An Analysis of the Transforming Functions of Bovine Papillomavirus type 4 in an *in vitro* assay System.

Thesis submitted for the degree of Ph.D.

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by

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3. Abbreviations

%(v/v)	volume in mls per 100ml water
%(w/v)	weight in g per 100ml water
A	adenine
aa	amino acid
ATP	adenosine triphospate
bp	base pairs
BPV	bovine papillomavirus
C	cytosine
cm	centimetres
CTP	cytosine triphosphate
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetra-acetic acid
g	grammes
G	guanine
HBS	HEPES-buffered saline
HEPES	N-2-hydoxyethyl piperazine-N'-2-ethane sulphonic acid
HPV	human papillomavirus
hr	hours
kb	kilobase pairs
kDa	kiloDaltons
M	molar
M.Wt.	molecular weight
µg	microgrammes
mg	milligrammes

min	minutes
μl	microlitres
ml	millilitres
μM	micromolar
mm	millimetres
mM	millimolar
neo^r	neomycin resistant
ng	nanograms
OAc	acetate
ORF	open reading frame
PBS	phosphate buffered saline
pg	picogrammes
PV	papillomavirus
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
sec	seconds
T	thymine
TAE	Tris Acetate/EDTA
TBS	Tris buffered saline
TE	Tris\EDTA
TPA	12-O-tetradecanoyl phorbol-13-acetate
Tris	tris(hydroxymethyl)aminomethane
V	volts

4. Abstract

The transformation biology of bovine papillomavirus type 4 (BPV-4) was investigated using an *in vitro* assay system. *In vivo* the virus cooperates with bracken to cause alimentary canal carcinoma in cattle and transforms via a "hit-and-run" mechanism; viral DNA is observed in papillomas but not in the resulting tumours. Molecularly cloned BPV-4 genome and subgenomic fragments were transfected into primary bovine palate fibroblasts (PalF) and their ability to induce morphological transformation assessed. BPV-4 is incapable of transformation without the introduction of a cooperating activated *ras* oncogene and the resultant cells are neither immortal nor tumorigenic. The major transforming function was found to be the E7 open reading frame (ORF) with a possible role for the E8 ORF. The majority of BPV-4 transformed lines are found to lose viral DNA on passage, as observed *in vivo*. BPV-4 and the other BPV subgroup B viruses BPV-3 and BPV-6 lack an E6 ORF, a transforming function in other papillomaviruses. Introducing an E6 from human papillomavirus type 16 in cooperation with BPV-4 E7 leads to immortalisation. Immunocytochemistry was used to determine the cellular localisation of the transforming proteins of the virus. A possible interaction of the E8 protein with a structural component of gap junctions is demonstrated. A bracken

mutagen, quercetin, is able to initiate PalF cells, allowing full malignant transformation by BPV-4 and *ras*.

5. Introduction

5.1 Carcinogenesis

5.1.1 Multistage Carcinogenesis

Carcinogenesis is a multistage process. In human cancers, the statistical analysis of age-dependent tumours indicates kinetics dependent on the fifth or sixth power of elapsed time, suggesting that five or six distinct sequential stages are involved in the disease (Nordling, 1953; Peto, 1977). The hypothesis of multistage carcinogenesis has been confirmed *in vivo* (notably in colorectal carcinoma (Fearon and Vogelstein, 1990)) and by many workers using *in vitro* models (see for example Land et al, 1983; Newbold and Overall, 1983).

The carcinogenesis process has been subdivided into at least three sequential steps in animal models such as mouse skin tumorigenesis: *initiation*, *promotion* and *progression* (Hecker et al, 1982). An outline of the major steps involved is shown in **Fig 5.1.** The progression of benign lesions to malignancy has been studied in this system by the use of chemical initiators and tumour promoters. In experiments of this type the initiator is an agent (typically 7,12-dimethylbenz(a)anthracene (DMBA)) which gives rise to genetic damage, whilst the promoter compound's (typically 12-O-tetradecanoylphorbol-

13-acetate (TPA)) effects are initially reversible suggesting epigenetic events (see Yuspa and Poirier, 1988). Thus the initiation step gives rise to mutations, rearrangements and/or amplification of cellular DNA, then multiple applications of promoter give rise to a clonal expansion of initiated cells which may then progress to carcinoma. It has been observed that initiation is dependent upon cell proliferation, perhaps requiring fixation of genetic damage before repair occurs (for example see Cayama et al, 1978) and thus viral or chemical agents which increase cell division rate may accelerate the initiation step.

Each step in the multistage process of carcinogenesis can be thought of as the removal of a physiological barrier against the development of the malignant phenotype. Epidemiology has identified broad classes of agent important in the development of malignancies. These include viruses (both RNA and DNA tumour viruses), environmental carcinogens (including radiation, chemical carcinogens / mutagens), host immune status and inheritance of predisposition to a particular tumour type (Eg. childhood retinoblastoma).

Fig 5.1. Model for tumorigenesis in mouse skin.

Duplication of chromosome carrying H-ras mutation

Loss of normal H-ras genes

Normal cell → Initiated cell → Papilloma → Squamous Carcinoma → Spindle cell Carcinoma

Mutational activation of H-ras protooncogene

Mutational inactivation of p53 genes

Genetic alterations occurring during skin tumorigenesis.

Schematic representation of chemical induced carcinogenesis in mouse skin. Treatment with an initiating compound (here DMBA) gives rise to initiated cells by mutation of the Harvey-ras oncogene. The initiated cells can be expanded by the action of a tumour promoting agent (here TPA) to give rise to benign proliferative lesions (papillomas) within 6-8 weeks. These benign lesions may develop into carcinomas if further genetic damage occurs (here mutation of the p53 tumour suppressor gene).

5.1.2 Oncogenes and Tumour Suppressor Genes

The theory of *oncogenes* has evolved over the last decade to explain the nature of the carcinogenesis process at the molecular level. Oncogenes are the activated form of *proto-oncogenes* which are part of the genetic makeup of all cells. Each step in the tumorigenesis process would thus be the result of an oncogene activation. The oncogenes encode products such as growth factors, growth factor receptors, protein kinases, signal transducers and nuclear replication and transcription factors (for review see Klein, 1988). The initial discovery of oncogenes was largely due to work on highly transforming RNA tumour viruses. These viruses can disrupt normal cellular regulation by the DNA intermediate of the virus integrating in or near a gene involved in normal cell growth regulation, the integration event leading to a deregulation of the gene (insertional mutagenesis). Alternatively the DNA stage of the retrovirus can combine with the host genome and then become excised after recombination with a cellular gene. This can give rise to an activated cellular oncogene under strong retroviral control. A large number of genes which, when mutated, can give rise to activated oncogenes were identified as a result of the study of cancer-causing retroviruses (for review see Cooper, 1990).

At this point it is interesting to consider oncogene activation in papillomavirus associated malignancies. Reports exist of papillomaviruses (PVs) consistently integrating near the locus of the c-*myc* nuclear proto-oncogene (Durst et al, 1987; Couturier et al, 1991), and in some cases sequences flanking the integration site are amplified suggesting a mechanism for oncogene activation in these cases (Lazo et al, 1989; Wagatsuma et al, 1990). Cellular oncogene involvement in these malignancies could also be either via epigenetic disruption of the regulation of these genes by papillomavirus proteins, or by genetic mutation by environmental factors. The c-Ha-*ras* proto oncogene (a GTP binding protein involved in signal transduction) is found to be mutated in a significant percentage of PV associated cervical carcinomas, as is the c-*myc* nuclear proto oncogene (for example see Riou et al, 1988) suggesting that these oncogenes may cooperate with the PV genome in the progression of cervical carcinoma. This is confirmed by the observation that HPV-16 E7 oncoprotein can cooperate with activated c-Has-*ras* in an *in vitro* transformation assay (Matlashewski et al, 1987). In addition the Ha-*ras* 1 gene is activated and occasionally amplified in papillomavirus associated cancer in cattle (Campo et al, 1990).

Another class of genes involved in cancer pathogenesis are the tumour suppressor genes. The observation that fusion of tumour cells with normal cells invariably gave rise to a nontumorigenic phenotype suggested that the genetic component of normal cells was able to suppress the tumorigenic phenotype of the tumour cell partners (reviewed in Weinberg, 1991). The fact that somatic cell fusions have an unstable karyotype and lose chromosomes allowed the correlation of the presence of particular chromosomes with tumor suppressing functions and from this observation it was suggested that the development of a tumorigenic phenotype may be the loss of tumor suppressor gene function, a hypothesis shown to be true for the inherited eye tumour retinoblastoma (Benedict et al, 1983; Sparkes et al, 1983). In this example the carrier inherits a mutated copy of the retinoblastoma gene, predisposing the individual to the disease (should the second copy become mutated).

The mechanism of action of tumour suppressor genes is thought to be as transducers of anti-proliferative signals controlling cell cycle progression, differentiation pathways and cell mortalilty (senescence). Thus the loss of tumour suppressor gene functions leads to loss of responsiveness to extracellular growth-inhibitory signals. One example of these exogenous signals is the phenomenon of contact inhibition thought to be mediated by membrane protein interactions (see Folkman and Mascona, 1978). Another class are those signals mediated by gap junction pores; structures which allow the passage of small molecules between cells. There is a substantial body of evidence suggesting that gap junctions allow the passage of growth-inhibiting molecules between cells, although the nature of these molecules is at present unclear (Pitts et al, 1988). The action of macromolecular growth factors, such as tumour growth factor-ß (TGF-ß), has also been shown to influence normal cell growth and differentiation (for review see Aaronson, 1991). Within the context of papillomavirus research, it is interesting to note that a putative suppressor of transformation by papillomaviruses exists on the short arm of chromosome 11, as human embryo fibroblasts with a deletion in this chromosome become susceptible to transformation by HPV-16 alone (Smits et al, 1988), and the tumorigenic phenotype of HeLa cells (HPV 18 positive cervical carcinoma cell line) is suppressed by the introduction of chromosome 11 (Saxon et al, 1986). Indeed a model has been proposed by zur Hausen that the development of HPV associated malignancy is as a result of the breakdown of cellular mechanisms which suppress papillomaviral genome functions (see zur Hausen, 1991). In addition, two tumour suppressor gene products are of particular interest, as they have been shown to be the targets of papillomaviral "oncoproteins". These molecules are the retinoblastoma gene product $p105^{Rb}$, and the p53 protein.

The retinoblastoma gene product is a nuclear protein existing in both phosphorylated and unphosphorylated states (Lee et al, 1988). Phosphorylation of the protein is regulated throughout the cell cycle and it is believed that only the hypophosphorylated form of the protein suppresses cell proliferation (for example see Ludlow et al, 1990). The hypophosphorylated form of the protein is found to be bound by the transforming proteins of several DNA tumour viruses including Adenovirus E1A, SV40 large T antigen and papillomavirus E7 protein, via a conserved region of these proteins (reviewed in Green, 1989), see **Fig 5.2.** Indeed studies in tumour cells have shown mutated forms of $p105^{Rb}$ which maintain phosphorylation sites but cannot undergo phosphorylation are unable to bind SV40 large T, suggesting conformational changes or alterations in interactions with other cell components prohibiting phosphorylation (Kaye et al, 1990). In addition the stimulation of quiescent cells leads to phosphorylation of $p105^{Rb}$ (Mihara et al, 1989) and hyperphosphorylation of the protein is observed in differentiating cells of the haemopoietic system

(Furukawa et al, 1990) which provides further evidence that the phosphorylation state of the protein is of crucial importance in cell proliferation and differentiation control.

The p53 tumour suppressor nuclear phosphoprotein was first identified as a result of its complexing with SV40 large T and has also been shown to complex with Adenovirus E1B and human papillomavirus E6 (Lane and Crawford, 1979; Sarnow et al, 1982; Werness et al, 1990). In the cases of SV40 and adenovirus, the complexing has been shown to stabilise p53 (Oren et al, 1981; Reich et al, 1983) and presumably inactivate it, while the E6 binding causes a rapid and specific degradation of p53 destroying its growth suppressing potential (Scheffner et al, 1990). While wild-type p53 has tumour suppressor properties and reduces transformation by certain oncogenes, activating mutations can cause mutant p53 to act as an oncogene and augment transformation (Finlay et al, 1989; Eliyahu et al, 1989) and the mutant form has been found in a number of human malignancies, notably those of the colon (Vogelstein et al, 1989) and lung (Takahashi et al, 1989).

Fig 5.2. Adenovirus Ela, SV40 LT and HPV E7 homology.

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BPV4  E7 aa1  MKGQNVTLQD.IAIELEDTISPINLHCEEEI.E.TEEVDT.......PNP.FA
HPV16 E7 aa1  MHGDTPTLHEYM.LDLQ..PETTDLYCYEQLNDSSEEEDEIDGPAGQAEPDRA
Ad E1a   aa36 SHFEPPTLHE.L.YDL 118 EVIDLTCHEAGFPPSDDEDE
SV40 LT  aa6  NREESLQLMD.L.LGL  99 NEENLFCEEEM.PSSDDEAT
                  Region 1          Region 2

BPV4  E7 aa43 ...ITATCYACEQVLRLAVVTST.EGIHQLQQLLFDNLFLLCAACSKQVFCNRRPERNGP
HPV16 E7 aa51 HYNIVTFCCKCDSTLRL.CVQSTHVDIRTLEDLLMGTLGIVCPICSQKP
                   Zn++        hydrophobic             Zn++
```

Notes: Identical or similar amino acids are denoted in bold type. **Region 1** and **Region 2** are the domains important in binding the tumour suppressor gene product $p105^{Rb}$. **Zn^{++}** refers to the **Cys-X-X-Cys** zinc binding domain. (The arrow in he BPV-4 E7 sequence denotes the engineered 3' deletion which removes the second of the two $Zn=^{++}$ binding domains in the $E7^{+}E7_{-}$ constructs which are used in transfection studies in this thesis (see Results and Discussion section 7.2)).

Suggested roles of p53 in neoplastic transformation include transcriptional activation of genes which suppress proliferation and control of initiation of DNA synthesis (reviewed in Marshall, 1991).

The observation that adenovirus, SV40 and the oncogenic human papillomaviruses all interact with both the p105Rb and p53 tumour suppressors makes it tempting to speculate that the dysfunction of these two molecules can act in concert, and indeed recent work has suggested that they may be involved in two stage *in vitro* cellular senescence (Shay et al, 1991). Thus the DNA tumour viruses outlined above would provide for two steps in the multistage transformation process which *in vitro* may only require the disruption of three or four oncogenes / tumour suppressor genes (for example see Voglestein et al, 1989).

5.1.3 Viruses in Carcinogenesis

Recent estimates suggest that viral infection may have a significant and specific role in approximately 15 percent of human malignancies (see **Table 5.1.**), with cervical and hepatocellular carcinomas accounting for some 80 percent of these tumours (reviewed in zur Hausen, 1991). In each case viral presence alone is insufficient to induce cancer, and the long latency periods (often several decades) of these diseases suggest that additional factors are required after viral infection. Examples of viral involvement in carcinogenesis by indirect mechanisms such as the immunosuppression induced by human immunodeficiency virus (HIV) or the general mutagenic / DNA amplifying activity of herpes simplex virus (HSV) are not relevant to this thesis and will not be considered further.

A major group of viruses implicated as having a direct role in cancer development, (both on the basis of epidemiological data and *in vitro* experimental models) are the papillomaviruses, particularly the anogenital HPVs, whose role in multistage carcinogenesis will be considered for the remainder of the introduction.

Table 5.1. Viruses in human cancers.

Virus type	Benign proliferations	Malignant tumor
EBV	Hairy leukoplakia Infectious mononucleosis	Burkitt's lymphoma Nasopharyngeal cancer B-lymphomas in immunosuppressed individuals
HBV	Focal liver hyperplasia	Hepatocellular carcinoma
HPV types 5, 8, 14, 17, 20	Cutaneous plaques and papillomas in patients with EV	Skin carcinomas usually at sun-exposed sites in renal allograft and in EV patients
HPV types 16, 18, 31*, 33, 35*, 39*, 45*, 51*, 52, 56*, 58*, 59*, 61*	Cervical intraepithelial neoplasia; vulvar, penile, and perianal intraepithelial neoplasias	Cervical cancer Vulvar cancer Penile cancer Perianal and anal cancer
HPV types 6, 11	Condyloma acuminatum	Verrucous carcinoma of vulva and penis, Buschke-Löwenstein tumors
HTLV-I	Smouldering leukemia	Adult T cell leukemia

*Only rarely found.

From: zur Hausen (1991)

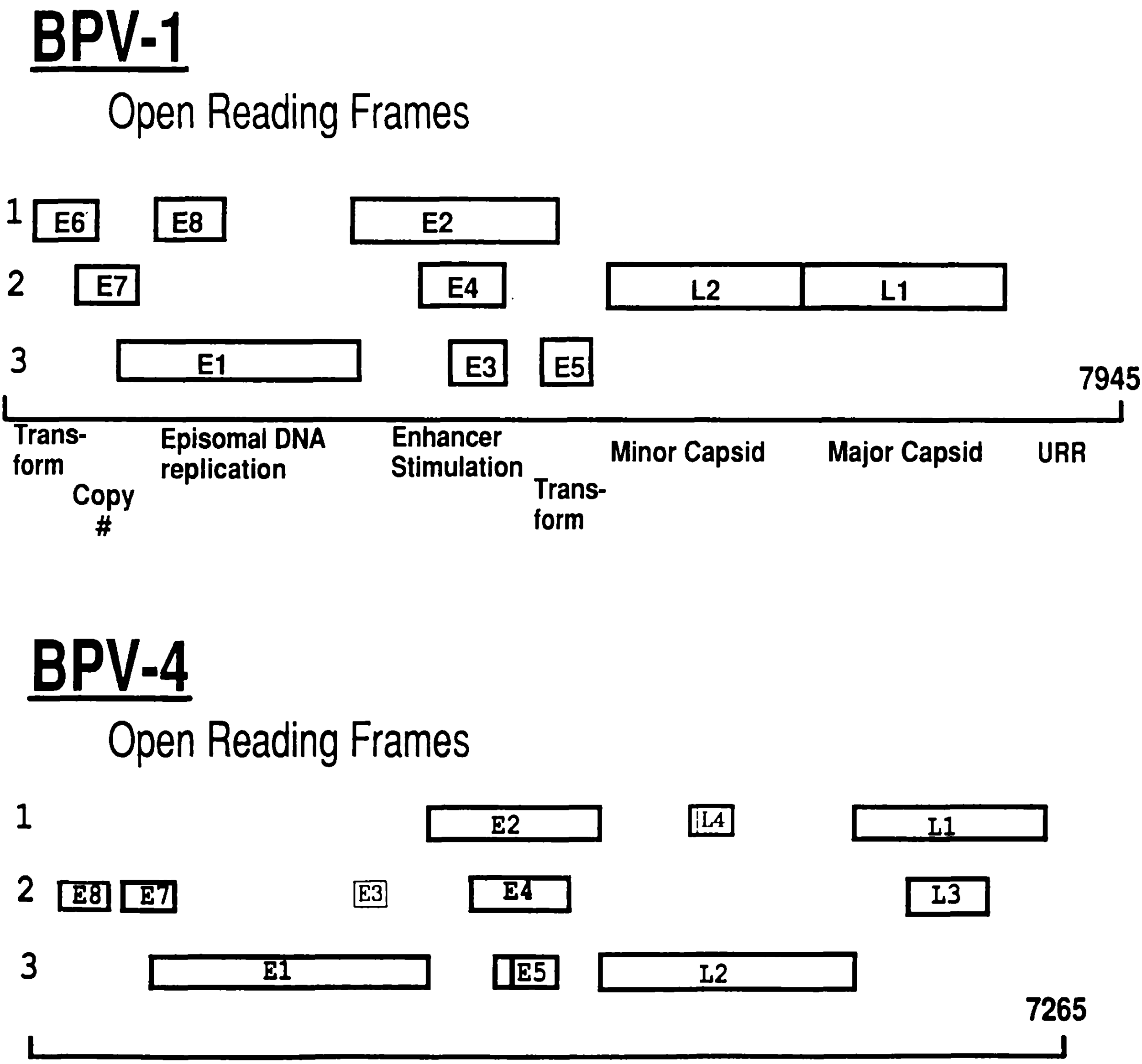
5.2 The Papillomaviruses

The papillomaviruses are small DNA tumour viruses which cause mainly benign lesions (papillomas or "warts") in a wide variety of animals including man. The benign papillomas induced by the viruses usually regress but with the synergistic co-operation of cofactors they may persist and develop into a carcinoma. Not all papillomavirus types are able to sustain this transition however, suggesting the involvement of specific viral factors. Clearly the papillomavirus system is an excellent model for investigation into the multistage carcinogenesis process. For example, Cottontail Rabbit (Shope) Papillomavirus (CRPV) was the first DNA tumour virus to be isolated and characterised (Shope and Hurst, 1933). Much of the groundwork on co-carcinogenesis was provided by the CRPV model system where virus-induced benign papillomas in rabbits were observed to progress to carcinomas with the co-operation of chemical cofactors (Rous and Friedewald, 1944).

The overall genome organization is similar in many of the papillomavirus types (see **Fig 5.3.**). The viral genome is in the form of a closed double stranded circle of 7-8 Kb. The genetic information is encoded in one strand only, consisting of large overlapping open reading frames (ORFs), the other strand being punctuated with stop codons. ORFs often show conservation of position and also homology between different virus species. Transcription is unidirectional and the mRNAs are generated by complex splicing mechanisms which juxtapose non-contiguous ORFs leading in some cases to fusion proteins.

Research in the papillomavirus field has been hindered to a great extent by the lack of an *in vitro* tissue culture system permissive for lytic propagation of the viruses. Papillomaviruses only grow in differentiated mucosal or cutaneous epithelium at highly specific anatomical sites with papillomavirus production thought to be tightly coupled to cell differentiation during epithelia development. With the advent of molecular biology techniques this limitation has been overcome to some extent. In particular the ability of some of the animal papillomaviruses (notably Bovine Papillomavirus type 1) to transform rodent fibroblasts in culture together with molecular biology techniques has allowed the assignment of functions to discrete areas of the viral genome.

Fig 5.3. Papillomavirus genome organisation.



Schematic representation of the genomes of BPV-1 and BPV-4. Major viral open reading frames are shown as boxes and functions demonstrated for BPV-1 ORFs are described beneath them. Significantly BPV-4 lacks an E6 ORF, an important transforming ORF in both BPV-1 and the oncogenic human papillomaviruses. The above diagram is not drawn to scale.

These functions include promoters, enhancers, the origin of DNA replication, plasmid maintenance sequences, transforming regions, sequences involved in trans-activation/repression and the genes encoding viral capsid proteins. The viral genome can be classified into three general areas:

Early: the genes involved in transformation, plasmid maintenance etc.

Late: The viral capsid protein genes.

Long Control Region (LCR): Sequences involved in transcriptional regulation of the virus.

Although the early region of PVs is involved in transformation, recent evidence has suggested that the late region may play a significant role in some viruses. Thus although the bovine virus BPV-1 suffers no loss of transforming potential of established cells when its entire late region is deleted (Lowy et al, 1980; Campo and Spandidos, 1983) the CRPV genome contains two late elements which influence transformation efficiency. The first found within the 3' end of the L1 ORF is required for transformation of keratinocytes, while the second element, which localises to the 5' portion of L2 is found to have an inhibitory effect on transformation of NIH 3T3 cells (Meyers and Wettstein, 1991).

More recent (and sophisticated) tissue culture techniques such as the use of collagen rafts (for example see McCance et al 1988) and the nude mouse xenograft system (Kreider et al, 1985) have allowed investigation into *in vitro* transformation by the HPVs, but the use of the animal papillomaviruses model systems uncovered the initial molecular transformation mechanisms of papillomaviruses. In addition, certain aspects of papillomaviral transformation, such as the role of environmental cofactors and host immune response can only readily be addressed using animal models where direct experimentation is possible. Bearing these points in mind, an interesting animal system is bovine papillomavirus type 4 (BPV-4) associated carcinoma of the alimentary canal in cattle. In this case the cofactors of the disease are well characterised (discussed in detail later in this introduction), and the system is providing an understanding of host immune response against papillomaviruses with a view to anti-papillomavirus vaccine development (Jarrett et al, 1991).

Recently a primate papillomavirus from rhesus monkeys (RhPV) has been isolated and characterised. This system has exciting possibilities as a model for HPV in anogenital carcinoma as RhPV infects the genital tract and has been found associated with a range of lesions from benign to malignant (Ostrow et al, 1990). In addition the *in vitro* transformation characteristics of RhPV (Schneider et al, 1991) are very similar to HPV 16 (Matlashewski et al, 1987) in that the virus requires a cooperating *ras* oncogene to transform primary cells. DNA sequencing has established that both RhPV and HPV-16 have very similar genomes (Ostrow et al, 1991). Although other primate PVs have been isolated and characterised (Kloster et al 1988; Resza et al, 1991), the similarities between RhPV and HPV-16 make the RhPV system the most suitable model for the human genital PVs at present.

The papillomaviruses can be characterised into those that cause fibropapillomas and those which cause purely epithelial lesions. The use of in vitro transformation assays has demonstrated that in general the fibropapillomaviruses such as the bovine viruses BPV-1 (Morgan and Meinke, 1980) and BPV-2 (Jarrett, 1985) contain sufficient genomic information to transform primary cells. The viruses induce a readily detectable morphological transformation in culture, and thus much of the groundwork in determining the oncoproteins of papillomaviruses was provided using the BPV-1 virus (see below). In contrast the epithelial papillomaviruses such as RhPV and HPV-16 require an activated oncogene (*ras*) for transformation of cultured cells and do not induce striking changes in morphology.

5.3 Human Papillomaviruses

The driving force behind the explosion in papillomavirus research in the last decade or so comes from the observation that papillomaviruses are found to be associated with a number of human malignancies. The first association of papillomaviruses with a human malignancy, reported in 1978, was in the hereditary condition Epidermodysplasia verruciformis (EV) which is characterised by disseminated skin warts. Squamous cell carcinomas arising from these warts were found to harbour a papillomavirus: HPV-5 (Orth et al, 1978 and reviewed in Orth, 1987). More recently, the discovery that HPVs 16 and 18 are aetiologically associated with cervical carcinoma greatly encouraged research in papillomavirus biology (Durst et al. 1983, Boshart et al. 1984). Cervical cancer is a major killer of women presenting at greater than 500,000 cases per year world-wide and is fatal in approximately 45% of these (even with medical intervention).

Epidemiological studies on cervical cancers suggest that a sexually transmitted agent is involved in the disease.

For example, it has been known for over a century that the disease does not present in nuns or other virgins. Another observation is that the sexual partners of men whose first wives had cervical cancer are themselves at much greater risk of contracting the disease (Kessler 1986). While some controversy exists at the moment as to the prevalence of HPV infection in normal tissue (see for example Munoz et al, 1988; de Villers et al, 1987), biopsies from genital and anal cancers regularly reveal the presence of HPV DNA (see for example zur Hausen 1988). HPV 16 DNA accounts for around 50% of these biopsies, HPV 18 20% and HPV 33 a little less than 10% (the remaining 10% being made up of other HPV types). Despite an accumulating body of epidemiological evidence for HPV involvement in cervical cancer, some workers have expressed concern over the apparently ubiquitous presence of HPV in the general population (de Villers et al, 1987) and the design of existing epidemiology studies (for example Munoz et al, 1988). Currently the strongest evidence for HPV oncogenicity comes from *in vitro* transformation studies.

5.3.1 HPV *In Vitro* Transformation Studies

There is good evidence from these *in vitro* studies that papillomavirus infections are responsible for the premalignant lesions which precede genital carcinoma. The development of a typical condyloma morphology in heterografted human foreskin tissue infected by HPV 11 demonstrates that this virus is capable of inducing this type of lesion (Kreider et al, 1985). *In vitro* transformation of primary human keratinocytes with HPV 16 and 18 has been shown to lead to an "immortalised" phenotype, although these transformed cells are not tumorigenic in athymic mice, unlike cell lines derived from malignant cervical tumours (Durst et al 1987, Pirisi et al 1987). Recent work utilising differentiating human keratinocytes in a "raft" system has demonstrated that HPV 16 transfection leads to the development of a morphology sharing histological similarity with cervical intraepithelial neoplasia (CIN) (McCance et al 1988).

In general, HPV DNA when detected in non malignant tissue is found to be in an episomal state. In HPV associated malignancies, however, the viral DNA is found integrated in the host genome, often at high copy number (Durst et al 1985). Integration of the viral DNA does not appear to favour any particular location in the host genome, but the integration event usually occurs within the 3' end of the E1 ORF or the 5' end of the E2 (Schwartz et al 1985) an event which is postulated to disrupt the viral regulatory mechanisms (for review see

Ward et al 1989). There are, however, exceptions to this general rule. HPV DNA is has been observed to remain episomal in fully developed carcinoma and premalignant lesions with integrated viral DNA have been observed (for example see Matsukua et al 1989).

5.3.2 Oncoproteins of The Human Papillomaviruses

When HPV related tumour biopsies are assayed for the expression of HPV genes, the E6 and E7 ORFs are found to be consistently retained and expressed, implying a direct role for these ORFs in maintaining the transformed phenotype (Schwartz et al 1985). The E6 and E7 proteins have been shown to contain **Cys-X-X-Cys** repeats which have been shown to mediate zinc binding in HPV 18 and BPV 1 (Barbosa et al, 1989). These ORFs are required for the morphological transformation of rodent cells in culture (Bedel et al 1987) and also in cooperation with activated ras for the transformation of primary keratinocytes (Matlashewski et al, 1987).

HPV is found to be capable of transforming both human primary fibroblasts and keratinocytes (Pirisi et al 1987). The transformed fibroblasts have an extended life-span but are not immortal while the transformed keratinocytes appear almost identical to control cells but have an indefinite life-span in tissue culture. The expression of **both** HPV-16 E6 and E7 is required for the extended life-span observed in fibroblasts although partial extension can be achieved using E6 alone (Watanabe et al 1989) *(The lack of a quantitative in vitro bioassay for HPV E6 has limited work on this gene and most of the work on transformation properties of the E6 have been performed using BPV-1 and rodent cells).* Using E7 alone in these assays is ineffective in extending the life-span of primary human fibroblasts despite the observation that the E7 product is sufficient to immortalise and transform rodent cells (Kanda et al 1988). In primary genital epithelial cells however, both E6 and E7 are required for efficient immortalisation (see for example Munger et al, 1989a). HPV-16 transformation of human cells appears to be different from HPV-16 transformation of rodent cells as the human cells lack morphological characteristics of transformed cells and are not tumorigenic in athymic mice.

Once the E7 ORF was identified as a transforming region of oncogenic HPVs, workers began to attempt to define a function for the E7 protein. A number of domains have been assigned to the HPV-16 E7. These include a $p105^{Rb}$ binding domain, a casein kinase II (CKII) phosphorylation domain and two **Cys-X-X-Cys** zinc binding domains (Dyson et al, 1989; Munger et al 1989b; Barbosa et al, 1990). The transformation potential of HPV-16 E7 depends primarily on the Rb and zinc binding domains as mutations in either of these two domains abolishes transformation (Edmonds and Vousden, 1989; Chesters et al, 1990).

The HPV-16 E7 product has been shown to encode transactivation and transformation functions similar to those of adenovirus E1A (Phelps et al, 1988) and the amino acid sequence of the E7 shows striking sequence homology with the p105*Rb* binding domains 1 and 2 of the Adenovirus E1A protein (see **Fig 5.2.**). These studies demonstrated that E7 is capable of acting as a transcriptional transactivator, having the ability to transactivate the adenovirus E2 promoter. E7 also shares sequence homology with large T antigen of SV40. As previously mentioned, both Adenovirus E1A and SV40 large T have been shown to bind the product of a tumour suppressor gene, the retinoblastoma gene product ($p105^{Rb}$), the depression of which is observed in a number of human malignancies. The removal of $p105^{Rb}$ by these viruses has been postulated to allow uncontrolled cell proliferation (Linzer, 1988). It has been shown that HPV 16 E7 can bind the Rb product (Dyson et al, 1989) with greater affinity than HPV 6b which is associated with benign lesions and this may explain the difference in malignant potential between these two viruses (Gage et al 1990). The binding of E7 to p105^{Rb} prevents the cellular protein from interacting with its normal targets (Defeo-Jones et al, 1991; Rustgi et al, 1991).

The E6 protein of HPV-16, like E7, has **Cys-X-X-Cys** motifs and binds zinc (Barbosa et al, 1989). In common with the BPV-1 E6 (Lamberti et al, 1990), HPV-16 E6 can function as a transcriptional activator (Sedman et al, 1991), and a possible oncogenic function is therefore transcriptional activation of target cellular genes. The HPV-16 E6 also binds a tumour suppressor protein, p53 (Werness et al, 1990) and enhances its degradation by the ubiquitin dependant pathway (Scheffner et al, 1990). The removal of p53 in this way will thus negate its role as a suppressor of cell proliferation. Studies on the relationship between p53 binding and degradation have demonstrated that degradation requires E6 sequences which are different from those required for complexing and transactivation functions (Crook et al, 1991d). In addition these studies showed a possible reason for the difference in oncogenic potential of different HPV types. While the benign HPV types (6 and 11) encode a conserved C-terminal p53 binding domain, the N-terminal domain (shown in these studies to direct p53 degradation) is not conserved, explaining why HPVs 6 and 11 show some p53 binding capacity, but no degradation of the protein is detectable. The importance of p53 dysfunction in anogenital cancer is demonstrated by the finding that while HPV positive carcinomas contain wild type p53 sequences, in HPV negative lesions mutant p53 is present (Crook et al 1991a, 1991b; Scheffner et al, 1990). In addition, the presence of wild type p53 is found to reduce the ability of E7 and *ras* to transform baby rat kidney cells, while mutant p53 transfection increases transformation in this system (Crook et al, 1991c).

In summary there is good experimental evidence for the involvement of certain papillomavirus types in anogenital malignancies. This involvement is insufficient for cancer development, and chemical and physical cofactors affecting the infected cell are likely to play an important role in the carcinogenesis process. The role of environmental cofactors will be considered later in the Introduction.

5.4 The Bovine Papillomaviruses

The bovine papillomaviruses are a valuable model for work on the human viruses as both systems share a number of common factors:

a) Large number of viral types

b) High lesion specificity of the different virus types

c) Examples of malignant progression in both systems

d) Proposed role of cofactors

To date six types of Bovine Papillomaviruses (BPVs) have been isolated and characterised and these have been divided into two subgroups , A & B, on the basis of their pathology, site specificity and genome homology (Campo et al., 1980, 1981; Campo and Coggins, 1982; Jarrett et al 1984):

SUBGROUP A: Contains BPVs 1,2 & 5. These viruses cause fibropapillomas.

SUBGROUP B: Contains BPVs 3,4 & 6. These viruses cause true epithelial papillomas.

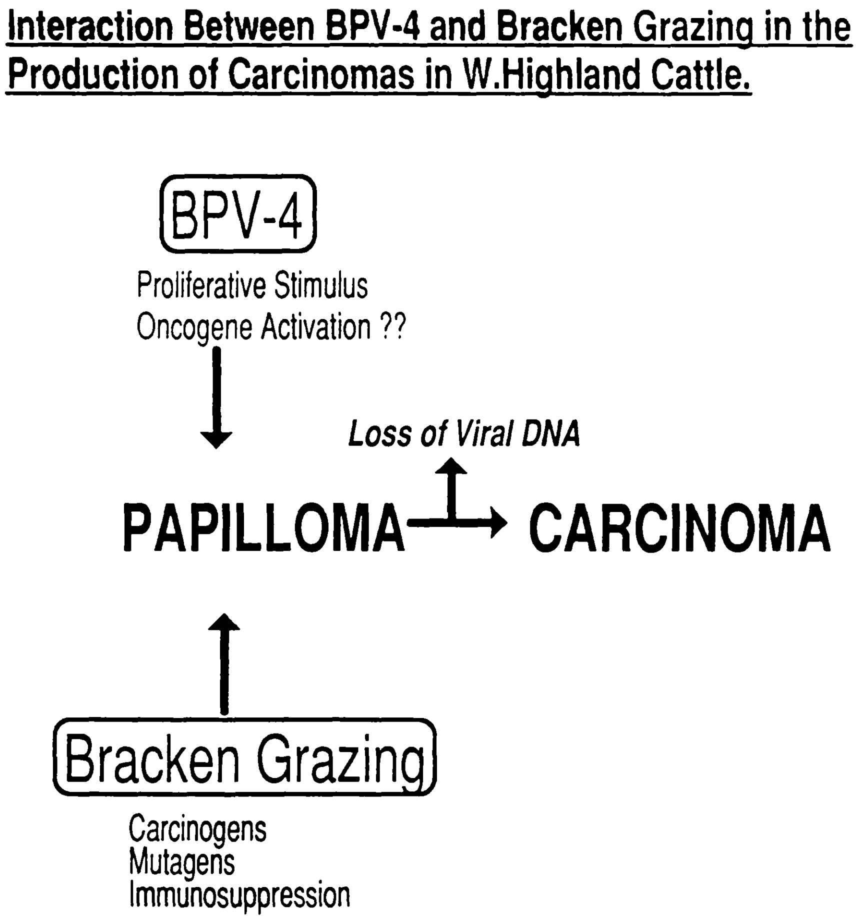
BPV type 4 is found associated with true carcinoma (of the alimentary canal) *in vivo* and this virus shows a very high target specificity for the mucosal epithelia of the alimentary canal, only inducing tumours when injected into this site and not when innoculated into cutaneous epithelia (Campo et al, 1980; Jarrett, 1985). The virus is evolutionarily distinct from the subgroup A viruses as demonstrated by the lack of any cross reactivity between antisera raised against BPV-4 and any of the subgroup A viruses (Jarrett et al., 1980, 1984). Similarly the DNAs from members of different subgroups do not cross hybridize in Southern blot analysis, even at low stringency.

There are two naturally occurring cancers in cattle which have been shown to be associated with papillomavirus infection; those of the urinary bladder and upper alimentary canal.

Early observations showed that extract made from bovine cutaneous warts contained a transmissible agent which when injected into the bovine bladder could induce carcinoma (Olson et al, 1959). In the reciprocal experiment, extracts from naturally occurring bladder carcinomas had the potential to cause papillomas (of skin and vagina) and polyps and fibromas in the bladder of experimental cattle (Olson et al, 1965). These experiments were performed before the existence of multiple bovine papillomavirus types was established (see above), and hence the particular virus involved was not established. In addition, bracken fern grazing was shown later (by epidemiological analysis) to be a significant risk factor in the disease progression (Pamukcu et al, 1967) but was not considered in the original experiments. Experimental animals fed bracken fern develop bladder tumours indistinguishable from those observed in field cases (Price and Pamukcu, 1968). Bracken fern is known to contain carcinogens, mutagens (Evans I.A. et al, 1982) and immunosuppressants (Evans W.C. et al., 1982). More recent experimentation by Campo and Jarrett has addressed both the viral type involved and the cooperative effects of bracken grazing in long term experiments. In these studies bracken fed cattle were innoculated with BPV-2 and developed cutaneous warts at the site of injection. In addition, the animals (which were chronically immunosuppressed due to the bracken diet) developed bladder carcinomas indistinguishable from field cases (Campo and Jarrett, 1986; MS Campo, personal communication). Bladder carcinoma did not develop in animals with a bracken-free diet which developed skin warts only. Animals which did not receive virus but were fed bracken did however develop tumours of the bladder and subsequent analysis revealed that these tumours contained viral DNA, which may have been activated by bracken induced immunosuppression. In these experiments Southern blot analysis established that 69% of the induced bladder carcinomas contained BPV-2 DNA while 46% of field cases were positive for BPV-2 (or the closely related BPV-1 virus). This frequent association of viral genomes with these tumours strengthens the hypothesis that there is a viral component in the disease progression.

BPV-4 has been shown to be the causative agent of papillomatosis of the alimentary canal in cattle (Campo et al, 1980). The benign papillomas usually regress, but in cattle of the West Highlands of Scotland, which continuously ingest bracken fern, the papillomatosis is more widespread and persistent, showing a much greater incidence of progression to carcinoma (Jarrett et al., 1978; Jarrett, 1981). A schematic outline of the disease progression is shown in **Fig 5.4.** Immunosuppression and widespread papillomatosis have been reproduced experimentally in animals using the immunosuppressant azathioprine and virus alone (Campo and Jarrett, 1986). Scarification of bovine epithelium followed by infection with BPV-4 leads to the development of benign papillomas which regress with time due to the host immune response. Immunosuppressed animals given virus had much more widespread and persistent papillomatosis.

Fig 5.4. Model for BPV-associated carcinoma *in vivo*.



Schematic representation of BPV-4 associated carcinoma of the alimentary canal in cattle. Viral infection gives rise to benign papillomas which usually regress. In the presence of chemical cofactors (bracken fern) these lesions may progress to carcinoma however. The viral DNA, while present in the papillomas, is undetectable in the resultant carcinomas suggesting that the virus has a role in the initiation of the disease but is not required for the maintenance of malignancy.

Development of alimentary canal carcinoma was found to occur in experimental animals which were given BPV-4 and fed on a diet of bracken confirming the observations of field cases. Clearly this system is an excellent model for the study of multistage carcinogenesis.

An interesting observation is that while viral DNA is detectable as high copy number episomes in benign lesions, it is absent in both naturally occurring and experimentally induced carcinomas (Campo et al., 1985). The viral DNA is therefore required for the *initiation* <u>but not the maintenance</u> of the transformed state. The "Hit and Run" action of BPV-4 is consistent with the mechanism postulated for transformation by herpes simplex virus, human cytomegalovirus and Adenovirus where viral sequences are not consistently maintained or expressed in transformed cells (for review see Macnab, 1987). This situation is in contrast with the human papillomaviruses HPV 16 and HPV 18 which are involved in carcinoma of the cervix. In this case the viral genome is generally found to be episomal in pre-malignant lesions and in an integrated state in the carcinomas (Durst et al., 1985; Schwartz et al., 1985). The fidelity of loss of BPV-4 DNA in alimentary canal carcinoma is perhaps the most interesting aspect of BPV-4 transformation biology and will be discussed in some detail later in this thesis.

5.4.1 Bovine Papillomavirus Type 1 Transformation *In Vitro*

The discovery that BPV-1 (or its naked DNA) could efficiently transform rodent fibroblasts *in vitro* was a major breakthrough in papillomavirus research. Although recently research has shifted largely to the oncogenic human papillomaviruses HPV 16 and HPV 18, much of the groundwork in papillomavirus transformation biology was performed using BPV-1 transformation of rodent fibroblasts. The oncoproteins of BPV-1 have been identified as the E5 and E6 (Schiller et al, 1984, 1986; Yang et al, 1985; Schlegel et al, 1986).

The major transforming ORF of BPV-1 appears to be the E5 which encodes a 44 amino acid protein which exists as a transmembrane dimer (Schlegel and Wade-Glass, 1987). The protein contains two cysteine residues which are important for dimerisation, but mutation of these residues does not totally abolish transformation potential, suggesting that dimerisation is not crucial for this property (Horwitz et al, 1988). The E5 is required for efficient transformation of C127 cells and can transform in the absence of other viral ORFs (see for example Burkhardt et al 1987). The protein is found to be associated with the plasma membrane and Golgi apparatus (Burkhardt et al, 1989). Rawls and co-workers have demonstrated that DNA synthesis is induced in quiescent contact inhibited C127 cells when cloned BPV-1 E5 is introduced (Rawls et al, 1989). Microinjection of synthetic E5 peptides into the nucleus of quiescent C127 cells will also produce this effect, while a cytoplasmic injection will not (Green and Lowenstein 1987), a result seemingly at odds with the observed cytoplasmic location of the E5 protein (Burkhardt et al, 1989). The ability of E5 to stimulate DNA synthesis has been shown to reside in the terminal third of the protein (Green and Lowenstein, 1987) while the hydrophobic core of the protein increases the efficiency of the induction of DNA synthesis but can be replaced by a random sequence of hydrophobic amino acids (Horwitz et al, 1989) suggesting that a transmembrane localisation may be important. E5 has been shown to affect the activity and metabolism of the receptors for epidermal growth factor (EGF) and colony stimulating factor 1 (CSF-1) (Martin et al, 1989). It has been shown to interact with another receptor , the platelet derived growth factor (PDGF) receptor. The observation that treatment of quiescent cells with PDGF results in DNA synthesis and that PDGF receptor activation occurs following infection (Petti et al, 1991) supports the hypothesis that a principal role of the E5 protein is to directly bind and activate this receptor. The E5 binding to PDGF receptor may be via the region of homology between the E5 of fibropapillomaviruses and the PDGF molecule (Petti et al, 1991). BPV-1 E5 has also been shown to bind a 16Kd cellular protein, the binding of which may be important for the process of transformation (Goldstein and Schlegel 1990). The 16kd cellular protein shares the physical properties of a protein identified as a structural component of gap junctions (Finbow et al, 1990) and it is feasible that removal of the gap junction protein and subsequent disruption of intercellular communication may be a major role of the BPV-1 E5 although this has yet to be demonstrated.

The BPV-1 E6 has also been defined as a transforming ORF; expression of E6 from a strong heterologous promoter can induce focus formation and anchorage independence in C127 cells while mutations in E6 reduce the transformation efficiency of the whole viral genome (Schiller et al, 1984). BPV-1 E6 contains **Cys-X-X-Cys** Zinc binding domains (Barbosa et al, 1989) and has been proposed to act as a transcriptional activator (Lamberti et al, 1990). In this respect one function of the BPV-1 E6 may be to transcriptionally activate crucial cellular genes as part of the transformation programme.

The E7 region of BPV-1 partially overlaps the E6 with the E6/E7 region being complex and potentially encoding a number of products. Some dispute exists as to whether BPV-1 E7 mutation has any effect on transformation although recent work has suggested that both the E6 and E7 ORFs are required for full transformation of C127 cells by BPV-1 (Neary and DiMaio, 1989). The E7 proteins of both BPV-1 and the benign human HPV-8 lack independent transformation potential (Iftner et al, 1988) possibly because the retinoblastoma binding domain and the CKII site present in HPV-16 E7 are missing (Iftner et al, 1990). It should be noted however, that CRPV E7 lacks the CKII domain but is still essential for tumorigenesis in vivo (Brandsma et al, 1991). In addition the CKII sites of HPV-16 E7 have been shown to be less critical than the Rb binding domains and **Cys-X-X-Cys** motifs (Watanabe et al, 1990; Storey et al, 1990).

5.4.2 Bovine Papillomavirus Type 4 Transformation *In Vitro*

Early attempts to investigate the transforming properties of BPV-4 *in vitro* used established murine NIH-3T3 and C127 fibroblasts These studies showed that BPV-4 encoded all the required information to transform NIH-3T3 cells to a full malignant phenotype at a frequency of focus formation similar to BPV-1 (Campo and Spandidos, 1983). The transformation of C127 cells required low cell density or the presence of the tumour promoter TPA (Smith et al, 1987; Smith and Campo 1988). TPA has been shown to cause a disruption of intracellular communication (Murray and Fitzgerald, 1979), and these observations suggest that the presence of neighbouring normal cells may inhibit the manifestation of the transformed phenotype in this cell type. As TPA induces a host of cellular changes, however, the precise mechanism by which the drug enhances transformation is unclear. Serum stimulation was found to increase transformation by BPV-4 and this suggests that growth factors present in the serum may allow manifestation of the transformed phenotype. The physical state of the input DNA was shown to be important, as circular BPV-4 genome was less capable of transformation; linear genome (interrupting the E1 ORF) or a cloned 3.6 kb fragment containing the 3' half of the L1, E7, E8 and the 5' half of the E1 ORFs were found to be effective. Interruption of the E1 ORF may be the crucial factor here, as functions which negatively control DNA replication and transcription have been mapped to the E1 and E2 ORFs respectively in BPV-1 (Berg et al, 1986; Roberts and Weintraub, 1986; DiMaio, 1986; Lambert et al, 1987) and similar functions may exist in BPV-4.

BPV-4 DNA is **absent** from the majority of transformed C127 clones, a situation similar to that found in the *in vivo* cancers.

The experiments using C127 cells have provided interesting data concerning which areas of the BPV-4 genome may be important in transformation and highlighting some of the cofactors and cellular responses which may play a role in this system. The cell type being employed is quite remote from the natural host cell of the virus, however, being established, fibroblastic and of a different species. A more relevant system would be the use of primary bovine cells in similar experiments. Initial observations using primary bovine fibroblasts have shown that BPV-4 is capable of transforming these cells only in the presence of an activated *ras* oncogene, and while the cells appear morphologically transformed, they are unable to induce tumours in athymic mice (Jaggar et al., 1990).

Recent work has shown that bovine epithelial cells from the palate can be infected in vitro with BPV-4 and when placed in the renal capsule of athymic mice will produce virus-producing papillomas with identical morphology to those induced in cattle (Gaukroger et al., 1989). This system obviously has exciting possibilities for research into BPV-4 transformation biology.

5.5 Environmental Factors in Papillomavirus Associated Malignancies

The long latency period of papillomavirus associated carcinomas (notably HPV in anogenital carcinoma, and BPV-4 in alimentary canal carcinoma) implies that the dysfunction of a number of crucial proto-oncogenes or tumour suppressor genes must occur before the onset of carcinoma, and it is likely that environmental factors play a role in the disease progression. The major environmental cofactors in BPV-4 associated alimentary canal carcinoma have been identified as the chemicals found in bracken fern (outlined above) although the precise bracken constituents required for cooperation with the virus have not yet been identified. In other papillomavirus systems the cooperation of environmental carcinogens is less clear.

Historically, the first observations that PVs could cooperate with chemical cofactors in carcinogenesis came from work on CRPV in the 1930s and 40s. In this system it was observed that when tar and/or methylcholanthrene were administered in conjunction with the virus the rate of progression of papillomas to carcinomas was greatly accelerated from that observed with either virus or chemicals alone (see Rous and Friedewald, 1944).

Epidemiological studies on human cervical carcinoma has suggested that several different cofactors may be involved in disease progression. The major chemical cofactor suggested to play a role in this disease is cigarette smoking, first proposed following a study by Winklelstein (1977) and subsequently confirmed by other epidemiologists (the evidence for this association has been reviewed in Winklestein, 1990). Further epidemiological studies have also identified smoking as a significant risk factor in anal cancer (for example Holly et al, 1989) and the progression of laryngeal papillomas to carcinoma (Lindenberg et al, 1986). Using the *Salmonella typhimurium* mutagenicity test, specific fractions from cigarette smoke have been shown to be mutagenic (Keir et al, 1974), these fractions may accumulate in the cervix as the cervical mucus of smokers has been shown to be mutagenic by this assay (Holly et al, 1986). Indeed, some non-smokers have mutagenic cervical mucus (Holly et al, 1986) which may be the result of passive smoking (Slattery et al, 1989) or other environmental mutagens. Another significant consequence of cigarette smoking is the impairment of the immune response, including a reduction in the activity of both natural killer and T cells, and a significant reduction in immunoglobulin concentration (reviewed in Holt, 1987).

Other chemical cofactors implicated in cervical carcinoma include oral contraceptives (see for example Beral et al, 1988) which may act by activating the glucocorticoid responsive element in the HPV genome, increasing viral transcription (Gloss et al, 1987) which it turn has been shown to increase transformation in an *in vitro* assay (Durst et al, 1989). Also, in one individual dietary factors, (namely fried pork intake), were shown to cause prolonged HPV-11 associated papilloma growth of the urinary tract (Schneider et al, 1990), which may be due to the elevated mutagenic potential of human urine following fried pork consumption (Baker et al, 1982).

Both epidemiological studies and *in vitro* models have demonstrated that chronic irritation and/or inflammation may contribute to carcinogenesis. Epidemiological work on anal cancer identified these factors as a significant risk (Holly et al, 1989) and cervical deformation from surgical lacerations during child birth were found more frequently in women presenting with cervical carcinoma (Stanimirovic et al, 1990). Mechanical irritation of the skin has been found to activate latent genomes of the rodent *Mastomys nataliensis* papillomavirus (MnPV) (Seigsmund et al, 1991) confirming early observations by Rous and Beard (1935) that inflammation resulting from the use of chemical irritants encouraged the malignant conversion of CRPV induced papillomas.

Another risk factor in certain papillomavirus associated tumours is radiation. Patients with the inherited disease epidermodysplasia verruciformis (EV), a skin condition characterised by multiple papillomavirus induced lesions, have a high chance of developing carcinomas in sun exposed areas, implicating UV radiation as a cofactor (Orth 1987). Radiotheraphy has also been shown to elevate the risk of malignant conversion of laryngeal papillomas (Lindeberg et al, 1986).

The ability of viral genomes to cooperate with chemical promoters has been considered using the chemical TPA in *in vitro* model systems. Viral replication has been shown to increase for a number of virus types including BPV-1 (Amtmann and Sauer, 1982) and BPV-4 (Smith et al, 1987; Smith and Campo, 1988). Also of relevance is the observation that gene expression can be upregulated in the presence of TPA as demonstrated for the *ras* oncogene (Dotto et al, 1985), and for BPV-1 (Amtmann and Sauer, 1982), BPV4 (Smith et al, 1987) and HPV 18 (Gius and Laimins, 1989) viral genes. In addition TPA presence has been shown to increase the transforming potential of BPV-4 (Smith and Campo, 1988) and BPV-1 (Tsang and Stich, 1988). The viral replication and transcriptional activation phenomena may be as a result of the ability of TPA to activate the transcription factor AP1 (Angel et al, 1987). Levels of expression of papillomaviral oncoproteins may determine the efficiency of morphological transformation by these genes as shown for HPV-16 (Lees et al, 1990) and proposed for BPV-4 (Jaggar et al, 1990). The relationship between efficiency of *in vitro* transformation and levels of BPV-4 gene products will be considered in more detail later in this thesis.

Another consequence of TPA treatment may be the disruption of junctional communication. Co-culturing experiments have shown that normal cells have an inhibitory effect on the growth of co-cultured transformed cells, an effect which is abolished by TPA treatment (Sivak and van Duuren, 1970). TPA has been shown to inhibit junctional communication in *in vitro* studies by a number of workers (for example see Newbold and Amos, 1981). More recent work on mouse skin *in vivo* has suggested that TPA treatment may serve to disrupt the junctional communication patterns of the skin leading to inappropriately formed communication compartments (Kam and Pitts, 1988).

The observation that papillomaviruses can cooperate with TPA would suggest that the viral genomes can act as initiating agents. The possibility that papillomaviruses could also act as promoters has not been addressed by many workers and indeed experiments using the rodent papillomavirus MnPV demonstrated that this virus had no observable capacity to cooperate with DMBA (Amtmann et al, 1987). It seems plausible, however that the proliferative stimulus afforded by papilloma production *in vivo* may serve to amplify a population of cells which have been initiated by environmental mutagens and in this sense the papillomaviral genome could function as a classical tumour promoter. Indeed experiments using BPV-4 have demonstrated that the virus can cooperate with both initiators and tumour promoters *in vivo*. In these experiments fetal bovine palate tissue was infected with BPV-4 and subcutaneously implanted in nude mice. The animals then had slow releasing TPA or DMBA pellets implanted subcutaneously at different sites, and in both cases a carcinoma developed from the infected bovine tissue (25% and 59% of animals respectively) (J.M. Gaukroger, personal communication), a result not observed in control animals implanted with uninfected tissue and considerably higher than the spontaneous carcinoma development observed in animals which received infected tissue but no chemical implant of either kind (less than 2%) (Gaukroger et al, 1991). These results suggest that BPV-4 can act as a promoter on a population of cells which have been chemically initiated. Indeed the observation that BPV-4 DNA is absent from carcinomas *in vivo* would suggest that either the viral genome acts as an initiator by causing host DNA damage before the viral genome is lost (for example as a DNA amplifying agent as demonstrated by Smith and Campo, 1989) or as a promoter of initiated cells during viral presence. The latter hypothesis will be considered in the Results and Discussion section of this thesis.

5.6 Summary; Cellular Targets in Papillomavirus Associated Malignancy

In summary, a variety of PV types have been implicated in multistage carcinogenesis on both epidemiological evidence and on the basis of their transformation potential *in vitro*. The associated long latency of these tumours implies that papillomaviruses do not contain sufficient genetic information to induce these tumours on their own, and indeed activated oncogenes have been found to be involved in a significant number of cases (e.g. c-Ha-*ras* and *c-myc* frequently found in HPV associated anogenital cancer and Ha-*ras* 1 activation in BPV-4 associated alimentary canal carcinoma). Further evidence for the role of oncogene activation in these systems are the requirement for activated *ras* cooperation for transformation of primary cells *in vitro*, and the discovery of the suppressor of HPV transformation on chromosome 11.

In vitro assays have identified the oncoproteins of some papillomaviruses, notably the E6 and E7 proteins of the oncogenic HPV types 16 and 18. The targets of these oncoproteins are the tumor suppressor gene products $p105^{Rb}$ and p53 which are bound (E7-$p105^{Rb}$) or bound and degraded (E6-p53 complex). The dysfunction of these regulatory proteins by papillomaviral oncoprotein binding is postulated to lead to deregulated cell division, one factor in the multistage carcinogenesis process. The importance of disabling these regulatory proteins in anogenital cancer is shown by the observation that HPV negative lesions have alterations in the genes for these proteins or altered patterns of their expression (Wrede et al, 1991).

The interaction of environmental cofactors is likely to play a crucial role in the PV associated malignancies as the primary lesion caused by papillomavirus infection is benign, and in the absence of cofactors will usually regress. These cofactors have been identified in BPV-4 associated alimentary canal carcinomas (the mutagens, carcinogens and immunosuppressants present in bracken fern) and in sufferers of the rare condition EV (UV radiation exposure), although the cofactors in HPV associated anogenital carcinoma are less well understood.

Thus although recent studies have demonstrated the direct cellular targets of papillomaviral oncoproteins, an examination of the role of contributing factors (such as environmental mutagens, host immune status etc) should provide a deeper understanding of the papillomavirus associated tumours as a multistage progression.

5.7 Aims of Ph.D. Research

The effects of the products of CRPV, BPV-1 and the oncogenic human papillomaviruses in *in vitro* assay systems have been characterised in many laboratories over the last decade (as outlined in this Introduction) and the ORFs involved in transformation and immortalisation have been identified. Initial studies on BPV-4 have indicated only which general regions of the viral genome may encode transformation functions. The aims of the work presented in this thesis were to determine more precisely the viral genes with transformation potential (both alone and in cooperation with other ORFs) and to characterise the phenotype of cells transformed by these genes. In addition, it was envisaged that the cellular localisation of these gene products would be determined, in order to provide an initial indication of possible interaction between viral proteins and cellular structures. The multifactorial nature of BPV-4 transformation *in vivo* would also be considered by the use of a bracken mutagen, quercetin, in cooperation with BPV-4 genes in an *in vitro* transformation assay.

6. Materials and Methods

6.1 General Laboratory Practice

6.2 Solutions and Reagents

Chemicals were of analytical grade where possible and were weighed out to an accuracy of 0.01g in plastic boats using a Mettler PM3000 balance. Chemical suppliers were BCL, BDH Ltd., Gibco-BRL Ltd., Pharmacia or the Sigma Chemical company.

Water was distilled using a MilliQ Reagent Grade Water Purification System (Millipore).

Phenol, "phenol/chloroform" and chloroform were prepared and stored as recommended in Sambrook *et al* (1989)

6.2.1 Solution Preparation

Chemicals were dissolved by stirring at room temperature (or 65^{o}C if necessary) using a magnetic stirring bar. Solutions were adjusted to correct pH using appropriate acids and bases and the pH was monitored using a Tris electrode Kent 7045/46 (Russell). Solutions were sterilised by autoclaving in a pressure cooker for 20 mins. Heat labile solutions were sterilised by passing through a Millipore 0.22μm "Acrodisc" filter.

6.3 Materials

6.3.1 Enzymes

Restriction enzymes were purchased from Pharmacia and came complete with appropriate buffer.
Calf Intestinal Phosphatase (CIP) was purchased from BCL.
T4 DNA Ligase was purchased from BRL.
Lysozyme, proteinase K and ribonuclease A (RNase A) were published from Sigma.

6.3.2 Radiolabelled Compounds

Radioisotopes were obtained from Amersham International plc.

[^{35}S]dATPαS (cat No. SJ1304)
(Specific activity>111T Bqmmol^{-1})

[α-^{32}-P]dCTP (cat No. PB10205)
(Specific activity>37T Bqmmol^{-1})

6.3.3 Plasticware

Nunclon tissue culture flasks and 1.9ml ampoules for storage of cells in liquid nitrogen were obtained from Gibco.

Petri dishes, disposable pipettes and polypropylene tubes were obtained from Falcon.
Sterile universal tubes were obtained from Millipore.
1.5ml microcentrifuge tubes were obtained from Sarstedt.
Disposable micropipette tips were obtained from Labsystems Group.

6.3.4 Molecular Biology Kits

A number of molecular biology techniques were performed using reagents supplied in kit form. Manufacturer's instructions for storage and use were adhered to throughout unless stated otherwise.

Random Prime DNA Labelling kits were obtained from BCL.
Geneclean ^{tm} kits were obtained from Bio-101 Inc.
"Sequenase" ^{tm} DNA sequencing kits were obtained from USB.
"VectorStain" Alkaline Phosphatase detection kits were obtained from Vector Laboratories.

6.3.5 Plasmids

All plasmids were propagated in E.coli strain DH5-α^{tm} competent bacteria. Generation of new plasmids will be discussed in the results section.

pSV2neo is a PBR322 derived vector encoding the neomycin (neo) resistance gene of Tn5 cloned under the control of SV40 early promoter with 3'poly(A) site (Southern and Berg, 1982).

pZipneoSV(X)1 (pZipneo) is a Mo-MuLV derived vector with a unique BamHI cloning site and a selectable neo resistance gene (Cepko et al, 1984).

pT24 is a pUC13 derived plasmid containing the activated human c-Ha-*ras* oncogene from the T24 human bladder carcinoma line originally cloned in pBR322 (M. O'Prey unpublished results, Santos et al, 1982).

pBV4B1 is the whole BPV-4 genome cloned in the BamH1 site of pAT153 (Campo and Spandidos, 1983).

pSVE8^{+}E7^{+} comprises the intact E7 and E8 ORFs (XhoII fragment) cloned as a 2.0Kb fragment (nt 6487-1274) in the BamHI site of pSV2neo (Jaggar et al, 1990).

pSVE8+E7- is as pSVE8^{+}E7^{+} but the cloned fragment has a 200bp EcoR1 fragment deleted in the 3'third of E7 (Jaggar et al 1990) see **Fig 5.2.**

pZipE8$^+$E7$^+$ as pSVE8$^+$E7$^+$ but the fragment is cloned in pZipneoSV(X)1 (Jaggar et al, 1990).

pZipE8$^+$E7$^-$ as pSVE8$^+$E7$^-$ but the fragment is cloned in pZipneoSV(X)1 (Jaggar *et al* 1990).

pZipE2 contains the E2 ORF of BPV-4 (nt 2741-4334) cloned in the pZipneoSV(X)1 vector (Jaggar *et al* 1990).

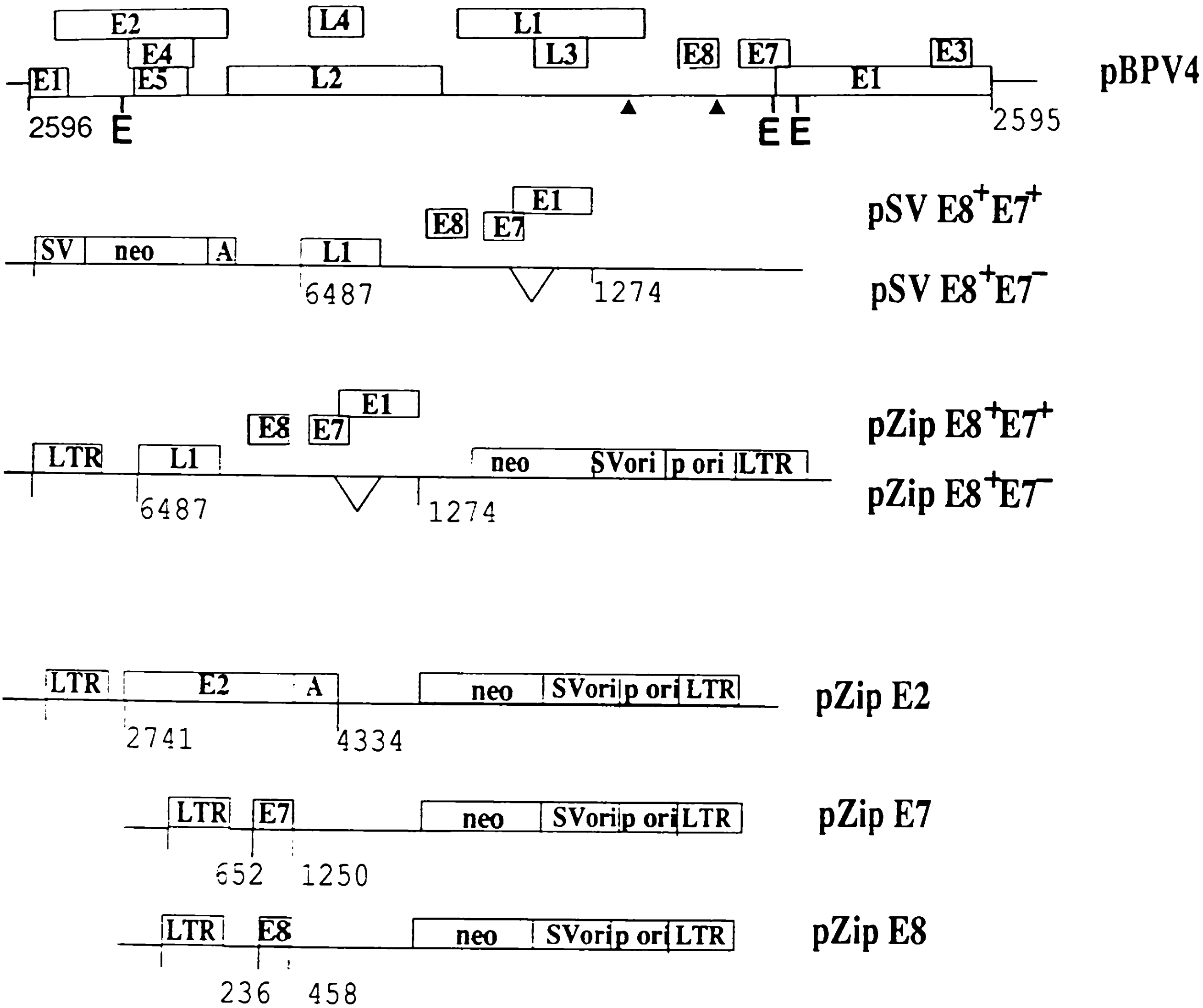
pZipE7 contains the E7 ORF (nt 652-1250) of BPV-4 cloned in pZipneoSV(X)1 (G.J. Grindlay, unpublished).

pZipE8 contains the E8 (nt 236-458) ORF of BPV-4 cloned in pZipneoSV(X)1 (G.J. Grindlay, unpublished).

pJ4Ω16.E6 was a gift from Lionel Crawford. The plasmid is a pBR322 derivative . The vector has the HPV-16 E6 700bp fragment cloned into the BamH1/BglII site being driven by the Mo-MULV LTR and containing the SV40 T poly(A) site. (Storey et al, 1987).

A diagrammatic map of each plasmid used is given in **Fig 6.1.** for BPV-4 containing plasmids and in **Fig 6.2.** for the pJ4Ω16.E6 and pT24 plasmids.

Fig 6.1. Structure of BPV-4 containing vectors.

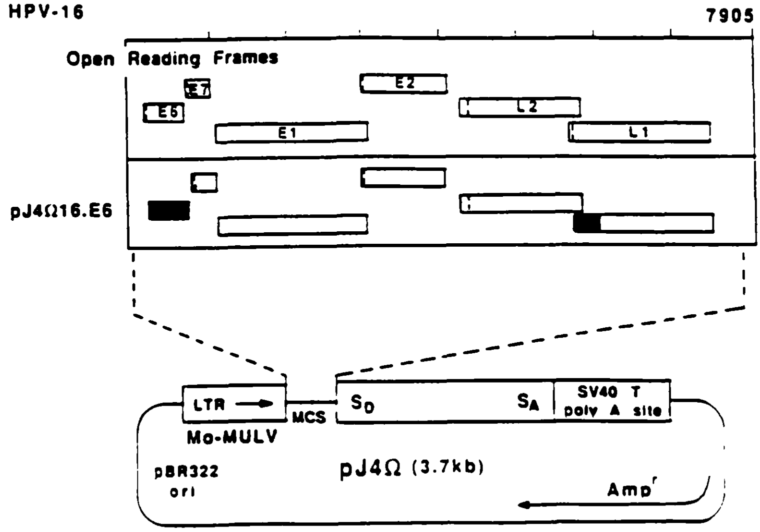


Notes: BPV-4 recombinant plasmids. pBV4 contains the full viral genome cloned in the BamH1 site of pAT153 (Campo and Coggins, 1982). pSVE8$^+$E7$^+$ and pZipE8$^+$E7$^+$ contain the XhoII fragment (nt 6487 to 1274) cloned in the BamH1 site of pSV2neo and pZipneoSV(XI) respectively. the pSVE8$^+$E7$^-$ and pZipE8$^-$E7$^-$ plasmids contain the same fragment but with a deletion spanning nt 905 to 1138 (symbolised by an inverted triangle). pZipE2, pZipE7 and pZipE8 contain the E2, E7 and E8 ORFs of BPV-4 cloned in the pZipneoSV(XI) vector respectively. The filled arrowheads indicate the BPV-4 LCR (nt 7000 to 300). Vector and insert sequences are not drawn to scale. **SV**=SV40 promotor; **LTR**=MoLV LTR; **A**=poly(A) site; **neo**=g418 resistance gene; **SVori**= SV40 DNA replication origin; **p ori**=plasmid DNA replication site. The polyadenylation site in pZipE2 is the early poly(A) site of BPV-4. Numbers refer to BPV-4 map coordinates.

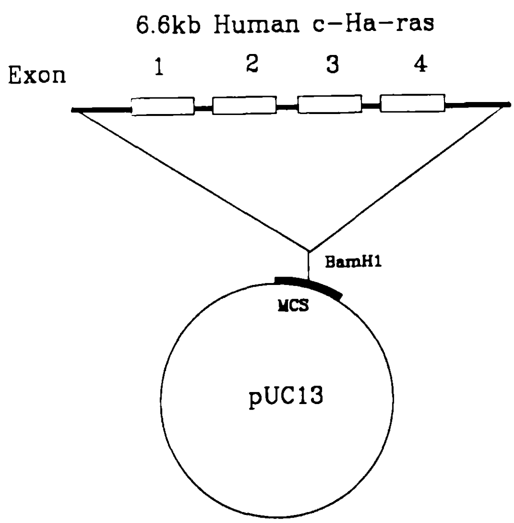
E=EcoRI restriction enzyme site.

Fig 6.2. Structure of pJ4Ω16.E6 and pT24 vectors.

a



b



Notes: A) structure of the HPV-16 E6 containing plasmid pJ4Ω16.E6. A map of the HPV genome is shown (ORFs are represented by closed boxes) and below it a diagramm where the regions of the genome present in this construct are represented by solid bars. **MCS**=multiple cloning site; **SD**=splice donor site; **SA**=splice acceptor site. The diagram is not to scale and is adapted from Storey et al, 1988. **B)** Structure of pT24 plasmid. The 6.6kb insert was isolated from a human bladder carcinoma (T24) and was originally cloned in pBR322 (Santos et al, 1982) and transferred to pUC13 (M. O'Prey, unpublished results). **MCS**= multiple cloning site. Plasmid and vector sequences are not to scale.

6.3.6 Other materials

DNA Markers: HindIII digested bacteriophage lambda
HaeIII digested bacteriophage PX174

Biodyne nylon membrane for use in Southern transfers was obtained from Pal Process Filtration Ltd.

X-Omar XAR5 film for autoradiography was obtained from Kodak.

6.4 General Methods

Phenol/Chloroform Extraction: Removal of contaminating protein from DNA-containing solutions was performed by the addition of an equal volume of phenol/chloroform followed by vortex mixing for 15 secs. The tube was then centrifuged for 5 mins in a microfuge and the aqueous DNA containing phase transferred to a fresh tube. Care was taken not to transfer any of the interphase in the process.

Ethanol Precipitation: DNA in solution was precipitated with salt and ethanol to facilitate concentration of dilute solutions or a change of sample buffer. To the solution 1/10 volume of 3M NaOAc pH5.2 and 2 volumes of absolute ethanol were added. The sample was vortexed and allowed to stand on ice for 10mins before centrifugation in a microfuge at $4^{O}C$ for 15-30 mins. The supernatant was then carefully removed and the pelleted DNA washed free of salt by adding 1ml of 70% ethanol and recentrifuging for 10mins. The supernatant was carefully removed, excess solvent drained onto a paper towel and the DNA pellet dried in a freeze drier before being resupended in a suitable volume of TE.

6.5 Centrifugation

Small samples of less than 1.5ml were routinely centrifuged in an Eppendorf 5414 bench-top microfuge. The pelleting of small bacteria samples or trypsinised cells in plastic universal tubes was performed in a MSE Mistral 4L low speed centrifuge. Large volume, low speed centrifugation was performed in a Sorvall RC-5B centrifuge. Ultracentrifugation, as used in CsCl density gradient purification of plasmids, was performed using a Kontron Instruments Centrikon T-2070.

6.6 Handling of Bacteria

Work with bacteria was carried out in a category 1 containment work area. Contamination of cultures was avoided by following standard microbiological handling techniques as described in Sambrook *et al* (1989).

6.6.1 Bacterial Media and Antibiotics

Luria Broth (L-broth):		1%(w/v) bactotryptone (Difco)
		0.5%(w/v) yeast extract (Difco)
		170mM NaCl
L-Agar:		L-broth containing 1.5% agar
Superbroth:	Solution A:	1.33%(w/v) bactotryptone
		2.66% (w/v) yeast extract
		0.44% (v/v) glycerol
	Solution B:	0.54M KH_2PO_4
		0.27M K_2HPO_4

Solution A and B are autoclaved separately. Superbroth is made by adding 100mls of solution B to 900mls of solution A.

SOC:	2% (w/v) bactotryptone
	0.5%(w/v) yeast extract
	10mM NaCl
	2.5mM KCl

10mM $MgCl_2$

10mM $MgSO_4$

20mM glucose

If required, ampicillin was added at a concentration of 50 mg/ml to growth media following autoclaving and cooling to 50^oC. Ampicillin was kept as a stock solution: 50mg/ml in dH_2O stored at -20^oC until use.

6.6.2 Preparation of DNA for cloning

Vector DNA was prepared for cloning as follows. 50ug of vector DNA was digested for 4 hours at 37^oC with 200 U of appropriate restriction enzyme in a volume of 200 µl. The vector was then incubated at 37^oC twice for 30 mins with 1U of calf intestinal phosphatase. Proteinase K was added to a concentration of 100 µg/ml and the sample incubated at 37^oC for a further 15 mins. The now cut, dephosphorylate and deproteinased vector was phenol chloroform extracted, ethanol precipitated and then resuspended to approximately 200 ng/ml in TE pH8.0 and stored at -20^oC.

Insert DNA was prepared as follows. Parental plasmid was restricted with appropriate restriction enzyme(s) and run on a 1-3% agarose gel. Fragments to be cloned were identified by size comparison with marker DNA and cut out of the gel as a discrete band. The DNA was extracted from this band using the "Geneclean" tm kit (Bio-101 Inc.) using manufacturer's instructions. This method involves melting the agarose at 55°C in the presence of a high concentration sodium iodide which acts as a chaotropic agent. The DNA in the melted agarose is bound to a silica matrix at this high salt concentration. The silica matrix and bound DNA are pelleted in a microfuge and washed several times in an ice cold salt/ethanol solution before the matrix is pelleted and the DNA eluted in a low salt solution. Approximately one fifth of the extracted DNA was then checked for size and purity by running on a 1% agarose minigel.

6.6.3 Ligation of Insert and Vector DNA

Linear vector DNA (typically 100-200 fmol) was mixed with a threefold excess of insert DNA, ethanol precipitated and resuspended in 8 µl of TE pH8.0. In order to denature any self annealed cohesive ends the mixture was heated to 65°C for 5 mins then annealing was allowed to occur by incubating the mixture on ice for 1 hour. To the annealed mixture 1 µl of 10 X ligase buffer (0.66M Tris.Cl pH 7.6, 0.1M $MgCl_2$ and 10mM DTT), 0.5 µl of 20 mM ATP and 0.5 U of T4 DNA ligase were added and the mixture was incubated for 18-20 hrs at 15^{o}C.

6.6.4 Transformation of Bacteria

All plasmids were propagated in the E.coli strain DH-5α (tm) supplied as frozen stocks by BRL and stored at -70^{o}C until use. Bacteria were transformed as per manufacturers instructions. After thawing on ice 50 μl of bacteria were added to a polypropylene Falcon 2059 tube on ice and 1μl of ligation reaction previously diluted 1 in 5 with TE pH7.4 was added and mixed by gently moving a pipette tip through the mixture. The tube was left on ice for 30 mins before being heat shocked for 45 seconds at 42^{o}C. The tube was returned to ice for 2 mins then 950 μl of SOC medium was added. The tube was then transferred to a shaking 42^{o}C incubator (275 rpm) for 1 hour. Following this the bacteria were spread on an L-agar plate containing appropriate antibiotics and transformants selected by incubating the plate overnight at 37^{o}C.

6.6.5 Small Scale Preparation of Plasmid DNA

Transformant colonies were screened to identify correct clones by performing a small scale DNA extraction as described by Birnboim and Doly (1979). Colonies were picked and grown overnight in 5 ml cultures in sterile universal tubes. 1.6mls of the culture was then used to prepare DNA, the remainder being stored at $4^{O}C$. The bacteria were pelleted by centrifugation for 2 mins in a microfuge. The supernatant medium was poured off and all traces of medium removed by a micropipette. The pellet was resuspended in 100μl of Solution I (25 mM Tris, 10mM EDTA pH8.0, 50 mM glucose and 4 mg/ml lysozyme) and left at room temp for 5 mins. 200 μl of freshly prepared Solution II (0.2N NaOH, 1% SDS) was then added and the tube was inverted rapidly to mix the contents and then placed on ice for 10 mins. 150 μl of Solution III (3M KOAc pH4.8) was added, the tube shaken rapidly and then returned to ice for 10 mins. The chromosomal DNA was pelleted by spinning the tube for 15 mins in a microfuge and the supernatant was transferred to a fresh tube. The plasmid was phenol/chloroform extracted then ethanol precipitated at room temperature. The pellet was resuspended in 50 μl TE pH8.0 containing boiled RNase A at a concentration of 50 μg/ml. The sample was then digested with diagnostic restriction enzymes to verify the clone.

6.6.6 Large Scale Preparation of Plasmid DNA

Large quantities of plasmid DNA were prepared using a modification of that described by Birnboim and Doly (1979) in that the growth medium used was Superbroth. Solutions I-III were as described in the previous section.

Bacteria containing the plasmid of interest were streaked onto an L-agar plate containing appropriate antibiotics and grown overnight at 37^{o}C. A single colony was picked from this plate and used to inoculate 5mls of Superbroth medium which was then put in a shaking incubator at 225rpm for approximately 8 hours at 37^{o}C. This culture was then added to 500mls of Superbroth then returned to the shaking incubator for 36 hours.

The culture was spun at 4000rpm for 10 mins at 4^{o}C to pellet the bacteria. The supernatant medium was removed and the bacterial pellet was resuspended in two 30 ml aliquots of Solution I. The suspensions were left at room temperature for 5 mins. To each aliquot was added 60 mls of ice-cold Solution II and the solutions were mixed by inverting the bottles gently. This allows the lysis of the bacteria and denaturation of the chromosomal DNA. The bottles were placed on ice for 10 mins then 45mls of Solution III was added. The bottles were shaken rapidly then returned to ice for a further 10 mins allowing the precipitation of the renatured chromosomal DNA. The chromosomal DNA was then pelleted by centrifugation at 10,000 for 10 mins at $4^{o}C$. The supernatant was then carefully removed and retained by filtration through several layers of sterile tissue. The nucleic acid in this cleared lysate was precipitated by the addition of 72 mls (i.e. 0.6 volumes) of isopropanol. The lysates were mixed and left for 10 mins at room temperature after which the nucleic acid was pelleted by centrifugation for 10 mins at 1000rpm at $4^{o}C$. The supernatant was then withdrawn and the nucleic acid pellet washed with 50mls of 70% ethanol to remove any salt. The solution was then centrifuged again for 10 mins at 1000rpm at $4^{o}C$ and the supernatant withdrawn. The pellet was allowed to dry for 10 mins before being resuspended in 8 mls of TE.

Caesium chloride equilibrium centrifugation was then used to further purify the plasmid DNA. 9.3 g of CsCl was added to the solution and allowed to dissolve at room temperature. Ethidium bromide was then added to a final concentration of 600 µg/ml. The refractive index of this solution was then determined and adjusted to 1.4. The solution was transferred to 13.5 ml Kontron tubes and centrifuged at 38,000 rpm for 48 hrs. After this period contaminating RNA is pelleted to the bottom of the tube, while protein floats to the top. Two distinct bands are observed; the lower band contains residual chromosomal DNA while the upper contains the plasmid DNA. The upper band was removed by carefully inserting a syringe needle below it and gently drawing the band off.

Ethidium bromide was removed by several extractions with CsCl saturated isopropanol. The CsCl was then removed by dialysing the solution in dialysis tubing (Sartorius) against 3 changes of TE. The DNA was then precipitated with ethanol, resuspended in 1-3 mls of TE (dependent on size of pellet), before being quantitated by spectrophotometry. The plasmid solution was aliquoted and stored at $-20^{o}C$.

6.7 Tissue Culture

6.7.1 Media and Antibiotics

Cells were grown routinely in Dulbecco's Modified Eagle's Medium DMEM supplemented with 100mls foetal calf serum and 10mls of 200 mM L-glutamine per 1000mls. Penicillin (50 units/ml), streptomycin (50 μg/ml) and amphotericin B (2.5 μg/ml) were added to inhibit microbial contamination. All tissue culture reagents were supplied by Gibco with the exception of foetal calf serum (NBL). All reagents were stored at $4^{o}C$ except glutamine, foetal calf serum, antibiotics and trypsin which were stored frozen at -20^{o}C until needed.

6.7.2 Stock Solutions

Phosphate Buffered Saline (PBS) pH7.3: 137 mM NaCl, 44 mM KCl, 1.4 mM KH_2PO_4 and 8.4 mM $Na_2HPO_4.7H_2O$

PBS/EDTA (PE): As PBS but with the addition of EDTA to 1 mM.

Trypsin Solution: made fresh from a 2.5%(w/v) frozen stock by diluting 1 in 10 with PE. Trypsin solution was stored at 4^{o}C and used for up to 2 weeks.

Geneticin (G418): Made to 100 mg/ml in dH_2O and stored frozen at -20^{o}C until use.

Quercetin: A stock 10mM solution of quercetin was made up in ethanol and stored frozen at -20^{o}C until use.

6.7.3 Isolation of Primary Bovine Fibroblasts

Primary bovine palate fibroblasts (PalF cells) were isolated as follows. Short term bovine foetuses (less than 5 months gestation term) were obtained from the Glasgow University Veterinary College. The soft palate tissue was removed by dissection and surface sterilised by a 30 second wash in 70% ethanol. The tissue was then dissected into small cubes of less than 2 mm using crossed scalpels. The cubes of tissue were placed in Falcon 10 cm diameter petri dishes (approximately 12 cubes per dish) and placed in a dry 37^{o}C incubator for 5 mins to allow the pieces of tissue to adhere to the plastic. Following this, 5 mls of medium were added to each petri dish, care being taken not to disturb the explants. The explants were fed twice weekly for two weeks to allow fibroblasts to grow out and keratinocytes to differentiate and die. After this time the fibroblasts were trypsinised and reseeded in 125 cm^2 flasks. At this stage aliquots of cells were frozen down in liquid nitrogen for future experiments.

6.7.4 Maintenance of Cells in Culture

Cells were fed twice weekly as described in Freshney (1987). Old medium was withdrawn into a buschner flask and fresh medium added. PalF cells were grown until just sub confluent when they were split and replated 1 in 10. Replating was performed as follows: medium was withdrawn and cells were washed in PBS. To a 75 cm^2 flask 1-2 mls of trypsin solution (pre-warmed to 37^{o}C) was added. Flasks were transferred to the 37^{o}C hot room until the cells had detached from the flask. The trypsin / cell suspension was then transferred to a sterile universal, 10 mls of complete medium added and the cells pelleted by centrifugation at 1000rpm for 10 mins. The pellet was then resuspended in fresh medium and the cells replated at the appropriate density.

6.7.5 Quercetin Initiation of Primary Fibroblasts

PalF cells were initiated with quercetin with a modified technique to that described by Sakai *et al* (1990). Actively growing PalF cultures were exposed to quercetin at one of the following concentrations: 0 μM (ethanol only), 5 μM, 20 μM or 45 μM. Cells were incubated for 48 hours after which the cultures were washed twice with fresh medium. The cells were then split and replated at appropriate density and allowed to settle for a further 24 hours before transfection by calcium phosphate precipitation.

6.8 DNA Transfection of Primary Bovine Cells

DNA transfection was performed using the calcium phosphate precipitation technique. PalF cells were trypsinised and seeded at a density of 5×10^5 cell in an 80 cm^2 flask. The cells were fed with 9 mls of growth medium 24 hrs prior to addition of the calcium phosphate-DNA precipitate.

The coprecipitate was formed as follows. DNA (20 µg) was diluted in 450 µl of 0.1 X TE pH8.0 and 50 µl of 2.5M $CaCl_2$ was added. This mixture was slowly added drop-wise to an equal volume of 2 X HBS (HEPES 50 mM, NaCl 280 mM, Na_2HPO_4 1.5 mM pH7.12) with gentle shaking. The mixture was then left for 30 mins to allow the precipitate to form. After this time the precipitate was slowly added to the flask of cells and the flask incubated overnight. After withdrawal of the medium the flask was washed twice in serum free medium and then fresh complete medium was added.

6.9 Selection of Transfected Cells

Following removal of the precipitate, cultures of transfected PalF cells were left in complete medium for 48hrs. The cultures were selected in medium containing 500µg /ml G418 for 21-28 days being fed twice weekly during this period. After this time G418 resistant colonies were scored and either picked or stained.

6.10 Isolation of Clonal Populations

Neomycin resistant clones were identified and marked using a permanent marker pen. The cells were then washed in sterile PBS. The top of the flask was removed using a red hot scalpel and the identified colonies were ringed using sterile 6mm stainless steel cloning rings with high vacuum silicone grease. 100 µl of trypsin solution was pipetted within the cloning ring using a micropipette, and the cell suspension was then transferred to a multiwell plate for expansion into cell lines.

6.11 Staining of Cells

Cells were washed twice in sterile PBS then fixed in methanol for 10 mins. The methanol was removed then 5-10 mls of freshly filtered Geimsa stain was added. The flask was shaken gently for 5-10 mins after which the Geimsa was removed and the flasks rinsed in tap water until it ran clear. The flasks were air dried overnight before being scored and/or photographed.

6.12 Growth of Cell Lines in Methocel

The extent of transformation of cell populations was assayed by their ability to grow in a methocel based medium. The medium was prepared as follows: 3 g of Methocel 4000CP (Fluka) was added to 200 mls of dH_2O and autoclaved. The methocel was left to dissolve with stirring for 2-3 days at $4^{o}C$. To the methocel solution the following was added: 22 mls of 10 X SF12 medium (Gibco), 4 mls of 50x essential amino acids, 4 mls of 0.1M sodium pyruvate, 2.5 mls of 0.2M glutamine, 5 mls of 7.5% sodium bicarbonate and 100 mls of foetal calf serum. Cells to be assayed were trypsinised and counted and 10^5 cells were added to 15mls of methocel mix in universals by shaking. The mixture was added to bacterial petri dishes to discourage cells adhering to the bottom of the dish and left at $37^{o}C$ for 7-10 days before being scored.

6.13 Storage of Cells under Liquid Nitrogen

Long term storage of cells under liquid nitrogen at $-196^{o}C$ retains growth viability and cell characteristics. Cells were trypsinised, pelleted and resuspended at a concentration of 10^6 cells /ml in growth medium. DMSO was then added at a concentration of 10% (v/v) to act as a cryoprotectant, inhibiting the growth of ice crystals. The cell suspension was transferred to plastic ampoules in 1ml aliquots, the ampoules wrapped in cotton wool, placed inside a polystyrene box and then placed in a $-70^{o}C$ freezer overnight to ensure a slow rate of cooling. The ampoules were then transferred to a liquid nitrogen storage rack and stored until required. Recovery of cells was performed by removing the ampoules from the nitrogen and immediately transferring them to a beaker containing water at 37^{o}C. After thawing the cells were pelleted by centrifugation then resuspended in fresh medium and transferred to a 80cm^2 tissue culture flask. The cultures were then left for 48hrs before being refed.

6.14 Athymic Mouse Tumorigenicity Assay

The malignant potential of a population of cells was assayed by subcutaneous injection into athymic nude mice. Cells were trypsinised, counted and pelleted. The cells were then resuspended in sterile PBS to give a concentration of 10^8 cells/ml. Mice (three per assay) were each injected with 0.1 mls of this suspension (10^7 cells) and examined for tumour growth at one week intervals following injection. If no tumours developed at the injection site by 15-20 weeks after injection the injected cells were considered to be non-tumourigenic.

6.15 Immunocytochemistry

To determine localisation of the E7 and E8 gene products, immunocytochemistry was performed. PalF cells were seeded out at $5x10^5$ cells per 75cm^2 tissue culture flask, left to settle overnight, washed and fed with fresh medium and then transfected by calcium phosphate precipitation. The medium was replaced 24 hours later. After a further 24 hours the cultures were washed in PBS, trypsinised, pelleted by centrifugation (1000 rpm for 5 mins) then resuspended in 1 ml of PBS. The suspension was counted using a haemocytometer and PBS was added to give a concentration of 2×10^4 cells / ml. 500 µl of this suspension was then spun onto glass slides using a Cyto-Tek cytospin centrifuge for 5 mins at 500 rpm.

Slides were air dried before being treated with goat serum for 1 hour to remove non-specific background. All antibody incubations were performed at room temperature in a humid box and slides were washed in freshly prepared TBS (0.5M Tris/HCL diluted 1/10 in 0.15M NaCl) for 5 mins between each incubation step. Positive controls used were anti-bovine MHC monoclonal antibody or anti-rat *ras* monoclonal antibody which is known to bind the bovine *ras* gene product. All antisera were raised in rabbits with the exception of the anti-*ras* antibody which was raised in mouse. As a negative control, no primary antibody was used (TBS only). Slides were treated with (in order); primary antiserum (1 hour), secondary anti-rabbit (or anti-mouse for *ras* detection) in the monoclonal antibody conjugated to an alkaline phosphatase enzyme (1 hour), alkaline phosphatase substrate (Vector laboratories) 30 mins. Slides were then washed, air dried and mounted using a water based resin.

6.16 Microinjection

Neighbouring cells can communicate with each other by allowing the passage of small molecules through specialised membrane structures called gap junctions. Cells which can communicate by allowing passage of molecules through these structures are said to be chemically coupled. Microinjection dye-transfer experiments can be used to see if a culture of cells are communicating in this way. A fluorescent dye, lucifer yellow, is injected into a single cell using microinjection apparatus. After some time the culture of cells can be examined under fluorescent light: the extent of spread of the injected dye from the original injected cell to its neighbours is a measure of the extent of dye coupling and hence junctional communication. Microinjection was kindly performed by Dr J.D. Pitts of the Beatson Institute.

Cultures to be examined were grown until almost confluent. A single cell was then iontophoretically injected using microelectrodes made from Quick-fil capillaries (1.2 o.d., Clark electro-Medical Instruments, Reading, England, with a tip resistance of 10 Mohm when filled with 3M KCL), back filled with a 4% aqueous solution of Lucifer Yellow CH (Sigma). Each injection was monitored using a Leitz Orthoplan microscope equipped with both UV and visible light sources. Iontophoresis was performed with the aid of a Neurolog system (Digitimer, Welwyn Garden City, England) using pulses of 20nA, 1 Hz and 500 ms duration for 5 mins. The injection site was then photographed under both visible and UV light.

6.17 Eukaryotic DNA analysis

Cell lines transfected with BPV-4 DNA were examined by preparing high molecular weight genomic DNA and examining this DNA for the presence of viral sequences by the technique of Southern blotting (Southern 1975). The principle of this technique is as follows. The DNA was digested with an appropriate restriction enzyme, separated on an agarose gel and then transferred to a nylon membrane. The membrane was then examined by treating with a radiolabelled single stranded BPV-4 DNA probe to allow the detection of viral sequences in the genomic DNA.

6.17.1 Preparation of Genomic DNA from Cell Lines

Genomic DNA was prepared from cell lines using a modification of the technique of Miller *et al* (1988). The cells from a confluent 75 cm^2 tissue culture flask were trypsinised, pelleted and then washed with PBS. The cell pellet was resuspended in 3 mls of lysis buffer (10 mM Tris-HCl, 400 mM NaCl and 2 mM Na_2EDTA, pH8.2) and digested overnight at 37^oC with 0.5 mls of a protease K solution (1 mg protease K in 1% SDS and 2 mM Na_2EDTA). After digestion, 1 ml of a saturated NaCl solution was added and the tubes shaken vigorously for 15 seconds. The samples were then spun at 2500 rpm for 15 mins to precipitate the protein pellet. The supernatant was carefully transferred to another tube and 2 volumes of absolute ethanol (room temperature) were added. The tubes were inverted several times until the DNA precipitated and the DNA strands were removed using a pasteur pipette. Excess ethanol was removed then the pellet was allowed to air dry before being resuspended in 100-200 µl of TE. The DNA was allowed to dissolve for 2 hours before being quantitated.

6.17.2 DNA Restriction and Agarose Gel Electrophoresis

Genomic DNA (typically 20 µg) was digested overnight with 100U of restriction enzyme (usually EcoRI) in the appropriate buffer supplied by the manufacturer. The digested DNA was then phenol / chloroform extracted and precipitated in ethanol. The DNA was then resuspended in 60 µl of gel loading buffer, loaded onto a 1% agarose gel and electrophoresed at 50V until the marker dye (bromophenol blue) had run approximately 2 cm from the bottom of the gel. To allow fragment sizes to be assessed, one lane of the gel was loaded with 1 µg bacteriophage lambda DNA cut with the restriction enzyme HindIII. The gel was then stained by the addition of ethidium bromide and photographed using a UV transilluminator.

6.17.3 Southern Transfer

The restricted genomic DNA was transferred from the gel to a nylon membrane using the blotting technique described by Southern (1975). The gel was placed in 0.25 N HCl for 10 mins causing partial depurination of the DNA. The gel was then transferred to a solution containing 0.5 N NaOH, 1.5 M NaCl for 30 mins. This allows hydrolysis of the DNA at the sites of depurination causing fragmentation of the DNA which in turn facilitates efficient transfer. Denaturation of the DNA also occurs with this treatment. The gel was then transferred to a neutralising solution of 0.5M Tris.Cl pH7.4, 3 M NaCl for 1 hour.

A large tray was filled with a reservoir of 20 X SSC transfer buffer (3 M NaCl, 3 M sodium citrate pH7.0). In this tray was placed an inverted baking tray such that the bottom of the tray was above the level of transfer buffer. A piece of 3M paper was then placed on the inverted dish so that the sides of the paper extended down into the reservoir and any air bubbles under the paper were removed by gently rolling a pipette over the paper. The gel was placed on top of the 3M paper and the area around the gel made water tight by placing pieces of X-ray film around it. This prevents "short circuiting" of the blotting apparatus so that the only transfer of buffer is through the gel. A piece of nylon membrane (Pal Biodyne) was cut to the size of the gel, pre-soaked in 2 X SSC and then placed over the gel. Air bubble were avoided by rolling a pipette gently over the gel surface. On top of the gel, three thicknesses of 3M paper were placed followed by a 10 cm thick pile of paper towels. An inverted baking dish was placed on top of the apparatus followed by a 2 kg weight. The apparatus was then left overnight. This process facilitates the transfer of gel buffer from the reservoir into the paper towels by capillary action, the DNA being transferred in the process. After transfer was complete the nylon membrane was baked in an 80^{o}C oven for 2hrs to cause the DNA to cross link to the membrane.

6.17.4 Radiolabelled Single Stranded DNA Probes

DNA probes for hybridisation were prepared using a "random prime" kit purchased from BCL which utilises the method of Feinberg and Vogelstein (1983). DNA probes were prepared from their parental plasmid by digestion with appropriate restriction enzymes, electrophoresis on a 1% agarose gel and then purification of the probe from the agarose slice using the "Geneclean" kit as per manufacturer's instructions. 50ng of the probe DNA was used per labelling reaction.

The DNA was made up to a volume of 9 µl with dH_2O and denatured by boiling for 10 mins followed by cooling on ice. 2 µl of reaction buffer was added (0.5 M Tris.HCl pH7.4, 50 mM $MgCl_2$, 2 M HEPES pH6.6, 120 mM ß-mercaptoethanol and 600 µg/ml random hexanucleotides) followed by 3 µl of labelling mixture (500 µM dATP, dGTP and dTTP). To this 1.85 MBq of [^{32}P-]dCTP was added followed by 1U of E.Coli DNA polymerase I (Klenow fragment). The mixture was incubated at 37^{o}C for 30mins. Unincorporated nucleotides were removed from the mixture by passing it through an Elutip-d column as described in the manufacturers instructions. Prior to use in hybridisation the probe was denatured by boiling for 10 mins.

6.17.5 Prehybridisation and Hybridisation of Membranes

To reduce non-specific binding of probe to membrane the membrane was prehybridised in 10 mls of prehybridisation solution [5 X Denhardt's (100 X Denhardt's solution is 2%(w/v) Ficoll, 2%(w/v) Polyvinylpyrrolidone and 2%(w/v) bovine serum albumin), 5 X SSC, 0.1% SDS and 500 μg/ml of denatured salmon sperm DNA] for 3 hours at 65^{o}C before the labelled probe was added. The hybridisation was then carried out overnight at 65^{o}C. Prehybridisation / hybridisation was carried out in heat sealed plastic bags, all air bubbles being carefully removed.

6.17.6 Washing of Hybridised Membranes and Autoradiography

In order to remove non-specifically bound probe, membranes were washed initially in a solution of 2 X SSC, 0.1% SDS for 10 mins at room temperature to remove unbound probe. The membrane was then washed with 0.1 X SSC, 0.5% SDS for 1 hour at 65^{O}C with changes of buffer every 15 minutes. After washing the filters were air dried then exposed to X-ray film at -70^{O}C.

Blots were stripped for reprobing by washing them for 1 hour in a stripping solution (0.5 x SSC, 0.1% SDS) at 90^{O}C) with changes of buffer every 15 mins. The blot was then autoradiographed overnight to ensure all the radioactive probe had been removed.

6.18 DNA Sequencing

6.18.1 Preparation of Plasmid for DNA Sequencing

Plasmids were sequenced using the "Sequenase" (tm) kit from USB. Manufacturers instructions were adhered to throughout. Plasmid (approx 4 μg) was diluted to a final volume of 18 μl before being denatured by the addition of 2 μl of 2 M NaOH. 8 μl of 5M ammonium acetate (pH7.5

filter sterilised) was added followed by 100 µl of ethanol. The plasmid was precipitated by spinning in a microcentrifuge for 15 mins at 4^{o}C then the ethanol was carefully removed using a micropipette. The pelleted plasmid was washed with 70% ethanol, air dried, then resuspended in 7 µl of water immediately before use.

6.18.2 Preparation of Synthetic Oligonucleotide Primers

Initial sequence was obtained using M13 "universal" forward and reverse primers as supplied in the "Sequenase" (tm) kit. This strategy allow for approximately 200-300 base pairs to be sequenced from each end of the insert. Further sequence was obtained by designing synthetic oligonucleotide primers of 15-20 base pairs to anneal near the ends of the sequence obtained using the universal primers. Oligonucleotides were synthesized using an Applied Biosystems oligonucleotide synthesizer. The synthesized oligonucleotide was removed from the cartridge by repeatedly passing 5mls of ammonia through the cartridge for 2-3 hours. The collected ammonia was left in a glass vial at 45^{o}C overnight, then the oligonucleotide was precipitated with ethanol. The pellet was resuspended in 1 ml of TE then the concentration of oligonucleotide was quantitated by spectrophotometer. The concentration of oligonucleotide was ajusted to 4 μg/μl by the addition of TE.

6.18.3 Sequencing Plasmid DNA

Plasmid DNA was sequenced using a "Sequenase" tm kit (USB) which employs the dideoxy sequencing technique of Sanger et al (1977). To denatured plasmid DNA (4 μg in 7 μl TE) 2 μl of "Sequenase" buffer and 1 μl (4ng) of appropriate primer were added. The primer was allowed to anneal to the template by heating to 65°C for 2 mins then allowing to cool slowly. "Sequenase" labelling mix (dGTP) was diluted 1:5 in dH^2O. "Sequenase" enzyme was diluted 1:8 in ice-cold TE. To the annealed DNA mixture the following was added: 10 μl of DTT (0.1M), 2 μl of diluted "Sequenase" labelling mix, 0.5 μl of [^{35}S] dATP and 2 μl of diluted "Sequenase" enzyme. The reaction was mixed and allowed to stand at room temperature for 5 mins. The labelling reaction was then terminated by transferring 3.5 μl of the labelling reaction to separate eppendorf tubes containing 2.5 μl of either G, A, T or C "Sequenase" termination mixes. These tubes were then incubated at 37°C for five mins. The reaction was stopped by adding 4 μl of "stop" solution. The samples were heated to 75°C for 2 mins immediately before loading. Samples were run on a 6% acrylamide gel as described in Sambrook *et al* (1989). Following electrophoresis the gel was fixed for 30 mins in a 10% methanol / 10% acetic acid solution. The gel was then transferred to a piece of filter paper and dried using a gel drying apparatus. Dried gels were then autoradiographed.

6.18.4 Computer Analysis of DNA Sequence Data

Sequence data were aligned and compared to other sequences using the Genetics Computer Group sequence analysis software (University of Wisconsin Biotechnology Centre, Madison, WI).

7. Results and Discussion

7.1 DNA Sequencing

The six bovine papillomaviruses isolated to date have been divided into two subgroups: Subgroup A (BPVs type 1,2 & 5) cause fibropapillomas while the Subgroup B viruses (BPVs 3,4 & 6) cause true epithelial papillomas. An important transforming gene in the well characterised BPV-1 virus and in the oncogenic human papillomaviruses is E6. Previous sequencing analysis of BPV-4 predicted that no E6 ORF is present (GenEMBL Accession No. X59063) making BPV-4 unique among sequenced papillomaviruses in this respect (Jackson et al, 1991). The significance of the absence of E6 in BPV-4 is exemplified by the fact that integrity of the BPV-1 E6 is essential for the development of a fully transformed phenotype (Neary and DiMaio, 1989). In order to address if the lack of an E6 ORF was a general feature of the subgroup B BPVs, molecular cloning of the L1 to E1 genome areas of BPV-3 and BPV-6 was performed and followed by DNA sequence analysis .

BPV-3 Sequencing: The appropriate region of the BPV-3 genome was subcloned from the pBV3 plasmid which is described in Coggins et al, 1983. The 950bp EcoRI to HindII B' and the 250bp BamHI D fragments of pBV3 were isolated by restriction enzyme digestion and subcloned into pAT153 (Twigg and Sherratt, 1980). Each recombinant was sequenced initially using pBR322 multiple cloning site sequencing primers. Synthetic oligonucleotides were used to extend sequence readings as described in the Materials and Methods section and the sequence was extended further across the LCR and into the L1 region by sequencing using synthetic primers designed to anneal to the parental pBV3 plasmid. Sequencing reactions were performed on one strand only, but a minimum of three gel runs were performed for each primer to ensure the reading of sequence was accurate.

BPV-6 Sequencing: BPV-6 DNA was subcloned from the pBV6 plasmid as described in Jarrett et al 1984. The 1.4kb BamHI fragment of pBV6 was subcloned into pIC20H (Marsh et al 1984). Initial sequence was obtained with M13 forward and reverse primers. The sequence was extended using synthetic primers as in the BPV-3 sequencing and part of the E1 ORF sequence was obtained by sequencing the parental pBV6 plasmid. As in the case of BPV-3, sequencing was usually on one strand only, but several gel runs were performed using each primer to ensure accuracy of sequence.

The sequences of the subgroup B BPVs were found to have strong homology throughout most of the region analysed (**Fig 7.1.**). The E7 and E8 ORFs and part of the E1 and L1 ORFs of BPVs 3 and 6 were identified by the homology of their putative translation products to those of BPV-4 (**Fig 7.2.**). The sequence homology within the E8, E7, E1 and L1 ORFs is high, with only a few deletions or insertions of exactly three or six nucleotides maintaining the reading frames. Similarly, the LCR region, in general the least conserved region between different PV types, shows considerable sequence identity among the three subgroup B BPVs (**Fig 7.3.**). Open reading frame analysis demonstrates that the subgroup B BPVs have no ORF resembling an E6 between the E8 and E7 ORFs (**Fig 7.1.; Fig 7.4.**). Indeed sequence homology between the three viruses breaks down in this region and large deletions or insertions are found suggesting that this region is not used to encode a function common to all three viruses, an argument strengthened by the three viruses possessing strong conservation of sequence in all other regions examined (E1, E7, E8, L1 and LCR) in the three viral genomes.

The ORF analysis of the subgroup B viruses (**Fig 7.4.**)

shows the existence of a number of small ORFs with potential coding capacities of between 36 and 46 amino acids from the first methionine codon. Although the "a" and "b" ORFs of BPV-4 and BPV-6 appear unrelated in terms of sequence data, the "c" ORFs of BPV-3 and BPV-4 have considerable homology, and in the same position in BPV-6 there is a short ORF of 25 codons from the first methionine codon with homology to the other "c" ORFs (Fig 7.2.**c.**). As the first five codons of the putative "c" ORFs encode the C-terminal four amino acids and stop codon of the E8 ORF, the sequences are necessarily constrained in this region. Nucleotide and amino acid homology breaks down after this short region however, with large deletions/insertions prohibiting the existence of an E6 ORF (**Fig 7.1.**).

Fig 7.1. Nucleotide homology between B group BPVs.

```
      1                                                  50
Bpv3  CGCACCGTTT GAGGTACATA TAAAAGG.CT GCAGCTCACT TTCACTACAT
Bpv4  CGTACCGAAT CGGGTGCATA TATAAGGAGA GCAGTGCGGA TTTGGTGCAT
Bpv6  TGCACCGTTT CAGGTCGATA TAAAA....A GCAGACAAAG CTAGTTTCTG
Cons  -G-ACCG--T --GGT--ATA TA-AA----- GCAG------ -T---T-C--

      51                                                100
Bpv3  GGACCTTTGG GCTCCATCTG CATTATGAGC TTGACTCTAA TTTACTGGTT
Bpv4  GAGGCAGTAG CTCTCATC.. ....ATGTCT TTGTGGCTTA TCTATGTTTT
Bpv6  TGGAGCTTAG GTTCCATC.. GATCATGCGC TTAACTCTGA TCTTTTGGTT
Cons  -------T-G ----CATC-- ----ATG--- TT----CT-A T-T-----TT

      101                                               150
Bpv3  ATTGCTGCTC TGGGTTTCAT TTCACTTTCT GTCATTGTGC CTTGCTATTA
Bpv4  GTTGCTTTTC TGGTGTGCTT TTAATTTTCT TGCACTGTTA TTTGCAATTA
Bpv6  GTTGTTGCTC TGGTGTGGGT TTCACTTGGC TGCTCTCTGC ATAGCCATTA
Cons  -TTG-T--TC TGG--T---T TT-A-TT--- --C--T-T-- -T-GC-ATTA

      151                                               200
Bpv3  TATTGTATTT GCTGTTAATG TCTACTATTA CTAGTTTACA TGGATGGGAT
Bpv4  TTGTGTACCT TCTGTTAATT TCTACTATTA CTCGGTTAGA TGGATGGGAT
Bpv6  TTCTTTTTTT GCTTTTACTA TCTGCTATTG ATGAACTAAA TGGATGGGAC
Cons  T--T-T---T -CT-TTA-T- TCT-CTATT- -T----TA-A TGGATGGGA-

      201                                               250
Bpv3  TGAAGCTGTT TTATTTATAC TGTTAATCAT CTTTATTTGC GA.CGCGATT
Bpv4  TGAATTTTTA TTTATTGTGT TTATTATTTG CTTGATTTCT GATTTGCTTT
Bpv6  TGAATCGCAC ATGTTTGTAT TACTATTGAT ATTGGTTTGT GATCGATTTT
Cons  TGAA------ -T--TT-T-- T--T--T--- -TT--TTT-- GA------TT

      251                                               300
Bpv3  TTCTTGAATG TTAGGGAGTT TCTGCAGACA TTGCTGTTTT TGTCTTTGTT
Bpv4  TG.CTATATA TAGTGGAGAT TATGCGTTTG GTGTTATTGC TGTCTTTGCT
Bpv6  TGTTTAAATG TGCATGATTT GTTAGAGGGT TTCGTGTTGC TTTCTGTATT
Cons  T---T--AT- T----GA--T --T------- -T--T-TT-- T-TCT-T-

      301                                               350
Bpv3  ATATGGGGAG GACTAGCTTG .......... .......... ..........
Bpv4  AGATGAATTA GAACAGGAGG AACAGTAAAT AGAATATTGC TGTGCTTTTA
Bpv6  AGTTGGTGAC GAGGA..... .......... .......... ..........
Cons  A--TG----- GA--A----- ---------- ---------- ----------

      351                                               400
Bpv3  ...TTAACAC CTTAAGGGTT TGGCCACTTA TAATAGCTGA CCTTTGCTCT
Bpv4  CATTTGAGAA GGATTGTAGG TGTGGGCATT TAAAAGCTGA CCTTCCAGTC
Bpv6  .......... CTAGTTTAGG TGTGGACATT TAAGAGCAAT CTTGACCTGT
Cons  ---------- ---------- TG----C-T- TAA-AGC--- C-T-------

      401                                               450
Bpv3  GCAATT..AA GTGGCGTCTT AAAGGGAGTT ATTTAAGCCG TGTAGAGTTT
Bpv4  TTAATTGCAG TAGGCGCCTA AGAGGGTGGT GGTGGTATAA GTTCAAGTTT
Bpv6  GTAATTAAAA AAGGCGCTTA A......... .........G TGTAGCGT..
Cons  --AATT--A- --GGCG--T- A--------- ---------- --T---GT--

      451                                               500
Bpv3  TGCAATTAAA AATGAAGGGC CAGGACGTGA CTTTGAAAAA CGTTGCTGTA
Bpv4  TTCAA..GAA AATGAAGGGG CAGAACGTGA CATTACAGGA CATTGCAATA
Bpv6  ....CAGAAA AATGAAGGGC CAGAGCATGA TTTTGAAAGA CTTAGCTGCA
Cons  --------AA AATGAAGGG- CAG--C-TGA --TT--A--A C-T-GC---A

      501                                               550
Bpv3  GAATTAGAGG ATGTAGTCAG TCCAATTATA TTGGATTGCG AGGAGGAGAT
Bpv4  GAATTAGAGG ATACAATTAG TCCAATTAAC TTGCATTGTG AAGAGGAGAT
Bpv6  GAATTAGAAG AGGTAGTCAG TCCAATTAAC TTGGACTGTG AAGAAGAGAT
Cons  GAATTAGA-G A---A-T-AG TCCAATTA-- TTG-A-TG-G A-GA-GAGAT

      551                                               600
Bpv3  AGAAACTGAG GAAGTAGACT GTCCGGCCCC GTAT...GCT GTAGAGGCTG
Bpv4  TGAAACTGAG GAGGTGGACA CCCCTAACCC TTTTGCAATA ACAGCAACT.
Bpv6  TGCAAATGAG GAAGTTGACT GTCCTGTCAC CTTTTGCTTA GTTGAAGCTG
Cons  -G-AA-TGAG GA-GT-GAC- --CC---C-C -T-T------ ---G---CT-

      601                                               650
Bpv3  TTTGTTATGT TTGTGAAAAC CCCTTACGTT TAGCTCTCGT CTCCTCACCG
Bpv4  ..TGTTATGC TTGCGAGCAA GTCCTTCGTT TAGCTGTTGT AACGTCAACA
Bpv6  TGTGTCATGT TTGTGAACAA GTTTTGCGTT TAGCCGTCGT TGCCTCGCCA
Cons  --TGT-ATG- TTG-GA--A- ----T-CGTT TAGC--T-GT --C-TC--C-

      651                                               700
Bpv3  GACGGGATCC ATCAACTGCA TCAA...CTG CTGTTGGACT GCATCTCCTT
Bpv4  GAAGGAATTC ATCAACTGCA GCAA...CTG CTGTTCGACA ACCTCTTTCT
Bpv6  GACGGGATAC TGCAATTGCA GCAACTCCTG CTGACGGACT CCCTGTCGTT
Cons  GA-GG-AT-C --CAA-TGCA -CAA---CTG CTG---GAC- -C-T-T---T

      701                                               750
Bpv3  ACTGTGTGCA AACTGCTCTA GAGAGGTCTA CTCTAACCGC AGACCCCAAC
Bpv4  ACTGTGTGCA GCTTGCTCCA AACAAGTGTT CTGTAACCGC AGACCCGAGC
Bpv6  TCTGTGTACA AGCTGCTCTA GAGAAGCGTT CTGTAACCGC AGACCACAAC
Cons  -CTGTGT-CA ---TGCTC-A -A-A-G--T- CT-TAACCGC AGACC--A-C

      751                                               800
Bpv3  GAAATGGACC CTAA...... AGGTATTACC GGGCAAAGTT TTCTTGACGA
Bpv4  GAAATGGACC CTAA...... AGGTACTACC GTGCTTGACT TTATTGAGGA
Bpv6  GCAATGGATC CTAATGAAAA AGGTATTGAA GTGCTGAGTT TTATAGATGA
Cons  G-AATGGA-C CTAA------ AGGTA-T--- G-GC-----T TT-T-GA-GA

      801                                               850
Bpv3  CCAAGCAGAA TGTAGTGAAT CTGATAATAG CGAGCAAGGG TGTGAAGAAA
Bpv4  ACAAGCAGAA TGTAGTCAAT CTG...ATAG CGAGCAAGGC AGTGAAGAAA
Bpv6  ACAAGCAGAA TGTAGAGGGT CAG...ATAG CGAGGAAGGG TATGAAGAAA
Cons  -CAAGCAGAA TGTAG----T C-G---ATAG CGAG-AAGG- --TGAAGAAA

      851                    874
Bpv3  GCCTGTCTGA TTTGTCTGAC CTAA
Bpv4  ATTGTTCTGA TATATCTGAC CTAA
Bpv6  GCCAGTCTAG TCTGTCCGAC CTAA
Cons  -----TCT-- T-T-TC-GAC CTAA
```

Comparison of E8 to L1 regions of the subgroupB BPVs. The E8 and E7 encoding regions are indicated in bold type and the initiation codons for thw "c" polypeptides and E1 are underlined. Dots represent insertions/deletions and the consensus indicates regions of homology between the viruses. The regions of sequence analysed are BPV-3 697-1521, BPV-4 264-1113 and BPV-6 747-1524.

Fig 7.2. B group BPVs putative translation product homology.

A

```
            1                                                               60
BPV3.E7     MKGQDVTLKN VAVELEDVVS PIILDCEEEI ETEEVDCPAP YA.VEAVCYV CENPLRLALV
BPV4.E7     MKGQNVTLQD IAIELEDTIS PINLHCEEEI ETEEVDTPNP FA.ITATCYA CEQVLRLAVV
BPV6.E7     MKGQSMILKD LAAELEEVVS PINLDCEEEI ANEEVDCPVT FCLVEAVCHV CEQVLRLAVV
Consens     MKGQ---L-- -A-ELE---S PI-L-CEEEI --EEVD-P-- -----A-C-- CE--LRLA-V
                 ......SV40 LT/Ad E1A homology......                CXX C

            61                                  100
BPV3.E7     SSPDGIHQLH QLLL.DCISL LCANCSREVY SNRRPQRNGP
BPV4.E7     TSTEGIHQLQ QLLF.DNLFL LCAACSKQVF CNRRPERNGP
BPV6.E7     ASPDGILQLQ QLLLTDSLSF LCTSCSREAF CNRRPQRNGS
Consens     -S--GI-QL- QLL--D---- LC--CS---- -NRRP-RNG-
                                   CXXC
```

B

```
           -57                                                          -1
BPV3.E8     anctvcgavshtkvsvvinnslfnMlllapdsvapfevhikgcsslslhgplgsici
BPV4.E8                                 gkyqlhpirsyrigciykessadlvheavali
BPV6.E8                                                             vpsi

            1                                  42
BPV3.E8     MSLTLIYWLL LLWVSFHFLS LCLAIILYLL LMSTITSLHG WD
BPV4.E8     MSLWLIYVLL LFWCAFNFLA LLFAIIVYLL LISTITRLDG WD
BPV6.E8     MRLTLIFWLL LLWCGFHLAA LCIAIILFLL LLSAIDELNG WD
Consens     M-L-LI--LL L-W--F---- L--AII--LL L-S-I--L-G WD

            1                                          44
BPV1.E5     MPNLWFLLFL GLVAAMQLLL LLFLLLFFLV YWDHFECSCT GLPF
```

C

```
              1                                            45
BPV3.ORFc     MDGIEAVLFI LLIIFICDAI FLNVREFLQT LLFLSLLYGE D*
BPV4.ORFc     MDGIEFLFIV FIICLISDLL LLYIVEIMRL VLLLSLLDEL EQEEQ*
BPV6.ORFc     MDGTESHMFV LLLILVCDRF LFKCA*
Consensus     MDGIE---FV LLIILICD-- LL---E---- -L-LSLL--- -----

HPV8 E6       MDGQDKASYS LDTNKDELPS TIKELAAALG IPLQDCSVPC NFCGN...
```

Notes: A) Comparison of the E7 products of BPVs 3, 4 and 6 showing conserved **Cys-X-X-Cys** motifs and region of homolgy to SV40 LT/ Adenovirus E1a (ie potential p105^{Rb} binding site). Consensus indicates amino acids common to all three proteins. **B)** Putative E8 comparison. Upstream sequences to the proposed product (upper case) are shown in lower case letters and the first methionine of BPV-3 E8 is undelined and in uppercase. Amino acids common to all three products are given in the consensus sequence. **C)** Comparison of the potential products of the putative "c" ORFs of the B group BPVs with the E6 of HPV-8. The consensus indicates identical amino acids shared by two of the three ORFs. * represents a stop codon.

The protein encoded by the E7 ORF of all three viruses contains the putative p105^{Rb} binding domains and the two **Cys-X-X-Cys** zinc binding motifs but lacks the CKII phosphorylation site in common with CRPV, HPV-5 and HPV-8 (**Fig 7.2.a**). The consequences of the absence of the CKII phosphorylation site will be discussed in more detail later in this thesis.

The E8 ORFs of all three viruses share a common methionine codon, with BPV-3 having an additional methionine codon 5' to the common one (**Fig 7.2.b**). If translated from the first AUG codon, the putative BPV-3 E8 peptide would be extended by 33 residues. However, the amino acid homology breaks down sharply upstream of the common methionine, suggesting that translation begins at the common AUG and that the major product from this region is 42 amino acids long in all three viruses. This protein is 52% homologous between the three viruses. All three of the postulated peptides are highly hydrophobic with a leucine content similar to BPV-1 E5 (BPV-3 E8 33%, BPV-4 E8 29%, BPV-6 E8 33%, BPV-1 E5 34%). Indeed when the hydrophobicity of the three E8 peptides and the BPV-1 E5 peptide are compared (Kyte and Doolittle, 1982) a striking similarity is observed (**Fig 7.5.**) suggesting that the subgroup B E8 peptides and BPV-1 E5 may share a common function.

Fig 7.3. LCR homology between B group BPVs.

```
      1                                                    50
Bpv3  AATACAGATA CCTACAGTCA ATTGCAACTA GATGCCCCCC TAAAGAAGAT
Bpv4  ACTACAGATT TATTACCTCA TTAGCTACTA GATGCCCCAA AAAGACAGAC
Bpv6  AGTACAGGTT TATTAAGTCT TTAGCCACTA AATGCCCTGG TAAAGAGGAA
Cons  A-TACAG-T- --T----TC- -T-GC-ACTA -ATGCCC--- -AA----GA-

      51                                                  100
Bpv3  GCTGCTGCAA CTGAGGACCC TTATGCAAAG TACACATTTT GGGATGTGGA
Bpv4  ACTCAGGTTA AAGAAGACCC CTATAAAGAC CTAAAGTTCT GGGACGTGGA
Bpv6  ACTTGCAGAA AAGAAGACCC ATATGCTAAA TATAAATTCT GGGATGTTAA
Cons  -CT------A --GA-GACCC -TAT----A- ---A--TT-T GGGA-GT--A

      101                                                 150
Bpv3  CCTTACAGAA CGATTTTCTA TGAATTTAGA TCAGTATTCC TTAGGTAGAA
Bpv4  CCTAACAGAA AAGTTTACCA CAAACCTGAA CCAGCACTCC CTGGGTAGAA
Bpv6  CCTAACAGAA AGGTTTCTTC TAATTTGGAA CATGTACT.. .TGGGTCGCA
Cons  CCT-ACAGAA ---TTT---- --A------A ---G-A-T-- -T-GGT-G-A

      151                                                 200
Bpv3  AGTTCTTATT TCAAATAGGA AAGAAAAGCA GAGGAATCAA ACGGTCTGCA
Bpv4  AATTTTTGTT CCAGATAGG. .....TAGAA AAGCTACCAA ACGCTCTGCA
Bpv6  ATTTTTTGTT TCAAATAGG. .....TAAAA GAGGTAGCAA ACGGCCTGCA
Cons  A-TT-TT-TT -CA-ATAGG- ------A--A -AG--A-CAA ACG--CTGCA

      201                                                 250
Bpv3  CCGAAAGCGG TCACTTTTGA AAGTAGCAGC CGCAGCAAAA AGGCGCCAAA
Bpv4  CCGAAAACGG TCACATTTGA AAAT...ACT GAAGGTAAAA AGGCGCCAAA
Bpv6  CCGAAAACGG TCACCTTTG. ..ATAGCAGT ...AGCAAAA AAGCGCCAAA
Cons  CCGAAA-CGG TCAC-TTTG- ---T---A-- ----G-AAAA A-GCGCCAAA

      251                                                 300
Bpv3  GCGTAGGCGT AAGAATGTCT AG.AGCCAAA ACTATGAGCA ATATGTACAT
Bpv4  GCGTAGGCGG AAAAATGTGT AGCTGCCAAC ACCTGCAGCA GTATGAGCAA
Bpv6  GCGTAGGCGG AAAAATGCAT AACTGCCAA. GTGTGAATGA GCAACATGTA
Cons  GCGTAGGCG- AA-AATG--T A---GCCAA- ------A--A --A-------

      301                                                 350
Bpv3  CAACAACAAG CATTGACAAA GAA...TAAA TTCATGTAAG CCTTTGTGAA
Bpv4  TATG...... .....ATAAA TAAACTTTTC TTTATGTAAG A...TATGAA
Bpv6  AATGTTTTGC TGCACACACC TATAATTCTT TTTGTGCAAG ACTTTGTGAA
Cons  -A-------- -----A-A-- -A----T--- TT--TG-AAG ----T-TGAA

      351                                                 400
Bpv3  TCTTGCCAAT AAAATGCTGA TCTGAGCCAC CCTTCTTATC TTCCTTGTGA
Bpv4  TGTTGGCAAT AAAA...TGC AGACAAACAA AGCACTGTTA TCTTTGGTTT
Bpv6  TCTTCCAAAT AAAATGTTGC GGTTATACAC CCTCCTTATC TCCTTTGATT
Cons  T-TT---AAT AAAA---TG- ----A--CA- ----CT--T- T---T-G---

      401                                                 450
Bpv3  TTTGTTACCC CCTCCCAAAT ATTCGTGCAA AGCGGTGCAC CTGGTTGAAC
Bpv4  TTTTTC...A TCTCCGGCAC TTGTGTGTTT GTGCAAGCAC CCGCCT.TGC
Bpv6  TTTTTCCAGC CCTTCTCCCA GTGTTTGCAA AACTGTGCAC CTG.GTGTGC
Cons  TTT-T----- -CT-C----- -T---TG--- ------GCAC C-G--T---C

      451                                                 500
Bpv3  ACCTGCATGC TTAATAAACC TTAAAGTATG CAGAGGCACA CCTAAGTAAA
Bpv4  ACCTGGACGC TGTGTAAAGC AAAAACTATG CAGGCACACC TAAAGGCTGT
Bpv6  ACCTGGCTGC TTTCTAACAC GGAAACTATG .TGGCACACC CTAATATTTC
Cons  ACCTG---GC T---TAA--C --AAA-TATG --G---CAC- ---A------

      501                                                 550
Bpv3  CACCTTTAAG G..TTATTGG CTCCAAAAAA GCTGCAAGCT GCCAAGCCAA
Bpv4  CAACTTTGAA GTTTTATTGG CCACAAAATT AATTGGCCGA GCCAAGTTGG
Bpv6  TTTTTTGCAA GTTTTATTGG CTCTAAAACA TAATTGCATT GCCAACTTGG
Cons  ----TT--A- G--TTATTGG C---AAAA-- ---------- GCCAA-----

      551                                                 600
Bpv3  GCTATAAAAA AGTACTCAAA AGCACATACT GGCAGTACGC CCGGGCAAGC
Bpv4  CATATAAAAC TGTTCTTGAA TAGTCACACT GGCTTTTGGC CATAACAAGC
Bpv6  C...TATAAA AGTTACTTAG AAGCAGCGGC TGGCGTACCG CCAGACAAGC
Cons  ----TA-AA- -GT-----A- ---------- -G---T---- C----CAAGC

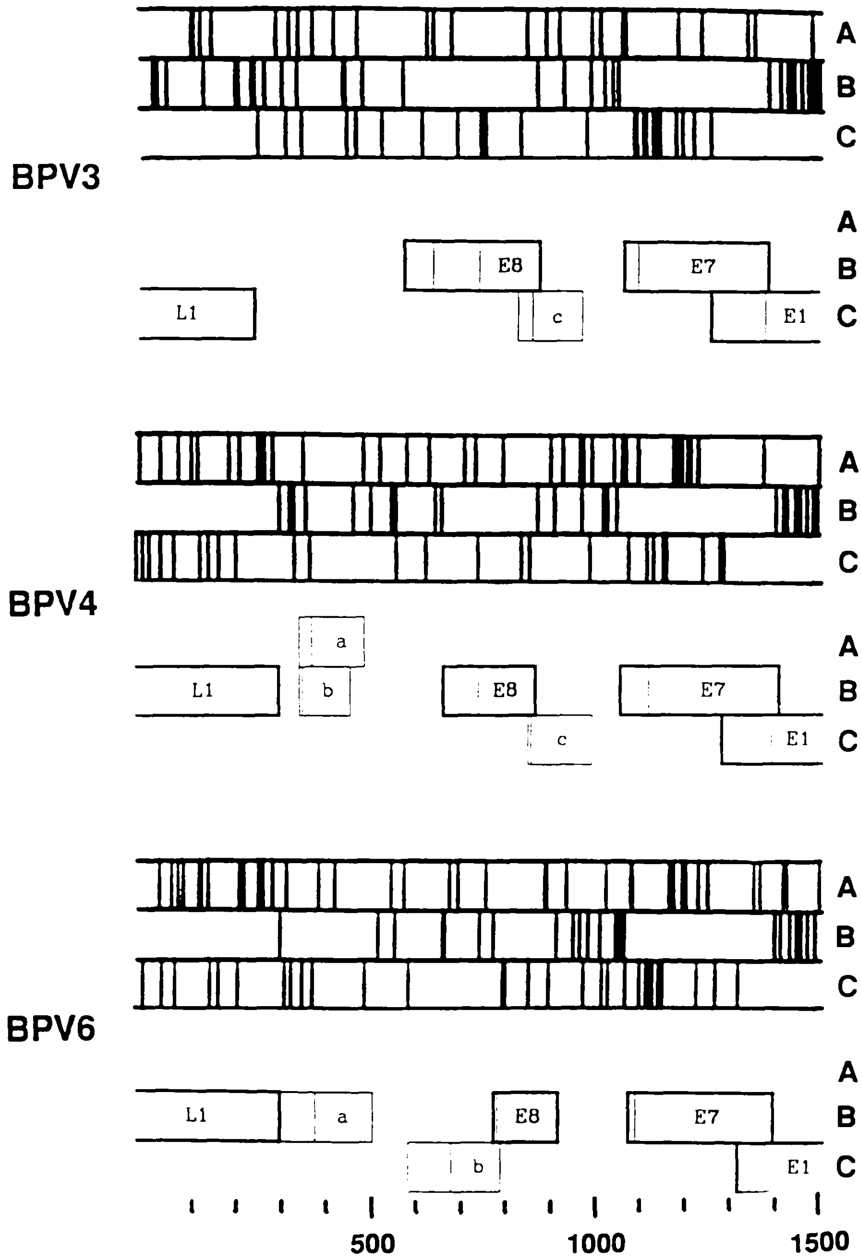
      601                                                 650
Bpv3  TTAGGCAAAC TGTACCGTTT GCGGTGC.AG TTTCTCACAC AAAGGTAAGT
Bpv4  TCAGACAAAG TGTACCGATT GCGGT.CGAA ACTCTCACGC TAGGTAAGT.
Bpv6  ACAGACAAAA CATACCGGAT TCGGTGCACC GCTCTCACGC TTAGTAAGTC
Cons  --AG-CAAA- --TACCG--T -CGGT-C--- --TCTCAC-C ---G--A---

      651                                                 700
Bpv3  GTTGTTATTA ACAAC.AGTC TCTTTAATAT GTTATTACTT GCACCCGATT
Bpv4  GTTGTACCTA ACAACTATTT ACCTAGGGAA AATATCAGTT GCATCCCATT
Bpv6  ATTATTATTA ACAACTATGT ..GTTGTATA AATTATTACA GTATCGAATA
Cons  -TT-T---TA ACAAC-A--- ---T------ --T------- G-A-C--AT-

      701                              731
Bpv3  CGGTCGCACC GTTTGAGGTA CATATAAAAG G
Bpv4  CGGTCGTACC GAATCGGGTG CATATATAAG G
Bpv6  CGGTTGCACC GTTTCAGGTC GATATAAAAA G
Cons  CGGT-G-ACC G--T--GGT- -ATATA-AA- G
```

Comparison of the LCR regions of the subgroup B bovine papillomaviruses. The 3' portion of the L1 ORF is shown in bold type. The numbering used is arbitrary and does not refer to specific map coordinates.

Fig 7.4. ORF analysis of B group BPVs.

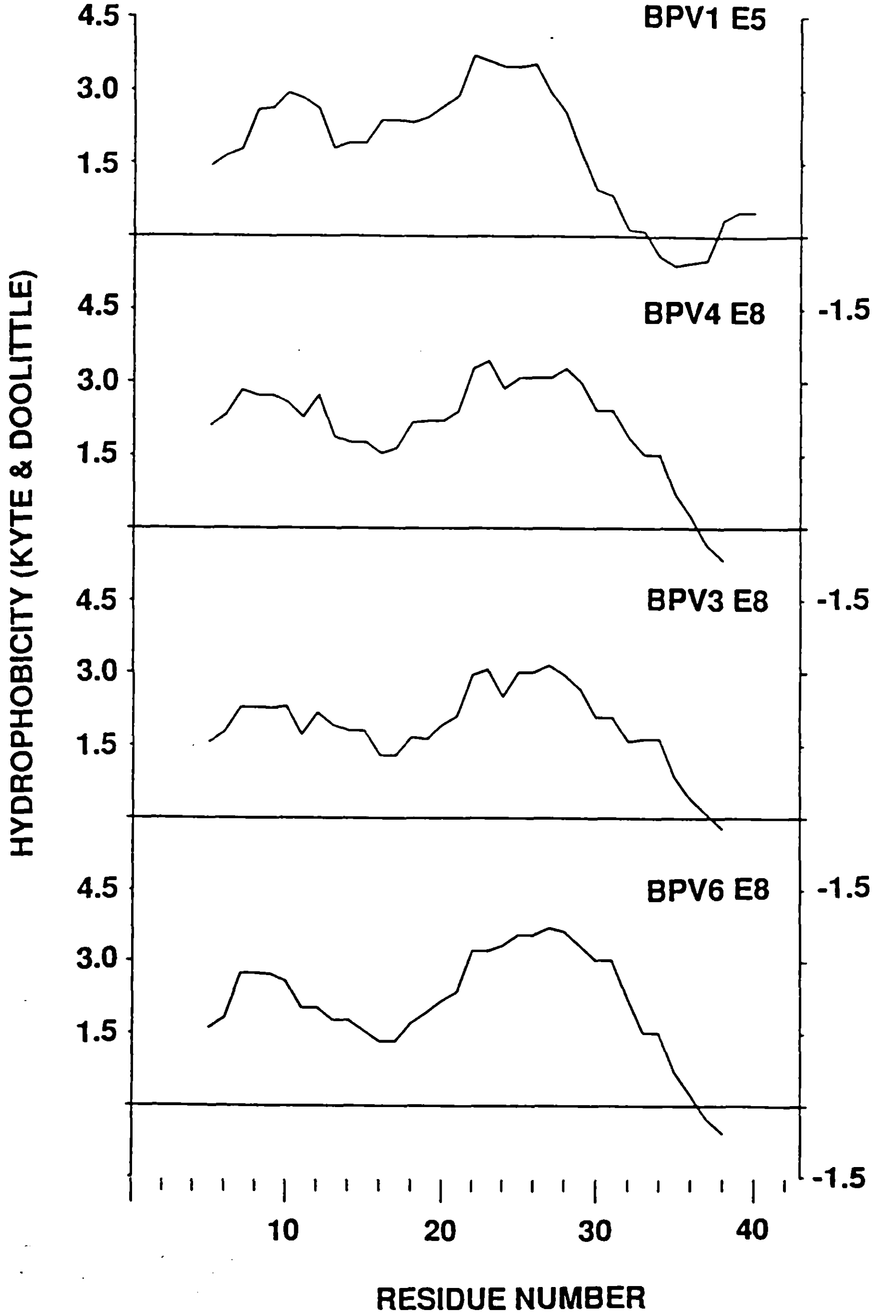


ORF analyses for the L1 to E1 regions of BPVs 3, 4 and 6. Stop codons in all three reading frames (A, B and C) are given by vertical lines. Below each stop codon analyses is a representation of the ORFs where the first ATG is indicated by a vertical line. Only ORFs of greater than 35 amino acids from the first ATG are shown. Major ORFs (whose products are identifiable papillomaviral proteins) are shown with bold lines, minor ORFs by finer lines. Nucleotides 6789-1112 of the BPV-4 sequence are displayed with the corresponding areas of the other two viruses.

The BPV-1 sequence **V-X-W-D-X-F-X-C-X-C,** shown to be crucial for transformation is absent from the putative E8 peptide of BPVs 3, 4 and 6. The E5a peptide of HPV-6 however, has been shown to have transformation activity despite lacking this sequence, although it does contain a **C-X-C** motif in the C-terminus (Chen and Mounts 1990). The similarity of BPV-4 E8 to the BPV-1 E5 protein suggests a possible role of the E8 peptide in the transformation mechanism of BPV-4. This question will be addressed more fully both in transformation assays (Section 7.2.) and in possible interactions between BPV-4 E8 and cellular proteins (Section 7.4.).

As mentioned previously, the E6 gene is important in the transformation mechanism of the oncogenic human papillomavirus types. The mode of action of the E6 protein in these viruses has been shown to be the complexing with, and subsequent degradation of, the p53 tumour suppressor gene product, thus deregulating the cell cycle (Werness et al, 1990; Scheffner et al, 1990). The observation that both the E7 and E6 genes are often maintained and transcribed in papillomavirus associated carcinomas is evidence for the role of the E6 in tumorigenesis *in vivo* (Schwartz et al, 1985). The lack of an apparent E6 function raises interesting questions

Fig 7.5. Hydrophobicity plots (BPV-1 E5 / B virus E8s).



Kyte and Doolittle hydrophobicity plots for the E5 protein of BPV-1 and the proposed E8 proteins of the subgroup B BPVs. The residue specific hydrophobicity indices were averaged over nine amino acids. Regions above the x-axis (positive) are hydrophobic, those below the axis (negative) are hydrophillic. Average hydrophobicity values of > +1.6 are indicative of membrane-integrated domains.

regarding the transformation biology of BPV-4 both *in vivo* and *in vitro*. A viral E6 function is obviously not required for virus infection and propagation leading to the conclusion that either the subgroup B BPVs do not require E6 functions, or that these functions are provided by other viral or host proteins. It may prove interesting to examine the status of the bovine p53 gene and its expression in papillomas and carcinomas. Recent work on anogenital lesions has shown that the progression of squamous cell carcinoma (SCC) requires p53 dysfunction either through binding and degradation by HPV E6 or by mutation in the host p53 protein in HPV negative lesions (Crook et al, 1991a, b). If p53 dysfunction is crucial in the development of alimentary canal SCC in cattle, then p53 mutations might be expected to be found in papillomas and especially carcinomas. At present nothing is known about the bovine p53 gene or protein, but work is currently underway using PCR primers against the conserved consensus regions of the human p53 gene to attempt to sequence the bovine p53 homolog and examine its status in normal, papilloma and carcinoma biopsies (M. Jackson, personal communication). Another question is whether the introduction of an E6 function from a heterologous virus could enhance the transformation potential of BPV-4 in *in vitro* transformation assays. This point was addressed experimentally, and will be considered later in this thesis.

7.2 Transfection of PalF cells

The transforming properties of BPV-4 were investigated by transfecting various constructs containing cloned BPV-4 sequences into PalF cells. All the plasmids used in these studies are described in the Materials and Methods (Section 6.3.5) and a summary of the plasmids is given in **Figures 6.1.** and **6.2.** A cooperating *ras* gene function was provided by the pT24 plasmid. Transformation was assessed by using a neomycin resistance assay. Where G418 resistance was not conferred by the plasmids being used, the pZipneo plasmid was cotransfected at a ratio of one to ten of the other transfected constructs. PalF cells were explanted from fetal palate tissue and grown in 10% FCS DMEM medium, cells being split before reaching confluence. Early passage cells were transfected using the calcium phosphate transfection protocol, then selected in G418 containing medium as described in the Materials and Methods section. The susceptibility of PalF cells to G418 has previously been assessed (Jaggar, 1990; Jaggar et al, 1990) and 500 µg/ml was found to be suitable. Individual experiments were scored on the number of G418 resistant macrocolonies (greater than 5mm in diameter) present at the end of the 21-28 day selection period. Small G418^r microcolonies of less than 5mm were observed in all transfections. These small colonies had a flat morphology and appeared identical to untransfected PalF cells. Expansion of these colonies proved to be impossible, except as a pool of colonies from a single flask, and with continued passage these pooled cells senesced and died. This microcolony formation was taken to represent cells which were G418 resistant but untransformed and microcolonies were not scored.

Most individual transfection experiments were repeated between four and six times but transfection efficiency was found to vary considerably from experiment to experiment. A number of precautions were taken to minimise changes in conditions between experiments. Whenever possible cells derived from the same original palate biopsy and of an equivalent passage number were used in each repetition of an experiment. Cells were only deemed suitable for transfection up until the fifth passage following isolation as preliminary experiments not presented here demonstrated that later passage cells were more difficult to transfect. In addition transfection solutions were stored at 4^{o}C for a maximum of 4-6 weeks before being discarded and fresh solutions made. Newly made reagents were tested before use, by transfection of pZipneo into PalF cells and subsequent G418 selection.

To allow comparison between experiments, the results from multiple repetitions of transfections were averaged before being standardised to the number of macrocolonies observed on transfection with pBV4 + *ras*, which is used as a "baseline". Thus the average number of macrocolonies obtained with pBV4 + *ras* is taken to be 100% and the average values for other transfections were scaled accordingly (**Table 7.2.**). It was hoped that assigning average values would facilitate the identification of general trends. In all cases the raw data of numbers of macrocolonies are given before normalisation (**Table 7.1.**). It is recognised that, as transfection efficiency appears to be highly variable, the average of multiple experiments is only useful to identify these general trends rather than assign strict quantitative values to them. It may be possible in the future to repeat the experiments using a more reliable and consistent transfection protocol, such as lipofection. However, whilst the quantitative aspects of transformation experiments were often variable, qualitative aspects were not: transformation parameters such as colony morphology and growth in semi-solid media were totally reproducible from experiment to experiment.

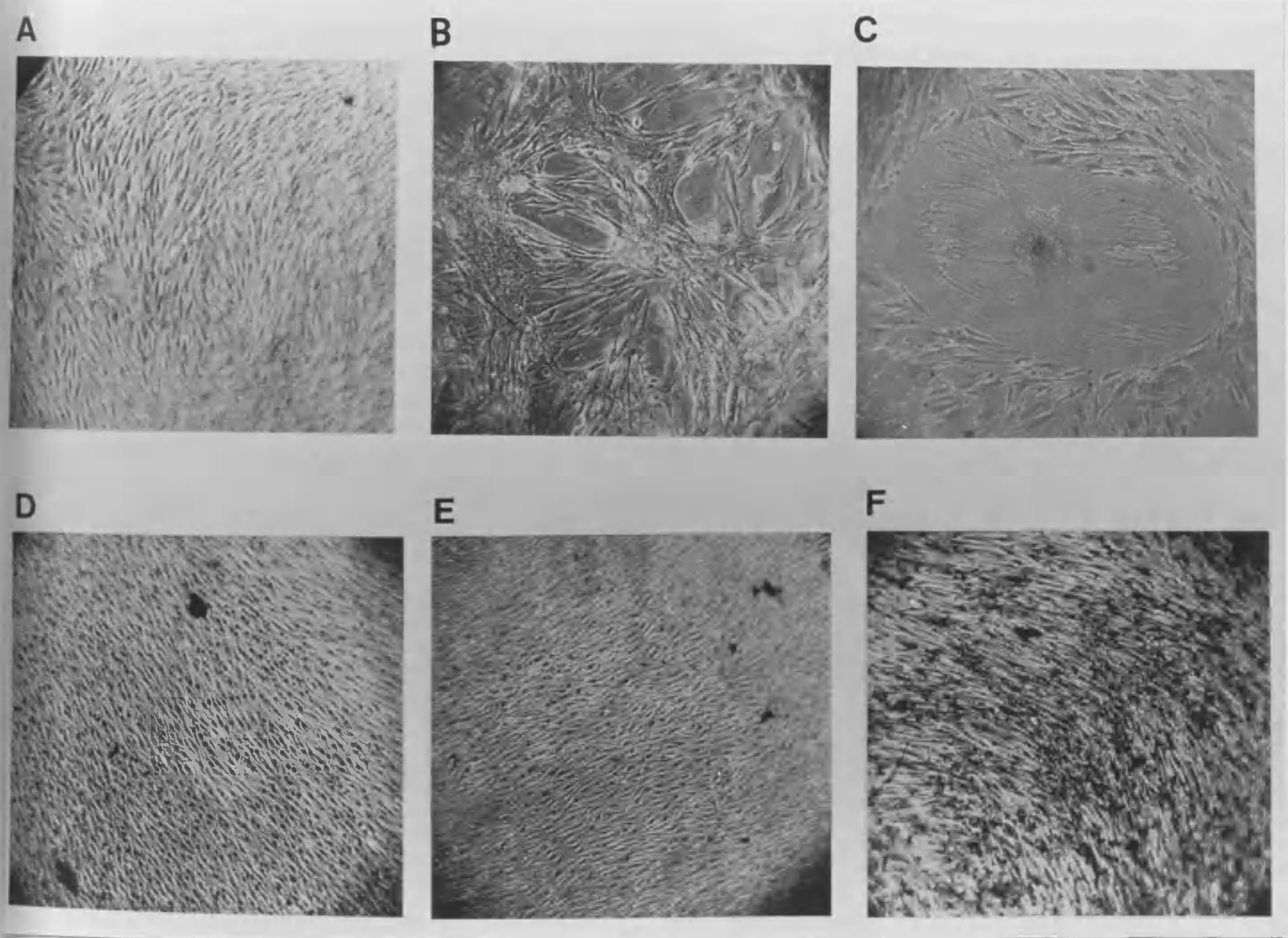
7.2.1 Introduction of pBV4 into PalF Cells

Introduction of the plasmid pBV4 into PalF cells consistently had very little effect. Morphologically, the cells were almost identical to controls, and like control cells senesced on passage. This is in marked contrast to the subgroup A fibropapillomaviruses, BPV-1 and BPV-2, which are capable of transforming PalF cells efficiently and to full malignancy (W.F.H. Jarrett, unpublished results). Results using HPV-16 transformation of primary rat kidney epithelial cells have shown that an activated *ras* is required for transformation (Matlashewski et al 1987) confirming previous observations that primary cells can only be transformed with the cooperation of two or more oncogenes (Land et al 1983). This is interesting in the case of BPV-4, as *in vivo* BPV-4 associated carcinomas have an activated *ras* gene (Campo et al 1990) suggesting an important role for cellular oncogenes in the disease progression. Work in our laboratory by a previous PhD student has shown that PalF cells cannot be transformed by a single oncogene hit (Jaggar, 1990) confirming the oncogene cooperation theory in this cell type.

7.2.2 Cotransfection of pBV4 with the pT24 activated ras plasmid.

Cotransfection of pBV4 with pT24 gave rise to a number of macrocolonies with a partially transformed phenotype. The colonies consisted of elongated cells, had a "tighter" appearance and were larger than control (neo^r only) colonies (**Fig 7.6.**). The cells occasionally adopted a "criss-cross" morphology, especially at the fringe of colonies, but in general the colonies appeared to be contact inhibited (**Fig 7.6.**). This effect is not solely due to *ras* as transfection of the pT24 *ras* plasmid with a pZipneo selectable marker gave a result identical to control pZipneo only transfected cultures as reported in Jaggar et al, 1991. The pBV4 + *ras* transformed colonies could also be expanded individually, unlike control $G418^r$ colonies which could only be expanded as a pool. This indicates that while BPV-4 + *ras* cotransfection does not lead to a full morphological transformation, the macrocolonies have a considerable growth advantage over control cells. This growth advantage is limited however, as expanded pBV4 + *ras* colonies were not immortal and eventually senesced and died after prolonged subculture.

Fig 7.6. Morphology of BPV-4 + *ras* transformed PalF cells.



Notes: Panels above are; **A)** control PalF cells; **B)** BPV-4+*ras*, giant cells; **C)** a BPV-4+*ras* "giant" cell; **D)** BPV-4+*ras* ; **E)** pSVE8^{+}E7^{+}+ras; **F)** pZipE8^{+}E7^{+}+*ras*. All cells were G418 selected. The cells were fixed in methanol and stained as described in the Materials and Methods section. **Magnification=x18**

Table 7.1. Transformation efficiency of BPV-4 constructs with co-operating *ras* and HPV-16 E6 constructs.

Constructs	**Exp1**	**Exp2**	**Exp3**	**Exp4**	**Exp5**	**Exp6**	**Average**
Neo	0	0	0	0	0	0	**0**
BPV-4+ras+neo	17	20	10	20	14	7	**14.67**
BPV-4+ras+ZipE2	26	8	nd	24	25	9	**18.40**
$SVE8^{+}E7^{+}$+ras	18	28	nd	8	nd	7	**15.25**
$SVE8^{+}E7^{+}$+ras+ZipE2	23	23	10	9	9	7	**13.50**
$ZipE8^{+}E7^{+}$+ras	nd	10	9	nd	36	15	**17.50**
$ZipE8^{+}E7^{+}$+$SVE8^{+}E7^{-}$+ras	nd	10	8	nd	nd	18	**12.00**
ZipE7+ras	19	11	7	16	23	18	**15.67**
ZipE8+ras	nd	2	0	0	2	4	**1.60**
ZipE7+$SVE8^{+}E7^{-}$+ras	nd	8	10	nd	20	19	**14.25**
ZipE7+ZipE8+ras	3	15	10	2	24	5	**9.83**
ZipE7+$ZipE8^{+}E7^{-}$+ras	nd	19	16	nd	nd	5	**13.33**
16E6+neo	0	0	0	1	4	0	**0.83**
16E6+ras+neo	0	14	9	8	0	13	**7.33**
16E6+ZipE7+ras	13	17	20	9	18	28	**17.50**
16E6+ZipE7+ZipE8+ras	10	14	17	9	30	24	**17.33**

Notes: **nd**=not done, **16E6**=HPV-16 E6 containing construct pJ4Ω16.E6.
Exp1= experiment 1, **Exp2**=experiment 2 etc.
Column marked **Average** is the numerical average of experiments 1-6.
Transfections were performed as outlined in Materials and **Methods**.

Addendum

Constructs	**Exp A**	**Exp B**	**Exp C**
Neo	0	0	0
BPV-4+ras	14	6	16
BPV-4+ras+pZipE2	19	13	26

Table 7.2. Standardised average transformation efficiency of BPV-4 constructs with co-operating *ras* and HPV-16 E6 constructs.

Construct(s)	Transformation Efficiency
Neo	0%
BPV-4+*ras*+neo	100%
BPV-4+*ras*+E2	125%
SVE8^{+}E7^{+}+*ras*	104%
SVE8^{+}E7^{+}+*ras*+E2	92%
ZipE8^{+}E7^{+}+*ras*	119%
ZipE8^{+}E7^{+}+SVE8^{+}E7^{-}+*ras*	82%
ZipE7+*ras*	107%
ZipE8+*ras*	11%
ZipE7+SVE8^{+}E7^{-}+*ras*	97%
ZipE7+ZipE8+*ras*	67%
ZipE7+ZipE8^{+}E7^{-}+ras	91%
16E6+neo	6%
16E6+ras+neo	50%
16E6+ZipE7+ras	119%
16E6+E7+E8+ras	118%

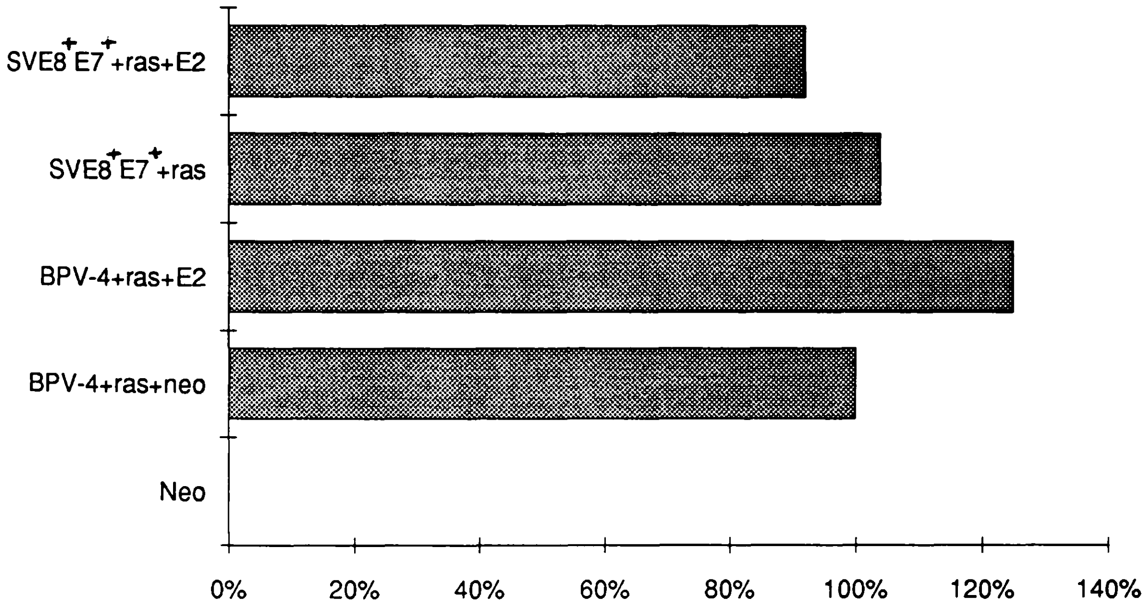
The above data were obtained by standardising the average macrocolony (**Table 7.1**) numbers obtained to BPV-4+*ras* which is taken as 100%.

These observations for BPV-4 are in accordance with the transforming potential of other strictly epitheliotrophic viruses such as HPV-16 and HPV-18. In general the epitheliotrophic PVs do not cause obvious morphological transformation and focus formation and induce more subtle effects on cell morphology and growth potential than do the fibroblastic viruses such as BPV-1 (Burnett and Gallimore, 1985; Chesters and McCance, 1985; Nesseri and Wettstein, 1986).

7.2.3 Formation of "Giant" Cells on Transfection with pBV4 and pT24

An interesting observation is that when primary cells were transformed by full genome BPV-4 in cooperation with activated *ras* a proportion of the transformed colonies contain greatly enlarged cells with correspondingly enlarged nuclei (**Fig 7.6.**). These areas of "giant" cells appeared approximately 2 to 3 weeks after transfection and persisted for approximately 10-14 days before developing into a regular tight focus. A similar giant cell morphology has been reported by other workers investigating BPV-1 transformation of murine C127 fibroblasts and in this case the giant morphology has been shown to be associated with very high levels of viral DNA replication (Burnett et al 1989). It is therefore conceivable that the giant cell morphology observed with BPV-4 and *ras* transfected cells is similarly due to maximal viral DNA replication in this system. An initial investigation into this possibility proved to be inconclusive and time constraints prohibited pursuing this matter further. It should be possible, however, to assess if maximal DNA replication is taking place in these "giant" cells by employing the technique of *in situ* DNA hybridisation.

Fig 7.7. Standardised transformation efficiency of BPV-4 transforming region with cooperating *ras*.



Note: The above graph represents macrocolony formation expressed as a percentage of that observed on transfection of BPV-4 + ras. These results were obtained by using the average of all experiments (see **Table 7.1.**) and standardising them to the average value obtained by BPV-4 (see **Table 7.2.**). **E2** refers to the construct pZipE2.

7.2.4 Transformation of PalF Cells with the 2.0kb E8-E7 Fragment in Cooperation with ras

Previous work using mouse fibroblasts has demonstrated that in these cells transformation by BPV-4 can occur when using a subgenomic 3.6 kb fragment encoding the E8 and E7 ORFs (Smith and Campo 1988). The efficiency of transformation using the subgenomic E8-E7 clone in this system is essentially the same as that observed when using complete BPV-4 genome suggesting that the major transforming region of BPV-4 is encoded by E7 and/or E8. The E8 and E7 ORFs, cloned as a 2.0 kb fragment in the pSVE8^{+}E7^{+} vector give no transformation on its own, but when cotransfected with *ras* give essentially the same efficiency of transformation as full genome BPV-4 (**Fig 7.7.**). The morphology of the resultant colonies transformed by pSVE8^{+}E7^{+} + *ras* is essentially identical to that observed with pBV4 + *ras* (**Fig 7.6.**) suggesting that in this system, as in the established murine fibroblast experiments, the transforming function resides in the E8-E7 region. Efficiency of transformation may be linked to the level of expression of the transforming E8-E7 region as the 2.0 kb fragment is more efficient in cooperating with *ras* when under LTR control in the pZipE8^{+}E7^{+} plasmid than when under BPV-4 transcriptional control (**Fig 7.7.**). As with pSVE8^{+}E7^{+}, pZipE8^{+}E7^{+} was ineffective in morphological transformation without a cooperating *ras*. When under the powerful pZip retroviral transcriptional control, the transformation of the E8-E7 region + *ras* gives rise to colonies with a more tightly packed and aggressive appearance (**Fig 7.6**). As observed with pBV4 + *ras*, PalF cells transformed by pSVE8$^+$E7$^+$ + *ras* or pZipE8$^+$E7$^+$ + *ras* are not immortal and senesce on passage.

7.2.5 Transactivation functions of the BPV-4 E2 Open Reading Frame

The E2 ORF encodes a protein with transactivation properties and cotransfection of this ORF with pBV4 and pT24 increases the number of G418^r macrocolonies (Table 7.1. and Addendum p118) . Although the efficiency of transformation is increased when using the E2 construct the morphology of the resulting colonies is identical to that observed with pBV4 + *ras* (**Fig 7.6.**). This suggests that the E2 ORF can influence the level of expression of the transforming ORFs, but does not directly influence the transformed phenotype. This is seen to be the case for HPV 16 where elevated expression of E2 leads to an increased expression of the E7 product and enhanced cellular transformation, abolishing the requirement for the E7 ORF to be introduced under the transcriptional control of a strong heterologous promoter (Lees et al 1990). Workers in our group have also shown that BPV-4 E2 can transactivate the BPV 4 LCR (Jackson and Campo 1991). Curiously, E2 enhancement of transformation does not occur when the pZipE2 construct is cotransfected with pSVE8$^+$E7$^+$ and pT24 (**Fig 7.7.**). In this case, efficiency of transformation is essentially identical in the presence or absence of the pZipE2 plasmid. This raises the possibility that there is a previously unidentified E2 responsive element contained in the pBV4 plasmid but not in the pSVE8$^+$E7$^+$ subclone. Alternatively, it is possible that the SV40 element in pSVE8$^+$E7$^+$ interferes in some way with the E2 transactivation of the BPV-4 LCR perhaps by competing for the E2 product.

7.2.6 The Role of E7 Integrity in Transformation of PalF Cells

An intact E7 open reading frame is crucial for transformation as deletion of the 3' third of the E7 ORF in both the pSV and pZip cloned E8$^+$E7$^-$ constructs results in a loss of transformation capability (**Table 7.3.**). Indeed, the transforming function seems to be encoded by the E7 ORF as pZipE7 will cooperate with *ras* to transform cells with high efficiency. These results show that an intact E7 ORF together with an activated *ras* gene is absolutely required for BPV-4 mediated transformation.

Table 7.3. Requirement of E7 integrity for macrocolony formation.

Transfected DNA	Macrocolony Formation Exp1	Exp2
$pSVE8^{+}E7^{+}$	0	0
$pSVE8^{+}E7^{+}$ + ras	<10	<10
$pSVE8^{+}E7^{-}$	0	0
$pSVE8^{+}E7^{-}$ + ras	0	0
$pZipE8^{+}E7^{+}$	0	0
$pZipE8^{+}E7^{+}$ + ras	>50	>40
$pZipE8^{+}E7^{-}$	0	0
$pZipE8^{+}E7^{-}$ + ras	0	0

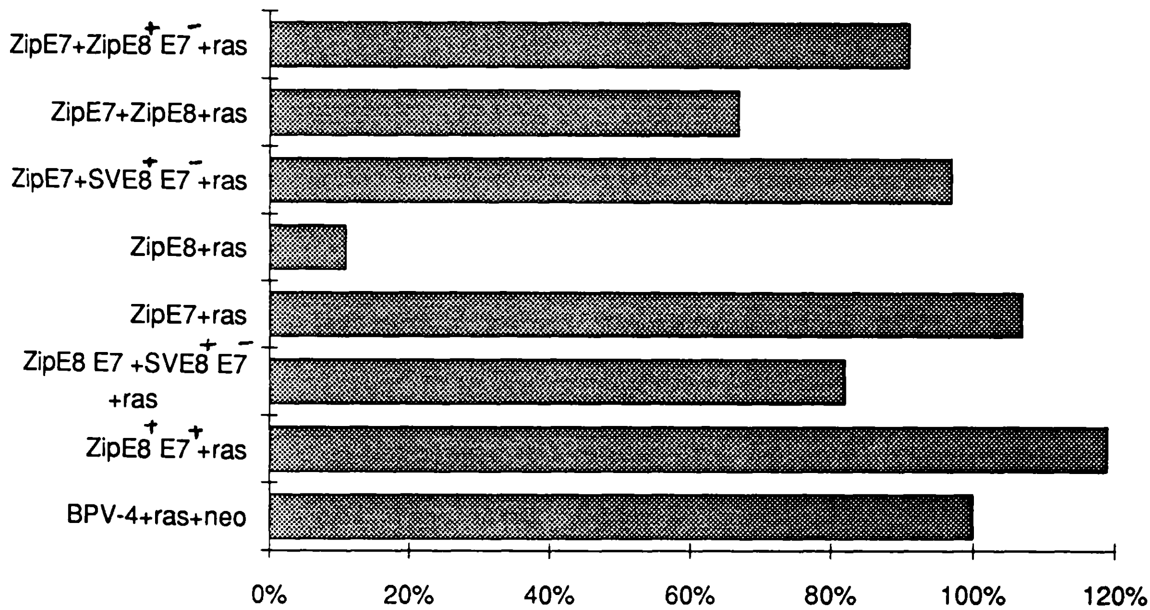
Notes: Macrocolony formation was assessed in PalF cells by G418 selection as described in the Materials and Methods section. Only colonies larger than 5mm in diameter and with a morphologically transformed appearance were scored. The above results suggest that both an intact E7 and a cooperating ras plasmid are required for morphological transformation.

Where no. of macrocolonies is given as <10, this indicated that between 5 and 10 macrocolonies were scored.

This is in agreement with the observation that HPV-18 E7 is capable of transforming primary rat embryo fibroblasts only in the presence of an activated *ras* gene (Bedell et al, 1989). Deletion of 3' third of E7 in the $E8^{+}E7^{-}$ clones confirms the inability of EcoRI digested BPV-4 to transform C127 and NIH-3T3 cells (Smith and Campo 1988). This deletion destroys the second of the two putative DNA binding motifs of E7 (**Fig 5.2.; Fig 6.1.**), the mutation of which abolishes transformation mediated by HPV 16 (Storey et al, 1990; Watanabe et al, 1990). This leads us to conclude that like HPV 16, the E7 is the ORF responsible for morphological transformation.

BPV-4 sequence analysis has shown that BPV-4 E7 contains the two $p105^{Rb}$ binding domains shown to be important in HPV 16 transformation (**Fig 5.2.**) and it is feasible, although as yet undemonstrated, that BPV-4 E7 indeed binds the bovine equivalent of $p105^{Rb}$ as part of the transformation mechanism. There have been three important domains identified in papillomavirus E7 sequences; the $p105^{Rb}$ binding domain, two **Cys-X-X-Cys** "zinc finger" putative DNA binding motifs and a casein kinase II (CKII) phosphorylation site. (**Fig 5.2.**). The most crucial of these domains in terms of transformation biology are the $p105^{Rb}$ binding and the **Cys-X-X-Cys** motifs. Site directed mutagenesis of these domains gives rise to a functionally inactive E7 in transformation assay (Edmonds and Vousden, 1989; Chesters et al, 1990). In addition E7 products of the more oncogenic HPV types are found to bind the p105Rb protein with higher affinity than the more benign HPV types (Munger et al, 1989b; Gage et al 1990). It has been shown that the binding of $p105^{Rb}$ by E7 prohibits the Rb protein from complexing with its cellular targets and this is the postulated mechanism for the transforming function of E7, while the **C-X-X-C** motifs have been shown to be crucial for the transactivation functions of E7 (Phelps et al, 1988). Sequence analysis of BPV-4 E7 demonstrates that it lacks the serine residues at amino acid positions 31 and 32 which constitute the CKII domain (**Fig 5.2.**) and which are phosphorylated in HPV-16 E7 (Smotkin and Wettstein, 1987). Nevertheless the results of transfecting PalF cells indicate that BPV-4 E7 is still capable of causing morphological transformation. There is some evidence that the presence of a CKII site is not indispensable for E7 activity. Like the BPV-4 E7, the CRPV E7 protein also lacks the CKII domain (Barbosa and Wettstein, 1988) but despite this is absolutely required for tumorigenicity *in vivo* (Brandsma at al, 1991).

Fig 7.8. Cooperation between BPV-4 E7 and E8 ORF with *ras* in transformation.



Note: The above graph represents macrocolony formation expressed as a percentage of that observed on transfection of BPV-4 + *ras*. These results were obtained by using the average of all experiments (see **Table 7.1.**) and standardising them to the average value obtained by BPV-4 (see **Table 7.2.**)

In addition, deletion or mutation of the serine 31 and 32 residues in HPV-16 do not abolish transformation (Watanabe et al, 1990; Storey et al 1990) and the lack of these residues in BPV-4 is not expected to interfere with the putative binding to $p105^{Rb}$. No information is currently available on a bovine $p105^{Rb}$ equivalent, but with newly developed anti-E7 antisera (G.J. Grindlay, personal communication), it should be possible to perform immunoprecipitation experiments to see if the BPV-4 E7 complexes with any cellular proteins.

7.2.7 Effects of BPV-4 E8 on Transformation Efficiency

In experiments performed to date the E8 ORF has no direct transforming ability and indeed when the average of standardised results are considered, E8 appears to have a negative effect on transformation efficiency under certain conditions. Cells transfected with E8 and *ras* alone (either under its own transcriptional control in the pSVE8^{+}E7^{-} plasmid, or under MoLV LTR in pZipE8^{+}E7^{-} or pZipE8) die more quickly than control cells suggesting that the E8 gene product may be lethal (**Fig 7.8.**). In these experiments a number of colonies with an abnormal vacuolated phenotype are observed approximately 2 weeks after transfection. These colonies very rarely survive until the experiment is scored for foci and by 7-10 days after appearing, they had usually died.

Although the BPV-4 E8 appears to accelerate cell death when transfected in the absence of an E7 ORF, the data presented show that E8 had no demonstrable quantitative effect on macrocolony formation in the presence of a transforming E7 ORF.

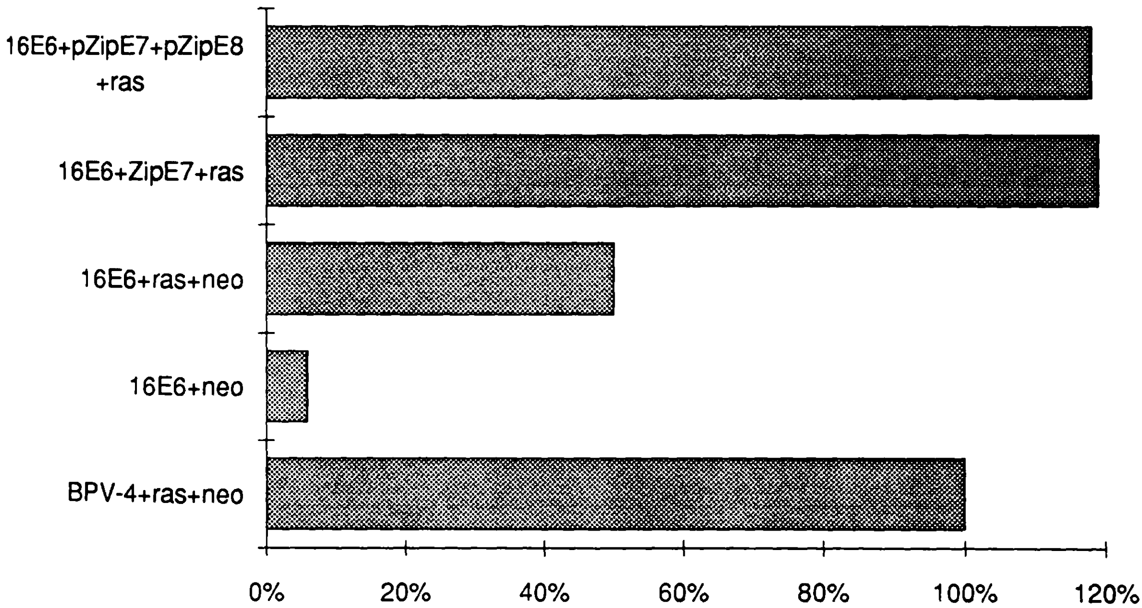
As previously discussed, the predicted BPV-4 E8 peptide shares considerable sequence similarity to the BPV-1 E5. BPV-1 E5 is a transforming gene of the virus (reviewed in DiMaio and Neary, 1990) in stark contrast to BPV-4 E8. Although they share the same hydrophobicity (see **Fig 7.5.**) and predicted membrane orientation (see **Fig 7.10.**), BPV-4 E8 lacks all the amino acid residues shown to be crucial for transformation by BPV-1 E5 (Horowitz et al, 1988, 1989) as shown in **Fig 7.10.** It is thus perhaps unsurprising that E8 does not act as a transforming protein, although the hydrophobicity plots, amino acid similarity and predicted trans-membrane folding of the two peptides suggest that they may share some common function.

As outlined in the introduction, a principal activity of BPV-1 E5 has been demonstrated to be the activation of membrane receptors including platelet derived growth factor (PDGF) receptor and the epidermal growth factor (EGF) receptor (Petti et al 1991; Martin et al 1989). Recent work has also demonstrated that the E5 binds to a 16kd cellular protein (Goldstein and Schlegel 1990) which has been shown to be the 16k component of vacuolar proton channel-forming ATPases and gap junctions (Leitch and Finbow, 1990; Finbow et al, 1991). It has been postulated that E5-16k complexing would disrupt the normal 16k H^+ATPase functions of acidification of endosomal vesicles. This disruption in turn would lead to extended binding of growth factor to growth factor receptor and recycling of receptors to the cell surface, leading to sustained receptor stimulation and ultimately contributing to cell transformation (Goldstein et al, 1991; Finbow et al, 1991). It should be noted however, that studies using sets of E5 mutants have shown that complexing of BPV-1 E5 with the 16k protein is insufficient in itself for transformation. Although complexing is not sufficient, it may still be required for transformation as analysis of transformation competent E5 mutants has shown that they all retain the capacity to bind the 16k protein (Goldstein and Schlegel 1990). This observation suggests that BPV-4 E8 may contribute to transformation by binding the 16k protein although in itself it has no strict transforming ability. As the critical residues for the interaction of E5 and the 16k protein have not yet been fully mapped, it is not possible to predict if BPV-4 E8 meets the requirements to complex with the 16k protein. The use of E8 and 16K specific antisera in co-immunoprecipitation experiments on BPV-4 E8 transiently transfected cells may address this question. The toxicity of overexpressed E8 may prove to cause technical difficulties in this respect, and with this in mind BPV-4 E8 is currently being cloned in an inducible mammalian expression vector system (R. Anderson, personal communication). The alternative E5 or E8 interaction with the 16k could be to the gap junction form, leading to a disruption of junctional communication. By inhibiting junctional communication in this way it can be postulated that the normal growth control of surrounding cells via junctional communication would be "silenced" thus contributing to cell transformation. This phenomenon, if it exists, could in part explain the observation that E8 has no strict transformational activity, but may be able to contribute to transformation mediated by E7. The possibility that BPV-1 E5 and/or BPV-4 E8 can disrupt junctional communication will be considered in detail in a later section.

7.2.8 Cooperation of BPV-4 Transforming Functions, *ras* and HPV-16 E6 In Immortalisation

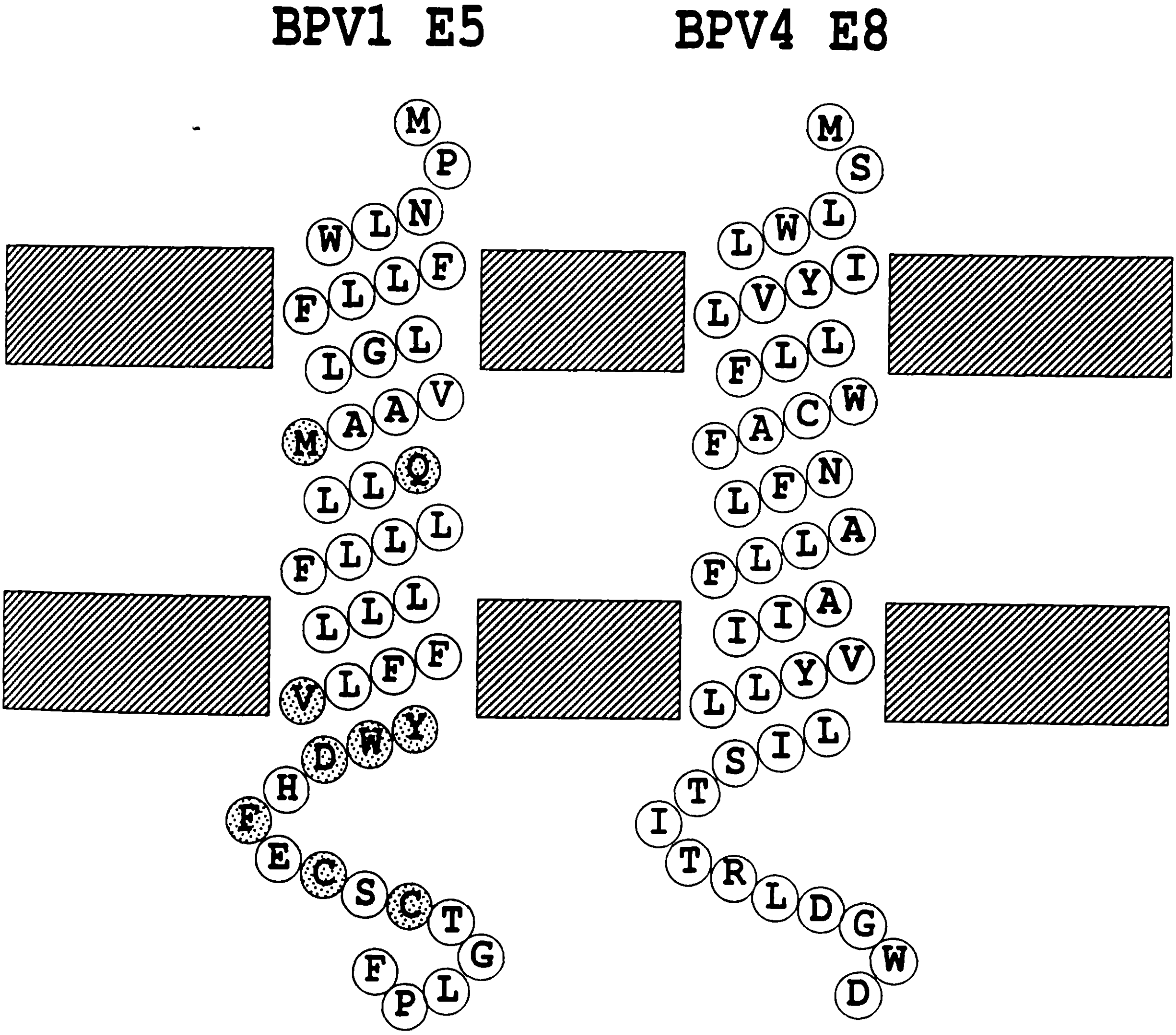
The lack of an E6 ORF in BPV-4 may explain why the viral DNA is unable to confer immortality to primary cells even in the presence of activated *ras*. The E6 ORF from HPV 16 (under MoLV LTR transcriptional control) was cotransfected with the transforming region of BPV-4 and activated *ras* into primary bovine fibroblasts to assess if the presence of an E6 ORF will allow the BPV-4 DNA to morphologically transform these cells to a greater degree and / or give rise to an immortal phenotype. The introduction of HPV-16 E6 ORF alone gives rise to no morphologically transformed colonies but partial transformation is observed when the a cooperating pT24 construct is introduced **(Fig 7.9)**. Cells transformed in this way have an elongated and refractile appearance, but grow very slowly and are contact inhibited **(Fig 7.11.)**. In addition, of ten colonies of pJ4Ω16.E6 + *ras* transformed cells picked, only a single colony was able to be expanded. Due to the sparsity of different clones and the slow growth rate of these cells, the immortality of this clone of cells has not been tested, but observations that HPV-18 E6 + *ras* transfected primary rat embryo fibroblasts are not immortal suggests that these cells may senesce if continually passaged (Bedell et al, 1989).

Fig 7.9. Cooperation of HPV-16 E6 with BPV-4 E7 and E8 ORF with *ras* in transformation.



Note: The above graph represents macrocolony formation expressed as a percentage of that observed on transfection of BPV-4 + ras. These results were obtained by using the average of all experiments (see **Table 7.1.**) and standardising them to the average value obtained by BPV-4 (see **Table 7.2.**). **16E6** refers to the HPV-16 E6 containing construct pJ4Ω16.E6.

Fig 7.10. Predicted membrane orientations of BPV-1 E5 and BPV-4 E8 proteins.



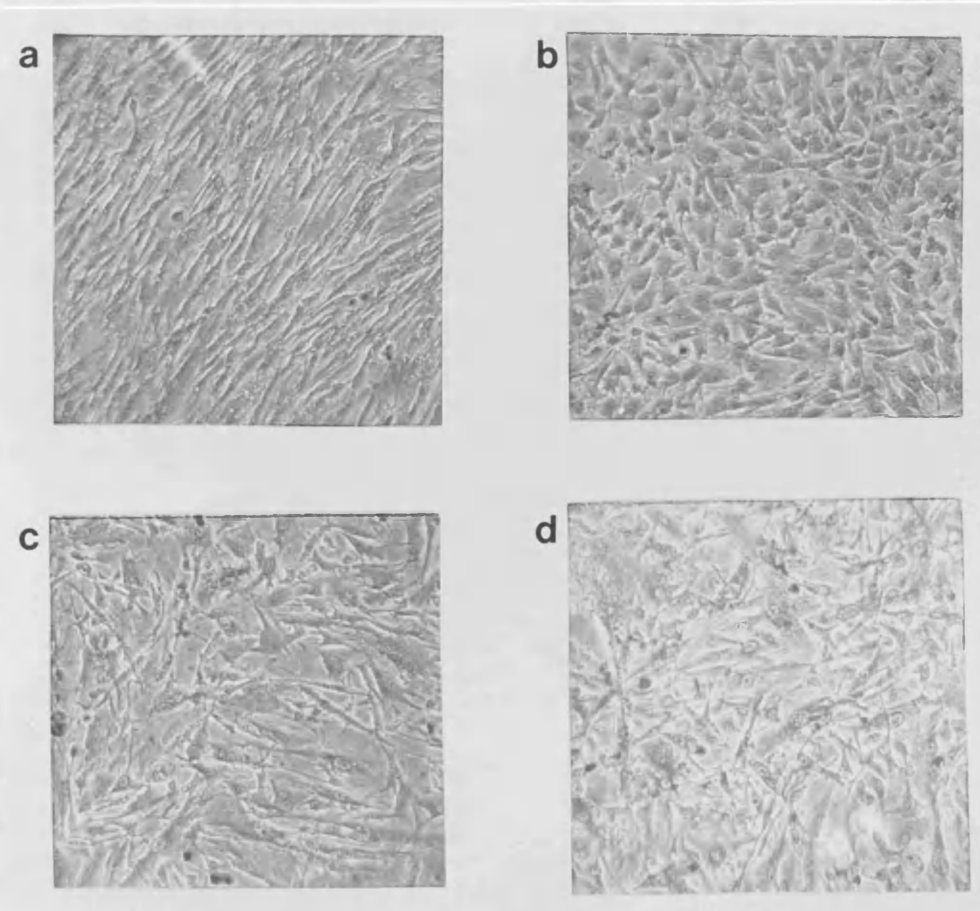
Predicted membrane folding of the BPV-1 E5 and BPV-4 E8 proteins. Mutation of the shaded residues in E5 diminishes the transformation potential of this protein (Horwitz et al, 1988, 1989).

Cotransfection of pJ4Ω16.E6, pT24 and pZipE7 induces colonies which have an aggressive transformed morphology, cells being more tightly packed and piled up than colonies obtained using pZipE7 and *ras* alone (**Fig 7.11.**). These colonies can readily be expanded. This morphology is also observed when pJ4Ω16.E6, pZipE7, pZipE8 and pT24 are transfected together. When pJ4Ω16.E6, pZipE7 and pT24 are used in transfections (with or without a pZipE8 construct) the resultant colonies do not senesce on continued subculture and have been passaged routinely once weekly for ten months with no obvious retardation of growth potential. This is in contrast to cells transformed in the absence of HPV-16 E6. It appears therefore that the combined action of BPV-4 E7, HPV-16 E6 and *ras* can lead to immortalisation of PalF cells. This observation is in accord with experiments involving immortalisation of rat embryo fibroblasts by BPV-1 where morphological transformation required an E5 gene, but the long term viability of these cells in culture required an additional E6 (Cerni et al, 1989).

7.2.9 Growth of BPV-4 Transformed Cells in Methocel.

An experimental technique to establish the extent of transformed phenotype of a cell line is anchorage independence; its ability to form colonies in a soft agar

Fig 7.11. Morphology of PalF cells transformed in the presence of HPV-16 E6.



Magnification=x60

Notes: A) untransfected control PalF cells; B) PalF cells transformed by pJ4Ω16.E6+*ras*; C) PAlF cells transformed by pJ4Ω16.E6+pZipE7+*ras*; D) PAlF cells transformed by pJ4Ω16.E6+pZipE7+pZipE8+*ras*.

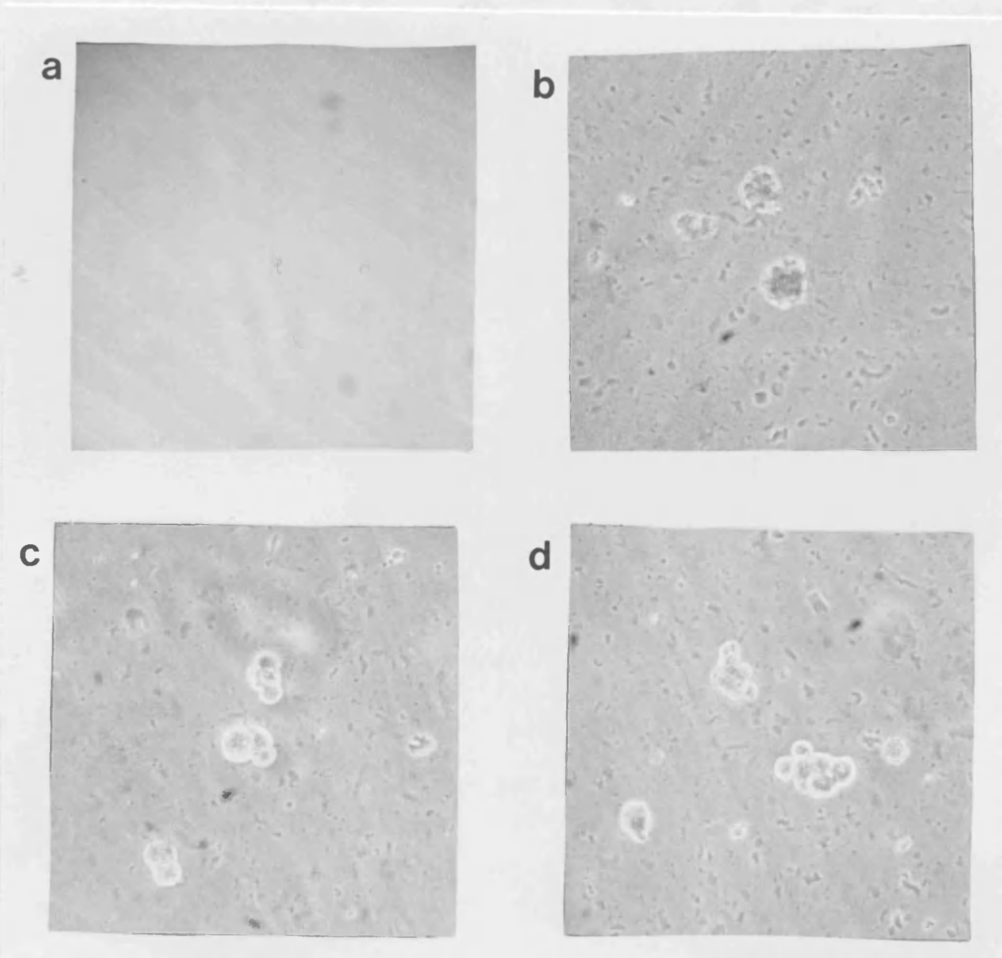
or methocel based growth medium. Some BPV-4 transformed cell lines were thus plated out in methocel and their growth in this medium assessed (**Table 7.4.**). Untransfected PalF cells have no capacity for methocel growth whatsoever. A positive control for the experiment was the established murine fibroblast line NIH-3T3 transformed by BPV-2 (Campo and Spandidos, 1983). These cells are highly tumorigenic in nude mice and form colonies in methocel with high efficiency (**Fig 7.12.**). PalF cells transformed by BPV-4 and cooperating *ras* do form colonies in methocel (Fig) but at considerably lower frequency than the positive control. This was also found to be the case for pSVE8^{+}E7^{+}+*ras*, pZipE7+pZipE8+ras and pJ4Ω16.E6+pZipE7 +pZipE8+*ras* transformed PalF cells which have essentially the same growth potential in methocel as do whole genome BPV-4+*ras* transformed cells. In contrast lines morphologically transformed by either pZipE7+*ras* or pJ4Ω16.E6+pZipE7+*ras* had no capacity for growth in methocel whatsoever (see **Table 7.4.**).

Table 7.4. Methocel growth of transformed PalF cells.

Cell Line	**Growth in Methocel**
PalF control (untransfected)	negative
NIH3T3-BPV2	positive
pBV4+*ras* (5 lines)	positive
SVE8^{+}E7^{+}+*ras* (3 lines)	positive
SVE8^{+}E7^{+}+*ras*+E2	positive
pZipE7+*ras* (2 lines)	negative
pZipE7+pZipE8+ras	positive
16.E6+pZipE7+*ras* (2 lines)	negative
16.E6+pZipE7+pZipE8+*ras* (2 lines)	positive

Notes: The ability of cell lines to grow in methocel was assessed as described in the Materials and Methods section. **16.E6=** HPV-16 E6 containing construct pJ4Ω16.E6. **ras** refers to the pT24 construct. Positive growth in methocel was assigned if the cell lines examined produced colonies after 7-10 days growth in methocel (see Materials and Methods). The morphology of the methocel colonies is shown in **Fig. 7.14.**

Fig 7.12. Methocel growth of transformed PalF cells.



Magnification=x60

Notes: Colony formation of transformed PalF cells in methocel. **A)** Untransfected PalF cells; **B)** BPV-2 transformed NIH3T3 cells (positive control); **C)** BPV-4 + *ras* transformed PalF cells; **D)** pSVE8^{+}E7^{+}+*ras* transformed PalF cells. Methocel Growth assay was performed as outlined in the Materials and Methods section 6.12.

From these preliminary observations it can be speculated that the presence of BPV-4 E8 is required for anchorage independence, especially as HPV16.E6+E7+*ras* transformed cells do not form colonies in methocel, but cells from the same experiment with an additional E8 containing construct (pJ4Ω16.E6+pZipE7+pZipE8+*ras*) do. As transfection of BPV-4 E8 without E7 leads to premature cell death, it is not possible to speculate whether a combination of E7 and E8 is required for this effect.

Other experiments using HPV-16 to transform human embryonic lung fibroblasts have shown that E6 and E7 expressed from an SV40 early promoter give poorly transformed cells with an extended life-span, but anchorage independence was only observed in one out of four isolates tested (Watanabe et al 1989). In addition a study by Smits and coworkers showed that HPV-16 could only cause anchorage independence of embryonic fibroblasts when the cells had a deletion in the short arm of chromosome 11 (Smits et al, 1988), the site of a postulated tumor suppressor gene known to inhibit the tumorigenicity of the HPV-18 containing cell line HeLa (Saxon et al 1986; Boshart et al 1984). It should be noted however that HPV-18 E7 alone in cooperation with *ras* is able to induce anchorage independence in rat embryo fibroblasts.

7.2.10 Tumorigenicity of BPV-4 Transformed Cells

The tumorigenicity of PalF cell lines transformed by BPV-4 containing constructs and pT24 was assessed by subcutaneous injection into athymic mice. Without exception, cells transformed under any conditions with BPV-4 or subgenomic constructs and *ras* proved to be non-tumorigenic in nude mice (**Table 7.5.**). This is also found to be true for the immortal cell lines transformed in the presence of HPV-16 E6. The lack of tumorigenicity of resultant cell lines suggests that other cellular factors are required before a fully malignant phenotype develops; a situation analogous to the long delay observed between BPV-4 infection and carcinoma in cattle. This is also in accordance with data obtained from the transfection of primary human fibroblasts and rat embryo fibroblasts with HPV 16 where the transfected cells gain an extended life-span and become morphologically transformed but are non-tumorigenic in nude mice (Cerni et al, 1989; Matlashewski et al 1988; Pirisi et al 1987).

Table 7.5. Tumorigenicity of transformed PalF cells.

Athymic Mouse Tumorigenicity Assay

Cell Line	**Tumorigenicity**
PalF control	negative
C4Ta2a control	positive
4+*ras*(5 lines)	negative
SVE8^{+}E7^{+}+*ras*(3 lines)	negative
SVE8^{+}E7^{+}+*ras*+E2	negative
pZipE7+*ras*(2 lines)	negative
pZipE7+pZipE8+ras	negative
16.E6+pZipE7+*ras*(2 lines)	negative
16.E6+pZipE7+pZipE8+*ras*(2 lines)	negative

Athymic mice injected as described in Materials and Methods section. **16.E6** refers to the HPV 16 E6 containing construct pJ4Ω16.E6. **ras** refers to the pT24 construct.

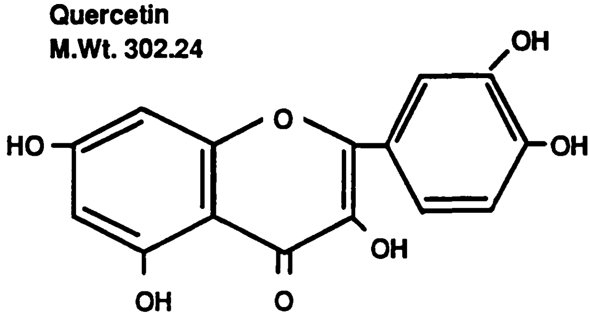
7.2.11 Morphological transformation of quercetin initiated cells by BPV-4 + *ras*.

The progression of BPV-4 associated lesions to carcinoma is associated with chemical cofactors present in bracken fern (see Introduction). The progression of virally induced papillomas to carcinoma in cattle which have not been exposed to bracken has never been observed indicating the crucial role of chemical cofactors in this system. One of the best characterised mutagens present in bracken fern is the flavonoid quercetin, (5,7,3',4'-tetrahydroxyflavone) the chemical structure of which is shown in **Fig 7.13.** Quercetin is a widely distributed flavonoid, also found in most edible fruits and vegetables (Ambrose et al, 1951). Quercetin has been shown to bind DNA (Rahman et al, 1990) and induce a spectrum of genetic lesions in bacteria (Bjielanes and Chang, 1977; Maruta et al, 1990) and cultured mammalian cells (Amacher et al 1979; Nakayasu et al, 1986; Ishikawa et al, 1987) including clastogenic damage (Ishidate, 1988), an observation of particular significance as bracken grazing cattle have been found to have a wide spectrum of chromosomal abnormalities (Moura et al, 1988). In addition, quercetin has been shown to activate protein kinases and competitively inhibit ATP binding by phosphatases (Van Wart-Hood et al, 1989).

Fig 7.13. Molecular structure of quercetin.

QUERCETIN

Quercetin
M.Wt. 302.24



- **Plant Flavonol, found in bracken**
- **Mutagenic in Salmonella typhimurium**
- **Shown to act as an initiator in two stage transformation in vitro**
- **Induces chromosomal aberrations in cultured mammalian cells**
- **Bracken grazing cattle have cytogenetic abnormalities**

Work in a number of laboratories has generally shown that quercetin alone does not act as a carcinogen (Ambrose et al 1951, Hirono et al 1981; Morino et al, 1981). It has been shown that quercetin can act as an initiator in a two stage transformation assay in mammalian cells *in vitro*, although in the same assay it has an inhibitory effect when used as a promoter (Sakai et al, 1990). In this respect it is interesting to note that quercetin may act as a cytostatic agent by interfering with a putative cell cycle regulatory molecule (Hosokawa et al, 1990) and has been demonstrated to inhibit the growth of gastric cancer cells in culture (Yoshida et al, 1990).

The effects of quercetin as an initiating agent on PalF cells was examined by treating cultures of PalF cells with a single dose of quercetin for 48 hrs before washing the mutagen out and transfecting the cells with BPV-4 and/or *ras* (see Materials and Methods 6.7.5). In the discussion of the following experiments, cultures treated with a single dose of quercetin in the manner outlined will be described as "initiated". In all cases the pZipneo construct was cotransfected to allow selection of cells in G418. Following transfection and selection protocols, the colonies in each experiment were pooled and morphology, growth in methocel and tumorigenicity in nude mice determined. The use of a single dose of quercetin in this assay ensures that any observed effects on morphology or growth characteristics are due to long term mutational damage and not short term phenomena such as protein kinase activation.

Treatment of PalF cells with a single dose of quercetin has no observable changes in cell morphology of cells transfected with pZipneo only (**Table 7.6.**). This observation was found to be consistent for cultures initiated with 5 μM, 20 μM or 45 μM quercetin. In these cultures, only small contact-inhibited microcolonies were observed after the selection period and after these colonies were pooled and expanded, the resultant cultures were indistinguishable from untransfected PalF cells or uninitiated pZipneo transfected cells (see **Fig 7.14.**). This result suggests that at the concentrations examined, quercetin does not appear to induce any significant long term changes in cell morphology. This result was also found to be the case in cultures transfected with BPV-4 DNA. At all three concentrations of quercetin, transfection with pBV4 and pZipneo gave rise to microcolony formation only, a result identical to non-initiated cultures (**Table 7.6.**). These cultures were indistinguishable from pZipneo only transfected cells (**Fig 7.14.**). This result is similar to those presented in

Table 7.6. Effect of quercetin initiation on macrocolony formation by BPV-4 + *ras*.

		Concentration of Quercetin			
		0μM	5μM	20μM	45μM
A	Neo	0	0	0	0
B	BPV4+neo	0	0	0	0
C	*ras*+neo	0	0	0	0
D	BPV4+*ras*+neo	17	17	18	20

Cultures of PalF were initiated with quercetin then transfected as described in the Materials and Methods section. Macrocolony formation was assessed by neomycin resistance assay: only colonies of >5mm and with a transformed appearance were scored.

the previous section, where pBV4 alone was incapable of inducing morphological transformation, and demonstrates that initiation of PalF cells with 5 µM, 20 µM or 45 µM quercetin did not overcome this limitation. The pooled microcolonies from these experiments had no growth potential in the methocel colony formation assay (**Table 7.7.**).

Transfection of quercetin initiated cells with *ras* and neo also followed the pattern observed for non-initiated cells; *ras* alone was incapable of morphological transformation in both initiated and non-initiated cultures (**Table 7.6.**). Again, at the three quercetin concentrations tested only microcolony formation was observed, and when pooled the resultant colonies were morphologically indistinguishable from control (pZipneo only) transfected cells (**Fig 7.14.**). The cells had no potential for growth in methocel irrespective of the concentration of quercetin used to initiate them (**Table 7.7.**).

The above results demonstrate that these concentrations of quercetin did not cause transformation, or predispose PalF cells to transformation by pBV4 or *ras* alone. Cotransfection of initiated PalF with pBV4, *ras* and pZipneo gave rise to macrocolony formation as previously

Table 7.7. Effect of quercetin initiation on anchorage independence in PalF cells.

Quercetin (μM)	DNA Transfected	Growth in Methocel	Efficiency
0	neo	negative	-
0	NIH-BPV2	positive	$1.54(+/-0.21)\times10^{-4}$
0	pBV4+ras+neo	positive	$4.41(+/- 0.6)\times10^{-4}$
5	neo	negative	-
5	pBV4+ras+neo	positive	$4.08(+/-0.32)\times10^{-4}$
20	neo	negative	-
20	pBV4+ras+neo	positive	$6.95(+/-0.45)\times10^{-4}$
45	neo	negative	-
45	pBV4+neo	negative	-
45	ras+neo	negative	-
45	pBV4+ras+neo	positive	$6.75 (+/-0.15)\times10^{-4}$

Notes: **neo**=pZipneo; **ras**=pT24
NIH-BPV2 are NIH-3T3 cells transformed by BPV2.

Efficiency of methocel colony formation was determined as follows. 106 cells were plated (duplicates) in a 60cm^2 petri dish and incubated for 7-10 days. Each plate was then scored for colonies by counting six 1cm^2 areas from each plate, averaging this number and then multiplying by 60 to give an estimate of total numbers of colonies. The duplicates were then averaged and the result is given as number of colonies per 10^6 cells.

described for non-initiated cells. At all quercetin concentrations examined the number of macrocolonies produced was similar, between 17 and 20 macrocolonies per transfection, implying "initiation" does not enhance the overall transformation efficiency of pBV4 and *ras* (**Table 7.6.**).

When the macrocolonies produced after non-initiated cells and cells initiated with 5 µM quercetin, were pooled the resultant cultures in both cases were morphologically identical; cells were elongated and refractile but remained contact inhibited with very few cells piling up or adopting a "criss-cross" morphology as shown in **Fig 7.14.**, in keeping with previous experiments where PalF cells were transfected with pBV4 + *ras*. These cells were found to grow in methocel with the same efficiency as previously reported for PalF cells transformed with pBV4 and *ras* (**Table 7.4.**).

In the process of expansion, the pooled pBV4 + *ras* macrocolonies from cultures initiated with either 20 µM or 45 µM quercetin became dominated by cells with a more aggressively transformed morphology. These highly transformed cells were in striking contrast to observations made on PalF cells which had been treated with the lower doses of quercetin. The cells were very elongated and refractile, appeared not to be contact-inhibited and adopted a "criss-cross" morphology, the cells piling up to form dense "foci" (**Fig 7.14.**). This morphology was observed in expanded populations from both the 20 μM and 45 μM quercetin initiated transformants.

On plating in methocel, these cells were found to grow with much higher efficiency than non-initiated pBV4 + *ras* cells; colony formation was higher (**Table 7.7.**) and the resultant colonies were of a much larger size (**Fig 7.14.**). The enhanced growth of these populations of cells in methocel, together with the striking morphological differences observed over controls suggests that the quercetin initiation procedure gave rise to a population of cells which can be transformed to a higher degree by the action of BPV-4 and *ras*. In addition to enhanced plating efficiency and growth in methocel, both pooled populations of cells were found to induce fibrosarcomas in the athymic nude mouse assay approximately 4 weeks after injection. These tumours initially grew to a larger size than those induced by the positive control cells (BPV-2 transformed NIH-3T3 cells), attaining maximum size by weeks 6-7. After attaining maximum size they regressed to a considerable extent although were still present at 12-14 weeks (**Fig 7.15.**). The fact that the tumours regressed to some extent implies either the tumours were susceptible to the vestigial immune response present in the animals, and/or the injected cells lost their capacity for growth whilst in the animal. Surprisingly, the cells had a high capacity for growth in vitro, and were passaged weekly for approximately 16 weeks. During this time the cells showed no reduction in growth capacity, and time constraints prevented an assessment as to whether these cells were immortal.

The efficiency of the initiation step cannot be established from this experiment however, as the macrocolonies were pooled before growth in methocel was assessed, and no obvious differences in phenotype between individual macrocolonies were noted when the numbers of macrocolonies were scored. In order to address this limitation, the experiment was repeated for quercetin concentrations of 0 μM, 20 μM and 45 μM and the morphology of resultant macrocolonies was carefully assessed. This was done in the hope that any macrocolonies with an unusual morphology could be scored and compared to the morphology normally encountered in non-initiated cells transformed by pBV4 and *ras*.

As with previous experiments, no macrocolony formation was observed in cultures transfected with pBV4 or *ras* alone, irrespective of the concentration of quercetin used to initiate the cells (**Table 7.8.**). Transfection of the two constructs together gave rise to a number of macrocolonies as expected from previous experiments. Of the 34 macrocolonies observed following transfection of a flask of non-initiated cells with pBV4 + *ras*, none appeared to have a morphology any different from that observed in similar earlier experiments (shown in **Fig 7.6.**). This confirmed the previous observation that in this system the action of pBV4 and *ras* does not lead to dramatic changes in cell morphology. The macrocolonies produced on pBV4 + *ras* transfection of cells initiated with quercetin at a 20 μM concentration were mostly of a morphology indistinguishable from that observed with non-initiated cells but between the two individual experiments scored, less than 5% (4 out of 91) of macrocolonies had a highly transformed morphology similar to that shown in **Fig 7.14.** This implies that the initiation by quercetin has less than a 5% chance of causing genetic lesions which predispose a cell to full morphological transformation by the action of pBV4 and *ras*. A similar situation was found in cells initiated with quercetin at the higher concentration (45 μM) where less than 3% (2 out of 70) of pBV4 + *ras* induced macrocolonies had a morphology significantly different from pBV4 + *ras* transfected non-initiated cells (**Table**

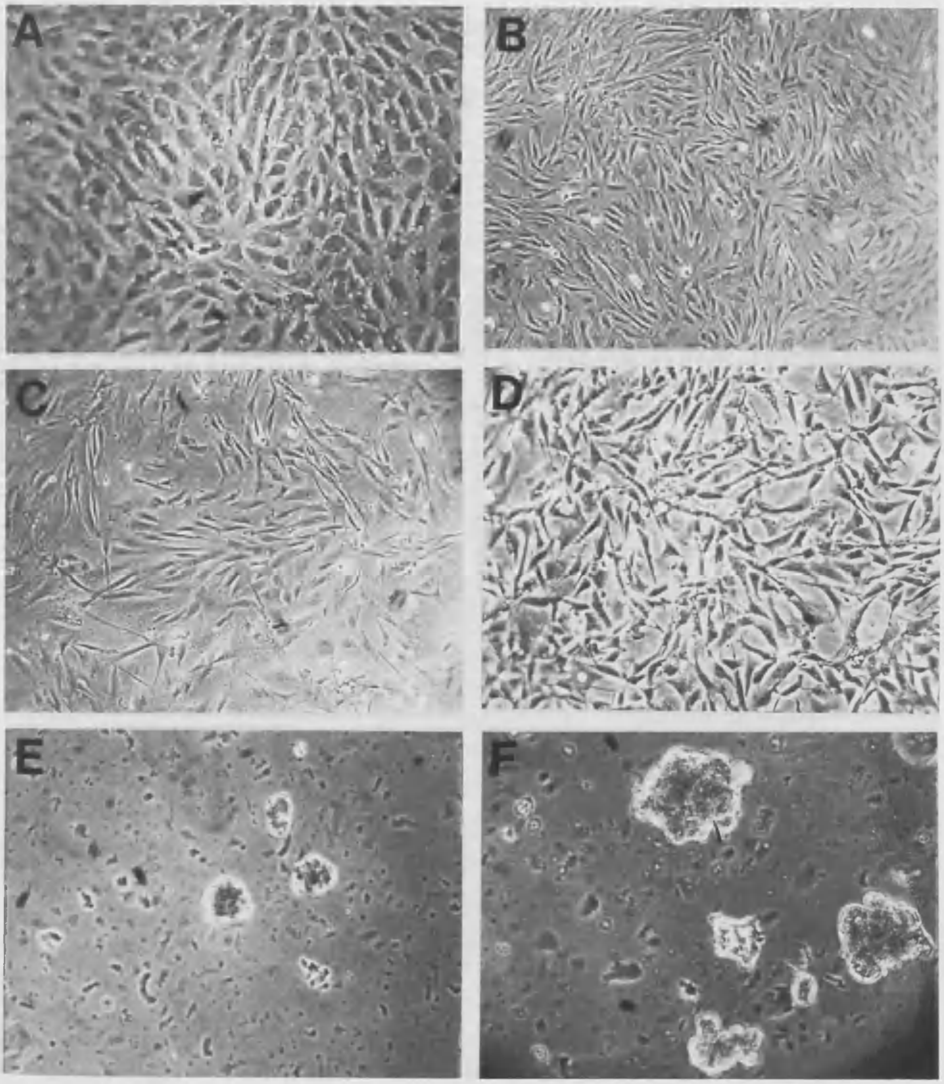
7.8.). The observation that the higher concentration of quercetin does not increase the chances of predisposing a cell to high morphological transformation may be a consequence of toxicity, or of the cells sustaining excessive genetic damage giving rise to loss of viability. Neither of these points were addressed experimentally in this body of work however.

Table 7.8. Efficiency of formation of abnormal macrocolonies by BPV-4 + ras in quercetin initiated cells.

		Quercetin Concentration		
		0µM	20µM	45µM
A	Neo	0	0	0
B	BPV4	0	0	0
C	ras	0	0	0
D	BPV4+ras(i)	34(0)	45(3)	33(2)
E	BPV4+ras(ii)	-	46(1)	27(0)

Numbers given are macrocolonies formed as described in Materials and Methods section. Numbers given in brackets are macrocolonies with a very highly transformed morphology (see **Fig 7.14.D**).

Fig 7.14. Comparison of macrocolony morphology and growth in methocel between initiated and non-initiated cells transformed by BPV-4 + *ras*.



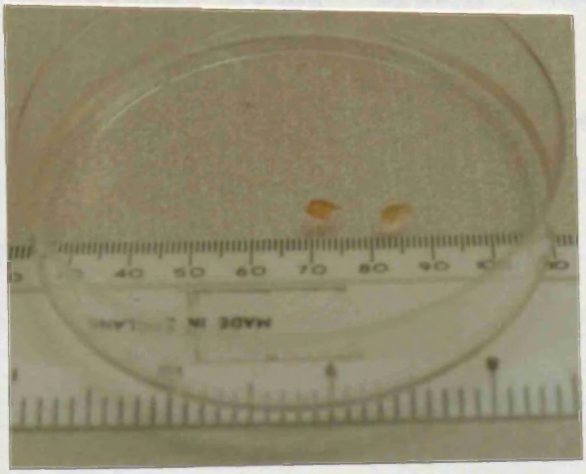
Magnification=x60

A) Control PalF cells **B)** PalF cells initiated with 20μM quercetin. **C)** PalF cells transformed by BPV-4+ras (G418 selected) **D)** Quercetin initiated PalF cells transformed by BPV-4+ras (G418 selected). **E)** Growth of BPV-4+ras transformed cells in methocel. **F)** Growth of 20μM initiated BPV-4 + ras transformed PalF cells in methocel.

Fig 7.15. Tumorigenicity of quercetin initiated BPV-4 + *ras* transfomed cells.

A) Nude mouse tumour 5 weeks after injection. Similar sized tumours were observed in control (C4-Ta2a) and BPV-4 +ras transformed PAlF cells which had been initiated with either 20μM or 45μM quercetin.

B) Twelve weeks following injection the tumours induced by quercetin initiated BPV-4+ras transformed cells had regressed to a considerable extent.



C) Excised regressed tumours (12 weeks) from quercetin initiated BPV-4+ras transformed cells.

The scale of genetic damage caused by quercetin was examined in PalF cells by cytogenetic analysis, the results of which are given in **Table 7.9.** Karyotypic analysis was kindly performed in the laboratory of Dr. Ruedi Fries and Sabina Solinas in ETH, Zurich. Non-transfected PalF cells were found to have a normal complement of 60 chromosomes with no rearrangements taking place on establishment and passaging of the cells (**Table 7.9.**). A normal karyotype was also observed in SVE8^{+}E7^{+}+*ras* transformed cells and in cells which had been treated with quercetin, at a concentration of 20 µM or 45 µM but not transfected (**Table 7.9.**). It can be seen from the table that some metaphases were observed to have a depletion in chromosome number. The chromosomes lost in each case were not consistent and were deemed to be an artefact of the karyotyping procedure (R. Fries and S. Solinas personal communication), and the statistically small number of metaphases typed prevented any valid conclusions being drawn. When the initiated PalF populations were transfected with pBV4 + *ras*, the resultant macrocolony pools were found to be tumorigenic as outlined earlier. The tumorigenic populations were also karyotyped and found to be normal, with no consistent chromosome loss (**Table 7.9.**).

Table 7.9. Karyotypic analyses of quercetin initiated cells.

Line	**Metaphases Scored**	**Number of chromosomes**	**Sex**	**Rearranged**
PalF	5	60	XY	No
		59 (28)		No
		60		No
		60		No
		60		No
SVE8$^+$E7$^+$+*ras*	5	60	XX	No
		58 (2,19)		No
		60		No
		60		No
		60		No
PQ3	5	57 (1,7,12)	XY	No
		60		No
		60		No
		59 (26)		No
		60		No
PQ2	5	60	XY	No
		60		No
		60		No
		60		No
		60		No
Q3D	5	60	XY	No
		60		No
		59 (8)		No
		60		No
		59 (2)		No
Q2D	5	60	XY	No
		56 (1,4,17,28)		No
		60		No
		60		No
		58 (unknown)		No

Cell lines karyotyped are as follows:
PalF= untransfected/non-initiated control; **SVE8$^+$E7$^+$+*ras***= uninitiated PalF cells transformed by pSVE7$^+$E8$^+$+*ras*; **PQ3**= PalF cells initiated with 45μM quercetin; **PQ2**= PalF cells initiated with 20μM quercetin; **Q3D**= as PQ3 but cells were transformed with pBV4+*ras*; **Q2D**= as PQ2 but cells were transformed with pBV4+*ras*. Numbers in brackets refer to chromosomes lost.

In all initiated and tumorigenic lines examined, the chromosome banding pattern was indistinguishable from normal PalF, suggesting that no large scale genetic rearrangements had occurred (R. Fries, personal communication). These preliminary results would indicate that neither initiation by quercetin or the subsequent transformation by BPV-4 and *ras* causes large scale genetic damage. It should be noted however that reports of quercetin acting as a clastogenic agent required considerably higher dosages than utilised in these studies (see Ishidate M. 1988), and its seems likely therefore that quercetin acts as an initiating mutagen. It would however be interesting to treat PalF cells with increasing quercetin concentrations and to monitor their cytogenetic status, as it has been noted that bracken grazing cattle display large scale chromosomal abnormalities (Moura et al, 1988).

In conclusion BPV-4 was incapable of transforming PalF cells without a cooperating activated *ras* plasmid in any experiments performed. The data presented would suggest that the E7 ORF of BPV-4 encodes the major transforming function of the virus. There appears to be a role for the E8 ORF in anchorage independence although the E8 has no direct transforming ability on its own. HPV-16 E6 can cooperate with BPV-4 E7 to lead to immortalisation of

PalF cells but the cells are non-tumorigenic. In addition, the mutagen quercetin can initiate PalF cells, allowing subsequent transformation by BPV-4 + *ras* to a malignant phenotype. The results of this section are summarised in **Table 7.10**.

Table 7.10. Summary of BPV-4 + *ras* transformation experiments.

Characteristics of BPV-4 Transformed Cells

Viral Genes + Activated *ras*	Morphological Transformation	Growth in Methocel	Immortalisation	Tumorigenic in Nude Mice
BPV-4	+	+	-	-
E7	+	-	-	-
E8	--	-	-	-
E7+E8	+	+	-	-
16E6	-	-	-	-
16E6+E7	+	-	+	-
16E6+E7+E8	+	+	+	-
BPV-4 (Quercetin)	+++	+++	?	+++

An overview of the characteristics of BPV-4 transformed PAlF cells.

-= does not display characteristic
--= negative for characteristic
+= positive for characteristic
+++= very strongly positive for characteristic.
?= characteristic not fully determined

7.3 Cellular Localisation of BPV-4 Oncoproteins

7.3.1 Control Reactions

In order to establish the cellular localisation of the BPV-4 E7 and E8 proteins immunocytochemistry was used. PalF cells were transfected with various constructs containing the genes for these viral proteins. The cells were not selected in G418 containing medium and were grown for 48 hrs before immunocytochemistry was performed. Details of antisera used are given in the **Table 7.11.** The technique involves spinning a cell suspension on to a glass microscope slide, incubating this slide with an antiserum raised against the peptide to be localised, then visualising specifically-bound antibodies using a secondary antibody. This secondary affinity-purified anti-IgG antibody (Sera Lab) binds specifically to the primary antibody. The secondary antibody also has a conjugated alkaline phosphatase enzyme covalently linked to it. This allows the visualisation of a positive result by incubating the slide with the alkaline phosphatase substrate (3-hydroxy-2-napthonic acid 2,4 dimethylanilide / 4-chloro-O-toluidine-1, 5^1 diazonium napthalene disulfonate).

Table 7.11. Antisera used in immunolocalisation studies.

Antiserum	**Species**
anti-ras (249) (Furth et al, 1982)	RAT
anti bovine MHC class 1 (Alan Teale, ILRAD)	MOUSE
anti BPV-4 E7 (G.J. Grindlay, unpublished)	RABBIT
anti BPV-4 E8 (G.J. Grindlay, unpublished)	RABBIT

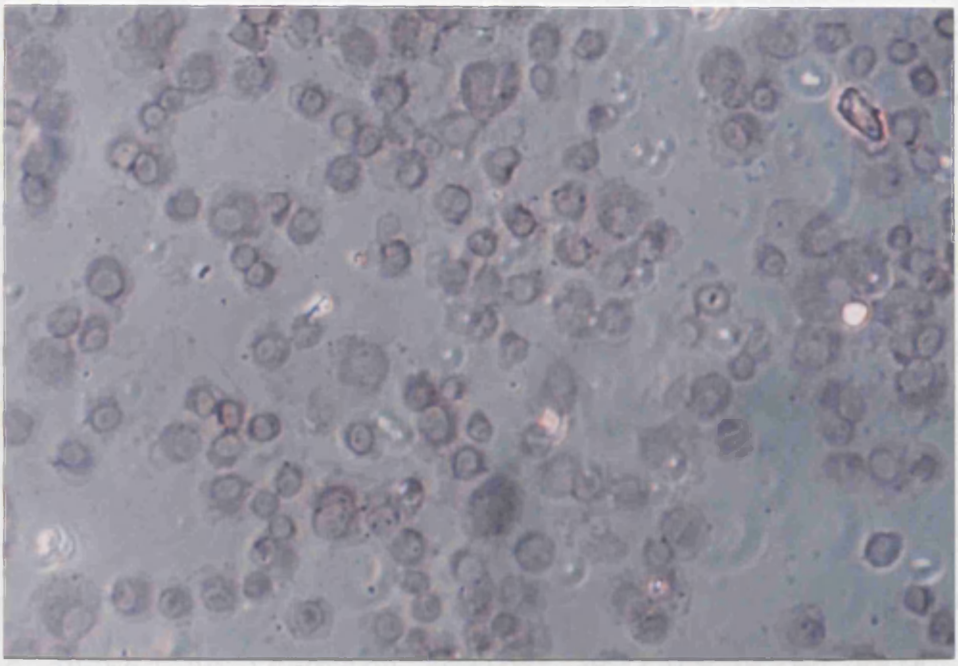
Bound antibody was detected by using species specific secondary antibodies conjugated to an alkaline phosphatase enzyme (see Materials and Methods).

Anti E7 and Anti E8 antisera were raised in rabbits as follows. The E7 antiserum was raised against a bacterially produced ß-galactosidase E7 fusion protein and the E8 antiserum was raised against a synthetic nonapeptide corresponding to the C-terminus of E8 conjugated to keyhole limpet haemocyanin. The specificity of the antisera was determined by Western blot analyses (G.J. Grindlay, personal communication).

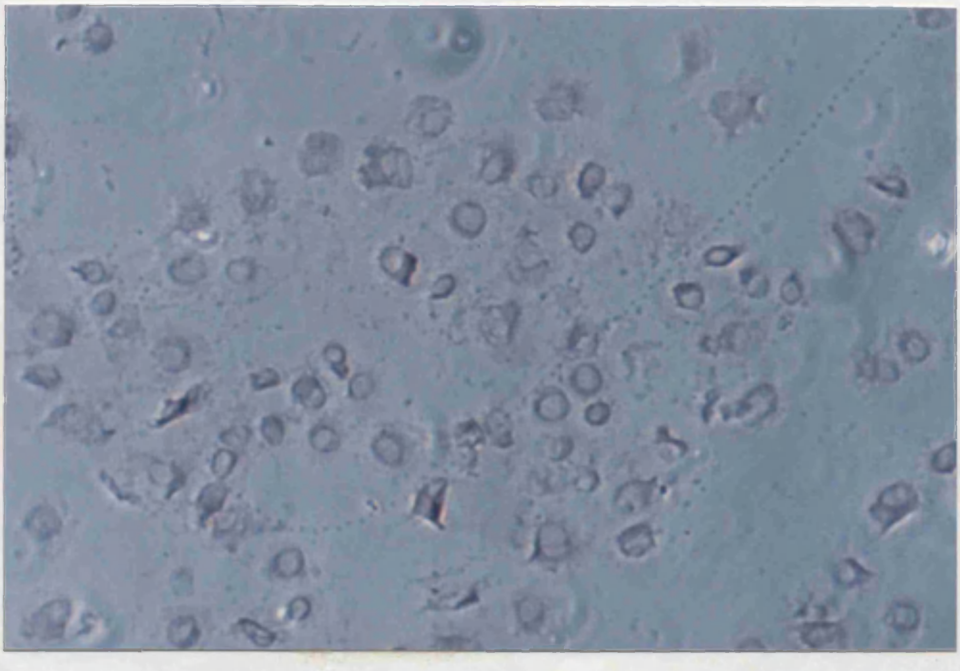
A local colour reaction takes place where the secondary antibody is bound due to the enzymatic conversion of the substrate to an insoluble red precipitate. This colour reaction can then be visualised by light microscopy.

A number of controls were employed to ensure that the reactions were working properly, positive results were not a result of background staining, and to establish that the antisera used did not cross-react. Two positive controls were used to establish if the reagents in the detection kit were functioning properly. Firstly a monoclonal antibody which recognizes antigens of the bovine major histocompatibility complex (MHC) class I was used (this was a gift from Dr Alan Teale of ILRAD, Kenya through Dr Liz Glass, AFRC, Edinburgh). The MHC complex should be expressed on the surface of primary cells and indeed using this antiserum as a primary antibody resulted in strong staining of all cells (**Fig 7.16.**). An additional control was a monoclonal antibody which recognises conserved regions of the Ha-*ras* gene product (Furth et al, 1982) and is therefore expected to cross react with the bovine Ha-*ras* protein. This antiserum also gives a strong positive staining (**Fig 7.16.**) confirming its cross-reactivity and the general suitability of this method for the detection of cellular proteins.

Fig 7.16. Immunocytochemistry: positive control reactions.



A) Untransfected PalF cells stained with an anti bovine MHC class I antibody.



B) Untransfected PAlF cells stained with an anti-*ras* antibody.

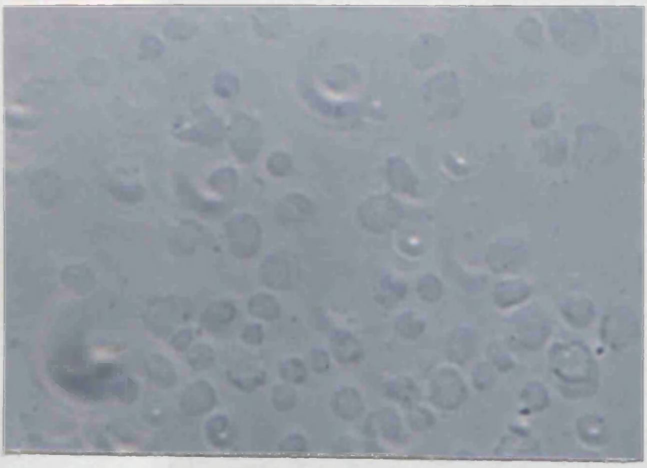
Magnification=x100

To ensure that the secondary antiserum was not binding non-specifically, a negative control which had not been incubated with a primary antiserum was included. When the primary antiserum was replaced with TBS only, no staining occurred (**Fig 7.17.**) demonstrating that the reaction was indeed specific.

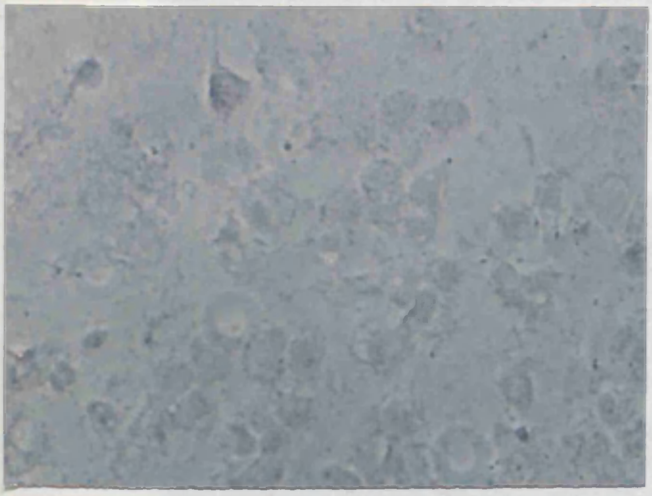
The specificity of the primary antisera was tested by using untransfected PalF cells. When treated with the anti-E7 antiserum these cells showed no staining (**Fig 7.17**) confirming that the antiserum does not display any non-specific binding. Similarly, the anti-E8 antiserum also showed no reaction against untransfected PalF cells (**Fig 7.17.**).

Possible cross-reactivity of the antisera was examined as follows. Cells transfected with pZipE7 were examined using the anti-E8 antiserum. Under these conditions there was no staining, demonstrating that the E8-antiserum does not recognise the E7 protein (**Fig 7.18.**). The E7-antiserum was also examined in this way and no staining was observed when this antiserum was used on pZipE8 transfected cells (**Fig 7.18.**).

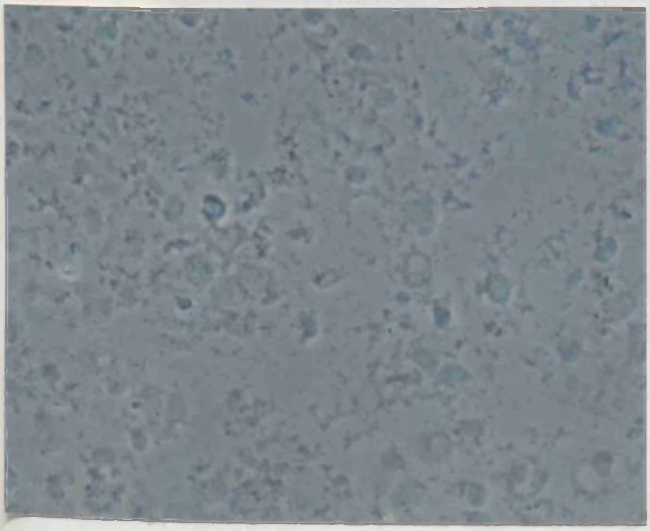
Fig 7.17. Immunocytochemistry: negative control reactions



A) Negative control; untransfected PalF cells, no 1^{o} antibody.



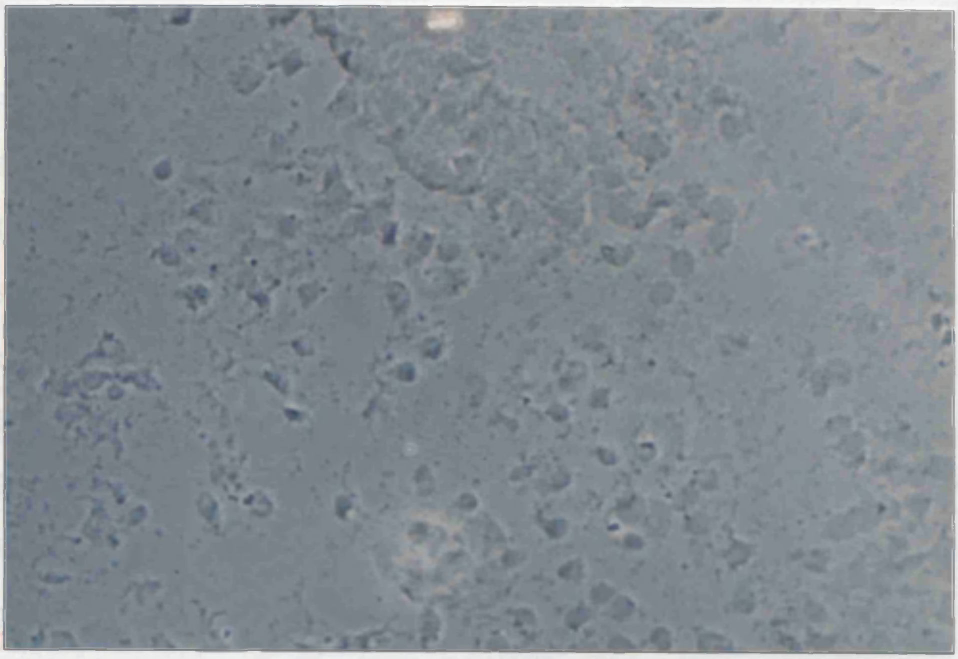
B) Negative control; untransfected PalF cells, anti-E7 1^{o} antiserum.



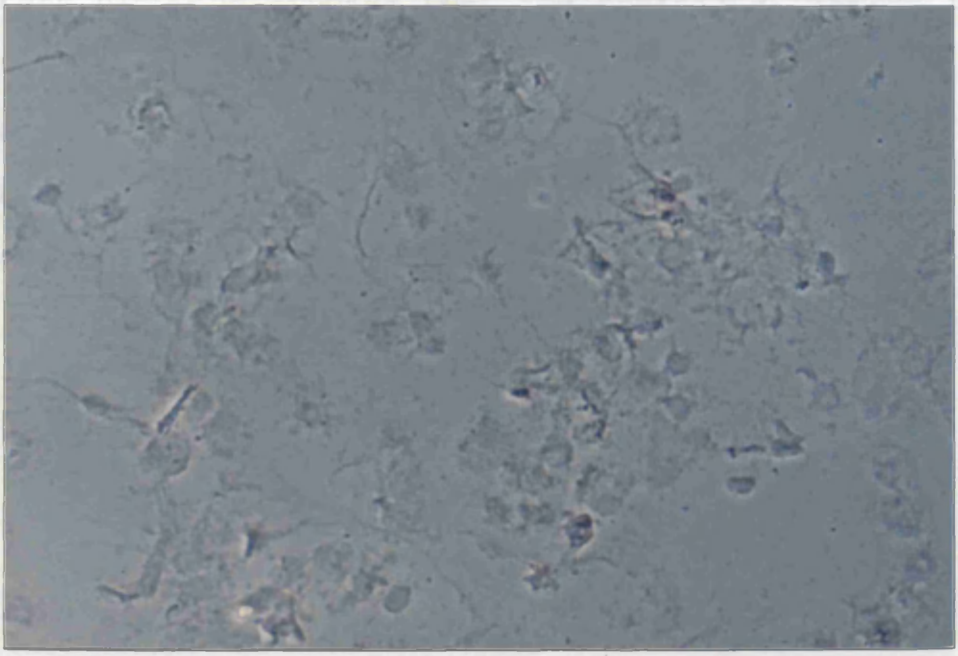
C) Negative control; untransfected PalF cells, anti-E8 1^{o} antiserum.

Magnification=x100

Fig 7.18. Immunocytochemistry: cross-reactivity control reactions.



A) PalF cells transfected with pZipE8 and treated with anti-E7 1^{o} antiserum.



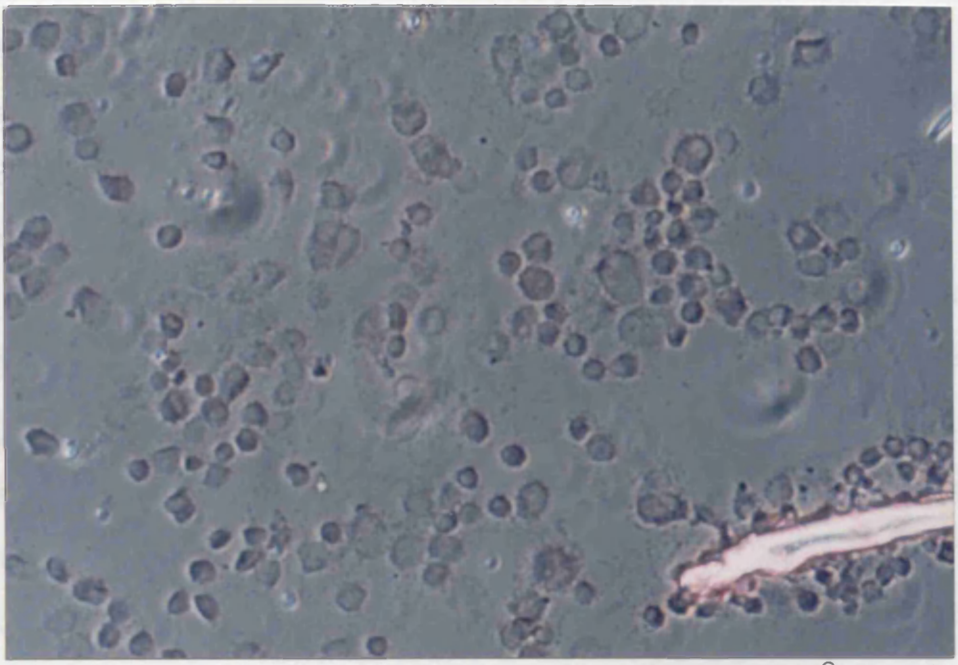
B) PalF cells transfected with pZipE7 and treated with anti-E8 1^{o} antiserum.

Magnification=x100

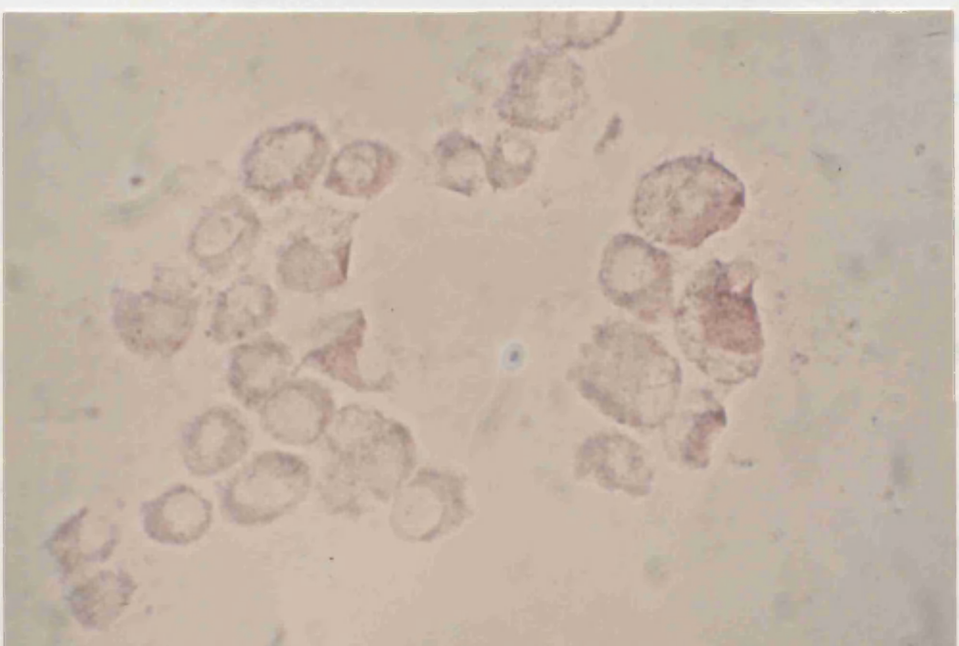
7.3.2 BPV-4 E7 Localisation

The cellular localisation of BPV-4 E7 was investigated in PalF cells transfected with either pZipE7, pBV4 or pSVE8^{+}E7^{+}. Immunocytochemical staining was essentially equivalent between cells transfected by either pBV4 or pSVE8^{+}E7^{+} but cells transfected with pZipE7 had a higher degree of staining which may be a reflection on the more efficient transcriptional control of the pZip vector (**Fig 7.19.**). The low level of staining observed with pBV4 or pSVE8^{+}E7^{+} was sufficiently higher than background to suggest that these constructs are expressing E7 at levels detectable by light microscopy. It is also known from western blotting studies that the anti-E7 antiserum is of low titre (G.J. Grindlay, personal communication) and this may be why the E7 protein staining in the pBV4 or pSVE8^{+}E7^{+} transfectants was not as strong as that observed in cells transfected by pZipE7.

Fig 7.19. Immunocytochemistry: BPV-4 E7 cellular localisation.



A) pZipE7 transfected PalF cells. Anti-E7 1^{o} antiserum (low magnification). **Magnification=x100**



B) pZipE7 transfected PalF cells. Anti-E7 1^{o} antiserum (high magnification). **Magnification=x350**

In the pZipE7 transfectants the staining demonstrates that this protein localises chiefly to the cytoplasm with some staining being present in the nucleus (**Fig 7.19.**). This observation is in accordance with the observed functions of HPV 16 E7 namely transcriptional transactivation (Phelps et al, 1988) and complexing with the product of the retinoblastoma anti-oncogene (Dyson et al 1989). In addition it was observed that only 40-60% of cells displayed a positive staining. This would indicate that, as expected, only transfected cells producing E7 protein stained with this technique. The observation that staining occurs in only a subset of cells is further evidence that the staining is not artefactual.

It was not always possible to observe a nuclear E7 localisation in repeated experiments which may be due to the relative insensitivity of the technique. It has been observed, however, that levels of E7 expression in HPV-associated lesions *in vivo* are generally low. A more sensitive approach, such as using heavy metal tagged secondary antibodies followed by electron microscopy visualisation may be necessary to establish the extent of nuclear localisation of E7. This may be especially true when the E7 is under the normal transcriptional control of the BPV-4 LCR rather than when artificially overexpressed in the pZipE7 vector.

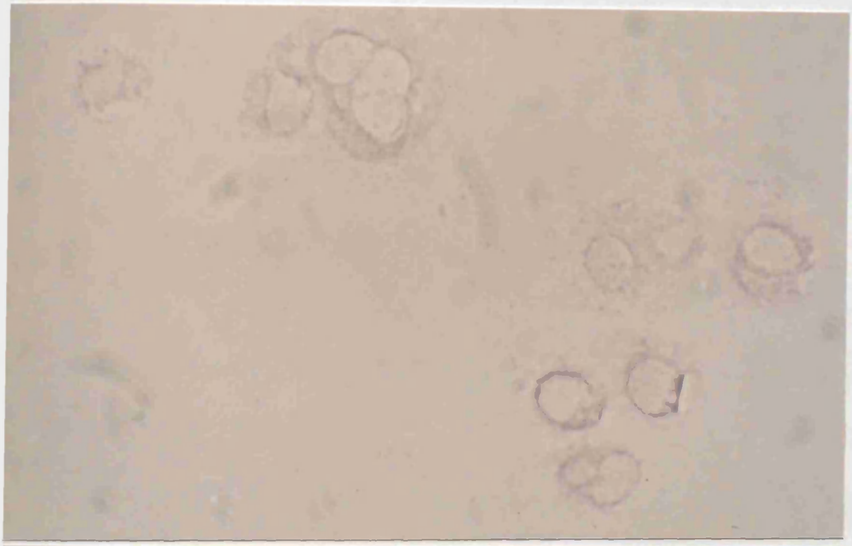
The BPV-4 E7 localisation is in accordance with amino acid sequence data which would predict interaction with both DNA and the nuclear tumour suppressor protein $p105^{Rb}$. The localisation of BPV-4 E7 is also in agreement with previous studies on the HPV-16 E7 which localises to the cytoplasm by immunocytochemistry (Smotkin and Wettstein, 1987) and to the nucleus by immunofluorescence (Sato et al, 1989). The effects of BPV-4 E7 in transformation assay, the conservation of crucial domains between the E7s of oncogenic HPVs and BPV-4 and the similar cellular localisation of both proteins make it reasonable to speculate that the E7 may function in very similar ways in both the human and bovine viruses.

7.3.3 BPV-4 E8 Localisation

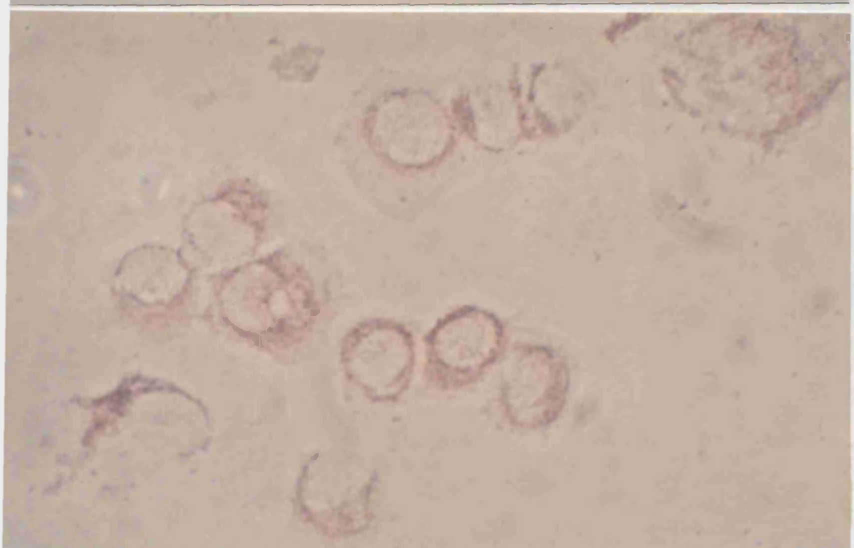
PalF cells were transfected with either pZipE8, pBV4 or $pSVE8^{+}E7^{+}$ 48 hrs before immunocytochemistry was performed to determine the localisation of the BPV-4 E8 protein. The E8 protein proved to be easier to detect using this technique than did the E7, which is probably due to the anti-E8 antiserum being of higher titre as demonstrated by its efficacy in Western blotting (G.J. Grindlay, personal communication).

Transfected cells showed E8 was localised primarily in the membrane compartments, particularly the plasma membrane, endoplasmic reticulum (ER) and Golgi apparatus. This was true for cells transfected with pBV4 and pSVE8^{+}E7^{+} with particularly good staining observed in pZipE8 transfected cells (**Fig 7.20.**). This membrane localisation is in accordance with the ER and Golgi apparatus localisation of the BPV-1 E5 (Burkhardt et al. 1989) which shows considerable sequence similarity and an almost identical hydrophobicity profile to the BPV-4 E8 (**Fig 7.5.**). Determination of the orientation of the E5 in membrane fractions has been achieved by the use of immunoelectron microscopy (Burkhardt et al, 1989). In this work, using baculovirus expressed E5 in Sf9 insect cells, the E5 was found to localise in cytoplasmic as well as Golgi membranes, with the carboxyl-terminus facing either extracellularly or interluminarly respectively. This localisation is in contrast to the suggestions that BPV-1 E5 acts predominantly in the nucleus as a mitogenic stimulator (Green and Lowenstein, 1987). However the microinjection of synthetic E5 in C127 and NIH3T3 cells has shown that it acts as a mitogenic stimulator only when introduced directly into the nucleus and has no effect when microinjected into the cytoplasm (Green and Lowenstein, 1987).

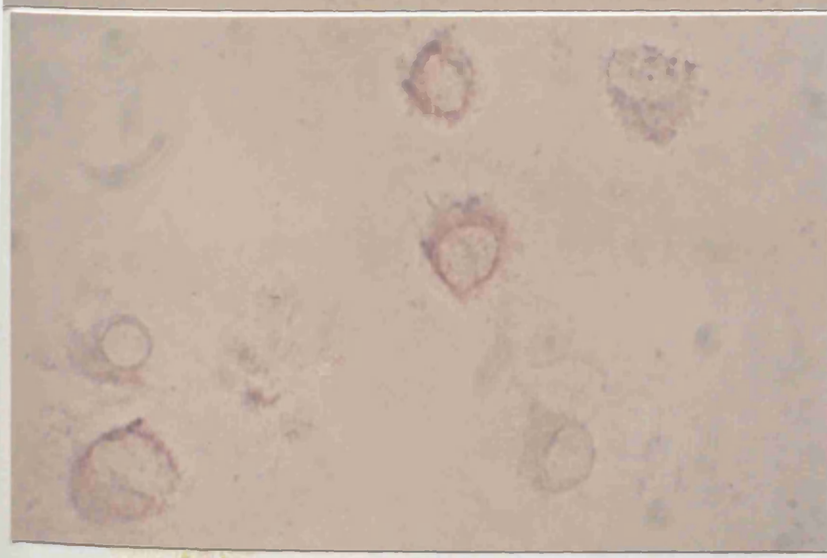
Fig 7.20. Immunocytochemistry: BPV-4 E8 cellular localisation.



A) PalF cells transfected with pBV4. Anti-E8 1° antiserum.



B) PalF cells transfected with pSVE8$^+$E7$^+$. Anti-E8 1° antiserum.



C) PalF cells transfected with pZipE8. Anti-E8 1° antiserum.

Magnification=x350

BPV-1 E5 has not been demonstrated to have a nuclear localisation by immunolocalisation techniques. Indeed, it has been predicted, from studies of the membrane orientation of E5, that if any E5 were present in the nucleus, it would be orientated with the interactive carboxyl-terminus projecting into the space between the two nuclear membranes (Burkhardt et al, 1989) precluding the carboxyl-terminal from having a nuclear function.

The BPV-1 E5 localisation was confirmed by other workers using the technique of epitope addition (Field et al, 1988) to engineer a functional BPV-1 E5 fusion protein which could be localised by the use of monoclonal and polyclonal antibodies against the inserted epitopes, followed by immunofluorescence microscopy (Goldstein and Schlegel, 1990). Indeed, when this method was employed in coprecipitation assays, the BPV-1 E5 was found to be complexed with a cellular protein of 16kd, a complex not observed when antisera against the carboxyl-terminal domain of E5 are used. The authors suggest that antibodies raised against the E5 carboxyl-terminal domain may potentially displace any BPV-1 E5 bound proteins or select for non-bound E5, problems not encountered when using the epitope addition technique. The 16Kd cellular protein complexed with E5 was later identified to be the component vacuolar proton channel-forming ATPases and gap junctions (Finbow et al 1988; Leitch and Finbow, 1990; Goldstein et al, 1991).

The similar localisation of BPV-4 E8 and BPV-1 E5, together with their amino acid similarity and hydrophobicity profiles further suggests the possibility that the two viral proteins may share some common function(s). It is therefore feasible that the BPV-4 E8 protein might interact with the 16k protein in a manner similar to BPV-1 E5. The role of BPV-1 E5 on membrane receptor activation has been documented (Petti et al, 1991; Martin et al 1989) but studies on observable consequences of 16k complexing had not been performed at the time of commencement of this thesis. It can be hypothesised that the capacity of BPV-1 E5 expressing cells to communicate with adjacent cells via their gap junctions might be decreased as the 16k protein, a component of gap junctions (Finbow et al. 1991), would be bound by E5. By similar reasoning, cells producing BPV-4 E8 may have a reduced communication capacity if this viral protein is also capable of binding the 16k protein. As the ability of papillomavirus genomes to affect junctional communication had not been documented, it was decided to examine the communication potential of a variety of papillomavirus transformed cell lines. The results of this investigation will be presented in the following section.

7.4 Microinjection/Dye Transfer

The possibility of papillomavirus proteins interacting with the gap junctional form of the 16k protein (Finbow et al, 1991) was assessed by dye transfer studies. The principle of the dye transfer technique is as follows. Cells are grown until subconfluent on glass coverslips. A single cell is iontophoretically microinjected with a solution of the fluorescent dye Lucifer Yellow CH (see Materials and Methods section 6.16.) which will pass freely through gap junction pores but will be retained by cells which do not possess these structures. The extent of passage of the dye from the injected cell to neighbouring cells in direct physical contact is a measure of how efficient the "chemical coupling" or gap junctional communication is between these cells.

The role of gap junctions in cellular growth control and tumorigenesis is at present unclear, with no general pattern in defects of junctional communication in tumorigenesis having been demonstrated (Weinstein and Pauli, 1987). Recent studies have suggested that in epidermal tumorigenesis an important aspect may be the breakdown of the patterns of junctional communication (Pitts et al, 1988). Dye-transfer experiments on normal mouse epidermis have produced a map of normal junctional communication patterns (Kam et al, 1986; Pitts et al, 1988). The stroma forms a large apparently limitless compartment of cells while the epidermal layer is composed of smaller communicating compartments. There is a distinct communication boundary between the stromal and epidermal layers marked by the basement membrane.

A chemical known to affect gap junctions is the tumour promoter 12-O-tetradecanoyl phorbol-13-acetate (TPA) which has been shown to inhibit junctional communication of sensitive cells in culture (Yotti et al, 1979) by reducing the rate of gap junction formation (Pitts and Burk, 1987). As discussed in the Introduction, TPA is effective in a two stage mouse skin tumorigenicity model by acting as a promoting agent on populations of cells initiated with the mutagen dimethyl benzanthracene (DMBA) (Balmain et al, 1984). Work using cultured keratinocytes has suggested that TPA acts by activation of the differentiation pathway giving rise to prematurely differentiating basal cells which are in turn replaced by expansion of stem cells, some of which will have been initiated by DMBA treatment. The release of these initiated cells from normal growth control after several treatments with the promoter constitutes the observed hyperplasia (Parkinson, 1985). The disturbance of the basal cells (which manufacture the constituents of the basal membrane) in this scheme may explain the loss of the basal membrane and could explain why the dermal-epidermal communication boundary is frequently observed to break down in TPA treated mouse skin (Kam and Pitts, 1987).

Junctional communication is a likely candidate for a feedback mechanism required to control the division rate of basal cells to match the loss of terminally differentiated cells by desquamation, and both positive and negative feedback mechanisms utilising junctional communication have been proposed for this control (Sheridan, 1976; Loewenstein, 1979). For example, the observation that *ras* proto oncogene products are expressed more strongly in proliferating layers than differentiated cells (Furth et al 1987) gives rise to the hypothesis that the upper layers of communicating cells act as a sink for second messengers (Loewenstein 1979), preventing threshold levels of inositol phosphates and other second messengers accumulating in the basal layers. This model has special significance in the case of BPV-4 E8, as this viral protein is found to be expressed in the basal and suprabasal layers of BPV-4 induced papillomas

(R. Anderson, personal communication). Should BPV-4 E8 complex with the 16k gap junction protein and lead to a loss of junctional communication, the "sink" effect of the upper differentiating layers would be abolished, levels of second messenger would increase in basal and supra basal layers and consequently aberrant proliferation in these layers could occur. An assessment of the potential of papillomavirus transformed cells to communicate via their gap junctions was performed by microinjection / dye-transfer experiments.

In the first instance, the consequences of BPV-1 transformation of primary bovine conjunctiva fibroblasts (CON cells) was examined (Smith et al, 1987). The binding of BPV-1 E5 to the 16K protein has been well documented (Goldstein and Schlegel, 1990) and therefore it is reasonable to assume that BPV-1 transformed cells will display any biochemical consequences of 16k-E5 complexing. Control CON cultures showed moderate dye coupling, with the dye spreading to all cells in physical contact with the injected cell (**Table 7.12.**). This demonstrates that control, untransformed CON cells have functional gap junction structures. CON cells transformed by BPV-1 showed a completely disrupted junctional communication status however; no dye transfer occurred and the dye remained in the original injected cell (**Table**

7.12.). This suggests that one consequence of transformation of CON cells by BPV-1 is the disruption of cell-cell communication. This disruption may well be a result of E5 and 16k complexing although these observations do not provide direct evidence for this. A possible way of providing a direct correlation between E5-16k complexing and junctional communication inhibition would be the use of inducible vector systems encoding the BPV-1 E5. In this way a population of normally communicating cells would be transfected with an inducible BPV-1 E5 construct. The gap junctional status of this transfected cell population could be assessed before the construct encoding BPV-1 E5 was induced. Following the induction stimulus, the same population of cells (now expressing the E5 transcript) could be examined for chemical coupling, and any loss of communication status attributed to E5 expression. This type of experiment is currently being initiated at the Beatson Institute (M. Finbow and M.S. Campo, personal communication).

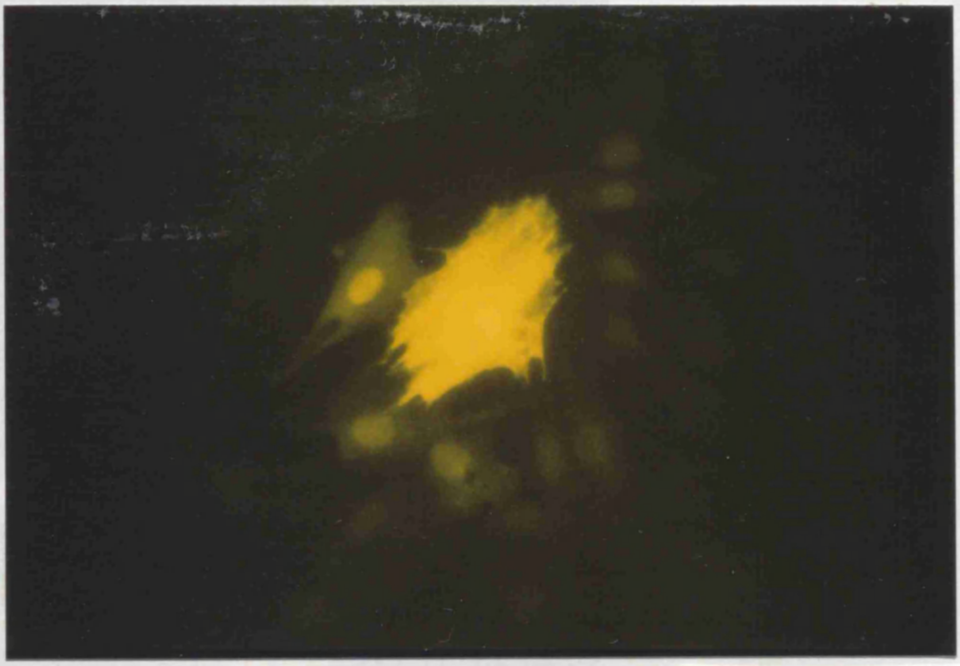
Table 7.12. Dye transfer capacity of papillomavirus transformed cells.

Cell line	**% of injections displaying dye-spread**	**Avg No. coupled cells per group**
PalF control	100	14.4
Con20	100	7.9
Con BPV-1	20	0.4
pBV4+ras	90	2.8
pSVE8$^+$E7$^+$+ras(1)	40	1.3
pSVE8$^+$E7$^+$+ras(2)	10	1.2
pSVE8$^+$E7$^+$+ras+pZipE2	80	2.3
pZipE7+ras	100	9.2
PZipE7+pZipE8+ras	95	6.2
pJ4Ω16.E6+pZipE7+ras	100	3.2
pJ4Ω16.E6+pZipE7+pZipE8+ras	100	3.2
C12K (HPV-ve)	100	3.9
C10K (HPV+ve)	8	0.1

Notes: **Con20 BPV-1** are Con20 cells transformed by BPV-1. **C10K** cells were isolated from an HPV+ve cervical lesion and **C12K** cells were isolated from HPV-ve dysplastic tissue.

Each culture was injected at between 5 and 12 individual sites.

Fig 7.21. Microinjection / dye-transfer experiments on PalF cells.

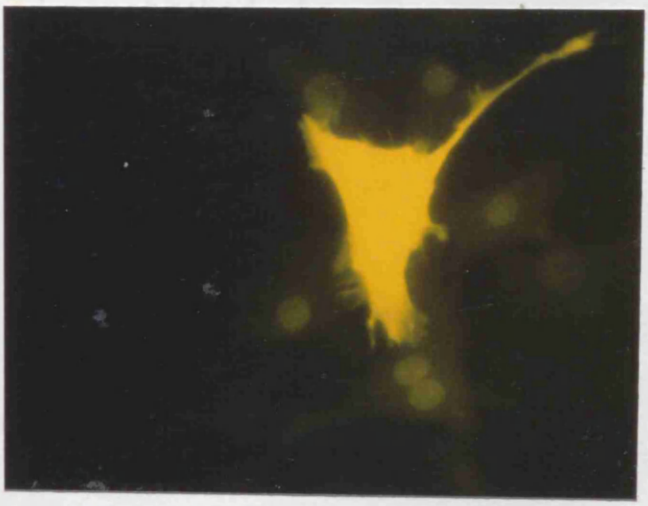


Magnification=x350

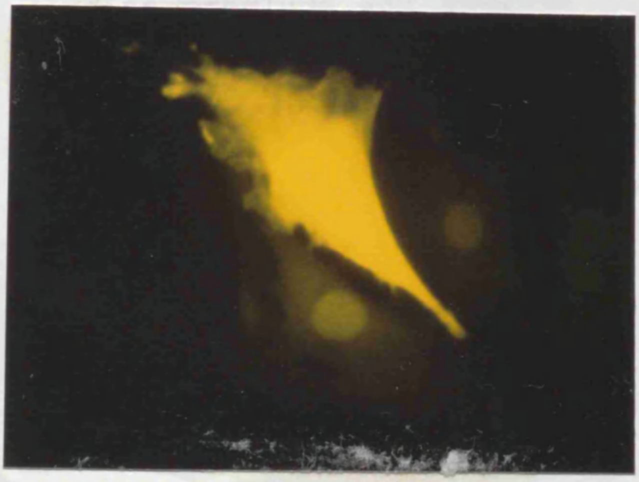
Dye-coupling in untransfected PalF cells.

PalF cells were microinjected with the the fluorescent dye Lucifer Yellow CH and photographed under UV light as described in Materials and Methods section 7.4.

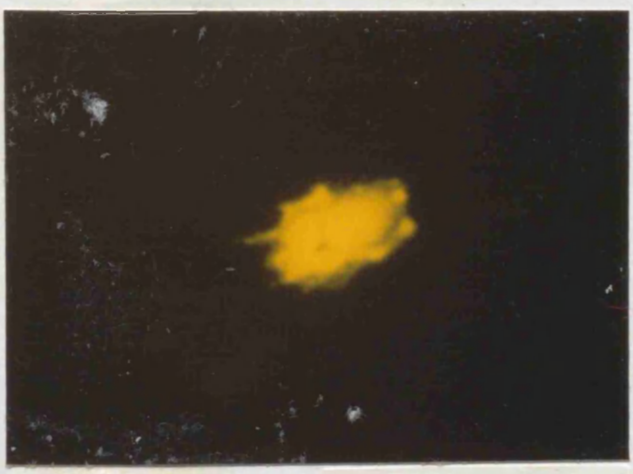
Fig 7.22. Microinjection / dye-transfer experiments on BPV-4 + *ras* transformed cells.



A) PalF cells transformed by pSVE8^{+}E7^{+} + *ras* + pZipE2 and examined for dye-coupling as described in Materials and Methods section 7.4.



B) Dye transfer in PalF cells transformed by pJ4Ω16.E6 + pZipE7 + pZipE8 + *ras*.



C) Dye transfer in PalF cells transformed by pSVE8^{+}E7^{+} + *ras*(2).

Magnification=x350

Disruption of junctional communication was also observed in human papillomavirus associated biopsy material. Cells from a squamous cell carcinoma sample, previously demonstrated to be positive for HPV-16 DNA by the polymerase chain reaction (G.McGarvie, personal communication) were found to have disrupted communication potential when compared to cells from HPV -ve dysplastic tissue (**Table 7.12.**). In addition, the HPV-16 transformed keratinocyte line W12 (Stanley et al, 1989) was found to be uncoupled (G.Sibbett personal communication, **Table 7.12.**). The small number of samples examined in these studies preclude any general conclusions but it would be interesting to examine a larger number of papillomavirus associated cell lines and biopsies to see if the loss of junctional communication is a common feature of papillomavirus transformation biology.

BPV-4 transformed PalF cell lines were also examined for their communication potential. Clonal populations of cells (originally isolated as single $G418^r$ colonies from transfection experiments) were expanded and grown on coverslips to a suitable density. This analysis was limited to clones of cells which recovered well from liquid N_2 storage and had sufficient life-span when recovered to obtain healthy cultures of cells. This precaution was taken to avoid metabolically inactive, senescing or dead cells being scored as non-communicating. Control untransfected PalF cells displayed moderate dye coupling; the injected dye spreading freely to neighbouring cells (**Table 7.12, Fig 7.21**). BPV-4+*ras* transformed cells showed an almost normal coupling with only a very slight reduction in communication (**Table 7.12., Fig 7.22.**). This situation was also found to be true for cells transformed by pSVE8^{+}E7^{+}+*ras*+pZipE2 (**Table 7.12.**). In addition, pZipE7+pZipE8+*ras* transformed PalF cells also had essentially normal communication patterns (**Table 7.12, Fig 7.22**).

Immortalisation of cultures by the joint action of HPV-16 E6, BPV-4 E7 and *ras* was not accompanied by a disruption of junctional communication in the case of pJ4Ω16OE6+ZipE7+*ras* transformed cells (**Table 7.12.**) as these cells communicated freely. The addition of the E8 encoding pZipE8 construct (in pJ4Ω16.E6+ZipE7+ZipE8+*ras* transformed PalF cells) did not detectably alter this communication (**Table 7.12.**). This implies that sustained loss of junctional communication is not a necessary feature of immortalisation of this cell type.

A very interesting result was observed in the case of a clone of cells transformed by pSVE8^{+}E7^{+}+*ras* (2). In this population of cells, junctional communication was totally abolished (**Table 7.12, Fig 7.22.**); the dye remained in the injected cell only. Lack of communication was not due to cell death or senescence, as the pSVE8^{+}E7^{+}+*ras* cells could be expanded for several population doublings after the stage at which they were used for the microinjection studies.

The observation that only one cell line transformed by BPV-4 genes + *ras* (of six examined) shows an abnormal junctional communication pattern suggests that sustained loss of junctional communication is not important for the transformed phenotype of these cells and indeed may not take place at any stage in the transformation process. It could be proposed that the BPV-4 E8 protein does not complex with the 16k protein and therefore does not normally interfere with junctional communication. If this hypothesis was correct, the lack of junctional communication observed in the pSVE8^{+}E7^{+}+*ras* non-communicating line would possibly be due to a random genetic mutation, giving rise to an alteration in the production, modification or localisation of the 16k protein and thus an aberrant communication pattern. Such a hypothesis is unlikely however, as such a genetic event is likely to be very uncommon and searches for such mutants in cultured cells are rarely successful (J. Pitts, personal communication).

An alternative possibility is that the communicating and non-communicating cell lines differ in their maintenance of viral DNA or patterns of expression. As outlined in the Introduction, BPV-4 mediated cell transformation *in vitro* has been found to mimic the *in vivo* situation; viral DNA is not required for the maintenance of the transformed state (Smith and Campo, 1988; Campo et al, 1985). In earlier Southern blot analyses I demonstrated that in pBV4 + *ras* transformed PalF cells BPV-4 DNA was often lost on continued passage (Jaggar et al, 1990). It is possible therefore, that the pSVE8^{+}E7^{+}+*ras*(2) non-communicating cell line had maintained a significant copy number of BPV-4 DNA while the non-communicating lines had lost the majority of the transfected DNA. The status of the E8 gene, encoding the putative 16k interacting protein, is of particular interest in this respect. It is reasonable to assume that E8 may exert any effects at an early stage in the transformation process, as immunolocalisation experiments of E8 production in BPV-4 induced papillomas has demonstrated that the E8 is produced at early stages of papilloma development (R. Anderson, personal communication).

The possibility of a difference in maintenance of viral DNA between the communicating and non-communicating cell lines was addressed by preparation of genomic DNA from the transformed cell lines and the analysis of their viral DNA status by Southern blotting and DNA hybridisation. This will be presented in the following section.

7.5 Southern Blotting

As discussed in the Introduction, presence of BPV-4 DNA is not required either for the maintenance of malignancy *in vivo* (Campo et al, 1985) or for the maintenance of the transformed phenotype in C127 cells *in vitro* (Smith and Campo, 1988). The presence of viral DNA in BPV-4 transformed PalF cells was investigated by Southern blotting. The DNAs from fifteen cell lines were digested with EcoR1 and 20μg of each of the digested samples was electrophoresed and blotted as described in the Materials and Methods section.

Four cell lines transformed by pBV4+*ras* (out of seven) were found to contain no detectable viral DNA (**Fig 7.23.**) when probed with a BPV-4 full genome radioactive probe. One genome equivalent of pBV4 DNA could easily be detected in a reconstruction experiment performed at the same time. The reconstruction control experiment consisted of adding 1, 5 or 10 genome equivalents of BPV-4 to EcoR1 digested untransfected PalF DNA (**Fig**

7.23). The reconstruction blot was incubated in the same prehybridisation and hybridisation solutions as the transformed cell DNA blot. In addition, multiple integrations of neo sequences were detected in the BPV-4 negative cell lines when the blot was probed with a radioactive probe prepared from the neo resistance gene of pZipneo (**Fig 7.23**), a result which demonstrates the ability to detect transfected sequences in PalF cells.

Seven of the fifteen genomic DNA samples contained detectable viral DNA at various levels (**Fig 7.24.**, **Table 7.13.**). Of the positive cell lines, three contained less than one genome equivalent per cell, as judged by comparison to a reconstruction experiment performed at the same time (such as that shown in **Fig 7.23.**). These lines were transformed by pBV4+*ras*+pZipE2, pZipE7+*ras* and pJ4Ω16.E6+pZipE7+*ras*. The line transformed by pBV4+*ras*+pZipE2 appeared to have three discernible bands at approximately 10-15kb, 4.5kb and 1.8kb while the pZipE7+*ras* and the pJ4Ω16.E6+pZipE7+*ras* transformed cell DNAs both had a barely detectable smeared high molecular weight band (>15kb). The high molecular weight of this band would suggest an integrated and rearranged E7 in these samples or partial digestion of the sample DNA.

The three DNA positive BPV-4+ras transformed lines all appeared to maintain viral DNA at around 1 ge per cell as judged by the reconstruction experiment. In all three lines three bands of approximately 10-15kb, 4.5kb and 1.8kb were detected. The line transformed by pSVE8$^+$E7$^+$+ras (2) maintained a very high level of viral DNA compared to the other cell lines. This is particularly interesting as this was the line which displayed a reduced junctional communication potential (see **Table 7.12.** and **Fig 7.22.**). This line appeared to maintain viral DNA at greater than 100 ge per cell (**Fig 7.24.**). A short exposure autoradiograph of this blot (see **Fig 7.24.** panel b) allowed the identification of three major bands: two high molecular weight bands which may represent incompletely digested DNA and/or integration events, a strongly positive band at approximately 9kb and a smaller molecular weight band (approximately 800bp).

The pAT153 vector contains a single EcoR1 site (Twigg and Sherratt, 1980) while the BPV-4 genome contains three EcoR1 sites at nucleotides 906, 1139 and 3147. EcoRI digestion of pBV4 is known to produce the following fragments (Jaggar, 1990):

4.9 kb containing the major portion of the BPV-4 genome encompassing the 3' portion of the E2 ORF, the late ORFs, the non coding region, the complete E8 and the 5' two thirds of the E7 ORF; **5 kb** encompassing the pAT153 EcoR1 site to BPV-4 nucleotide 3147 encoding a 3' portion of E1 and a 5' portion of the E2 ORF; **200 bp** from BPV-4 nucleotides 906-1139 which represent the 3' third of the E7 ORF and a 5' part of the E1 ORF; **1.65 kb** from BPV-4 nucleotide 1139 to pAT153 EcoR1 site which encodes the a portion of the E1 ORF.

Thus in the pBV4 transfected cells which are positive for viral DNA the 4.5kb band may be the 3147-906 fragment of pBV4 (ie major portion of the genome), the 1.8kb band would correspond to the expected 1.65kb fragment (E1) while the high molecular weight band would represent the remainder of the recombinant plasmid integrated in the host genome. The differences between the observed and the expected band sizes is within the limits of measurement error. The lack of detection of the 200bp fragment (E7/E1) is probably due to this fragment having migrated off the gel.

EcoR1 digestion of the pSVE8^{+}E7^{+} plasmid generates three fragments (Jaggar, 1990): **6.7kb** (approx 4.9 kb of the vector with 1.8kb of BPV-4 representing the 3' end of the L1, the ncr, E8 and 5' two thirds of the E7 ORFs), **0.8kb** (approx 700bp of vector and 100bp of the BPV-4 E1 ORF) and **200bp** (the 3' third of the E7 ORF and the 5' end of the E1 ORF). In the genomic digest therefore, the observed band of 800bp may be the fragment containing the E1 portion, the band at 9kb may represent one arm of an integration event, and the high molecular weight band(s) may represent integrated and/or rearrangement events.

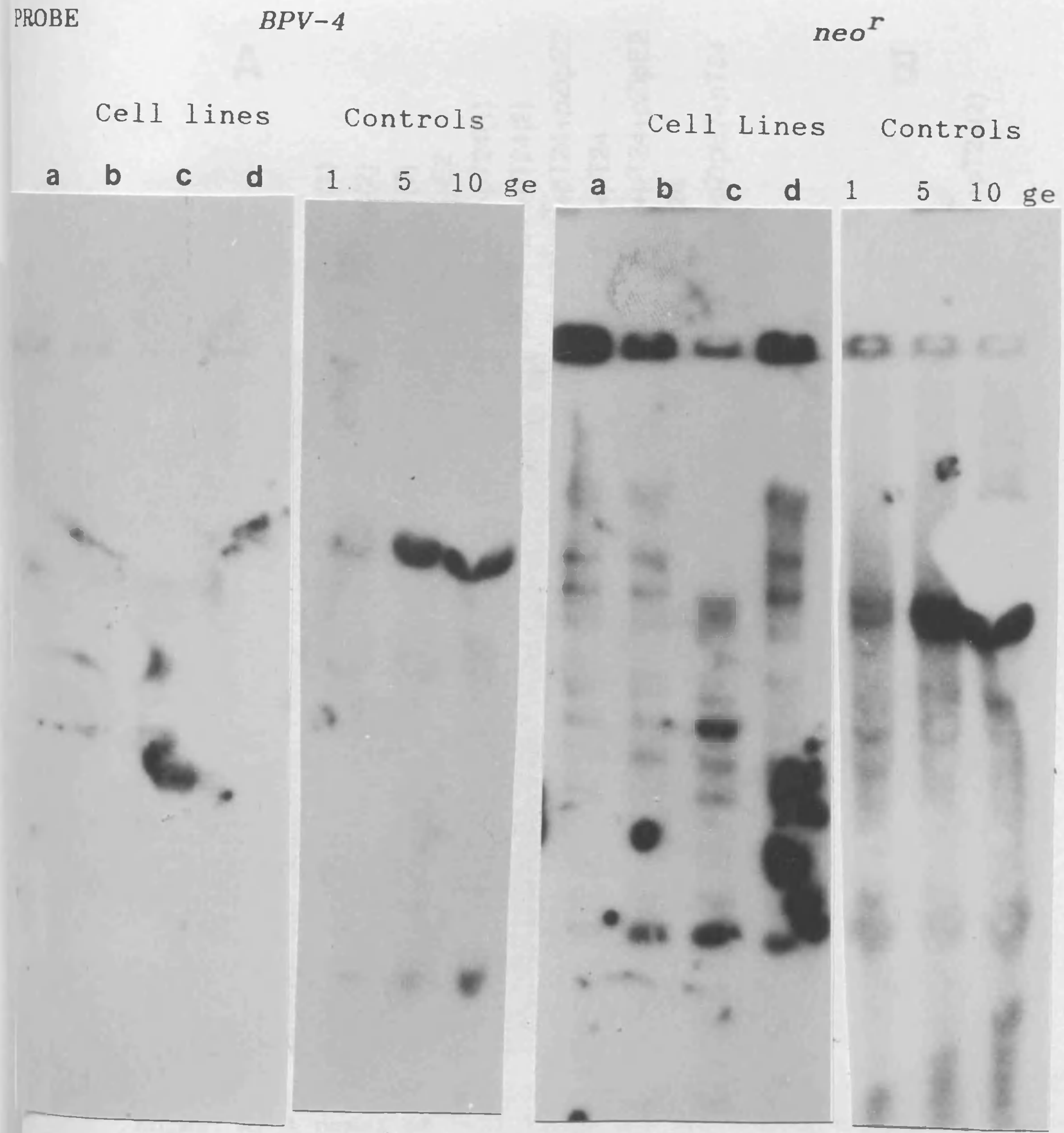
To assess in more detail which regions of the viral genome were present in these cell lines the blot was stripped clean and reprobed as described in the Materials and Methods section and rehybridised using an E7 probe (prepared by BamH1 digestion of the pZipE7 plasmid). The high molecular weight bands observed in the pZipE7+pT24 and pJ4Ω16.E6+pZipE7+pT24 transformed cell lines were detectable as a stronger signal strengthening the hypothesis that this band represents pZipE7 integration event(s) (**Fig 7.25**). The high molecular weight bands in the pSVE8$^+$E7$^+$+pT24(2) lane again were detectable as was the band at 9kb. The 800bp band detectable by a full genome probe (**Fig 7.24**) (postulated to contain 100bp of the E1) was not detected (**Fig 7.25**). In the pBV4 containing positive lanes, no distinctively positive bands were observed. It is possible, however that the shorter E7 probe is not as sensitive as the full genome BPV-4 probe, and therefore did not pick up the expected band at 4.5kb which would contain the 5' two thirds of E7.

After being stripped a second time, the blot was reprobed using a BPV-4 E8 probe which was prepared by BamH1 digestion of pZipE8 (**Fig 7.26**). The high molecular weight bands in the pZipE7+pT24 and pJ4Ω16.E6+pZipE7+pT24 transformed cell lines were not detectable as expected, as these cell lines had not been transfected with E8 DNA (**Fig 7.26**). The 9kb and high molecular weight bands of the pSVE8^{+}E7^{+}+T24 cell line were very strong when using this probe, suggesting that this line maintains a large amount of the E8 ORF. This observation is in accordance with this line's abnormal junctional communication potential, postulated to be the result of E8-16k complexing (see **Fig 7.22** and **Table 7.12**). Curiously the 800bp band, (which is proposed to contain 100bp of E1 and no E8), gave a strong signal when probed with E8. Sequence analysis has shown no significant homology between the E1 and E8 ORFs (data not shown) and the observation that this band is detectable with an E8 probe remains unexplained but could possibly be due to residual vector sequences in the probe. In the DNA positive pBV4 containing lines, the 1.8kb (E1 containing) band is undetectable and high molecular weight bands (proposed to represent the integrated portions of the recombinant) are only readily detectable in the pBV4+pT24(3) lane. The 4.5kb band (observed with the whole genome probe) which contains the major portion of the genome, including the E8 ORF, is easily detectable with the E8 probe.

The observation that several cell lines maintain DNA but are not immortal (**Table 7.13; Table 7.10**) would suggest that the senescence of these lines in culture is not a consequence of viral DNA loss. Indeed both DNA positive and negative cell lines appeared morphologically transformed at the time they were harvested for genomic DNA preparation.

In summary the Southern analyses would suggest that maintenance of viral DNA is not required for the transformed phenotype of PalF cells. The observation that the cell line transformed by pSVE8^{+}E7^{+}+*ras*(2) maintains an unusually high copy number of viral DNA (in particular the E8 ORF) and has an abnormal junctional communication pattern strengthens the hypothesis that the BPV-4 E8 protein may interact with the 16k component of gap junctions in a manner similar to that demonstrated for BPV-1 E5.

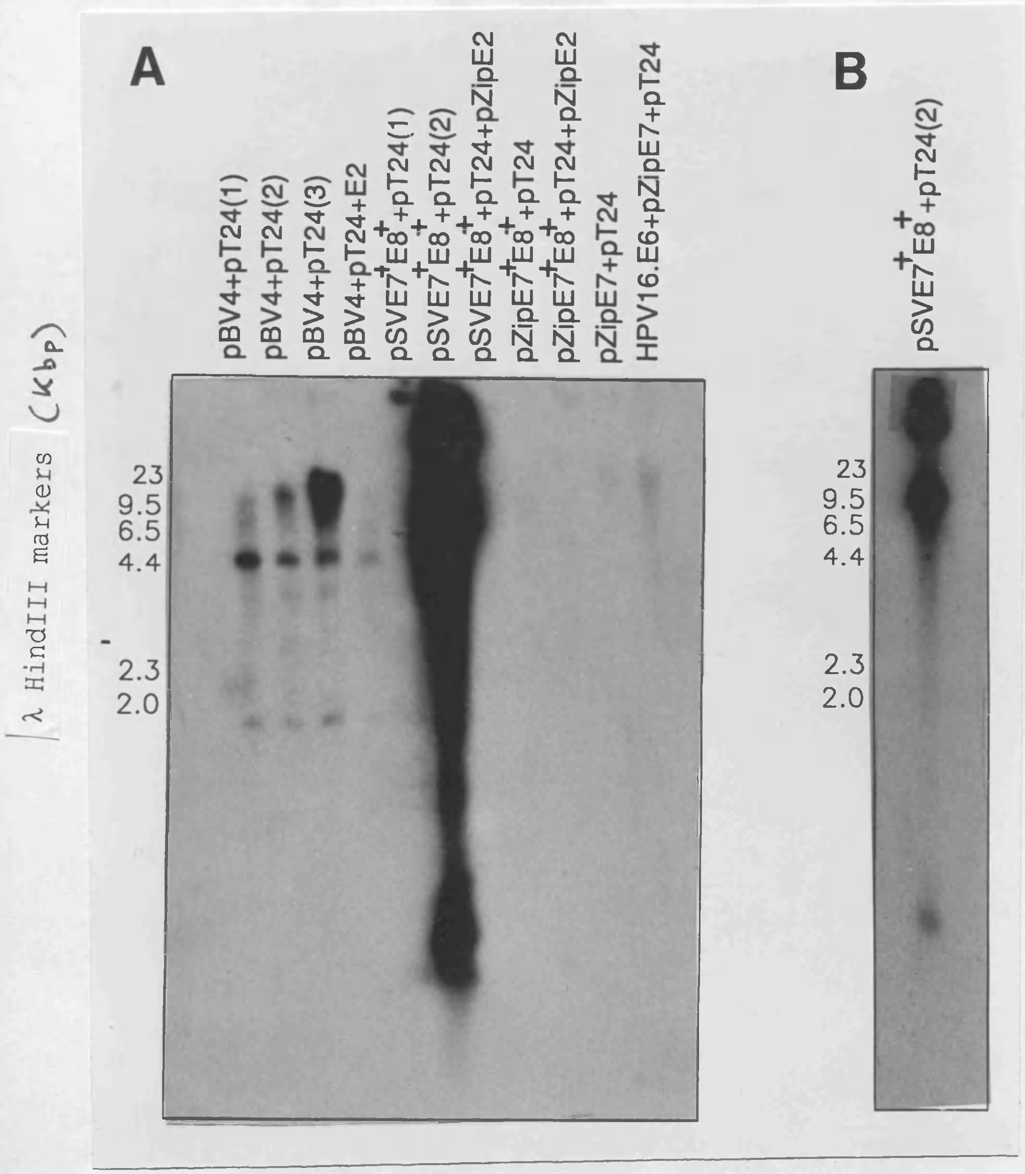
Fig 7.23. Loss of viral DNA in pBPV4+*ras* transformed PalF cells.



Notes: PalF cells examined for the presence of viral DNA by Southern blotting. After transfection with pBV4, pT24 and pZipneo, individual G418^r clones were picked and expanded and genomic DNA prepared as described (Section 7.5). The blot was probed with a full genome BPV-4 DNA probe isolated by BamH1 digestion of pBV4. A control reconstruction experiment was performed at the same time to ensure that the experimental conditions were correct. As an additional control, the blot was probed to detect pZipneo sequences.

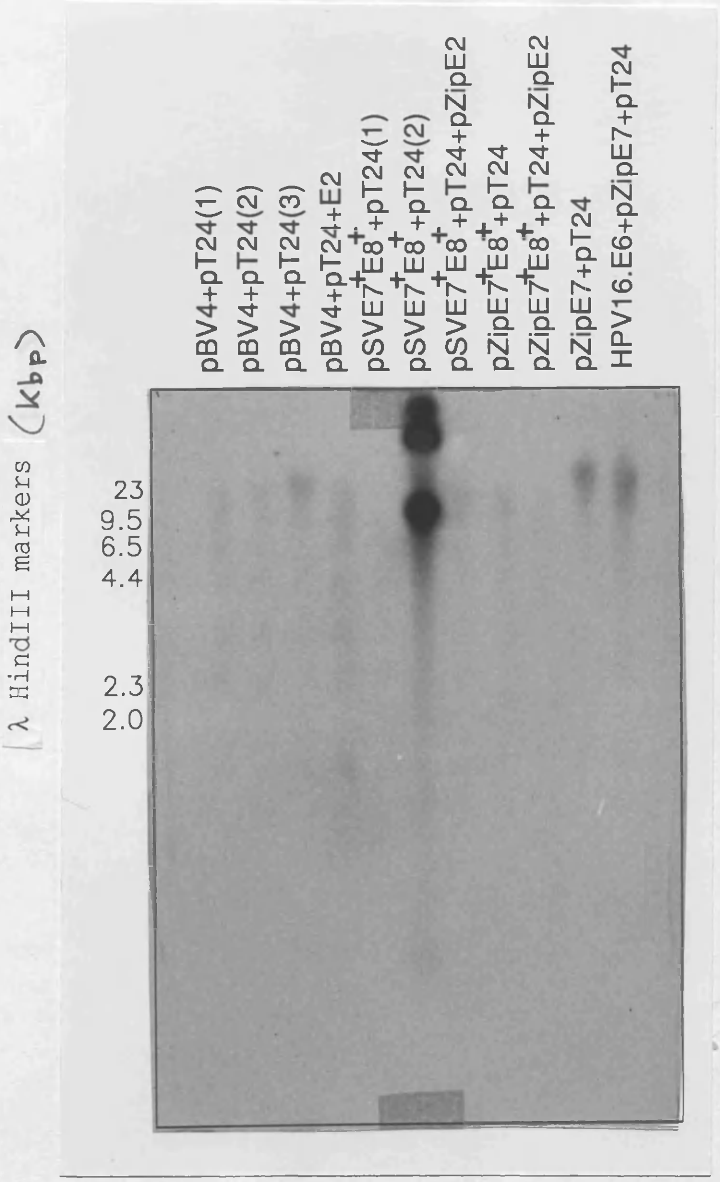
g.e.= number of genome equivalents.

Fig 7.24. Southern blot analysis of transformed PalF cells: presence of BPV-4 DNA.



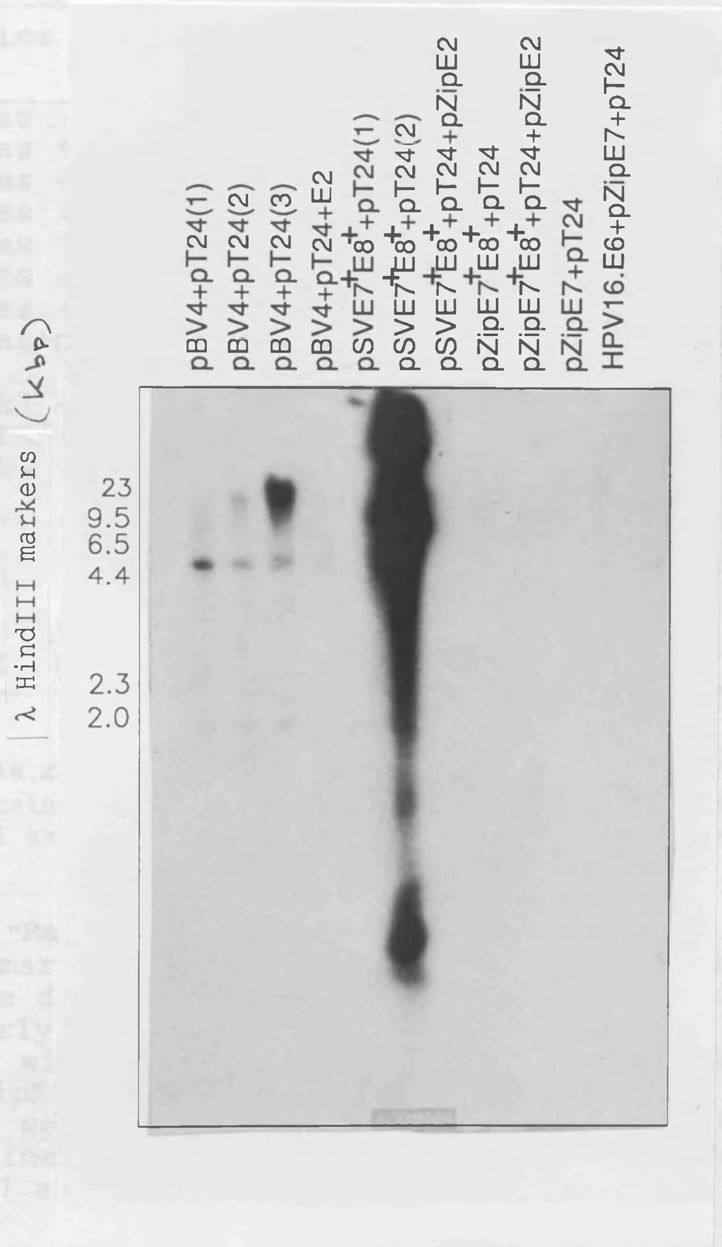
Notes: A) A panel of cell lines was examined for maintenance of BPV-4 sequences by Southern blotting. The probe used was a full genome BPV-4 probe isolated from pBV4 by BamH1 digestion and the blot was exposed for 3 days. Control reconstruction experiments (such as that shown in **Fig 7.23.**) were performed to ensure the procedure had worked and to assess copy number of maintained DNA (reconstruction not shown). All lanes contain 20μg of EcoR1 digested DNA. **B)** The result of an overnight exposure allowed the individual bands in the $SVE8^{+}E7^{+}$+T24 (2) lane to be sized.

Fig 7.25. Southern blot analysis of transformed cells: presence of BPV-4 E7 sequences.



Notes: The blot was probed with a BPV-4 E7 DNA probe isolated by BamH1 digestion of pZipE7. A control reconstruction experiment (as in **Fig 7.23.**) was performed at the same time to ensure that the experimental conditions were correct (reconstruction not shown). All lanes contain 20μg of EcoR1 digested DNA and the blot was exposed for two days.

Fig 7.26. Southern blot analysis of transformed cells: presence of BPV-4 E8 sequences.



Notes: The blot was probed with a BPV-4 E8 probe isolated from the plasmid pZipE8 by BamH1 digestion. The blot was exposed for two days.

Table 7.13. Summary of Southern blot analyses of transformed PalF cells.

Cell Line	**Viral DNA**	**Region(s) of Viral Genome Retained**
pBV4+ras (a)	-ve	
pBV4+ras (b)	-ve	
pBV4+ras (c)	-ve	
pBV4+ras (d)	-ve	
pBV4+ras (1)	+ve	4^*, E7, E8
pBV4+ras (2)	+ve	4^*, E7, E8
pBV4+ras (3)	+ve	4^*, E7, E8
pBV4+ras+pZipE2	+ve	4^*, E7, E8
$pSVE8^+E7^+$+ras (1)	-ve	
$pSVE8^+E7^+$+ras (2)	+ve	E7, E8
$pSVE8^+E7^+$+ras+pZipE2	-ve	
$pZipE8^+E7^+$+ras	-ve	
$pZipE8^+E7^+$+ras+pZipE2	-ve	
pZipE7+ras	+ve	E7
16.E6+pZipE7+ras	+ve	E7

Notes: 16.E6 refers to the plasmid pJ4Ω16.E6. Cell lines marked as **+ve** contained detectable viral DNA of at least 1 genome equivalent per cell as judged by reconstruction control experiments (see **Fig 7.23.**).

In the "**Region(s) of Viral Genome Retained**" column, cell lines marked **E7** were shown to contain E7 sequences using a probe derived by BamH1 digestion of the pZipE7 plasmid. Similarly those cell lines marked **E8** were positive when probed with the E8 fragment isolated by BamH1 digestion of pZipE8. Where cells transfected with whole genome (pBV4) were DNA positive (using a probe from pBV4), the cell line is marked $\mathbf{4}^*$ to indicate that sequences other than E7 and E8 may be present.

8. Conclusions and Speculations

Bovine Papillomavirus type 4 cooperates *in vivo* with the mutagens, carcinogens and immunosuppressants found in bracken to cause alimentary canal carcinoma in bracken-grazing cattle. The virus functions via a "hit and run" mechanism, being present in premalignant papillomas but absent from carcinomas. The transformation biology of BPV-4 was examined in a tissue culture system utilising primary bovine palate fibroblasts (PalF cells).

BPV-4 is incapable of transforming PalF cells without the introduction of a cooperating activated *ras* gene. In cooperation with *ras*, BPV-4 can morphologically transform PalF cells and give rise to cells which have an extended life-span and are able to grow in methocel. The cells are not immortal and do not cause tumours when injected into athymic mice. A 2.0kb subgenomic fragment encoding the E7 and E8 open reading frames (ORFs) can substitute for the whole BPV-4 genome in these assays and efficiency of transformation may be linked to the level of expression of this region. The main transforming protein of BPV-4 in this assay system was shown to be the E7 protein. The E7 product of most papillomaviruses has an important role in cellular transformation due to its ability to bind the product of the retinoblastoma gene $p105^{Rb}$. BPV-4 E7 has the necessary $p105^{Rb}$ binding domains and is thus postulated to act primarily in this way. The BPV-4 E8 has no independent transforming potential in this assay and indeed is lethal when over-expressed. When expressed at normal levels in cooperation with E7 however, the E8 gives an additional growth advantage to the transformed cells, allowing anchorage independent growth. The E8 ORF encodes a small hydrophobic protein with sequence similarity to the transforming BPV-1 E5, a protein shown to interact with the 16Kd component of gap junctions suggesting that BPV-4 E8 may also act in this way. As *in vivo*, the BPV-4 DNA is lost from the majority of BPV-4 transformed cultures with passage.

A curious aspect of the BPV-4 genome is that it lacks an E6 ORF. Sequence analysis of the other epitheliotrophic bovine papillomaviruses (BPVs 3 and 6) demonstrated that these viruses also lack an E6 ORF. In other papillomaviruses the E6 protein has been shown to have a major role in cell transformation by binding to and enhancing the degradation of the cellular p53 protein, thus deregulating the cell cycle. The absence of an E6 function in BPV-4 poses interesting questions regarding the way in which the virus transforms cells. PalF cells were co-transfected with the E6 ORF from HPV-16, various BPV-4 constructs and an activated *ras* gene, to see what effect introducing an E6 function has on the transformation efficiency of the virus and on the phenotype of resultant transformed clones. HPV-16 E6 does not transform PalF cells on its own, and has a low transformation rate when introduced in cooperation with activated *ras*. However, in conjunction with BPV-4 E7 and activated *ras*, transformation efficiency is increased over that observed with either BPV-4 E7 plus *ras* or that of HPV-16 E6 plus *ras*. In all cases the requirement for an activated *ras* is essential for cell transformation. Cells transformed with BPV-4 E7 and HPV-16 E6 are immortal in culture but not tumorigenic in nude mice. These lines do not grow in methocel but the introduction of BPV-4 E8, which has no transformation potential *per se* and is lethal when over-expressed in the absence of other BPV-4 genes, allows methocel growth, confirming that E8 plays an important part in cell transformation. These results, while showing that BPV-4 encoded functions cooperate with HPV-16 E6 *in vitro*, raise the question of whether E6 functions are unnecessary for BPV-4 transformation *in vivo*, or whether these functions are provided by another viral or host protein. Alternatively BPV-4 may have evolved another mechanism of evading p53 growth suppression.

Given the involvement of the E7 and E8 proteins in cell transformation, the cellular localisation was determined. PalF were transfected with either the E7 or the E8 ORF under the strong transcriptional control of the MoMLV promoter. Immunocytochemistry was utilised to allow the localisation of the E7 and E8 proteins to be determined. In all cases, control untransfected cells showed no staining and the antisera of E7 and E8 did not cross react. An anti-MHC class I antibody was used as a positive control. The E7 protein, which contains DNA binding motifs, was found to localise both to the cytoplasm and to the nucleus, which confirms other workers' localisation of the HPV E7 product. The E8 gene encodes a very hydrophobic protein and this was found to localise exclusively in membrane fractions, chiefly the Golgi, endoplasmic reticulum and perinuclear membranes and staining was also observed in the plasma membrane. The localisation of E8 is very similar to that reported for BPV-1 E5, and the two protein show sequence similarity and almost identical hydrophobicity profiles.

The E5 oncoprotein of BPV-1 has recently been shown by other workers to directly bind the 16K component of vacuolar ATPases. The 16K protein is also a major structural component of gap junctions, cellular structures which mediate cell-cell communication by allowing the passage of small molecules from one cell to another and are therefore implicated in growth control. In order to establish if the presence of this papillomavirus product can interfere with junctional communication, microinjection dye transfer experiments were performed. While non transformed control cells communicated freely, cells transformed by BPV-1 showed dramatically reduced junctional communication capacity. This was also found to be the case for HPV-containing cells explanted from cervical biopsies; HPV-positive cells had much reduced communication potential compared to HPV-negative controls. In the case of BPV-4, most transformed cell lines lose the viral genome on continuous passage, a phenomenon akin to the loss of BPV-4 DNA during malignant progression *in vivo*. The viral DNA negative cell lines invariably communicated with similar efficiency to control, untransfected cells. However, a few cell lines maintain viral DNA and those harbouring the BPV-4 E8 gene, which displays high sequence similarity to BPV-1 E5, were found to have reduced junctional communication capacity. These observations strengthen the hypothesis that the E5/E8 protein interferes with junctional communication, probably through its binding to the 16K component, and suggests that the abolition of junctional communication may be an important aspect of papillomavirus transformation biology. Direct evidence for BPV-4 E8 complexing the 16k protein will only come when immunoprecipitation studies similar to those used to identify the BPV-1 E5 to 16k interaction are performed. The use of inducible vectors (considered in the Results and Discussion section) encoding the E8, and immunoprecipitation assays should provide a suitable system for these studies. This avenue is currently being explored by other workers in the Beatson Institute and may provide formal proof of the E8-16k complexing in the near future.

The consequences of the postulated complexing of BPV-4 E8 and the 16k gap junction may be a disruption of the control of basal and supra-basal cell proliferation. A model proposed by Loewenstein (1979) and extended by Pitts (see Pitts et al, 1988) suggested that the junctional communication pattern in epithelia could give rise to a concentration gradient of second messenger molecules. The dividing populations in epithelia are known to produce greater quantities of second messenger molecules (Furth et al, 1987) which could diffuse through gap junction pores to the differentiating, non-dividing layers above. In this way the upper layers of the epithelium, which have no receptors for second messenger molecules and hence do not respond to them (discussed in Pitts et al, 1988) would act as a "sink", preventing the second messenger concentration in the basal layers reaching a threshold concentration for mitogenesis. This "epithelial brake" could represent one mechanism for control of regeneration following for example a wound; when the upper "sink" layer of cells is absent, the second messenger concentration would rise in the proliferative layers, giving rise to mitogenesis and repair of the damaged tissue. BPV-4 E8 has been localised in papillomas by immunocytochemistry to the basal and suprabasal cells, and if it disrupts junctional communication by binding the 16k protein as proposed, it is feasible that it could release the "epidermal brake" thus preventing diffusion of second messengers through the gap junction pores. The resultant hyperplasia would be the equivalent of a deep wound response by the basal/supra-basal layers of the epithelium, giving rise to hyperplasia. In this respect E8 may turn out to be an important oncoprotein in BPV-4 and indeed could conceivably be the major protein involved in papilloma formation.

This proposed mode of action of E8 would require a differentiated epithelium and would not be evident in a monolayer culture of PalF fibroblasts, suggesting why E8 is unable to transform these cells. The use of collagen raft cultures of epithelial cells would provide an ideal system to test this hypothesis. Epithelial cells would be transfected with an E8 construct and allowed to stratify on a collagen gel. Induction of E8 expression would give rise to hyperproliferation of the basal and supra basal layers, leading to a disruption of stratification. The hypothesis could be further strengthened by the use of constructs containing a mutant E8 with changes in the residues equivalent to those shown to be crucial for BPV-1 E5 binding to 16k. The model would predict no hyperproliferation in this case and these experiments are currently being considered by workers at the Beatson Institute.

One of the best characterised bracken mutagens is quercetin, a flavonoid which has been shown to cause a number of genetic lesions *in vitro* including point mutations and gross chromosomal abnormalities. Possible synergism between quercetin and BPV-4 has been examined. As previously observed, morphological transformation of cells requires the presence of both BPV-4 and *ras*, regardless of presence or absence of quercetin. However, cells which had been treated with quercetin and then transfected with BPV-4 plus *ras* showed dramatic changes in their growth potential compared to similar cultures which received no quercetin. These cells grew exceptionally well in methocel, displayed a very high cell division rate and a much more aggressive transformed appearance. More strikingly, they were fully tumorigenic in nude mice, a feature previously unreported for primary cells transformed *in vitro* by papillomavirus. From these results, it appears that quercetin initiates cells with high frequency, which are subsequently transformed by BPV-4 and *ras*. Initial studies suggest that the initiation event by quercetin does not appear to be at the level of large scale chromosomal damage (Rueudi Fries, personal communication), and is thus likely to be the result of dysfunction of individual genes perhaps by point mutation. Future work on the initiating role of quercetin may serve to identify possible target genes, perhaps by utilising a "shotgun" cloning strategy; examining the transformation potential of cloned DNA from tumorigenic PalF lines may serve to identify activated oncogenes, although the dysfunction of tumour suppressor genes would not be identified by this technique. The status of candidate genes could thus be examined by *in situ* chromosome hybridization of initiated cells or by the polymerase chain reaction followed by DNA sequencing. Thus the use of quercetin in investigating the transformation biology of BPV-4 provides us with a system which more closely mimics the *in vivo* situation and should help us reach a better understanding as to the roles of all the contributing factors in disease progression.

In the light of observations on the action of BPV-4 *in vivo* and in the *in vitro* situations discussed in this thesis, it is tempting to propose a tentative model for the role of BPV-4 in carcinogenesis. In the field of papillomaviruses, BPV-4 appears very much to be the black sheep. The accepted paradigm for HPV-associated malignancies is that the viral DNA often integrates into the host genome as part of the progression to carcinoma. This integration event (discussed more fully in the Introduction) is highly specific in that the E6 and E7 genes are usually maintained and transcriptionally active and the transcription regulatory regions of the virus (encompassing the LCR, E1 and E2 regions) are usually interrupted. Thus although host genome site into which the viral DNA integrates does not appear to be important, the portions of the viral genome retained are, in that the reading frames of the virus encoding the "oncogenic" functions are generally maintained and their expression deregulated. This fairly consistent pattern of viral integration suggests that there exists a plethora of possible integration events which give no selective growth advantage to the host cell. Thus the integration patterns observed in both pre-malignant and malignant lesions would therefore be the result of a particular integration event giving rise to a selective growth advantage (over cells with a less favourable integration or an episomal state of viral genome), in turn giving rise to a clonal expansion of that cell. It has been suggested that viral gene expression and cell growth may vary among cancers of different clonal origins due to viral integration pattern (Doeberitz et al, 1991).

When the involvement of BPV-4 in "hit-and-run" carcinogenesis is considered in the light of this hypothesis it becomes apparent that BPV-4 integration does not give this selective growth advantage, as viral DNA is never encountered in malignant lesions. The BPV-4 genome is postulated to be involved in the initiation of transformation, but not its maintenance and the viral DNA must therefore only proffer a growth advantage when in the episomal state. Indeed it may also be feasible that integration gives a positive disadvantage under many circumstances. For example the work presented in this thesis has shown that under certain conditions the over-expression of BPV-4 E8 can prove toxic. Little is known about the transcriptional machinery of BPV-4, but it is reasonable to hypothesise that possible deregulated expression of E8 following an integration event (following a pattern as described for the human PVs) may be disadvantageous.

It can be envisaged that the BPV-4 viral DNA may provide an initial growth stimulus to a cell, causing cell proliferation and perhaps the expansion of populations of cells initiated by the action of bracken mutagens such as quercetin. The expansion of such cell populations, with associated increase in DNA replication, would in turn provide a further target for the mutagens leading to additional genetic damage. Significant in this respect is the observation that cell turnover is required for the action of chemical initiators (Cayama et al, 1978). With the acquisition of several "hits" in this fashion the "oncogenic" functions provided by the viral genome could be replaced by a genetic lesion in the host genome, Eg. a mutation in the $p105^{Rb}$ gene. Under these circumstances, the episomal viral genome would be under no pressure to be maintained and would be lost by clonal expansion. This model would account for the long latency period of the disease progression, as the carcinoma development would require a number of distinct, and most probably rare genetic mutations.

In summary the proposed model for BPV-4 associated carcinogenesis is as follows. Viral infection gives rise to a proliferative lesion, possibly expanding a population of cells initiated by the chemical mutagens, such as quercetin, present in bracken fern. The proliferative action of BPV-4, only effective while the viral genome is in an episomal state, serves to give a target of replicating DNA for the mutagens to act on. The postulated E8-16k coupling may be a principal mechanism by which this proliferation occurs, by leading to the disruption of junctional communication and thus causing an increase in second messenger concentration in the basal and supra basal cells of the epithelium (by dysfunction of the suggested "epithelial brake" system). In addition the action of E7 in complexing with the tumour suppressor gene product $p105^{Rb}$ would further deregulate cell division. With time, further genetic damage is sustained, such as the mutation of *ras* genes and possibly p53 genes, which offer a further growth advantage thus abolishing the requirement for BPV-4 maintenance. The BPV-4 genome is lost by a process of clonal expansion and is not observed in frank carcinoma.

Possible cellular targets for interaction with BPV-4 proteins are thus being identified by sequence comparisons of BPV-4 products with those of the better characterised PVs, and by the use of *in vitro* models such as the PalF cell system (presented) or collagen rafts (proposed). It is therefore becoming feasible to speculate on the role of BPV-4 in multistage carcinogenesis in terms of actual cellular targets, as indeed I have attempted in this thesis.

As mentioned earlier, the general paradigm for HPV involvement in carcinoma follows the pattern of integration, maintenance and expression of particular viral functions and is thus very different from that observed for BPV-4 both *in vivo* and *in vitro*. There is however an increasing body of work from various laboratories which suggests that other papillomavirus types may occasionally transform by a "hit and run" type mechanism. For example, the loss of viral DNA sequences in lymph-node metastases from invasive HPV-16 containing cervical cancer has been reported, suggesting that in this case HPV-16 is involved in tumour formation but not required for maintenance of the tumorigenic phenotype of the metastatic cells, even when the viral genome was integrated in the primary cancer (Fuchs et al, 1989; Matsukuru et al, 1989). In two independent studies of the transcriptional activity of HPV-16 in cervical carcinomas, it was observed that viral mRNAs were not detected in all the tumours examined, suggesting that malignancy of these tumours does not require continuous expression of the papillomavirus genome (Lehn et al, 1985; Schwarz et al, 1985).

In vitro observations have also demonstrated that HPV can be lost from cultures. Cells from laryngeal papillomas containing either HPV 6 or 11 have been observed to lose viral DNA with repeated passage (DiLorenzo et al, 1992). Similar results have been observed for HPV-5 where malignant cells from an EV patient lost the extrachromasomal viral genome with passage in culture which is discussed in Fuchs and Pfister, 1990. Indeed these authors suggest that in immunocompetent patients the absence of HPV 5 or 8 in skin carcinomas may indicate that "the HPV DNA is not essential for the maintenance of the malignant state, but leaves the possibility of a role in cancer induction" (p3).

It seems to me reasonable to postulate that the genital HPVs may have an even greater involvement in carcinogenesis than the incidence of HPV positive carcinomas may suggest. Currently the evidence, as outlined above, for HPV "hit and run" is mostly indirect and circumstantial and consequently the possibility is not widely considered. Indeed, many HPV workers feel that viral DNA "negative" carcinomas may in fact harbour uncharacterised papillomavirus types not detectable by the probes used in current hybridization schemes.

The model for BPV-4 associated carcinogenesis predicts that the episomal viral DNA is maintained only until crucial target genes have been mutated by environmental factors. After these events the proliferative stimulus afforded by BPV-4 presence is redundant and the viral DNA is lost. In a similar way episomal HPV genomes in pre-malignant cervical lesions may serve to induce cell proliferation. It is interesting to note that cervical mucus from cigarette smoking women has been shown to have mutagenic properties (Holly et al, 1989), a probable explanation for smoking as a risk factor in the progression of cervical carcinoma. In this respect, the cervix is comparable to the alimentary canal of bracken grazing cattle which is subject to high local concentrations of mutagens during rumination. Should HPV genomes remain episomal in this environment there is a chance that the crucial cellular genes, possibly including the targets of HPV oncoproteins, will be mutated leading to redundancy and loss of the HPV genome (Indeed, the HPV-16 E7 has been demonstrated to induce cytogenetic abnormalities in cultured keratinocytes (Hashida and Yashimoto, 1991)). In these proposed cases the HPV genome would, as suggested for BPV-4, serve to give an initial proliferative stimulus (possibly leading to expansion of initiated cells) which would in turn give rise to a greater "target" for mutagenesis.

Should the situation outlined above occur, the behaviour of the resultant HPV-negative cancer might be expected to be more aggressive; the epigenetic dysfunction of target gene products by papillomavirus oncoprotein binding and/or enhancing degradation will surely be less effective than if the genes for these products have been mutated irreversibly. This situation has been observed in HPV negative anogenital carcinomas where the p53 gene is found to be mutant (Crook et al, 1991a, b) and in HPV negative cervical carcinoma cell lines where there in a disruption in the expression of both the retinoblastoma and the p53 genes (Wrede et al, 1991). An *in vitro* study has demonstrated that loss of the requirement for E7 expression in transformed cells is associated with elevation of the c-*myc* protein, suggesting that alterations in the expression of cellular genes can free transformed cells from their dependence of papillomaviral functions (Pim and Banks, 1991). A similar situation has been found in BPV-1 transformed primary mouse fibroblasts; expression of BPV-1 was required for transformation, but did not correlate with tumorigenicity and tumorigenic cell lines were found to have elevated c-*myc* expression (R.A.Mantyjarvi, personal communication). This observation confirms earlier results using hamster cell lines derived from BPV-1 induced transplantable tumours where neither the presence of viral DNA or levels of transcription correlated directly with tumorigenicity

(Jaureguiberry et al, 1983).

The above would explain why HPV negative carcinomas have a poorer prognosis, in terms of relapse rate and metastases formation, than carcinomas harbouring HPV genomes (Riou et al, 1990). Thus should the oncogenic genital papillomaviruses be shown to occasionally transform by the suggested mechanism *in vivo*, this could give rise to the most serious presentations of cancer. Direct *in vivo* evidence for HPV "hit and run" may prove difficult to attain, but indirect evidence may be obtained by attempting to establish the viral history of HPV negative tissue. For example, presentations of HPV negative carcinomas from individuals with a previous history of HPV positive premalignant lesions may imply an involvement for HPV in the initial stages of these tumours. In addition, the examination of premalignant dysplastic and / or normal tissue from a patient presenting an HPV negative carcinoma may indicate that the negative carcinoma arose from HPV positive tissue, although a direct correlation may prove difficult to establish. Interestingly, a recent report has demonstrated the presence of HPV 31 DNA in a cervical dysplasia but not in the resultant metaplasia (Shimano et al, 1991).

I should stress at this point that I suspect that if HPV "hit and run" does occur, it does so at a low frequency, with viral DNA maintenance being the "preferred" mode of action. In this respect the oncogenic HPVs and BPV-4 could be thought of as being at opposite ends of the viral DNA maintenance spectrum; HPV usually is maintained and expressed and often integrates, while BPV-4 is never observed to integrate and is lost in the conversion of papilloma to carcinoma. Interestingly several papillomaviruses, including BPV-1, have been shown to exist primarily as an extrachromosomal episome (for example see Lancaster, 1981). It is conceivable that the apparently contrasting mechanisms of transformation are due to differences in integration preference and that non-integrated HPV may behave in the manner as proposed for BPV-4. The fidelity of loss of the BPV-4 genome on progression to carcinoma may thus prove to be a trump card; indicating a possible mechanism of transformation by the oncogenic HPVs which would otherwise be overlooked. More epidemiological study and the analysis of viral DNA presence and status in normal, premalignant and malignant lesions may in the future confirm or deny this hypothesis.

"A clash of doctrines is not a disaster, it is an opportunity"

Alfred North Whitehead

Science and the Modern World

9. References

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