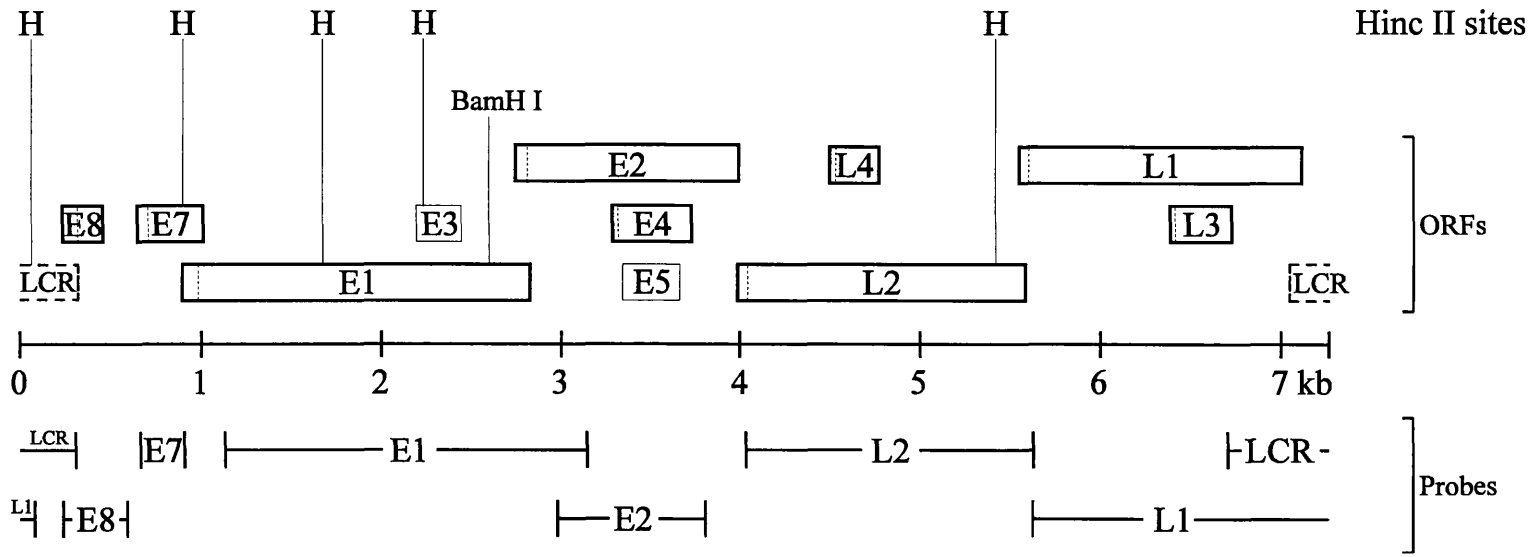


Figure 3.2 Genomic organisation of BPV-4 together with Hinc II restriction sites (above scale) and specificity of sub-genomic BPV-4 DNA probes (below scale). The viral genome (7.265 kb) is represented as linear. Closed boxes represent ORFs and the dashed box the long control region (LCR). The early ORFs are designated E, and the late ORFs L. The first ATG codon in an ORF is indicated by a vertical dashed line; ORFs without an ATG codon are represented as thin lined boxes. The position of the single BamH I site is also indicated.

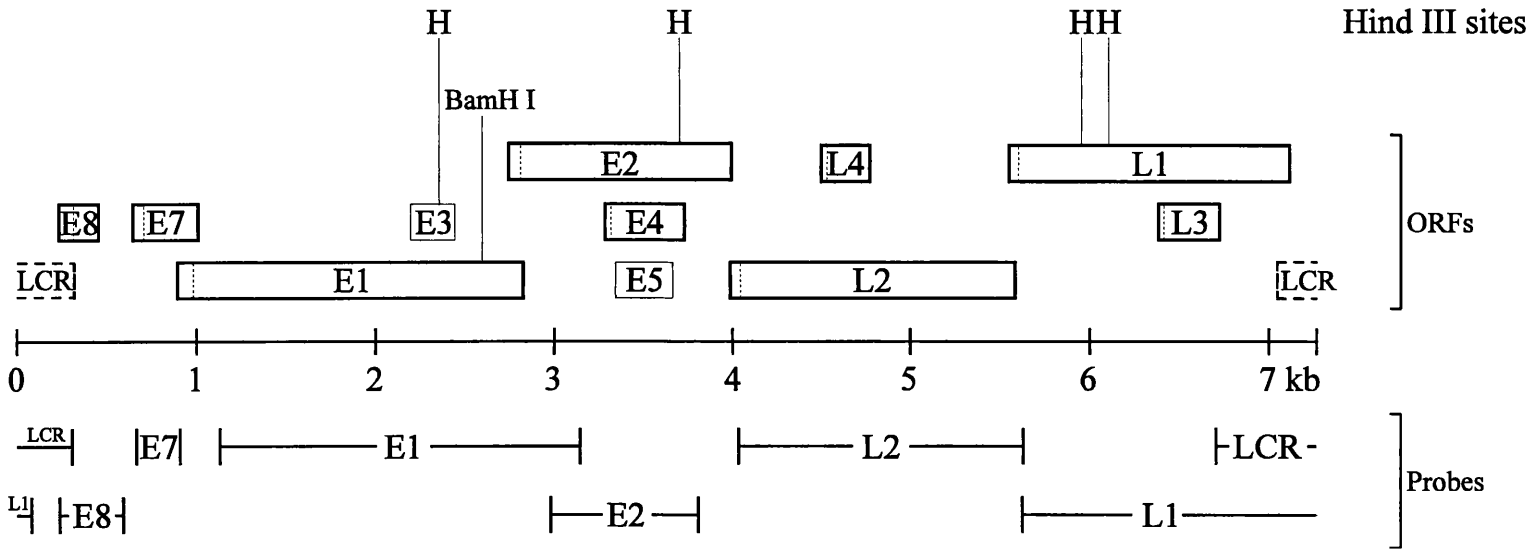


Figure 3.3 Genomic organisation of BPV-4 together with Hind III restriction sites (above scale) and specificity of sub-genomic BPV-4 DNA probes (below scale). The viral genome (7.265 kb) is represented as linear. Closed boxes represent ORFs and the dashed box the long control region (LCR). The early ORFs are designated E, and the late ORFs L. The first ATG codon in an ORF is indicated by a vertical dashed line; ORFs without an ATG codon are represented as thin lined boxes. The position of the single BamH I site is also indicated.

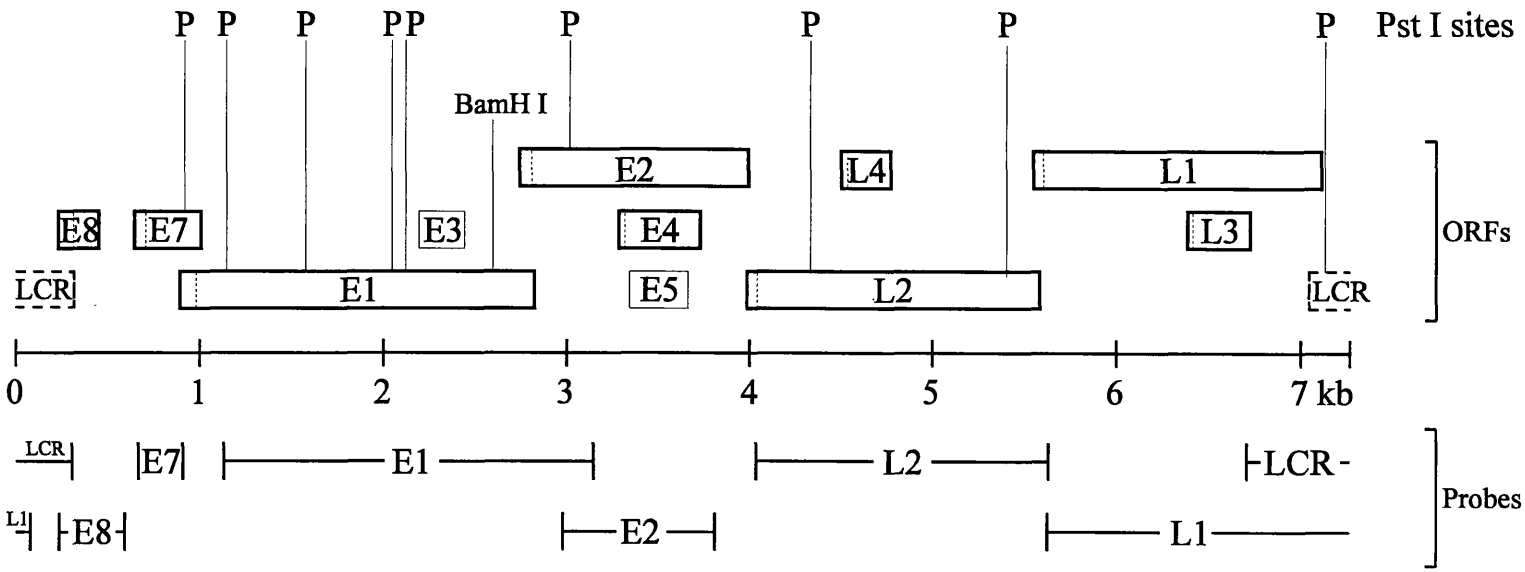


Figure 3.4 Genomic organisation of BPV-4 together with Pst I restriction sites (above scale) and specificity of sub-genomic BPV-4 DNA probes (below scale). The viral genome (7.265 kb) is represented as linear. Closed boxes represent ORFs and the dashed box the long control region (LCR). The early ORFs are designated E, and the late ORFs L. The first ATG codon in an ORF is indicated by a vertical dashed line; ORFs without an ATG codon are represented as thin lined boxes. The position of the single BamH I site is also indicated.

Table 3.2 Restriction fragment lengths of episomal BPV-4 DNA expected to be detected with whole and sub-genomic BPV-4 DNA probes. (Values are given in kilobase pairs. Probes are more fully described in Table 3.1 and Figures 3.1-3.4).

	BPV-4 probes							
Digest	BPV-4	LCR	E8	E7	E1	E2	L2	L1
BamH I	7.265	7.265	7.265	7.265	7.265	7.265	7.265	7.265
EcoR I	0.233	5.024	5.024	0.233	2.008	2.008	5.024	5.024
	2.008			2.008		5.024		
	5.024			5.024				
Hinc II	0.562	0.835	0.835	0.775	0.562	3.186	1.907	0.835
	0.775	1.907		0.835	0.775		3.186	1.907
	0.835				3.186			
	1.907							
	3.186							
Hind III	0.152	3.511	3.511	3.511	1.345	1.345	2.257	0.152
	1.345				3.511	2.257		2.257
	2.257							3.511
	3.511							
Pst I	0.075	1.052	1.052	0.227	0.075	0.891	1.077	1.052
	0.227	1.723		0.430	0.227	1.317	1.317	1.723
	0.430			1.052	0.430		1.723	
	0.473				0.473			
	0.891				0.891			
	1.052				1.317			
	1.077							
	1.317							
	1.723							

duplicate gels were prepared. Subsequent Southern blotting, hybridisation and autoradiographic conditions were carried out as detailed in Chapters 2.2.2.9-2.2.2.13. In all cases hybridised membranes were washed to a final stringency; 0.1 x SSC, 0.1% SDS for 30 min (2 x 15 min) at 65°C.

3.2.2 BPV-4-mediated amplification

Analysis of an amplified DNA fragment observed in C127sc cells transfected with BPV-4 DNA showed that this amplified region consisted of BPV-1 sequences flanked by cellular sequences homologous to autonomously replicating sequences (ARS) (Smith & Campo, 1988; Smith *et al.*, 1993). The BPV-1 sequences present encompassed the E6, E7, E1 and E2 ORFs, the origin of viral DNA replication (mapped to nt 7730 ± 100; Yang & Botchan, 1990) and part of the upstream regulatory region (the long control region; LCR). Cloning and later transfection of this amplified fragment (designated HL-10) showed that it was capable of inducing focus formation in C127 cells but, unlike BPV-1 DNA, could only transform primary bovine fibroblasts (PalF cells) in co-operation with an activated human Ha- *ras* gene (Smith *et al.*, 1993). This suggested that although the transformation capability of HL-10 resided within the BPV-1 ORFs, this ability may be influenced by the incomplete nature of the BPV-1 sequences and / or surrounding mouse sequences. The ability of BPV-4 DNA to induce amplification in these cells could be due to interaction with the ARS-like sequences or a cellular intermediate. However, possible interaction between BPV-4 products and BPV-1 sequences cannot be excluded. Therefore the following study was carried out in another cell line (ΔM9) which also contained integrated BPV-1 sequences in order to investigate whether BPV-4 was capable of amplifying BPV-1 DNA resident in these cells.

3.2.2.1 Cell lines

As already described for the ID14 work, the following transfected cell lines and DNA samples prepared from these lines had been previously isolated by Dr. K. Smith. The

parental line, ΔM9, (a gift from Dr. S. Burnett, Dept. of Medical Genetics, University of Uppsala) was derived by transformation of C127 cells with BPV-1 DNA. The BPV-1 DNA transfected into these cells had a 277 bp deletion between nts 4128-4406. This deletion involved the early gene mRNA polyadenylation site and part of the L2 ORF. The viral DNA had been digested with EcoR I prior to transfection, thus disrupting the E1 ORF. ΔM9 cells were found to contain 2-4 integrated copies of the mutant BPV-1 genome and displayed a flat non-transformed morphology (Burnett *et al.*, 1988).

Nine test clonal lines derived from transfection of ΔM9 cells with various BPV-4 genes were analysed. The viral regions transfected included:

whole genome BPV-4	- one clone
a sub-genomic fragment containing BPV-4 E8, E7	- three clones
a sub-genomic fragment containing BPV-4 E8	- two clones
a sub-genomic fragment containing BPV-4 E7	- two clones
a sub-genomic fragment containing BPV-4 E8, E7 plus part of the L1 and the E1 ORFs	- one clone

All the above viral genes were transfected as recombinant plasmids, (detailed in Figure 3.5) using a calcium phosphate precipitation protocol (K. Smith, personal communication). The whole genome BPV-4 construct and the sub-genomic construct pAT H-H3.6 (see Figure 3.5) were each transfected along with the selectable marker gene for neomycin resistance (pZipneo; Chapter 2.1.7). All other constructs already contained a gene encoding neomycin resistance. Each reaction contained 10 μg of the relevant plasmid DNA (plus, if appropriate, 1 μg pZipneo DNA). Control cell lines included two clones from a 'neo only' transfection and cells from a 'mock' (i.e. no DNA) transfection. Transformed cells were selected by growing cultures in DMEM supplemented with 2% foetal calf serum and 800 μg / ml G418 (see Chapter 2.1.3).

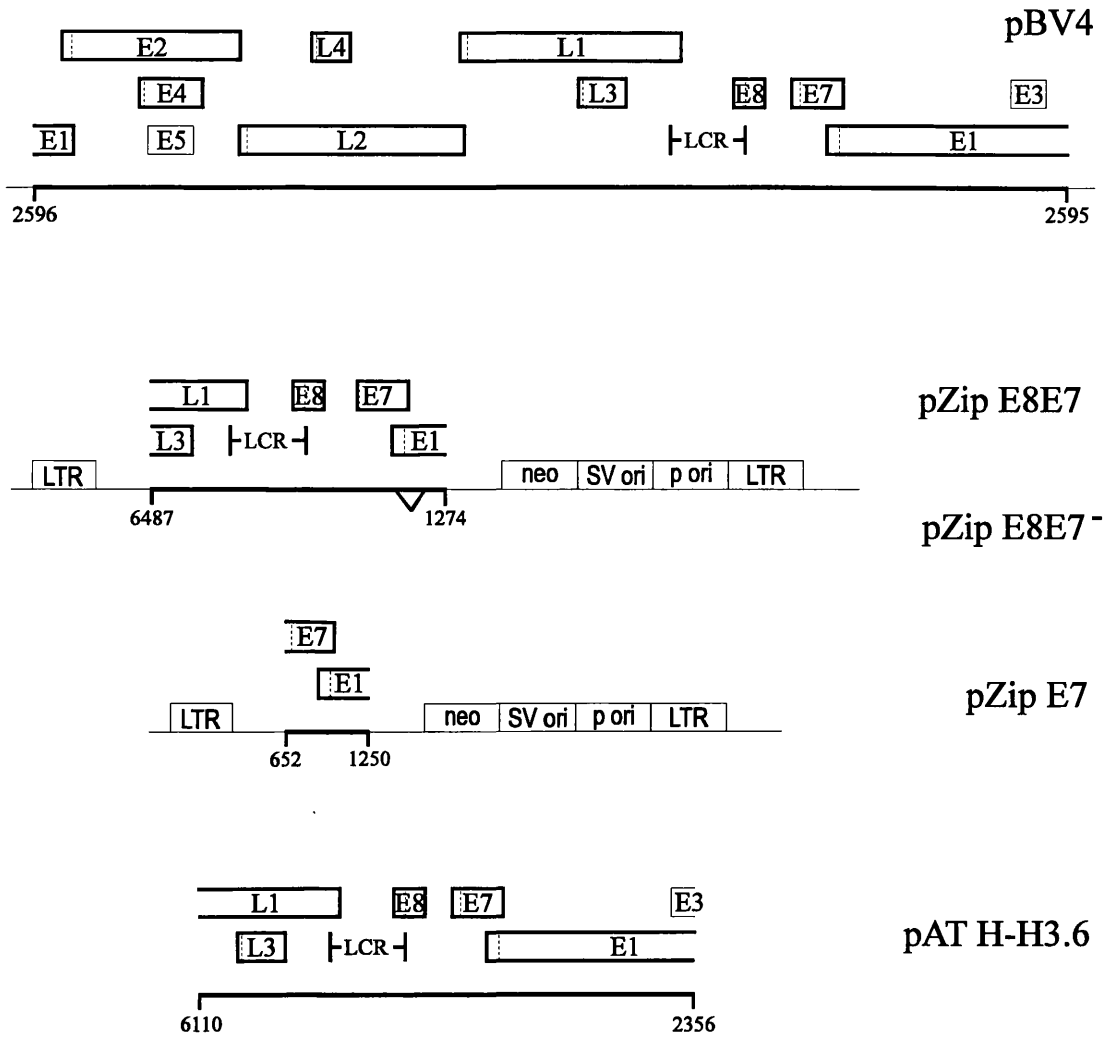


Figure 3.5 BPV-4 sequences present in transfected plasmid constructs.

Notes: pBV4 contains the whole genome cloned into the BamH I site of pAT153 (Campo & Coggins, 1982). pZipE8E7 contains the Xho II fragment cloned into the BamH I site of pZipneoSV(XI) (Jaggar *et al.*, 1990). pZipE8E7⁻ contains the Xho II fragment cloned into the BamH I site of pZipneoSV(XI) with a deletion spanning nts 905-1138 - symbolised by an inverted triangle (Jaggar *et al.*, 1990). pZipE7 contains nts 652-1250 cloned into the BamH I site of pZipneoSV(XI) (Pennie *et al.*, 1993). pAT H-H3.6 contains a Hind III fragment cloned into the Hind III site of pAT153 (Smith *et al.*, 1986).

E = early ORFs; L = late ORFs; LCR = long control region; LTR = MoLV LTR; neo = neomycin / G418 resistance gene; p ori = plasmid DNA replication origin; SV ori = SV40 DNA replication origin. Numbers refer to BPV-4 map co-ordinates, thick lines = viral sequences; thin lines = vector sequences.

3.2.2.2 DNA Analysis

Southern blot membranes of digested DNAs from the above cell lines were screened with BPV-4 DNA, BPV-1 DNA, murine ARS sequences and a murine c-Ha-*ras* DNA probe (acting as a loading control).

3.2.2.3 DNA probes

Whole genome BPV-4

This probe consisted of the full BPV-4 genome (7.265 kb). It was isolated by BamH I digestion of the plasmid construct pBV4. Details of this construct are given in Chapter 2.1.7.

Whole genome BPV-1

This probe consisted of the full BPV-1 genome (7.945 kb). It was isolated by Hind III digestion of the plasmid construct pBV1a (Campo & Coggins, 1982). Details of this construct are given in Chapter 2.1.7.

murine ARS

This probe consisted of a 719 bp fragment containing the ARS-like sequences 3' to the BPV-1 sequences in HL-10. It was isolated by Pst I / BamH I digestion of the plasmid construct pIC-ARS (Smith *et al.*, 1993). Details of this construct are given in Chapter 2.1.7.

c-Ha-ras

This probe consisted of a 576 bp cDNA fragment covering exons 1-4 of the murine c-Ha-*ras* gene. It was isolated by Not I / BamH I digestion of the construct pBS*ras* (gift from W. Lambie, Beatson Institute, Glasgow). This probe was used as an indicator of DNA loading levels. Details of this construct are given in Chapter 2.1.7.

3.2.2.4 Assay conditions

Three restriction enzyme DNA digests (BamH I, EcoR I and Hind III) were carried out per cell line. These enzymes were chosen as they have single restriction enzyme sites in BPV-1 and have relatively few enzyme sites in the BPV-4 genome. Generation of simple banding patterns was required to allow assessment of whether BPV-1 DNA amplification had occurred. Likewise, screening of membranes with the BPV-4 probe was to investigate the presence of BPV-4 DNA in these cells. Mapping of BPV-4 genes was not required.

Total genomic DNA was extracted from the cell lines as described in Chapter 2.2.2.4. Approximately 10 μg of each restriction enzyme-digested test cell line DNA and appropriate controls were run on 0.8% agarose gels (14 tracks) under standard electrophoretic conditions. Each gel contained a λ Hind III (500 ng) marker lane and a number of control tracks. These included similarly digested DNA from the parental cell line, ΔM9, and digested DNAs from both neo alone transfectants and cells exposed to the transfection solutions (in the absence of DNA). Positive control tracks for BPV-1 DNA contained 10 μg of digested ID14 DNA, while those for BPV-4 DNA contained 1 μg of similarly digested bovine oesophageal papilloma DNA which is known to contain episomal BPV-4 DNA. Tracks with papilloma DNA also contained 10 μg BamH I digested C127 DNA which acted as a carrier. On average two BPV-4-transfected cell line DNAs were analysed per gel with each gel being prepared in duplicate. Subsequent Southern blotting, hybridisation and autoradiographic conditions were carried out as detailed in Chapters 2.2.2.9-2.2.2.13. In all cases hybridised membranes were washed to a final stringency; 0.1 x SSC, 0.1% SDS for 30 min (2 x 15 min) at 65°C.

3.3 Results and Discussion

3.3.1 ID14 transfectants

In common with previous *in vitro* studies (Smith & Campo, 1988), BPV-4 DNA was found to be initially present in the ID14 transfectants, but in clones where different passages were looked at (i.e. ID4 and ID5) it was found to be progressively lost on continued sub-culture (Figure 3.6; tracks 9-11 and 6-8 respectively). In both clonal lines no detectable BPV-4 DNA was found by passage 19. These late passage DNAs were not analysed further. That the result of BPV-4 transfection was similar in both C127sc cells (Smith & Campo, 1988) and ID14 cells (i.e. BPV-4 DNA is lost on continual sub-culture) suggests that the level of BPV-1 expression does not influence the maintenance of BPV-4 DNA. Whatever the selection process against BPV-4, it is still operational regardless of the level of BPV-1 expression.

The amount of BPV-4 DNA present differed between cell lines, ranging from approximately 1-2 copies per diploid genome (lines ID2 and ID3) to greater than 100 genome equivalents (g.e.) in line ID4 (Figure 3.7). The high molecular weight bands detected in four out of the five cell lines suggests that the BPV-4 DNA is integrated into the cellular genome (Figures 3.6 and 3.7). The remaining cell line (ID4) showed multiple bands (Figure 3.6; track 9) which is also indicative of integration. However the most prominent band observed, which migrated as for oesophageal papilloma (episomal BPV-4) DNA, could be due to either episomal or integrated tandemly repetitive viral sequences.

Results of the viral mapping of all lines showed no obvious rearrangements within the LCR and the E8, E7, L1 and L2 ORFs. As previous studies had shown that overexpression of the E8 ORF was detrimental to PalF cells (Jaggar *et al.*, 1990; Pennie *et al.*, 1993), this ORF was considered to be a prime candidate for negative selection. However, as noted above, no evidence from the Southern blot analyses specifically

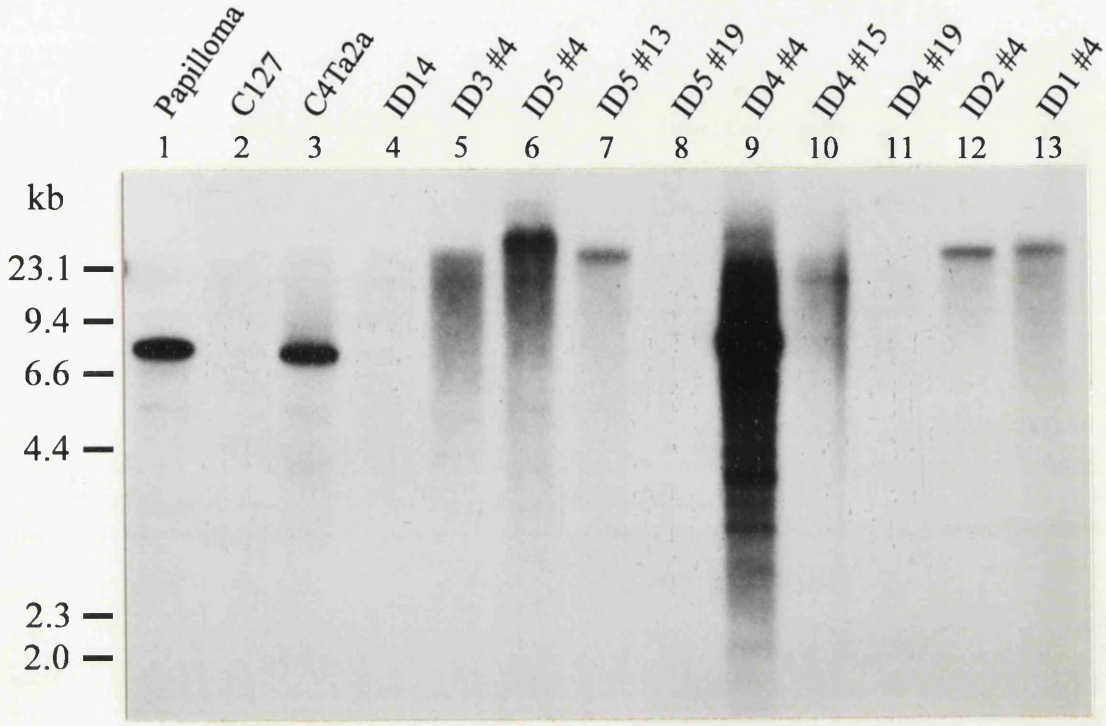


Figure 3.6 Southern blot analysis of ID14 transfectants and cell line C4Ta2a for maintenance of BPV-4 DNA.

Tracks 2-13 contain 10 μg of BamH I digested DNA. Track 1 contains 1 μg of BamH I digested bovine oesophageal papilloma DNA (which contains episomal BPV-4 DNA) + 9 μg of BamH I digested carrier DNA (C127 DNA). The probe used was whole genome BPV-4 DNA (see Table 3.1). The positions of λ Hind III digested DNA molecular weight markers are indicated in the left margin.

#x denotes passage number post transfection.

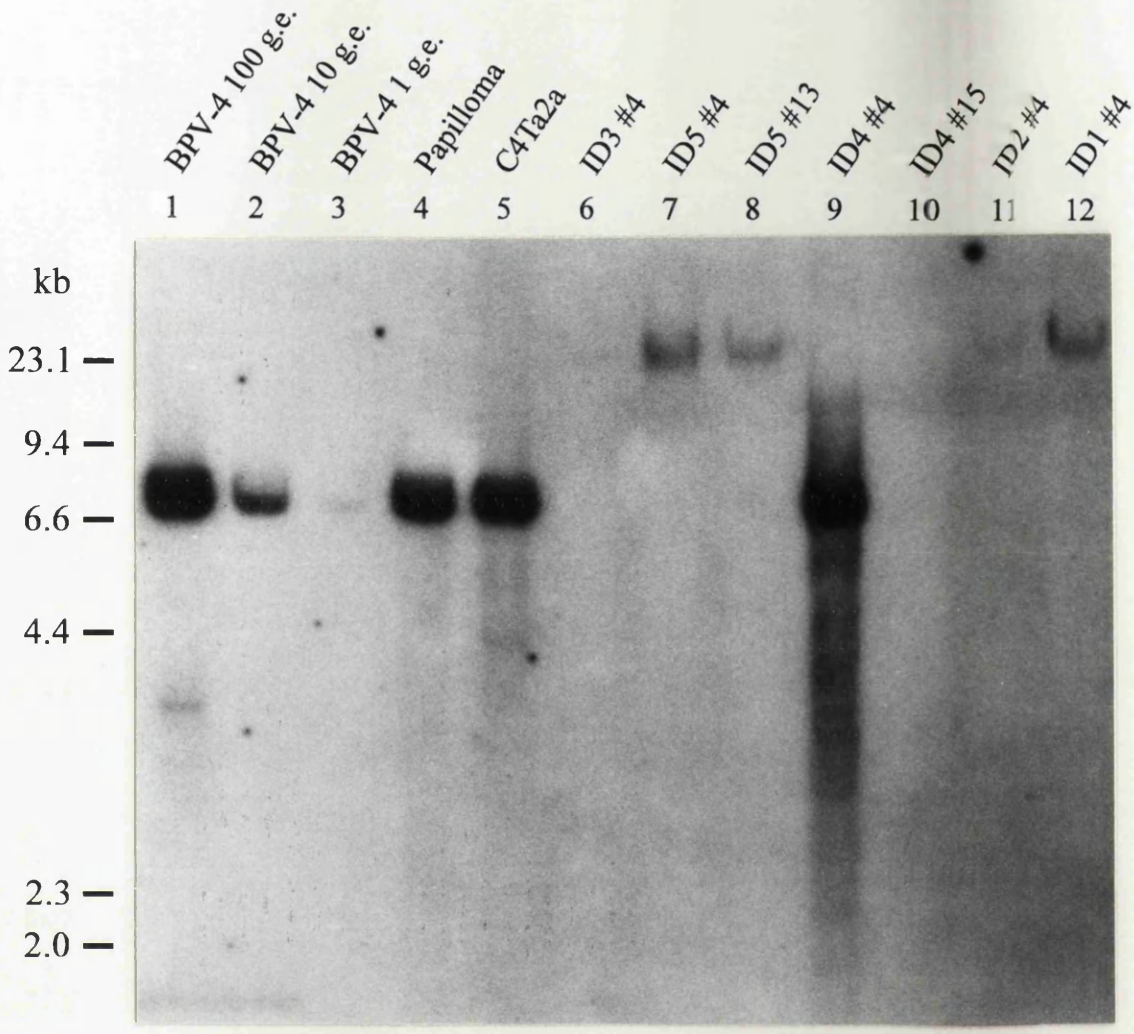


Figure 3.7 Southern blot analysis of ID14 transfectants and cell line C4Ta2a for the presence and quantification of BPV-4 sequences.

Tracks 1-3 contain respectively 100, 10 and 1 genome equivalents (g.e.) of BamH I digested BPV-4 DNA + 5 μg BamH I digested carrier (ID14) DNA. Track 4 contains 200 ng of BamH I digested bovine oesophageal papilloma DNA (which contains episomal BPV-4 DNA) + 5 μg of BamH I digested carrier (ID14) DNA. Tracks 5-12 contain 5 μg of BamH I digested DNA from the indicated cell lines. The probe used was whole genome BPV-4 (see Table 3.1). The positions of λ Hind III digested DNA molecular weight markers are indicated in the left margin.

#x denotes passage number post transfection.

implicated the E8 ORF. Thus if E8 functions are selected against this does not happen through major deletions of this ORF. However the analyses showed that in all lines major rearrangements had taken place between the 3' end of the E1 ORF and the 5' end of the E2 ORF. For example the result of E1 probing of digested DNAs from line ID4 is shown in Figure 3.8. Note that the BPV-4-specific bands observed in track 7 are markedly different from those seen in the papilloma positive control (track 1) for Pst I digested DNA. Similarly, unexpected bands were observed in other digests. The site of disruption could be narrowed down to a 661 bp region between the Hind III site (BPV-4 genome nt 2356) and the Pst I site (BPV-4 genome nt 3017). As, in all cases, the viral DNA had been linearised within the E1 ORF (BamH I site nt 2597) prior to transfection this would suggest that the BPV-4 DNA had integrated into the mouse genome via this site. It must be noted that this mapping technique is relatively insensitive and that small deletions and / or point mutations will be undetected. Likewise small inversions or duplications will also remain undetected if they do not affect the sequence recognition sites of the particular restriction enzyme used. All that can be stated is that there are no major rearrangements within the BPV-4 sequences present in these lines apart from in the E1 ORF which contains the BamH I site.

As regards the secondary transfectant line C4Ta2a, probing of BamH I digested DNA from this line with either whole genome BPV-4 or sub-genomic fragments showed a band of approximately 7 kb, which is less than the expected 7.265 kb (e.g. Figure 3.6; tracks 1 & 3 and Figure 3.9; tracks 1 & 6). This suggested the C4Ta2a line carries a deleted form of the viral genome. Digestion of C4Ta2a DNA with Kpn I, an enzyme which does not cut within the viral genome, and comparison of the sample's electrophoretic mobility to that of similarly digested bovine oesophageal papilloma DNA, indicated that the BPV-4 DNA present in this cell line had integrated into the cellular genome (Figure 3.9; tracks 13 & 14). This confirmed findings of Smith & Campo (1988). Although most ORFs appeared wildtype, digestion of C4Ta2a DNA with

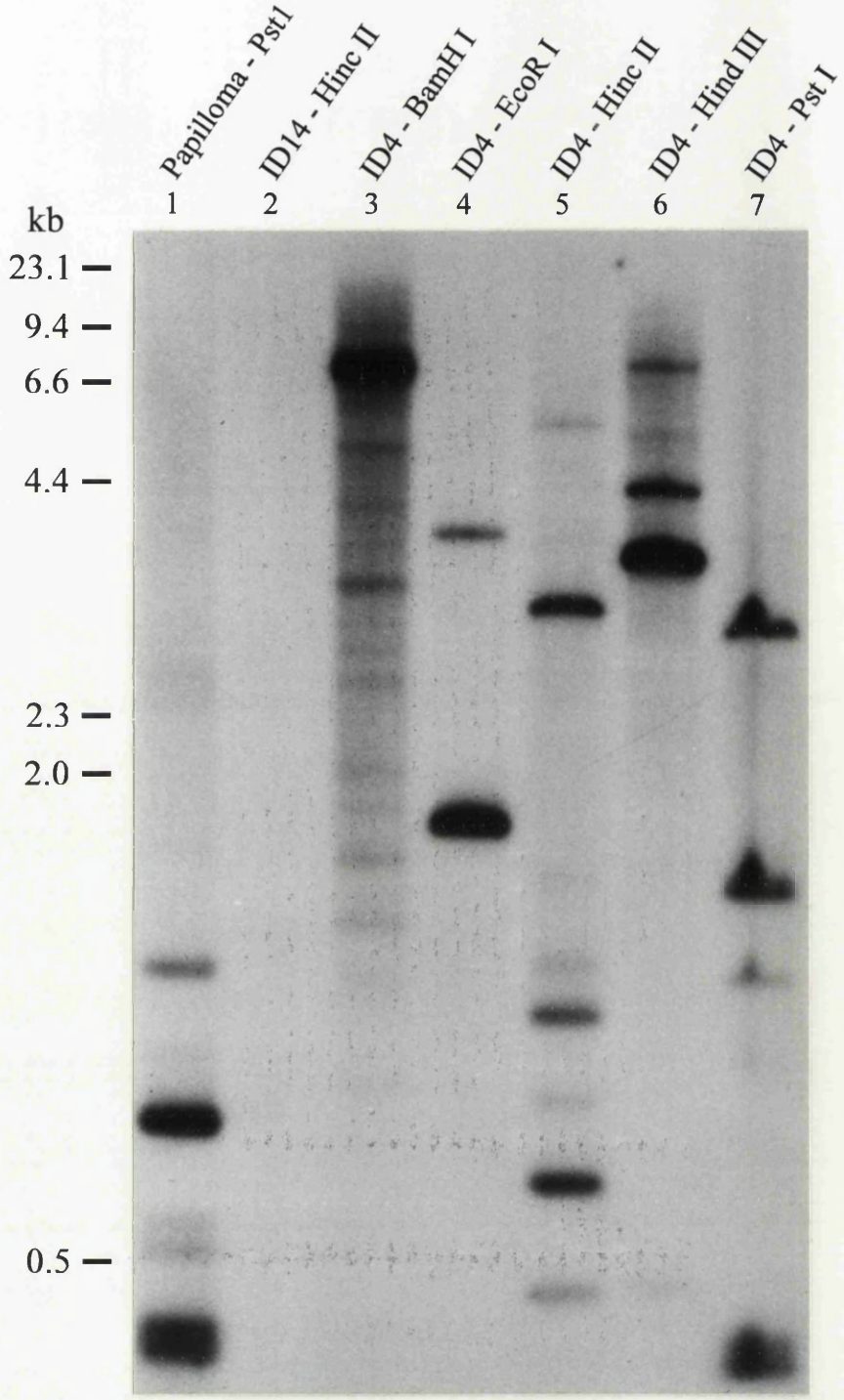


Figure 3.8 Southern blot analysis of cell line ID4 (passage 4) for the presence and status of BPV-4 E1 DNA.

Tracks 2-7 contain 5 μg of ID4 DNA digested with the indicated restriction enzymes. Track 1 contains 1 μg of Pst I digested bovine oesophageal papilloma DNA (which contains episomal BPV-4 DNA) + 4 μg of BamH I digested carrier DNA (C127 DNA). The probe used was BPV-4 E1 DNA (see Table 3.1). The positions of λ Hind III digested DNA molecular weight markers are indicated in the left margin.

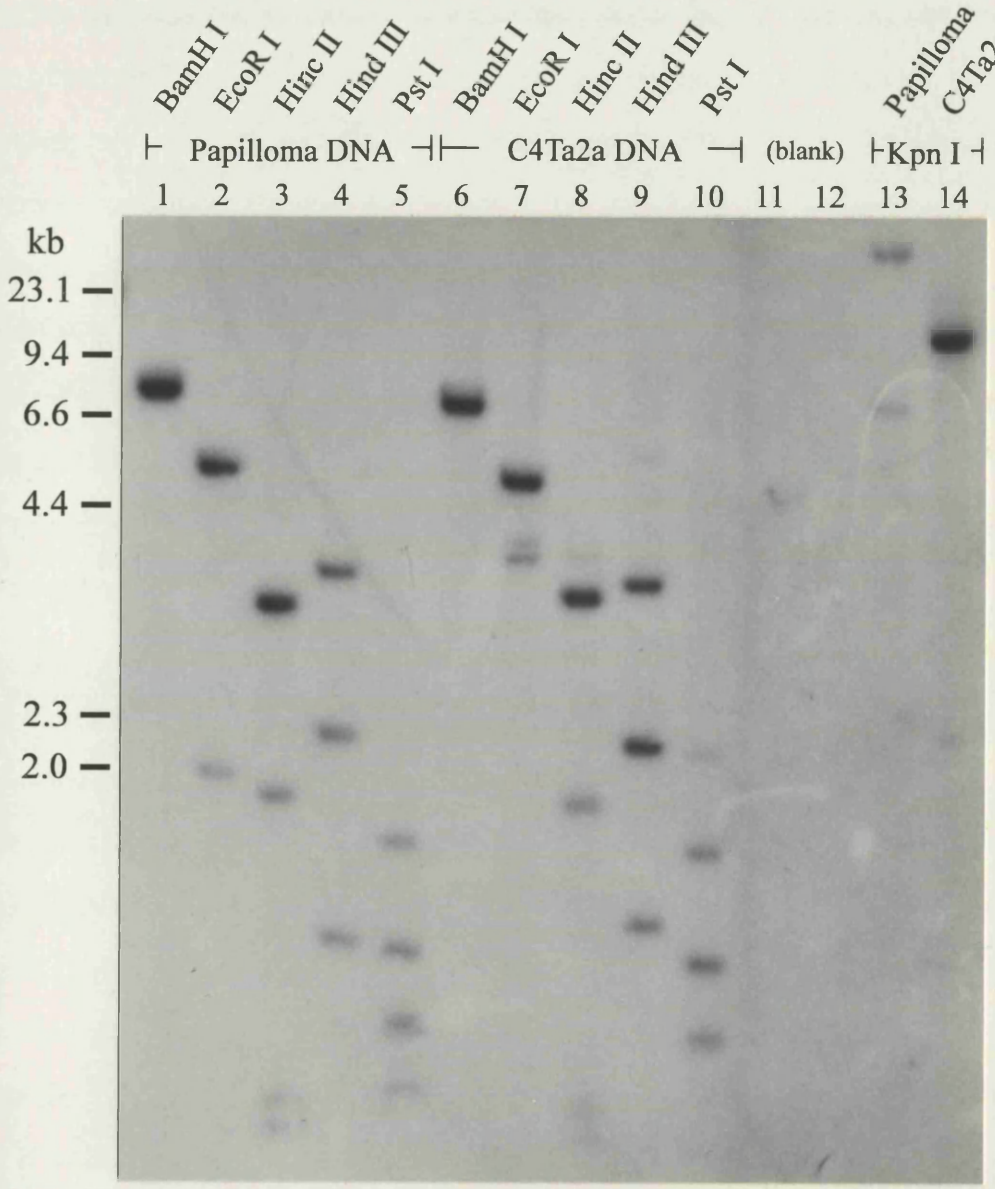


Figure 3.9 Southern blot analysis of cell line C4Ta2a for the presence and status of BPV-4 DNA.

Tracks 6-10 & 14 contain 5 μg of C4Ta2a DNA digested with the indicated restriction enzymes. Tracks 1-5 & 13 contain 200 ng of bovine oesophageal papilloma DNA (which contains episomal BPV-4 DNA) digested as indicated + 5 μg of BamH I digested carrier DNA (C127 DNA). The probe used was whole genome BPV-4 DNA (see Table 3.1). The positions of λ Hind III digested DNA molecular weight markers are indicated in the left margin.

restriction enzymes that cut within or on either side of the E1 ORF showed that this region of the viral genome was extensively disrupted, suggesting that the viral DNA had integrated into the cellular genome at the viral BamH I site. Hybridisation of membranes to whole genome BPV-4 DNA revealed that the expected 2008 base pair (bp) EcoR I fragment was absent and a new band of approximately 3800 bp was detected (Figure 3.9; cf. tracks 2 & 7). This is in agreement with Smith & Campo (1988) who found that this fragment contained BPV-4, BPV-1 and ARS sequences (Smith & Campo, 1988; 1989). The authors showed the BPV-4 DNA present in this secondary transfectant line appeared to have integrated within the region found to have been amplified post BPV-4 transfection.

Using the experimental technique described above it has not been possible to pin-point the deleted region suggested by BamH I digestion. As observed for the ID14 transfectants, the viral mapping of C4Ta2a detected only a single site of disruption within the BPV-4 genome, namely a 661 bp region between nts 2356-3017 of the viral genome. This would indicate that the suggested deletion of viral sequences in this cell line had occurred within this 661 bp region. This would affect the 3' end of the E1 ORF and /or the 5' end of the E2 ORF. As the C4Ta2a line is unusual in maintaining BPV-4 DNA it would be of great interest to further identify the suggested deletion site as it could be argued that if there is active selection against the virus, lack of a particular viral region in C4Ta2a might render the remainder 'immune' from such selection. Alternatively there may be active selection for maintenance of BPV-4 DNA in this line due to its site of integration within the cellular genome. Definitive experiments to address whether the C4Ta2a cell line does indeed contain a deleted form of the BPV-4 genome would include running a series of gels both longer in length and at lower percentage agarose than those used in the above experiments. If this result is confirmed it would be of interest to undertake fine mapping and / or sequencing of the identified disrupted 661 bp region.

3.3.2 BPV-4-induced cellular amplification

Results from the whole genome BPV-4 probing showed that none of the ΔM9 transfectants retained BPV-4 DNA (data not shown). Interestingly, although not retaining BPV-4 DNA, ΔM9 cells transfected with the E8 construct survived. This is in contrast to the behaviour of primary bovine fibroblasts (PalF cells) transfected with the same plasmid construct (Jaggar *et al.*, 1990; Pennie *et al.*, 1993). Such transfected primary cells die more rapidly than control classes. This may be due to inherent differences between primary and established lines, most clearly manifest in that established lines are immortal whereas primary cultures have limited lifespan. It could also be proposed that transfection of the BPV-4 E8 gene into ΔM9 cells causes increased expression of the BPV-1 sequences resident in these cells, resulting in transformation and subsequent tolerance of the otherwise 'toxic' effect of BPV-4 E8 expression.

There was no evidence that amplification of resident BPV-1 sequences had occurred. This was quantitatively determined by densitometric scanning of autoradiographs, comparing signal intensity of bands seen on probing membranes with whole genome BPV-1 DNA (Figure 3.10; Panel A) with those resulting from the murine c-Ha-*ras* probing (Figure 3.10; Panel B). The BPV-1 sequences in the C127sc line described in Smith & Campo (1989) and Smith *et al.* (1993) have an intact origin of replication. As such it is possible that the amplification observed in the C127sc transfected cells was initiated from this origin by direct interaction with a BPV-4-encoded function, or indirectly through BPV-4-directed transactivation of a BPV-1 protein required for DNA replication, probably the E1 protein (Lusky & Botchan, 1986). ΔM9 cells contain 2-5 copies of integrated BPV-1 DNA with an intact origin of replication but a disrupted E1 ORF. That no amplification of these BPV-1 sequences occurs after BPV-4 transfection gives weight to the proposal that BPV-4 products do not act directly on the BPV-1 replication origin but may involve BPV-1 E1. The ability of BPV-4 to amplify DNA is

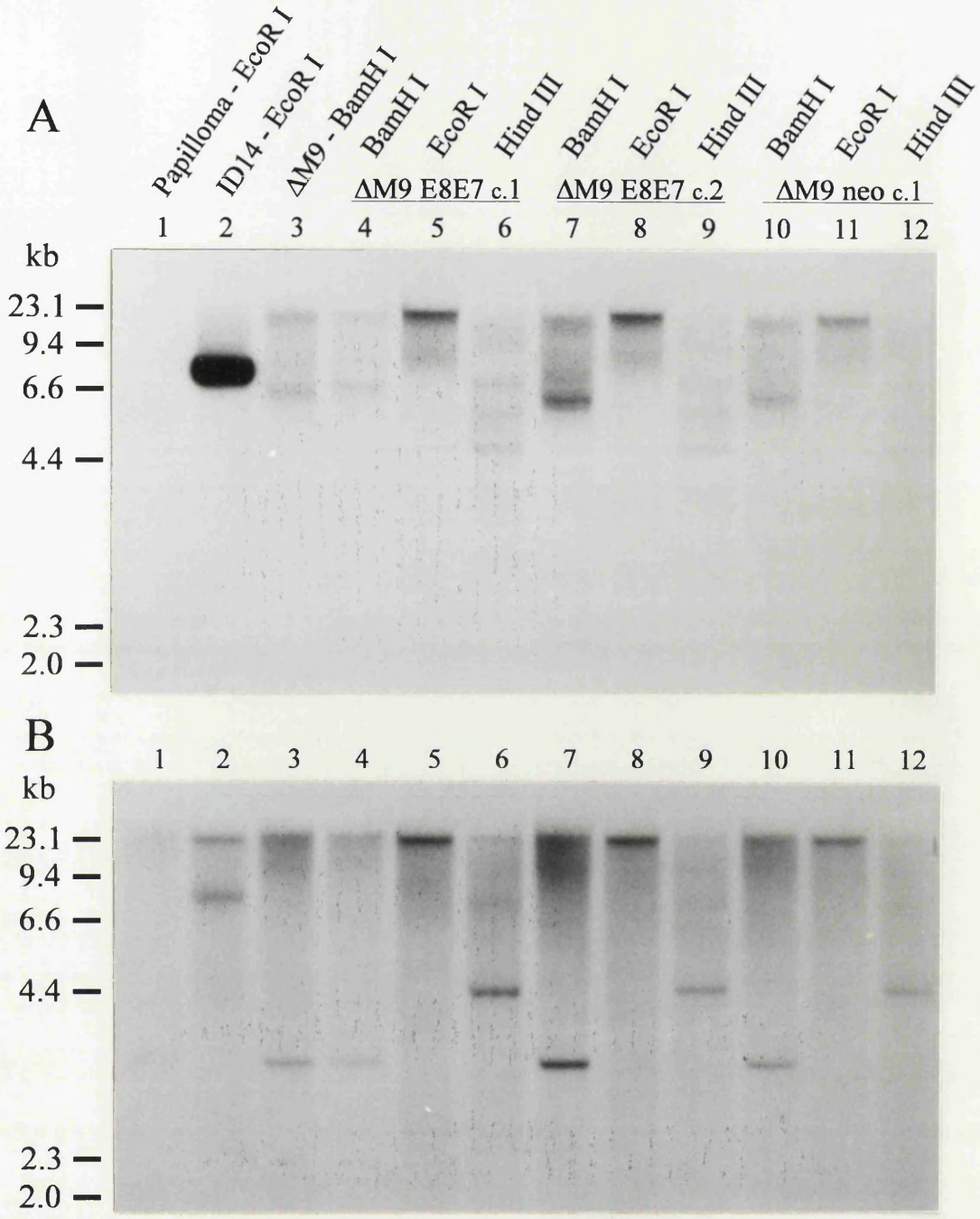


Figure 3.10 Southern blot analysis of ΔM9 transfectants for evidence of BPV-4-mediated amplification of resident BPV-1 sequences.

Tracks 2-12 contain 10 μg of DNA digested with the indicated restriction enzymes. Track 1 contains 1 μg of bovine oesophageal papilloma DNA (which contains episomal BPV-4 DNA) digested with EcoR I + 9 μg of BamH I digested carrier DNA (C127 DNA). c = clone. **Panel A**: Probed with whole genome BPV-1 DNA (see Chapter 3.2.2.3). **Panel B**: Probed with murine c-Ha-*ras* DNA as loading control (see Chapter 3.2.2.3).. The positions of λ Hind III digested DNA molecular weight markers are indicated in the left margin.

not confined to BPV-1 as it has been observed that transfection of an SV40-transformed cell line CL10 (Campo *et al.*, 1978) results in amplification of the SV40 DNA (K. Smith, personal communication).

Probing with the murine ARS probe gave general smeared signal with no obvious amplification of any region. This inconclusive result does not rule out a role for ARS-like elements in BPV-4-induced amplification. Viral integration into ARS-like sequences has been described in cells derived from C57BL/6 mice (Agrawal *et al.*, 1992). The subsequent amplification of both viral and cellular sequences in these cells led the authors to suggest that viral integration into ARS sequences may be important in establishing the transformed state. Although probing of the ΔM9 transfectants, which are known to contain integrated BPV-1 DNA, with a murine ARS probe did not reveal any amplification, the cellular sites of BPV-1 integration have not yet been identified in these cells and thus may not even involve ARS-like sequences.

Results from the above two studies involved investigation of the transformation biology of BPV-4 in the presence of BPV-1 sequences. Although examination of the presence and status of BPV-4 DNA and possible virally-induced amplification of cellular sequences in cells which do not contain BPV-1 DNA would be of interest, it was decided to focus on the transformation biology of BPV-4 in primary cells derived from the viral host species. This formed the main body of work of this thesis, as detailed in the following chapters.

CHAPTER 4

CO-OPERATION BETWEEN PAPILLOMAVIRUSES AND CHEMICAL CO-FACTORS IN CARCINOGENESIS

Chapter 4

CO-OPERATION BETWEEN PAPILLOMAVIRUSES AND CHEMICAL CO-FACTORS IN CARCINOGENESIS

4.1 Introduction

Cancer is a multistage, multifactorial process.

As most cancers are somatic in origin identification of environmental factors and their contribution to the carcinogenic process are important goals, in that this may allow greater understanding of the cellular and molecular changes involved as well as helping direct treatment strategies and thus hopefully reduce numbers affected.

Chemicals have been identified as causative factors in both human and animal cancers (Farber & Cameron, 1980; Yuspa & Harris, 1982; Yuspa & Poirier, 1988). With regard to papillomaviruses, synergism between papillomaviruses and chemical carcinogens leading to induction of malignancy was first described by Rous and Beard (1935). The particular virus under investigation was an aetiological agent of skin warts in rabbits, cottontail rabbit papillomavirus (CRPV) identified by Shope (1933). This virus can induce papillomas which can progress to carcinomas in domestic as well as cottontail rabbits. Rous and Beard's study showed that painting rabbit skin with tar could result in the development of papillomas. However these papillomas usually regressed when treatment stopped. Subsequent introduction of CRPV resulted in development of malignant tumours. That progression to malignancy was enhanced after treatment with both virus and carcinogen suggested some sort of interaction between the two agents. Similar results were found using methylcholanthrene (Rous & Friedewald, 1941; 1944).

Virus recovered from 'viral plus carcinogen' experiments showed no greater transformation potential than 'virus only' classes suggesting that the chemical carcinogens caused cellular modifications rather than affecting the virus directly.

With regard to bovine papillomaviruses, epidemiological evidence has identified bracken fern as an environmental co-factor in both BPV-2 and BPV-4-associated carcinogenesis. The fern contains a cocktail of chemicals including immuno-suppressants and mutagens. The immunosuppressants - the pterosides and pterosins - belong to the sesquiterpene group while one of the major mutagens, quercetin, belongs to the flavonoids, a large and diverse group of organic molecules which are widespread throughout the plant kingdom (Evans W.C. *et al.*, 1982). The chemical structure of quercetin (3,3',4',5,7-pentahydroxyflavone) is shown in Figure 4.1. Isoquercitrin, quercetin's glycoside, constitutes a large part of the flavonoid component in bracken (Evans I.A. *et al.*, 1982). On hydrolysis to quercetin, isoquercitrin was found to be mutagenic in a variety of *Salmonella* strains (Evans W.C. *et al.*, 1982). *In vitro* studies have shown that quercetin can bind DNA and induces a variety of genetic lesions in both bacterial and mammalian cells (Jackson *et al.*, 1993 for review), including clastogenic damage (Ishidate, 1988). This last observation is of particular significance as bracken-grazing cattle show a wide variety of cytogenetic abnormalities (Moura *et al.*, 1988). Quercetin competitively inhibits binding of ATP by phosphotyrosine phosphatases in chick embryo fibroblasts (Van Wart-Hood *et al.*, 1989). Conversely this compound has been shown to inhibit the phosphotransferase activity of the Rous sarcoma virus *src* gene product, $pp60^{src}$, both *in vitro* and *in vivo* (Graziani *et al.*, 1983). Although not carcinogenic by itself (Morino *et al.*, 1982; Hirono *et al.*, 1987), quercetin can also act as an initiator in an *in vitro* two stage transformation assay in mammalian cells (Sakai *et al.*, 1990).

Mol. Wt. 302.23

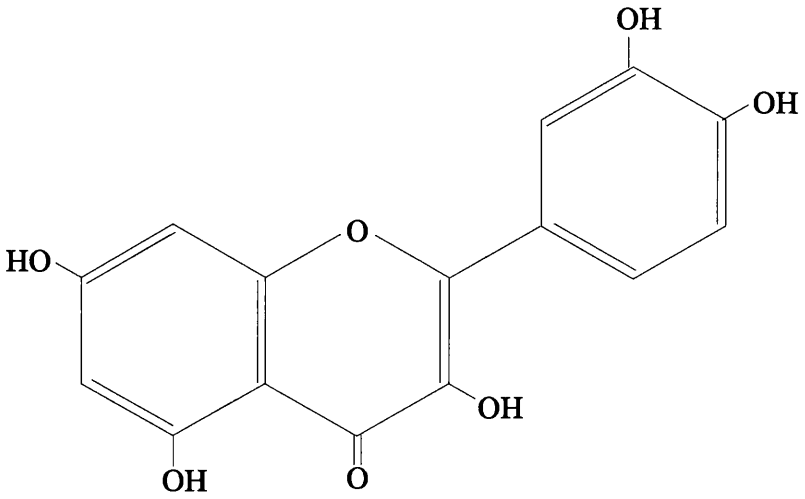


Figure 4.1 The molecular structure of quercetin.

Previous work has demonstrated the advantages in studying the transformation biology of BPV-4 in PalF cells as these cells are primary and are derived from the natural host and site of infection. BPV-4 transfection studies in PalF cells have confirmed results from similar studies carried out in established murine cell lines and have allowed functional mapping of various ORFs of this papillomavirus (described in more detail below). Morphological transformation of PalF cells by BPV-4 DNA was found to be dependent on co-transfection with an activated *ras* oncogene (Jaggar *et al.*, 1990). This requirement confirmed previous observations that transformation of primary cells requires co-operation between two or more oncogenes (Land *et al.*, 1983). Studies showed that PalF cells transformed by BPV-4 + *ras* had an extended lifespan but were not immortal. Although capable of anchorage-independent growth, they were not tumourigenic in nude mice indicating that additional factors were required for full transformation (Jaggar *et al.*, 1990). This appeared to imitate the natural history of upper alimentary canal carcinomas in cattle (Jarrett *et al.*, 1978). In the field the progression of BPV-4-induced papillomas to carcinomas occurs only in animals which are exposed to the mutagenic and immunosuppressive effects of chemicals present in bracken fern. Even then this progression may take years to develop (Campo & Jarrett, 1987). As already stated, quercetin is one of the major mutagens present in bracken fern. Results from *in vivo* studies have shown that although quercetin-treated calves infected with BPV-4 virus developed papillomas these did not progress to carcinomas, whereas two out of six animals infected with BPV-4 and fed on bracken fern did develop carcinomas (Campo *et al.*, 1994b). Animals fed on bracken fern became immunosuppressed whereas animals treated with quercetin did not. That 'quercetin-treated plus virally-infected' animals did not develop carcinomas may well be explained by the immunocompetence of these animals. Transformed cells in these animals may well be removed by normal immunosurveillance mechanisms. These *in vivo* results do not negate a role for quercetin in the progression of BPV-4-induced papillomas to

carcinomas but rather confirm that both immunosuppressants and co-carcinogens are required for such progression.

A series of experiments were carried out *in vitro* to examine the possible role of quercetin as an initiating agent in full transformation of primary bovine fibroblasts. Preliminary experiments, described more fully below, showed that quercetin synergises with BPV-4 *in vitro* (Pennie & Campo, 1992) thus providing a useful and important experimental model system for analysis of viral / chemical co-operation in carcinogenesis.

4.2 Phenotypic effects of quercetin treatment

4.2.1 Background

In the initial study looking at the effects of quercetin on PalF cells (Pennie & Campo, 1992), cells were treated with a single dose of quercetin for 48 hours at final concentrations of 5, 20 or 45 μM quercetin. Cells were washed, seeded into flasks and one day later were transfected with various test and control DNAs. Co-transfection with a plasmid encoding neomycin resistance allowed selection of cells in medium containing the neomycin analogue G418. Quercetin treatment did not affect the morphology of untransfected, 'neo alone', '*ras* + neo' or 'BPV-4 DNA + neo' transfectants. As found for untreated BPV-4-transfected cells, there was a requirement for co-transfection with an activated *ras* gene. This indicated that quercetin could not substitute for this activated oncogene or for BPV-4 genes, as cells transfected with '*ras* + neo' were also untransformed. In this initial analysis (Pennie & Campo, 1992), quercetin-treated BPV-4-transformed cells were examined with respect to transformation efficiency, morphology, ability to grow in semi-solid media and tumourigenicity in nude mice. Results from the 5 μM quercetin treatment class were identical to untreated BPV-4-transformed cells, in that cells showed the same transformation efficiency as untreated transfectants, showed similar colony morphology,

were capable of anchorage-independent growth but were non-tumourigenic. Results for 20 μM and 45 μM quercetin treatment classes showed that although quercetin did not increase the number of transformed colonies the cells showed a more aggressively transformed morphology than untreated transfectants. That is, these colonies showed a greater degree of overlying and piled up cells than untreated transfectants; such colonies were described as showing a 'criss-cross' morphology. Results from anchorage-independent growth assays showed that transformed cells originally exposed to these higher concentrations of quercetin, grew more efficiently in semi-solid media and the resultant colony size was much larger than untreated transformed cells. The two polyclonal populations, derived from treating PalF cells with 20 μM or 45 μM quercetin prior to transfection with whole genome BPV-4 DNA, were also found to be tumourigenic in nude mice. This showed that quercetin synergises with BPV-4 contributing to the full transformation of transfected PalF cells.

The following work not only confirms these results but also extends these findings by examining possible synergism between quercetin and sub-genomic fragments of BPV-4. Clonal populations were examined to investigate whether they displayed the same phenotypic manifestations as previously observed for the two tumourigenic polyclonal populations. It was proposed that use of clonal populations would allow more detailed analyses and indicate the extent and frequency of quercetin / BPV-4 synergism. Four different quercetin treatment protocols were used to address whether the observed synergism between virus and chemical was dependent on the timing of BPV-4 / quercetin exposure. A number of experimental approaches were employed to examine this synergistic relationship more closely and to elucidate possible mechanisms of quercetin action, whether these be genetic, epigenetic or indeed a combination of the two. Transfection of ethanol-treated (the quercetin solvent) PalF cells with BPV-4 genes served as a suitable control as well as confirming previously determined viral ORF functions in primary cells.

4.2.2 Experimental Rationale

4.2.2.1 Quercetin Treatment

A stock solution of 10 mM quercetin dissolved in ethanol was stored at -20°C until use. The general tissue culture conditions used for PalF cells are detailed in Chapter 2.2.1.3. For the majority of cell studies examining quercetin effects, a standard quercetin treatment was implemented. This involved pre-treating PalF cells with 20 μM quercetin (i.e. 80 μl quercetin stock solution per 40 ml culture medium) for 48 hr, followed by a one day interval between removal of quercetin and viral DNA transfection (termed protocol QA). Control-treatment consisted of addition of 80 μl ethanol (the quercetin solvent) to PalF cells for the same period of time prior to DNA transfection. The concentration of 20 μM quercetin was chosen as standard in all experiments as such cells looked healthier yet gave the same results as those treated at 45 μM quercetin for the same period (Table 4.1; W. Pennie, personal communication). Quercetin-induced growth inhibitory effects have been observed in a variety of cell lines (Hosokawa *et al.*, 1990; Yoshida *et al.*, 1990). There was no significant cytostatic effect on the PalF cells treated with 20 μM quercetin over the 48 hour incubation period used in the QA treatment. However, differences in growth rates between quercetin-treated, control-treated and untreated PalF cells were apparent on longer incubation (Figure 4.2).

4.2.2.2 Transfection Classes

Eight lipofection (DOTAP)-mediated test transfection classes were performed (with appropriate controls). These were:

1. PalF cells with whole genome BPV-4 (BPV-4 LCR promoter)
2. PalF cells with E7 and E8 ORFs (BPV-4 LCR promoter)
3. PalF cells with E7 and E8 ORFs (MoLV LTR promoter)
4. PalF cells with E7 ORF alone (MoLV LTR promoter)

5-8. Each of the above classes with addition of HPV-16 E6 (MoLV LTR promoter)

Table 4.1 Effect of quercetin concentration on PalF cells transfected with whole genome BPV-4 DNA. Data from Pennie (1992).

Quercetin (μM)	Viral genes	Morphological transformation	Anchorage independence	Immortalisation	Tumours in nude mice
0	BPV-4 + *ras*	+	+	-	0 / 3
5	BPV-4 + *ras*	+	+	nd	0 / 3
20	BPV-4 + *ras*	+++	+++	+	3 / 3
20	*ras*	-	-	-	0 / 3
45	BPV-4 + *ras*	+++	+++	+	3 / 3
45	*ras*	-	-	-	0 / 3
	NIH-BPV2	+	+	+	3 / 3

+ = positive; +++ = very strongly positive; - = negative for characteristic. nd = not determined

ras = pT24; NIH-BPV2 are NIH-3T3 cells transformed by BPV-2

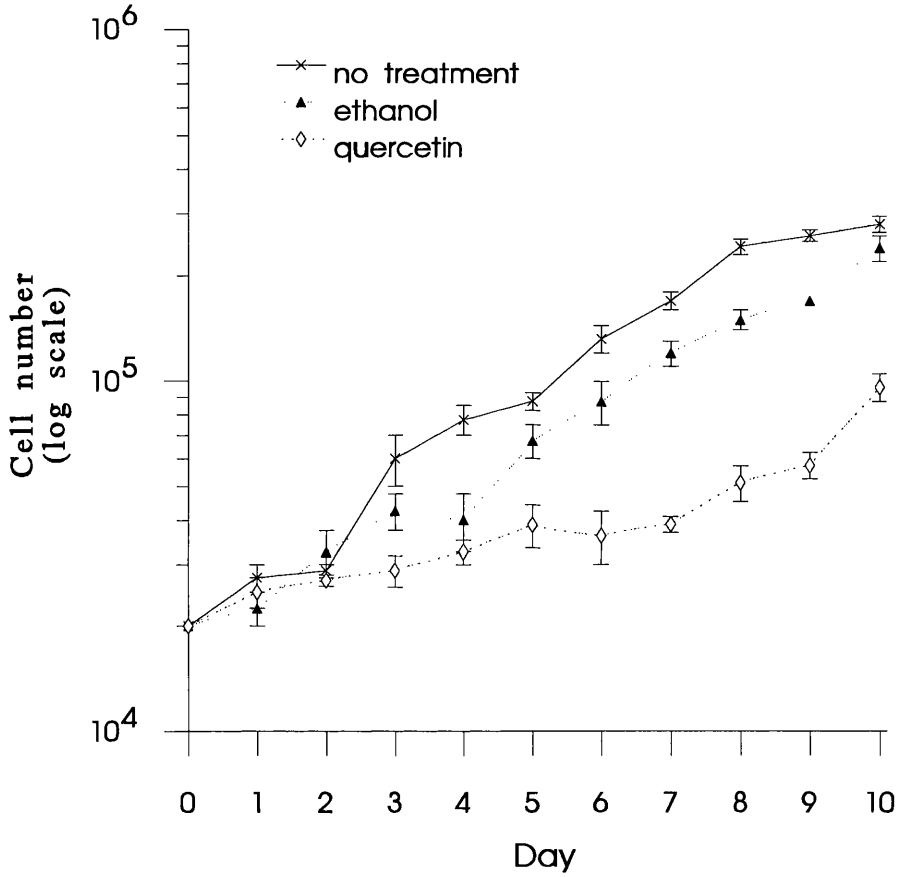


Figure 4.2 Growth curves of PalF cells treated with 20 μM quercetin relative to untreated and ethanol-treated controls. Growth was measured in normal growth medium and cell numbers correspond to the average cell count value from two T25 tissue culture flasks. Vertical bars indicate standard errors of mean values.

All the above viral genes were transfected as recombinant plasmids along with the selectable marker gene for neomycin resistance (pZipneo) and a plasmid containing an activated *ras* gene (pT24). All plasmids and conditions for transfection and selection in medium containing the neomycin analogue G418 are detailed in Chapters 2.1.7, 2.2.1.8 and 2.2.1.9 respectively. For simplicity, the three different BPV-4 DNA transfection types are designated as whole genome BPV-4, E8/E7 and E7 alone throughout the text, with the implicit recognition, unless otherwise stated, that they were all co-transfected with neo and *ras*.

Transfection of whole genome BPV-4 confirmed previous work. E7 and E8 genes were investigated as they have been identified as encoding the transforming products of BPV-4 (Jaggar *et al.*, 1990). While E7 alone transfectants are morphologically transformed, PalF cells cannot sustain transfection of the E8 gene alone (Jaggar *et al.*, 1990; Pennie *et al.*, 1993) and therefore had to be transfected in the presence of the E7 ORF. Two different constructs containing E8/E7 were available. One was under BPV-4 transcriptional control (pSVE8E7), the other under the control of a retroviral promoter (pZipE8E7). This allowed examination as to whether the efficiency of transformation was linked to the level of expression of this viral fragment. The E6 ORF has been identified as the second major oncogene of many papillomaviruses including both BPV-1 and HPV-16 (Schiller *et al.*, 1984; Yang *et al.*, 1985; Münger *et al.*, 1989; Watanabe *et al.*, 1989). Interestingly BPV-4 and the other sub-group B bovine papillomaviruses do not possess an E6 ORF and BPV-4-transformed cells are not immortal (Jackson *et al.*, 1991; Pennie *et al.*, 1993). However the addition of an exogenous HPV-16 E6 gene has been shown to confer immortality on PalF cells transformed by BPV-4 genes (Pennie *et al.*, 1993). Addition of an exogenous E6 gene in the present study (transfection classes 5-8 above) confirmed previous results and provided novel observations in quercetin-treated (QA) transfectants.

4.2.2.3 Methods

Control-treated and experimental (QA) transfections were carried out using the same passage of PalF cells (approximately 5 passages post-isolation). Each transfection class was performed twice in duplicate; i.e. for each transfection class two DOTAP / DNA mixes were made, each mix being split 50:50 between two flasks of cells - giving four transfected flasks per class. G418-resistant colonies which showed a transformed morphology were picked from each class (taking representative colonies from all four replicate flasks, - except in the case of BPV-4 whole genome transfectants; see below) in order to clonally expand them. *In vitro* BPV-4 is not a particularly powerful transforming virus and this in combination with the difficulties of working with primary cells limited the number of clones available for final analyses. Due to the large number of different transfection classes being investigated it was decided, for practical reasons, to aim to characterise 4-5 clonal lines from each transfection class. In the event, generally between 2-4 clones were expanded from each class to the stage where they could be fully assayed (some lines were discarded due to contamination problems). In each case clones from both transfection mixes were represented. There were a few instances among the control-treated transfection classes where this target was not met. Due to contamination problems no pSVE8E7-transfected cells were available for characterisation and only a single clone from transfection class pZipE8E7 + HPV-16 E6 was successfully expanded.

In order to repeat the preliminary polyclonal experiment of Pennie & Campo (1992) colonies from one of the four BPV-4 transfection flasks were pooled. Individual clones were picked from the remaining three flasks. Two quercetin-treated BPV-4-transfected polyclonal lines were isolated in similar fashion. Individual clones from this class were picked from the remaining two flasks.

Transformed cell lines from both control-treated and QA transfection classes were characterised with respect to morphological transformation, anchorage independence, immortalisation and tumourigenicity as detailed below:

Morphology :

Transfection experiments were examined for G418-resistant colonies present at the end of a 21-28 day selection period. Two types of colony morphology were observed in the transfections. The smaller of the two types measured less that 5 mm in diameter and such colonies were termed microcolonies. These colonies were flat and did not look morphologically transformed. Furthermore, although G418-resistant, these microcolonies could not be expanded and therefore were not scored. The second, and more prevalent, type of colony morphology observed consisted of colonies equal or greater than 5 mm in diameter. These colonies were termed macrocolonies and showed a piled up, transformed morphology. These colonies were counted as having been successfully transformed by BPV-4 genes + *ras*.

Anchorage Independence (Growth in Methocel)

The ability of a cell line to form colonies in semi-solid media is taken as a phenotypic measure of its degree of transformation. Virally-transfected lines were plated in 0.9% Methocel to test for anchorage-independent growth (see Chapter 2.2.1.11 for methodology). PalF cells treated with ethanol (80 µl per 40 ml culture medium for 48 hr) were used as a control in this assay. The BPV-4-transfected C127sc cell line C4Ta2a (Smith & Campo, 1988), which is tumourigenic and grows very efficiently in Methocel, was used as a positive control. Cells were examined after 10 days in Methocel. The efficiency of Methocel colony formation was determined by plating 2.5 x 10^5 viable cells in duplicate 60 mm bacterial petri dishes. Each plate was scored for colonies by counting six 1 cm^2 areas from each plate. The mean of these twelve

counts was used to calculate the total number of anchorage-independent colonies, with final results expressed as number of colonies per 10^3 cells seeded.

Immortalisation

Normal cells in culture undergo senescence. It is thought that cellular immortalisation (unlimited lifespan) may be one of the major events in neoplastic progression and thus was assayed in the present study. PalF cells are primary cells and, as expected, senesce on continued culture. Cells from each transfection class were passaged routinely for a period of 4-6 months, corresponding to 25-30 passages, or until senescence. Time constraints prevented a longer assay period. Control-treated (i.e. ethanol alone) PalF cells (+ neo + *ras*) were used as a control in this assay. These cells were not transformed and senesced approximately 4 weeks post-transfection. Cultures that continued to proliferate for at least four months after senescence of control cells and which survived to assay termination were scored as immortal.

Tumourigenicity

The ability to form tumours is an indicator of full cellular transformation. The malignant potential of transformed cells was assayed in athymic nude mice. Four-week old female MF1 nu/nu mice (Harlan-Olac, Bicester, England) were each injected subcutaneously with 1×10^7 viable cells suspended in 0.1 ml of complete medium. Each cell line was injected into at least three animals. The mice were examined for tumour growth at one week intervals up to 15 - 20 weeks post injection. If no tumour had developed by then, the cells were considered to be non-tumourigenic.

4.2.3 Results and Discussion

4.2.3.1 Control (ethanol-treated) BPV-4 transfections

Morphology

None of the transfection control classes were capable of transforming PalF cells (Table 4.2). At best a few microcolonies were observed, but these could only be maintained for 1-3 passages. These controls included PalF cells transfected with BPV-4 + neo, but without *ras*, which confirms the requirement for a co-operating oncogene for successful transformation of primary cells with BPV-4. Morphologically-transformed macrocolonies, however, were observed in all whole genome BPV-4 and sub-genomic fragment transfection classes (Figure 4.3). Mean number of G418-resistant macrocolonies ranged between 11.25-19.25 per 5 x 10^5 cells among transfection classes (Table 4.2). These mean values were not significantly different from each other (one way ANOVA for equality among eight transfection classes, $P = 0.207$). The results show that presence of the BPV-4 E7 gene alone (+ neo + *ras*) is sufficient to morphologically transform PalF cells.

This finding agrees with previous studies. Smith & Campo (1988) reported that an approximately 2 kb fragment of the BPV-4 genome containing the complete E8 and E7 ORFs induced transformation of established cells with the same efficiency as whole genome BPV-4. Disruption of the E7 ORF abolished morphological transformation, strongly suggesting that this ORF encodes a necessary transforming function. Further work has studied this phenomenon more closely. Jaggar *et al.* (1990) showed that a similar fragment of the BPV-4 genome containing the complete E8 and E7 ORFs induced transformation in co-operation with activated *ras* in PalF cells. Deletion of the 3' end of the E7 ORF abolished morphological transformation, providing further evidence of this gene's vital transforming function. PalF cells transfected with this E8/E7 fragment gave similar results to whole genome BPV-4 in both focus and G418-resistance assays. This indicated that the morphological transformation observed

Table 4.2 The transformation efficiency [a] of control (ethanol-treated) PalF cells transfected with whole or sub-genomic BPV-4 DNA fragments, with or without HPV-16 E6 DNA.

Viral genes (+ neo + *ras*)	Transfection #1		Transfection #2		Mean	S.D.
	Flask 1	Flask 2	Flask 3	Flask 4		
BPV-4	17	31	19	10	19.25	± 8.73
BPV-4 + 16E6 [b]	22	15	19	16	18.00	± 3.16
E8/E7 (Zip) [c]	16	12	15	11	13.50	± 2.38
E8/E7 (Zip) + 16E6	13	9	18	6	11.50	± 5.20
E8/E7 (SV) [d]	6	9	18	12	11.25	± 5.12
E8/E7 (SV) + 16E6	11	17	12	14	13.50	± 2.65
E7	23	14	18	17	18.00	± 3.74
E7 + 16E6	18	27	17	9	17.75	± 7.36
controls [e]						
neo only	0	0	0	0	0	-
ras only	0	0	0	0	0	-
neo + *ras*	0	0	0	0	0	-
BPV-4 + neo	0	0	0	0	0	-
16E6 + neo	0	0	0	0	0	-
16E6 + neo + *ras*	0	0	0	0	0	-

[a] Results are expressed as the number of G418-resistant macrocolonies per 5×10^5 cells

[b] 16E6 = HPV-16 E6

[c] refers to pZipE8E7 construct

[d] refers to pSVE8E7 construct

[e] As for viral DNA transfections, PalF cells were control-treated with ethanol for 48 hrs

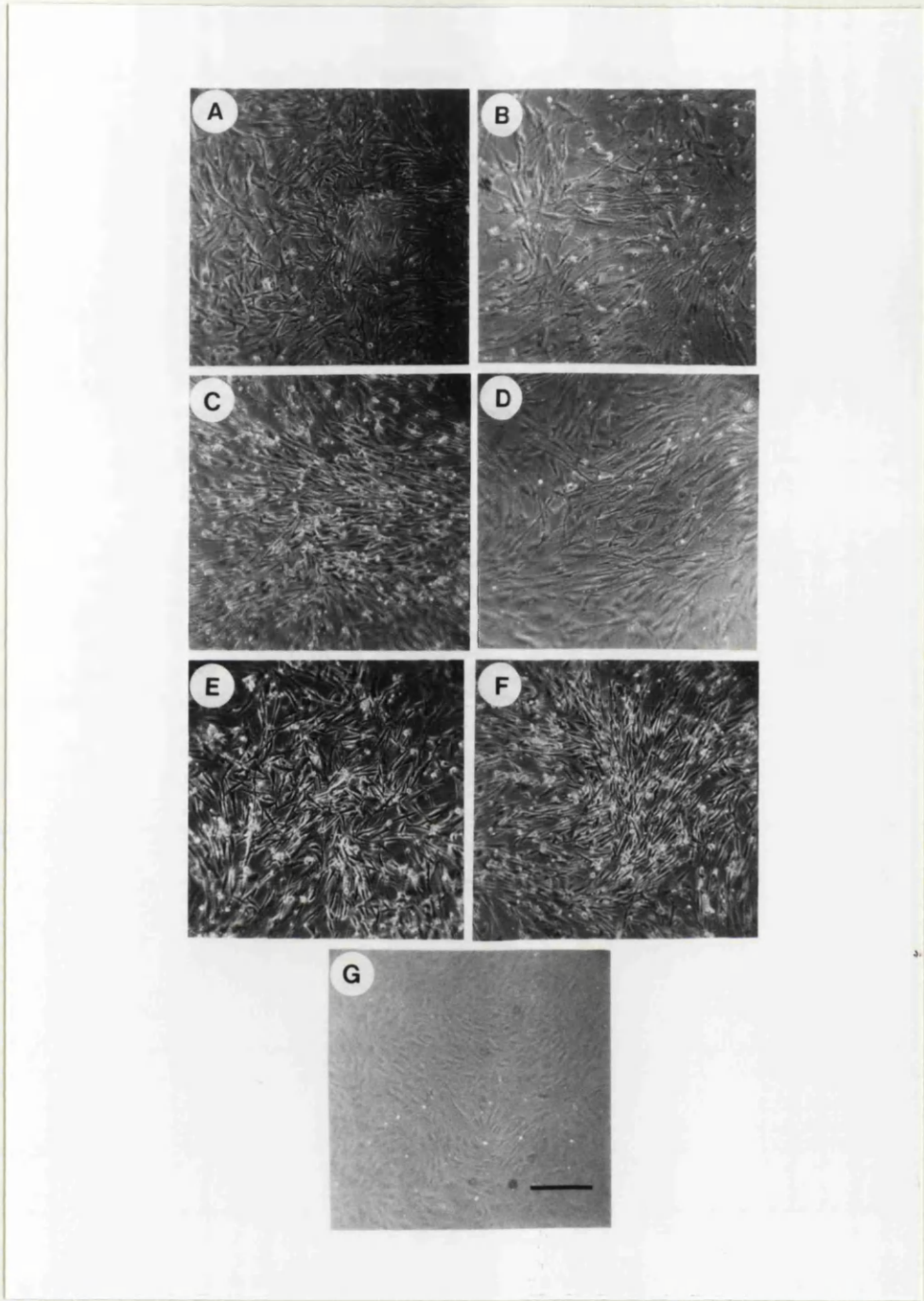


Figure 4.3 Morphology of ethanol-treated PalF cells transformed by whole or sub-genomic fragments of BPV-4 DNA, with or without HPV-16 E6 DNA.

A: BPV-4 + *ras*
B: BPV-4 + *ras* + HPV-16 E6
C: ZipE8E7 + *ras*
D: ZipE8E7 + *ras* + HPV-16 E6
E: E7 + *ras*
F: E7 + *ras* + HPV-16 E6
G: Ethanol-treated PalF cells

The bar indicates 100 μm in all panels.

in the whole genome BPV-4 transfectants was due to the action of the E8 / E7 products. Experiments described in this chapter confirm the above findings and clearly demonstrate that the E7 ORF is the major morphological transforming gene of BPV-4 *in vitro*.

Anchorage Independence

The assays of anchorage-independent growth are summarised in Table 4.3. Whereas the positive control C4Ta2a cells showed growth, normal PalF cells did not grow in Methocel. Among the transformed PalF cells only the whole genome BPV-4 and E8/E7 transfection classes were capable of growth in Methocel (Figure 4.4). All cell lines from these four transfection classes (including the single polyclonal line) showed anchorage-independent growth (Table 4.3; mean values: 2.12 - 4.04 Methocel colonies per 10^3 cells) but at a lower efficiency than the positive control line (6.76 per 10^3 cells) as indicated by non-overlapping 95% confidence ranges. Where multiple clones were scored within transfection classes, statistically significant differences in Methocel growth among clones were recorded:

BPV-4	5 clones, 2.82 - 4.04 per 10^3 cells;	one way ANOVA $P = 0.016$
BPV-4 + 16E6	3 clones, 2.64 - 3.64 per 10^3 cells;	one way ANOVA $P = 0.012$
E8/E7 (Zip)	2 clones, 2.14 - 3.38 per 10^3 cells;	two tailed *t*-test $P = 0.009$

Given the demonstrated variability among clones within transfection classes, and the relatively few clones assayed per class, appropriate caution should be exercised in interpreting a quantitative analysis of these results. It was clear, however, that none of seven clones transfected with the E7 gene alone showed anchorage-independent growth (Table 4.3; Figure 4.4), although all were morphologically transformed (Table 4.2; Figure 4.3). This showed that the E7 ORF does not, by itself, encode functions required for growth in semi-solid media. Rather it is the addition of an E8 ORF which confers anchorage independence on cells as the only transfectants capable of growth in Methocel are those classes containing an E8 ORF. However it is not possible to

Table 4.3 Summary of anchorage independence assay of control (ethanol-treated) PalF cells transfected with whole or sub-genomic BPV-4 DNA fragments, with or without HPV-16 E6 DNA.

Viral genes (+ neo + *ras*)	Clone	Anchorage independence[a] Mean ± S.D.	95% confid. range
BPV-4	Polyclonal	3.02 ± 0.90	2.45 - 3.59
BPV-4	1	4.04 ± 0.76	3.56 - 4.52
	2	3.04 ± 0.95	2.44 - 3.64
	3	3.38 ± 0.83	2.85 - 3.91
	4	3.06 ± 1.10	2.36 - 3.76
	5	2.82 ± 0.82	2.30 - 3.34
BPV-4 + 16E6[b]	1	3.64 ± 0.88	3.08 - 4.20
	2	2.64 ± 0.75	2.16 - 3.12
	3	3.16 ± 0.67	2.73 - 3.59
E8/E7 (Zip)[c]	1	2.14 ± 0.72	1.68 - 2.60
	2	3.38 ± 1.28	2.57 - 4.19
E8/E7 (Zip) + 16E6	1	2.12 ± 0.67	1.69 - 2.55
E7	1	did not grow	
	2	did not grow	
	3	did not grow	
E7 + 16E6	1	did not grow	
	2	did not grow	
	3	did not grow	
	4	did not grow	
control cell lines			
C4Ta2a (positive)		6.76 ± 3.32	4.65 - 8.87
PalF		did not grow	

[a] Mean number of colonies formed in methocel per 10^3 cells seeded.

[b] 16E6 = HPV-16 E6

[c] refers to pZipE8E7 construct

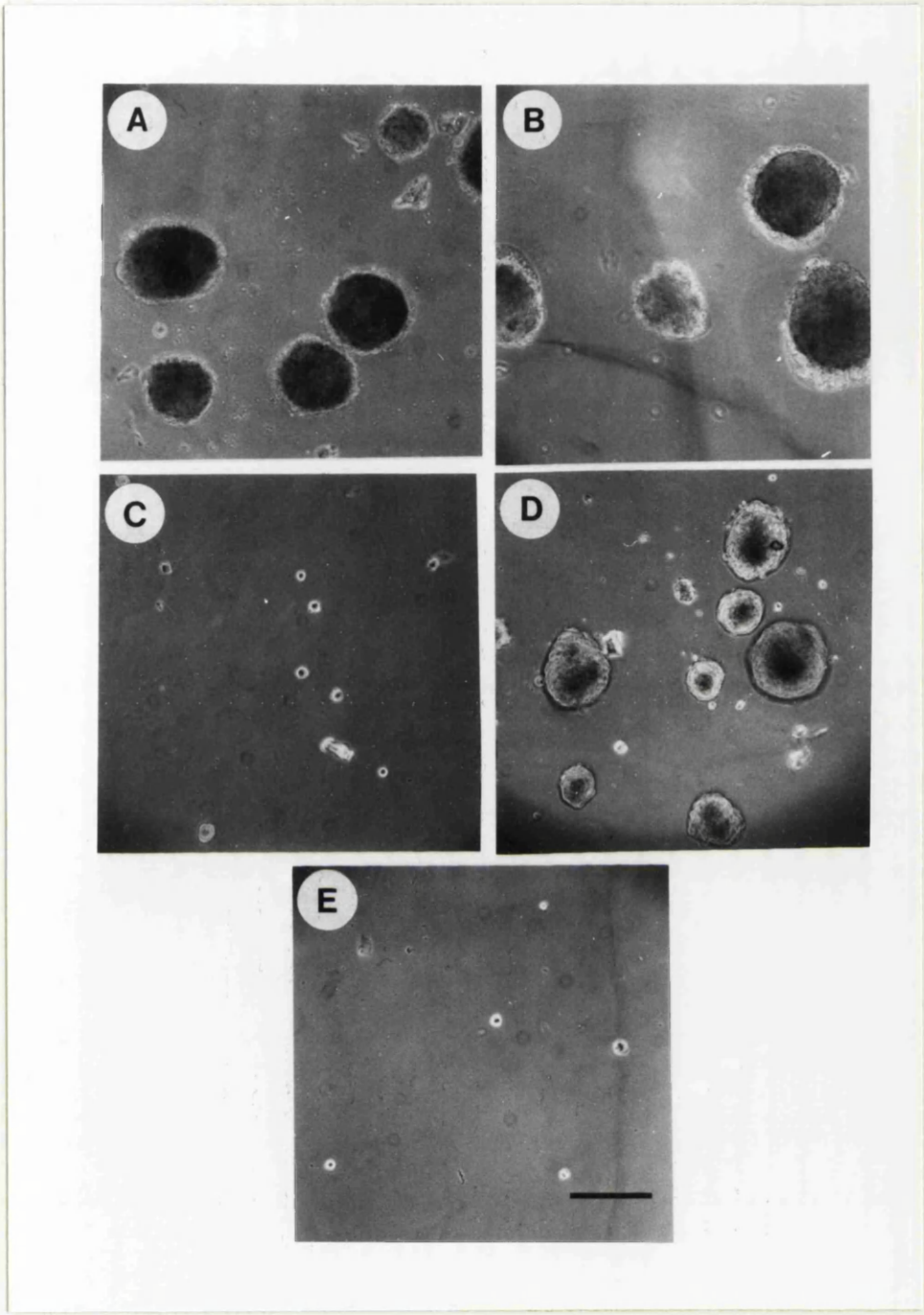


Figure 4.4 Anchorage-independent growth of ethanol-treated PalF cells transformed by whole or sub-genomic fragments of BPV-4 DNA.

A: BPV-4 + *ras*
B: ZipE8E7 + *ras*
C: E7 + *ras*
D: C4Ta2a (positive control)
E: Ethanol-treated PalF cells

The bar indicates 100 μm in all panels.

determine whether expression of E8 alone is sufficient to confer anchorage independence as PalF cells transfected with BPV-4 E8 DNA (+ neo + *ras*) die under G418 selection (Jaggar *et al.*, 1990; Pennie *et al.*, 1993). The lethal effect of E8 transfection may well be due to inappropriate levels of expression of the viral product. When transfected alone (+ neo + *ras*) the E8 ORF would obviously not be subject to regulatory control by other viral genes e.g. the BPV-4 E2 protein (Jackson & Campo, 1991; 1995). If the level of E8 expression does indeed dictate whether transfected cells survive or not, one possible way of circumventing this, thus allowing determination of protein function(s), would be use of an inducible mammalian gene expression system. However, until such experiments are undertaken, it cannot be deduced whether anchorage independence is determined solely by the E8 product or results from combined action of the E8 and E7 (and possibly other) viral proteins.

Immortalisation

One clone from each transfection class was assayed. Of the seven classes (including the BPV-4 polyclonal line) examined, three showed evidence of immortalisation, i.e. were still proliferating after continual subculture of 4-6 months. These were all transfections which included HPV-16 E6 DNA. Despite showing other evidence of phenotypic transformation, the four transfection classes without HPV-16 E6 did not exhibit immortalisation. However the life span of these non-immortalised BPV-4 transfectants was extended (to approximately 3 months) compared to PalF (+ neo + *ras*) controls.

Although the data are limited these results support previous work. Pennie *et al.* (1993) reported that, while incapable of transforming PalF cells by itself or when co-transfected with an activated *ras*, addition of HPV-16 E6 to BPV-4 DNA (+ neo + *ras*) conferred immortality to transformed cells. They also noted that there was still a requirement for E8 before cells containing HPV-16 E6 were capable of anchorage-independent growth.

This was also suggested by the present study (see E7 + HPV-16 E6 transfection class; Summary Table 4.4).

Tumourigenicity

Tumourigenicity was detected only in the positive control cell line (Table 4.4). Despite showing other evidence of phenotypic transformation none of the virally-transformed PalF cells were tumourigenic (Table 4.4). These results concur with the study of Pennie *et al.* (1993) and suggest that additional factors are needed for full transformation of PalF cells. This is similar to the *in vivo* situation where the progression of BPV-4-induced papillomas to carcinomas is co-factor dependent.

4.2.3.2 Quercetin-treated transfections

Morphology

Treatment of untransfected PalF cells with 20 μM quercetin for 48 hours did not affect the morphology of these cells. Likewise treatment with quercetin prior to DNA transfection (protocol QA) did not lead to morphological transformation of PalF cells in any the six control classes (Table 4.5). As was the case for control-treated cells, morphologically-transformed macrocolonies were observed in all QA whole genome BPV-4 and sub-genomic fragment transfection classes of PalF cells (Table 4.5; Figure 4.5). Mean number of G418-resistant macrocolonies ranged between 12.5-16.5 among transfection classes (Table 4.5). These mean values were not significantly different from each other (one way ANOVA for equality among eight transfection classes; $P = 0.748$). Thus, there was no apparent difference between the two E8/E7 constructs which differed in the origin of their promoters. There was also no significant difference in transformation efficiency between QA and ethanol-treated transfectants (two tailed t-test for equality between pooled data; $P = 0.576$). However, there was a very obvious qualitative difference between transformed cells from the two treatments. QA transformed cells exhibited a more aggressive morphology compared to

Table 4.4 Control transfections: Summary of phenotypic characterisation of control (ethanol-treated) PalF cells transfected with whole or sub-genomic BPV-4 DNA fragments, with or without HPV-16 E6 DNA.

Viral genes (+ neo + *ras*)	Clone	Morph. trans.	Anch. indep.	Immortal-isation	Tumours in nude mice [a]
BPV-4	Polyc.	+	+	-	0 / 3
BPV-4	1	+	+	-	0 / 3
	2	+	+	nd	0 / 3
	3	+	+	nd	0 / 3
	4	+	+	nd	0 / 3
	5	+	+	nd	0 / 3
BPV-4 + 16E6	1	+	+	+	0 / 3
	2	+	+	nd	0 / 3
	3	+	+	nd	0 / 3
E8/E7 (Zip) [b]	1	+	+	-	0 / 3
	2	+	+	nd	0 / 3
E8/E7 (Zip) + 16E6	1	+	+	+	0 / 3
E7	1	+	-	-	0 / 3
	2	+	-	nd	0 / 3
	3	+	-	nd	0 / 3
E7 + 16E6	1	+	-	nd	0 / 3
	2	+	-	nd	0 / 3
	3	+	-	+	0 / 3
	4	+	-	nd	0 / 3
<u>control cell lines</u>					
C4Ta2a (positive)		+	+	+	3 / 3
PalF		-	-	-	0 / 3

+ = positive; - = negative for characteristic. nd = not determined

[a] No. of tumour bearing animals / number of injected animals
10^7 cells were injected subcutaneously into each mouse

[b] refers to pZipE8E7 construct

Table 4.5 The transformation efficiency [a] of PalF cells treated with 20 μM quercetin prior to transfection (protocol QA) with whole or sub-genomic BPV-4 DNA fragments, with or without HPV-16 E6 DNA.

Viral genes (+ neo + *ras*)	Transfection #1		Transfection #2		Mean	S.D.
	Flask 1	Flask 2	Flask 3	Flask 4		
BPV-4	10	19	24	12	16.25	± 6.45
BPV-4 + 16E6 [b]	20	17	14	15	16.50	± 2.64
E8/E7 (Zip) [c]	17	10	12	11	12.50	± 3.11
E8/E7 (Zip) + 16E6	18	16	17	14	16.25	± 1.71
E8/E7 (SV) [d]	12	14	19	6	12.75	± 5.38
E8/E7 (SV) + 16E6	12	8	16	21	14.25	± 5.56
E7	18	10	14	11	13.25	± 3.59
E7 + 16E6	14	20	11	17	15.50	± 3.87
controls [e]						
neo only	0	0	0	0	0	-
ras only	0	0	0	0	0	-
neo + *ras*	0	0	0	0	0	-
BPV-4 + neo	0	0	0	0	0	-
16E6 + neo	0	0	0	0	0	-
16E6 + neo + *ras*	0	0	0	0	0	-

[a] Results are expressed as the number of G418-resistant macrocolonies per 5 x 10^5 cells

[b] 16E6 = HPV-16 E6

[c] refers to pZipE8E7 construct

[d] refers to pSVE8E7 construct

[e] Note that these controls used 20 μM quercetin-treated PalF cells

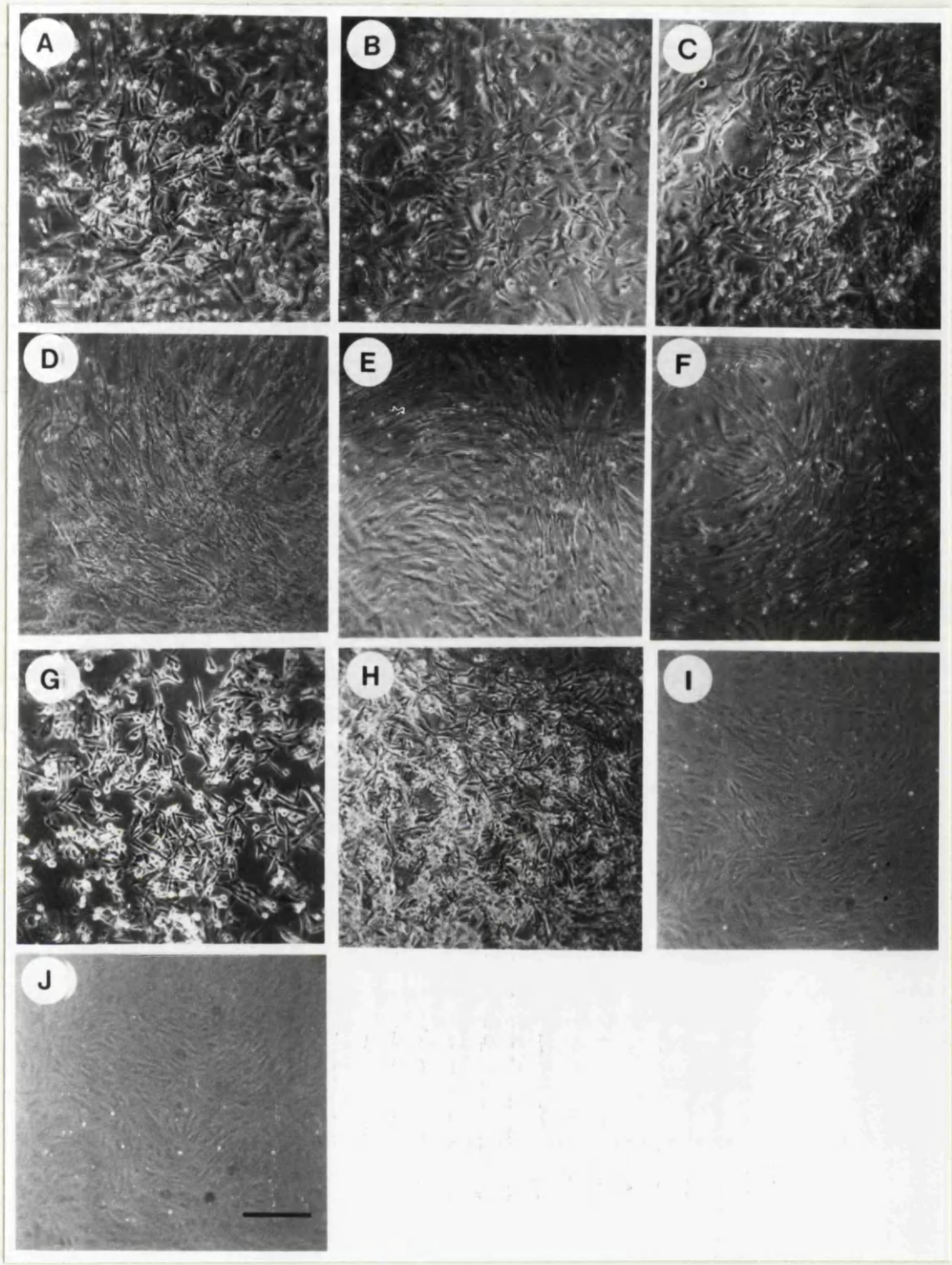


Figure 4.5 Morphology of PalF cells treated with 20 µM quercetin prior to transfection (protocol QA) with whole or sub-genomic fragments of BPV-4 DNA, with or without HPV-16 E6 DNA.

A: BPV-4 + *ras*
B: BPV-4 + *ras* + HPV-16 E6
C: ZipE8E7 + *ras*
D: ZipE8E7 + *ras* + HPV-16 E6
E: SVE8E7 + *ras*
F: SVE8E7 + *ras* + HPV-16 E6
G: E7 + *ras*
H: E7 + *ras* + HPV-16 E6
I: quercetin-treated PalF cells:
J: ethanol-treated PalF cells

The bar indicates 100 µm in all panels.

ethanol-treated transformants. This was the case in all classes, regardless of whether cells had been transfected with whole genome BPV-4 or only sub-genomic fragments E8/E7 (both constructs) or E7 alone (cf. Figures 4.3 & 4.5).

The results for whole genome BPV-4 transfectants concur with those of Pennie & Campo (1992) in that cells exposed to quercetin prior to transfection with BPV-4 genes show a more aggressive transformed morphology than untreated transfectants. As was found for their ethanol-treated counterparts (Table 4.2), transfection of quercetin-treated cells with BPV-4 E7 alone is sufficient to cause morphological transformation (Table 4.5).

Anchorage independence

The assays for anchorage-independent growth in QA transfectant classes are summarised in Table 4.6. The positive control C4Ta2a cells (not quercetin-treated) grew well in Methocel whereas the negative control, untransfected PalF cells treated with 20 μM quercetin for 48 hours, did not.

Growth efficiency among virally-transformed clones varied both within and among QA transfection classes, as found for ethanol-treated transfectants (cf. Table 4.3). Only clones within whole genome BPV-4 (one way ANOVA for equality; $P = 0.867$) and BPV-4 + HPV-16 E6 (two tailed t-test for equality; $P = 0.075$) classes showed similar growth. For all other classes there were significant differences among clones of the same class (one way ANOVAs or two tailed t-tests for equality; max. $P = 0.02$). Clones within these classes also displayed non-overlapping 95% confidence ranges and / or showed no growth at all. As with the results from ethanol-treated transfectants, appropriate caution needs to be exercised in interpreting these growth efficiency values. Large differences were also noted among the different QA transfection classes. In general, transformed clones containing whole genome BPV-4 grew well

Table 4.6 Summary of anchorage independence assay of PalF cells treated with 20 μM quercetin prior to transfection (protocol QA) with whole or sub-genomic BPV-4 DNA fragments, with or without HPV-16 E6 DNA.

Viral genes (+ neo + *ras*)	Clone	Anchorage independence [a] Mean ± S.D.	95% confid. range
BPV-4	Polyclonal 1	4.68 ± 2.27	3.24 - 6.12
	Polyclonal 2	2.88 ± 0.47	2.58 - 3.18
BPV-4	1	4.52 ± 1.84	3.35 - 5.69
	2	4.84 ± 2.28	3.39 - 6.29
	3	4.88 ± 1.08	4.20 - 5.56
BPV-4 + 16E6 [b]	1	3.88 ± 0.80	3.37 - 4.39
	2	4.80 ± 1.47	3.86 - 5.74
E8/E7 (Zip) [c]	1	0.68 ± 0.54	0.34 - 1.02
	2	1.08 ± 0.79	0.58 - 1.58
	3	did not grow	
E8/E7 (Zip) + 16E6	1	did not grow	
	2	did not grow	
E8/E7 (SV) [d]	1	0.66 ± 0.59	0.29 - 1.04
	2	0.78 ± 0.56	0.43 - 1.14
	3	did not grow	
E8/E7 (SV) + 16E6	1	did not grow	
	2	did not grow	

(continued over page)

Table 4.6 (continued)

Viral genes (+ neo + *ras*)	Clone	Anchorage independence [a] Mean ± S.D.	95% confid. range
E7	1	2.94 ± 1.56	1.95 - 3.93
	2	1.02 ± 0.91	0.44 - 1.60
	3	0.74 ± 0.64	0.33 - 1.15
	4	1.32 ± 0.58	0.95 - 1.69
E7 + 16E6	1	2.44 ± 0.59	2.07 - 2.81
	2	2.58 ± 1.11	1.87 - 3.29
	3	1.36 ± 1.06	0.69 - 2.03
	4	did not grow	
control cell lines			
C4Ta2a (positive)		6.76 ± 3.43	4.58 - 8.94
PalF		did not grow	
PalF (quercetin-treated)		did not grow	

[a] Mean number of colonies formed in methocel per 10^3 cells seeded.

[b] 16E6 = HPV-16 E6

[c] refers to pZipE8E7 construct

[d] refers to pSVE8E7 construct

(3.88 - 4.84 colonies per 10^3 cells), though not as efficiently as C4Ta2a positive control cells (6.76 colonies per 10^3 cells). Clones transfected with BPV-4 sub-genomic fragments showed poorer growth (0.00 - 2.94 colonies per 10^3 cells). There was no demonstrable difference between E8/E7 (Zip) classes and E8/E7 (SV) classes.

The mean growth efficiency values of QA whole genome BPV-4 clonal classes were at the higher end of the range of the combined mean values of ethanol-treated and QA transfectants. Although this suggests a tendency for QA derived clones to show increased growth efficiency in Methocel, a robust statistical analysis was not possible due to the intrinsic variability described above. These QA clones also appeared to be larger than their ethanol-treated counterparts, although this was not quantitatively analysed (cf. Figures 4.4 & 4.6).

Of particular note was the finding that of eight quercetin-treated clones containing the BPV-4 E7 gene (with or without an exogenous HPV-16 E6 gene) seven were now capable of growth in semi-solid media (Table 4.6). This contrasted with the results of the ethanol-treated PalF transformants (Table 4.3) which showed that transfection with E8 was required for anchorage-independent growth of PalF cells. This result suggests that quercetin substitutes, in part, for the action of the E8 gene in these transformants. Conversely, and contrary to expectation, quercetin-treated cells transfected with E8/E7 constructs grew very poorly or not at all in semi-solid media (Table 4.6; Figure 4.6). The differences between E7 and E8/ E7 cells suggest there is antagonism between E8 and quercetin.

As described earlier (Chapter 4.2.3.1 - *Anchorage Independence*) transfection of E8 DNA alone (+ neo + *ras*) is toxic to cells. This may be due to unregulated E8 expression as BPV-4 control elements are not present. Co-transfection with an E7 ORF, or when the E8 is in the context of the full BPV-4 genome, overcomes this toxicity.

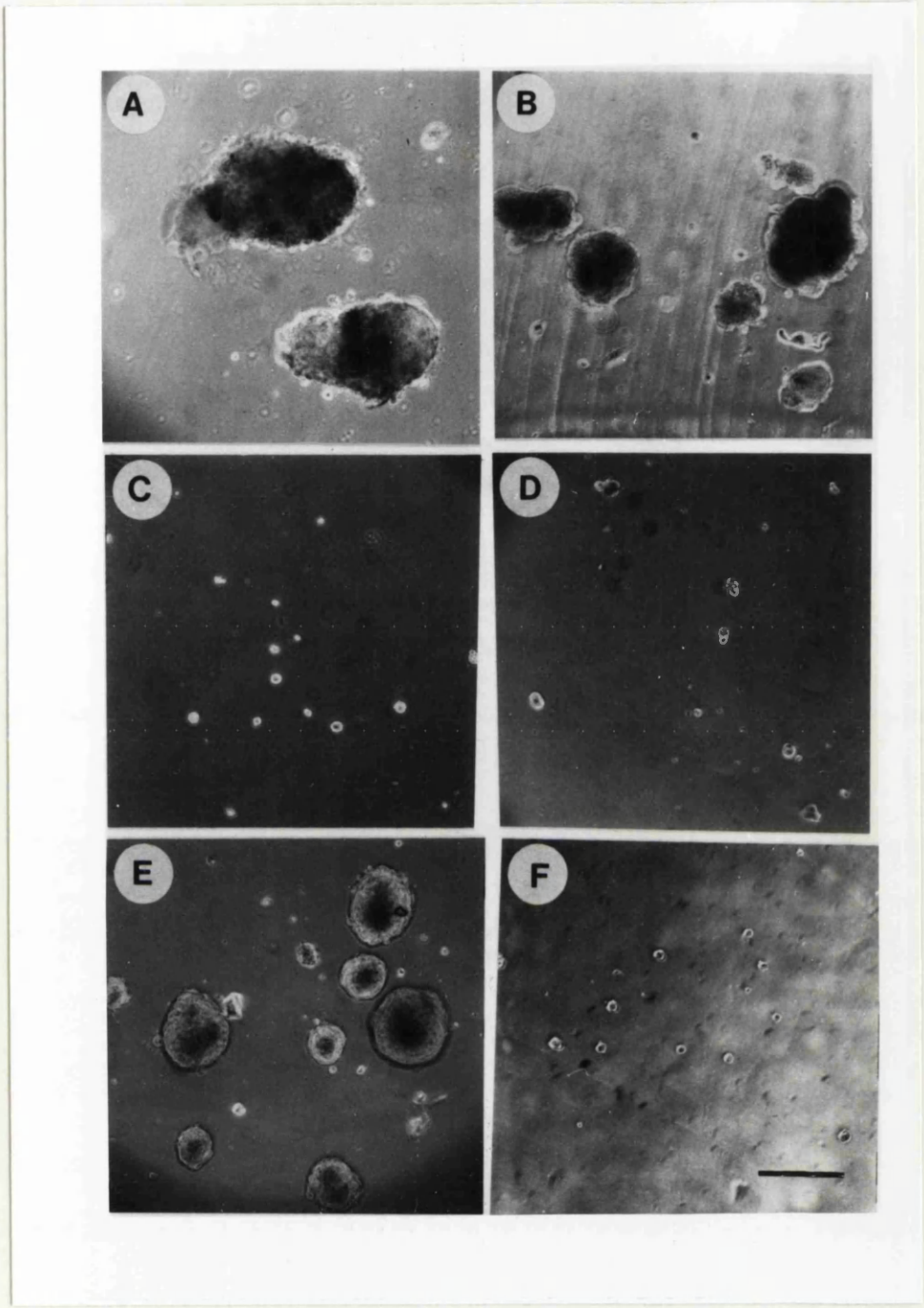


Figure 4.6 Anchorage-independent growth of PalF cells treated with 20 μM quercetin prior to transfection (protocol QA) with whole or sub-genomic fragments of BPV-4 DNA.

A: BPV-4 + *ras*
B: E7 + *ras*
C: ZipE8E7 + *ras*
D: SVE8E7 + *ras*
E: C4Ta2a (positive control)
F: Quercetin-treated PalF cells

The bar indicates 100 μm in all panels.

In E8/E7 classes, overcoming this toxicity may be due to differential expression of these two viral genes, or that the effects of E7 expression are dominant to those of E8 permitting continued cell survival. As quercetin has been shown to interfere with phosphorylation / dephosphorylation mechanisms (Van Wart-Hood *et al.*, 1989; Matter *et al.*, 1992) this may result in increased viral transcription. There may be a threshold for E8 expression in cells, above which the toxic effects of E8 become increasingly apparent. Although normally controlled by BPV-4 regulatory elements (and also cellular transcription factors, (Jackson & Campo, 1995), quercetin may increase E8 expression levels above this threshold. Current work has shown that transcription from the BPV-4 LCR is increased in the presence of quercetin (J. Connolly, personal communication). The net result of quercetin / E8 interaction, in the experimental conditions employed, may result in selection for cells, which although showing an aggressively transformed morphology, are not as transformed as ethanol-treated E8/E7 transfectants. This hypothesis is supported by the immortality and tumourigenicity data described below.

Immortalisation

The immortalisation data for QA transfectant classes are given in Table 4.7, together with a summary of all other phenotypic assays undertaken. The negative control, quercetin-treated PalF cells, showed no evidence of immortality. At least one clone from each transfection class was assayed. All cells tested from transfection classes involving whole genome BPV-4 (one polyclonal and two clonal lines) and E7 (two clonal lines) showed evidence of immortalisation, irrespective of whether HPV-16 E6 was co-transfected. In contrast no E8/E7 transfectants (seven clones tested) were found to be immortal, yet all showed morphological transformation, and some also a degree of anchorage independence. There was no apparent difference in results between the two E8/E7 constructs used.

Table 4.7 Test transfections: Summary of phenotypic characterisation of PalF cells treated with 20 μM quercetin prior to transfection (protocol QA) with whole or sub-genomic BPV-4 DNA fragments, with or without HPV-16 E6 DNA.

Viral genes (+ neo + *ras*)	Clone	Morph. trans.	Anch. indep.	Immortal-isation	Tumours in nude mice [a]
BPV-4	Polyc. 1	++	+	+	3 / 8
	Polyc. 2	++	+	nd	0 / 3
BPV-4	1	++	+	nd	0 / 3
	2	++	+	+	0 / 3
	3	++	+	nd	0 / 3
BPV-4 + 16E6 [b]	1	++	+	+	0 / 3
	2	++	+	nd	0 / 3
E8/E7 (Zip) [c]	1	++	(+)	-	0 / 3
	2	++	(+)	-	0 / 3
	3	++	-	-	0 / 3
E8/E7 (Zip) + 16E6	1	++	-	-	0 / 3
	2	++	-	nd	0 / 3
E8/E7 (SV) [d]	1	++	(+)	-	0 / 3
	2	++	(+)	-	0 / 3
	3	++	-	nd	0 / 3
E8/E7 (SV) + 16E6	1	++	-	-	0 / 3
	2	++	-	nd	0 / 3

(continued over page)

Table 4.7 (continued)

Viral genes (+ neo + *ras*)	Clone	Morph. trans.	Anch. indep.	Immortal-isation	Tumours in nude mice [a]
E7	1	++	+	nd	0 / 3
	2	++	+	nd	0 / 3
	3	++	+	nd	0 / 3
	4	++	+	+	0 / 3
E7 + 16E6	1	++	+	nd	0 / 3
	2	++	+	+	0 / 3
	3	++	+	nd	0 / 3
	4	++	-	nd	0 / 3
<u>control cell lines</u>					
C4Ta2a (positive)		+	+	+	3 / 3
PalF (ethanol-treated)		-	-	-	0 / 3
PalF (quercetin-treated)		-	-	-	0 / 3

(+) = weakly positive; + = positive; ++ = strongly positive;
- = negative for characteristic. nd = not determined

[a] No. of tumour bearing animals / number of injected animals
10^7 cells were injected subcutaneously into each mouse

[b] 16E6 = HPV-16 E6

[c] refers to pZipE8E7 construct

[d] refers to pSVE8E7 construct

These overall results differed markedly from the ethanol-treated viral transfectants in that presence of HPV-16 E6 was no longer a prerequisite for immortality in whole genome BPV-4 and E7-transfected cells. Thus quercetin treatment can substitute for addition of HPV-16 E6. That quercetin-treated E8/E7 transfected cells were not immortal, even in the presence of an exogenous E6 gene, is further evidence of antagonism between quercetin and E8 (see anchorage independence results above). It should be noted that this antagonism is not manifest when the E8 ORF is present in the context of the full BPV-4 genome. As proposed above, the level of E8 expression at any particular point in time may therefore dictate whether quercetin and E8 interact synergistically or antagonistically.

Tumourigenicity

Unlike ethanol-treated transfectants, which were all non-tumourigenic (Table 4.4), one population of cells from QA viral DNA transfectants was shown to be tumourigenic in nude mice (Table 4.7). This was one of the two whole-genome BPV-4 polyclonal lines. Synergism between quercetin and BPV-4 can therefore result in the full transformation of PalF cells. Tumours arising from injection of these polyclonal cells reached maximum size by week 10 and the mice maintained these tumours until the end of the 20 week experimental period. There was no partial regression of tumours as had been previously reported for the tumourigenic polyclonal line described by Pennie & Campo (1992). All other classes of transfectants were non-tumourigenic. Although one out of two polyclonal populations of BPV-4 cells were tumourigenic, five randomly picked clones (with or without HPV-16 E6) were not. That individual clones were not fully oncogenic suggests that progression to full tumourigenicity is not a frequent step under these conditions of quercetin exposure.

4.3 Timing of quercetin treatment

4.3.1 Background

The deleterious effects of continued ingestion of bracken by cattle have been well documented. Where possible farmers will move livestock to fern-free pastures. However shortages of good pasture can result in cattle feeding on bracken. As has already been described, BPV-4 can induce papillomas in the upper alimentary canal of cattle. These papillomas can progress to carcinoma in cattle grazing on bracken fern. It is thought the fern's contribution to progression is due to the mutagens, carcinogens and immunosuppressants present in the plant. Prior to exposure to bracken some cattle will be BPV-4 free, others may have a latent BPV-4 infection and yet others will have BPV-4-induced benign papillomas. The likelihood of papillomas progressing to carcinomas may be dependent on the order and time the animals are exposed to the fern and virus. In an attempt to mimic this natural temporal variation between fern ingestion and BPV-4 infection, preliminary *in vitro* studies examining four different quercetin treatment regimes were undertaken. These experiments were designed to assess whether varying the order and timing of quercetin treatment and viral DNA transfection had any effect on the degree and / or frequency of PalF transformation.

4.3.2 Experimental Rationale

4.3.2.1 Treatments

Four different protocols, QA-QD, were used in which the order and time interval between quercetin-treatment and viral DNA transfection varied (summarised in Table 4.8), although the duration of the single dose quercetin treatment was constant (48 hr). In QA, as previously described (Chapter 4.2.2.1) there was an interval of one day between removal of quercetin and viral DNA transfection. QA protocol repeated the conditions under which synergism between quercetin and whole genome BPV-4 had first been described (Pennie & Campo, 1992). Under protocol QB there were 10 days between removal of quercetin and viral DNA transfection. If the effect of quercetin was

Table 4.8 Key to codes relating to timing of quercetin [a] treatments.

Code	Treatment description
QA	single dose of quercetin, removed 24 hours before DNA transfection
QB	single dose of quercetin, removed 10 days before DNA transfection
QC	single dose of quercetin, given 24 hours after removal of DNA transfection mix
QD	single dose of quercetin, given 24 days post-transfection (i.e. after 21 days G418 selection)
Con	no quercetin treatment (ethanol only)

[a] In all cases cells were exposed to 20 μM quercetin for 48 hours.

epigenetic then by having this extended quercetin-free period, cells would have returned to 'normal' state prior to viral DNA transfection. In protocols QC and QD, PalF cells were treated with quercetin after transfection. QC cells were treated one day after removal of the DNA transfection mix. This examined the phenotypic consequence of quercetin treatment on PalF cells which presumably contained actively transcribing viral genes. QD cells were treated with quercetin 24 days after viral DNA transfection, which included a 21 day G418 selection period. Post-selection the majority of cells would contain viral DNA unaffected by any transformation-suppressive behaviour of surrounding normal (i.e. untransformed) cells. In certain cases non-transformed cells have been reported to inhibit growth of transformed cells (Dotto *et al.*, 1988). In addition levels of viral expression and / or virally-induced mutation of cellular genes are likely to be different from those found in QC cells. QD protocol allowed the examination of the effect of quercetin on cells selected for virally-induced morphological transformation.

4.3.2.2 Methods

The same viral DNA transfection classes, experimental procedure (i.e. each transfection done twice, and in duplicate), and characterisation criteria as detailed for QA protocol (see Chapter 4.2.2 above) were undertaken with QB - QD treatments. A combination of clonal and polyclonal populations were examined. Due to time constraints, however, resultant QB, QC, and QD transfectants were not characterised to the same extent as cells transfected under QA conditions.

4.3.3 Results and Discussion

4.3.3.1 Morphological transformation

Untransfected cells or cells transfected with *ras* or BPV-4 DNA alone were non-transformed, regardless of the timing of quercetin treatment (Table 4.9). This showed that quercetin could not substitute for an activated *ras* gene. Morphological

Table 4.9 Effect of timing of quercetin treatment (protocols QA-QD) on morphological transformation efficiency of PalF cells transfected with whole or sub-genomic BPV-4 DNA fragments with and without HPV-16 E6 DNA.

Viral genes	Without HPV-16 E6		With HPV-16 E6	
(+ neo + *ras*)	Mean [a]	95% Confid. range	Mean [a]	95% Confid. range
BPV-4				
QA [b]	16.25	5.99 - 26.51	16.50	12.30 - 20.70
QB	15.50	12.75 - 18.25	nd [c]	
QC	18.25	13.33 - 23.17	15.75	11.77 - 19.73
QD [d]	16.00	10.97 - 21.03	13.50	5.86 - 21.14
Control [e]	19.25	5.36 - 33.14	18.00	12.97 - 23.03
E8/E7 (Zip)				
QA	12.50	7.55 - 17.45	16.25	13.53 - 18.97
QB	11.50	5.07 - 17.93	nd	
QC	12.75	5.13 - 20.37	15.75	11.57 - 19.93
QD	14.00	6.54 - 21.46	11.00	5.05 - 16.95
Control	13.50	9.71 - 17.29	11.50	3.23 - 19.77
E8/E7 (SV)				
QA	12.75	4.19 - 21.31	14.25	5.4 - 23.10
QB	nd		nd	
QC	15.50	8.95 - 22.05	11.00	8.76 - 13.24
QD	13.00	3.99 - 22.01	13.00	10.76 - 15.24
Control	11.25	3.10 - 19.40	13.50	9.28 - 17.72
E7				
QA	13.25	7.54 - 18.96	15.50	9.34 - 21.66
QB	16.00	9.13 - 22.87	nd	
QC	16.50	11.06 - 21.94	16.50	6.91 - 26.09
QD	11.75	8.22 - 15.28	14.75	6.19 - 23.31
Control	18.00	12.05 - 23.95	17.75	6.04 - 29.46

(continued over page)

Table 4.9 (continued)

Viral genes (+ neo + *ras*)	Without HPV-16 E6		With HPV-16 E6	
	Mean [a]	95% Confid. range	Mean [a]	95% Confid. range
Control lines (+ neo)				
Quer. only	0		0	
ras only	0		0	
Quer. + *ras*	0		0	
BPV-4 only	0		0	
QA + BPV-4	0		0	
QB + BPV-4	0		0	
QC + BPV-4	0		0	

[a] Results are expressed as the number of G418-resistant macrocolonies per 5 x 10^5 cells. Mean values and 95% confidence ranges derived from counts from four different flasks.

[b] Key to quercetin treatments detailed in Table 4.8
QA data summarised from Table 4.5

[c] not determined

[d] In this class, transformation efficiency was scored prior to quercetin treatment.

[e] Control refers to non quercetin-treated transfectants (i.e. summary of Table 4.2)

transformation was assessed after 21 days G418-selection following DNA transfection. Therefore, control (ethanol-treated) PalF transfectants and QD transfection classes (which were treated with quercetin after this G418-selection period) were equivalent with regard to transformation efficiency scores.

Protocols QA-QC had no appreciable effect on the number of transformed colonies observed no matter the class of transfection (Table 4.9). However, qualitative differences among timing treatments were noted in that QA and QC cells exhibited a more aggressive transformed morphology than QB and QD transfectants (Figure 4.7). The latter, (QB and QD) displayed a transformed morphology similar to that of ethanol-treated transfectants (Figure 4.3). These trends were apparent in all classes of transfection (whole genome BPV-4 or sub-genomic fragments). Thus, the shorter the interval between quercetin treatment and viral DNA transfection, the greater the degree of morphological transformation irrespective of the sequential order of the treatments.

4.3.3.2 Anchorage Independence

The multiple transfection experiments gave rise to a large number of assay classes (and cell lines within each class). Only a partial characterisation of these was feasible in the time available. Summary data on growth in Methocel of virally-transfected PalF cells are given in Table 4.10. As presence of HPV-16 E6 showed no demonstrable role in anchorage independence (Chapter 4.2.3.2 above), data from BPV-4 DNA transfections with and without exogenous HPV-16 E6 were pooled. Similarly, since comparable results were obtained with pZipE8E7 and pSVE8E7 data from these transfection classes were also pooled in the summary table.

PalF cells transfected with whole genome BPV-4 originating from protocols QA and QC tended to grow more efficiently and form larger colonies in Methocel than their ethanol-treated counterparts (see Table 4.10; Figure 4.8). This suggests that

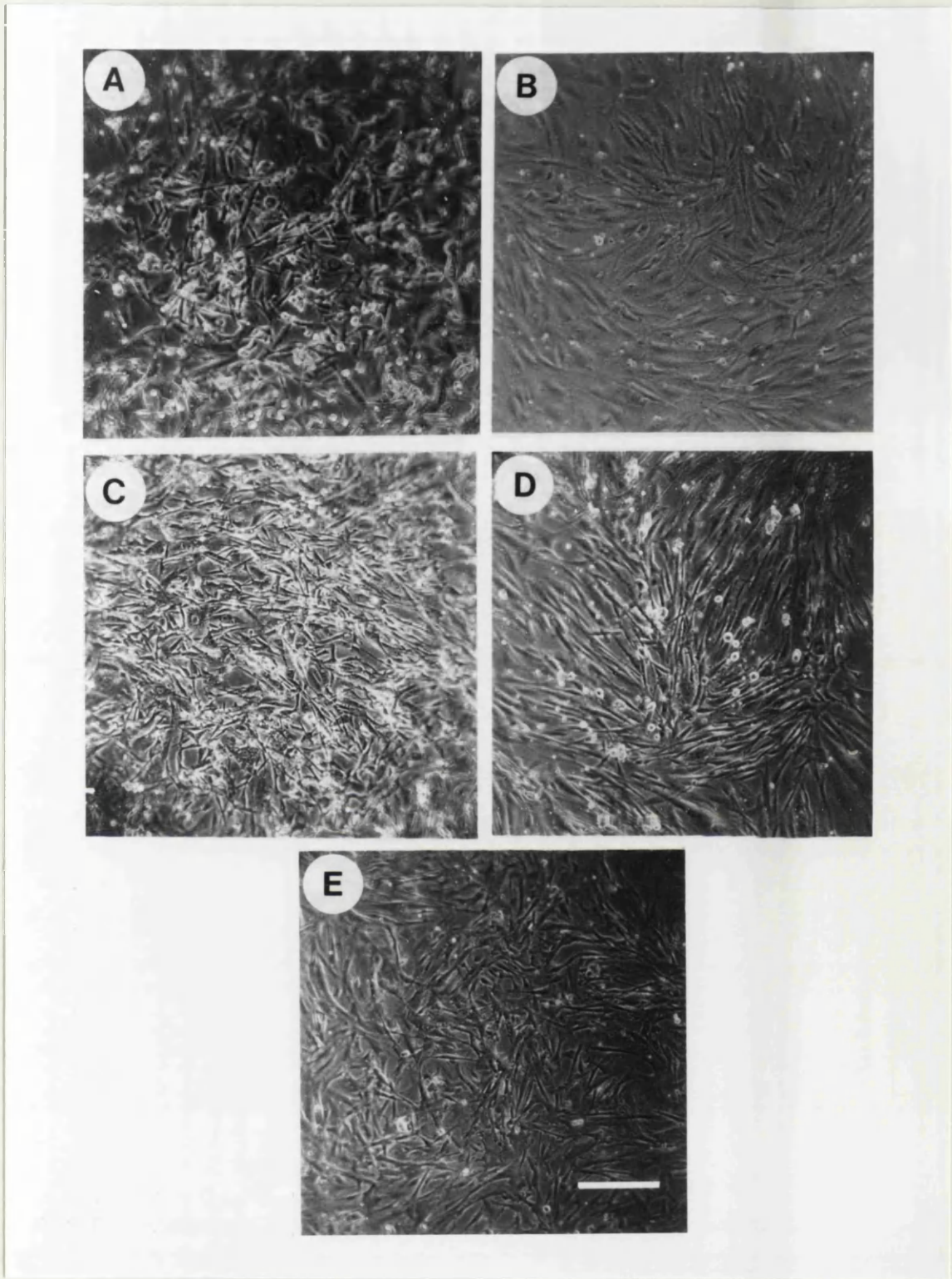


Figure 4.7 Morphology of quercetin-treated PalF cells transformed by BPV-4 DNA and activated *ras*.

(**A**) treated with 20 μM quercetin 24 hr or (**B**) 10 days **before** DNA transfection; (**C**) treated with quercetin 24 hr or (**D**) 3 weeks **after** DNA transfection. (**E**) DNA transfection but no quercetin.

The bar indicates 100 μm in all panels.

Table 4.10 Effect of timing of quercetin treatment (protocols QA-QD) on anchorage independence (A.I.) of PalF cells transfected with whole or sub-genomic BPV-4 DNA fragments.

Viral genes (+ neo + *ras*)	Methocel colonies per 10^3 cells: Range of Means [a]	No. of A.I. lines / No. of lines tested: Polyclonal	Clonal
BPV-4 (Pooled Data - with and without HPV-16 E6)			
QA [b]	2.88 - 4.88	2 / 2	5 / 5
QB	3.42	1 / 1	-
QC	4.70 - 5.30	2 / 2	-
QD	3.00 - 4.08	2 / 2	-
Control [c]	2.64 - 4.04	1 / 1	8 / 8
E8/E7 (Pooled Data - Zip and SV constructs with and without HPV-16 E6)			
QA	0.00 - 1.08	-	4 / 10
QB	2.74	1 / 1	-
QC	0.00 - 1.14	2 / 4	1 / 5
QD	0.00 - 1.94	2 / 2	1 / 2
Control	2.21 - 3.38	-	3 / 3
E7 (Pooled Data - with and without HPV-16 E6)			
QA	0.00 - 2.94	-	7 / 8
QB	0.16	1 / 1	-
QC	0.90 - 3.08	2 / 2	2 / 2
QD	1.16 - 3.32	3 / 3	2 / 2
Control	0.00	-	0 / 7
Control lines			
C4Ta2a (positive)	6.76	-	-
PalF (negative)	0	-	-

[a] Minimum and maximum mean values recorded from both polyclonal and clonal lines.

[b] key to quercetin treatments detailed in Table 4.8
QA data summarised from Table 4.6

[c] Control refers to non quercetin-treated transfectants (i.e. summary of Table 4.3)

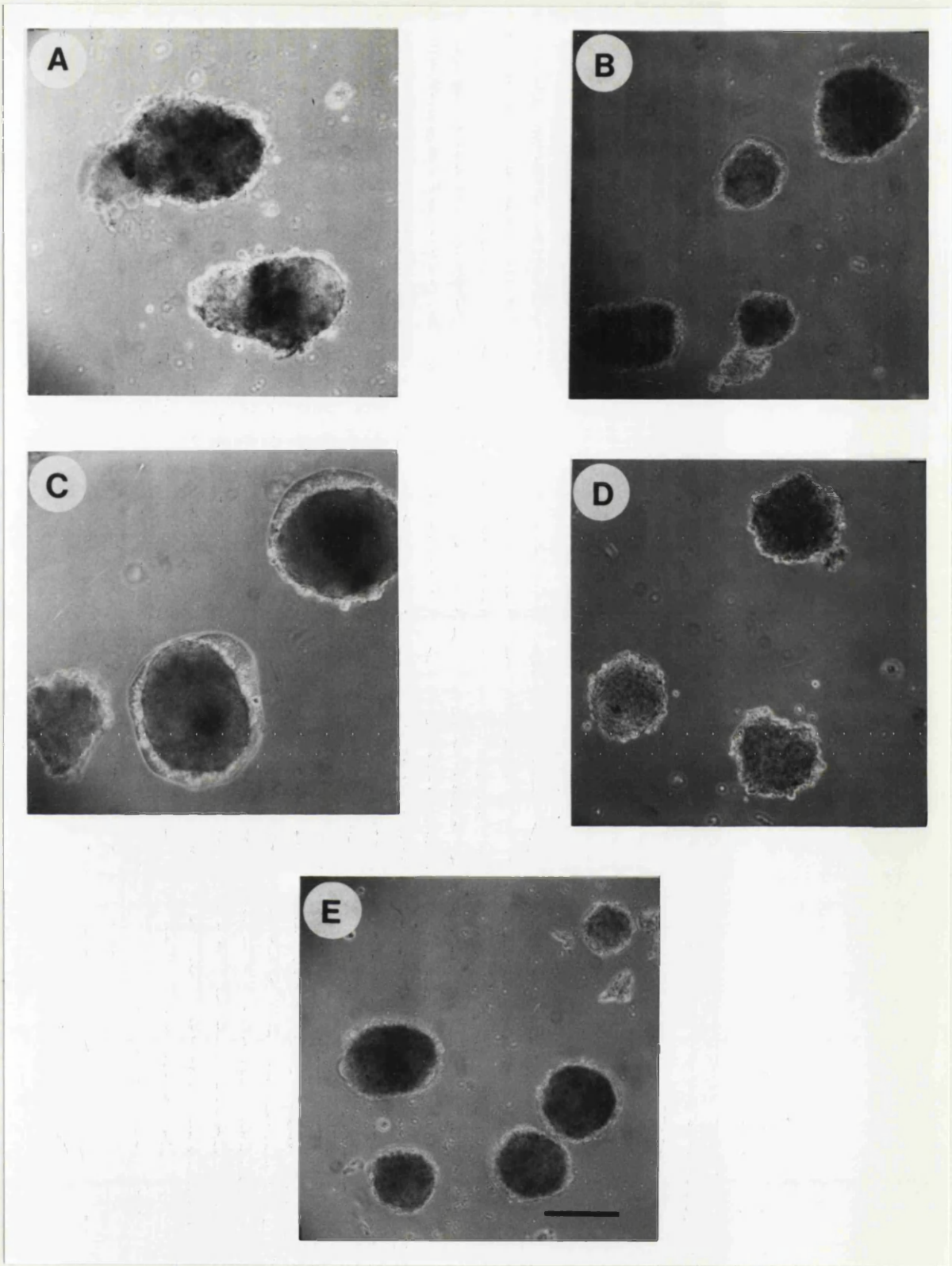


Figure 4.8 Anchorage-independent growth of quercetin-treated PaF cells (protocols QA - QD) transformed by whole genome BPV-4 DNA and activated *ras*.

(**A**) treated with 20 μM quercetin 24 hr or (**B**) 10 days **before** DNA transfection; (**C**) treated with quercetin 24 hr or (**D**) 3 weeks **after** DNA transfection. (**E**) DNA transfection but no quercetin. The bar indicates 100 μm in all panels.

quercetin / BPV-4 synergism is strongest the shorter the time interval between treatment and DNA transfection, regardless of their sequential order. Quercetin-treated PalF cells from all protocols transfected with E7 alone (with or without HPV-16 E6) became anchorage independent (Table 4.10; Figure 4.9). This was in contrast to their non-quercetin treated counterparts (Table 4.3; Figure 4.4). This shows that quercetin is capable of substituting for an E8 gene in conferring anchorage independence on E7-transformed cells regardless of time of treatment. This appears to be a relatively long lasting effect as QD populations were capable of such growth. Although quercetin-treated E7 classes grew in Methocel, they tended to do so with reduced efficiency as compared to the whole genome BPV-4 classes. This suggests that quercetin does not fully substitute for an E8 gene. QA, QC and QD E8/E7-transformed populations tended to grow poorly or not at all compared to ethanol-treated transfectants (Table 4.10; Figure 4.10). No trend could be projected for protocol QB as only a single population was assayed. The calculated growth efficiency for this population was, however, comparable to that of ethanol-treated E8/E7 transfectants. It may be that a long interval between quercetin treatment and viral DNA transfection minimises manifestation of quercetin / E8 antagonism.

4.3.3.3 Immortalisation

Selected populations from protocols QA and QC were assayed for immortalisation (Table 4.11). As already described for QA cells (Chapter 4.2.3.2 above), QC whole genome BPV-4 and QC E7 alone transformed cells showed evidence of immortalisation in the absence of exogenous HPV-16 E6. These populations continued to grow vigorously after long-term (4-6 months) continual growth. Although the experiments were terminated at this point none of the cultures displayed any signs of senescence. Thus quercetin can confer immortality on non-HPV-16 E6-containing cells, both treated immediately before (QA) and also immediately after (QC) transfection with BPV-4 genes. The single QC E7 + HPV-16 E6 polyclonal line assayed was also observed to be

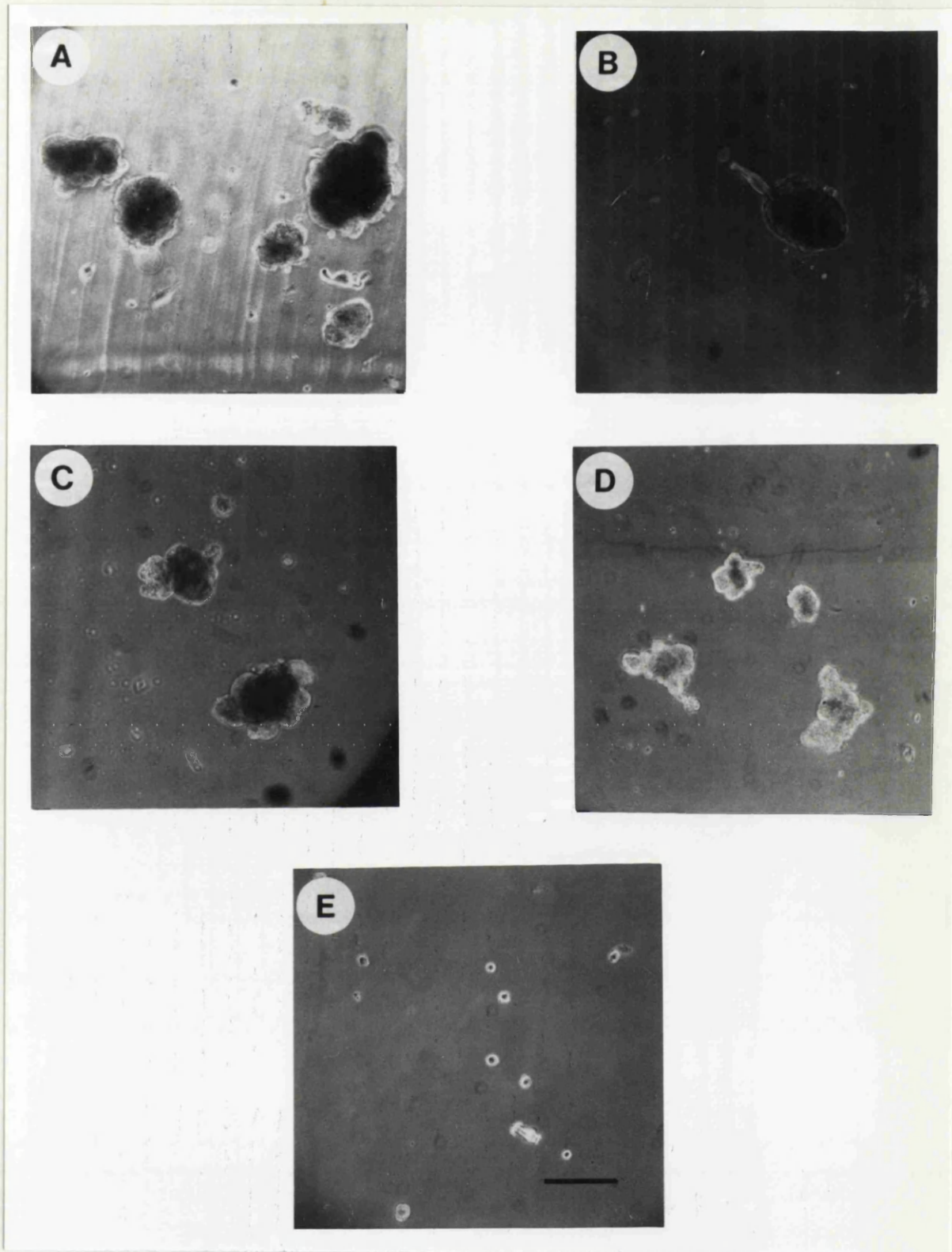


Figure 4.9 Anchorage-independent growth of quercetin-treated PalF cells (protocols QA - QD) transformed by BPV-4 E7 DNA and activated *ras*.

(**A**) treated with 20 μM quercetin 24 hr or (**B**) 10 days **before** DNA transfection; (**C**) treated with quercetin 24 hr or (**D**) 3 weeks **after** DNA transfection. (**E**) DNA transfection but no quercetin. The bar indicates 100 μm in all panels.

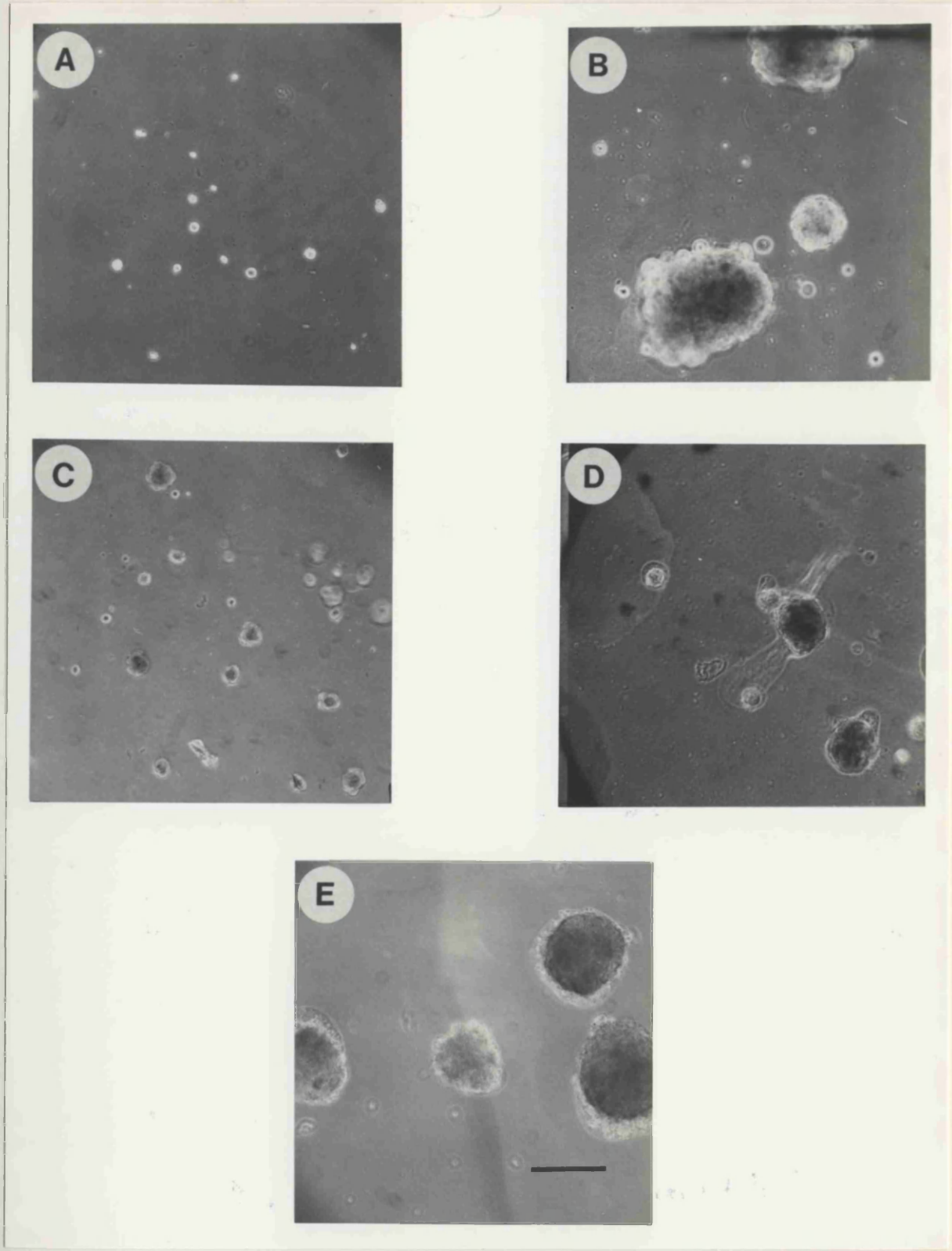


Figure 4.10 Anchorage-independent growth of quercetin-treated PalF cells (protocols QA - QD) transformed by BPV-4 E8E7 DNA and activated *ras*.

(**A**) treated with 20 μM quercetin 24 hr or (**B**) 10 days **before** DNA transfection; (**C**) treated with quercetin 24 hr or (**D**) 3 weeks **after** DNA transfection. (**E**) DNA transfection but no quercetin. The bar indicates 100 μm in all panels.

Table 4.11 Effect of timing of quercetin treatment (protocols QA-QD) on immortalisation of PalF cells transfected with whole or sub-genomic BPV-4 DNA fragments.

Viral genes (+ neo + *ras*)	Number of immortal lines / Number of lines tested			
	without HPV-16 E6		with HPV-16 E6	
	Polyclonal	Clonal	Polyclonal	Clonal
<u>BPV-4</u>				
QA [a]	1 / 1	1 / 1	-	1 / 1
QB	nd [c]	-	-	-
QC	1 / 1	-	nd	-
QD	nd	-	nd	-
Control [b]	0 / 1	0 / 1	-	1 / 1
<u>E8/E7</u> (Pooled Data - Zip and SV constructs)				
QA	-	0 / 5	-	0 / 2
QB	nd	-	-	-
QC	nd	nd	nd	nd
QD	nd	nd	nd	nd
Control	-	0 / 1	-	1 / 1
<u>E7</u>				
QA	-	1 / 1	-	1 / 1
QB	nd	-	-	-
QC	1 / 1	1 / 1	1 / 1	-
QD	nd	nd	nd	nd
Control	-	0 / 1	-	1 / 1

[a] key to quercetin treatments detailed in Table 4.8
QA data summarised from Table 4.6

[b] Control refers to non quercetin-treated transfectants (i.e. summary of Table 4.3)

[c] Not determined

immortalised. This suggests that quercetin and E6 do not act antagonistically. Lines transfected with E8/E7 constructs, with or without an exogenous E6 gene, have yet to be tested although, as already described, the ability of E8/E7 transfectants to grow in Methocel is abolished or reduced in all the quercetin treatment protocols used.

4.3.3.4 Tumourigenicity

Neither PalF cells nor quercetin-treated PalF cells induced tumours in nude mice. However five populations of quercetin-treated transfectants were tumourigenic (Table 4.12). One of these was the QA whole genome BPV-4 polyclonal line described earlier (Chapter 4.2.3.2). Although this polyclonal population was tumourigenic, randomly picked clonal lines were not, suggesting that although quercetin and BPV-4 synergise, resulting in full transformation of PalF cells, this is not a frequent event. One polyclonal line of QC cells transformed by whole genome BPV-4 was found to be tumourigenic, as were all three populations of QC cells transfected with E7. These comprised two polyclonal populations (one with and one without HPV-16 E6) and a clonal population (without HPV-16 E6). Tumours arising from injection of these cells had growth rates similar to tumours produced by whole genome BPV-4 QC cells. All QC tumours grew far more aggressively than QA transfectants, reaching maximum permitted size by week 4 (Figure 4.11). In fact the mice had to be killed at this time-point due to tumour burden.

Indications are that QC conditions provide the optimal conditions for viral / chemical synergism. Three lines of evidence point to this conclusion: a) both clonal and polyclonal QC cells are fully oncogenic; b) both whole and sub-genomic BPV-4 fragments can induce tumours; and c) such growths are far more aggressive than tumours arising from QA cells. These results suggest that the timing of exposure to quercetin is important in this experimental system.

Table 4.12 Effect of timing of quercetin treatment (protocols QA-QD) on tumourigenicity of PalF cells transfected with whole or sub-genomic BPV-4 DNA fragments.

Viral genes (+ neo + *ras*)	No. tumour bearers / No. mice tested [a] (No. of lines tested)			
	without HPV-16 E6		with HPV-16 E6	
	Polyclonal	Clonal	Polyclonal	Clonal
<u>BPV-4</u>				
QA [b]	3 / 8 and 0 / 3	0 / 9 (3)	-	0 / 6 (2)
QB	nd [c]	-	-	-
QC	3 / 3 (1)	-	0 / 3 (1)	-
QD	0 / 3 (1)	-	0 / 3 (1)	-
Control [d]	0 / 3 (1)	0 / 15 (5)	-	0 / 9 (3)
<u>E8/E7</u> (Pooled Data - Zip and SV constructs)				
QA	-	0 / 18 (6)	-	0 / 12 (4)
QB	nd	-	-	-
QC	0 / 6 (2)	0 / 3 (1)	nd	0 / 9 (3)
QD	0 / 3 (1)	0 / 3 (1)	nd	0 / 3 (1)
Control	-	0 / 6 (2)	-	0 / 3 (1)
<u>E7</u>				
QA	-	0 / 12 (4)	-	0 / 12 (4)
QB	nd	-	-	-
QC	2 / 3 (1)	3 / 3 (1)	2 / 3 (1)	-
QD	0 / 6 (2)	0 / 3 (1)	0 / 3 (1)	0 / 3 (1)
Control	-	0 / 9 (3)	-	0 / 12 (4)
<u>control cell lines</u>				
C4Ta2a (known positive tumourigenic line): - 3 / 3				
PalF (ethanol-treated): 0/ 9				
PalF (quercetin-treated): 0 / 9				

[a] 10^7 cells were injected subcutaneously into each mouse

[b] key to quercetin treatments detailed in Table 4.8
QA data summarised from Table 4.6

[c] Not determined

[d] Control refers to non quercetin-treated transfectants (i.e. summary of Table 4.3)

- = cell lines not generated

A) Nude mouse tumour 20 weeks after injection with 10^7 transformed cells (protocol QA).

B) Nude mouse tumour 4 weeks after injection with 10^7 transformed cells (protocol QC).

Figure 4.11 Tumourigenicity of quercetin-treated, whole genome BPV-4 + *ras* transformed PalF cells.

4.4 Effect of BPV-4 and / or quercetin on c-Ha-*ras* and c-*myc*

4.4.1 Background

As demonstrated above and elsewhere (Pennie & Campo, 1992), BPV-4 will only fully transform PalF cells *in vitro* in the presence of an activated c-Ha-*ras* gene and chemical co-factors. Activation of this cellular proto-oncogene has been observed in naturally occurring bovine alimentary canal cancers (Campo *et al.*, 1990), suggesting that this gene is a target for chemical carcinogenesis in this system. BPV-4 is not alone in requiring co-transfection of an activated oncogene for transformation of primary cells as this is also found to be the case for the monkey papillomavirus RhPV and HPVs 16 and 18 (Matlashewski *et al.*, 1987; Storey *et al.*, 1988; DiPaolo *et al.*, 1989; Schneider *et al.*, 1991). The human papillomavirus HPV-16 can transform baby mouse kidney epithelial cells when co-transfected with the oncogene v-*fos*, however these transformed cells require glucocorticoid hormones for proliferation (Crook *et al.*, 1989). Continued passage of such cell lines can result in hormone-independent lines, analysis of which reveals amplification / overexpression of the cellular proto-oncogene c-*myc* (Crook *et al.*, 1989). Both c-Ha-*ras* mutations and rearrangement / overexpression of c-*myc* have been reported in cervical cancers (Riou *et al.*, 1988; Crook *et al.*, 1991; Milde-Langosch *et al.*, 1991). These two cellular genes would thus appear to be common targets in papillomavirus / chemical co-carcinogenesis.

That quercetin treatment does affect phenotypic transformation of BPV-4-transfected PalF cells has been clearly shown (Chapters 4.2 and 4.3). However, the mechanism(s) of this synergism, be they genetic and / or epigenetic in origin, remain to be established. Disruption of cellular genes is a prime consideration, with two attractive candidates being the c-Ha-*ras* and c-*myc* proto-oncogenes. With this in mind the DNA and RNA status of both these proto-oncogenes was examined in both quercetin-treated (condition QA) and ethanol-treated PalF transfectants.

4.4.2 Experimental Rationale

4.4.2.1 Transfectant lines examined

All polyclonal and clonal lines derived from ethanol-treated and quercetin-treated (protocol QA) experiments described in Chapter 4.2 were investigated for the presence, integrity and expression levels of both c-Ha-*ras* and c-*myc* proto-oncogenes. These consisted of 19 ethanol-treated PalF transformed lines (1 polyclonal + 18 clonal; see Table 4.3) and 25 quercetin-treated lines (2 polyclonal + 23 clonal; see Table 4.6). Genomic DNA and total RNA were extracted as detailed in Chapters 2.2.2.4 and 2.2.3.1 respectively. Controls for these analyses were ethanol-treated PalF cells and PalF cells treated with 20 μM quercetin for 48 hr, removed and grown for one further day prior to extraction.

4.4.2.2 Probe derivation

c-Ha-ras

Although three different Ha-*ras* genes have been identified in the bovine genome only the c-Ha-*ras* 1 gene encodes a functional p21 *ras* protein (McCaffery *et al.*, 1989). The *ras* probe used in the present study was 1.5 kb in length and consisted of intron 1a, exon 1 and some satellite-like sequences. The construct from which this fragment was isolated was termed pA-Sma1.5 (see Chapter 2.1.7 for details).

c-myc

A bovine-specific c-*myc* probe was not available for this study. However, as exon 2 is highly conserved among species (Van Beneden *et al.*, 1986), a probe (415 bp in length) derived from exon 2 of human c-*myc*, was deemed a suitable alternative. Details of the probe and construct (pME2) are given in Chapter 2.1.7.

Ductin

An indicator of loading levels for both DNA and RNA experiments was required. A major difficulty in carrying out work on bovine cells is the current paucity of species-specific probes available for this purpose. It was decided to use a bovine ductin probe already available in the laboratory. This consisted of the complete ductin cDNA sequence (size 465 bp). The relevant construct pBov.16K is described in Chapter 2.1.7. Ductin is a major component of gap junctions. These junctions are involved in intercellular communication.

4.4.2.3 Assay conditions

DNA

Two restriction enzyme DNA digests were carried out per cell line, one using BamH I and the second EcoR I. Approximately 5 μg of each digest and appropriate controls were run on 0.8% agarose gels under standard electrophoretic conditions (Chapter 2.2.2.8). Each membrane was also loaded with similarly digested DNA from PalF cells and quercetin-treated PalF cells. In addition two further controls were present. One of these consisted of 5 μg HL-60 DNA, acting as positive control for the *myc* probe.. As these membranes were also hybridised to BPV-4 DNA probes, the second control was 200 ng bovine papilloma DNA (which contains episomal BPV-4 DNA) with 5 μg of BamH I digested murine cell line DNA. Subsequent Southern blotting, hybridisation and autoradiographic conditions were carried out as detailed in Chapters 2.2.2.9-2.2.2.13. A total of seven membranes were required to accommodate all samples. These were probed sequentially in the order *ras, myc* and ductin. In all cases hybridised membranes were washed to a final stringency; 0.1 x SSC, 0.1% SDS for 30 min (2 x 15 min) at 50°C. The membranes were later reprobed and washed at a higher stringency; 0.1 x SSC, 0.1% SDS for 30 min (2 x 15 min) at 65°C for reasons described below (Chapter 4.4.3.1).

RNA

RNA was immobilised onto supported nitrocellulose membrane (Hybond-C Extra; Amersham International plc) using a Bio-Dot Microfiltration Apparatus (Bio-Rad Ltd). RNA dot blots were made using 20 μg total RNA per well. Samples from each line were loaded in duplicate in adjacent wells. This replication allowed for possible inefficient vacuum filtration in individual wells. RNAs from any one particular class of ethanol-treated transfections were loaded onto the same membrane as their quercetin-treated counterparts. For example, RNA from ethanol-treated whole genome BPV-4-transformed cells were present on the same membrane as RNA from QA whole genome BPV-4-transformed cells. Each membrane was also loaded with RNA from PalF cells and quercetin-treated PalF cells. In addition two further controls were present on each blot. One of these consisted of 10 μg HL-60 RNA, acting as positive control for the *myc* probe. As these membranes were also hybridised to BPV-4 E7 and E8 DNA probes, the second control was papilloma DNA (200 ng), which contains BPV-4 DNA and therefore acted as a positive control for these genes. Post-hybridisation wash conditions were identical to those finally carried out for the DNA membranes i.e. 0.1 x SSC, 0.1% SDS for 30 min (2 x 15 min) at 65°C.

4.4.3 Results and Discussion

4.4.3.1 DNA Analyses

Stringency considerations

It was decided to use the *ras* DNA probe first, following the stringency wash conditions (0.1 x SSC, 0.1% SDS, 50°C) previously described for this bovine-specific probe in McCaffery *et al.* (1989). Similar results were obtained in that both presumed *ras*-specific and repetitive sequences were observed. The same conditions were also used for the ductin probe. The *myc* probe was human-derived, and as a consequence membranes hybridised with this probe were initially washed to a lower stringency (0.5 x SSC, 0.1% SDS for 30 min (2 x 15 min) at 50°C) than the *ras* and ductin DNA

probes. An overnight exposure indicated that more stringent washes were appropriate and were thus carried out to the same stringency as both *ras* and ductin. Expected hybridisation banding patterns resulting from *myc* and ductin probing were unknown for bovine DNA. However, later comparison of the resulting autoradiographs revealed that all three DNA probes shared a high proportion of identical bands and also a relatively high degree of background smearing (above approximately 6 kb in size) within tracks, observed after five day exposure. This was considered to be indicative of excessive repetitive sequence detection, which could obscure probe-specific interpretations. Following additional probings of these membranes with BPV-4-derived DNA probes (used for a separate analysis of these cell lines) it was decided to repeat the sequential probings with *ras*, *myc* and ductin, increasing the final wash to 0.1 x SSC, 0.1% SDS for 30 min (2 x 15 min) at 65°C. This dramatically reduced the background smearing, revealing presumed probe-specific bands. It is these results which are discussed below. Six rounds of probing / removal of probe had been undertaken on these membranes prior to repetition of the *ras*, *myc* and ductin hybridisations. As a result, although discernible, signal intensity was sub-optimal. Representative autoradiographs from this final set of probings are given in Figure 4.12.

c-Ha-ras *probing*

Probing revealed a band approximately 13 kb in size in the BamH I tracks and one of approximately 16 kb in the EcoR I tracks of control, quercetin-treated and / or transfected PalF DNAs (Figure 4 12; Panel A). Specific signal was observed in all bovine DNA tracks but was not detected in either mouse (C127sc; tracks 19 and 20) or human (HL-60; track 21) DNAs, confirming the bovine-specific nature of this probe. The faint bands of 7.3 kb and 5.0 kb seen in tracks 19 & 20 respectively were residual signal remaining from a previous hybridisation with a BPV-4 DNA probe. These control tracks were loaded with DNA (200 ng) from a papilloma containing BPV-4 DNA with 5 μg of mouse (carrier) DNA. The resultant BPV-4 signal in these tracks

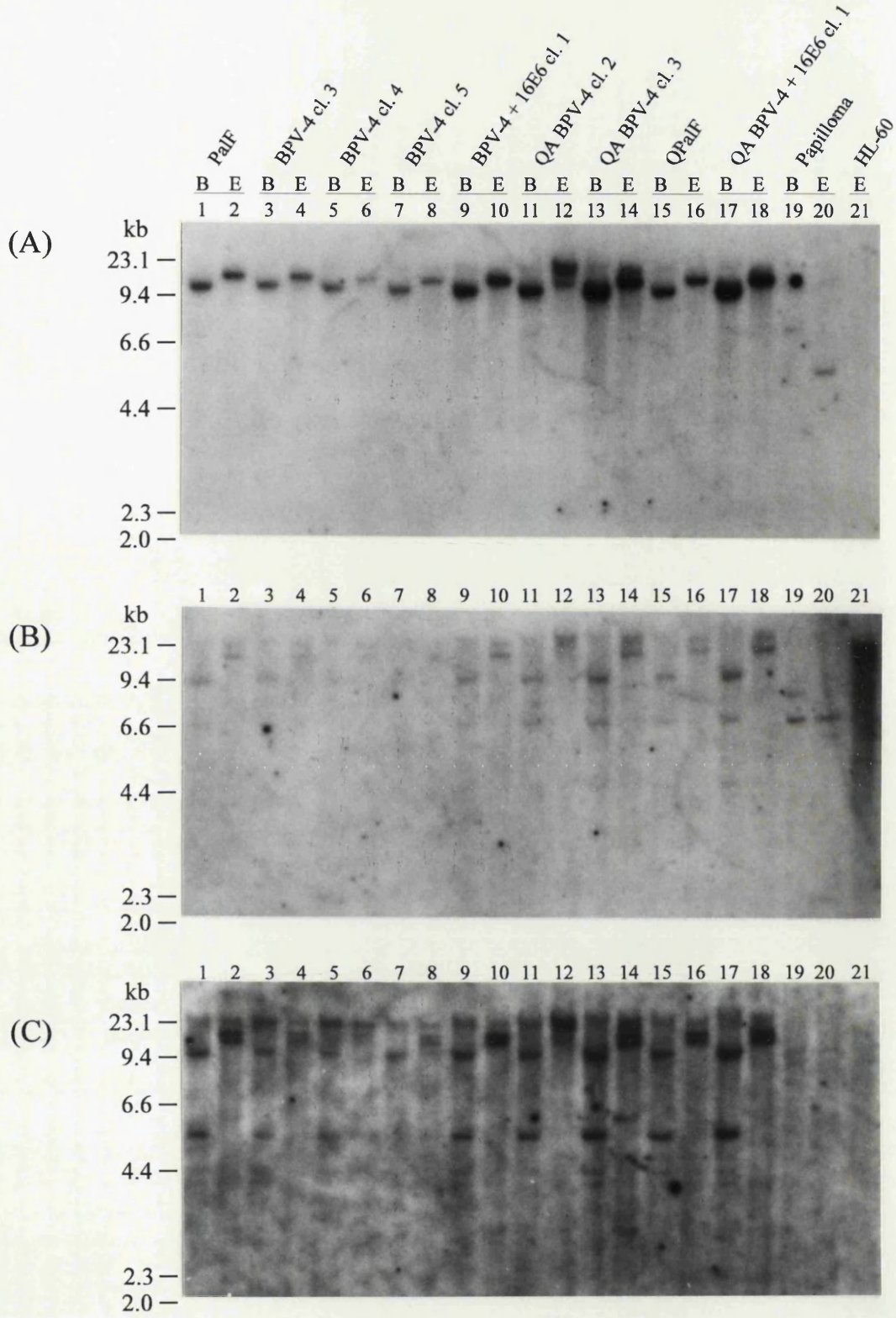


Figure 4.12 Southern blot analysis of control (ethanol-treated) and quercetin-treated (protocol QA) PalF transfectants: status of *ras* and *myc* sequences.

Tracks 1-18 & 21 contain 5 µg DNA digested with BamH I (B) or EcoR I (E). Tracks 19-20 contain 200 ng bovine oesophageal papilloma DNA (which contains episomal BPV-4 DNA) digested with BamH I and EcoR I respectively + 5 µg BamH I digested carrier DNA (C127 DNA). cl. = clone. **Panel A**: probed with bovine c-Ha-*ras* DNA. **Panel B**: probed with human c-*myc* DNA. **Panel C**: probed with bovine ductin DNA as loading control. (probes detailed in Chapter 2.1.7). The positions of λ Hind III digested DNA molecular weight markers are indicated in the left margin.

was extremely intense, and was not completely removed by the stripping protocol. It must be stressed that this did not interfere with results in the cell line DNA tracks and that any signal observed in these samples was probe-specific. The high molecular weight band of approximately 23 kb seen in track 12 was due to the incomplete digestion of that particular cell line DNA sample. Differences in band intensities among tracks could be solely attributed to variations in the amount of DNA loaded in each track. The banding profiles obtained from DNA from all virally-transfected lines, whether treated with quercetin (protocol QA) or not, were indistinguishable from those obtained from untransfected PalF cells or untransfected PalF cells treated with quercetin. This indicated that neither transformation of PalF cells by BPV-4 viral genes nor treatment with quercetin (with or without BPV-4 genes) affected the DNA status of the *ras* sequences detected by this probe.

c-myc *probing*

Typical banding patterns observed using *myc* probe are shown in Figure 4.12; Panel B. Overall signal intensity was poor, requiring long autoradiographic exposure times (> 10 day). The fact that a heterologous (human) probe was used, yet required high stringency post-hybridisation washes to strip off repeat DNA signal, was probably a major reason for this diminution of signal. Nevertheless, inspection of all autoradiographs revealed band signal in all tracks containing bovine or control (mouse or human) DNA.

HL-60 DNA (track 21) was run as a positive *myc* control on all membranes as this human promyelocytic leukaemia cell line is known to contain an amplified c-*myc* gene (Dalla Favera *et al.*, 1982). In all cases this human DNA gave the strongest signal. BamH I digests gave an approximately 23 kb band while EcoR I digests (e.g. track 21 Figure 4.12; Panel B) gave a band of approximately 14 kb in size. In both digests 2-3 smaller and weaker bands, along with background smearing, were observed. Similar

size and hybridisation patterns have been described for other studies involving human c-*myc* (Dalla Favera *et al.*, 1982; Alitalo *et al.*, 1983). Mouse carrier DNA was present as BamH I digests on all membranes (control tracks pertinent to other hybridisations - see Chapter 5.2.2). DNA from this murine fibroblast established line resolved a single band of approximately 6 kb on c-*myc* probing (Figure 4.12; Panel B, Tracks 19 & 20). This size estimate is close to that (5.6 kb) reported previously for c-*myc* probed mouse DNA BamH I digests (Crook *et al.*, 1989). (The weak 7.3 kb and 5.0 kb bands also seen in tracks 19 & 20 are residual signal from a previous probing as explained above.)

All bovine DNAs gave specific signal (Figure 4.12, Panel B, tracks 1-18), albeit weak in some cases. BamH I digested PalF DNAs resolved two probe-specific bands, approximately 9.5 kb and 6.2 kb in size. EcoR I digests similarly resolved two fragments, though of larger size (approximately 20 kb and 15 kb). In the absence of any detailed study on bovine c-*myc* the status of detected bands (whether active *myc* gene, a pseudogene, or *myc*-like sequences) cannot be established with certainty. However their detection following high stringency washes, coupled with the predicted detection of appropriately sized c-*myc* bands in both human and mouse DNAs at this stringency, would suggest that bovine c-*myc* was being detected. There was no observable difference in number / size of bands or intensity of signal (allowing for DNA loading variations) among control DNAs (ethanol-treated and quercetin-treated PalF) and all test DNAs (ethanol-treated and quercetin-treated (protocol QA) PalF viral transformant classes). Thus neither transformation of PalF cells by BPV-4 viral genes nor treatment with quercetin (with or without BPV-4 genes) resulted in a detectable change in the DNA status detected by this c-*myc* probe.

Ductin probing

Ductin was screened solely as a DNA gel loading and Southern transfer efficiency control. BamH I digestion of bovine genomic DNA revealed two bands, one of

approximately 9.5 kb, the other of approximately 5.0 kb. EcoR I digestion also revealed two bands, one of approximately 13.5 kb, the other of approximately 11.5 kb (see Figure 4.12, Panel C, tracks 1-18). Human and mouse DNA (tracks 19-21) gave very little or no signal. Strength of ductin signal across gels in bovine tracks correlated well with other measures of DNA loading (i.e. original ethidium bromide picture of gels, intensity of satellite sequences across tracks as observed in initial low stringency washes). Thus there was no reason to suspect that observed variation in signal among tracks with *ras* or *myc* probes was due to anything other than differential DNA loadings.

4.4.3.2 RNA Analyses

Figure 4.13 shows a representative example of results from one of the RNA dot blots probed sequentially with c-Ha-*ras*, (A), ductin (B) and c-*myc* (C). This particular blot predominantly contained RNA from clonal lines derived by transfection of PalF cells with BPV-4 E7 DNA, with or without HPV-16 E6 and with or without prior quercetin treatment (protocol QA). (See Figure 4.13 legend for full details.) As detailed in Chapter 4.4.2.3 each sample is duplicated on the blot. All blots contained RNA from PalF cells (dots A1 & A2) and quercetin-treated PalF cells (dots A4 & A5). These gave positive signal with all three probes indicating the expression of all three genes.

Each blot also contained duplicate samples of 200 ng BPV-4 bovine papilloma DNA (dots F11 & F12) and HL-60 (human cell line) RNA (dots H11 & H12). As illustrated in the example autoradiographs (Figure 4.13; Panels A-C), all blots probed with *ras* gave a signal for the bovine papilloma DNA dots (F11 & F12), and a weaker signal with ductin and c-*myc*. Signal from these dots is not unexpected given the bovine DNA present. The c-*myc* probe consistently gave the weakest signal, consistent with the use of a heterologous probe and the results of the DNA analyses described above. The HL-60 RNAs (H11 & H12) exhibited strong signal on c-*myc* probing as expected, but also gave strong signal with *ras* and ductin - despite the bovine-specific nature of both

	1	2	3	4	5	6	7	8	9	10	11	12
A	PalF			QPalF			QA E7 cl.4			QA E7+16E6 cl.1		
B	E7 cl.1			E7+16E6 cl.4			QA E7 cl.1			ZipE8E7 + 16E6 cl.1		
C	E7 cl.2			E7+16E6 cl.4			QA E7 cl.2			BPV-4 cl.3		
D	E7 cl.3			E7+16E6 cl.3			QA E7 cl.2					
E	BPV-4 +16E6 cl.2			E7+16E6 cl.2			QA E7 cl.3					
F	ZipE8E7 cl.2			E7+16E6 cl.1			QA E7+16E6 cl.2				Papilloma DNA	
G				ZipE8E7 cl.2			E7 + 16E6 cl.1					
H							QA E7+16E6 cl.4				HL-60	

(Key to dot blot autoradiographs on facing page)

Figure 4.13 RNA dot blot analysis of control (ethanol-treated) and quercetin-treated (protocol QA) PalF transfectants: status of *ras* and *myc* expression.

20 μg total RNA were loaded per well. Samples from each line were loaded in duplicate in adjacent wells unless otherwise indicated. Controls included RNAs from ethanol-treated PalF cells, quercetin-treated PalF cells, HL-60 cells (positive control for the *myc* probe) and 200 ng bovine oesophageal papilloma DNA, which contains episomal BPV-4 DNA (positive control for viral probing - see Chapter 5.2.2) cl. = clone. **Panel A**: probed with bovine c-Ha-*ras* DNA. **Panel B**: probed with bovine ductin DNA as loading control. **Panel C**: probed with human c-*myc* DNA. (probes detailed in Chapter 2.1.7).

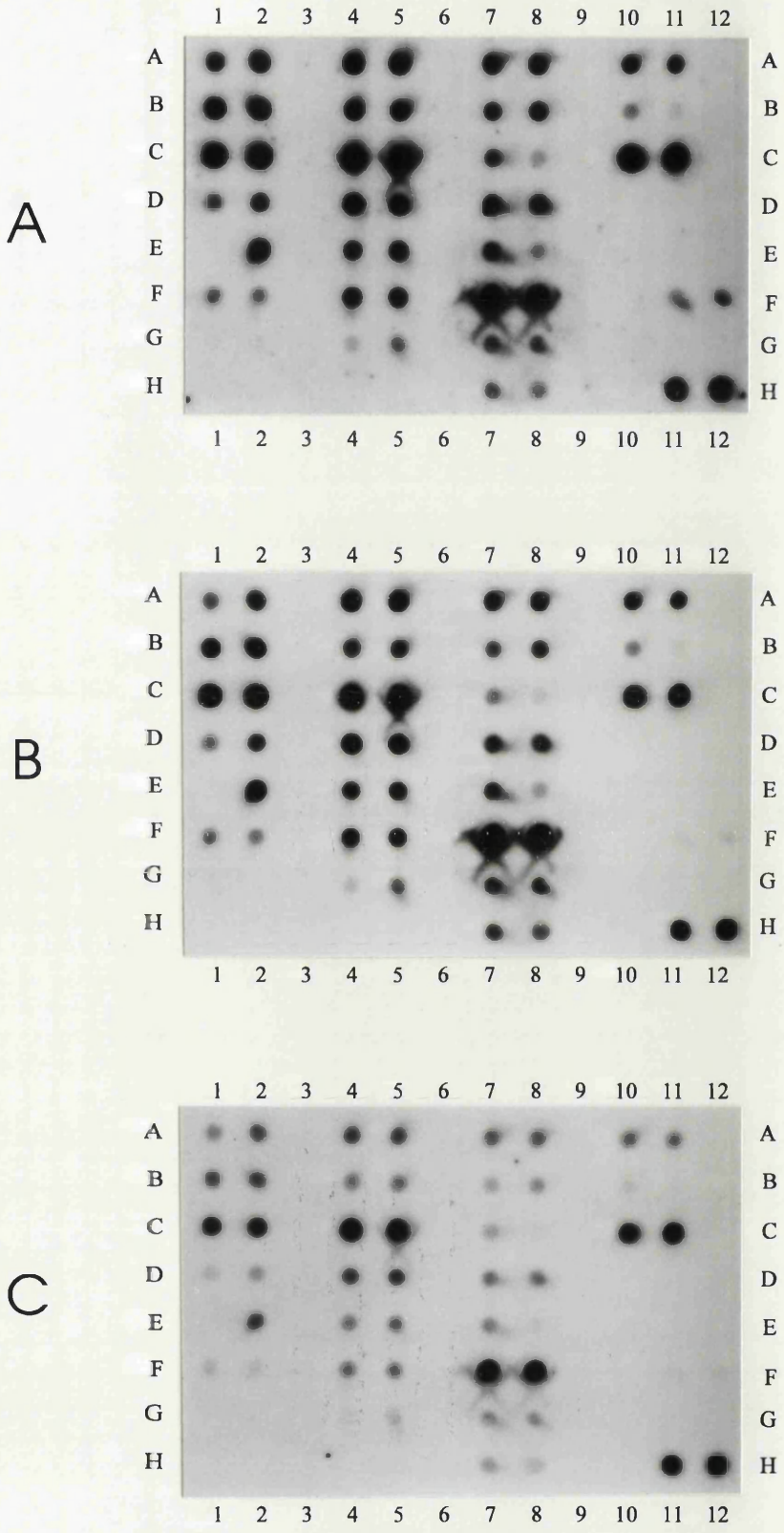


Figure 4.13

these probes. Previous DNA analyses (outlined above) did not detect homology between either the c-Ha-*ras* or ductin probes and HL-60 DNA under the same high stringency post-hybridisation washes used in the RNA analyses. These results may be due to the presence of a larger quantity of *ras* or ductin sequences in 20 µg total RNA compared to 5 µg DNA. (If this were the case then it would not be possible to distinguish between endogenous *ras* (bovine) and exogenous (human activated) *ras* expression in any of the transfectant lines.) An alternative explanation may be that the signal detected was from non-specific hybridisation to some other component of the total RNA solution that was dot-blotted. Probing of the same dot-blot membranes with BPV-4 sub-genomic fragments (Chapter 5.3; Figures 5.5 & 5.6) gave no signal from HL-60 dots. If detected signal was due to non-specific probe retention then it would be unlikely that both *ras* and ductin probes would 'hybridise' yet neither E7 nor E8 DNA would. Nevertheless, without further investigation, the possible non-specific nature of signal must be recognised.

Given the observed differences in RNA loading / membrane retention (e.g. Figure 4.13; Panels A-C; A1 vs A2 or C7 vs C8) and also the concerns expressed above, only a very basic quantitative analysis was considered. By comparing signal strength for any individual RNA dot across the three autoradiographs it was possible to comment on whether relative levels of *ras*, *myc* and ductin were changed compared to control PalF RNA. No obvious differences were apparent among any of the transformant classes analysed. This would suggest either no change in expression levels, or that expression of all three genes was affected to an equal extent.

4.4.3.3 Comments

This aspect of the project was planned as a preliminary investigation of DNA and RNA status of c-Ha-*ras* and c-*myc* in the various PalF transformed and quercetin-treated cell lines. While the DNA analyses indicated that there were no apparent major

rearrangements or amplification of these genes, under the conditions employed, one cannot rule out smaller genetic alterations (e.g. point mutations, inversions, doubling or tripling of gene copy number). This would require a much more detailed approach including a much better knowledge than is currently available from the literature of RFLP / sequences of these particular genes in the bovine genome.

Data from initial RNA studies suggested no major changes in expression of *ras* or *myc* genes, however a more detailed analysis of these cell line RNAs is required. Repetition of these RNA experiments using Northern blots of mRNA from these various cell lines would obviously be a more informative approach. Due to time constraints this much more labour intensive methodology was beyond the scope of the present study.

4.5 Mechanisms of quercetin action

4.5.1 Introduction

It has been reported that quercetin treatment can result in genetic mutation, including clastogenic damage, and in epigenetic events, for example this chemical has been shown to inhibit RNA polymerases (Nose, 1984) and disrupt phosphorylation / dephosphorylation mechanisms (Van Wart-Hood *et al.*, 1989; Matter *et al.*, 1992). The following set of experiments were carried out to investigate whether genetic or epigenetic changes arose subsequent to treatment of PalF cells with quercetin, independently of any contribution from BPV-4 genes and an exogenous activated *ras*.

4.5.2 Cytogenetic Analysis

4.5.2.1 Background

Consistent chromosomal aberrations have been associated with particular types of cancers (Solomon *et al.*, 1991). Bracken-grazing animals display large scale chromosomal abnormalities (Moura *et al.*, 1988) and it has been shown that quercetin, a component of bracken fern, can induce clastogenic damage. Ishidate (1988) observed

that treatment of a Chinese hamster fibroblast cell line (CHL) with quercetin concentrations of between 200-400 μM, induced both an increased level of tetraploidy and observable chromatid gaps, breaks and exchanges.

In a previous study of PalF cells, Pennie (1992) found no evidence of clastogenic damage at quercetin levels (20 μM or 45 μM) at which synergism between this chemical and BPV-4 had been observed. It was decided to extend this work by examining whether varying the concentration of quercetin and / or the length of chemical exposure had any detectable chromosomal effects on PalF cells.

4.5.2.2 Experimental Rationale

Actively growing (sub-confluent) cultures of PalF cells were treated with five different quercetin concentrations, namely 20, 45, 100, 200 and 300 μM for 48 hours. These concentrations spanned the levels observed to detect synergism with BPV-4 DNA in PalF cells and those which were reported to induce structural chromosome aberrations in Chinese hamster cells (Ishidate, 1988). For the 20 μM and 45 μM concentrations the quercetin solvent used was ethanol (stock solution: 10 mM quercetin) while for the higher concentration experiments (100-300 μM quercetin) dissolution in DMSO was necessary (stock solution: 100 mM quercetin). In all cases the final volume of ethanol or DMSO was made up to 180 μl per 40 ml culture medium. After removal of treatment, cultures were washed twice with PBS and fresh medium added. Controls were PalF cells and PalF cells exposed to 180 μl ethanol or DMSO (per 40 ml medium).

The karyotypic analysis was carried by Drs. Ruedi Fries and Sabina Solinas (ETH, Zürich) who specialise in bovine karyotypic analysis. These same workers had performed the previous karyotypic analysis of quercetin-treated PalF cells described by Pennie (1992). Sub-confluent flasks of PalF cells were air-freighted to the Zürich laboratory after treatment and were examined after one further passage. Q-banded

metaphase spreads were prepared for all classes and scored both for ploidy level and possible chromosomal rearrangements / damage.

4.5.2.3 Results and Discussion

Most PalF cells have a normal diploid complement of 60 chromosomes. The percentage of tetraploid genomes observed in these control cells was 7.3 % (Table 4.13). This was considered to be within the normal expected range for primary tissue culture cells (S. Solinas, personal communication). Both solvent treatments (irrespective of presence or level of quercetin) increased the percentage of observed tetraploidy approximately two to threefold (Table 4.13). These increases were statistically significant when compared to normal PalF cells (χ^2 tests on diploid : tetraploid ratios; PalF cells and ethanol-exposed cells, $P = 0.001$, PalF cells and DMSO-exposed cells; $P << 0.001$). However within each solvent class there was no statistical difference among control (no quercetin) and different quercetin concentrations (χ^2 tests on diploid : tetraploid ratios; ethanol class, $P = 0.137$; DMSO class, $P = 0.862$). Similarly there was no significant difference in ploidy levels between ethanol- and DMSO-exposed PalF cells (χ^2 tests on diploid : tetraploid ratios; ethanol and DMSO classes, $P = 0.123$). Thus, while differences in ploidy level were observed in the experiment these could be attributed to solvent effects rather than quercetin itself. In both cases quercetin-treated PalF cells showed higher percentage tetraploidy as compared to their solvent controls. Although not statistically significant, one cannot rule out the possibility that this trend may have a biological significance. There was no obvious chromosomal rearrangements, gaps or breaks observed among any of the karyotypes analysed.

While ethanol has a demonstrable effect on ploidy levels in these cells, this alone does not appear to contribute to transformation of PalF cells, as evidenced by phenotypic comparison of ethanol-treated versus quercetin-treated virally-transformed PalF cells (cf. Tables 4.4 and 4.7). It should be pointed out, however, that the ethanol-treated

Table 4.13 Results of the karyotypic analysis of quercetin-treated PalF cells.

PalF Cells Treatment	Metaphases Total scored	Number Diploid	Number Tetraploid	Percentage Tetraploid
PalF cells (untreated)	178	165	13	7.3
Ethanol only control	197	169	28	14.2
+ 20 μM Quercetin	144	118	26	18.1
+ 45 μM Quercetin	117	90	27	23.1
DMSO only control	156	122	34	21.8
+ 100 μM Quercetin	159	118	41	25.8
+ 200 μM Quercetin	96	72	24	25
+ 300 μM Quercetin	63	48	15	23.8

conditions used in the transfection studies contained only 80 µl ethanol per 40 ml medium compared to the 180 µl per 40 ml used in the above karyotypic analysis. Perhaps at this lower concentration ethanol would have a reduced effect on the proportion of tetraploid cells observed. No obvious structural chromosomal damage was observed even at quercetin concentrations 15 x greater than that used in all quercetin-treated transfection experiments. At these high levels, such damage was noted by Ishidate (1988) in a Chinese hamster cell line (CHL). This may in part be explained by differences in assay conditions. CHL cells were assayed immediately following quercetin treatment whereas the PalF cells were in transit for several days after removal of quercetin and underwent at least one further passage before analysis in Zürich. This would inevitably lead to selection against the most damaged cells.

4.5.3 Minisatellite Analysis

4.5.3.1 Background

The human genome contains hypervariable regions of DNA consisting of tandem repeats of a short sequence termed a minisatellite (Jeffreys *et al.*, 1985). Many of these minisatellites are highly polymorphic, this resulting from allelic differences in the number of repeats present. The observed allelic variation is presumed to arise by mitotic or meiotic unequal exchanges or by DNA slippage during replication leading to gain or loss of repeat units (Jeffreys *et al.*, 1985; 1987). This variation in length can be detected by digesting genomic DNA with a restriction enzyme which does not cleave the repeat unit, blotting the gels and probing the resulting membranes with radioactively labelled minisatellite DNA. The complex pattern of the resulting restriction fragment length polymorphism is called the DNA fingerprint (Jeffreys *et al.*, 1985). Highly variable minisatellite sequences appear to be ubiquitous among animals and plant genomes, including cattle (e.g. Vassart *et al.*, 1987; Georges *et al.*, 1988; 1990).

Vagnarelli *et al.* (1993) have described the development of a mutation assay in cultured mammalian cells based on direct analysis of minisatellite DNA. Chinese hamster cells (V79) were treated with the mutagen nitrosoguanidine and individual colonies were isolated and expanded. DNA analysis showed minisatellite variation in a number of these cell lines leading the authors to suggest that minisatellite sequences are hypermutable sites that can be used to detect the mutagenic effect of chemical agents. Suzuki *et al.* (1991) observed similar changes in mouse fibrosarcoma cells treated with 55 μM quercetin for 48 hours. In light of such applications and findings it was decided to examine minisatellite sequences in quercetin-treated PalF cells to determine whether or not the flavonoid quercetin causes direct mutation of PalF DNA.

4.5.3.2 Experimental Rationale

Cell treatments

Control and quercetin treatments (20, 45, 100, 200 and 300 μM for 48 hours) of PalF cells followed conditions described in Chapter 4.5.2.2. Following removal of treatment, cells were washed twice with PBS and allowed to grow for one further day in fresh medium before harvesting and subsequent DNA extraction. This mirrored the quercetin treatment used in protocol QA prior to transfection with BPV-4 DNA. DNA was extracted from the cells as described in Chapter 2.2.2.4.

Minisatellite probes

Three readily available minisatellite probes were used; human minisatellite probes J33.6 and J33.15 (Jeffreys *et al.*, 1985) and M13 bacteriophage DNA (Vassart *et al.*, 1987). The J33.6 (720 bp) and J33.15 (600 bp) probes were obtained from plasmid constructs pJ33.6 and pJ33.15 as detailed in Chapter 2.1.7. Wild type single strand M13 (Pharmacia) was used as probe template for M13 fingerprinting. In a pilot experiment a series of enzymes were used with the above probes to establish which enzyme / probe combination gave the most informative banding pattern for analyses. Four enzymes

commonly employed in DNA fingerprinting studies (Alu I, Hae III, Hinf I and Mbo I) were used to digest untreated PalF DNA. Mbo I digests were judged to give the most informative DNA fingerprints, i.e. maximising the number of discernible bands, while minimising background signal.

Assay conditions

Standard electrophoretic (5 μg digested DNA; 0.5 x TBE, 0.8% agarose) and Southern blotting procedures for Mbo I digests of control-treated and quercetin-treated PalF cells were undertaken (detailed in Chapter 2.2.2.14). Subsequent assay conditions, specifically optimised for the various minisatellite probes (Wells, 1988) were used. The J33.6 and J33.15 probes were radioactively labelled (^{32}P dCTP) by random priming (Chapter 2.2.2.11), while a primer extension protocol was used for M13 labelling (Chapter 2.2.2.14). A formamide based hybridisation solution was used for all three probings (Chapter 2.2.2.14). In all cases hybridised membranes were washed to a low final stringency; 1.5 x SSC; 0.1% SDS, for 20 min at 45°C.

4.5.3.3 Results and Discussion

The human minisatellite probe J6.3 detected only 3-4 bands, identical in size in all control-treated and quercetin-treated Mbo I digested DNAs (data not shown). This is not a particularly uncommon occurrence as individual minisatellite families do not occur at the same frequency in all species. Hybridisation of membranes with the remaining two probes J33.15 and phage M13 gave more complex DNA fingerprints (Figure 4.14). The J33.15 probe was particularly informative with in excess of 60 bands being discernible from the autoradiograph. Phage M13 probe resolved 14 bands under the same hybridisation conditions (Figure 4.14). In both cases, no difference in the number or intensity of detected bands could be observed between test and control tracks. Thus this assay provided no evidence of quercetin-induced chromosomal damage / mutation in PalF cells even at concentrations 15 x higher than that shown to synergise

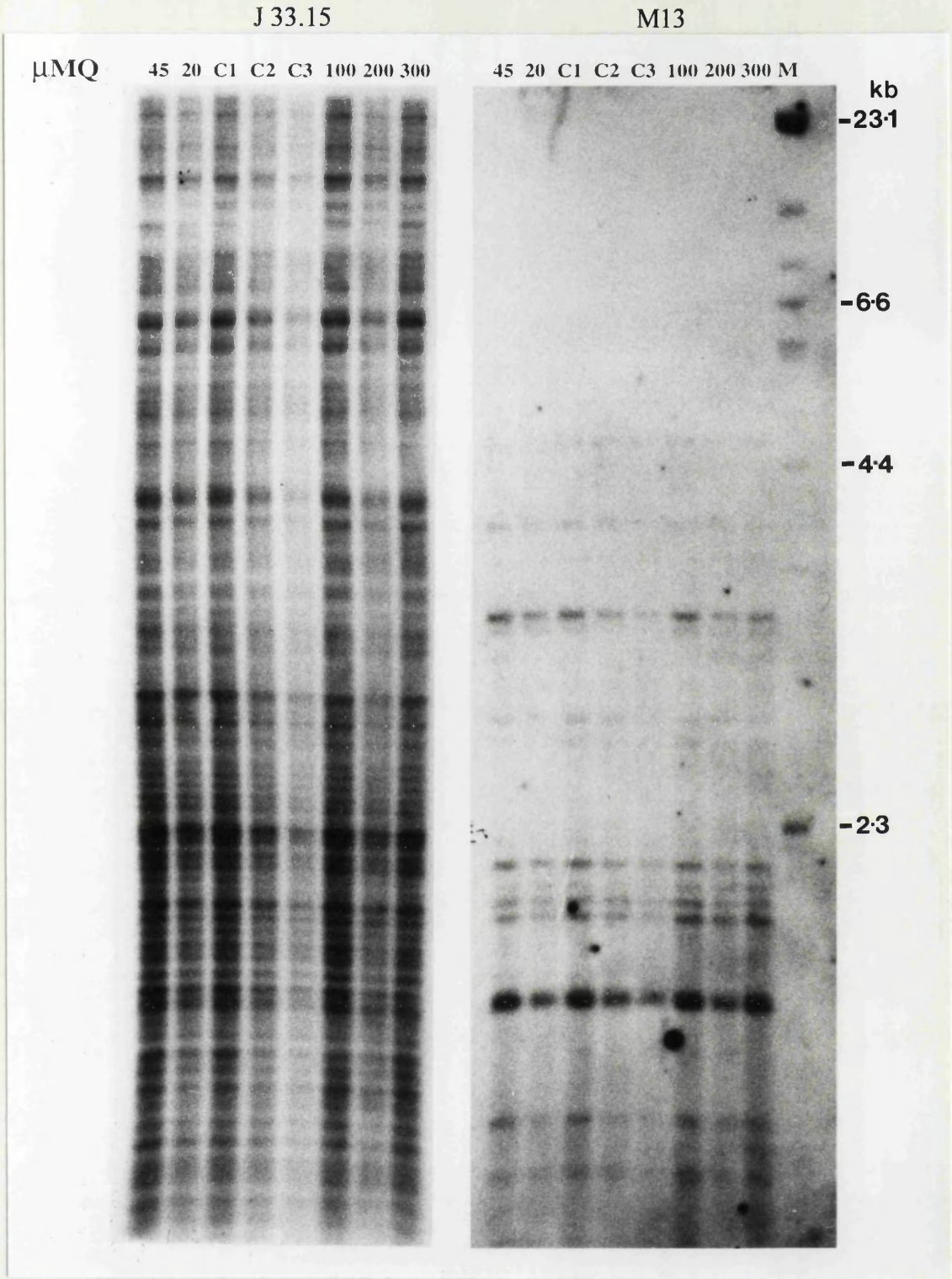


Figure 4.14 DNA fingerprints of control and quercetin-treated PalF cells produced by Jeffreys 33.15 and M13 bacteriophage DNA minisatellite probes.

All tracks contain 5μg Mbo I digested DNA. Numbers refer to quercetin concentrations used (μM). C1, C2 and C3 are ethanol-treated, untreated and DMSO-treated controls receptively. M = marker DNA (λ Hind III + λ EcoR I digested DNA).

with BPV-4 genes resulting in full transformation of PalF cells. However, it should be emphasised that a minisatellite assay of the magnitude employed above would only be likely to detect gross and widespread genetic alterations caused by quercetin treatment.

4.5.4 DNA Strand Break Analysis

4.5.4.1 Background

Quercetin is known to bind to both single and double strand DNA (Rahman *et al.*, 1990). It has also been shown to cause single strand scission in bacteriophage DNA, though this is dependent on the presence of both Cu(II) ions and oxygen. This strand scission reaction was demonstrated to account for the genotoxic activity of quercetin as assayed by bacteriophage inactivation (Fazal *et al.*, 1990). Quercetin has also been reported to induce DNA single strand breaks in mouse lymphoma (L5178Y) cells (Meltz & MacGregor, 1981).

In the present study the role of quercetin in inducing DNA strand breaks was investigated at two levels. First, the ability of quercetin to induce DNA breaks (at concentrations known to synergise with BPV-4 genes in PalF cells) was investigated using a plasmid DNA mobility assay system (Fitzsimmons *et al.*, 1994). Second, possible quercetin-induced DNA damage was measured in PalF cells using both alkaline and neutral filter elution assays. This approach has been successfully employed to measure single and double strand DNA breaks in irradiated mammalian cells (Kohn *et al.*, 1976; Bradley & Kohn, 1979).

4.5.4.2 Experimental Rationale

Plasmid DNA mobility assay

The ability of agents to induce DNA strand breaks can be detected and partially classified by means of a plasmid DNA mobility assay (Fitzsimmons *et al.*, 1994). Supercoiled plasmid (sc) is reduced to relaxed circular form (rc) by DNA single strand

breaks and to linear form (l) by double stranded breaks. These three types of plasmid forms have different electrophoretic mobilities in agarose gels and can therefore be differentiated, allowing detection of induced DNA damage.

The plasmid mobility experiments were carried out by Charles Walker and Dr. A. Lewis (CRC Department of Medical Oncology, Beatson Laboratories, University of Glasgow) who routinely carry out such assays. Incubation mixtures contained 1.6 μg supercoiled pBR322 DNA in a total volume of 60 μl 100 mM sodium phosphate buffer pH 7.4. Various concentrations of quercetin ranging from 0.5-20 μM were added to this reaction mix in the presence or absence of 333 μM $CuCl_2$. All quercetin doses were administered in 5 μl ethanol. Two control classes were also included, namely plasmid in buffer alone and plasmid in buffer with 5 μl ethanol. All reactions were incubated at 37°C for 60 minutes under aerobic conditions. The reactions were stopped by addition of 15 μl stop buffer (5 mM EDTA, 0.5% SDS, 60% glycerol, 0.1% bromophenol blue). Aliquots of 25 μl of stopped reaction mixtures were separated on a 1% agarose gel. DNA in gels was stained with ethidium bromide and photographed under UV transillumination. Densitometric analysis of gel negatives was performed using a Molecular Dynamics Laser Densitometer with image analysis software.

Filter elution assays

DNA damage (whether single or double strand) has been successfully measured in mammalian cells using the technique of filter elution under either alkaline or neutral conditions (Kohn *et al.*, 1976; Bradley & Kohn, 1979). This technique quantifies the level of DNA damage by measuring differential elution characteristics of intact versus fragmented DNA through a polycarbonate filter. Alkaline pH elution conditions measure both single and double strand DNA breaks, whereas neutral pH elution conditions measure only DNA double strand breaks.

For this preliminary analysis elution assays of quercetin-treated and control-treated PalF cells were carried out by Dr. J. Russell (Department of Radiation Oncology, Beatson Laboratories, University of Glasgow). The technique requires the radioactive labelling of DNA in cells for later detection and quantification of possible DNA damage. Actively growing (i.e. subconfluent) PalF cells were labelled with ^{3}H-thymidine for 20 hours. Subsequently radioactive medium was removed, cultures were washed twice with PBS, and fresh medium containing control or quercetin treatments added for a further 48 hr. Following removal of treatment medium, cultures were washed in PBS, trypsinised, resuspended in medium at a concentration of 5 x 10^5 cells per ml and placed immediately on ice to inhibit possible DNA repair. To each elution column one ml of cell suspension was loaded per nucleopore polycarbonate filter (pore size 2 μm), and the cells lysed (lysis buffer: 2 M NaCl, 0.02 M Na_2EDTA, 2% Sarkosyl, pH 10.2). Filters were subsequently rinsed with 0.02 M Na_2EDTA, pH 10.2. The elution columns were then attached to a peristaltic pump and 25-30 ml of either neutral (Tris glycine , pH 7.0) or alkaline (0.1 M tetra propylammonium hydroxide, 0.02 M Na_2EDTA, pH 12.2) eluting buffer was added. Buffer was pumped at 2 ml per hour (i.e. each run lasted 12-15 hours). At the end of the run, the filters and the eluate were counted using a scintillation counter. The fraction eluted (and hence the proportion of the sample containing DNA damage) was calculated by comparing this value to the total amount of radioactivity present in both filter and eluate. The positive control used throughout these experiments was control-treated PalF cells irradiated with 15 Gy (^{60}Cobalt source, dose rated 2.7 Gy / min).

Initials attempts at elution assays which involved a full set of quercetin concentration treatments (20-300 μM) for two different exposure times (48 and 96 hours), gave unsatisfactory results. The recommended ^{3}H-thymidine labelling dose (3.7 kBq / ml of culture medium) proved to be too efficient in PalF cells. Counts were considered to be too high to quantify accurately. Due to resulting time constraints it was decided to

repeat the assay only on PalF cells treated with 300 μM quercetin, the highest concentration considered. The negative control was PalF cells treated with an equal volume of DMSO (the quercetin solvent). For these latter assays the ^{3}H-thymidine labelling dose was reduced to 0.74 kBq / ml of culture medium.

4.5.4.3 Results and Discussion

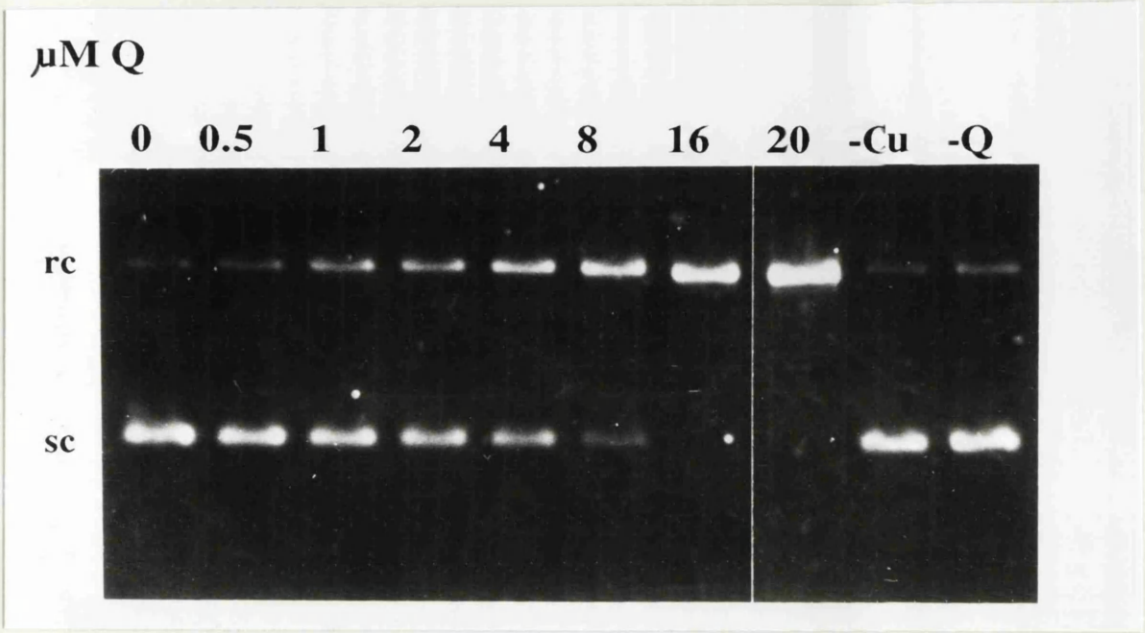
Plasmid DNA mobility assay

Results showed that quercetin induced single strand breaks in plasmid DNA (Figure 4.15). This was visualised by the conversion of supercoiled plasmid DNA to the relaxed circular form. No linear plasmid was observed indicating that no double strand DNA breaks were induced in this assay. The two control samples, namely plasmid alone (track 1) and plasmid plus ethanol (track 10), gave similar background levels (approximately 10%) of relaxed circular (rc) form. Increasing doses of quercetin led to increasingly higher levels of relaxed circular (i.e. nicked) plasmid. Results from the two highest concentrations of 16 μM and 20 μM showed that conversion to the relaxed circular form was complete. At the highest concentration it was found that total conversion from supercoiled to relaxed circular form was complete within 15 minutes post addition of quercetin (Figure 4.16). All reactions were carried out in the presence of $CuCl_2$. This requirement for Cu(II) ions was demonstrated by the lack of plasmid form conversion in conditions where 20 μM quercetin was included but $CuCl_2$ was absent (Figure 4.15; track 9). Although this is an *in vitro* assay it does indicate that quercetin is capable of inducing DNA damage at concentrations equal to and even more dilute than that found to synergise with BPV-4 genes contributing to the full transformation of PalF cells.

Filter elution assays

Results from both alkaline and neutral filter elution assays of PalF cells treated with 300 μM quercetin are summarised in Figure 4.17. In both cases there was no

A)



B)

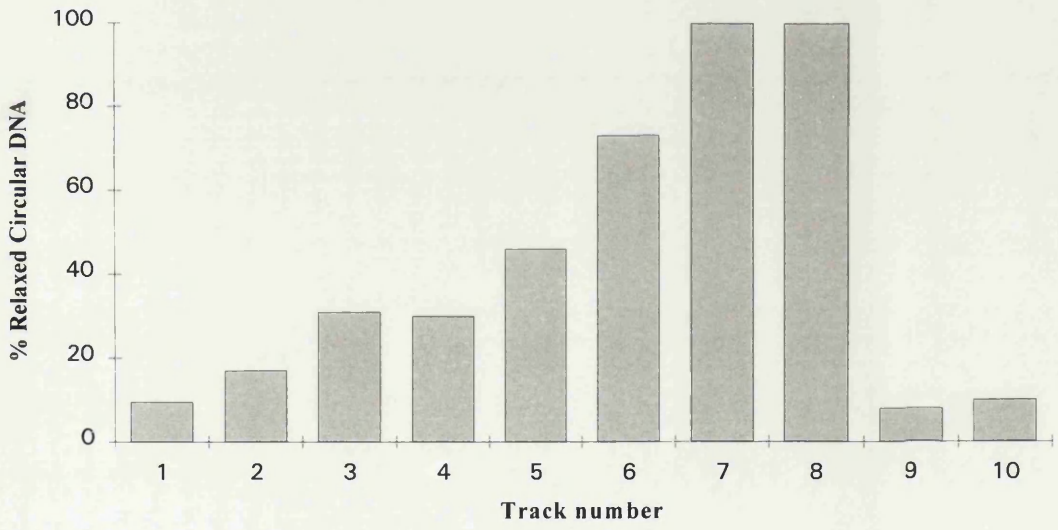


Figure 4.15 Ability of quercetin to induce plasmid pBR322 DNA damage in the presence of Cu(II) ions and oxygen. **A:** ethidium bromide stained gel; **B:** densitometric analysis of relative percentage of relaxed circular form to total plasmid DNA for each treatment / control.

Track 1: plasmid and buffer
Track 2: + 0.5 μM quercetin
Track 3: + 1.0 μM quercetin
Track 4: + 2.0 μM quercetin
Track 5: + 4.0 μM quercetin
Track 6: + 8.0 μM quercetin
Track 7: + 16.0 μM quercetin
Track 8: + 20.0 μM quercetin
Track 9: + 20.0 μM quercetin (no Cu)
Track 10: + ethanol

rc = relaxed circular form
sc = supercoiled form

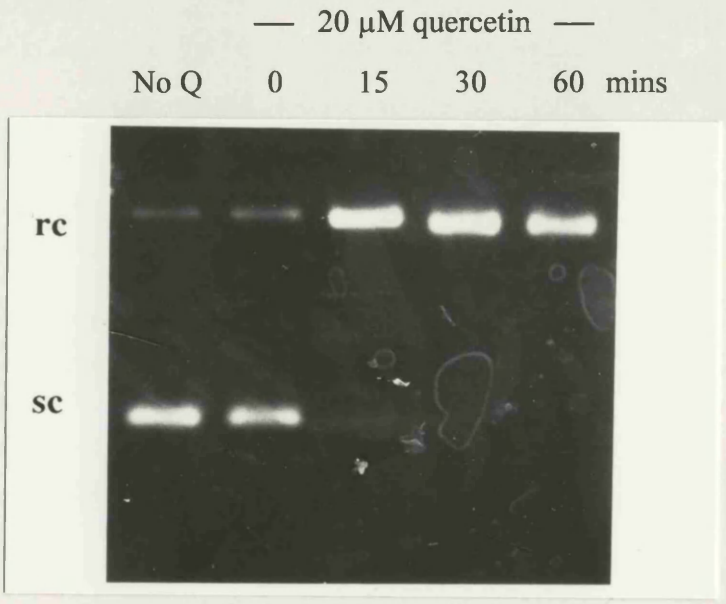


Figure 4.16 Ethidium bromide stained agarose gel demonstrating the conversion of pBR322 plasmid DNA by 20 μM quercetin from supercoiled (sc) to relaxed circular (rc) form over time. All reactions were carried out in the presence of Cu(II) ions and oxygen.

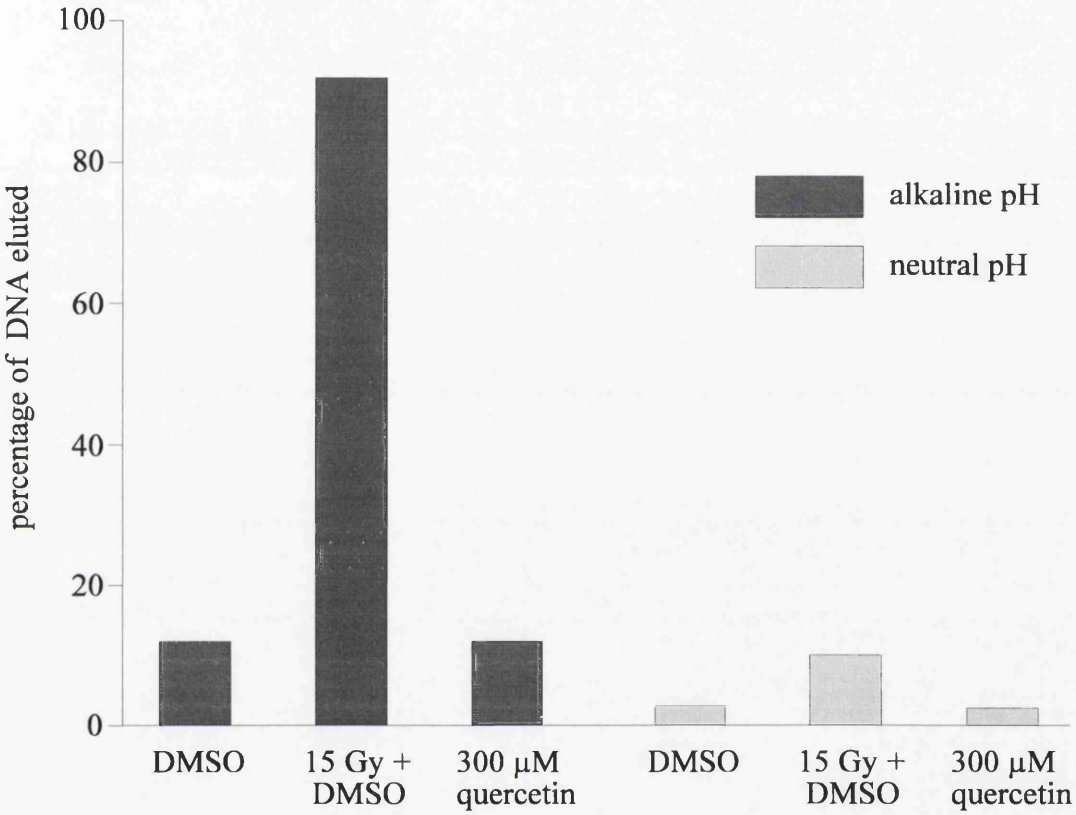


Figure 4.17 Elution assay to examine possible quercetin-induced DNA damage in PalF cells.

DNA damage is expressed as the percentage of ^{3}H-labelled DNA eluted. DMSO-treated PalF cells irradiated with 15 Gy ^{60}Co acted as a positive control for DNA damage.

discernible difference between solvent-treated and quercetin-treated classes. This was in contrast to results obtained with the positive control, DMSO-treated PalF cells irradiated with 15 Gy ^{60}Co. It has been estimated that approximately 1000 single strand breaks and 40 double strand DNA breaks are produced per Gray per cell (Elkind & Redpath, 1977), low levels of DNA damage resulting from quercetin treatment may therefore not be detectable. Also, some degree of DNA repair cannot be ruled out during quercetin treatment and / or prior to lysing of cells on filters. Since no detectable difference was found between control and 300 μM quercetin-treated classes repetition of this assay at the lower quercetin concentrations was not considered appropriate.

At first, comparison of results from the plasmid mobility and filter elution studies seem difficult to reconcile. Whereas quercetin concentrations as low as 0.5 μM induce single strand nicks in plasmid DNA, no DNA damage was detectable in cells at 300 μM. This apparent discrepancy may be explained not only by detection sensitivity of the respective assays but also due to the fact that one is an *in vitro* assay while the other is a measure of DNA damage in a cell system. As the intracellular concentration of quercetin in PalF cells is not known the apparent lack of detectable quercetin-induced DNA damage could be due to poor uptake of this chemical into the cells. This is supported by Hatcher & Bryan (1985) who suggested that apparently contradictory quercetin mutagenicity results in *Salmonella typhimurium* could be explained by the fact that oxidation of quercetin outside the cell led to the formation of products that were unable to cross the cell envelope.

4.5.5 DNA Adduct Formation

4.5.5.1 Background

The majority of known or potential environmental chemical carcinogens are biotransformed before they can interact with DNA, whereupon they form addition products or adducts (Miller & Miller, 1981). DNA adducts can be removed by DNA

repair processes, or in extreme cases by cell death. However, if neither of these regulatory processes come into play these DNA lesions may cause mutations which become fixed in the genome by DNA replication. Mutations caused by DNA adduct formation can subsequently play a part in multistage carcinogenesis (Miller & Miller, 1981). Quantification of the amount of DNA adducts formed has been used to estimate the initiating potential of both known and unknown genotoxic agents (Gupta, 1993).

Exposure to reactive oxygen species (ROS; e.g. $OH\cdot$, H_2O_2) has been implicated in carcinogenesis (Cross, 1987). Although first studies suggested that ROS could be involved in tumour promotion (Kensler & Taffe, 1986), exposure to ROS has shown to be genotoxic (Meneghini, 1988). This has stimulated work on the role of ROS in the process of initiation (Meneghini, 1988). ROS exposure results in the formation of various modified DNA bases (adducts), one of the major products being the promutagenic lesion 8-hydroxy-deoxyguanosine (8OHdG).

The genotoxicity of quercetin was reported to correlate with its ability to cause DNA damage in the presence of Cu(II) ions and oxygen (Rahman *et al.*, 1989). This reaction was associated with the generation of ROS including the formation of hydroxyl radicals. Fazal *et al.* (1990), using a bacteriophage model, concluded that the hydroxyl radical was the genotoxic agent in this system. Therefore quercetin-treated PalF cells were analysed for possible DNA adduct formation in order to investigate whether this chemical's contribution to transformation of PalF cells could be explained by chemically-induced DNA modification.

4.5.5.2 Experimental Rationale

Cell treatments

The sample classes tested included; untreated PalF cells, cells treated with either 20 or 45 μM quercetin for 4 days and their solvent controls (i.e. cells treated with ethanol for

4 days). Genomic DNA was extracted from all cultures immediately after removal of quercetin and washing of cultures in PBS, using standard DNA extraction methods (as described in Chapter 2.2.2.4).

DNA analyses

This work was carried out by Dr. A. Povey (Paterson Institute for Cancer Research, Manchester). DNAs from all classes were analysed for the presence of both bulky aromatic adducts and also small ROS-induced adducts using a modification of the ^{32}P-postlabelling technique (Gupta & Earley, 1988). In essence this technique involved enzymatic hydrolysis of DNA to 3'-monophosphates, enrichment of aromatic and / or hydrophobic adducted nucleotides by butanol extraction (resulting in elimination of normal nucleotides) and attachment of a ^{32}P-label to the 5'-hydroxyl end of adducted nucleotides. Separation and detection of DNA adducts was by thin layer chromatography and subsequent autoradiography. High pressure liquid chromatography (HPLC) of samples from the aqueous phase resulting from butanol extraction were analysed for possible oxidative damage, specifically the formation of 8OHdG. Fractions proposed to contain 8OHdG were subsequently processed as for the detection of bulky aromatic adducts. For both assays, spots present in test but not control samples indicated the presence of adducts. The assay sensitivity is approximately 1 adduct / 10^{10} nucleotides for detection of bulky aromatic adducts (Gupta, 1993) and approximately one 8OHdG residue per 10^6-10^7 dG residues for detection of 8OHdG (Povey *et al.*, 1993).

After detection by autoradiography, adduct spots were excised from the chromatogram and radioactivity was measured by Cerenkov counting. Blank spots equal in size to adduct spots were also counted and their count rates subtracted from adduct count rates to obtain true count values. Adduct levels were calculated by relative adduct labelling (RAL) and values finally expressed as attomoles of adduct / μg DNA (Gupta, 1985).

For the 8OHdG assay, quantification of adduct levels was based upon Cerenkov counting of adduct spots from experimental classes excised from the chromatograms, compared to those of known standards. Results were expressed as μmoles 8OHdG / mol dG (Povey *et al.*, 1993).

4.5.5.3. Results and Discussion

Analysis of the quercetin-treated samples by ^{32}P-postlabelling did not reveal adduct formation in either of the quercetin-treated classes (data not shown). Although there was not enough sample material to analyse all classes for 8OHdG levels three classes were assayed. These were 20 C (ethanol alone), 20 Q and 45 C (ethanol alone), giving values of 8.0, 12.6 and 11.2 μmoles 8OHdG / mol dG respectively. Although there was a slight increase in 8OHdG levels in the 20 μM quercetin-treated sample compared to its control this was not considered to be significant. Thus results from the above assays did not show significant experimental evidence of quercetin-induced DNA adduct formation in PalF cells.

4.5.6 Epigenetic Effects

4.5.6.1 Introduction

Quercetin has been reported to have diverse biological activities. In addition to the mutagenic activity of quercetin in bacterial and mammalian cells (Jackson *et al.*, 1993 for review) other studies have demonstrated epigenetic effects. For example, this flavonoid is known to affect many cellular enzymatic processes such as metabolism of cAMP (Graziani *et al.*, 1983). Quercetin has also been shown to inhibit protein kinase C (Gschwendt *et al.*, 1984). Contradictory effects on tyrosine phosphorylation have been described. For example, quercetin has been shown to inhibit the phosphotyrosine activity of the Rous sarcoma virus *src* gene product, pp60^{src}, both *in vitro* and *in vivo* (Graziani *et al.*, 1983). Conversely quercetin has been shown to synergise with the phosphotyrosine phosphatase inhibitor sodium orthovanadate,

resulting in greatly increased levels of protein-tyrosine phosphorylation in both uninfected and Rous sarcoma virus-transformed chick embryo fibroblasts (Van Wart-Hood *et al.*, 1989).

Given the important role tyrosine phosphorylation plays in cell growth, proliferation, differentiation and transformation (see Fischer *et al.*, 1992) and the reported effects quercetin can have on such phosphorylation it was decided to look at levels of phosphotyrosine in quercetin-treated PalF cells.

4.5.6.2 Experimental Rationale

Transfection classes

PalF cells were transfected with a construct containing the BPV-4 LCR and a reporter gene (luciferase) and a second plasmid construct containing a gene encoding β-galactosidase. Both constructs are detailed in Chapter 2.1.7. Test classes were incubated in medium containing 20 μM quercetin for 48 hours before or after DNA transfection (i.e. protocol QA & QC conditions respectively). The control class was treated with ethanol alone. Unlike the long-term transfection assays described in this chapter, cells from these experiments were harvested 2 days after removal of the DNA transfection solution. The experimental protocol is outlined in Table 4.14.

It was planned to carry out dual analyses of these transient transfections to examine both protein tyrosine phosphorylation levels in quercetin-treated transfections and to also examine whether quercetin's contribution to transfected PalF cells involved changes in the expression levels of BPV-4 genes. This latter study was to be tested by monitoring the transcriptional activity of the BPV-4 LCR, a region of the viral genome known to contain both promoter and enhancer sequences which regulate viral gene expression (Jackson & Campo, 1991; 1995). However, expression assays measuring both luciferase activity (driven by the BPV-4 LCR as promoter) and β-galactosidase activity

Table 4.14 Summary of experimental protocol used to examine possible quercetin-induced effects on protein phosphotyrosine levels and BPV-4 LCR activity in transfected PalF cells .

Day	QA Treatment	QC Treatment	Control
1	+ 20 μM quercetin (48 hours)	+ ethanol only (48 hours)	+ ethanol only (48 hours)
3	wash / seed 6 flasks cells @ 5 x 10^5 cells (Leave for 24 hours)	wash / seed 6 flasks cells @ 5 x 10^5 cells (Leave for 24 hours)	wash / seed 6 flasks cells @ 5 x 10^5 cells (Leave for 24 hours)
4	DNA Transfections: Flasks 1, 2, 5 - 15 μg p0luc [a] + 5 μg HSVβgal [c] Flasks 3, 4, 6 - 15 μg pLCRluc [b] + 5 μg HSVβgal		
5	wash cells + ethanol only (48 hours)	wash cells + 20 μM quercetin (48 hours)	wash cells + ethanol only (48 hours)
7	Harvest all classes Flasks 1-4: for luciferase and β-galactosidase assays Flasks 5-6: for protein phosphotyrosine assay		

[a] p0luc - vector alone control

[b] pLCRluc - vector + BPV-4 LCR (nucleotides 6710-331)

[c] HSVβgal - β-galactosidase plasmid (transfection control)

(driven by an HSV promoter; acting as a positive transcription control for transfected DNA) gave very low values. This may suggest that the transfection protocol had been unsuccessful. However, similarly poor assay results obtained throughout the laboratory at this time, using both PalF cells and other lines and also using constructs which had previously shown measurable reporter gene activity, pointed to a more likely problem with the common (commercial) lysis buffer used. This work was not repeated, although it is now currently being carried out by others. Examination of protein phosphotyrosine levels in these cells was still possible.

Protein phosphotyrosine assay

Experimental conditions for the following are detailed in Chapters 2.2.4.1-2.2.4.3. Total cell protein was extracted from all classes and equal amounts run on SDS-PAGE gels. Samples were loaded onto duplicate gels together with a track containing standard protein markers (Rainbow markers; Amersham International plc) and a control track of protein from a cell line known to contain elevated protein phosphotyrosine levels (LA29 cells: chick embryo fibroblasts (CEF) expressing a temperature sensitive v-*src* Rous sarcoma virus mutant; a gift from B. Haefner, Beatson Institute, Glasgow). One gel was stained with Coomassie brilliant blue stain to provide a check for equal protein loading among samples. The other gel was used for Western transfer onto nitrocellulose membranes using a Sartorius semi-dry blotting apparatus according to the manufacturers instructions. Subsequent to transfer, membranes were blocked in the appropriate buffer prior to incubation with a mouse phosphotyrosine monoclonal antibody (UBI, New York). An anti-mouse secondary antibody conjugated with alkaline phosphatase (Sigma) was added followed by further buffer washes and final visual detection of cellular protein phosphotyrosine levels by development with a chromogenic alkaline phosphatase substrate.

4.5.6.3 Results and Discussion

Preliminary results showed that there appeared to be a change in protein phosphotyrosine levels. Phosphotyrosine levels of an as yet unidentified protein (approximately 50-65 kD) were slightly increased in the quercetin-treated transfected cells in comparison to the ethanol-treated control. Data is not shown as due to high background / weak signal it was not possible to discern this band photographically. This experiment was repeated on two further occasions. However, on repetition there was notable degradation of protein samples preventing analyses. Suitable caution must therefore be applied in interpretation of results from a single experiment. However continuation of this work by a current PhD student in the laboratory (J. Connolly) has indicated both that quercetin treatment (protocol QC) increases BPV-4 LCR activity in PalF cells, as measured by luciferase reporter gene activity (see above), and also that several proteins show altered phosphotyrosine levels in response to quercetin treatment (J. Connolly, personal communication). Thus quercetin appears to have discernible epigenetic effects in BPV-4-transfected PalF cells.

CHAPTER 5

GAP JUNCTIONAL INTERCELLULAR COMMUNICATION

Chapter 5

GAP JUNCTIONAL INTERCELLULAR COMMUNICATION

5.1 Introduction

5.1.1 Gap Junctional Intercellular Communication (GJIC)

Gap junctions are specialised intercellular channels which provide cell-cell communication between the cytoplasms of adjacent cells in multicellular organisms. They can be open or closed (gated). Cell types tend to form homologous rather than heterologous gap junctions (Fentiman *et al.*, 1976; Pitts & Bürk, 1976) and 'sort out' into separate domains (Pitts, 1980). This results in the formation of communication compartments with well-defined boundaries (Pitts & Kam, 1985). For example, studies of junctional communication pathways in the mouse skin have shown that the stromal cells of the dermis form a large, apparently limitless, communication compartment, whereas the epidermis is divided into many small compartments (Kam *et al.*, 1986; Pitts *et al.*, 1988a). Mapping of these pathways was achieved by microinjection of individual cells with a fluorescent dye (Lucifer Yellow CH), which passes through gap junctions but not across the non-junctional membrane, and subsequent observation of the extent of dye spread. Similar patterns of communication have been observed in human skin (Salomon *et al.*, 1988). The permeability of gap junctions to small molecules, such as nucleotides, oligosaccharides and second messengers, is thought to be important in the homeostatic control of groups of cells in a tissue or organ (Yamasaki, 1990).

Proliferative control in normal epidermis is thought to operate within small units (epidermal proliferative units; Mackenzie, 1975) which are very similar, if not identical to the epidermal communication compartments (Pitts *et al.*, 1988a) described above. Each unit contains one stem cell surrounded in the basal layer by a small number of

committed cells. These committed cells divide a limited number of times to produce the overlying cells which on upwards migration will be eventually lost by desquamation. Stocks of committed cells are thought to be maintained by controlled division of stem cells. This suggests that there must be some form of feedback mechanism such that the division rate of basal cells matches the loss of terminally differentiated cells. Junctional communication is a likely candidate for such a scheme and both positive and negative mechanisms for growth control utilising junctional communication have been proposed (Sheridan, 1976; Loewenstein, 1979). It has been hypothesised that in the epidermis the proliferative activity of the basal cells can be modulated, in a skin thickness-dependent manner, by loss of growth factor-induced second messengers into the overlying, non-dividing cells. If there are sufficient overlying cells the level of second messengers (production of which occurs primarily in the basal layers) may not rise to the threshold required for initiation of cell division. However loss of overlying cells due to terminal differentiation will reduce the cytoplasmic volume available for diffusion of second messengers and cell division will occur. In summary, the upper layers act as a sink for second messengers and consequently as an effective 'brake' on basal layer proliferation (Loewenstein, 1979; Pitts *et al.*, 1988b).

GJIC has been implicated in a number of fundamental cellular processes, for example development, differentiation and growth control. Such processes are disrupted in cancer. Thus, study of the components of intercellular communication has been a focus of recent research. Many cancer cells show decreased GJIC levels (Loewenstein, 1979) and such disruption may also be involved in metastasis (Hamada *et al.*, 1988). A number of tumour promoters have also been shown to decrease GJIC both *in vitro* (Yamasaki, 1990; Fitzgerald & Yamasaki, 1990) and *in vivo* (Sugie *et al.*, 1987; Mesnil *et al.*, 1988).

5.1.2 Gap Junction Components

Each gap junction is composed of two connexons, one contributed from each communicating cell. A number of proteins are involved in gap junction formation. Connexins play a part in gap junction structure and / or control of channel permeability (Beyer *et al.*, 1987; Nicholson *et al.*, 1987; Kistler & Bullivant, 1988). Another family of molecules involved in gap junction formation are the cadherins (calcium-dependent cell adhesion molecules). The cadherins, like the connexins, are expressed in a tissue-specific manner (Jongen *et al.*, 1991) and can act as morphogenetic regulators conferring specific cell-cell adhesions and cell sorting mechanisms (Takeichi, 1991). However a 16 kD protein called ductin is considered to be the major structural component of gap junctions (Finbow & Pitts, 1993). This protein is very hydrophobic and is thought to contain four transmembrane segments arranged as a four α-helical bundle (Finbow *et al.*, 1992). Ductin is also the 'subunit c' or '16 kD proteolipid' of the vacuolar H^+-ATPase (V-ATPase; Mandel *et al.*, 1988). The vacuolar H^+-ATPase is a transmembrane proton pump and is responsible for the acidification of organelles such as endosomes, lysosomes, synaptic vesicles and Golgi bodies (Nelson, 1992). The vacuolar H^+-ATPases play a central role in down-regulation of activated growth factor receptors as they generate an acidic endosomal pH required for dissociation of ligand-receptor complexes as well as targeting these complexes for lysosomal degradation (Brown *et al.*, 1983).

5.1.3 Interaction Between Viral Oncoproteins And Ductin

It is known that the E5 oncoprotein of bovine papillomavirus type 1 (BPV-1) binds to ductin (Goldstein *et al.*, 1991). This binding appears to be necessary for the transforming activity of the E5 polypeptide (Goldstein *et al.*, 1992). Other viral oncoproteins such as HPV-6 E5, HPV-16 E5 (Conrad *et al.*, 1993) and HTLV-1 $p12^I$ (Franchini *et al.*, 1993) also bind to ductin. This suggests that ductin is a common cellular target for these viral proteins.

BPV-1 E5-transformed cells show constitutive activation of receptors for epidermal growth factor (EGF) and platelet derived growth factor (PDGF). E5 expression both stimulates the phosphorylation of the EGF receptor and decreases the down-regulation of these occupied receptors (Martin *et al.*, 1989). Similarly this protein has been shown to stimulate phosphorylation of the PDGF receptor (Petti *et al.*, 1991). Goldstein *et al.* (1991) proposed that a potential site for E5 action was the endosomal compartment and that the association between the BPV-1 E5 protein and ductin in this compartment might interfere with proton pump function, resulting in prolonged growth factor / receptor interaction and recycling of receptors to the cell surface. It was proposed that such a mechanism of viral protein action might well explain the observed activation of cell surface receptors and subsequent mitogenesis in BPV-1 E5-transformed cells. A recent study has reported that cells expressing HPV-16 E5 exhibit inhibition of endosomal acidification (Straight *et al.*, 1995). However, although yet to be demonstrated, the biological activity of BPV-1 E5 protein may depend equally on interactions within the Golgi apparatus and / or disruption of intercellular communication through binding of BPV-1 E5 protein to the ductin component of gap junctions.

The BPV-4 E8 ORF encodes a small hydrophobic polypeptide similar in length (42 residues) and hydrophobicity to the BPV-1 E5 protein (Jackson *et al.*, 1991). Immunostaining of PalF cells transfected with BPV-4 genes revealed that the BPV-4 E8 protein localised in the membrane compartments. The viral protein was found in the plasma membrane, the endoplasmic reticulum (ER) and the Golgi apparatus (Pennie *et al.*, 1993). This is similar to the BPV-1 E5 protein which is also found in the ER, Golgi apparatus and, to a lesser extent, in the plasma membrane (Burkhardt *et al.*, 1989). From these observations it is tempting to suggest that BPV-1 E5 and BPV-4 E8 proteins share some common function(s).

The reported binding of BPV-1 E5 protein to ductin prompted preliminary investigations in this laboratory as to whether such binding affected GJIC in cultured cells (Pennie, 1992). Gap junction mediated cell-cell communication was measured by dye transfer analysis. In this study primary bovine fibroblasts explanted from conjunctival tissue (CON cells; Smith *et al.*, 1987) were transformed with BPV-1 DNA. Results showed that these virally-transformed cells displayed completely disrupted gap junctional communication. Untransfected CON cells communicated well. Although not direct proof that disrupted cell-cell communication was due to the BPV-1 E5 / ductin proteins complexing, it did suggest that reduced communication was a consequence of viral transformation. GJIC was also found to be reduced in a cell line (C10K) which although derived from normal cervical tissue was found to be HPV-16 positive (Pennie, 1992). Cell line C12K, derived from HPV-16 negative normal cervical tissue communicated well (G. Sibbet, personal communication). This provided further circumstantial evidence of a viral component to disruption of GJIC. Examination of eight PalF lines which had been transfected with various BPV-4 genes + *ras* showed that one cell line displayed disrupted communication (Pennie, 1992). This was a clonal line derived from transfection of PalF cells with BPV-4 E8 and E7 ORFs (construct pSVE8E7). As the majority of the cell lines examined communicated well this implied that either BPV-4 E8 protein did not complex with ductin or that communicating and non-communicating cell lines differed in their maintenance of viral DNA or patterns of viral expression. The status of viral DNA in the various cell lines was examined by Southern blotting followed by hybridisation with BPV-4 DNA probes. Results showed that the cell line which showed greatly reduced GJIC maintained multiple copies of BPV-4 DNA (including the E8 ORF) while the other cell lines contained no or very little viral DNA (less than one genome equivalent per cell).

These results strengthen the hypothesis that the BPV-4 E8 protein complexes with ductin resulting in reduced GJIC and that this may be an important aspect of the

transformation biology of the BPV-4 virus. This led to the following more detailed study of GJIC in BPV-4-transformed PalF cells. Due to the observed contribution of quercetin to transformation of virally-transfected PalF cells the possible disruption of GJIC by this chemical was also investigated.

5.2 Experimental rationale

5.2.1 GJIC Measurement

Microinjection dye transfer can be used to measure GJIC. The small molecular weight fluorescent tracer compound Lucifer Yellow CH passes through gap junctions but not the non-junctional membrane. After injection into a single cell the extent of spread of this dye from the original injected cell to its neighbours provides a measure of dye coupling and hence gap junctional intercellular communication. The dye is retained in cells that do not possess functional gap junctions or, in cases where GJIC is reduced but not abolished, the dye spreads to fewer cells than in well coupled populations.

The technique of iontophoretic injection has been described by Pitts and Kam (1985). Single cells were injected with an aqueous solution of 4% w/v Lucifer Yellow CH (Sigma). Glass capillary tubes (outside diameter 1 mm) were used to form micropipettes which were back filled with this dye solution. The micropipette was mounted on a micromanipulator with both manual and electronic control. A silver electrode was connected to the micropipette and a second electrode was placed in the cell culture medium. When the micropipette tip had entered the cell the dye was injected by passing hyperpolarising current pulses (10 nA, 1 Hz, 500 ms duration) through the micropipette over a two minute period. All microinjection dye spread experiments were carried out by Dr. J.D. Pitts (Beatson Institute, Glasgow).

A variety of PalF transfectants was assayed for the extent of functional gap junction intercellular communication. These predominantly comprised the ethanol-treated and

quercetin-treated (protocol QA) cell lines previously described in Chapter 4.2. Cell lines derived from the three other quercetin / BPV-4 protocols (QB-QD; Table 4.8) were also examined. Untransfected PalF cells were assayed for the baseline GJIC level. All microinjection experiments were carried out on actively growing cultures. A minimum of 10 separate injections of single cells, in different areas of each tissue culture dish, were carried out. The amount of dye spread was measured on completion of the two minute injection and a GJIC value for each cell line was calculated by averaging counts from the individual injection sites. Approximately 2 x 10^6 viable cells were seeded per duplicate 60 mm petri dish for each cell line. Cells were allowed to settle overnight prior to microinjection. Cells were assayed when sub-confluent but in contact, to minimise the proportion of quiescent or dead cells being counted as non-communicating.

5.2.2 BPV-4 DNA and RNA status in GJIC assayed cell lines

To investigate whether GJIC levels in transformed PalF cells correlated with the presence and expression of BPV-4 genes , virally-transformed cell lines (ethanol-treated or protocol QA treated) were examined at both the DNA and RNA level with BPV-4-derived DNA probes.

5.2.2.1 Probe derivation

Whole genome BPV-4

This probe consisted of the full BPV-4 genome (7.265 kb). It was isolated by BamH I digestion of the plasmid construct pBV-4. Details of this construct are given in Chapter 2.1.7.

E8

This probe contained nts 236-590 of the BPV-4 genome. This covered the entire E8 ORF (nts 236-458) and did not contain any other viral ORF sequences. The construct

from which this fragment was isolated was termed pALTER-E8 (detailed in Chapter 2.1.7).

E7

This E7 probe contained nts 652-1250 of the BPV-4 genome. This covered the E7 ORF (nts 647-1009) and 357 bp of the E1 ORF. This E7-containing fragment was released by BamH I digestion of the plasmid construct pURE7 (detailed in Chapter 2.1.7).

5.2.2.2. Assay conditions

DNA

Total genomic DNAs from each cell line were assayed for the presence of BPV-4 genes by Southern blot analysis. GJIC levels were measured in cultures of similar passage number to those used for the DNA analysis. The Southern membranes employed in this analysis were also used for detection of c-Ha-*ras* and c-*myc* sequences and have been described fully in Chapter 4.4.2.3. Briefly, both BamH I and EcoR I DNA digests (5 μg) of each cell line were examined. Each membrane contained a number of test lines plus a series of control DNA samples. Controls included similarly digested DNA from untransfected PalF cells and quercetin-treated PalF cells. (Digested DNA from the human cell line HL-60 was used as a positive control for the c-*myc* probe.) Positive control tracks for viral DNA contained 200 ng bovine papilloma DNA (known to contain episomal BPV-4 DNA) digested with either BamH I or EcoR I. Five micrograms of BamH I digested DNA from a murine established fibroblast line (C127) was used as carrier DNA in these viral DNA control tracks. DNAs extracted from cell lines derived from transfection of PalF cells with whole genome BPV-4 DNA were screened with all three BPV-4 probes. E8/E7 transfectant classes were screened with both E8 and E7 probes while E7 transfectants were only screened with the E7-specific probe. Probe labelling (^{32}P dCTP) and conditions of hybridisation and autoradiography were carried out as detailed in Chapters 2.2.2.11-2.2.2.13. In all cases hybridised

membranes were washed to a final stringency of 0.1 x SSC, 0.1% SDS for 30 min (2 x 15 min) at 65°C.

RNA

GJIC levels in cell lines were measured in cultures of similar passage number to those used for the RNA analysis. The RNA dot blots (20 µg total RNA per well) were also used for c-Ha-*ras* and c-*myc* expression assays and have previously been described in Chapter 4.4.2.3. RNA from each cell line was loaded in duplicate in adjacent wells, with RNAs from any particular class of ethanol-treated transfections being present on the same membrane as their quercetin-treated counterparts. Each membrane also contained a set of control samples. RNAs from both untransfected PalF cells and untransfected quercetin-treated PalF cells were present. Two further control samples were 10 µg HL-60 RNA (a positive control for c-*myc* expression) and 200 ng bovine papilloma DNA (a positive control for BPV-4 probe hybridisation). Post-hybridisation conditions were identical to those carried out for the DNA membranes, i.e. 0.1 x SSC, 0.1% SDS for 30 min (2 x 15 min) at 65°C.

5.3 Results and Discussion

5.3.1 GJIC: ethanol-treated PalF transfectants

In untransfected PalF cells injected dye spread to an average of 18.4 cells (Table 5.1; Figure 5.1). Results from cells transfected with whole-genome BPV-4 (with or without HPV-16 E6) showed reduced GJIC compared to control cells (Table 5.1; Figure 5.1). This was the case both for all six clonal lines and the single polyclonal line assayed (student's *t*-test pairwise comparisons for equality; maximum $P < 0.001$). Transfection of PalF cells with either E8/E7 or E7-containing plasmids (with or without HPV-16 E6) showed a range of GJIC values which were much more similar to control cells than to whole genome BPV-4-transformed cells. Some of the cells transfected with E8/E7 and E7 alone appeared to show increased coupling as compared to PalF controls (Table 5.1).

Table 5.1 Levels of gap junctional intercellular communication (GJIC) in control (ethanol-treated) PalF cells transfected with whole or sub-genomic BPV-4 DNA fragments, with or without HPV-16 E6 DNA: Comparison with morphological transformation (M.T.), anchorage independence (A.I.) and immortalisation (Imm.) studies of the same cell lines.

Viral genes		GJIC	Phenotypic effects		
(+ neo + *ras*)	Clone	Mean[a] ± S.D.	M.T.	A.I.	Imm.
BPV-4	Polyc.	4.7 ± 2.3	+	+	-
BPV-4	2	1.7 ± 1.8	+	+	nd
	3	6.8 ± 3.3	+	+	nd
	4	7.9 ± 2.3	+	+	nd
	5	8.0 ± 4.0	+	+	nd
BPV-4 + 16E6 [b]	1	0.8 ± 1.1	+	+	+
	2	3.7 ± 1.6	+	+	-
E8/E7 (Zip) [c]	1	29.0 ± 5.8	+	+	-
	2	13.0 ± 3.6	+	+	nd
E8/E7 (Zip) + 16E6	1	35.0 ± 6.3	+	+	+
E7	1	23.9 ± 5.4	+	-	-
	3	11.6 ± 4.4	+	-	nd
	4	31.3 ± 7.5	+	nd	nd
E7 + 16E6	2	18.3 ± 9.7	+	-	nd
	3	15.5 ± 6.5	+	-	+
	5	23.6 ± 7.3	+	nd	nd
control cell line					
PalF (ethanol-treated)		18.4 ± 7.3	-	-	-

+ = positive, - = negative for phenotype; nd = not determined

[a] Mean number of fluorescently coupled cells ± standard deviation

[b] 16E6 = HPV-16 E6

[c] refers to pZipE8E7 construct

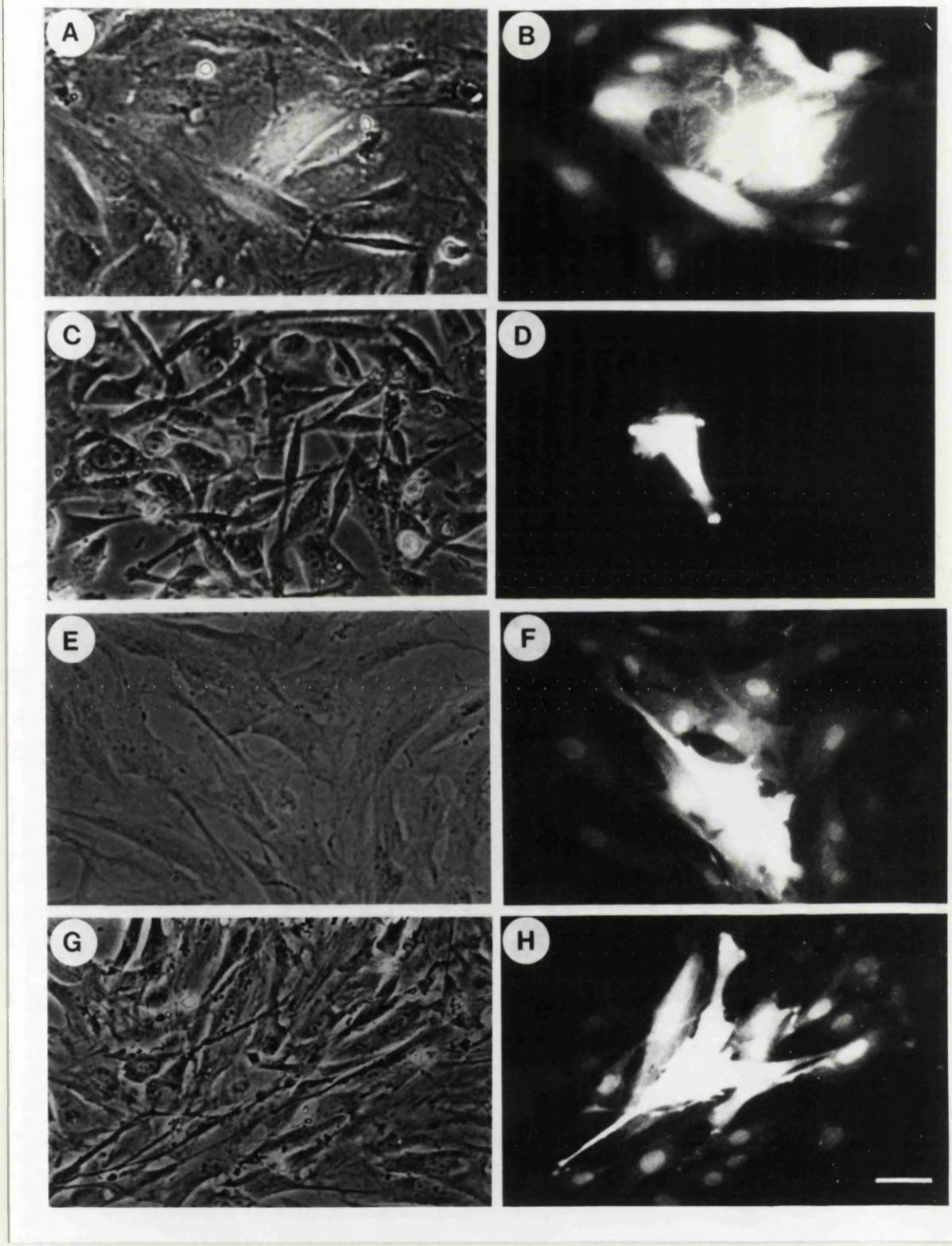


Figure 5.1 Down-regulation of GJIC in virally-transformed PalF cells as assayed by dye transfer analysis.

A & B Control PalF cells
C & D PalF cells transformed by BPV-4 + *ras*
E & F PalF cells transformed by ZipE8E7 + *ras*
G & H PalF cells transformed by E7 + *ras*

A, **C**, **E** & **G** are phase contrast and **B**, **D**, **F** & **H** are fluorescence micrographs. The bar indicates 10 μm in all panels.

This can be explained by the morphology of transformed cells. Such cells tend to be smaller than untransformed cells. Over a given time period, in well coupled cell cultures the dye will be able to diffuse into a greater number of small cells as compared to cells of larger volume. In the few cases where lower than normal GJIC values were recorded (e.g. Table 5.1 E7 clone 3; E8/E7 clone 2) this coincided with observations indicating that these particular cell cultures were not as densely populated as others. Thus, GJIC values recorded for all E8/E7 and E7 alone cell populations were considered as showing unimpaired intercellular communication. While the greatly reduced GJIC levels observed for whole genome BPV-4 transformants and the normal GJIC levels seen in E7 transformants could support the hypothesis that the E8 protein complexes with ductin, the lack of disruption of GJIC in E8/E7 transformants was surprising in that it appeared to contradict this theory.

Gap junctional intercellular communication was measured in two cell lines transfected with whole genome BPV-4 + HPV-16 E6. Both these lines displayed greatly reduced GJIC. Results from Table 5.1 indicated that PalF cells transformed by E8/E7 + HPV-16 E6 and E7 + HPV-16 E6 communicated well. Thus there does not appear to be a direct link between co-transfection of an E6 gene and disruption of GJIC. Previously it has been shown that addition of an HPV-16 E6 gene to PalF cells transfected with BPV-4 genes confers immortality (Chapter 4.2.3.1). A direct link between immortality and GJIC levels is, thus, not apparent. However, GJIC was assayed 5-6 weeks post DNA transfection whereas immortality was assessed over a 4-6 month period. A number of clones would have to be tested at the immortality assay endpoint before such a causative linkage could be confidently refuted.

As described earlier in Chapter 4.2.3.1 anchorage-independent growth of ethanol-treated PalF cells was dependent on transfection with viral DNA containing the E8 ORF (i.e. whole genome BPV-4 or E8/E7 constructs). In the above assays whole genome

transfectants displayed reduced communication while E8/E7 transfectants showed normal levels of GJIC (Table 5.1). Thus there is no direct correlation between GJIC levels and anchorage independence. Verification of this would require measurement of GJIC in cell lines derived from expansion of individual Methocel colonies.

5.3.2 GJIC: quercetin-treated (QA) PalF transfectants

Treatment of untransfected PalF cells with 20 μM quercetin for 48 hr did not appear to affect GJIC as compared to ethanol-treated controls (Table 5.2). Three QA BPV-4 clonal lines and one polyclonal line were assayed (Table 5.2). All four cell lines showed greatly reduced GJIC levels compared to quercetin-treated control cells (student's *t*-test pairwise comparisons for equality; maximum $P < 0.001$). The mean values obtained for GJIC in these cells lines were not markedly different from those observed in their ethanol-treated counterparts (cf. Table 5.1). Therefore, under both experimental conditions (i.e. with or without quercetin treatment) transfection of PalF cells with whole genome BPV-4 resulted in greatly reduced GJIC levels. Among all other transformant classes only one other clone showed evidence of disrupted intercellular communication. This was a line derived from transfection of PalF cells with the E8/E7 ORFs under the control of the BPV-4 LCR + HPV-16 E6 (construct pSVE8E7; Table 5.2) (student's *t*-test pairwise comparison for equality; $P < 0.001$). While this E8/E7 result (in conjunction with the whole genome BPV-4 data) tends to support the hypothesis that there is direct interaction between the E8 and ductin proteins resulting in disruption of GJIC, it should be noted that five other E8/E7-transfected clones showed normal levels of GJIC, a result mirrored by all ethanol-treated counterparts.

In the ethanol-treated transformant classes there was no correlation between co-transfection with HPV-16 E6 DNA and change in degree of intercellular communication. Similar results were also obtained for quercetin-immortalised

Table 5.2 Levels of gap junctional intercellular communication (GJIC) in PalF cells treated with 20 μM quercetin prior to transfection (protocol QA) with whole or sub-genomic BPV-4 DNA fragments, with or without HPV-16 E6 DNA: Comparison with morphological transformation (M.T.), anchorage independence (A.I.) and immortalisation (Imm.) studies of the same cell lines.

Viral genes (+ neo + *ras*)	Clone	GJIC Mean[a] ± S.D.	Phenotypic effects M.T.	A.I.	Imm.
BPV-4	Polyc. 1	2.1 ± 1.7	+	+	+
BPV-4	1	5.7 ± 3.3	+	+	nd
	2	3.9 ± 3.0	+	+	+
BPV-4 + 16E6 [b]	1	5.5 ± 2.9	+	+	+
E8/E7 (Zip) [c]	1	33.7 ± 9.8	+	(+)	-
	3	29.3 ± 8.5	+	-	-
E8/E7 (Zip) + 16E6	1	31.1 ± 7.8	+	-	-
E8/E7 (SV) [d]	2	23.7 ± 7.6	+	(+)	-
E8/E7 (SV) + 16E6	1	30.5 ± 10.4	+	-	-
	2	2.1 ± 2.1	+	-	nd
E7	1	19.6 ± 6.6	+	+	nd
	3	26.7 ± 8.6	+	+	nd
	4	38.8 ± 11.0	+	+	+
E7 + 16E6	2	25.0 ± 7.0	+	+	+
	4	22.6 ± 6.7	+	-	nd
control cell lines					
PalF (ethanol-treated)		18.4 ± 7.3	-	-	-
PalF (quercetin-treated)		19.9 ± 7.4	-	-	-

+ = positive, (+) = weakly positive,- = negative for phenotype; nd = not determined

[a] Mean number of fluorescently coupled cells ± standard deviation

[b] 16E6 = HPV-16 E6

[c] refers to pZipE8E7 construct

[d] refers to pSVE8E7 construct

transfectants i.e. both QA BPV-4 polyclonal and QA BPV-4 clone 2 showed reduced GJIC whereas QA E7 clone 4 exhibited normal levels of intercellular communication (Table 5.2). These observations suggest that there is no causative link between quercetin-influenced immortalisation and GJIC levels, though as stated above, more experimental data would be required to confirm this supposition. Quercetin conferred anchorage independence on QA E7-transfected PalF cells. These cells retained normal GJIC levels (Table 5.2). QA BPV-4 transfectants were capable of anchorage-independent growth yet displayed greatly reduced levels of intercellular communication (Table 5.2). Thus although quercetin contributes to transformation of virally-transfected PalF cells the action of this chemical does not appear to involve overt disruption of intercellular communication.

5.3.3 GJIC and BPV-4 DNA / RNA

Results from the microinjection experiments did not appear to support the hypothesis that a possible function of the BPV-4 E8 protein in cellular transformation was disruption of GJIC through direct complexing with ductin. Although results from whole genome BPV-4 (i.e. E8-containing) transfectants showed reduced levels of intercellular communication, hence supporting the above hypothesis, results from the E8/E7-transfected cells showed (with the exception of one population) that these transfectants communicated freely. It was therefore proposed that such apparently anomalous results could be explained by inherent differences in the maintenance or expression of the E8 gene between communicating and poorly communicating cell lines.

Seven whole genome BPV-4-transformed lines were assayed for both GJIC levels and viral DNA status. All of these cell lines contained detectable viral DNA when screened with the three viral probes (i.e. whole genome BPV-4, E8 and E7 probes). This was found to be the case irrespective of whether cells had been treated with quercetin prior

to transfection (QA conditions) or not (Tables 5.3 & 5.4). With only a single exception, viral DNA signal was weak suggestive of low copy number. This in itself was not unexpected as data from established cells (Smith & Campo, 1988; Chapter 3) and earlier preliminary studies on PalF cells (Jaggar *et al.*, 1990) had reported that viral DNA is lost on continued passage. An example of DNA from whole genome BPV-4 PalF transfectants probed with the E8 and E7 DNA probes is given in Figures 5.2 A & B respectively. Based on the known restriction map of the BPV-4-containing plasmid transfected (pBV4; Chapter 2.1.7), BamH I digestion should release a band of 7.265 kb, while EcoR I digestion should release a band of 5.024 kb detectable with the BPV-4 E8 probe. Similar sized bands should also be detected for episomal BPV-4 DNA control tracks. These expected banding patterns were observed in test and papilloma control tracks (Figure 5.2A). From similar probe / pBV4 construct / restriction enzyme considerations for the E7 probe screening, one would expect to detect a single BamH I band of 7.265 kb while EcoR I digestion should allow detection of three bands (5.024, 1.833 and 0.233 kb). As derivation of this whole genome BPV-4 recombinant plasmid involved linearisation of the viral DNA prior to cloning (Campo & Coggins, 1982), the expected detectable band sizes for the E7 probe are slightly different from those seen on probing papilloma DNA. Benign papillomas contain episomal BPV-4 DNA. Hybridisation of digested papilloma DNA with the E7 probe would detect a single band of 7.265 kb for BamH I digests and three bands (5.024, 2.008 and 0.233 kb) for EcoR I digests. The signal seen in BamH I tracks of both cell line DNAs and control papilloma DNA tracks was as expected (Figure 5.2 B). Although the 5.024 kb band was detectable in the EcoR I digested cell line DNA tracks, the 1.833 kb and 0.233 kb bands were not. This was thought to be due to lack of sensitivity of detection, explained by the small amount of viral DNA present in these lines. E7 probing of EcoR I digested papilloma DNA revealed the predicted bands (Figure 5.2 B: track 20).

Table 5.3 Comparison of gap junctional intercellular communication (GJIC) levels and BPV-4 DNA / RNA status in control (ethanol-treated) PalF cells transfected with whole or sub-genomic BPV-4 DNA fragments, with or without HPV-16 E6 DNA.

			DNA /RNA detection with BPV-4 probes				
Viral genes		GJIC	BPV-4	E8 probe		E7 probe	
(+ neo + *ras*)	Clone	Mean ± S.D.[a]	DNA	DNA	RNA	DNA	RNA
BPV-4	Polyc.	4.7 ±2.3	+	+	-	+	-
BPV-4	2	1.7 ± 1.8	+	+	+	+	+
	3	6.8 ± 3.3	+	+	-	+	+
	4	7.9 ± 2.3	+	+	nd	+	nd
	5	8.0 ± 4.0	+	+	-	+	-
BPV-4 + 16E6 [b]	1	0.8 ± 1.1	+	+	-	+	-
	2	3.7 ± 1.6	+	+	-	+	+
E8/E7 (Zip) [c]	1	29.0 ± 5.8		-	-	-	-
	2	13.0 ± 3.6		-	-	-	-
E8/E7 (Zip) + 16E6	1	35.0 ± 6.3		-	-	-	-
E7	1	23.9 ± 5.4				+	+
	3	11.6 ± 4.4				+	+
	4	31.3 ± 7.5				nd	nd
E7 + 16E6	2	18.3 ± 9.7				-	-
	3	15.5 ± 6.5				+	+
	5	23.6.± 7.3				nd	nd
control cell line							
PalF (ethanol-treated)		18.4 ± 7.3	-	-	-	-	-

+ = detected, - = not detected; nd = not determined

[a] Mean number of fluorescent cells ± standard deviation

[b] 16E6 = HPV-16 E6

[c] refers to pZipE8E7 construct

Table 5.4 Comparison of gap junctional intercellular communication (GJIC) levels and BPV-4 DNA / RNA status in PalF cells treated with 20 μM quercetin prior to transfection (protocol QA) with whole or sub-genomic BPV-4 DNA fragments, with or without HPV-16 E6 DNA.

			DNA/RNA detection with BPV-4 probes				
Viral genes		GJIC	BPV-4	E8 probe		E7 probe	
(+ neo + *ras*)	Clone	Mean[a] ± S.D.	DNA	DNA	RNA	DNA	RNA
BPV-4	Polyc.1	2.1 ± 1.7	+	+	-	+	-
BPV-4	1	5.7 ± 3.3	nd	nd	nd	nd	nd
	2	3.9 ± 3.0	+	+	-	+	-
BPV-4 + 16E6 [b]	1	5.5 ± 2.9	+	+	-	+	-
E8/E7 (Zip) [c]	1	33.7 ± 9.8		-	-	-	-
	3	29.3 ± 8.5		-	-	-	-
E8/E7 (Zip) + 16E6	1	31.1 ± 7.8		-	-	-	-
E8/E7 (SV) [d]	2	23.7 ± 7.6		-	-	-	-
E8/E7 (SV) + 16E6	1	30.5 ± 10.4		-	-	-	-
	2	2.1 ± 2.1		-	-	-	-
E7	1	19.6 ± 6.6				-	-
	3	26.7 ± 8.6				+	+
	4	38.8 ± 11.0				+	+
E7 + 16E6	2	25.0 ± 7.0				+	+
	4	22.6 ± 6.7				+	+
control cell lines							
PalF (ethanol-treated)		18.4 ± 7.3	-	-	-	-	-
PalF (quercetin-treated)		19.9 ± 7.4	-	-	-	-	-

+ = detected, - = not detected; nd = not determined

[a] Mean number of fluorescent cells ± standard deviation

[b] 16E6 = HPV-16 E6

[c] refers to pZipE8E7 construct

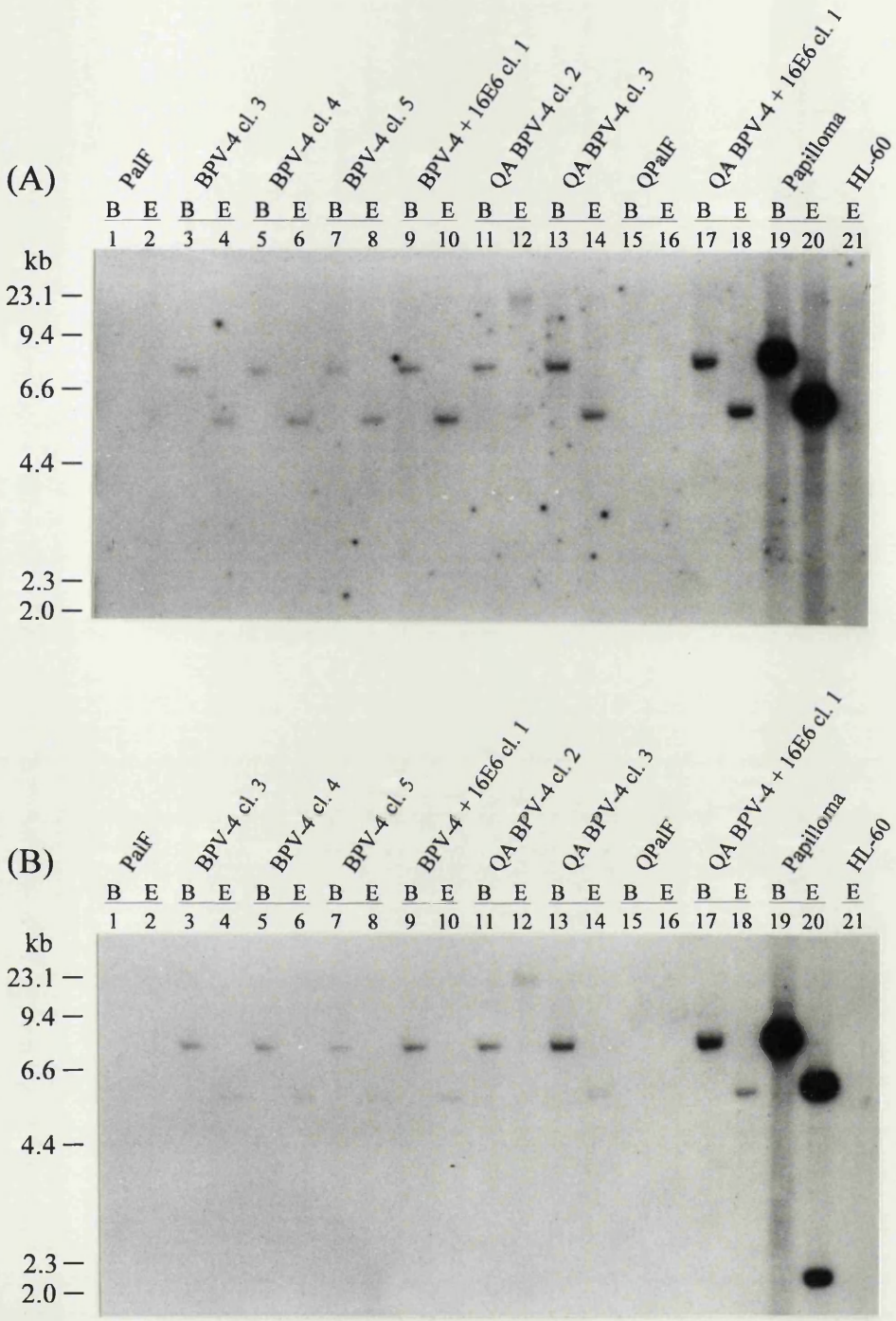


Figure 5.2 Southern blot analysis of control (ethanol-treated) and quercetin-treated (protocol QA) PalF transfectants for the presence of BPV-4 E8 and E7 DNA.

Tracks 1-18 & 21 contain 5 µg DNA digested with BamH I (B) or EcoR I (E). Tracks 19-20 contain 200 ng bovine oesophageal papilloma DNA (which contains episomal BPV-4 DNA) digested with BamH I and EcoR I respectively + 5 µg BamH I digested carrier DNA (C127 DNA). cl. = clone. **Panel A**: probed with BPV-4 E8 DNA. **Panel B**: probed with BPV-4 E7 DNA. (probes detailed in Chapter 2.1.7). The positions of λ Hind III digested DNA molecular weight markers are indicated in the left margin.

Further evidence that lack of signal intensity was due to the small amount of viral DNA present in these transformed PalF lines was shown by results from probing digested DNA from a cell line derived from transfection of ethanol-treated PalF cells with whole genome BPV-4. This cell line, called BPV-4 clone 2, was shown to contain multiple copies of BPV-4 DNA, and hybridisation with the above BPV-4 DNA probes revealed the expected bands (Figure 5.3 A & B; tracks 9 and 10). In order to determine the approximate copy number and state of the viral sequences present in this clone a further agarose gel / Southern blot was undertaken, and the resulting membrane probed with whole genome BPV-4. Approximate copy number was assessed by comparing hybridisation signal of BPV-4 clone 2 DNA with that of known standards; namely 1, 10 and 100 genome equivalents of BamH I digested pBV-4 DNA. Each standard was loaded in 5 μg BamH I digested murine carrier DNA. The status of the viral DNA (whether episomal or integrated) was assessed by comparing BamH I and Kpn I digests of cell line DNA. Results showed that BPV-4 clone 2 contained approximately 100 genome equivalents of viral DNA (Figure 5.4; tracks 3-5 vs 7 & 11). The Kpn I digest of this cell line (Figure 5.4; track 11) indicated that the majority (if not all) viral DNA present in this line had integrated into the cellular genome at multiple sites, a conclusion supported by the banding pattern seen in the BamH I digest track (Figure 5.4; track 7). Similar detailed assessment of viral DNA status or copy number was not carried out on the other transfected lines. Although these cell lines contained very little detectable viral DNA compared to BPV-4 clone 2 they exhibited a similar reduction in GJIC.

Results showed that on screening DNA samples from the nine E7-transfected cell lines (whether with or without quercetin treatment and / or an exogenous E6 gene) assayed for both GJIC levels and BPV-4 DNA status, seven retained E7 DNA (Tables 5.3 & 5.4). All nine lines were shown to have undisrupted levels of intercellular communication (Tables 5.3 & 5.4). These results showed that although transfection of

Figure 5.3 Southern blot analysis of control (ethanol-treated) and quercetin-treated (protocol QA) PalF transfectants for the presence of BPV-4 E8 and E7 DNA.

Tracks 1-18 & 21 contain 5 μg DNA digested with BamH I (B) or EcoR I (E). Tracks 19-20 contain 200 ng bovine oesophageal papilloma DNA (which contains episomal BPV-4 DNA) digested with BamH I and EcoR I respectively + 5 μg BamH I digested carrier DNA (C127 DNA). cl. = clone; polycl. = polyclonal. **Panel A**: probed with BPV-4 E8 DNA. **Panel B**: probed with BPV-4 E7 DNA. (probes detailed in Chapter 2.1.7). The positions of λ Hind III digested DNA molecular weight markers are indicated in the left margin.

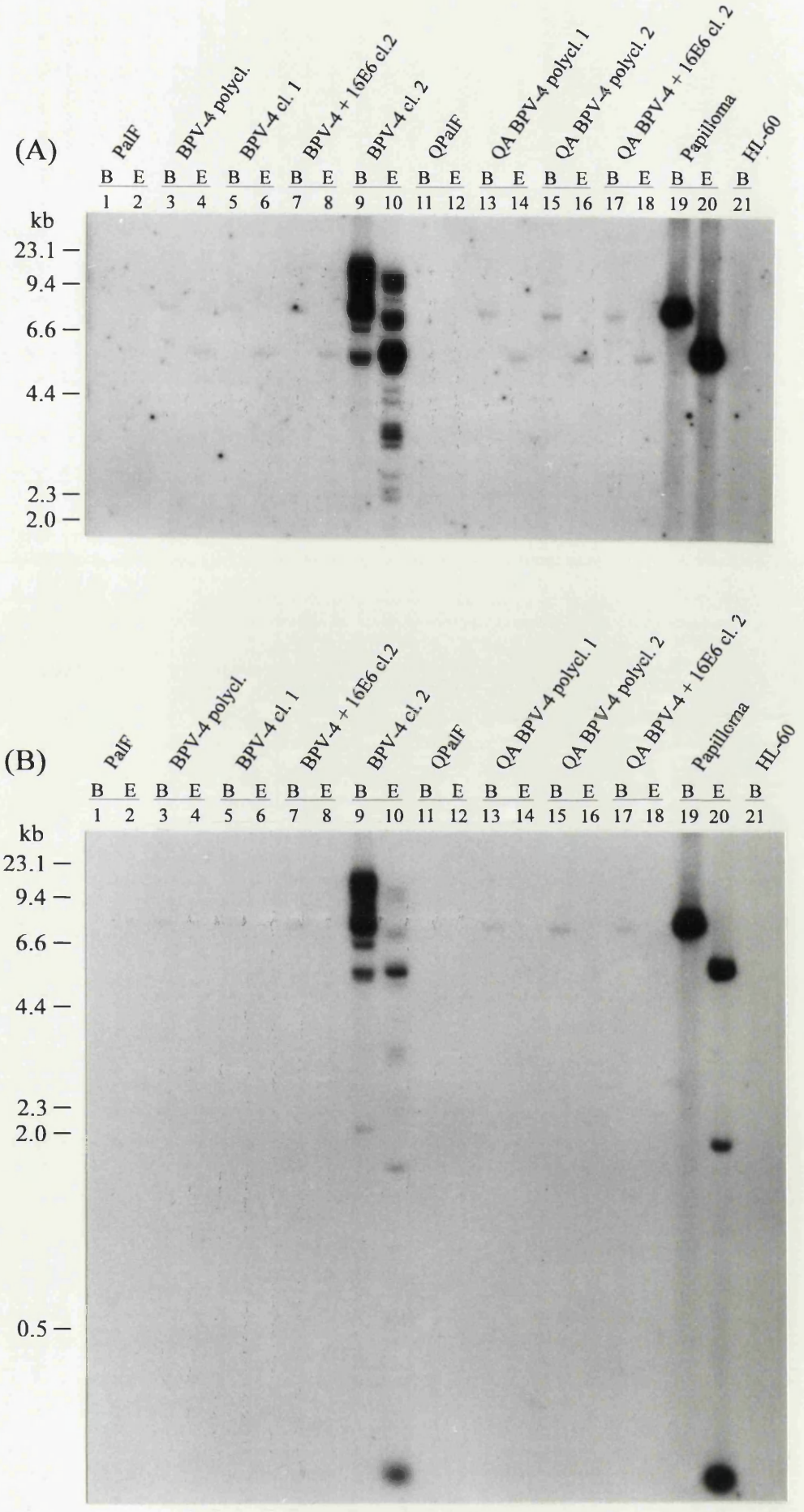

(A)
PalF
BPV-4 polycl.
BPV-4 cl. 1
BPV-4 + 16E6 cl.2
BPV-4 cl. 2
QPalF
QA BPV-4 polycl. 1
QA BPV-4 polycl. 2
QA BPV-4 + 16E6 cl. 2
Papilloma
HL-60
B E B E B E B E B E B E B E B E B E B E B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
kb
23.1
9.4
6.6
4.4
2.3
2.0
(B)
PalF
BPV-4 polycl.
BPV-4 cl. 1
BPV-4 + 16E6 cl.2
BPV-4 cl. 2
QPalF
QA BPV-4 polycl. 1
QA BPV-4 polycl. 2
QA BPV-4 + 16E6 cl. 2
Papilloma
HL-60
B E B E B E B E B E B E B E B E B E B E B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
kb
23.1
9.4
6.6
4.4
2.3
2.0
0.5

Figure 5.3

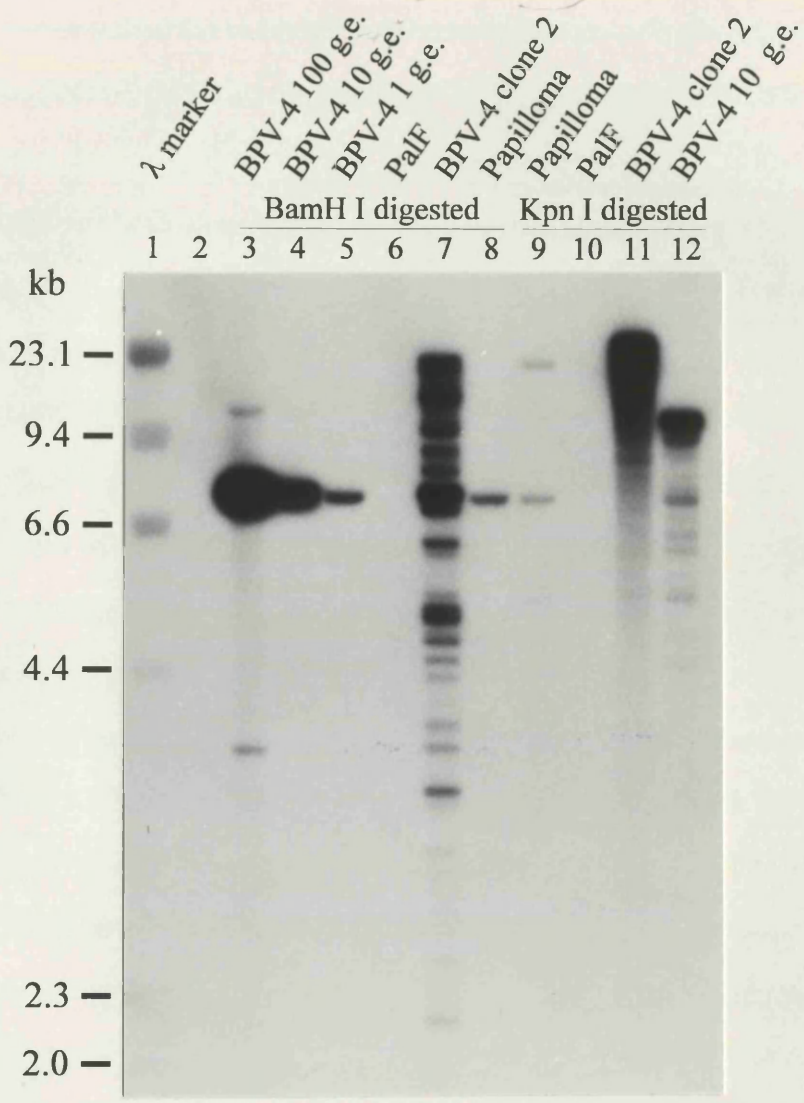


Figure 5.4 Determination of BPV-4 DNA status and approximate copy number in the transformed PalF cell line BPV-4 clone 2.

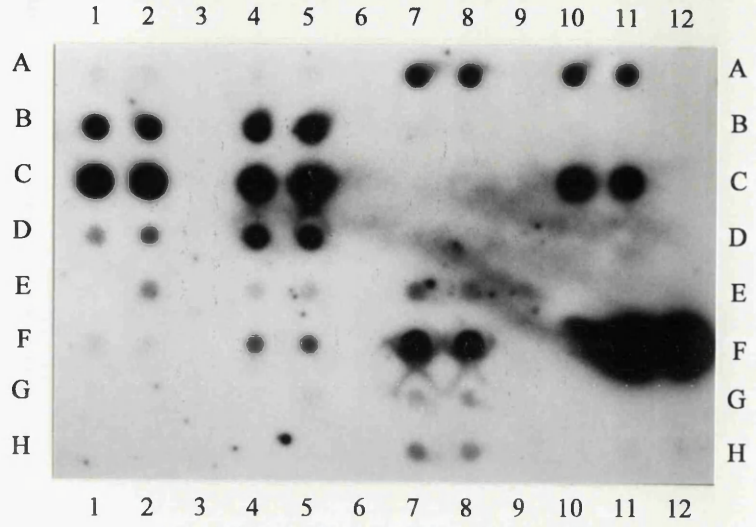
Tracks 3-5 contain standard BPV-4 genome equivalents (g.e.) digested with BamH I + 5 μg BamH I digested carrier DNA (C127 DNA). Tracks 6-7 & 10-11 contain 5 μg digested DNA as indicated. Tracks 8-9 contain 20 ng bovine oesophageal papilloma DNA (which contains episomal BPV-4 DNA) digested with BamH I and Kpn I respectively + 5 μg BamH I digested carrier DNA (C127 DNA). Track 12 contains 10 BPV-4 g.e. digested with Kpn I + 5 μg BamH I digested carrier DNA (C127 DNA). The positions of λ Hind III digested DNA molecular weight markers (Track 1) are indicated in the left margin. (Track 2 contains no DNA).

the E7 ORF was required for morphological transformation of PalF cells (Tables 4.4 & 4.7), presence of the E7 ORF had no significant effect on the level of GJIC.

Viral DNA could not be detected in any of the nine GJIC assayed cell lines derived from transfection of PalF cells with E8/E7-containing plasmids. This was regardless of whether these viral genes were under the control of their own promoter (BPV-4 LCR, construct pSVE8E7; see Chapter 2.1.7) or a heterologous promoter (MoLV LTR, construct pZipE8E7; see Chapter 2.1.7) or whether they had been treated with quercetin or not (Tables 5.3 & 5.4). This suggested that either transfection of viral DNA into these cells had been unsuccessful or that there was active selection against the E8/E7-containing constructs. The first hypothesis can be ruled out as such cells would not have been morphologically transformed and would have died during the 3 week selection period. With regard to the second hypothesis, previous work has reported that BPV-4 DNA was not detected in four PalF lines transformed by E8/E7 plasmids (Jaggar *et al.*, 1990). Similar examination of three E8/E7-transfected PalF clones showed that only one line retained viral DNA (Pennie, 1992). Thus, considered with the above data, PalF cells transfected with E8/E7-containing constructs are less likely to maintain viral DNA (or lose it more rapidly) than other classes of transfectants. As seven out of nine clones transfected with the BPV-4 E7 ORF retained E7 DNA it is tempting to propose that there may be active selection against E8. As such active selection is not apparent in whole genome BPV-4 transfectants (all assayed clones retaining E8 sequences) the relatively rapid loss of these sequences in E8/E7 transfectants may be due to regulation / expression differences of viral genes between these two transfection classes. Given the absence of E8 DNA in all E8/E7 lines tested, the normal levels of intercellular communication observed in these cells is no longer at variance with the hypothesis that a possible function of BPV-4 E8 protein in cellular transformation is the disruption of GJIC by virtue of its binding to the 16 kD ductin protein.

BPV-4 expression studies showed detectable levels of E7 RNA in three out of nine whole genome BPV-4-transformed lines and seven out of nine E7-transformed lines. These included both ethanol-treated and quercetin-treated (QA) transfectants (Tables 5.3 & 5.4). An example of results from hybridisation of membranes with the E7 probe is shown in Figure 5.5. This membrane predominantly contains total RNAs from E7-transformed PalF cells. The demonstrated expression of E7 RNA in seven out of nine E7-transformed lines, coupled with the observed normal GJIC levels, excludes a role for E7 in disruption of intercellular communication. Although E8 DNA was detected in all of the whole-genome BPV-4-transformed lines, E8 RNA was only detected in one line (BPV-4 clone 2; Table 5.3; Figure 5.6). This was the clone previously found to contain multiple copies of BPV-4 DNA (approximately 100 genome equivalents). The relatively weak signal evident for this sample suggests a lack of detection sensitivity for the E8 expression assay. Further evidence for this has been provided by the fact that E8 protein has since been detected in one of the whole genome BPV-4-transformed lines which showed no detectable E8 RNA (BPV-4 clone 5; R. Anderson, personal communication). No definitive conclusions, therefore, can be drawn from a comparison of overall E8 expression results and GJIC status of the various cell lines examined. However, both lines showing positive evidence of E8 RNA expression, either directly or indirectly (BPV-4 clonal lines 2 & 5, respectively), also show reduced GJIC levels. This is in agreement with the proposed hypothesis that E8 protein binds to ductin resulting in reduced cell-cell communication.

That GJIC is disrupted in BPV-4-transformed lines which are shown to retain the E8 gene supports the hypothesis that like the BPV-1 E5 protein, the BPV-4 E8 protein (which shares homology with this oncoprotein) binds to ductin disrupting normal levels of GJIC. Binding of the BPV-4 E8 protein to ductin has recently been demonstrated using an *in vitro* (cell-free) translation system (A. Faccini, personal communication). A direct cellular demonstration of E8 protein / ductin binding in a 'clean' background

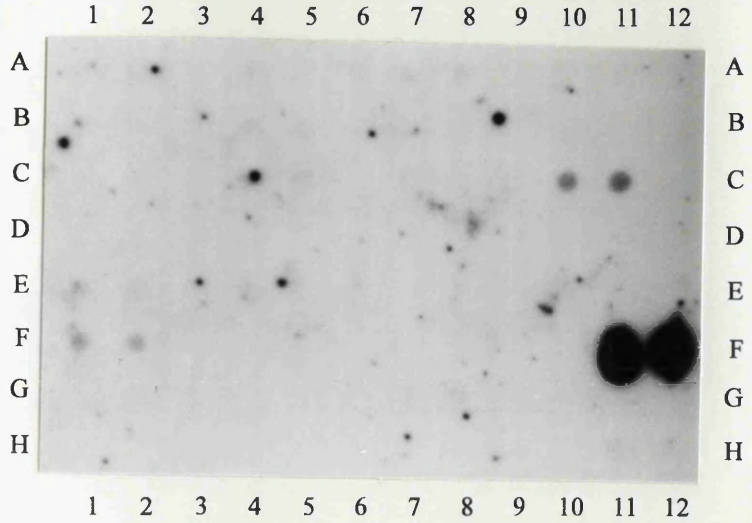


Key:

	1 2	3	4 5	6	7 8	9	10 11	12
A	PalF		QPalF		QA E7 cl.4		QA E7+16E6 cl.1	
B	E7 cl.1		E7+16E6 cl.4		QA E7 cl.1		ZipE8E7 + 16E6 cl.1	
C	E7 cl.2		E7+16E6 cl.4		QA E7 cl.2		BPV-4 cl.3	
D	E7 cl.3		E7+16E6 cl.3		QA E7 cl.2			
E	BPV-4 +16E6 cl.2		E7+16E6 cl.2		QA E7 cl.3			
F	ZipE8E7 cl.2		E7+16E6 cl.1		QA E7+16E6 cl.2		Papilloma DNA	
G			ZipE8E7 cl.2		E7 + 16E6 cl.1			
H					QA E7+16E6 cl.4		HL-60	

Figure 5.5 RNA dot blot analysis of control (ethanol-treated) and quercetin-treated (protocol QA) PalF transfectants: status of BPV-4 E7 expression.

20 μg total RNA were loaded per well. Samples from each line were loaded in duplicate in adjacent wells unless otherwise indicated. Controls included RNAs from ethanol-treated PalF cells, quercetin-treated PalF cells, HL-60 cells (positive control for the *myc* probe - see Chapter 4.4.3.2) and 200 ng bovine oesophageal papilloma DNA, which contains episomal BPV-4 DNA (positive control for viral probing) cl. = clone. The RNA dot blot was probed with BPV-4 E7 DNA. (probe detailed in Chapter 2.1.7).



Key:

	1	2	3	4	5	6	7	8	9	10	11	12
A	PalF			QPalF			QA SVE8E7 cl.3			QA ZipE8E7 cl.1		
B	BPV-4 polycl.			QA ZipE8E7 + 16E6 cl.1			QA SVE8E7 cl.2			QA ZipE8E7 cl.2		
C	QA BPV-4 polycl. 2			QA BPV-4 cl.2			SVE8E7 + 16E6 cl.1			BPV-4 cl.2		
D	BPV-4 cl. 1			QA BPV-4 cl.3			QA SVE8E7 cl.1			QA E7 + 16E6 cl.1		
E	BPV-4 cl. 5			QA BPV-4 + 16E6 cl.1			QA SVE8E7 + 16E6 cl.1					
F	BPV-4 cl. 2						QA BPV-4 polycl.1				Papilloma DNA	
G	BPV-4 + 16E6 cl. 3						QA ZipE8E7 cl.2					
H							QA ZipE8E7 cl.3				HL-60	

Figure 5.6 RNA dot blot analysis of control (ethanol-treated) and quercetin-treated (protocol QA) PalF transfectants: status of BPV-4 E8 expression.

20 µg total RNA were loaded per well. Samples from each line were loaded in duplicate in adjacent wells. Controls included RNAs from ethanol-treated PalF cells, quercetin-treated PalF cells, HL-60 cells (positive control for the *myc* probe - see Chapter 4.4.3.2) and 200 ng bovine oesophageal papilloma DNA, which contains episomal BPV-4 DNA (positive control for viral probing) cl. = clone; polycl. = polyclonal. The RNA dot blot was probed with BPV-4 E8 DNA. (probe detailed in Chapter 2.1.7).

i.e. in the absence of other viral products would be the ultimate test of the above hypothesis. However, this is not as yet possible due to the observed lethality on transfecting PalF cells with the E8 gene alone + *ras* (Jaggar *et al.*, 1990; Pennie *et al.*, 1993). As proposed in the previous chapter, a possible way of overcoming this could be the cloning of this viral gene into an inducible mammalian gene expression system. Although transformation is often associated with loss of GJIC (Mesnil & Yamasaki, 1993) and the BPV-4 E7 ORF encodes the major transforming protein of this virus (as measured by morphological transformation of PalF cells), loss of coupling cannot be ascribed to this oncoprotein as lines shown to contain and express the E7 gene display normal levels of GJIC.

The loss of GJIC through the binding of E8 protein to ductin is supported in a recent paper by Oelze *et al.* (1995). In this paper they describe how transfection of a keratinocyte line (HaCat cells) with a recombinant HPV-16 E5 gene also results in reduced GJIC. HPV-16 E5 protein localises to the cellular membrane and has also been shown to associate with ductin. As BPV-1 E5 (Goldstein *et al.*, 1991), HTLV-1 $p12^{I}$ (Franchini *et al.*, 1993) and the E5 protein of both HPV-6 and -16 (Conrad *et al.*, 1993) can also bind to ductin this suggests that ductin is a common cellular target for these viral proteins. Disruption of normal cell-cell communication may be an early though essential part of the transformation biology of these viruses, including BPV-4. Such deregulation of GJIC may well 'protect' virally-infected cells by isolating them from the tumour suppressive effects of surrounding normal cells creating a permissive cellular environment for expression of the viral oncogenic proteins. Once expression of the major transforming viral oncogenes has occurred, and a population of transformed cells established, there may well be no further selective pressure to directly inhibit GJIC, although disrupted intercellular communication may occur at later stages due to virally-independent mechanisms of transformation. Reduced cell-cell communication

CHAPTER 6

DISCUSSION

Chapter 6

DISCUSSION

6.1 Introduction

Bovine papillomavirus type 4 induces papillomas of the mucosa of the upper alimentary canal of cattle. In immunocompetent animals these benign lesions regress approximately one year post infection (Jarrett, 1985). However in cattle feeding on bracken fern, and consequently exposed to the mutagens, carcinogens and immunosuppressants present in the plant, some of these papillomas can progress to carcinomas. The critical roles played by both virus and fern in the aetiology of these squamous cell carcinomas are a good example of the multifactorial nature of carcinogenesis. Furthermore, BPV-4 lends itself to both *in vitro* and *in vivo* experimentation.

The work described in this thesis involved examination of the transformation biology of BPV-4 in both primary and, to a lesser extent, in established cells. Synergism between the virus and quercetin, a chemical found in bracken fern and proposed to act as a co-factor in BPV-4-associated carcinogenesis, was examined in PalF cells which are primary palate fibroblasts derived from the natural host species. Results confirmed previous preliminary work carried out in this laboratory describing viral / chemical synergism and extended these findings by showing that quercetin also synergised with sub-genomic BPV-4 fragments. In general, exposure to quercetin and transfection with BPV-4 genes increased the degree of cellular transformation of transfected cells, in some cases resulting in induction of tumours in nude mice. The phenotypic consequence of viral / chemical synergism was also found to be affected by the order of, and time interval between, treatment of cells with quercetin and transfection with viral

genes. Results from these *in vitro* studies suggested that this experimental system provides a useful and important model for analysis of viral / chemical co-operation in papillomavirus-associated carcinogenesis.

6.2 Cell transformation by BPV-4 genes *in vitro*

Morphological transformation of PalF cells by whole genome BPV-4 or sub-genomic fragments was found to be dependent on co-transfection with an activated *ras* gene. This is not unique to BPV-4 as similar conditions are required for transformation of primary rodent cells by other papillomaviruses, including the oncogenic HPVs 16 and 18 (Matlashewski *et al.*, 1987; Storey *et al.*, 1988). Throughout the text it is assumed, unless otherwise stated, that all viral DNA transfection classes included co-transfection with an activated *ras* gene and a selectable gene for neomycin resistance. PalF cells transfected with BPV-4 genes were only partially transformed as although they had an extended lifespan they were not immortal, and although capable of anchorage-independent growth they were not tumourigenic in nude mice. This indicated that additional factors were required for full transformation, thus mimicking the natural history of upper alimentary canal carcinomas in cattle.

6.2.1 E7 is the major transforming gene of BPV-4

The transforming functions of BPV-4 mapped to the E7 and E8 ORFs (Figure 1.1). The E7 ORF was identified as the major morphological transforming gene of BPV-4 *in vitro* as, in the absence of other viral genes, it induced morphological transformation. Furthermore, disruption of this ORF abolishes morphological transformation in both primary and established cells (Smith & Campo, 1988; Jaggar *et al.*, 1990). The BPV-4 E7 protein shows amino acid similarity with the HPV-16 E7 protein. Although lacking the casein kinase II (CKII) phosphorylation sites found in HPV-16 E7 (Barbosa *et al.*, 1990), BPV-4 E7 protein contains both putative binding domains for zinc and the cellular tumour suppressor protein p105Rb (Jaggar *et al.*, 1990). These domains are

important for the transforming capabilities of HPV-16 E7 (Edmonds & Vousden, 1989; Chesters *et al.,* 1990); the zinc fingers are essential for the transactivation activity of the E7 protein (Phelps *et al.*, 1988) and binding of E7 to p105Rb effectively prevents interaction of this cellular tumour suppressor protein with its normal targets (Defeo-Jones *et al.*, 1991; Rustgi *et al.*, 1991). The CKII sites of HPV-16 E7 also contribute to the transformation activity of this protein, although they are less critical than the p105Rb and zinc-binding domains (Barbosa *et al.*, 1990).

Although BPV-4 E7 protein binding studies have yet to be done, this viral oncogene product may well have similar functions to the HPV-16 E7 protein. This is indicated in that, as for HPV-16 (Defeo-Jones *et al.*, 1991; Rustgi *et al.*, 1991), mutations in either of the two cys-x-x-cys motifs or the p105Rb binding domain of the BPV-4 E7 protein abolish cell transformation (Jaggar *et al.*, 1990; G.J. Grindlay, personal communication). It could be envisaged that BPV-4 E7-mediated disruption of the normal functions of p105Rb, and the resulting deregulation of cellular proliferative control, would provide optimal conditions for the action of other BPV-4 genes by providing an expanded and more receptive cellular pool. Rb-binding may be a feature common to the oncogenic papillomaviruses as exemplified by the E7 proteins of HPVs 16 and 18. However, further qualification of this statement may be required in that the oncogenic potential of a specific viral type may be influenced not only by the presence of a particular virally-encoded product, but also by the biochemical properties displayed by the protein. For example, although the E7 proteins of HPVs 6, 16 and 18 all bind to p105Rb, HPV-6 E7 was found to bind this protein less efficiently than the E7 proteins of HPVs 16 or 18 (Barbosa *et al.*, 1990). This may correlate directly with the relative oncogenic potential of these viruses. Whereas HPVs 16 and 18 are associated with malignant cervical tumours (zur Hausen & Schneider, 1987), HPV-6 is associated predominantly with benign genital warts and is only rarely found in cervical cancers (zur Hausen, 1989b). The biological significance of the BPV-4 E7 protein is also

indicated *in vivo* as examination of papillomas revealed that E7 is expressed in all epithelial layers and in all stages of tumour development (Campo *et al.,* 1994c).

6.2.2 The BPV-4 E8 gene confers anchorage independence

E8 is the second transforming gene of BPV-4. Although BPV-4 E8 had no independent transforming potential in this experimental system, co-transfection with an E7 gene conferred anchorage independence on transfected cells (Figure 4.4; Table 4.3). Manifestation of this phenotype is dependent on transfection of cells with E8 DNA: cells transfected with whole genome BPV-4, or a sub-genomic fragment containing the E8/E7 ORFs, were capable of anchorage-independent growth whereas cells transfected with E7 alone were not. However E8's contribution to transformation can not be assessed in isolation as transfection of PalF cells with BPV-4 E8 DNA in the absence of other viral genes is lethal to cells (Jaggar *et al.*, 1990; Pennie *et al.*, 1993).

As papillomavirus gene expression is regulated by both virally-encoded and cellular transcription factors (e.g. Vande Pol & Howley, 1990) it could be proposed that the detrimental effect of transfection of PalF cells with the BPV-4 E8 gene alone is due to the absence of such regulatory control. The papillomavirus E2 ORF encodes a full-length protein which can act as a transactivator (Spalholz *et al.,* 1985) and also truncated proteins, lacking the N-terminal transactivation domain, which repress transcription (Cripe *et al.*, 1987). However, under some circumstances the full-length transactivator form of E2 can repress transcription (Thierry & Yaniv, 1987). A truncated form of the E2 protein has not been demonstrated for BPV-4 (Jackson & Campo, 1995), although minor transcripts which have the potential to encode shorter form E2 proteins have been detected in BPV-4-induced papillomas (Stamps & Campo, 1988). The BPV-4 LCR has been shown to contain positive and negative E2-dependent and E2-independent transcriptional control elements (Jackson & Campo, 1991; 1995). Absence of E2-mediated regulatory control may result in inappropriate levels of E8

expression, which in turn may result in cell death. Current determination of E8 function is restricted by the observed lethality on transfection of this gene, however if the level of E8 expression does dictate whether transfected cells survive or not, it may be possible to circumvent this experimental limitation by use of an inducible mammalian gene expression system. Until there is a suitable assay system in operation it is not known whether anchorage independence is determined solely by the E8 product or results from combined action of the E8 and E7 (and possibly other) viral proteins.

In contrast to the behaviour of PalF cells, the non-transformed murine fibroblast line ΔM9, which contains integrated BPV-1 sequences, is able to survive transfection with the BPV-4 E8 gene alone. Transfection of these cells with BPV-4 E8 DNA may cause increased expression of resident BPV-1 sequences, resulting in BPV-1-mediated transformation and subsequent tolerance of the otherwise toxic effect of BPV-4 E8 expression. Conversely, survival of E8-transfected ΔM9 cells may well be independent of any BPV-1 effect, and may be due to inherent differences between established and primary cells. For instance, unlike primary cells, transformation of established cells with BPV-4 genes does not require co-transfection with an activated *ras* gene. Current studies in the laboratory are examining whether other established cells, namely the murine fibroblast line NIH3T3, which does not contain BPV-1 DNA, can sustain transfection with an E8 gene. Initial results suggest that this is the case and future work proposes transfection of NIH3T3 cells with mutated forms of the E8 gene (V. O'Brien, personal communication). This should allow identification and detailed investigation of E8-encoded functions.

6.3 Progressive loss of BPV-4 DNA does not correlate with overt mutation or rearrangement of viral ORFs

Although similarly associated with a naturally occurring epithelial cancer and provisionally proposed to share some protein functions in common with the high risk

HPVs (e.g. binding of the E7 protein to the p105Rb tumour suppressor protein), BPV-4 DNA is not retained during tumour progression *in vivo* (Campo *et al.*, 1985). Similar results have been observed both in controlled *in vivo* experiments in nude mice (Gaukroger *et al.*, 1991) and in established (Smith & Campo, 1988) and primary transfected cells (Pennie, 1992). Results from the nude mice experiments, in which virus-producing papillomas were induced by implanting BPV-4-infected foetal palatine tissue beneath the renal capsule of nude mice, described the spontaneous malignant progression of one of 57 virally-induced papillomas. BPV-4 DNA was detected in the papilloma fronds but not in the carcinoma or the metastatic deposits found in the spleen (Gaukroger *et al.*, 1991).

More recent work used this nude mouse xenograft implant system to investigate the interaction of chemical co-carcinogens with BPV-4 in cell transformation (Gaukroger *et al.*, 1993). Virus-infected tissue was implanted into mice exposed to the tumour promoter TPA or the tumour initiator DMBA, and the growth and neoplastic progression of BPV-4-induced papillomas was monitored. Results showed that BPV-4 synergised with both TPA and DMBA, greatly enhancing both the production of papillomas and the frequency of malignant progression. Consistent with previous observations (Gaukroger *et al.*, 1991), BPV-4 DNA was not found in the carcinomas. These results confirmed the synergism between viral and chemical carcinogens, demonstrated that BPV-4 could interact with both a promoter and an initiator and showed that loss of viral DNA was observed in controlled experiments.

Results from *in vitro* transfection studies were similar to the *in vivo* findings. Analysis of established mouse fibroblasts transfected with whole genome BPV-4 revealed that although cells were transformed only nine out of 60 cell lines examined contained BPV-4 DNA (Smith & Campo, 1988). Comparison of cell line DNAs from early versus late passages showed that BPV-4 DNA was progressively lost on continued passage.

Although several of the transformed lines were found to be tumourigenic in nude mice, the majority did not contain detectable viral DNA, indicating that maintenance of BPV-4 DNA is not required for development of the fully transformed phenotype. In the few lines found to contain BPV-4 DNA, the viral sequences appeared to have integrated into the host cellular genome and were transcriptionally silent. These observations led to the proposal that presence of these BPV-4 sequences had no bearing on maintenance of the transformed state (Smith & Campo, 1988). Similarly, although transformation of PalF cells was dependent on transfection of cells with BPV-4 genes (plus an activated *ras* gene) not all cells retained viral DNA (Pennie, 1992).

The frequency with which BPV-4 DNA is lost both *in vivo* and *in vitro*, suggested not only that viral functions do not appear necessary for tumour progression but also that active selection against whole or part of the BPV-4 genome may occur. *In vivo*, the BPV-4 DNA found in benign papillomas is always present in the episomal state. It could be argued that the absence of viral DNA in frank cancers is due to non-segregation of BPV-4 DNA during successive cell divisions. However if this were so, one might expect BPV-4 DNA to be found fortuitously in carcinomas more frequently than studies indicate (Campo *et al.*, 1985). Selection against BPV-4-containing cells could also result from an immune response, in that cells retaining viral DNA might induce an immune response and be removed, whereas cells with no viral DNA would survive. Further evidence supporting the proposal that viral DNA may be lost as a result of negative selection is provided by data from *in vitro* studies. Analysis of lines derived from transfection of established mouse fibroblasts with BPV-4 genes showed that, when detected, the BPV-4 DNA had integrated into the cellular genome (Smith & Campo, 1988). Most of these lines lost viral DNA on continuous passage, however one line, C4Ta2a, stably maintained its viral DNA content. This line was found to contain a deleted form of the BPV-4 genome. If there is active selection against the BPV-4, lack of particular viral sequences in C4Ta2a may render this line

immune from such selection. Due to the observed detrimental effect on transfecting primary cells with BPV-4 E8 DNA, it was hypothesised that this viral ORF was a target for negative selection.

The BPV-4-transfected established cells in which loss of BPV-4 DNA had previously been described were derived from non-transformed parental cells subsequently found to contain transcriptionally silent, integrated BPV-1 sequences (Smith *et al.*, 1993). The origin of the BPV-1 sequences present in these cells is unknown. Characterisation of this line, termed C127sc, revealed that subsequent to transfection with BPV-4 genes, amplification of the integrated BPV-1 DNA and flanking cellular sequences had occurred (Smith & Campo, 1988; 1989). This phenomenon is discussed in Chapter 6.4. Loss of BPV-4 DNA from virally-transformed lines through the action of BPV-1-mediated effects could therefore not be discounted. This possibility was examined in this current study by using ID14 cells as the parental line. This line contains episomal, actively expressing BPV-1 DNA. Southern blots of DNAs from BPV-4-transfected ID14 cells were screened with a whole genome BPV-4 DNA probe to examine cells for the presence of BPV-4 sequences. Results showed that although BPV-4 DNA was found to be initially present in the transfectants, and appeared to have integrated into the cellular genome, it was lost on continued passage (Figure 3.6). These results were similar to those from the C127sc study. Furthermore they indicated that the level of BPV-1 expression did not influence the maintenance of BPV-4 DNA.

DNAs from ID14 transfectants were also screened with a panel of sub-genomic BPV-4 DNA probes to assess the presence and integrity of individual BPV-4 ORFs. Results from the viral mapping showed that there were no obvious rearrangements within the LCR and most of the viral ORFs screened, including the E8 ORF. Therefore, if E8 functions are selected against, as first hypothesised, this does not occur through major deletions or major rearrangements within the E8 ORF. Similarly, the proposed negative

selection against maintenance of viral genes does not act through major rearrangements of the LCR or the E7, L1, or L2 ORFs . Major rearrangements were detected over a 661 bp region between the 3' end of the E1 ORF and the 5' end of the E2 ORF (Figure 3.8). However, as all recombinant plasmids had been linearised within the E1 ORF (i.e. within this rearranged region), this suggested that BPV-4 DNA had integrated into the cellular genome via this site. As a result of this experimental manipulation the possible role this viral region might play in selection could not be assessed.

Although no major rearrangements could be detected within the BPV-4 sequences present in these lines, apart from the E1 ORF, it should be noted that the mapping technique used was relatively insensitive. Therefore it is possible that small deletions, and / or point mutations would remain undetected. Such DNA lesions might be of consequence as to whether BPV-4 DNA was lost or retained. Likewise, small inversions or duplications may also be of biological significance, affecting the maintenance or otherwise of BPV-4 sequences. Unless such lesions affect the sequence recognition sites of the particular restriction enzymes used in the mapping analysis, they would also remain undetected.

It was hoped that analysis of the cell line C4Ta2a might indicate possible viral targets for negative selection. This cell line is unusual in that, unlike other BPV-4-transfected lines, it stably maintains viral sequences. Comparison of BPV-4-specific hybridisation banding patterns to those of episomal BPV-4 DNA controls, showed that this line contained rearranged viral sequences which had integrated into the cellular genome. Although transfected with BamH I linearised whole genome BPV-4, this line appeared to contain a deleted form of the viral genome (Figures 3.6 & 3.9). This confirmed findings of Smith & Campo (1988). In common with the BPV-4-transfected ID14 cells, most of the viral ORFs, including E8, appeared wildtype, while disruption of the E1 ORF suggested that viral DNA had integrated into the cellular genome via the BamH I

site found in this ORF. As found for the ID14 transfectants, the rearranged region in C4Ta2a was mapped to a 661 bp region between the 3' end of the E1 ORF and the 5' end of the E2 ORF. This suggested that the deletion of viral sequences in this line had occurred within this region. Fine mapping and / or sequencing of this disrupted region may prove of great interest. If there is active selection against BPV-4, lack of particular viral sequences in C4Ta2a may render this line immune from such selection. It must also be considered that site of integration within the cellular genome may also influence whether BPV-4 is maintained. Analysis of the C4Ta2a line showed that the BPV-4 DNA in these cells had integrated within a cellular region containing integrated BPV-1 DNA flanked by sequences homologous to autonomously replicating sequences (ARS) (Smith *et al.,* 1993). ARS sequences have been implicated in DNA replication (e.g. Struhl *et al.*, 1979). Therefore, one cannot rule out the possibility that either or both types of sequence affected maintenance of BPV-4 DNA.

Although the analysis of virally-transformed mouse fibroblasts did not reveal selection against the E8 ORF, circumstantial evidence that this may happen was provided by analysis of PalF transfectants. Detailed viral mapping of these cell lines was not undertaken, however screening of their DNAs with BPV-4 DNA probes revealed that although the majority of lines derived from transfection with either whole genome BPV-4 or the E7 gene alone contained viral DNA at time of assay (Figure 5.2), viral sequences were not detected in E8/E7-transfected lines. Viral DNA was lost regardless of whether the E8/E7 genes were under the control of the BPV-4 LCR or the strong heterologous Moloney murine leukaemia virus long terminal repeat (MoLV LTR). As the whole genome BPV-4 transfectants contained E8 sequences, the loss of these sequences in E8/E7 transfectants may be due to differences in levels of viral expression and the control of such expression between these two transfection classes. Previous work has also reported that viral DNA was either absent (Jaggar *et al.*, 1990) or detected

only occasionally in PalF cells transfected with E8/E7-containing constructs (Pennie, 1992).

All the above DNAs were examined by Southern blotting. Analysis of these DNAs using the more sensitive polymerase chain reaction (PCR) technique with ORF-specific primers would allow examination as to whether E8/E7 sequences are indeed absent or present at very low levels. Even if such sequences were detected this would still indicate significant differences between class of transfection and amount of viral DNA present. These results also suggest that the presence of BPV-4 DNA in every cell is not required for the maintenance of the transformed state.

It should also be considered that cellular factors present in fibroblasts may not be optimal for the activity of BPV-4 genes and this may be of some consequence with regard to the maintenance of viral DNA. Repetition of these experiments in primary bovine keratinocytes, the natural target cell for BPV-4 infection, would allow examination as to whether the behaviour of BPV-4 differed between these two cell types. The importance of tissue-specific factors is implicated not only by the strict tissue-type specificity of infection observed for the epitheliotropic papillomaviruses *in vivo*, but also by molecular analysis of these viral genomes. Bernard *et al.* (1989) reported that although the promoter activity of the HPV-18 long control region (LCR) was observed in keratinocytes, similar activity was not detected in fibroblasts suggesting that this viral function was tissue specific.

6.4 BPV-4-mediated amplification

Amplification of cellular genes is a common event during malignant progression (Stark & Wahl, 1984; Alitalo *et al.*, 1986). Gene amplification can occur after treatment with chemical or physical carcinogens or viruses (Kleinberger *et al.*, 1986). For example, an analysis of cervical tumours containing integrated HPV-16 DNA has

reported amplification of both viral and cellular sequences (Wagatsuma *et al.*, 1990). Previous work revealed amplification and rearrangement of specific host sequences in BPV-4-transformed lines (Smith & Campo, 1988; 1989) and it was therefore proposed that induction of cellular DNA amplification may be an important aspect of BPV-4 transformation activity. Further characterisation of these lines unexpectedly revealed that the amplified 'cellular' locus consisted of 5.2 kb of the BPV-1 genome integrated into a cellular region of ARS-like sequences (Smith *et al.*, 1993). ARS elements were first described in yeast and have been identified as putative chromosomal origins of DNA replication (Struhl *et al.*, 1979; Stinchcomb *et al.*, 1979; Chan & Tye, 1980). Similar DNA sequences found in other eukaryotes were also assayed in yeast and it has been proposed that they may also be involved in DNA replication (Struhl *et al.*, 1979; Botchan & Dayton, 1982; Roth *et al.*, 1983; Montiel *et al.*, 1984). Although manifestation of this amplification was dependent on transfection of the C127sc parental cells with BPV-4 genes, the possibility that this phenotype resulted from interaction (whether direct or indirect) between BPV-4 and BPV-1, BPV-4 and the ARS-like sequences or BPV-4 and a cellular intermediate had to be considered. Examination of possible interaction between BPV-4 and BPV-1 was undertaken in this thesis using another non-transformed parental line (ΔM9) known to contain integrated BPV-1 sequences. Transfection of this line with various BPV-4 genes induced morphological transformation although none of the resulting transfectants retained BPV-4 DNA. Comparison of the BPV-1 sequences present in ΔM9 and C127sc cells showed that both lines contained intact origins of replication but that ΔM9 cells contained a disrupted BPV-1 E1 ORF. The observation that resident BPV-1 sequences were amplified in BPV-4-transfected C127sc cells but not in the ΔM9 transfectants led to the proposal that the transformed phenotype may have resulted from BPV-4 transactivation of a BPV-1 protein required for DNA replication. The BPV-1 E1 protein is involved in viral replication and would thus be a suitable candidate (Lusky & Botchan, 1986).

BPV-4-mediated amplification has also been observed in SV40-transformed cells indicating that the above results were not unique. The SV40 DNA in these cells was integrated into a cellular region of repetitive sequences and both SV40 DNA and these flanking sequences were amplified subsequent to transfection with BPV-4 genes (M.S. Campo & K. Smith, unpublished data). As already proposed for the maintenance or loss of integrated BPV-4 sequences *in vitro*, the site of viral integration within the cellular genome may also play a role in BPV-4-induced amplification. ARS-like sequences were part of the amplified region found in BPV-4-transfected C127sc cells (Smith *et al.*, 1993). Viral integration into ARS-like sequences and subsequent amplification of the viral and flanking cellular sequences has also been described in BPV-1-transformed primary mouse fibroblasts (Agrawal *et al.*, 1992). It is therefore possible that these cellular sequences could play a part in amplification (and establishment of the transformed state) by serving as origins of DNA amplification (Zastrow *et al.*, 1989). Although amplification of ARS-like sequences was not observed in any of the ΔM9 transfectants, a role for ARS sequences cannot be ruled out as the cellular site of integration in these cells was not known and may not even involve ARS-like sequences.

It has been suggested that integration of human papillomavirus DNA into the cellular genome may be an important and causative event in the progression of virus infection into malignant transformation (Dürst *et al.*, 1985; Wagatsuma *et al.*, 1990). Integration of viral DNA close to, and interaction with, cellular origins of DNA replication may affect transcription of both cellular and viral sequences. Although one could imagine this having phenotypic significance in the development of cervical lesions in humans it is more difficult to propose a similar mechanism of cellular transformation in BPV-4-induced lesions as BPV-4 DNA is episomal in benign papillomas and is rarely found in malignant tumours (Campo *et al.*, 1985).

6.5 E8 disrupts intercellular communication

The 42 amino acid protein product encoded by the BPV-4 E8 ORF is similar in length, hydrophobicity and putative secondary structure to the BPV-1 E5 protein (Jackson *et al.*, 1991). As reported for BPV-1 E5 (Burkhardt *et al.*, 1989), BPV-4 E8 protein was found to localise to membrane compartments (Pennie *et al.*, 1993). Due to the similarities between the BPV-1 E5 and BPV- 4 E8 proteins, it was proposed that these two oncoproteins may share some common function(s).

BPV-1 E5 oncoprotein has been shown to bind to ductin, a 16 kD cellular protein component of both vacuolar H^+-ATPase and gap junctions (Mandel *et al.*, 1988; Finbow *et al.*, 1992). Gap junctions are intercellular channels and provide cell-cell communication between neighbouring cells. These junctions are permeable to small molecules, such as nucleotides, oligosaccharides and second messengers, and this is thought to be important in the homeostatic control of groups of cells in a tissue or organ (Yamasaki, 1990). The vacuolar H^+-ATPase is a transmembrane proton pump and is responsible for the acidification of organelles such as endosomes, lysosomes, synaptic vesicles and Golgi bodies (Nelson, 1992). These proton pumps play a central role in down-regulation of activated growth factor receptors as they generate an acidic endosomal pH required for dissociation of ligand-receptor complexes as well as targeting these complexes for lysosomal degradation (Brown *et al.*, 1983).

Binding of the BPV-1 E5 protein to ductin appears to be necessary for the transforming activity of this viral polypeptide (Goldstein *et al.*, 1992). Other viral oncoproteins such as HPV-6 E5, HPV-16 E5 (Conrad *et al.*, 1993) and HTLV-1 $p12^I$ (Franchini *et al.*, 1993) also bind to ductin. This suggests that ductin is a common cellular target for these virally-encoded polypeptides and that abrogation of normal ductin function may be involved in induction of cellular transformation.

BPV-1 E5-transformed cells show constitutive activation of receptors for epidermal growth factor (EGF) and platelet derived growth factor (PDGF) (Martin *et al.*, 1989; Petti *et al.*, 1991). It has been proposed that the biological activity of the BPV-1 E5 protein may depend on interaction of this viral protein with ductin, resulting in disruption of normal proton pump function (Goldstein *et al.*, 1991). This may result in prolonged growth factor / receptor interaction and recycling of receptors to the cell surface, thus explaining the observed activation of cell surface receptors in BPV-1 E5-transformed cells. Increased recycling of growth factor receptors has also been observed in cells transformed by HPV-16 E5 (Straight *et al.*, 1993) and it has recently been reported that cells expressing HPV-16 E5 exhibit inhibition of endosomal acidification (Straight *et al.*, 1995). Activation of growth factor receptors may also be an important aspect of the transformation activity of BPV-4 as increased numbers of EGF receptors are observed in alimentary canal cancer cells (Smith *et al.*, 1987). However the biological activity of BPV-1 E5 protein may also depend on disruption of intercellular communication through binding to the ductin component of gap junctions. Gap junctional intercellular communication (GJIC) has been reported to be involved in cellular differentiation and growth control (Loewenstein, 1979). As such processes are disrupted in cancer, disruption of intercellular communication may be a common and critical step in establishing the transformed state. Alteration of GJIC is a common feature of many cancer cells (Loewenstein, 1979) and disruption of intercellular communication may also contribute to the early stages of metastasis (Hamada *et al.*, 1988). Further supportive evidence for the involvement of GJIC in carcinogenesis is the finding that many tumour promoters can disrupt normal levels of GJIC both *in vitro* (Yamasaki, 1990; Fitzgerald & Yamasaki, 1990) and *in vivo* (Sugie *et al.*, 1987; Mesnil *et al.*, 1988).

In light of the reported binding of BPV-1 E5 protein to ductin, levels of GJIC were measured in BPV-1-transformed primary bovine conjunctival fibroblasts

(Pennie, 1992). Results showed that BPV-1-transformed cells showed disrupted gap junctional communication as compared to untransfected controls. Although this did not provide direct proof that disruption of intercellular communication was due to ductin / BPV-1 E5 protein interaction it suggested that reduced GJIC was a consequence of viral transformation. Further indirect evidence from analysis of two human cervical cell lines-one positive for HPV-16 DNA, the other HPV-16 negative-implicated a viral component to disruption of GJIC. Although both lines were derived from normal cervical tissue, the cell line positive for viral DNA showed greatly reduced GJIC whereas the cell line which did not contain HPV-16 DNA communicated well (Pennie, 1992; G. Sibbet, personal communication).

The similarities between the BPV-1 E5 and BPV- 4 E8 proteins mentioned above and the proposal that these two oncoproteins may share some common function(s) led to the following studies. It was hypothesised that a possible function of the BPV-4 E8 protein in cellular transformation was the disruption of GJIC through direct interaction of this viral oncoprotein with ductin. BPV-4 E8 protein has recently been shown to bind ductin using an *in vitro* cell-free system (A. Faccini, personal communication). As BPV-1-transformed cells had been shown to have disrupted GJIC, PalF cells transformed by whole genome BPV-4 or sub-genomic fragments were assayed for levels of intercellular communication by microinjection of cells with a fluorescent tracker dye. A preliminary study had reported that one of eight lines derived from transfection of PalF cells with various BPV-4 genes displayed disrupted communication (Pennie, 1992). This led to the more detailed analysis of BPV-4-transformed PalF cells described in this thesis. Levels of functional gap junction intercellular communication were measured in transformed lines derived from transfection of PalF cells with either whole of sub-genomic fragments of BPV-4. These lines were also examined for the presence and expression of BPV-4 genes to investigate their possible correlation with observed levels of GJIC.

E7 transfectants communicated freely (Figure 5.1; Table 5.1) and analysis of their DNAs revealed that the majority of E7 transfectant lines tested contained E7 DNA (Table 5.3). These results showed that although transfection of the E7 ORF was required for morphological transformation of PalF cells, presence of the E7 ORF had no significant effect on GJIC levels. All whole genome BPV-4-transformed lines, which had previously been shown to have disrupted GJIC, contained viral DNA including E8 sequences (Table 5.3). With the exception of one whole genome BPV-4 transfectant which contained multiple copies of the viral genome (Figure 5.4), there was very little viral DNA present in these transfected lines. However there was no apparent correlation between amount of viral DNA and degree of intercellular communication as comparison of results obtained from cells containing low amounts of viral DNA to the single line containing multiple copies of the BPV-4 genome showed that these lines exhibited similar reduction in GJIC (Table 5.1; Figures 5.2 and 5.3).

None of the E8/E7 transfectants retained viral DNA. This was regardless of whether the transfected viral genes were under the control of their own promoter (BPV-4 LCR) or a heterologous promoter (MoLV LTR). That these lines were both negative for viral DNA and displayed normal levels of GJIC provided further circumstantial evidence that disruption of GJIC was mediated by viral proteins.

As regards analysis of viral gene expression, three out of nine whole genome BPV-4-transformed lines and seven out of nine E7-transformed lines showed detectable levels of E7 RNA (Tables 5.3 & 5.4). The observation that E7 transfectants expressed discernible levels of E7 RNA and displayed normal levels of GJIC suggested that BPV-4 E7 is not involved in disruption of intercellular communication. E8 RNA was only detected in one cell line. This line was derived from transfection of PalF cells with whole genome BPV-4. Failure to detect E8 RNA in other lines known to contain E8 sequences was thought to be due to lack of assay sensitivity as E8 protein was

subsequently detected in a BPV-4-transformed line which had been scored as negative for E8 RNA (R. Anderson, personal communication). Total RNA was used in these experiments and detection of viral RNA might be improved by use of mRNA or RT-PCR (reverse transcriptase polymerase chain reaction). No definitive conclusions can be made from comparison of overall E8 expression and the GJIC status of the various cell lines examined. However that the two cell lines shown to express BPV-4 E8 also showed reduced GJIC levels provides indirect evidence that BPV-4 E8 protein down-regulates intercellular communication.

Once again, due to the observed lethality of transfecting PalF cells with the E8 ORF alone, direct demonstration of E8 protein / ductin binding has not been possible. Cloning of this viral gene into an inducible mammalian gene expression vector may allow further functional analysis of the E8 ORF. Similarly, it may be possible to examine this proposed E8-encoded function by transfecting PalF cells with a whole genome BPV-4 plasmid construct containing a mutated E8 ORF and measuring GJIC levels in these cultures. If these transfectants were found to contain viral DNA, including the mutated E8 sequences, yet showed normal levels of GJIC this would indicate that disruption of intercellular communication is a function of the E8 protein.

The observation that a number of viral oncoproteins, including the E5 proteins of BPV-1 and HPVs 6 and 16, bind ductin suggests that this protein is a common cellular target for these viral products. Deregulation of normal GJIC may be an important aspect of the transforming biology of these viruses, isolating virally-transformed cells from the suppressive control of surrounding normal cells. The transformation biology of BPV-4 may also involve disruption of cell-cell communication as a means of establishing cellular transformation. This could result through complexing of the BPV-4 E8 protein with ductin. The BPV-4 E8 protein is similar in length and hydrophobicity to the BPV-1 E5 protein (Jackson *et al.*, 1991). The BPV-4 E8

oncoprotein binds ductin as assayed using an *in vitro* cell-free system (A. Faccini, personal communication) and, as described in this thesis, reduced GJIC is only observed in cells containing E8 sequences. The proposed mode of action of the E8 protein would therefore involve perturbation of GJIC thus effectively removing, or partially removing, virally-infected cells from the tumour suppressive effects of surrounding normal cells. Hyperproliferation of these cellular 'islands' could result due to impaired gap junction-mediated exchange of hormones, essential metabolites and secondary messengers with surrounding cells. Such dysregulation may provide a suitable environment for expression of other viral oncoproteins and further proliferation of virally-infected cells could ensue. The binding of BPV-4 E7 to the p105Rb protein could contribute to the clonal expansion of virally-infected cells by preventing p105Rb-mediated intracellular tumour suppressive effects. Once a population of transformed cells has been established, E8-mediated disruption of GJIC may be redundant for, or even deleterious to, the long-term survival of cells. Therefore there may be no further selective pressure to directly inhibit GJIC. Such a scheme would fit in with the cellular localisation and temporal expression of the E8 protein. *In vivo*, E8 is expressed only in the deep layers of papillomas, where little or no vegetative viral DNA replication takes place, and is only detected during the early stages of tumour development (Campo *et al.*, 1994c). This proposed model of E8 function would suggest that the E8 protein is critical for the induction of BPV-4-mediated cellular transformation and papilloma development.

6.6 BPV-4 lacks an E6 gene

BPV-4 lacks an E6 ORF as do the other subgroup B bovine viruses BPV-3 and BPV-6 (Jackson *et al.*, 1991). The E6 proteins of the oncogenic HPVs 16 and 18 bind to and degrade p53 protein and this is thought to be an important event in the transformation biology of these viruses (Scheffner *et al.*, 1990). Addition of an HPV-16 E6 ORF to BPV-4 genes contributes to cellular transformation by conferring immortality to

transfected PalF cells as reported by Pennie *et al.* (1993) and confirmed in this thesis. However the transfected cells are not tumourigenic. That the BPV-4 virus, although lacking an E6 gene, is capable of successful infection and propagation *in vivo* and malignant transformation both *in vivo* and *in vitro* in the presence of chemical co-factors raises the question whether E6 functions are necessary for BPV-4 transformation. Similar functions may be provided by another BPV-4 or host protein. As BPV-4 does not contain an E6 gene it might be predicted that p53 protein would be unaltered in BPV-4-induced papillomas but that p53 mutations, perhaps induced by environmental co-factors, may occur during the progression of benign papillomas to carcinomas. Alternatively BPV-4 may have evolved another mechanism of evading p53 growth suppression.

Recent work examining co-operation between BPV-4 and HPV-16 E6 genes has shown that 16E6 possess functions in addition to the inactivation of p53. Transfection studies showed that PalF cells transformed with BPV-4, *ras* and mutant p53 were tumourigenic in nude mice only when an HPV-16 E6 gene was also transfected (L. Scobie, unpublished data). BPV-4 and 16E6 were also found to co-operate to enhance transformation in p53 null mouse fibroblasts, providing further evidence that not all 16E6 transformation functions are mediated via inactivation of p53. However, p53 inactivation may play a role in BPV-4-induced cellular transformation as, unlike PalF cells, p53 null mouse fibroblasts can be transformed by BPV-4 + 16E6 in the absence of *ras* (L. Scobie, unpublished data).

The bovine p53 gene has been localised to chromosome 19q15 (Coggins *et al.*, 1995). Examination of p53 protein levels in BPV-4-induced papillomas and carcinomas showed that p53 protein levels were elevated in papillomas but not in carcinomas (L. Scobie, unpublished data). This coincided with expression of BPV-4 E7 protein in the basal and suprabasal cell layers. Papillomas and carcinomas were also screened for

mutations in the p53 gene using the technique of single strand conformation polymorphism (SSCP). Mutations were detected in several carcinomas, however no mutations were detected in the papillomas suggesting that mutation of this gene is a relatively late event in progression. The apparent stabilisation of p53 protein in papillomas and its coincidence with BPV-4 E7 protein expression may be a consequence of BPV-4 E7 expression. Elevated levels of p53 protein have also been observed in primary human foreskin epithelial cells immortalised by the HPV-16 E7 protein (Demers *et al.,* 1994). Similarly, stabilisation of p53 protein is induced in cells transformed by adenovirus 5 E1A (Lowe & Ruley, 1993).

Changes in ploidy have been observed in HPV-associated genital lesions, with the degree of genomic instability increasing with worsening histologic diagnosis (Reid *et al.*, 1984). Karyotypic analysis showed that biopsies classified as sub-clinical papillomaviral infections (SPI) contained cells with diploid genomes, whereas the majority of cells from biopsies characterised as cervical intraepithelial neoplasia (CIN-1) lesions were polyploid and CIN-2 and CIN-3 lesions were predominantly aneuploid (Reid *et al.*, 1984). Altered ploidy has also been observed in HPV-16-immortalised human epithelial cells (Dürst *et al.*, 1987; Hawley-Nelson *et al.*, 1989; Smith *et al.*, 1989). These data support a causal relationship between HPV-encoded functions and ploidy. Transfection of primary mouse epidermal keratinocytes with HPV-16 indicated that induction of chromosomal abnormalities, especially changes in ploidy, were linked to expression of the E7 gene (Hashida & Yatsumoto, 1991). The HPV-16 E7 protein may be directly involved in genome duplication as expression of this viral oncoprotein has been shown to induce DNA synthesis in rodent cells (Sata *et al.*, 1989). The observed induction and stabilisation of the p53 protein in HPV-16 E7-immortalised cells (Demers *et al.*, 1994) may therefore occur in response to genomic instability brought about by E7-encoded functions. SV40-transformed cells also show chromosomal aberrations (Oksala & Therman, 1974). As both SV40 LT and HPV-16

E7 proteins bind p105Rb (Ludlow *et al.*, 1988; Dyson *et al.*, 1989), genome instability may result through common mechanisms.

Tightly regulated mechanisms of G1 and G2 cell cycle control appear critical for mammalian genome stability (Almasan *et al.*, 1995 for review). As the p53 and Rb proteins act as a G1 / S checkpoint, disruption of normal tumour suppressor protein functions may lead to inappropriate entry into the S-phase of the cell cycle. If cellular DNA is damaged, further replication may result in chromosomal abnormalities. A role for p105Rb in G1 / S progression and genetic instability was suggested by Almasan *et al.* (1995). Using several human tumour lines and gene amplification as a measure of genomic instability, the authors found that DNA amplification potential correlated with inactivated Rb. Thus inactivation of normal p105Rb function by complexing of a viral protein to this tumour suppressor may not only result in cellular proliferation but may also affect genome stability.

HPV-16 and BPV-4 E7 functions are not identical, for example unlike HPV-16 E7 (Chesters *et al.*, 1990) transfection with the BPV-4 E7 gene alone does not immortalise primary cells. In spite of this, the BPV-4 E7 protein may similarly induce chromosomal instability. If this were to happen the apparent stabilisation of p53 protein in BPV-4-induced papillomas expressing the E7 protein (L. Scobie, unpublished data) may occur in response to E7-mediated chromosomal damage. Cytogenetic analysis of these papillomas would allow examination as to whether chromosomal abnormalities, including changes in ploidy, are apparent in tumours known to express BPV-4 E7 protein.

6.7 BPV-4 synergises with the flavonoid quercetin

Epidemiological analysis of human cancers has suggested that 35-40 % of cancer deaths in the USA could be linked to dietary factors (Doll & Peto, 1981). Such factors,

including dietary composition, alcohol consumption and cigarette smoking, contribute to the carcinogenic process at either the initiation or progression phases, although the exact nature of their contribution remain unknown. Dietary factors are also implicated in the development of upper alimentary canal cancers in cattle. Bracken-grazing animals affected by viral papillomatosis of the upper alimentary tract are at much higher risk for the subsequent development of squamous cell carcinomas than animals with a bracken-free diet (Jarrett *et al.*, 1978; Campo *et al.*, 1994b). Analysis of the chemical composition of this plant has revealed that it contains a complex mixture of mutagens, carcinogens and immunosuppressants (Evans I.A. *et al.*, 1982; Evans W.C. *et al.*, 1982). Progression of BPV-4-induced papillomas to malignancy is greatly influenced by the effects of these chemicals (e.g. Campo *et al.*, 1994b).

One of the major mutagens present in bracken is the flavonoid quercetin (Evans W.C. *et al.*, 1982). Flavonoids are a class of phenolic compounds and are ubiquitous in plants (Kühnau, 1976). Quercetin is one of the most widely found flavonoids and is present not only in bracken but also in tea, coffee, cereal grains and a variety of fruit and vegetables (Kühnau, 1976). Due to their widespread occurrence, humans and animals unavoidably ingest a large amount of these phenolic compounds daily. Numerous studies have been carried out to investigate the chemical and biological activities of quercetin in both bacterial and mammalian cells *in vitro* and also *in vivo* (Jackson *et al.*, 1993 for review). Results from these studies indicated that quercetin has diverse and at times apparently contradictory effects.

Quercetin can bind DNA (Rahman *et al.*, 1990) and has been found to be mutagenic in both prokaryotic (e.g. Bjeldanes & Chang, 1977) and eukaryotic (e.g. Nakayasu *et al.*, 1986) cells. The genotoxicity of quercetin was reported to correlate with its ability to cause DNA damage in the presence of Cu(II) ions and oxygen (Rahman *et al.*, 1989). These mutations were thought to result through intercalation of quercetin into DNA,

generation of reactive oxygen species (ROS), including hydroxyl radicals, leading to single-strand DNA breaks (Fazal *et al.*, 1990). Similarly quercetin has also been shown to bind to protein and in the presence of Cu(II) and other ions, cause fragmentation of the protein by a free radical mechanism (Ahmed *et al.*, 1994). Exposure to ROS has been implicated in carcinogenesis (Cross, 1987).

Quercetin has also been found to induce DNA rearrangements (Suzuki *et al.*, 1991) and clastogenic damage (Ishidate, 1988). This last observation may be of significance to the malignant transformation of BPV-4-induced upper alimentary canal papillomas *in vivo*, as cattle feeding on bracken fern show a wide variety of cytogenetic abnormalities (Moura *et al.*, 1988). This flavonoid has also been shown to interfere with phosphorylation / dephosphorylation mechanisms (Van Wart-Hood *et al.*, 1989; Matter *et al.*, 1992). Although quercetin has been shown to synergise with the phosphotyrosine phosphatase inhibitor vanadate increasing protein-tyrosine phosphorylation in avian cells, this compound has also been reported to inhibit several protein kinases including protein tyrosine kinases (Graziani *et al.*, 1983). and also protein kinase C (Gschwendt *et al.*, 1984). Such inhibition would be expected to interfere with signal transduction pathways.

Quercetin can also act as an initiator in an *in vitro* two-stage transformation assay in mammalian cells, yet the same study reported that this chemical also inhibited promotion of transformation by TPA, demonstrating the diverse action of quercetin (Sakai *et al.*, 1990). Further to its ability to inhibit promotion by TPA, and contrary to its mutagenic activity, quercetin has been shown to inhibit the growth of various cell lines derived from human cancers including colon and gastric cancers (Hosokawa *et al.*, 1990; Yoshida *et al.*, 1990) and squamous cell carcinomas of the head and neck (Castillo *et al.*, 1989). This compound has also been reported to arrest gastric tumour cells (Yoshida *et al.*, 1990) and leukaemic T-cells (Yoshida *et al.*, 1992) in the G1 phase

of the cell cycle. This growth inhibitory effect may be due to quercetin-mediated disruption of enzymatic processes involved in cellular proliferation.

Results from *in vivo* studies appear similarly contradictory as although the majority of studies reported that quercetin is not carcinogenic (e.g. Morino *et al.*, 1982), Pamukcu *et al.* (1980) reported that quercetin induced intestinal and bladder carcinomas in Norwegian rats and Ertürk *et al.* (1983) observed that quercetin significantly increased the frequency of liver tumours in Sprague-Dawley and Fischer 344 rats.

Although conflicting results from the various *in vitro* and *in vivo* studies examining quercetin activities have been observed, this does not negate further analysis of the chemical but rather highlights the problems in identifying and elucidating its effect on cells. It also indicates that results may be only pertinent to the particular experimental system used and that appropriate caution should be made in extrapolating findings to other systems.

Quercetin has discernible effect on BPV-4 transformation *in vitro* as it synergises with the virus to fully transform primary bovine fibroblasts (Pennie, 1992). An obvious and interesting question therefore was to address whether this compound contributes to the transforming process via genetic and / or epigenetic mechanisms. The observed and reproducible effects of quercetin treatment on BPV-4-transformed PalF cells *in vitro* led to the studies described in this thesis in order to dissect possible mechanisms of quercetin action.

One of the immediate phenotypic effects was that PalF cells treated with quercetin prior to transfection with BPV-4 genes showed a much more aggressively transformed morphology than untreated transfectants. This was observed in all transfection classes regardless of whether cells had been transfected with whole genome or sub-genomic

BPV-4 fragments (cf. Figures 4.3 & 4.5). Treatment with quercetin, without subsequent transfection with viral DNA, did not change the cellular morphology of PalF cells compared to untreated controls. Neither did quercetin increase average cellular lifespan of these primary cells, as treated PalF cells senesced on continued culture over the same time scale as untreated controls. To assay whether quercetin-treated BPV-4-transfected cells were more transformed than their non-treated counterparts, clonal, and in some cases polyclonal, populations were characterised with respect not only to morphological transformation but also with respect to anchorage independence, immortalisation and tumourigenicity.

The BPV-4 E8 gene is required for anchorage-independent growth. However quercetin-treated PalF cells transfected with E7 alone were also capable of anchorage-independent growth (Figure 4.6; Table 4.6). This showed that quercetin could substitute for an E8 gene. Quercetin and BPV-4 E8 appear to be antagonistic as quercetin-treated cells transfected with constructs containing E7 and E8 showed no or severely reduced growth in semi-solid media. Although this detrimental effect is most likely due to antagonism between E8 and quercetin this cannot be tested directly as cells do not survive transfection of the E8 gene alone (Jaggar *et al.*, 1990; Pennie *et al.*, 1993).

Treatment with quercetin also immortalised BPV-4-transformed cells (Table 4.7). An immortalised phenotype had previously only been seen in BPV-4-transformed cells containing an HPV-16 E6 gene (Pennie *et al.*, 1993). These results showed that quercetin could substitute for addition of an exogenous E6 gene. In the above experiments quercetin was found to confer immortality to cells transformed by either the whole genome BPV-4 or the E7 gene but not to cells transfected with E8/E7 constructs (Table 4.7). This was interpreted as further evidence of antagonism between quercetin and E8. These results could be due to inappropriate / unregulated levels of E8 expression. The two constructs containing the E8/E7 ORFs are driven in one case by

the BPV-4 LCR promoter while the other is under the control of the Moloney murine leukaemia virus LTR (see Chapter 2.1.7). In the construct containing the whole genome BPV-4, E8 and the other viral genes are under the control of the BPV-4 LCR and are subject to viral regulatory control, for example through the action of the E2 protein. The regulation of E8 expression at any particular point in time may therefore dictate whether quercetin and E8 interact synergistically or antagonistically.

The observed synergism between BPV-4 and quercetin is dependent on time of treatment with this chemical. Of the four protocols used, in which the order and the interval between quercetin treatment and viral DNA transfection were varied, cells achieved the most transformed state when treated with quercetin either immediately before or after DNA transfection (Table 4.12). Lengthening of the interval between quercetin and transfection reduced the degree of transformation, irrespective of the sequential order of the treatments. This suggested that the effect of quercetin was due, in part, to an epigenetic mechanism of action. PalF transfectants treated with quercetin either immediately prior or immediately post-transfection with BPV-4 DNA were tumourigenic in nude mice (Table 4.12). Interestingly, the apparent irrelevance of the sequential order of the two treatments in inducing full cellular transformation was at variance with previous findings that the effect of quercetin on two-stage transformation of BALB / 3T3 cells differed depending on the stage and specific experimental conditions under which quercetin was added to the cells (Sakai *et al.*, 1990). The results from the PalF system may be explained by the observation that BPV-4 can synergise with both tumour initiators and tumour promoters (Gaukroger *et al.*, 1993). However qualitative differences were apparent, in that tumours induced by cells treated with quercetin immediately post-transfection (protocol QC) grew far more aggressively than tumours resulting from injection of cells treated with quercetin immediately prior to DNA transfection (protocol QA). That E7 QC transfectants, with or without an exogenous E6 gene, were the only sub-genomic classes capable of inducing tumours in

nude mice (Table 4.12) confirms that the greatest synergism between quercetin and viral functions takes place in cells treated with quercetin immediately after transfection. Nevertheless, the number of anchorage-independent clones formed by quercetin-treated E7 cells were fewer than those obtained with whole genome BPV-4 (Table 4.10), suggesting that other viral genes may contribute to the transformed phenotype. It could be argued that quercetin's contribution to full cellular transformation is most clearly manifest when added immediately post BPV-4 transfection, as such cells will already be exposed to the action of viral proteins and significant loss of viral DNA will not yet have occurred.

In the presence of quercetin, the BPV-4 E7 gene showed itself to be the major viral oncogene as measured by anchorage independence, immortalisation and tumourigenicity assays. This may be relevant to papilloma induction and carcinoma progression *in vivo*. Quercetin can substitute for both an endogenous BPV-4 E8 and an exogenous HPV-16 E6 viral gene functions *in vitro*. BPV-4 does not possess an E6 gene (Jackson *et al.*, 1991) and the important functions carried out by this gene in other papillomaviruses (Scheffner *et al.*, 1990; Sedman *et al.*, 1991) may be provided by the quercetin present in bracken. The E8 ORF is expressed in the suprabasal epithelial layers during the early stages of papilloma development (R. Anderson *et al.* in preparation). The ability of quercetin to substitute for E8 *in vitro* may indicate long term consequences *in vivo* as the animals are exposed to the quercetin present in bracken long after viral E8 expression in the papillomas has stopped. However, quercetin can only substitute for E8 in part. Although quercetin conferred anchorage independence on virally-transformed cells in the absence of E8, this chemical had no apparent effect on GJIC (Table 5.2). Disruption of GJIC is ascribed to the direct physical interaction between BPV-4 E8 protein and ductin, the major structural component of such junctions.

Contrary to previous reports (Ishidate, 1988; Suzuki *et al.*, 1991) no chromosomal abnormalities, large DNA rearrangements, DNA adducts or single or double strand DNA breaks in quercetin-treated PalF cells were detected (e.g. Figure 4.14). However the genetic changes reported by others were observed in established rather than in primary cells. Furthermore, the reported chromosomal damage was observed at much higher concentrations of quercetin than those used in our experimental system. The failure to observe any quercetin-induced mutagenic effects in PalF cells may be due to lack of assay detection sensitivity. However, as quercetin was shown to induce single strand DNA breaks in a cell-free plasmid DNA mobility assay at concentrations equal to and even more dilute than that found to synergise with BPV-4 in PalF cells, this could suggest that the *in vitro* results reflect poor intracellular uptake of the chemical. Hatcher & Bryan (1985) and Fazal *et al.* (1990) suggested that apparently conflicting genotoxicity results for quercetin could be explained by oxidation of the chemical outside cells, leading to the formation of products unable to cross the cell envelope. The above experiments were carried out in order to screen for possible quercetin-specific effects. Similar analyses of both quercetin-treated and control (ethanol-treated) virally-transformed PalF cells would be of interest.

Activation of the cellular genes *ras* and *myc* has been implicated in the progression of papillomavirus-transformed cells (Ocadiz *et al.*, 1987; Riou *et al.*, 1988; Campo *et al.*, 1990; Couturier *et al.*, 1991). However, examination of both control and virally-transformed PalF cells, regardless of whether treated with quercetin or not, showed that the DNA and RNA status of these two genes remained unchanged (Figures 4.12 & 4.13). Although results showed that there was no overt change at either the DNA or RNA level in transfectants, findings in a recent paper by Krontiris *et al.* (1993) may prove of some interest as regards further analysis of *ras* sequences and expression in the PalF *in vitro* system. In this paper the authors describe an association between the risk of developing a number of different types of cancer in humans and

mutations in the HRAS1 minisatellite locus found approximately 1000 bp downstream of the H-*ras*-1 gene. Although they cannot as yet exclude that the rare alleles of the HRAS1 minisatellite observed in cancer patients are not just simply markers for risk of cancer development as opposed to having an active role in disease pathogenesis, Krontiris *et al.* (1993) propose that such minisatellite mutations disrupt expression of nearby genes. This disruption includes the c-Ha-*ras* 1 gene. Although results from the analysis did not point to any changes in *ras* expression levels in the transfected PalF lines, this was looked at using RNA dot blots of total RNA. A more detailed analysis of these cells may well be warranted using not only Northern blots of mRNA but also probes covering different regions of the c-Ha-*ras* 1 gene, including ones immediately downstream of the coding sequence.

The ability of quercetin to affect levels of protein-tyrosine phosphorylation (Graziani *et al.*, 1983; Van Wart-Hood *et al.*, 1989) was examined in PalF cells. Tyrosine phosphorylation is thought to play an important role in cell growth, proliferation, differentiation and transformation (Fischer *et al.*, 1992). Preliminary results showed that there was a change in phosphotyrosine levels of, as yet, unidentified proteins in quercetin-treated cells. This was seen as an increase in intensity of a band running approximately between 50-65 kD as compared to the same band in ethanol-treated controls. This work has been carried on by a current PhD student in the laboratory. Results from this continued work confirm that the phosphotyrosine levels in treated cells are altered and extend this initial study by showing that such alterations are transient as phosphotyrosine profiles return to normal within eight hours of quercetin being removed (J. Connolly, personal communication). The concentration of quercetin used and time of exposure in the phosphotyrosine experiments were identical to conditions used in the transfection work. That changes in phosphotyrosine levels were transient, returning to normal over time, may explain why the synergism between BPV-4 genes and quercetin *in vitro* weakens the longer the time interval between them.

Recent work has shown that the activity of the BPV-4 LCR is 2-3 times higher in cells treated with quercetin after transfection with BPV-4 genes (protocol QC) than cells treated with quercetin immediately before transfection (protocol QA) (J. Connolly, unpublished data). As these experimental conditions are the most likely to result in full transformation of PalF cells these results would appear to support the proposal that the effects of quercetin are most clearly manifest in cells already actively expressing viral genes.

6.8 Summary

In summary, quercetin appears to cause epigenetic changes in PalF cells as measured by transient alteration in phosphotyrosine levels of, as yet, unidentified proteins. Identification of these proteins may well provide insight as to the action of quercetin in this *in vitro* system. It may even provide us with a feasible model of the chemical's *in vivo* contribution to cellular transformation in BPV-4-infected bracken-grazing cattle. Previous studies have proposed epigenetic mechanisms of quercetin action (Ishikawa *et al.*, 1987). In this study mouse fibrosarcoma cells were treated with quercetin in order to examine whether this flavonoid could alter the tumourigenic and metastatic activity of these cells in mice. Quercetin treatment was observed to have diverse effects in that it was found to decrease the tumourigenic potential of some clones while promoting the metastatic potential of others (Ishikawa *et al.*, 1987). The authors proposed that quercetin might act via an epigenetic mechanism affecting DNA methylation. Quercetin was found to cause hypermethylation in these cells, presumably inducing changes in gene expression which in some instances resulted in tumour regression whereas in others tumour progression was unimpeded.

Although no discernible mutation was found either as a result of treatment with quercetin and / or transfection with BPV-4 genes in PalF cells, this may be linked to the sensitivity of the experimental assays used. Therefore, at the present time, genetic

mechanisms of quercetin action cannot be ruled out. What can be said with confidence is that the synergism between quercetin and BPV-4 genes in PalF cells is indisputable as such cells become fully transformed forming tumours in nude mice. Although molecular mechanisms of quercetin action have yet to elucidated, the observed contribution to full transformation of BPV-4-transfected cells strengthens the hypothesis that this flavonoid is a possible co-factor in BPV-4-associated carcinogenesis *in vivo*.

Obviously it would be of great interest to repeat these studies in primary bovine keratinocytes as these are the natural target cell for infection by BPV-4. Furthermore, collagen rafts could be used to examine the effects of quercetin and / or BPV-4 genes on keratinocyte differentiation.

This *in vitro* experimental system has allowed partial functional characterisation of BPV-4 genes and provides a useful model system for analysis of viral / chemical co-operation in papillomavirus-associated carcinogenesis. The exact nature of the observed synergism between BPV-4 and the flavonoid quercetin is not as yet known, however a model of cellular transformation could be envisaged in which BPV-4 provides an initial proliferative stimulus, possibly by BPV-4 E8-encoded disruption of cell-cell communication, resulting in removal of normal proliferative control or isolation of virally-transformed cells from the growth inhibitory effects of surrounding non-transformed cells. Inactivation of p105Rb through binding to the BPV-4 E7 protein will also contribute to proliferation of transformed cells. This expanded cellular population would provide a further receptive target for the action of both viral and environmental factors, thus increasing the chances of additional genetic damage. Quercetin's contribution to the carcinogenic process has yet to be determined. It may, in concert with other chemicals present in bracken fern, cause direct mutation of cellular genes and / or act via epigenetic mechanisms such as disruption of phosphorylation and DNA methylation levels.

REFERENCES

REFERENCES

Agrawal R., Pelkonen J., Rytkönen M. and Mäntyjärvi R.A. (1992) Integration of bovine papillomavirus type 1 DNA and analysis of the amplified virus-cell junctions in transformed primary mouse fibroblasts. *J. Gen. Virol.* **73**, 201-206

Ahmed M.S., Ainley K., Parish J.H. and Hadi S.M. (1994) Free radical-induced fragmentation of proteins by quercetin. *Carcinogenesis* **15**, 1627-1630

Alitalo K. and Schwab M. (1986) Oncogene amplification in tumor cells. *Adv. Cancer Res.* **47**, 235-281

Alitalo K., Schwab M., Lin C.C., Varmus H.E. and Bishop J.M. (1983) Homogeneously staining chromosomal regions contain amplified copies of an abundantly expressed cellular oncogene (c-*myc*) in malignant neuroendocrine cells from a human colon carcinoma. *Proc. Natl. Acad. Sci. USA* **80**, 1707-1711

Almasan A., Linke S.P., Paulson T.G., Huang L-c and Wahl G.M. (1995) Genetic instability as a consequence of inappropriate entry into and progression through S-phase. *Cancer and Met. Reviews* **14**, 59-73

Androphy E.J., Dvoretzky I. and Lowy D.R. (1985) X-linked inheritance of epidermodysplasia verruciformis: Genetic and virologic studies of a kindred. *Arch. Dermatol.* **121**, 864-868

Barbosa M.S., Lowy D.R. and Schiller J.T. (1989) Papillomavirus polypeptides E6 and E7 are zinc-binding proteins. *J. Virol.* **63**, 1404-1407

Barbosa M.S., Edmonds C., Fischer C., Schiller J.T., Lowy D.R. and Vousden K.H. (1990) The region of the HPV E7 oncoprotein homologous to adenovirus E1a and SV40 large T antigen contains separate domains for Rb binding and casein kinase II phosphorylation. *EMBO J.* **9**, 153-160

Bernard B.A., Bailly C., Lenoir M.C., Darmon M., Thierry F. and Yaniv M. (1989) The human papillomavirus type 18 (HPV18) E2 gene product is a repressor of the HPV18 regulatory region in human keratinocytes. *J. Virol.* **63,** 4317-4324

Beverley P. (1991) Immunology of cancer. In, *Introduction to the Cellular and Molecular Biology of Cancer*, pp. 406-433. Edited by L.M. Franks and N.M. Teich. Oxford University Press, Oxford

Beyer E.C., Paul D.L. and Goodenough D.A. (1987) Connexin 43: A protein from rat heart homologous to the gap junction protein from liver. *J. Cell. Biol.* **105**, 2621-2629

Birnboim H.C and Doly J. (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* **7**, 1513-1523

Bishop J.M. (1991) Molecular themes in oncogenesis. *Cell* **64**, 235-248

Bishop J.M. and Varmus H.E. (1982) Functions and origins of retroviral transforming genes. In, *Molecular Biology of Tumour Viruses. Part III, RNA Tumor Viruses*, pp. 999-1108. Edited by R. Weiss, N. Teich, H. Varmus and J. Coffin. Cold Spring Harbor Laboratory Press, New York

Bjeldanes L.F. and Chang G.W. (1977) Mutagenic activity of quercetin and related compounds. *Science* **197**, 577-578

Botchan P.M. and Dayton A.I. (1982) A specific replication origin in the chromosomal rDNA of *Lytechinus variegatus*. *Nature* **299**, 453-456

Bradley M.O. and Kohn K.W. (1979) X-ray induced DNA double strand break production and repair in mammalian cells as measured by neutral filter elution. *Nucleic Acids Res.* **7**, 793-804

Breitburd F., Croissant O. and Orth G. (1987) Expression of human papillomavirus type 1 E4 gene product in warts. *Cancer Cells* **5**, 115-122

Brinton L.A. (1992) Epidemiology of cervical cancer-an overview. In, *The Epidemiology of Human Papillomavirus and Cervical Cancer*, pp. 3-23. Edited by N. Muñoz, F.X. Bosch, K.V. Shah and A. Meheus. Oxford University Press, Oxford

Broker T.R. and Botchan M. (1986) Papillomaviruses: Retrospectives and Prospectives. In, *Cancer Cells 4. DNA Tumor Viruses. Control of Gene Expression and Replication*, pp. 17-36. Edited by M. Botchan, T. Grodzicker and P.A. Sharp. Cold Spring Harbor, New York

Brown J.M., Anderson R.G.W. and Goldstein J.L. (1983) Recycling receptors: the round-trip itinerary of migrant membrane proteins. *Cell* **32**, 663-667

Burkhardt A., Willingham M., Gay C., Jeang K-T. and Schlegel R. (1989) The E5 oncoprotein of bovine papillomavirus is oriented asymmetrically in Golgi and plasma membranes. *Virology* **170**, 334-339

Burnett S., Moreno-Lopez J. and Pettersson U. (1988) A novel spontaneous mutation of the bovine papillomavirus 1 genome. *Plasmid* **20**, 61-74

Campo M.S. (1987) Papillomas and cancer in cattle. *Cancer Surveys* **6**, 39-54

Campo M.S. and Coggins L.W. (1982) Molecular cloning of bovine papillomavirus genomes and comparison of their sequence homologies by heteroduplex mapping. *J. Gen. Virol.* **63**, 255-264

Campo M.S. and Jarrett W.F.H. (1986) Papillomavirus infection in cattle: viral and chemical cofactors in naturally occurring and experimentally induced tumours. In *Papillomaviruses, CIBA foundation Symposium 120*, pp. 117-130. Edited by D. Evered and S. Clark. John Wiley and Sons, Chichester

Campo M.S. and Jarrett W.F.H. (1987) Papillomavirus and disease. In, *Molecular Basis of Virus Disease. Society of General Microbiology Symposium* **40**, 215-243. Edited by W.C. Russell and J.W. Almond. Cambridge University Press, Cambridge

Campo M.S. and Spandidos D.A. (1983) Molecularly cloned bovine papillomavirus DNA transforms mouse fibroblasts *in vitro*. *J. Gen. Virol.* **64**, 549-557

Campo M.S., Cameron I.R. and Rogers M.E. (1978) Tandem integration of complete and defective SV40 genomes in mouse-human somatic cell hybrids. *Cell* **15**, 1411-1426

Campo M.S., Moar M.H., Jarrett W.F.H. and Laird H.M. (1980) A new papillomavirus associated with alimentary tract cancer in cattle. *Nature* **286**, 180-182

Campo M.S., Moar M.H., Laird H.M. and Jarrett W.F.H. (1981) Molecular heterogeneity and lesion type specificity of cutaneous bovine papillomaviruses. *Virology* **113**, 323-335

Campo M.S., Moar S.H., Sartirana M.L., Kennedy I.M. and Jarrett W.F.H. (1985) The presence of bovine papillomavirus type 4 DNA is not required for the progression to, or the maintenance of, the malignant state in cancers of the alimentary canal in cattle. *EMBO J.* **4**, 1819-1825

Campo M.S., McCaffery R.E., Doherty I., Kennedy I.M. and Jarrett W.F.H. (1990) The Harvey *ras* gene 1 is activated in papillomavirus-associated carcinomas of the upper alimentary canal in cattle. *Oncogene* **5**, 303-308

Campo M.S., Jarrett W.F.H., Barron R., O'Neil B.W. and Smith K.T. (1992) Association of bovine papillomavirus type 2 and bracken fern with bladder cancer in cattle. *Cancer Res.* **52**, 6898-6904

Campo M.S., Jarrett W.F.H., O'Neil B.W. and Barron R.J. (1994a) Latent papillomavirus infection in cattle. *Res. Vet. Science* **56**, 151-157

Campo M.S., O'Neil B.W., Barron R.J. and Jarrett W.F.H. (1994b) Experimental reproduction of the papilloma-carcinoma complex of the alimentary canal in cattle. *Carcinogenesis* **15**, 1597-1601

Campo M.S., Anderson R.A., Cairney M. and Jackson M.E. (1994c) Bovine papillomavirus type 4: from transcriptional control to control of disease. In, *Viruses and Cancer. Society of General Microbiology Symposium* **51**, 47-70. Edited by A.C. Minson, J.C. Neil and M.A. McRae. Cambridge University Press, Cambridge

Capon D.J., Seeburg P.H., McGrath J.P., Hayflick J.S., Edman U., Levinson A.D. and Goeddel D.V. (1983) Activation of Ki-*ras*2 gene in human colon and lung carcinomas by two different point mutations. *Nature* **304**, 507-512

Castagna M., Takai U., Kaibuchi K., Sano S., Kikkawa U. and Nishizuka Y. (1982) Direct activation of calcium-dependent, phospholipid-dependent protein kinase by tumour promoting phorbol esters. *J. Biol. Chem.* **257**, 7847-7851

Castillo M.H., Perkins E., Cambell J.H., Doerr R., Hassett J.M., Kandaswami C. and Middleton E. (1989) The effect of the bioflavonoid quercetin on squamous cell carcinoma of the head and neck origin. *Am. J. Surg.* **158**, 351-355

Cepko C.L., Roberts B.E. and Mulligan R.C. (1984) Construction and applications of a highly transmissible murine retrovirus shuttle vector. *Cell* **37**, 1053-1062

Chan C.S.M. and Tye B.K. (1980) The chromosomal replication origins in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* **77**, 6233-6239

Chen P-L., Scully P., Shew J-W., Wang J.Y.J. and Lee W-H. (1989) Phosphorylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation. *Cell* **58**, 1193-1198

Chesters P.M., Vousden K.H., Edmonds C. and McCance D.J. (1990) Analysis of human papillomavirus type 16 open reading frame E7 immortalizing function in rat embryo fibroblast cells. *J. Gen. Virol.* **71**, 449-453

Coggins L.W., Scobie L., Jackson M.E. and Campo M.S. (1995) Assignment of the bovine p53 gene to chromosome 19q15 by fluorescence *in situ* hybridisation. *Mammalian Genome* **6**, 687-688

Conrad M., Bubb V.J. and Schlegel R. (1993) The human papillomavirus type 6 and 16 E5 proteins are membrane-associated proteins which associate with the 16-kilodalton pore-forming protein. *J. Virol.* **67**, 6170-6178

Cooper G.M. (1982) Cellular transforming genes. *Science* **218**, 801-806

Couturier J., Sastre-Garau X., Schneider-Maunoury S., Labib A. and Orth G. (1991) Integration of papillomavirus DNA near *myc* genes in genital carcinomas and its consequences for protooncogene expression. *J. Virol.* **65**, 4534-4538

Cripe T.P., Haugen T.H., Turk J.P., Tabatabai F., Schmid P.G., Dürst M., Gissmann L., Roman A. and Turek L.P. (1987) Transcriptional regulation of the human papillomavirus 16 E6-E7 promoter by a keratinocyte dependent enhancer, and by viral E2 transactivator and repressor gene products: implications for cervical carcinogenesis. *EMBO J.* **6**, 3745-3753

Crook T., Banks L., Smith K. and Crawford L. (1989) Amplification and overexpression of c-*myc* proto-oncogene correlate with the loss of glucocorticoid dependence in rodent cells transformed by human papillomavirus type 16. *Mol. Carcinogenesis* **2**, 308-313

Crook T., Wrede D., Tidy J., Scholfield J., Crawford L. and Vousden K.H. (1991) Status of c-*myc*, p53 and retinoblastoma genes in human papillomavirus positive and negative squamous cell carcinomas of the anus. *Oncogene* **6**, 1251-1257

Cross C.E. (1987) Oxygen radicals and human disease. *Ann. Inter. Med.* **107**, 526-545

Dalla Favera R., Wong-Staal F. and Gallo R.C. (1982) *onc* gene amplification in promyelocytic leukaemia cell line HL-60 and primary leukaemic cells of the same patient. *Nature* **299**, 61-63

Defeo-Jones D., Huang P.S., Jones R.E., Haskell K.M., Vuocolo G.A., Hanobik M.G., Huber H.E. and Oliff A. (1991) Cloning of cDNAs for cellular proteins that bind to the retinoblastoma gene product. *Nature* **352**, 251-254

Demers G.W., Halbert C.L. and Galloway D.A. (1994) Elevated wild-type protein levels in human epithelial cell lines immortalised by the human papillomavirus type E7 gene. *Virology* **198**, 169-174

DiPaolo J.A., Woodworth C.D., Popescu N.C., Notario V. and Doninger J. (1989) Induction of human cervical squamous cell carcinoma by sequential transfection with human papillomavirus 16 DNA and viral Harvey *ras*. *Oncogene* **4**, 395-399

Doll R. and Peto R. (1981) The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. *J. Natl. Cancer Inst.* **66**, 1191-1308

Doorbar J. (1991) An emerging function for E4. *Papillomavirus Report* **2**, 145-147

Doorbar J., Evans H.S., Coneron I., Crawford L.V. and Gallimore P.H. (1988) Analysis of HPV-1 E4 gene expression using epitope-defined antibodies. *EMBO J.* **7**, 825-833

Doorbar J., Ely S., Sterling J., McLean C. and Crawford L. (1991) Specific interaction between HPV-16 E1-E4 and cytokeratins results in collapse of the epithelial cell intermediate filament network. *Nature* **352**, 824-827

Dotto G.P., Weinberg R.A. and Ariza A. (1988) Malignant transformation of mouse primary keratinocytes by Harvey sarcoma virus and its modulation by surrounding normal cells. *Proc. Natl. Acad. Sci. USA* **85**, 6389-6393

Dürst M., Kleinheinz A., Hotz M. and Gissmann L. (1985) The physical state of human papillomavirus type 16 DNA in benign and malignant genital tumours. *J. Gen. Virol.* **66**, 1515-1522

Dürst M., Petrusevska T.D., Boukamp P., Fusenig N.E. and Gissmann L. (1987) Molecular and cytogenetic analysis of immortalized keratinocytes obtained after transfection with human papillomavirus type 16 DNA. *Oncogene* **1**, 251-256

Dyson N., Howley P.M., Münger K. and Harlow E. (1989) The human papillomavirus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science* **243**, 934-936

Edmonds C. and Vousden K.H. (1989) A point mutational analysis of human papillomavirus type 16 E7 protein. *J. Virol.* **63**, 2650-2656

Elkind M.M. and Redpath J.L. (1977) Molecular and cellular biology of radiation lethality. In, *Cancer: A Comprehensive Treatise* **6**, 51-99. Edited by F.F. Becker. Plenum, New York

Erikson J., ar-Rushdi A., Drowinga H.L., Nowell P.C. and Croce C.M. (1983) Transcriptional activation of the translocated c-*myc* oncogene in Burkitt lymphoma. *Proc. Natl. Acad. Sci. USA* **80**, 820-824

Ertürk E., Nunoya T., Hatcher J.F., Pamukcu A.M. and Bryan G.T. (1983) Comparison of bracken fern and quercetin carcinogenicity in rats. *Proc. Am. Assoc. Cancer Res.* **24**, 53

Evans H.J. (1993) Molecular genetic aspects of human cancers: The 1993 Frank Rose Lecture. *Br. J. Cancer* **68**, 1051-1060

Evans I.A., Prorok J.G., Cole R.C., Al-Salmani M.H., Al-Samarrai A.M.H., Patel M.C. and Smith R.M.M. (1982) The carcinogenic, mutagenic and teratogenic toxicity of bracken. *Proc. R. Soc. Edinburgh.* **81B**, 65-77

Evans W.C., Patel M.C. and Koohy Y. (1982) Acute bracken poisoning in homogastric and ruminant animals. *Proc. R .Soc. Edinburgh.* **81B**, 29-64

Farber E. and Cameron R. (1980) The sequential analysis of cancer development. *Ad. Cancer Res.* **31**, 125-226

Fauci A.S. (1988) The human immunodeficiency virus: infectivity and mechanisms of pathogenesis. *Science* **239**, 617-622

Favre M., Breitburd F., Croissant O. and Orth G. (1975) Structural polypeptides of rabbit, bovine and human papillomaviruses. *J. Virol.* **15**, 1239-1247

Fazal F., Rahman A., Greensill J., Ainley K., Hadi S.M. and Parish J.H. (1990) Strand scission in DNA by quercetin and Cu(II): identification of free radical intermediates and biological consequences of scission. *Carcinogenesis* **11**, 2005-2008

Fearon E.R. and Vogelstein B. (1990) A genetic model for colorectal tumorigenesis. *Cell* **61**, 759-767

Fentiman I., Taylor-Papadimitriou J.K. and Stoker M. (1976) Selective contact dependent cell communication. *Nature* **264**, 760-762

Ferenczy A., Mitao M., Nagai N., Silverstein S.J. and Crum C.P. (1985) Latent papillomavirus and recurring genital warts. *N. Engl. J. Med.* **313**, 784-788

Finbow M.E. and Pitts J.D. (1993) Is the gap junction channel - the connexon - made of connexins or ductin? *J. Cell Sci.* **106**, 463-472

Finbow M.E., Pitts J.D., Goldstein D.J., Schlegel R. and Findlay J.B.C. (1991) The E5 oncoprotein target; a 16 kDa channel-forming protein with diverse functions. *Mol. Carcinogenesis* **4**, 441-444

Finbow M.E., Eliopoulos E.E., Jackson P.J, Keen J.N., Meagher L., Thompson P., Jones P. and Findlay J.B.C. (1992) Structure of a 16 kDa integral membrane protein that has identity to the putative proton channel of the vacuolar H^+ATPase. *Protein Eng.* **5**, 7-15

Fischer E.H., Charbonneau H., Cool D.E. and Tonks N.K. (1992) Tyrosine phosphatases and their possible interplay with tyrosine kinases. In, *Interactions Among Cell Signalling Systems. CIBA foundation symposium,* **164,** 132-144. Edited by R. Sato, G.R. Bock and K. Widdows. John Wiley and Sons, Chichester

Fitzgerald D. and Yamasaki H. (1990) Tumor promotion: models and assay systems. *Teratog. Carcinog. Mutagen.* **10**, 89-102

Fitzsimmons S.A., Lewis A.D., Riley R.J. and Workman P. (1994) Reduction of 3-amino-1,2,4-benzotriazine-1,4-di-*N*-oxide (tirapazamine, WIN 59075, SR 4233) to a DNA-damaging species: a direct role for NADPH:cytochrome P450 oxidoreductase+. *Carcinogenesis* **8**, 1503-1510

Forman D., Sitas F., Newell D.G., Stacey A.R., Boreham J., Peto R., Campbell T.C., Li J. and Chen J. (1990) Geographic association of *Helicobacter pylori* antibody prevalence and gastric cancer mortality in rural China. *Int. J. Cancer* **46**, 608-611

Fournier R.E.K. and Ruddle F.H. (1977) Microcell-mediated transfer of murine chromosomes into mouse, Chinese hamster and human somatic cells. *Proc. Natl. Acad. Sci. USA* **74**, 319-323

Franchini G., Mulloy J.C., Koralnik I.J., Lo Monico A., Sparkowski J.J., Andresson T., Goldstein D.J. and Schlegel R. (1993) The human T-cell leukemia / lymphotropic virus type 1 $p12^I$ protein cooperates with the E5 oncoprotein of bovine papillomavirus in cell transformation and binds the 16-kilodalton subunit of the vacuolar H^+ATPase. *J. Virol.* **67**, 7701-7704

Franks L.M and Teich N.M. (1991) Preface. In, *Introduction to the Cellular and Molecular Biology of Cancer*, pp. vii-viii. Edited by L.M. Franks and N.M. Teich. Oxford University Press, Oxford

Frazer I.H. and Tindle R.W. (1992) Cell-mediated immunity to papillomaviruses. *Papillomavirus Report* **3,** 53-58

Friend S.H., Bernards R., Rogelj S., Weinberg R.A., Rapaport J.M., Alberts D.M. and Dryja T.P. A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. *Nature* **323**, 643-646

Gaffney D.F., McLauchlan J., Whitton J.L. and Clements J.B. (1985) A modular system for the assay of transcription regulatory signals: the sequence TAATGARAT is required for herpes simplex virus immediate early gene activation. *Nucleic Acids Res.* **13**, 7847-7863

Gaukroger J., Chandrachud L., Jarrett W.F.H., McGarvie G.E., Yeudall W.A., McCaffery R.E., Smith K.T. and Campo M.S. (1991) Malignant transformation of a papilloma induced by bovine papillomavirus type 4 in the nude mouse renal capsule. *J. Gen. Virol.* **72**, 1165-1168

Gaukroger J., Bradley A., Chandrachud L., Jarrett W.F.H. and Campo M.S. (1993) Interaction between bovine papillomavirus type 4 and cocarcinogens in the production of malignant tumours. *J. Gen. Virol.* **74**, 2275-2280

Georges M., Lequarré A.S., Castelli M., Hanset R. and Vassart G. (1988) DNA fingerprinting in domestic animals using four different minisatellite probes. *Cytogenet. Cell Genet.* **47**, 127-131

Georges M., Lathrop M., Hilbert P., Marcotte A., Schwers A., Swillens S., Vassart G and Hanset R.. (1990) On the use of DNA fingerprints for linkage studies in cattle. *Genomics* **6**, 461-474

Gissmann L., Wolnik L., Ikenberg H., Koldovsky U., Schnürch H.G. and zur Hausen H. (1983) Human papillomavirus types 6 and 11 DNA sequences in genital and laryngeal papillomas and in some cervical cancers. *Proc. Natl. Acad. Sci. USA* **80**, 560-563

Godbout R., Dryja T.P., Squire J., Gallie B.L. and Phillips R.A. (1983) Somatic inactivation of genes on chromosome 13 is a common event in retinoblastoma. *Nature* **304**, 451-453

Goldstein D.J. and Schlegel R. (1990) The E5 oncoprotein of bovine papillomavirus type 1 binds to a 16 kd cellular protein. *EMBO J.* **9**, 137-146

Goldstein D.J., Finbow M.E., Andresson T., McLean P., Smith K., Bubb V. and Schlegel R. (1991) Bovine papillomavirus E5 oncoprotein binds to the 16K component of vacuolar H^+-ATPases. *Nature* **352**, 347-349

Goldstein D.J., Kulke R., DiMaio D. and Schlegel R. (1992) A glutamine residue in the membrane-associating domain of the bovine papillomavirus type 1 E5 oncoprotein mediates its binding to a transmembrane component of the vacuolar H^+-ATPase. *J. Virol.* **66**, 405-413

Graziani Y, Erikson E. and Erikson R.L. (1983) The effect of quercetin on the phosphorylation activity of the Rous sarcoma virus transforming gene product *in vitro* and *in vivo*. *Eur. J. Biochem.* **185**, 583-589

Groff D.E. and Lancaster W.D. (1986) Genetic analysis of the 3' early region transformation and replication functions of bovine papillomavirus type 1. *Virology* **150**, 221-230

Gschwendt M., Horn F., Kittstein W., Fürstenberger G., Besemfelder E. and Marks F. (1984) Calcium and phospholipid-dependent protein kinase activity in mouse epidermis cytosol. Stimulation by complete and incomplete tumor promoters and inhibition by various compounds. *Biochem. Biophys. Res. Commun.* **124**, 63-68

Gupta R.C. (1985) Enhanced sensitivity of ^{32}P-postlabeling analysis of aromatic carcinogen-DNA adducts. *Cancer Res.* **5**, 5656-5662

Gupta R.C. (1993) ^{32}P-Postlabelling analysis of bulky aromatic adducts. In, *Postlabelling Methods for Detection of DNA Adducts*, pp. 11-23. Edited by D.H. Phillips, M. Castegnaro & H. Bartsch. Oxford University Press, Oxford

Gupta R.C. and Earley K. (1988) ^{32}P-Adduct assay: comparative recoveries of structurally diverse DNA adducts in the various enhancement procedures. *Carcinogenesis* **9**, 1687-1693

Hall C.V., Jacob P.E., Ringold G.M. and Lee F. (1983) Expression and regulation of *Escherichia coli lacZ* gene fusions in mammalian cells. *J. Mol. Appl. Genet.* **2**, 101-109

Hamada J-i., Takeichi N. and Kobayashi H. (1988) Metastatic capacity and intercellular communication between normal cells and metastatic cell clones derived from a rat mammary carcinoma. *Cancer Res.* **48**, 5129-5132

Hansen M.F. and Cavenee W.K. (1987) Genetics of cancer predisposition. *Cancer Res.* **47**, 5518-5527

Harris C.C. and Hollstein M. (1993) Clinical implications of the p53 tumor-suppressor gene. *New Engl. J. Med.* **329**, 1318-1327

Harris H. (1985) Suppression of malignancy in hybrid cells: the mechanism. *J. Cell Sci.* **79**, 83-94

Harris H., Miller O.J., Klein G., Worst P. and Tachibana T. (1969) Suppression of malignancy by cell fusion. *Nature* **223**, 363-368

Hashida T. and Yasumoto S. (1991) Induction of chromosome abnormalities in mouse and human epidermal keratinocytes by the human papillomavirus type 16 E7 gene. *J. Gen. Virol.* **72**, 1569-1577

Haskell K.M., Vuocolo G.A., Defeo-Jones D., Jones R.E. and Ivey-Hoyle M. 1993 Comparison of the binding of the human papillomavirus type 16 and cottontail rabbit papillomavirus E7 proteins to the retinoblastoma gene product. *J. Gen. Virol.* **74**, 115-119

Hatcher J.F. and Bryan G.T. (1985) Factors affecting the mutagenic activity of quercetin for *Salmonella typhimurium* TA98: metal ions, antioxidants and pH. *Mutation Res.* **148**, 13-23

Hawley-Nelson P., Vousden K.H., Hubbert N.L., Lowy D.R. and Schiller J.T. (1989) HPV-16 E6 and E7 proteins cooperate to immortalize human foreskin keratinocytes. *EMBO J.* **8**, 3905-3910

Hayward W.S., Neel B.G. and Astrin S.M. (1981) Activation of a cellular *onc* gene by promoter insertion in ALV-induced lymphoid leukosis. *Nature* **290**, 475-480

Hecker E., Fusenig N.E., Kung W., Marks F. and Thielman H.W. (eds). (1982) *Carcinogenesis-A Comprehensive Survey, Volume 7.* Raven Press, New York

Henderson B.E., Ross R.K. and Pike M.C. (1991) Toward the primary prevention of cancer. *Science* **254**, 1131-1138

Hennings H., Shores R., Wenk M.L., Spangler E.F., Tarone R. and Yuspa S.H. (1983) Malignant conversion of mouse skin tumours is increased by tumour initiators and unaffected by tumour promoters. *Nature* **304**, 67-69

Hirono I., Ogino H., Fujimoto M., Yamada K., Yoshida Y., Ikagawa M. and Okumara M. (1987) Induction of tumors in AC1 rats given a diet containing ptaquiloside, a bracken carcinogen. *J. Natl. Cancer Inst.* **79**, 1143-1149

Hosokawa N., Hosokawa Y., Toshiyuki S., Marui N., Nishino H., Kawai K. and Aoike A. (1990) Inhibitory effect of quercetin on the synthesis of a possibly cell-cycle-related 17 kDa protein, in human colon cancer cells. *Int. J. Cancer.* **45**, 1119-1124

Hug H. and Sarre T.F. (1993) Protein kinase C isoenzymes: divergence in signal transduction? *Biochem. J.* **291**, 329-343

Husøy T., Mikalsen S-O and Sanner T. (1993) Phosphatase inhibitors, gap junctional intercellular communication and [^{125}I]-EGF binding in hamster fibroblasts. *Carcinogenesis* **14**, 2257-2265

Ishidate M. (1988) *Data Book on Chromosomal Aberration Tests In Vitro*. Elsevier, Amsterdam

Ishikawa M., Okada F., Hamada J-i., Hosokawa M. and Kobayashi H. (1987) Changes in the tumorigenic and metastatic properties of tumor cells treated with quercetin or 5-azacytidine. *Int. J. Cancer* **39**, 338-342

Jablonska S. and Orth G. (1985) Epidermodysplasia verruciformis. *Clin. Dermatol.* **3**, 83-96

Jackson M.E. and Campo M.S. (1991) Positive and negative E2-independent regulatory elements in the long control region of bovine papillomavirus type 4. *J. Gen. Virol.* **72**, 877-833

Jackson M.E. and Campo M.S. (1995) Both viral E2 protein and the cellular factor PEBP2 regulate transcription via E2 consensus sites within the bovine papillomavirus type 4 long control region. *J. Virol.* **69**, 6038-6046

Jackson M.E., Pennie W.D., McCaffery R.E., Smith K.T., Grindlay G.J. and Campo M.S. (1991) The B subgroup bovine papillomaviruses lack an identifiable E6 open reading frame. *Mol. Carcinogenesis* **4**, 382-387

Jackson M.E., Campo M.S. and Gaukroger J.M. (1993) Cooperation between papillomavirus and chemical co-factors in carcinogenesis. *Crit. Rev. Oncogenesis* **4,** 277-291

Jaggar R.T., Pennie W.D., Smith K.T., Jackson M.E. and Campo M.S. (1990) Cooperation between bovine papillomavirus type 4 and *ras* in the morphological transformation of primary bovine fibroblasts. *J. Gen. Virol.* **71**, 3041-3046

Jarrett W.F.H. (1985) The natural history of bovine papillomavirus infection. *Adv. Viral Oncol.* **5**, 83-102

Jarrett W.F.H., McNeil P.E., Grimshaw T.R., Selman I.E. and McIntyre W.I.M. (1978) High incidence area of cattle cancer with a possible interaction between an environmental carcinogen and a papillomavirus. *Nature* **274**, 215-217

Jarrett W.F.H., Campo M.S., O'Neil B.W., Laird, H.M. and Coggins L.W. (1984) A novel bovine papillomavirus (BPV-6) causing true epithelial papillomas of the mammary gland skin: a member of a proposed new BPV subgroup. *Virology* **136,** 255-264

Jeffreys A.J., Wilson V. and Thein S.L. (1985) Hypervariable 'minisatellite' regions in human DNA. *Nature* **314**, 67-73

Jeffreys A.J., Wilson V., Kelly R., Taylor B.A. and Bulfield G. (1987) Mouse DNA fingerprint-analysis of chromosome localization and germline stability of hypervariable loci in recombinant inbred strains. *Nucleic Acids Res.* **15**, 2823-2836

Jongen W., Fitzgerald D., Makato A., Piccoli C., Slaga T., Gros D., Takeichi M. and Yamasaki H. (1991) Regulation of connexin 43-mediated gap junctional intercellular communication by Ca^{+} in mouse epidermal cells is controlled by E-cadherin. *J. Cell Biol.* **114**, 545-555

Kaltenbach J.P., Kaltenbach M.H. and Lyons W.B. (1958) Nigrosin as a dye for differentiating live and dead ascites cells. *Exp. Cell Res.* **15**, 112-117

Kam E., Melville L. and Pitts J.D. (1986) Patterns of junctional communication in skin. *J. Invest. Dermatol.* **87**, 748-753

Kaur P. and McDougall J.K. (1988) Characterization of primary human keratinocytes transformed by human papillomavirus type 18. *J. Virol.* **62**, 1917-1924

Kensler T.W. and Taffe B.G. (1986) Free radicals in tumour promotion. *Adv. Free Rad. Biol. Med.* **2**, 347-387

Kidd J.G. and Rous P. (1940) Cancers deriving from the virus papillomas of wild rabbits under natural conditions. *J. Exp. Med.* **71**, 469-472

Kistler J., Christie D. and Bullivant S. (1988) Homologies between gap junction proteins in lens, heart and liver. *Nature* **331**, 721-723

Kleinberger T., Etkin S. and Lavi S. (1986) Carcinogen-mediated methotrexate resistance and dihydrofolate reductase amplification in Chinese hamster cells. *Mol. Cell. Biol.* **6**, 1958-1964

Knudson A.G. (1971) Mutation and cancer: statistical study of retinoblastoma. *Proc. Natl. Acad. Sci. USA* **68**, 820-823

Kohn K.W., Erickson L.C., Ewig R.A.G. and Friedman C.A. (1976) Fractionation of DNA from mammalian cells by alkaline elution. *Biochem.* **15**, 4629-4637

Koufos A., Hansen M.F., Lampkin B.C., Workman M.L., Copeland N.G., Jenkins N.A. and Cavenee W.K. (1984) Loss of alleles at loci on human chromosome 11 during genesis of Wilms' tumor. *Nature* **309**, 170-172

Krontiris T.G., Devlin B., Karp D.D., Robert N.J. and Risch N. (1993) An association between the risk of cancer and mutations in the HRAS1 minisatellite locus. *N. Engl. J. Med.* **329**, 517-523

Kühnau J. (1976) The flavonoids. A class of semi-essential food components: their role in human nutrition. *Wld. Rev. Nutr. Diet* **24**, 117-191

Lam E. W-F. and La Thangue N.B. (1994) DP and E2F proteins: coordinating transcription with cell cycle progression. *Curr. Opin. Cell Biol.* **6**, 859-866

Lamberti C., Morrissey L.C., Grossman S.R. and Androphy E.J. (1990) Transcriptional activation by the papillomavirus E6 zinc finger oncoprotein. *EMBO J.* **9**, 1907-1913

Lancaster W.D., Castellano C., Santos C., Delgado G., Kurman R.J. and Jenson A.B. (1986) Human papillomavirus deoxyribonucleic acid in cervical carcinoma from primary and metastatic sites. *Am. J. Obstet. Gynecol.* **154**, 115-1190

Land H., Parada L.F. and Weinberg R.A. (1983) Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating genes. *Nature* **304**, 596-602

Law M-F., Lowy D.R., Dvoretzky I and Howley P.M. (1981) Mouse cells transformed by bovine papillomavirus contain only extrachromosomal viral DNA sequences. *Proc. Natl. Acad. Sci. USA* **78**, 2727-2731

Lee W-H., Bookstein R., Hong F., Young L-J., Shew J-Y., Eva Y-H. and Lee P. (1987) Human retinoblastoma susceptibility gene: cloning, identification, and sequence. *Science* **235**, 1394-1399

Loewenstein, W.R. (1979) Junctional intercellular communication and the control of growth. *Biochem. Biophys. Acta* **560**, 1-65

Lowe S.W. and Ruley H.E. (1993) Stabilization of the p53 tumor suppressor is induced by adenovirus 5 E1A and accompanies apoptosis. *Genes Dev.* **7**, 535-545

Ludlow J.W., DeCaprio J.A., Huang C.M., Lee W.H., Paucha E. and Livingston D.M. (1988) SV40 large T antigen binds preferentially to an underphosphorylated member of the retinoblastoma susceptibility gene product family. *Cell* **56**, 57-65

Lusky M. and Botchan M.R. (1985) Genetic analysis of bovine papillomavirus type 1 *trans*-acting replication factors. *J. Virol.* **53**, 955-965

Lusky M. and Botchan M.R. (1986) Transient replication of bovine papilloma virus type 1 plasmids: *cis* and *trans* requirements. *Proc. Natl. Acad. Sci. USA* **83**, 3609-3613

Lutzner M.A. (1978) Epidermodysplasia verruciformis: An autosomal recessive disease characterised by viral warts and skin cancer. A model for viral oncogenesis. *Bull. Cancer* **65**, 169-182

Mackenzie I.C. (1975) Ordered structure of the epidermis. *J. Invest. Dermatol.* **65**, 45-51

Macnab J.C.M. (1987) Herpes simplex virus and human cytomegalovirus: their role in morphological transformation and genital cancers. *J. Gen. Virol.* **68**, 2525-2550

Malkin D. (1993) p53 and the Li-Fraumeni syndrome. *Cancer Genet. Cytogenet.* **66**, 83-92

Malkin D., Li F.P., Strong L.C., Fraumeni J.F., Nelson C.E., Kim D.H., Kassel J., Gryka M.A., Bischoff F.Z., Tainsky M.A. and Friend S.H. (1990) Germline p53 mutations in a familial syndrome of breast cancer, sarcomas and other neoplasms. *Science* **250**, 1233-1238

Mandel M., Moriyama Y., Hulmes J.D., Pan Y-C.E., Nelson H. and Nelson N. (1988) cDNA sequence encoding the 16-kDa proteolipid of chromaffin granules implies gene duplication in the evolution of H^+-ATPases. *Proc. Natl. Acad. Sci. USA* **85**, 5521-5524

Mannens M., Slater R.M., Heyting C., Bliek J., de Kraker J., De Pagter-Holthuizen P. and Pearson P.L. (1988) Molecular nature of genetic changes resulting in loss of heterozygosity of chromosome 11 in Wilms' tumor. *Hum. Genet.* **81**, 41-48

Martin P., Vass W.C., Schiller J.T., Lowy, D.R. and Velu T.J. (1989) The bovine papillomavirus E5 transforming protein can stimulate the transforming activity of EGF and CSF-1 receptors. *Cell* **59,** 21-32

Matlashewski G., Schneider J., Banks L., Jones N., Murray A. and Crawford L. (1987) Human papillomavirus type 16 DNA cooperates with activated *ras* in transforming primary cells. *EMBO J.* **6**, 1741-1746

Matsungaga I. (1981) Genetics of Wilms' tumour. *Hum. Genet.* **57**, 231-246

Matter W.F., Brown R.F. and Vlahos C.J. (1992) The inhibition of phosphatidylinositol 3-kinase by quercetin and analogs. *Biochem. Biophys. Res. Commun.* **186**, 624-631

McCaffery R.E. and Jackson M.E. (1994) An element binding a C/EBP related transcription factor contributes to negative regulation of the bovine papillomavirus type 4 long control region. *J. Gen. Virol.* **75**, 3047-3056

McCaffery R.E., Coggins L.W., Doherty I., Kennedy I., O'Prey M., McColl L. and Campo M.S. (1989) Multiple Harvey-*ras* genes in the bovine genome. *Oncogene* **4**, 1441-1448

McIntyre M.C., Frattini M.G., Grossman S.R. and Laimins L.A. (1993) Human papillomavirus type 18 E7 protein requires intact Cys-X-X-Cys motifs for zinc binding, dimerization, and transformation but not for Rb binding. *J. Virol.* **67**, 3142-3150

Meltz M.L. and MacGregor J.T. (1981) Activity of the plant flavonol quercetin in the mouse lymphoma L5178Y $TK^{+/-}$ mutation, DNA single-strand break, and Balb / c 3T3 chemical transformation assays. *Mutation Res.* **88**, 317-324

Meneghini R. (1988) Genotoxicity of active oxygen species in mammalian cells. *Mutation Res.* **195**, 215-230

Mesnil M. and Yamasaki H. (1993) Cell-cell communication and growth control of normal and cancer cells: evidence and hypothesis. *Mol. Carcinogenesis* **7**, 14-17

Mesnil M., Fitzgerald D. and Yamasaki H. (1988) Phenobarbital specifically reduces gap junction protein mRNA level in rat liver. *Mol. Carcinogenesis* **1**, 79-81

Meyers C., Harry J., Lin Y-L. and Wettstein F.O. (1992) Identification of three transforming proteins encoded by cottontail rabbit papillomavirus. *J. Virol.* **66**, 1655-1664

Milde-Langosch K., Becker G. and Loning T. (1991) Human papillomavirus and c-*myc* / c-*erbB2* in uterine and vulvar lesions. *Virchows Arch A.* **419**, 479-485

Miller D.G. (1980) On the nature of susceptibility to cancer. The presidential address. *Cancer* **46**, 1307-1318

Miller E.C. and Miller J.A. (1981) Mechanisms of chemical carcinogenesis. *Cancer* **47**, 1055-1064

Miller S.A., Dykes D.D. and Polesky H.F. (1988) A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res.* **16,** 1215

Montiel J.F., Norbury C.J., Tuite M.F., Dobson M.J., Mills J.S., Kingsman A.J. and Kingsman S.M. (1984) Characterization of human chromosomal DNA sequences which replicate autonomously in *Saccharomyces cerevisiae*. *Nucleic Acids Res.* **12,** 1049-1068

Morgan D., Pecoraro G., Rosenberg I. and Defendi V. (1990) Human papillomavirus type 6b DNA required for initiation but not maintenance of transformation of C127 mouse cells. *J. Virol.* **64**, 969-976

Morino K., Matsukura N., Kawachi T., Ohgaki H., Sugimura T. and Hirono I. (1982) Carcinogenicity test of quercetin and rutin in golden hamsters by oral administration. *Carcinogenesis* **3**, 93-97

Moura J.W., Stocco dos Santos R.C., Dagli M.L.Z., D'Angelino J.L., Birgel E.H. and Becak W. (1988) Chromosome aberrations in cattle raised in bracken fern pasture. *Experientia* **44**, 785-788

Münger K., Phelps W.C., Bubb V., Howley P.M. and Schlegel R. (1989) The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. *J. Virol.* **63**, 4417-4421

Murray A.W. and Fitzgerald D.J. (1979) Tumour promoters inhibit metabolic cooperation in co-cultures of epidermal and 3T3 cells. *Biochem. Biophys. Res. Commun.* **91**, 395-401

Nagaya T., Nakamura T., Tokino T., Tsurimoto T., Imai M., Mayumi T., Kamino K., Yamamura K. and Matsubara K. (1987) The mode of hepatitis B virus DNA integration in chromosomes of human hepatocellular carcinoma. *Genes Development* **1**, 773-782

Nakayasu M., Sakamoto H., Terada M., Nagao M. and Sugimura T. (1986) Mutagenicity of quercetin in Chinese hamster lung cells in culture. *Mutation Res.* **174**, 79-83

Neil J.C., Hughes D., McFarlane R., Wilkie N.M., Onions D.E., Lees G. and Jarrett O. (1984) Transduction and rearrangement of the *myc* gene by feline leukaemia virus in naturally occurring T-cell leukaemias. *Nature* **308**, 814-820

Nelson N. (1992) Organellar proton-ATPase. *Curr. Opin. Cell Biol.* **4**, 654-660

Nicholson B., Dermietzel R., Teplow D., Traub O., Willecke K. and Revel J-P. (1987) Two homologous protein components of hepatic gap junctions. *Nature* **329**, 732-734

Nomura A., Stemmermann G.N., Chyou P.H., Kato I., Perez-Perez G.I. and Blaser M.J. (1991) *Helicobacter pylori* infection and gastric carcinoma among Japanese Americans in Hawaii. *N. Engl. J. Med.* **325**, 1132-1136

Nose K. (1984) Inhibition by flavonoids of RNA synthesis in permeable WI-38 cells and of transcription by RNA polymerase II. *Biochem. Pharmacol.* **33**, 3823-3827

Nowell P.C. (1976) The clonal evolution of tumor cell populations. *Science* **194**, 23-28

Ocadiz R., Sauceda R., Cruz M., Graef A.M. and Gariglio P. (1987) High correlation between molecular alterations of the c-*myc* oncogene and carcinoma of the uterine cervix. *Cancer Res.* **47**, 4173-4177

Oelze I., Katenbeck J., Crusius K. and Alonso A. (1995) Human papillomavirus type 16 E5 protein affects cell-cell communication in an epithelial cell line. *J. Virology* **69**, 4489-4494

Oksala T. and Therman E. (1974) Mitotic abnormalities and cancer. In, *Chromosomes and Cancer*, pp. 239-263. Edited by J. German. John Wiley, New York

Olson C., Pamukcu A.M., Brobst D.F., Kowalcyzk T., Satter E.J. and Price J.M. (1959) A urinary bladder tumour induced by a bovine cutaneous papilloma agent. *Cancer Res.* **19**, 779

Olson C., Pamukcu A.M. and Brobst D.F. (1965) Papilloma-like virus from bovine urinary bladder tumours. *Cancer Res.* **24**, 840-849

Orth G. (1987) Epidermodysplasia verruciformis. In, *The Papovaviridae, Volume 2. The Papillomaviruses*, pp. 199-243. Edited by N.P Salzman and P.M. Howley. Plenum Press, New York

Ostrow R., Bender M., Niimura M., Seki T., Kawaskima M., Pass F. and Faras A. (1982) Human papillomavirus DNA in cutaneous primary and metastasized squamous cell carcinoma from patients with epidermodysplasia verruciformis. *Proc. Natl. Acad. Sci. USA.* **79**, 1634-1638

Ostrow R.S., McGlennen R.C., Shaver M.K., Kloster B.E., Houser D. and Faras A.J. (1990) A rhesus monkey model for sexual transmission of a papillomavirus isolated from a squamous cell carcinoma. *Proc. Natl. Acad. Sci. USA.* **87**, 8170-8174

Pamukcu A.M., Price J.M. and Bryan G.T. (1976) Naturally occurring and bracken fern induced bovine urinary bladder tumours. *Vet. Path.* **13**, 110-122

Pamukcu A.M., Yalçiner S., Hatcher J.F. and Bryan G.T. (1980) Quercetin, a rat intestinal and bladder carcinogen present in bracken fern. *Cancer Res.* **40**, 3468-3472

Parsonnet J., Friedman G.D., Vandersteen D.P., Chang Y., Vogelman J.H., Orentreich N. and Sibley R.K. (1991) *Helicobacter pylori* infection and the risk of gastric carcinoma. *N. Engl. J. Med.* **325**, 1127-1131

Pennie W.D. (1992) An analysis of the transforming functions of bovine papillomavirus type 4 in an *in vitro* assay system. PhD Thesis, University of Glasgow

Pennie W.D. and Campo M.S. (1992) Synergism between bovine papillomavirus type 4 and the flavonoid quercetin in cell transformation *in vitro*. *Virology* **190**, 861-865

Pennie W.D., Grindlay G.J., Cairney M. and Campo M.S. (1993) Analysis of the transforming functions of bovine papillomavirus type 4. *Virology* **193**, 614-620

Petti L., Nilson L.A. and DiMaio D. (1991) Activation of the platelet-derived growth factor receptor by the bovine papillomavirus E5 transforming protein. *EMBO J.* **10,** 845-855

Pfister H., Hettich I., Runne U., Gissmann L. and Chilf G.N. (1983) Characterization of human papillomavirus type 13 from focal epithelial hyperplasia Heck lesions. *J. Virol.* **47**, 363-366

Phelps W.C., Yee C.L., Münger K. and Howley P.M. (1988) The human papillomavirus type 16 E7 gene encodes transactivation and transformation functions similar to those of adenovirus E1 A. *Cell* **53**, 539-547

Picksley S.M. and Lane D.P. (1994) p53 and Rb: their cellular roles. *Curr. Opin. Cell Biol.* **6**, 853-858

Pietenpol J.A., Tokino T., Thiagalingam S., El-Diery W.S., Kinzler K.W. and Vogelstein B. (1994) Sequence-specific transcriptional activation is essential for growth suppression by p53. *Proc. Natl. Acad. Sci. USA* **91**, 1998-2002

Pike M.C. and Forman D. (1991) Epidemiology of cancer. In, *Introduction to the Cellular and Molecular Biology of Cancer*, pp. 49-97. Edited by L.M. Franks and N.M. Teich. Oxford University Press, Oxford

Pitts J.D. (1980) The role of junctional communication in animal tissues. *In vitro*. **16**, 1049-1056

Pitts J.D. and Bürk R.R. (1976) Specificity of junctional communication between animal cells. *Nature* **264**, 762-764

Pitts J.D. and Kam E. (1985) Communication compartments in mixed cell cultures. *Exp. Cell Res.* **156**, 439-449

Pitts J.D., Finbow M.E. and Kam E. (1988a) Junctional communication and cellular differentiation. *Br. J. Cancer* **58**, Suppl. IX, 52-57

Pitts J.D., Kam E. and Morgan D. (1988b) The role of junctional communication in cellular growth control and tumorigenesis. In, *Gap Junctions*, pp. 397-409. Edited by R.G Johnson and E.L. Hertzberg. Alan R. Liss, New York

Ponder B.A.J. (1991) Inherited cancer syndromes. In, *Genes and Cancer*, pp. 99-106. Edited by D. Carney and K. Sikora. John Wiley & Sons Ltd., England

Povey A.C., Wilson V.L., Weston A., Doan V.T., Wood M.L., Essigmann J.M. and Shields P.G. (1993) Detection of oxidative damage by ^{32}P-postlabelling: 8-hydroxydeoxyguanosine as a marker of exposure. In, *Postlabelling Methods for Detection of DNA adducts*, pp. 105-114. Edited by D.H. Phillips, M. Castegnaro and H. Bartsch. Oxford University Press, Oxford

Price J.M. and Pamukcu A.M. (1968) The induction of neoplasms of the urinary bladder of the cow and the small intestine of the rat by feeding bracken fern. *Cancer Res.* **28**, 2247-2251

Quintanilla M., Brown K., Ramsden M. and Balmain A. (1986) Carcinogen-specific mutation and amplification of Ha-*ras* during mouse skin carcinogenesis. *Nature* **322**, 78-80

Rahman A., Shahabuddin, Hadi S.M., Parish J.H. and Ainley K. (1989) Strand scission in DNA induced by quercetin and Cu(II): role of Cu(I) and oxygen free radicals. *Carcinogenesis* **10**, 1833-1839

Rahman A., Shahabuddin, Hadi S.M. and Parish J.H. (1990) Complexes involving quercetin, DNA and Cu(II). *Carcinogenesis* **11**, 2001-2003

Reeve A.E., Sih S.A., Raizis A.M. and Feinberg A.P. (1989) Loss of heterozygosity at a second locus on chromosome 11 in sporadic Wilms' tumor cells. *Mol. Cell. Biol.* **9**, 1799-17803

Reid R., Crum C.P., Herschman B.R., Fu Y.S., Braun L., Shah K., Agronow S.J. and Stanhope C.R. (1984) Genital warts and cervical cancer. III. Subclinical papillomaviral infection and cervical neoplasia are linked by a spectrum of continuous morphologic and biologic change. *Cancer* **53**, 943-953

Richart R.M. (1968) Natural history of cervical intraepithelial neoplasia. *Clin. Obstet. Gynecol.* **10**, 748-784

Riou G., Barrois M., Dutronquay V. and Orth G. (1985) Presence of papillomavirus DNA sequences, amplification of c-*myc* and c-Ha-*ras* oncogenes and enhanced expression of c-*myc* in carcinomas of the uterine cervix. In, *Papillomaviruses*, pp. 47-56. Edited by P. Howley and T. Broker. Liss, New York

Riou G., Barrois M., Cheng Z.M., Duvillard P. and L'homme C. (1988) Somatic deletions and mutations of c-Ha-*ras* gene in human cervical cancers. *Oncogene* **3**, 329-333

Rodenhuis S. (1992) *ras* and human tumors. *Seminars in Cancer Biol.* **3**, 241-247

Roth G.E., Blanton H.M., Hager L.Z. and Zakian V.A. (1983) Isolation and characterization of sequences from mouse chromosomal DNA with ARS function in yeast. *Mol. Cell. Biol.* **3**, 1898-1908

Rous P. and Beard J.W. (1935) A comparison of the tar tumors of rabbits and the virus induced tumors. *Proc. Soc. Exp. Biol. Med.* **33**, 358-360

Rous P. and Freidewald W.F. (1941) The carcinogenic effect of methylcholanthrene and of tar on rabbit papillomas due to a virus. *Science* **94**, 495-498

Rous P. and Freidewald W.F. (1944) The effect of chemical carcinogens on virus-induced rabbit papillomas. *J. Exp. Med.* **79**, 511-537

Rustgi A.K., Dyson N. and Bernards R. (1991) Amino-terminal domains of c-*myc* and N-*myc* proteins mediate binding to the retinoblastoma gene product. *Nature* **352**, 541-544

Sakai A., Sasaki K., Mizusawa H. and Ishidate M. (1990) Effects of quercetin, a plant flavonol, on two-stage transformation *in vitro*. *Teratogen. Carcinogen. Mutagen.* **10**, 333-340

Salomon D., Saurat J-H. and Meda P. (1988) Cell-to-cell communication within intact human skin. *J. Clin. Invest.* **82**, 248-254

Santos E., Tronick S.R., Aaronson S.A., Pulciani S. and Barbacid M. (1982) T24 human bladder oncogene is an activated form of the normal homologue of BALB- and Harvey-MSV transforming genes. *Nature* **298**, 343-347

Sato H., Furuno A. and Yoshiike K. (1989) Expression of human papillomavirus type 16 E7 gene induces DNA synthesis of Rat3Y1 cells. *Virology* **168**, 195-199

Saxon P.J., Srivatsan E.S. and Stanbridge E.J. (1986) Introduction of human chromosome 11 via microcell transfer controls tumorigenic expression of HeLa cells. *EMBO J.* **5**, 3461-3466

Scheffner M., Werness B.A., Huibregtse J.M., Lavine A.J. and Howley P.M. (1990) The E6 oncoprotein encoded by human papillomavirus type 16 and 18 promotes the degradation of p53. *Cell* **63**, 1129-1136

Schiller J.T., Vass W.C. and Lowy D.R. (1984) Identification of a second transforming region of bovine papillomavirus. *Proc. Natl. Acad. Sci. USA.* **81**, 7880-7884

Schiller J., Vousden K., Vass W.C. and Lowy D.R. (1986) The E5 open reading frame of bovine papillomavirus type 1 encodes a transforming gene. *J. Virol.* **57**, 1-6

Schlegel R., Phelps W.C., Zhang Y.L. and Barbosa M. (1988) Quantitative keratinocyte assay detects two biological activities of papillomavirus DNA and identifies viral types associated with cervical carcinoma. *EMBO J.* **7**, 3181-3187

Schneider J.F., McGlennen R.C., LaBresh K.V., Ostrow R.S. and Faras A.J. (1991) Rhesus papillomavirus type 1 cooperates with an activated *ras* in transforming primary epithelial rat cells independent of dexamethasone. *J. Virol.* **65**, 3354-3358

Schwarz E., Freese U.K., Gissmann L., Mayer W., Roggenbuck B., Stremlau A. and zur Hausen H. (1985) Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. *Nature* **314**, 111-114

Sedman S.A., Barbosa M.S, Vass W.C., Hubbert N.L., Haas J.A., Lowy D.R. and Schiller J.T. (1991) The full length E6 protein of human papillomavirus type 16 has transforming and transactivating activities and cooperates with E7 to immortalise keratinocytes in culture. *J. Virol.* **65**, 4860-4866

Shah K.V. and Howley P.M. (1990) Papillomaviruses. In, *Virology, Second Edition*, pp. 1651-1676. Edited by B.N. Fields and D.M. Knipe. Raven Press Ltd., New York

Sheridan J.D. (1976) Cell coupling and cell communication during embryogenesis. In, *The Cell Surface in Animal Embryogenesis*, pp. 409-447. Edited G. Poste and G.L. Nicholson. Elsevier, New York

Shope R.E. (1933) Infectious papillomatosis of rabbits. *J. Exp. Med.* **58**, 607-623

Smith K.T. and Campo M.S. (1988) 'Hit and run' transformation of mouse C127 cells by bovine papillomavirus type 4: the viral DNA is required for the initiation but not for maintenance of the transformed phenotype. *Virology* **164**, 39-47

Smith K.T. and Campo M.S. (1989) Amplification of specific DNA sequences in C127 mouse cells transformed by bovine papillomavirus type 4. *Oncogene* **4**, 409-413

Smith K.T., Patel K.R. and Campo M.S. (1986) Transcriptional organisation of papillomavirus type 4. *J. Gen. Virol.* **67**, 2381-2393

Smith K.T., Campo M.S., Bradley J., Gaukroger J. and Jarrett W.F.H. (1987) Cell transformation by bovine papillomavirus: cofactors and cellular responses. *Cancer Cells* **5**, 267-273

Smith K.T., Coggins L.W., Doherty I., Pennie W.D., Cairney M. and Campo M.S. (1993) BPV-4 induces amplification of 'silent' BPV-1 in a sub-line of C127 cells. *Oncogene* **8**, 151-156

Smith P.P., Bryant E.M., Kaur P. and McDougall J.K. (1989) Cytogenetic analysis of eight human papillomavirus immortalized human keratinocyte cell lines. *Int. J. Cancer* **44**, 1124-1131

Solomon E., Borrow J. and Goddard A.D. (1991) Chromosome aberrations and cancer. *Science* **254**, 1153-1160

Southern P.J. and Berg P. (1982) Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. *J. Mol. Appl. Genet.* **1**, 327-341

Spalholz B.A., Yang Y.C. and Howley P.M. (1985) Transactivation of a bovine papillomavirus transcriptional regulatory element by the E2 gene product. *Cell* **42**, 183-191

Sparkes R.S., Murphree A.L., Lingua R.W., Sparkes M.C., Field L.L., Funderburk S.J. and Benedict W.F. Gene for hereditary retinoblastoma assigned to human chromosome 13 by linkage to esterase D. *Science* **219**, 971-973

Stamps A.C. and Campo M.S. (1988) Mapping of two novel transcripts of bovine papillomavirus type 4. *J. Gen. Virol.* **69**, 3033-3045

Stanbridge E.J. (1985) A case for human tumor-suppressor genes. *Bioessays* **3**, 252-255

Stark G.R. and Wahl G.M. (1984) Gene amplification. *Ann. Rev. Biochem.* **53**, 447-491

Stinchcomb D.T., Struhl K. and Davis R.W. (1979) Isolation and characterization of a yeast chromosomal replicator. *Nature* **282**, 39-43

Storey A., Pim D., Murray A., Osborn K., Banks L. and Crawford L. (1988) Comparison of the *in vitro* transforming activities of human papillomavirus types. *EMBO J.* **6**, 1815-1820

Straight S.W., Hinkle P.M., Jewers R.J. and McCance D.J. (1993) The E5 oncoprotein of human papillomavirus type 16 transforms fibroblasts and effects the downregulation of the epidermal growth factor receptor in keratinocytes. *J. Virol.* **67**, 4521-4532

Straight S.W., Herman B. and McCance D.J. (1995) The E5 oncoprotein of human papillomavirus type 16 inhibits the acidification of endosomes in human keratinocytes. *J. Virol.* **69**, 3185-3192

Struhl K., Stinchcomb D.T., Scherer S. and Davis R.W. (1979) Eukaryotic DNA segments capable of autonomous replication in yeast. *Proc. Natl. Acad. Sci. USA* **77**, 4559-4563

Sugarbaker J.P., Gunderson L.L. and Wittes R.E. (1985) Colorectal cancer. In, *Cancer: Principles and Practices of Oncology*, pp. 800-803. Edited by V.T. De-Vita, S.A. Hellman and S.A. Rosenberg. J.B. Lippincott, Philadelphia

Sugie S., Mori H. and Takahashi M. (1987) Effect of *in vivo* exposure to the liver promoters phenobarbital and DDT on the gap junctions of rat hepatocytes: a quantitative freeze-fracture analysis. *Carcinogenesis* **8**, 45-51

Suzuki S., Takada T., Sugawara Y., Muto T. and Kominami R. (1991) Quercetin induces recombinational mutations in cultured cells as detected by DNA fingerprinting. *Jpn. J. Cancer Res.* **82**, 1061-1064

Takeichi M. (1991) Cadherin cell adhesion receptors as a morphogenetic regulator. *Science* **251**, 1451-1455

Teich N.M. (1991) Oncogenes and cancer. In, *Introduction to the Cellular and Molecular Biology of Cancer*, pp. 230-268. Edited by L.M. Franks and N.M. Teich. Oxford University Press, Oxford

Thierry F. and Yaniv M. (1987) The BPV-1 E2 transacting protein can be either an activator or a repressor of the HPV-18 regulatory region. *EMBO J.* **6**, 3391-3397

Trichopoulos D., Papaevangelou G., Violaki M., Vissoulis C.H., Sparros L. and Manousos O.N. (1976) *Br. J. Cancer* **34**, 83-87

Vagnarelli P., Guilotto E., Fattorini P., Mucciolo E., Bensi M., Tessera L. and De Carli L. (1993) Variation of minisatellites in chemically induced mutagenesis and in gene amplification. In, *DNA Fingerprinting: State of the Science,* pp 71-77. Edited by S.D.J. Pena, R. Chakraborty, J.T. Epplen and A.J. Jeffreys. Birkhäuser Verlag Basel, Switzerland

Van Beneden R.J., Watson D.K., Chen T.T., Lautenberger J.A. and Papas T.S. (1986) Cellular *myc* (c-*myc*) in fish (rainbow trout): Its relationship to other vertebrate *myc* genes and to the transforming genes of the MC29 family of viruses. *Proc. Natl. Acad. Sci. USA* **83**, 3698-3702

Van Dyke T.A. (1994) Analysis of viral-host protein interactions and tumorigenesis in transgenic mice. *Sem. Cancer Biol.* **5**, 47-60

Van Ranst M., Kaplan J.B. and Burk R.D. (1992) Phylogenetic classification of human papillomaviruses: correlation with clinical manifestations. *J. Gen. Virol.* **73**, 2653-2660

Van Wart-Hood J. Linder M.E. and Burr J.G. (1989) TPCK and quercetin act synergistically with vandate to increase protein-tyrosine phosphorylation in avian cells. *Oncogene* **4**, 1267-1271

Vande Pol S. and Howley P.M. (1990) A bovine papillomavirus constitutive enhancer is negatively regulated by the E2 repressor through competitive binding for a cellular factor. *J. Virol.* **64**, 5420-5429

Vassart G., Georges M., Monsieur R., Brocas H., Lequarré A.S. and Christophe D. (1987) A sequence in M13 phage detects hypervariable minisatellites in human and animal DNA. *Science* **235**, 683-684

Wagatsuma M., Hashimoto K. and Matsukura T. (1990) Analysis of integrated human papillomavirus type 16 DNA in cervical cancers: amplification of viral sequences together with cellular flanking sequences. *J. Virol.* **64**, 813-821

Watanabe S., Kanda T. and Yoshiike K. (1989) Human papillomavirus type 16 transformation of primary human embryonic fibroblasts requires expression of open reading frames E6 and E7. *J. Virol.* **63**, 965-969

Watanabe S., Kanda T., Sato H., Furuno A. and Yoshiike K. (1990) Mutational analysis of human papillomavirus type 16 E7 functions. *J. Virol.* **64**, 207-214

Weinberg R.A. (1992) The retinoblastoma gene and gene product. *Cancer Surv.* **12**, 43-57

Weissman B.E., Saxon P.J., Pasquale S.R., Jones G.R., Geiser A.G. and Stanbridge E.J. (1987) Introduction of a normal chromosome 11 into a Wilms' tumor cell line controls its tumorigenic expression. *Science* **236**, 175-180

Wells R.A. (1988) DNA fingerprinting. In, *Genome Analysis - A Practical Approach*, pp. 153-170. Edited by K.E. Davies. IRL Press, Oxford

Werness B.A., Levine A.J. and Howley P.M. (1990) Association of human papillomavirus types 16 and 18 E6 proteins with p53. *Science* **248**, 76-79

Yamasaki H. (1990) Gap junction intercellular communication and carcinogenesis. *Carcinogenesis* **11**, 1051-1058

Yang L. and Botchan M. (1990) Replication of bovine papillomavirus type 1 DNA initiates within an E2-responsive enhancer element. *J. Virol.* **64**, 5903-5911

Yang Y-C., Okayama H. and Hawley P.M. (1985) Bovine papillomavirus contains multiple transforming genes. *Proc. Natl. Acad. Sci. USA.* **82**, 1030-1033

Yoshida M., Sakai T., Hosokawa N., Marui N., Matsumoto K., Fujioka A., Nishino H. and Aoike A. (1990) The effect of quercetin on cell cycle progression and growth of human gastric cancer cells. *FEBS Lett.* **260**, 10-13

Yoshida M., Yamamoto M. and Nikaido T. (1992) Quercetin arrests human leukemic T-cells in late G1 phase of the cell cycle. *Cancer Res.* **52**, 6676-6681

Yuspa S.H. and Harris C.C. (1982) Molecular and cellular basis of chemical carcinogenesis. In, *Cancer Epidemiology and Prevention,* pp. 23-43. Edited by D. Schottenfeld and J.F. Fraumeni. Saunders, Philadelphia

Yuspa S.H. and Poirier M.C. (1988) Chemical carcinogenesis: from animal models to molecular models in one decade. *Adv. Cancer Res.* **50**, 25-70

Zastrow G., Koehler U., Müller F., Klavinius A., Wegner M., Wienberg J., Weidle U.H. and Grummt F. (1989) Distinct mouse DNA sequences enable establishment and persistence of plasmid DNA polymers in mouse cells. *Nucleic Acids Res.* **17**, 1867-1879

Zavitsanos X., Hatzakis A., Kaklamani E., Tzonou A., Toupadaki N., Broeksma C., Chrispeels J., Troonen H., Hadziyannis S., Hsieh C-C., Alter H. and Trichopoulos D. (1992) Association between hepatitis C virus and hepatocellular carcinoma using assays based on structural and nonstructural hepatitis C virus peptides. *Cancer Res.* **52**, 5364-5367

zur Hausen H. (1976) Condylomata acuminata and human genital cancer. *Cancer Res.* **36**, 794

zur Hausen H. (1989a) Papillomaviruses in anogenital cancer as a model to understand the role of viruses in human cancer. *Cancer Res.* **49**, 4677-4681

zur Hausen H. (1989b) Papillomaviruses as carcinomaviruses. *Adv. Viral Onc.* **8**, 1-26

zur Hausen H. (1991) Viruses in human cancers. *Science* **254**, 1167-1173

zur Hausen H. and Schneider A. (1987) The role of papillomaviruses in anogenital cancer. In, *The Papovaviridae. Volume 2, The Papillomaviruses*, pp. 245-263. Edited by N.P. Salzman and P.M. Howley. Plenum Press, New York