Mutational analysis of the transforming protein E8 of bovine papillomavirus type-4 (BPV-4)

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ABSTRACT

The transforming genes of bovine papillomavirus type 4 (BPV-4) are encoded by the E7 and E8 open reading frames (ORFs). E7 is the transforming gene of BPV-4, in that, in co-operation with activated *ras*, it induces morphological transformation of primary bovine fibroblasts (PalF) in the absence of other viral genes, but the acquisition of anchorage independent growth requires the additional presence of E8.

The BPV-4 E8 is a small transforming protein localized to cellular membrane. It consists of two domains: a very hydrophobic region encompassing the first 30 amino acids of the protein, and a second region of mainly hydrophilic amino acids comprising the C terminal 12 residues. The results in this thesis demonstrated that in addition to the ability of E8 to grow independently of anchorage, PalF cells expressing E8 lose gap junction intercellular communication (GJIC), can grow in low serum, and are not contact inhibited. E8 also transactivates the cyclin A promoter in PalF cells.

Mutant forms of E8 were generated to establish if the transforming functions of the protein could be segregated and therefore to define its functional domains. Mutations were introduced both in the hydrophobic domain and in the hydrophilic C-terminal tail, and chimeras with BPV-1 E5 were constructed. Cells expressing either E8 wild type or its mutants were analysed for their ability to: morphological transformation, anchorage independent growth, focus formation, cell population growth in low serum, tumorigenicity in nude mice, trans-activation of the cyclin A promoter, binding to ductin and down regulation of GJIC.

The analysis of E8 mutants and chimeras constructed with BPV-1 E5 show that the multiple transforming function of E8 can be segregated and that both the hydrophobic domain and the hydrophilic C-terminal tail of E8 are critical for its functions and for the transactivation of the cyclin A promoter. These results

support the hypothesis that the different aspects of cellular transformation produced by E8 might be due to interaction with different cellular targets. The observation from the analysis of the transformation parameters of E8 and BPV-1 E5 expressing cells suggest that E8 acts differently from E5. This study also demonstrates that the separate domains of E5 and E8 are not functionally interchangeable. The short term co-transfection analysis of E8 mutants suggest that substitution of alanine with proline, which is expected to alter the conformation of the hydrophobic domain, may have an effect on cell transformation. Also the short-term co-transfection experiments of E8 mutants in the putative casein kinase II site support the possibility that BPV-4 E8 might be phosphorylated by CKII and that this phosphorylation could have an effect on the biological activities of this protein.

For my wife and parents

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

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ABBREVIATIONS

ATP	Adenosine triphosphate
bp	Base pair
BPV	Bovine papillomavirus
BSA	Bovine serum albumin
cm	Centimetres
CRPV	Cottontail rabbit papillomavirus
CsCl	Caesium chloride
DEPC	dimethyl sulphoxide
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	3' deoxyribonucleoside 5' triphosphate
E. Coli	Escherichia coli
ECL	Enhanced chemilluminesence
EDTA	Ethylenediamine tetra-acetic acid
EtBr	Ethidium bromide
EtoH	Ethanol
FCS	Foetal calf serum
FITC	Fluorescein-isothiocyanate
g	Gram
G418	Geneticin, G418-sulphate
GJIC	Gap junctional intercellular communication
HBS	HEPES buffered saline
HEPES	N-[2-Hyroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HPV	Human papillomavirus
hr	Hours
HZ	Hertz
kb	Kilobase pairs
kD	KiloDalton
kg	Kilogram
l	Litre
LCR	Long control repeat
M	Molar
mg	Milligram
MgCl2	magnesium chloride
min	Minute
ml	Millilitre
mM	Millimolar
MoLV	Moloney murine leukemia virus
mRNA	Messenger ribonucleic acid
nA	Nanoamp
neo	Neomycin
nts	Nucleotides
^{o}C	Degree centigrade
OD	Optical density (light absorbance)
ORF	Open reading frame

PalF	Foetal Bovine Palate Fibroblasts
PBS	Phosphate-buffered saline
PE	PBS plus EDTA
pg	picogram
RI	Refractive Index
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
sec	second
SV40	Semian virus 40
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	Tris (hydroxymethyl) aminomethane
Tween 20	Polyoxyethylene sorbitan monolaurate
UV	Ultraviolet
V	Volts
v/v	Volume per unit volume
W	Watts
w/v	Weight per unit volume
µg	Microgram
µl	Microlitre
µM	Micromolar
%	percentage

Single letter amino acid code

Alanine	Ala (A)
Argenine	Arg (R)
Asparagine	Asn (N)
Aspartic acid	Asp (D)
Cysteine	Cys (C)
Glutamic acid	Glu (E)
Glutamine	Gln (Q)
Glycine	Gly (G)
Histidine	His (H)
Isoleucine	Ile (I)
Leucine	Leu (L)
Lysine	Lys (K)
Methionine	Met (M)
Phenylalanine	Phe (F)
Proline	Pro (P)
Serine	Ser (S)
Threonine	Thr (T)
Tryptophan	Trp (W)
Tyrosine	Tyr (Y)
Valine	Val (V)

CHAPTER ONE

INTRODUCTION

1.1 The Multistage Nature of Carcinogenesis

Despite considerable progress, cancer is still the leading cause of death. It is recognised as being a complex, multistage, genetic disease, in which the regulation of growth and maturity of normal cells is disrupted. Generally a tumor develops from a single cell that begins to proliferate abnormally (Nowell 1976). The development of a fully malignant tumor involves complex interactions between several factors, both exogenous (environmental) and endogenous (host-related, such as genetic, hormonal, or immunological factors). In addition, carcinogenesis is assumed to proceed through multiple discernible stages. The stages include initiation, promotion, and progression.

Initiation involves exposure of normal cells to some form of genotoxic agent such as physical, chemical or microbial carcinogens, which causes a genetic change(s) and cell proliferation. The cells at this stage, although altered at the DNA level, are phenotypically normal. The second stage of carcinogenesis is promotion, which leads to the appearance of benign tumors. A third stage, progression, covers conversion of benign alterations to malignant ones as well as their further evolution to tumors with increasing degree of malignancy (Sugimura,

1992; Kinzler and Vogelstein, 1996b). The overall process can occupy most of the individual's lifespan.

Such a model for a multistage process was first proposed by Foulds in 1957, who pointed out that tumor progression occurred in a stepwise fashion, each step determined by the activation, mutation or loss of specific genes (Foulds, 1957). Evidence for the multistep nature of tumorigenesis has come from several independent sources such as epidemiology, clinical observation and pathology and, more recently, molecular genetic studies.

Epidemiological studies support the concept that cancer is a multistep process. Cancer development in humans shows a clear exponential relationship of cancer incidence and age. It can occur at all ages, but in most tumor types it becomes much more common with advancing age excluding the distinctive group of childhood tumor (Vogelstein and Kinzler, 1993). From such statistics it has been estimated that somewhere between three and seven independent events, each of low probability, are typically required to change a normal cell into a cancer cell (Renan, 1993). This supports the hypothesis that multiple independent events are necessary for the development of cancer.

A model for understanding the interplay of gene mutation and the evolution of a normal to cancerous cell has been elucidated in colorectal carcinoma (Fearon and Vogelstein, 1990; Kinzler and Vogelstein, 1996a). The Vogelstein model illustrates the progression from a benign adenoma to metastatic colorectal cancer. In this model four critical gene mutations not found in normal colonic epithelium have been noted in colorectal carcinomas. These processes of

activation and loss are the DNA changes which appear to have substantial effect on transformation from normal to malignant. Similar to the findings in colorectal tumors, molecular analyses of cervical tumors have indicated activation of cellular genes during malignant progression (Riou et al., 1985).

These evidences support the hypothesis that cancer is a complex genetic disease that may take years or even decades between the initial genetic damage and the eventual emergence of a tumor. Multiple genetic events are likely to be involved in tumor progression. It suggests that initial genetic damage by identified risk factor, by itself, is not sufficient to elicit cancer.

1.1.1 Oncogenes and Tumor Suppresser genes

There is increasing evidence that a malignant cancer predominantly arises through an accumulation of genetic events (Fearon and Vogelstein, 1990; Bishop, 1991). Notably, genes associated with carcinogenesis have been essentially categorized into two distinct classes.

One class is that of proto-oncogenes, these are normal cellular genes and are responsible for positive growth signals (Druker et al, 1989; Cooper, 1990; Hesketh, 1994). Activation of proto-oncogenes to oncogenes causes dysfunction of growth and differentiation pathways and enhance the probability of neoplastic transformation. Carcinogens can cause the genetic changes that can lead to activation of proto-oncogenes, including mutation (Singer and Grunberger, 1983), translocation (Preston R. J. 1990), and gene amplification (Lavi, 1981; Schimke,

1990), driving the process. For example, *ras* proto-oncogenes are activated primarily at codons 12, 13, and 61 by base substitutions caused by chemical (e.g. DMBA, dimethylbenzanthracene) and physical (e.g. radiation) carcinogens in both mammalian cells and animal models (Balmain and Brown, 1988; Brown et al., 1990). A translocation between chromosomes 2 and 8 is responsible for *myc* activation in Burkitt's lymphoma (Dalla-Favera et al., 1983). Gene amplification of *N-myc* is characteristic of many neuroblastomas (Brodeur et al., 1984).

The first oncogene to be discovered was the viral *v-src* gene, the transforming gene of a retrovirus named Rous sarcoma virus. Subsequent work established that this gene was derived from a normal cellular gene, *c-src*, picked up by the virus sometime during its evolution, a process termed transduction (Bishop and Varmus, 1982). Many other cellular proto-oncogenes have since been isolated and identified; they can be classified as nuclear, cytoplasmic, or membrane receptors on the basis of their site of action. Such genes encode products which are fundamental to the normal cell growth and development. Their role in the cell can be divided into four groups according to the point where they interfere with cell growth control: growth factors (e.g. *sis*), growth factor receptors (e.g. *erbB, fms, kit*), cytoplasmic transducer of growth factor responses (e.g. *src, ras, raf*) and transcription factors that mediate growth factor-induced gene expression (e.g. *jun, fos, myc*) i.e. they can be involved in the disruption of normal growth factor related signalling at any point on that pathway (Hunter, 1991; Teich, 1991).

The second class of genes which play an important role in tumorigenesis, often referred to as tumor suppressor genes, is responsible for negative growth signals (Weinberg 1991; Hinds and Weinberg, 1994). Like proto-oncogenes, these are also normal cellular genes that, when inactivated, cause dysregulation of growth and differentiation pathways and enhance the probability of tumor formation.

Early evidence for the existence of tumor suppressor genes came from somatic cell hybridization studies, which showed that fusion of tumor cells with normal cells results in loss of tumorigenicity (Harris et al., 1969; Sager, 1985; Harris, 1988). These experiments showed that the normal cells were donating genetic information capable of suppressing the transformed phenotype of their tumor cell partner. This suppression of malignancy was dependent on retention of specific chromosomes, loss of which contributes to cancer. The assumption was that the chromosomes which were lost contained tumor suppressor genes.

As a result, tumor suppressor research focused on the study of these key chromosomes for identification of candidate genes. Experiment with the technique of microcell transfer enabled the introduction of a single chromosome from a donor to a recipient cell (Fournier and Ruddle, 1977; Saxon and Stanbridge, 1987). The transfer of chromosome 11 derived from a normal human fibroblast cell into a Wilms tumor cell line (Weissman et al., 1987), or Hela cells, an established human cervical carcinoma line containing papillomavirus DNA (Saxon et al., 1986), resulted in the suppression of tumorigenicity. This provided

direct evidence for the existence of a tumor suppressor gene on chromosome 11. Similar experiments have shown that other chromosomes are also associated with tumor suppression (see below).

Two tumor suppressor genes are of particular interest to this work as they have been shown to be targets of papillomavirus oncoproteins. These genes are the retinoblastoma gene product, pRb, and the p53 protein.

The retinoblastoma (Rb) gene was the first tumor suppressor gene to be isolated and identified by Knudson in the studies of retinoblastoma, a childhood cancer of the retina. He suggested that more than one genetic mutation (hit) was necessary for either inherited (positive family history) or sporadic (no family history) cancer (Knudson, 1971). Patients with heritable retinoblastoma carry one germ-line mutation in Rb gene locus and develop tumor when the remaining normal allele undergoes a somatic mutation, hallmarks of tumor suppressor gene. In contrast, people born without an Rb mutation in their germ line cells must acquire two mutations (hits) in the same retinal cell to develop cancer. It was demonstrated that both copies of Rb genes are inactivated in the cancer. Inactivation of the retinoblastoma protein may also be involved in the development of a proportion of osteosarcomas, soft tissue sarcomas, and small cell lung, breast, and bladder carcinomas (Weinberg, 1992).

The Rb gene maps to chromosome 13 band q14, and encodes a 105kD (kiloDalton) nuclear phosphoprotein, that plays a role in proliferation, development and differentiation (Friend et al., 1986; Lee et al, 1987).

This protein is regulated in a cell cycle dependent manner by phosphorylation. During G1, Rb is predominantly hypophosphorylated and becomes increasingly more phosphorylated during progression through the cell cycle (Ludlow et al, 1990).

Further studies have shown that p105Rb mediates control over cell growth, differentiation, and development by interacting with certain transcription factors that play a role in these biological processes. The underphosphorylated form of Rb binds to and alters the transactivation function of the E2F transcription factor, which is required for DNA transcription and replication (Adams and Kaelin, 1995). Free E2F is a transcriptional activator, and the complex of Rb-E2F is a transcriptional repressor. On mitogenic stimulation and releases of a cell cycle block at G1, Rb becomes hyperphosphorylated, loses its affinity for E2F, and releases E2F to activate early response genes.

Rb also interacts with components of the cell cycle machinery, namely, the cyclins and cyclin dependent-protein kinases (CDKs), to control cell division. Cyclins functionally activate the CDKs and are expressed differentially during cell cycle progression (for review see Bottazzi and Assoian, 1997).

Interest in Rb increased substantially when it was discovered that this protein exists within DNA tumor virus-transformed cells in the form of complexes with various virus-encoded oncoproteins. Human adenovirus, SV40, and human papillomavirus (HPV) each specify an oncoprotein, E1A, large T antigen, and E7,

respectively, that forms complexes with the host cell Rb (for review see Green 1989).

The ability of these viral oncoprotein to complex with pRb suggests that these DNA tumor viruses transform cells through their ability to damage a vital cellular growth suppression mechanism.

Another well characterized tumor suppressor gene involved in cancer is the p53 gene, which is located on chromosome 17q13.1 (McBride et al., 1986) and encodes a 53,000-dalton protein (Levine, 1992). Consistent loss of normal protein function in a variety of different tumors indicates that this locus is also subject to somatic mutation and inactivation. Of particular interest is the finding that the individuals affected by the family cancer syndrome, called Li-Fraumeni syndrome (LFS), have germline mutations in their p53 gene. LFS patients inherit a predisposition to develop multiple forms of cancer, including breast carcinoma, sarcomas, and leukaemia at an early age (Malkin et al., 1990; Malkin, 1993).

The p53 protein exerts its antiproliferative activity by promoting a block at the G1 phase of the cell cycle. Several biochemical activities have been ascribed to p53 that may mediate the biological effects of the protein. This protein has an acidic amino terminal domain that can transactivate genes under the control of promoters containing specific p53 DNA binding motifs. An internal domain located between amino acids 115 and 295 has been identified as a region of specific DNA binding. The p53 protein thus may mediate its effect on cell processes by binding to specific DNA motifs at promoters, thereby repressing or activating transcription of genes involved in growth control (Kern et al., 1992).

Also of interest is the finding that the p53 protein, like the Rb protein is bound by viral transforming proteins, suggesting that tumor suppressor gene products may be early cellular targets for inactivation in the transformation process of both virally induced and naturally occurring cancers. For example, and of particular significance to this thesis, a number of DNA tumor viruses, including papillomaviruses encode proteins which bind to and inactivate the growth suppressor function of p53 and Rb. The significance of this in virus associated cellular transformation will be discussed more fully later in this chapter.

1.1.2 Viruses and Cancer

Epidemiological studies supported by clinical and molecular biological investigations of certain human cancers indicate that viruses contribute to one of several events that cause these malignancies. It is estimated that viral infections are responsible for approximately 15 percent of the human cancer world-wide incidence. Cancer of the cervix and hepatocellular carcinoma account for about 80 percent of these tumors (zur Hausen, 1991b). Virus infection alone is not sufficient to induce cancer. Long incubation periods of years to decades are needed, suggesting that other genetic or environmental co-factors may be crucial after viral infection. Viruses can contribute to the development of cancer by a variety of mechanisms, both direct or indirect.

An example of indirect mechanism is the immunosuppression induced by human immunodeficiency virus (HIV) which considerably increases the risk for

developing certain cancers, most notably Kaposi sarcomas and B cell lymphoma (Biggar et al., 1994; Schulz, et al., 1996). The virus weakens the immune system and increases the susceptibility to cancer. Some viruses, like Epstein-Bar virus (EBV), Hepatitis B virus, Human T cell leukaemia-lymphoma virus type 1(HTLV-1) and several type of papillomaviruses appear to play a direct etiologic role (zur Hausen, 1991b). This chapter focuses on the role of papillomaviruses which are directly associated with the work of this thesis, particularly bovine papillomavirus type 4.

1.2 The papillomaviruses

Papillomaviruses are classified as the genus papillomavirus of the papovaviridae family by virtue of their capsid structure and biochemical composition (Mathews, 1982). They contain only DNA and structural proteins. The DNA is closed circular double-stranded and has molecular weight of 5×10^6 Daltons corresponding to about 8kbp (kilobase pair) (Watson and Littlefield 1960; Crawford, 1965). They cause tumours of the skin and mucosa in many animals, including man. Papillomaviruses infect basal epidermal cells, leading to cell transformation, proliferation and papillomatosis. Generally, the tumours are benign and regress. Occasionally, with the synergistic co-operation of cofactors, they may persist and progress to malignant carcinomas (Jarrett et al., 1978: zur Hausen, 1982; Campo, 1988).

The overall genome organisation of many papillomaviruses is similar, a characteristic feature of papillomavirus genomes is that all major open reading

frames (ORFs) are located on the same DNA strand. The genome of papillomaviruses is divided into three functional regions, the first two being "early" genes which are expressed before the onset of viral DNA replications and the "late" genes which are expressed after viral DNA replications. Two major L genes designated L1 and L2 code for proteins which are present in the papillomavirus capsid. Eight E genes are designated E1 to E8, which encode proteins involved in aspects of virus replication and transformation. The third region is the long control region (LCR) or upstream regulatory region (URR), contains regulatory elements for DNA replication and transcription (Chen et al., 1982; Pfister, 1987; Ward et al., 1989).

Papillomaviruses have been identified in warts from many animal species: rabbits (Shope 1933), hamster (Graffi et al., 1969), sheep (Gibbs et al., 1975), goat (Davis and Kemper 1936), deer (Tajima et al., 1968), cattle (Olson and Cook 1951), horses (Montes and Vaughan 1975), dogs (Cheville and Olson 1964), monkeys (Koller and Olson 1972), and also chaffinches (Osterhaus et al., 1977).

The most commonly studied of these viruses are the human (HPV), bovine (BPV) and cottontail rabbit (CRPV) papillomaviruses (for review see Jackson et al., 1996) which will be discussed in greater detail below.

The advantages in studying animal papillomavirus is that some questions such as the role of the immune system in virus control and tumor progression, and the usefulness of antiviral or anti-tumor vaccines, can only be addressed in animal systems. For instance, only in animals can the immune response be studied following experimental infection and vaccination of the natural host (Jarrett et al.,

1991; Christensen et al., 1991, Campo, 1997a, b). In addition, these viruses cause serious agricultural and financial problems in domestic animals.

1.2.1 Cottontail rabbit papillomavirus

Cottontail Rabbits (Shope) papillomavirus (CRPV) was the first DNA tumor virus to be isolated and characterized, and was the first model for analysis of viral carcinogenesis in mammals (Shope and Hurst 1933; Rouse and Beard, 1935). The virus induces papillomas on the hairy skin of cottontail and domestic rabbits and these papillomas can progress to squamous skin carcinoma in up to 25% of cottontail rabbits (Kidd and Rous, 1940; Syverton, 1952).

Early studies (Rous and Beard, 1935; Rous and Friedewald, 1941; 1944) showed that 75% of domestic rabbits with benign papillomas ultimately developed cancers 6 months to one year after CRPV infection without any other treatment. The application of carcinogenic chemical such as tar and/or methylcholathrene in the absence of virus gave rise to benign papillomas, which rarely progressed to carcinoma after a period of 6 months to 2 years. However, infection by CRPV combined with tar and/or methylcholathrene treatment produced numerous carcinomas after only 1 to 2 months. From this extensive series of experiments, it has been concluded that CRPV and carcinogens synergize powerfully in inducing malignant conversion of papillomas. Study of this virus has proved useful for the analysis of the multifactorial nature of papillomavirus-associated carcinogenesis as co-factors are involved in malignant progression.

The important role played by environmental co-factors is demonstrated by the limited geographical distribution of CRPV infection, as the papillomatosis is endemic in rabbits in particular States in USA, especially the Midwestern states bordering the Mississippi river (Shope 1933; Kreider and Bartlett, 1981).

The ability of CRPV to induce tumors in experimental animals has allowed examination of papillomavirus induced cellular transformation. CRPV encodes three transforming proteins; two are encoded by the E6 ORF and one by the E7 ORF. Papilloma formation in rabbits, however, requires all three proteins (Meyers et al., 1992; Schmitt et al., 1994). The E7 protein has been shown to bind the retinoblastoma suppressor protein (pRb) (Haskell et al., 1993; Schmitt et al., 1994), but E6 protein most likely performs an as yet unknown transforming function (Harry and Wettstein, 1996).

The similarity which has been observed between HPV and CRPV E7 proteins demonstrated that a crucial activity of HPV E7, pRb binding, is also shown by the CRPV E7 protein. The conservation of the binding activity of the CRPV E7 protein argues for the conservation of functional activity as well. Thus, both of these viral oncoprotein may cause cellular transformation through their ability to alter the function of a critical growth suppressing protein, pRb (Haskell et al., 1993). This has led to increasing interest in use of CRPV as a model for HPV pathogenesis, and to understand the genetic factors, in particular immunogenetics, that condition the expression of the oncogenic potential of papillomavirus (Breitburd et al., 1997).

1.2.2 Human papillomavirus

There are more than 70 types of human papillomaviruses, many of which have been shown to be associated with anogenital carcinoma (zur Hausen, 1994), skin cancer (Pfister et al., 1983; Bunny et al., 1987) and upper respiratory tract cancer (Watts, et al., 1991). These can be divided into two major subgroups initially classified on the basis of their ability to infect specific anatomic regions, namely cutaneous and mucosal epithelia (de Villiers, 1989).

In 1979 specific cutaneus HPV types were identified in a rare form of human carcinoma arising in patients with epidermodysplasia verruciformis (EV) at sunlight exposed sites (Orth et al., 1979). EV is influenced by genetic and environmental factors, as well as by HPV.

Evidence for the importance of genetic factors includes a high frequency of consanguinity among EV patients and a frequent familial occurrence of the disease. Sunlight appears to be a particularly important environmental factor, as tumors develop primarily in areas of the body that are exposed to the sun. These tumors are example of co-operative effects of specific virus infections and other environmental carcinogens. More than 20 types have been isolated from EV associated skin lesions, however, HPV 5 and 8 are the predominant types, being found in 90% of skin carcinoma of patients with the disease (Arends et al., 1990).

The best characterized HPVs are those that infect the mucosa of the anogenital tract. According to their clinical lesions these can be divided into two groups:

One group, such as HPV 6 and 11 called "low risk", which are very rarely found in malignant tumors but induce benign genital warts; a second group, such as HPV 16, 18, 31 and 33, called "high risk", which are frequently found in cervical carcinomas (zur Hausen, 1991b).

HPV types 16 and 18 are the most common types found in malignant lesions of the genital tract. Several observation studies indicate that genital HPVs are transmitted primarily through contact with infected cervical, vaginal, vulvar, penile or anal epithelium (Gissman et al., 1982; Johnson et al., 1991; Nuovo et al., 1991; Zhu et al., 1993; Labropoulou et al., 1994).

DNA of HPV types 16, 18, and 33 and less frequently of other types can be found in about 90% of cervical, vulvar, and penile cancer biopsies, if such investigations are carried out under standarized experimental conditions. HPV-16 is present in about 50% of these biopsies, HPV-18 in 20% and HPV-33 may be seen in up to 10%. The remaining 10 % harbours other HPV types (zur Hausen and Schneider, 1987).

Cervical cancer is the second most frequent cancer in females on a world-wide scale exceeded only by breast cancer (Parkin et al., 1993). The world-wide annual incidence of cancer of the cervix is greater than 500,000 cases per year world wide with about 45% mortality, even with medical intervention (Broker and Botchan, 1986; Scheffner et al., 1994).

It is important to point out that most high risk HPV infections do not progress to cancer and, for the cases that do progress to cancer there is a long

latent period (between 5 and 20 years or several decades). Other cofactors are required for full malignant transformation, and HPV is essential, but not sufficient for it (zur Hausen, 1991b). This reasoning is in line with numerous observation supporting the multistep nature of carcinogenesis.

1.2.2.1 Functions of the viral gene products

The genome of HPVs is double-stranded DNA circle about 8 kbp long which can be divided into three regions: (1) the long control region (LCR) which contains control viral replication and transcription; (2) the region that encodes the early (E) genes encoding proteins involved in cell transformation, or in the replication and transcription of the viral genome; and (3) the region that encodes the late (L) genes encodes the major L1 and minor L2 capsid proteins (Fuchs and Pfister, 1996).

The E1 ORF of HPVs encodes a nuclear phosphoprotein essential for viral DNA replication that is 600-650 amino acids long. The E1 protein is a DNA-dependent ATPase and an ATP-dependent helicase that binds an AT-rich palindromic sequence within the region of replication (Chow and Broker, 1994; Howley, 1996). The E1 and E2 proteins form a complex which stabilizes the binding of E1 to the origin of replication. E1 associates with the cellular DNA polymerase α, a component of the cell replication machinery (Park et al., 1994).

The E2 ORF of HPVs encodes a nuclear phosphoprotein of about 350-500 amino-acids that regulates both viral DNA replication and transcription. The E2

protein is characterized by three functional domains comprising an amino-terminal transacting domain of about 200 amino-acids, a central flexible hinge highly divergent in size and sequence among PVs, and a carboxy-terminal DNA binding and protein dimerization domain of about 90 amino-acids (Thierry, 1996). E2 usually activates viral gene transcription when present as a full length molecule, but acts as a repressor of the early promoter in high risk HPVs, such as HPV-16 and HPV-18 (Romanczuk et al., 1990; Howely, 1996).

The E4 ORF of HPVs is entirely contained within the central portion of the E2 ORF, but it is translated in a different reading frame. The primary E4 product is expressed from spliced mRNAs encoding an E1-E4 fusion protein containing the first five residues of E1 (Doorbar ,1996; Doorbar et al., 1988). The size of E4 proteins ranges between 90-140 amino acids for genital (mucosal epithelia) HPVs and between 200-250 amino acids for cutaneous HPVs. The E4 proteins constitute a multispecies family arising from post-translational modifications and multimerizations that have the ability to aggregate into cytoplasmic and nuclear inclusions (Rogel-Gaillard et al., 1993; Doorbar, 1996).

The E4 proteins should be considered as late proteins, because their expression coincides with the onset of vegetative viral DNA replication (Croissant et al., 1985). The function of E4 is still a matter of speculation. A most probable role would be to interfere with normal keratinocyte differentiation to favour the production and release of viral particles (Doorbar et al., 1991; Rogel-Gaillard et al., 1993; Doorbar, 1996). The E4 protein of HPV-16 has been shown to associate

with tonofilaments, resulting the disruption of the cytokeratin filament network (Roberts et al., 1993; Doorbar, 1996).

The E5 ORF of HPVs encodes a small (75-100 amino acids long) membrane-associated hydrophobic protein (DiMaio et al., 1994; Banks and Matlasheweski, 1996). The E5 protein of genital HPVs has only a weak transforming activity *in vitro* (Howely, 1996). The HPV-16 E5 interacts with ductin and down-regulates gap junctions (Oelze et al., 1995). A probable consequence of E5 interaction with the vacuolar H^+-ATPase form of ductin is the inhibition of the acidification of endosomes, leading to the observed retention and recycling of undegraded epidermal growth factor (EGF) receptor from endosomal compartments (Straight et al, 1995). This provides a likely explanation for the observed co-operation between HPV-16 E5 and EGF receptors in cell transformation (Leechanachai et al., 1992; Straight et al., 1995). The HPV-16 E5 gene, unlike HPV-16 E6 and E7, is generally lost in advanced carcinomas often as a consequence of viral integration (Howely, 1996). Hence it is likely that the E5 protein would play a role in the early stages of cell transformation.

Two viral early genes, E6 and E7, play a crucial role in tumor formation. In several malignant lesion and in cell lines derived from cervical cancer, HPV DNA is integrated in the cellular genome (Yee et al., 1985; Durst et al., 1987; Popescu et al., 1987). This viral DNA integration does not appear to favour any particular location in the host genome but the integration event usually occurs within the E1/E2 region. As a consequence the E2 gene product(s) is either lost or altered. Since the E2 gene product suppress transcription of E6 and E7 (Cripe et

al., 1987; Thierry and Yaniv, 1987; Romanczuk et al., 1990), disruption of the E2 gene may enhance expression of E6 and E7 genes.

Further evidence for the function of HPV16/18 E6 and E7 proteins in the development of cancer has been obtained by *in vitro* cell transformation assays (Mansur and Androphy, 1993). Both protein have been shown to have *in vitro* transformation activity, although E6 is usually less active. E6 and E7 from the low risk HPV types 6 and 11 have very weak *in vitro* transformation activity, showing good correlation between *in vitro* transformation activity and clinical observation. The best evidence for the involvement of E6 and E7 comes from biochemical studies. E6 and E7 from the high risk HPV types have the ability to alter pathways involved in cell cycle control, interacting with and neutralizing the regulatory function of two tumor suppressor proteins, p53 and pRb, respectively (Dyson et al., 1989; Werness et al., 1990).

E7 is the major transforming protein of HPV. It is a small protein of 98 amino acids, which is localised in the nucleus and attached to the nuclear matrix (Greenfield et al., 1991). Also a recent report describes E7 in the nucleolus (Zatsepina et al., 1997).

Several domains have been identified in HPV-16 E7 by various laboratories (Dyson et al., 1989; Munger et al., 1989; Barbosa et al., 1990): the p105 retinoblastoma gene product (RB) binding domain, the casein kinase II (CKII) phosphorylation sites, and the two zinc binding Cys-X-X-Cys motifs. The transformation properties of HPV-16 E7 depend primarily on Rb-binding and zinc

binding; mutation in either of these two domains abolish transformation (Edmonds and Vousden, 1989; Chesters et al., 1990), highlighting their critical role. Binding of E7 to p105Rb prevents the interaction of p105Rb with its natural target, namely the transcription factor E2F (Defeo-Jones et al., 1991; Rustgi et al., 1991); the zinc fingers are essential for the transactivation activity of E7 (Phelps et al., 1988) and mutations that disrupt them destabilize the protein (Story et al., 1990). The CKII sites although less critical (Watanabe et al., 1990; Storey et al., 1990), nevertheless contribute to the transformation activity of HPV-16 E7 (Barbosa et al., 1990; Firtzlaff et al., 1991).

The E7 protein has been shown to associate with several other protein including the Rb-related protein, p107 (Davies et al., 1993), cyclin A and the protein kinase $p33^{CDK2}$. These proteins are believed to play important role in cell growth control and may be critical to the transformation activity of E7 (Tommasino et al., 1993).

In addition HPV-16 E7 binds the members of the AP1 family of transcription factors, including *c-Jun*, *JunB*, *JunD* and *c-Fos* through one of the CXXC motifs (Antinore et al., 1996); this binding is independent of p105Rb interaction, and appears to contribute to cell transformation by E7. A recent report has demonstrated that E7 can complex with the basal transcription factor, TBP, the TATA box binding protein, and that unlike the pRb interaction, the affinity of this interaction is increased upon phosphorylation of E7 by CKII (Philips and Vousden, 1997).

Furthermore, E7 expression in human keratinocytes can uncouple cellular differentiation and proliferation by interaction with $p21^{Cip1}$ (Jones et al., 1997). Also it has been demonstrated that HPV-16 E7 protein blocks the ability of p21 to inhibit CDK activity and PCNA-dependent DNA replication through direct binding to the carboxyl terminus of p21. Thereby E7 interaction with the carboxyl terminus of p21 modulates its dual inhibitory activity and disrupt normal cell cycle control (Funk et al., 1997).

E6 is second major oncogene of HPV-16, which encodes a 151 amino acid long protein localized to the nucleus and in non nuclear membrane. Like E7, E6 binds zinc, *in vitro*, through Cys-X-X-Cys repeats (Barbosa et al., 1989; Farthing and Vousden, 1994; Munger, 1995; Howley, 1996). HPV-16 E6 binds to the core structure of another negative regulator of the cell cycle, p53, promoting its degradation through the ubiquitin pathway (Werness et al., 1990; Scheffner et al., 1990; Li and Cofino, 1996) and therefore sequestering it from controlling cell proliferation. This function is believed to be important for E6 immortalizing and transforming activities.

However other roles for E6 in transformation have been suggested. Introduction of HPV-16 E6 into p53 null mouse fibroblasts was shown to confer immortality (Scobie et al., 1997). It has also been demonstrated that the ability of E6 to immortalize primary mouse fibroblasts was independent of its interaction with p53 (Pim et al., 1994). The ability of E6 to act as a repressor (Etscheid et al.,

1994) and/or activator of transcription (Akusta et al., 1996) has also been shown to be independent of p53.

It has been shown that the truncated E6*1 protein of HPV-16 trans-activates the HPV-16 early promoter p97 as well as the heterologous promoter of adenovirus E2. Full length HPV-16 E6 protein was found to trans-activate heterologous promoter, but repress transcription from the autologous p97 promoter. The transcription-modulatory activity of full length E6 and E6*1 proteins toward the autologous promoter suggests that the regulation of transcription, which might contribute to the latency of HPV-16, is finely regulated (Shirasawa et al., 1994).

Several other proteins have been shown to interact with E6, in particular E6-BP (E6 binding protein), a calcium binding protein which appears to associate only with high risk E6 proteins (Chen et al., 1995). The E6 protein of HPV-16 was also found to associate with a cellular protein (E6 associated protein, E6-AP) that links E6 and p53 in their degradation complex (Chen et al., 1995; Elston et al., 1998), however the significance of any of these interactions in relation to cellular transformation has not as yet been determined.

Other recognised functions of E6 which have been reported and can be associated with cellular transformation include the inhibition of human keratinocytes differentiation (Sherman and Shlegel, 1996) and the activation of telomerase (Klingelhutz et al., 1996); telomerase activation is linked to cell immortalisation and is characteristic of most cell lines and tumors.

1.2.3 Bovine papillomaviruses

Cattle are infected by six different types of bovine papillomavirus (BPV), each one associated with a specific disease. The viruses are divided into two subgroups A and B, on the basis of their genome homology, site specificity and clinical manifestations. Subgroup A comprises the fibropapillomaviruses, BPV 1, 2, and 5, which cause lesions of both dermal fibroblasts and keratinocytes. Subgroup B includes types 3, 4, and 6 which are wholly epitheliotropic, where only keratinocytes are involved (Campo et al., 1981; Jarrett et al., 1984). The fibropapillomaviruses have a larger genome (approximately 7.9kb) than the epithelial papillomaviruses, whose genome is approximately 7.3 kb. The viruses are evolutionarily more closely related to each other within a subgroup than they are between subgroups, and there is a little or no immune cross-reactivity between the viruses of the two subgroups (for review see Campo, 1995).

BPV-1 induces fibropapillomas of the penis of bulls and of the teats and udder of cows and can also infect adjacent skin, BPV-2 is the agent of common cutaneus warts and BPV-5 causes rice grain fibropapillomas of the udder. BPV-3 causes epithelial papillomas of the skin, BPV-6 causes teat frond papillomas, and BPV-4 induces papillomas of the alimentary canal (Campo and Jarrett, 1987).

1.2.3.1 Bovine papillomavirus type 1

Bovine papillomavirus type 1 is a fibropapillomavirus that infects both epidermal keratinocytes and the underlying dermal fibroblasts, resulting in the

production of benign tumors. The observation that BPV-1 or its DNA could transform mouse fibroblasts in culture (Dvoretzky et al., 1980) opened up an entire new field of investigation in papillomavirus research.

The DNA of BPV-1 was the first to be completely sequenced (Chen et al., 1982), further genetic dissection has identified E5 and E6 ORFs as the transforming genes of BPV-1 (Yang et al., 1985; Schiller et al., 1986). Both the E5 and E6 protein have been found in transformed cells (Androphy et al., 1985; Schlegel et al., 1986). The 15.5kD E6 protein is localized in both the nuclear and membrane fractions, whilst the 7kD E5 protein is only found associated with membranes.

E5 is the major transforming protein of BPV-1, is capable of transforming established mouse fibroblasts and keratinocytes in the absence of other viral gene products (Leptak et al., 1991), and has been shown to induce cellular DNA synthesis (Green and Leowenstein, 1987). It is a 44 amino acid long highly hydrophobic protein and contains two distinct domains: an amino terminal hydrophobic region that is predicted to traverse the cell membrane and a 14 amino acid hydrophilic carboxyl-terminal domain, which contains two cystein residues that mediate homodimer formation (Schlegel et al., 1986; Schlegel and Wade-Glass, 1987; Horwitz et al., 1988).

The protein is located mainly in the endoplasmic reticulum and Golgi membranes (Burkhardt et al., 1989), and targets a number of cellular proteins. It has been shown to activate the receptors for epidermal growth factor (EGF) and colony stimulation factor-1 (CSF-1) (Martin et al., 1989), to bind and activate the

receptor for platelet derived growth factor (PDGF) (Goldstein, et al., 1994; Petti et al., 1991; Petti and DiMaio, 1992), and to bind the 16kD (kiloDalton) protein called ductin (Goldstein et al., 1991; Holzenburg et al., 1993). Ductin is a component of both vacuolar H^+-ATPase and of gap junctions (Finbow et al., 1991). The interaction between E5 and 16kD ductin will be discussed in section 7.1.2.1.1.

BPV-1 E6 is the second transforming protein of the virus (Yang et al., 1985) but contrary to E5 it is only weakly transforming; it is capable of binding zinc through Cys-X-X-Cys motifs (Barbosa et al., 1989) and has transcriptional transactivator activity (Lamberti et al., 1990; Yang et al., 1991). In this respect it is possible that deregulation of cellular genes contributes to transformation by E6. As explained before (section 1.2.2) the main action of HPV-16 E6 appears to be the binding (Werness et al., 1990) and the subsequent deregulation (Scheffner et al., 1990) of the tumor suppressor protein p53, thus abrogating its control on cell proliferation.

Like the E6 proteins of high risk HPVs, BPV-1 E6 has been shown to bind *in vitro* to a calcium binding protein, E6-BP (Chen et al., 1995). Also BPV-1 E6 binds E6-AP but fails either to complex with p53 or to degrade associated proteins, implying that BPV-1 E6 might transform cells through a mechanism different from that of the HPVs (Scheffner et al., 1992). It has also been reported that transformation by BPV-1 E6 does not require transcription activation and that association of BPV-1 E6 with E6-AP is a function separable from transcriptional

activation by E6. Association of E6 with E6-AP appears to be necessary but not sufficient for transformation by E6 (Ned, R. et al., 1997).

Recently it has been reported that BPV-1 E6 interacts with paxillin. Paxillin is a protein involved in transducting signals from the plasma membrane to focal adhesions and the actin cytoskeleton, and disrupts the actin cytoskeleton. Disruption of the actin cytoskeleton is a characteristic of many transformed cells, and in BPV-1 E6 transformed cells may be mediated by E6 through its interaction with paxillin (Tong and Howley, 1997).

Taken together the observation that BPV-1 E5 activates the platelet-derived growth factor and epidermal growth factor receptors and that E6 disrupts paxillin function support the view that signals from cell adhesion and growth factor receptor are both required for regulated cell proliferation and that disruption of these pathways co-operatively contribute to tumorigenesis.

The E7 protein of BPV-1 contains neither Rb-binding domain or CKII sites and is consequently non-transforming (Iftner et al., 1990). Furthermore, It has been reported that BPV-1 E7 contributes to the control of viral DNA replication and copy number of viral genomes (Lusky and Botchan, 1986).

1.2.3.2 Bovine papillomavirus type 4 and cell transformation

Because of the clinical importance of papillomavirus infection, and the risk of malignant progression of some of the lesion induced by this group of viruses (zur Hausen, 1991a), the study of their transformation potential and characteristics acquires particular importance.

BPV-4 is a weakly transforming papillomavirus, it induces papillomas in the mucosal epithelium of the upper gastrointestinal tract of cattle; the papillomas are, in general, benign and eventually regress spontaneously (Jarrett, 1985). Lesions can progress to carcinoma in animals feeding on bracken fern. Bracken fern contains several mutagens one of which, quercetin, has been shown to cooperate with BPV-4 in *in vitro* transformation (Campo et al., 1994b).

It has been found that in naturally occurring bovine alimentary cancers the *ras* gene is rearranged and activated (McCaffery et al., 1989; Campo et al., 1990), p53 gene is mutated (Scobie, 1996) and the level of EGF receptors is increased (Smith et al., 1987). These observations support the hypothesis that multiple independent events are necessary for the development of cancer (Table 1.1).

Table 1.1 *In vivo* cell transformation by BPV-4

Present in papillomas	Yes
Present in carcinomas	No
Mutation of p53	Yes
High levels of EGF receptor	Yes
Activation of *ras* gene	Yes

Although infection by BPV-4 is a prerequisite for carcinogenesis, the viral DNA is lost during malignant progression (Campo et al., 1985). Loss of viral DNA during progression has also been demonstrated to occur both *in vitro*

systems (Smith and Campo, 1988) and in carcinoma induction in xenografts of BPV-4 infected bovine palate tissue. (Gaukroger et al., 1991). Thus, BPV-4 appears to act by a "hit and run" mechanism in carcinogenesis of the upper alimentary canal of cattle and that the presence of BPV-4 DNA is required for the initiation but not the maintenance of the malignant phenotype.

Early studies of the transforming properties of BPV-4 *in vitro* were conducted in established mouse fibroblast cells. The virus was found to transform both NIH-3T3 and C127 established cells *in vitro*. Complete morphological transformation of C127 mouse fibroblast cells requires cooperation between the viral DNA and the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Campo and Spandidos, 1983; Smith and Campo,1988). Results showed that, as *in vivo*, additional cofactors contribute to viral DNA-dependent morphological transformation.

An important observation from these *in vitro* experiments was that, as in cancer *in vivo* (Campo et al., 1985), no viral DNA could be detected in the majority of transformed C127 cell lines, and even when present the viral sequences were not expressed (Smith and Campo,1988).

Work on C127 cell lines defined the BPV-4 E7 and E8 ORFs as the transforming genes (Smith and Campo,1988). This was the first indication that E7 and E8 were the main transforming proteins of BPV-4, and was later confirmed in primary bovine cells (Jaggar et al., 1990).

In nature BPV-4 infection is restricted to the mucous epithelium of the alimentary canal of cattle (Jarrett, 1985) and therefore, *in vitro* transformation of

established mouse fibroblasts would appear a rather remote system from the natural one. Thus, more recent work has looked at BPV-4 transformation of primary bovine fibroblasts (PalF cells) from the foetal palate (Jaggar et al., 1990). These cells have the distinct advantage of being non established and are therefore presumed to be normal when placed in culture, belong to the natural host of BPV-4, and although fibroblastic (keratinocytes are the natural target cells) derive from one of the main sites of BPV-4 infection (Jarrett, 1985).

Primary bovine palate fibroblasts (PalF cells) when transfected with BPV-4 alone exhibit no morphological change. BPV-4 can achieve morphological transformation of PalF cells only when cotransfected with an activated *ras*. These partially transformed cells have an extended life span and are capable of anchorage independent growth, however are not immortal or tumorigenic in nude mice (Jaggar et al., 1990). This indicates that BPV-4 needs the co-operation of other factors such as quercetin, for full transformation *in vitro* (Pennie and Campo, 1992; Pennie et al., 1993; Cairney and Campo, 1995; Scobie et al., 1997).

For two reasons work with BPV-4 is valuable, the first being that the virus is involved in a naturally occurring cancer in its own host, therefore providing the opportunity for studying carcinogenesis in a natural system, and the second is that the bovine system presents itself as a valuable model for mucous epithelia papillomavirus infection in humans, particularly that of the anogenital tract and provides an experimental situation for the development of therapeutic and/or

prophylactic vaccines and the investigation of the immune response (Campo, 1994; Kirnbauer et al., 1996; Campo, 1997a, b).

1.2.3.2.1 Genomic Organisation of BPV 4 and function of viral protein

The BPV-4 genome is a double-stranded DNA circle 7.265 kbp long (Jackson and Campo, 1995), which can be divided into three regions; a long control region (LCR) which contains elements through which viral gene replication and expression are regulated and regions encoding the early and late gene products. The major open reading frames are shown in figure 1.1.

An unusual feature of BPVs of subgroup B is their lack of the E6 ORF, which has apparently been replaced by the E8 ORF (Jackson et al., 1991). Thus for these viruses an E6 ORF is not required for a successful infection cycle, and papillomas induced by BPV-4 can progress to alimentary canal carcinoma even though BPV-4 does not posses an E6 ORF.

There are four late open reading frames (ORFs), numbered L1 to L4. The L1 and L2 ORFs encode the major and minor structural protein of the virion respectively. Both proteins encode virus neutralizing epitopes, these are effective prophylactic vaccines which prevent infection (Campo et al., 1997; Campo, 1997a, b). Although the L3 and L4 ORFs do have ATG start codons, their functions are not known.

Between the early and late gene ORFs, there is a BPV-4 LCR, which contains regulatory elements for both viral DNA replication and transcription. Transcription from the LCR is regulated by E2 as well as by cellular factors, e.g.

PEBP2. Sequence analysis of the BPV-4 LCR revealed a number of potential binding sites for transcription factors, including the virally encoded E2 transactivator / repressor (Jackson and Campo, 1991, 1995). There are four E2 binding sites in the LCR, binding sites 1 (BS1), 2 (BS2), 3 (BS3), and 4 (BS4).

The E1 ORF of BPV-4 is transcribed into a number of separate mRNAs (Smith et al., 1986; Stamps and Campo, 1988; Campo et al., 1994a). However the function(s) of any of these transcripts has not yet been well characterized. The BPV-4 E1 ORF shows a high degree of homology with E1 ORFs from other papillomaviruses, such as BPV-1 E1, suggesting that this protein plays a role in the replication of viral DNA (Lambert, 1991).

The BPV-4 E2 protein is a transcription regulator, which acts on the BPV-4 LCR to regulate transcription. Mutational analysis of the LCR demonstrated that BS2 and BS4 mediated transactivation by E2, whereas BS1 and BS3 are responsible for repression by elevated levels of E2 (Jackson and Campo, 1995; Morgan et al., 1998). These results suggest that the four E2 sites each perform different functions in the control of transcription and that competition between cellular transcription factors and viral E2 protein is essential in regulating the level of viral gene expression during papilloma development (Jackson and Campo, 1995).

Two of the ORFs , E3 and E5, contain no ATG and are therefore suspected of having no function.

Similar to the E1 ORF, the E4 ORF is transcribed into several RNA species (Stamps and Campo, 1988). Two of these, 7E11 and 1.6 kb transcripts, encode a potential E1-E4 fusion peptide, although a different region of E1 is found in these two transcripts. The 7E11 transcript is most likely to encode the E1-E4 fusion peptide described for HPV-1 (Doorbar et al., 1988). The HPV-16 E1-E4 fusion protein interferes with cytokeratin assembly (Doorbar et al., 1991; Roberts et al., 1993), possibly upsetting the differentiation programme and favouring the production of virion progeny; accordingly the expression of BPV-4 E4 is greatest in the differentiated layers of papillomas (Anderson et al., 1997). This coincides with the vegetative replication of viral DNA (Campo et al., 1994a), in agreement with previous studies (Breiburd et al., 1987). The BPV-4 7E11 transcript may in some way contribute to BPV-4 virus production by interfering with normal epithelial differentiation.

E7 is one of the transforming gene of BPV-4, in that, in co-operation with activated *ras*, it induces morphological transformation of PalF cells in the absence of other viral genes. It is located in the cytoplasm and in the nucleus (Pennie et al., 1993; Campo et al., 1994b). *In vivo*, E7 is expressed in all layers of papillomas at all stages of development (Anderson et al., 1997). It possesses the two Cys-X-X-Cys motifs and potential p105Rb binding domain, although it lacks the casein kinase II site which is the feature of the E7 protein of the high risk HPVs (Jaggar et al., 1990). Mutation of either of these domains abolish the protein's transforming ability *in vitro* (Campo et al., 1994b; Jackson et al., 1996),

in line with previous demonstration of the importance of these domains in the transforming and immortalizing activity of HPV E7 (Vousden, 1994).

Nevertheless, although morphologically transformed, E7 expressing PalF cells are not capable of growing independently of anchorage, thus showing that other viral gene(s) encode function(s) that confer anchorage independent growth.

The E8 open reading frame of BPV-4 encodes a small, 42 amino acid long protein that is composed of two domains: a very hydrophobic region, theoretically capable of forming a transmembrane α-helix, encompassing the first 30 amino acids of the protein, and a second region of mainly hydrophilic amino acids comprising the C terminal 12 residues (Figure 1.2). It is localised in the nuclear membrane, the endoplasmic reticulum, the Golgi apparatus and occasionally the plasma membrane (Pennie et al., 1993). *In vivo*, E8 expression is limited to the basal, suprabasal layers of early papillomas, i.e., not in areas of vegetative viral DNA replication, with decreased expression in late stage papillomas (Anderson et al., 1997); E8 is therefore a true early protein.

In vitro E8 binds to ductin, the 16 kDa protein that forms transmembrane channels in both gap junctions and vacuoles. Overexpression of E8 is lethal to PalF cells *in vitro* (Campo, 1992) but when co-expressed with E7, it contributes to cell transformation by conferring anchorage independent growth (Pennie et al., 1993) and down-regulating gap junction intercellular communication (GJIC) in primary bovine fibroblasts (Faccini et al., 1996). These partially transformed cells however are not immortal or tumorigenic in nude mice (Pennie et al., 1993).

Recent work (O'Brien and Campo, 1998) on the specific role of E8 in cellular transformation has shown that when E8 expressed in an established NIH-3T3 cells, it is transforming: cells can grow in low serum and in suspension, and these cellular phenotypes are associated with the ability of E8 to transactivate cyclin A gene promoter, which will be discussed more fully in chapter six.

As mention above, the subgroup B papillomaviruses are unique among the papillomaviruses in not possessing an E6 ORF (Jackson et al., 1991). This gene is crucial to the transforming ability of other papillomaviruses, such as HPV-16 and 18. It raises the question of whether E6 functions are not required by BPV-4, or whether these functions are supplied by another viral or host protein. The demonstration that HPV-16 E6 confers immortality to PalF cells transformed by BPV-4 E7 and *ras* in the presence or absence of E8 suggests that HPV-16 E6 does indeed provide functions which are not supplied by BPV-4 E7 and E8 (Pennie et al., 1993). A summary of the transformation potential of BPV-4 in PalF cells is shown in table 1.2.

The E6 proteins of the oncogenic HPVs have been demonstrated to bind and promote deregulation of the p53 tumor suppressor gene product (Scheffner et al., 1990; Werness et al., 1990). Nevertheless, it has been demonstrated that E6 can transform cells independent of its interaction with p53 (Scobie et al., 1997). Transfection of p53-null mouse fibroblasts with the entire BPV-4 genome in the presence or absence of HPV-16 E6, demonstrated that an immortal phenotype was only achieved when the HPV-16 E6 was present.

Table 1.2 Summary of PalF cell transformation by BPV-4, *In vitro*

Transfected DNA (+ *ras*)	Morphological transformation	Anchorage independence	Immortality	Tumorigenicity in nude mice
BPV-4	Yes	Yes	No	No
E7	Yes	No	No	No
E7+E8	Yes	Yes	No	No
E7+16 E6	Yes	No	Yes	No
E7+E8+16 E6	Yes	Yes	Yes	No
Quercetin+BPV-4	Yes	Yes	Yes	Yes

These experiments indicated that BPV-4 lacks several functions to achieve complete *in vitro* transformation of PalF cells, and that BPV-4 transformed PalF cells, even in the presence of HPV-16 E6, are not tumorigenic in nude mice (Pennie et al., 1993), suggesting that additional cofactors such as quercetin (Pennie and Campo, 1992; Pennie et al., 1993; Cairney and Campo, 1995; Scobie et al., 1997) are necessary for BPV-4 transformed cells to gain a fully transformed phenotype. The dependence on additional cofactors to achieve a malignant state is supported by BPV-4 transformation *in vivo* (Jarret et al., 1978; Campo et al., 1994b).

GENOMIC ORGANISATION OF BPV-4

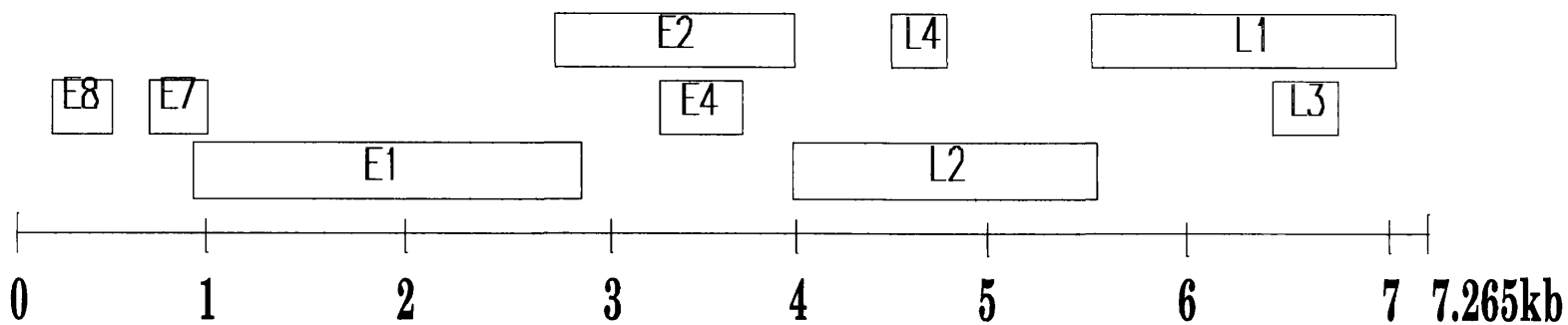


Figure 1.1 The genomic organization of BPV-4

The viral genome is represented as linear, and the boxes represent ORFs.

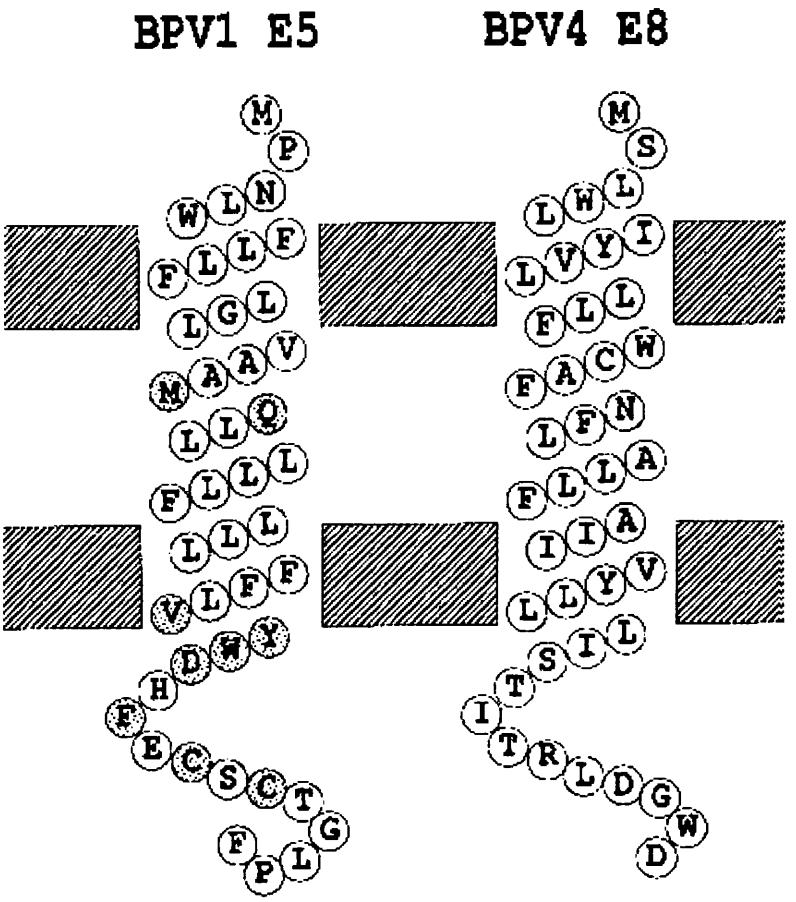


Figure 1.2 Putative transmembrane configuration of BPV-1 E5 and BPV-4 E8

The amino acids of BPV-1 E5 that have been shown to be critical for cell transformation (Horwitz et al., 1988, 1989) are shaded.

1.3 Aim of Ph.D. project

As discussed, initial studies from this laboratory have suggested that while E7 is the main transforming gene of BPV-4 in primary bovine cells, E8 provides significant contributions to cell transformation. However, our understanding of BPV-4 E8 and cell transformation is still limited.

In established murine fibroblast NIH-3T3 cells, it has been shown that cells expressing E8 are capable of anchorage independent growth. E8 deregulates cyclin A expression, trans-activates human cyclin A gene promoter, increases endogenous cyclin A levels in cells maintained in short-term suspension culture and in low serum, also NIH-3T3 cells expressing E8 continue to proliferate in low serum but do not activate autocrine mechanism (O'Brien and Campo, 1998).

Building on this information on the biology of BPV-4 E8 in both established and primary cells and in an attempt to expand on the information of E8 functions, the aim of the work in this thesis was to define and segregate different functional domains of the BPV-4 E8 protein in PalF cells, for their ability to:

- confer anchorage independent growth
- confer escape from contact inhibition
- allow growth in low serum
- trans-activate the cyclin A promoter
- bind 16k ductin, and down-regulate gap junction intracellular communication (GJIC).

To this end, several mutations have been introduced in the predicted α-helical transmembrane domain and in the C-terminus, along with the chimeric molecules formed between BPV-4 E8 and BPV-1 E5.

It has been shown that binding of BPV-1 E5 to ductin requires the N-terminal hydrophobic domain and that glutamine at position 17 is important for ductin interaction and for the transforming function of the protein (Goldstein et al 1992a, b; Sparowski et al., 1994; Sparowski et al., 1996). To test whether the corresponding residue, asparagine, in the E8 polypeptide of BPV-4 is also crucial for cell transformation, E8 mutants containing single amino acid substitutions at residue 17 were produced.

Since the BPV-4 E8 has the postulated α-helical domain, we were interested to determining whether this predicted α-helical domain of E8 is responsible for the biological activity of this protein. Proline has the property of forcing a bend in the main chain and of disrupting an α helix, thus alanine residues at positions 15 and 20 were changed to proline. The same two alanine residues were also mutated to the chemically similar amino acid glycine to control for any effect due to changes in residues rather than in structure.

To define the role of C-terminus hydrophilic tail of E8 a C-terminus truncated form of E8 comprising only the membrane-localised region was generated. A similar mutant form was obtained for the BPV-1 E5 to create a truncated form of this protein. Also two chimeras were constructed between E8 and E5 to test the relative contribution each domain of E8 made to cell

transformation and whether these domains were functionally interchangeable between the two peptides.

There is a potential CKII site in the C-terminus hydrophilic tail of E8 BPV-4 E8. To assess the possibility that E8 might be phosphorylated by CKII and whether this phosphorylation could affect the biological activities of this protein, three mutations were generated in the putative CKII site. The threonine in the putative CKII site was changed to aspartic acid, which is an acidic amino acid, mimicking a phosphorylated amino acid; alanine a nonpolar amino acid which cannot be phosphorylated; or serine (polar amino acid) which maintains the putative CKII site.

The results in this thesis demonstrate that in addition to anchorage independent growth, PalF cells expressing E8 loss GJIC, can grow in low serum, and are not contact inhibited. E8 also trans-activates the cyclin A promoter in PalF cells. The results obtained from the analysis of the transformation parameters of E8 and BPV-1 E5 expressing cells suggest that E8 acts differently from E5.

The analysis of E8 mutants and chimeras constructed with BPV-1 E5 show that the multiple transforming function of E8 can be segregated and that both the hydrophobic domain and the hydrophilic C-terminal tail of E8 are critical for its functions. Results also demonstrate that the separate domains of E5 and E8 are not functionally interchangeable.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

2.1.1 Antibodies

SUPPLIER	ANTIBODY
Amersham International plc, Amersham, Bucks, England	Anti-mouse IgG horseradish peroxidase linked whole antibody (raised in sheep)
Boehringer Mannheim UK Ltd., Lewes, East Sussex, England	Mouse monoclonal antibody (Clone 12CA5) to a peptide epitope drived from the hemagglutinin protein of human influenza virus

2.1.2 Bacterial Hosts

SUPPLIER	BACTERIAL HOSTS
Gibco Europe Life Technologies Ltd., Paisley, Scotland	E. coli DH5α competent cells
Promega ltd., Chilworth Research Centre, Southampton, England	E. coli JM109 competent cells

2.1.3 Buffers

TE	10mM Tris-HCl, 1mM EDTA pH 8.0
5× TBE buffer	40mM Tris base, 16mM acetic acid, 1mM EDTA, pH8.0
1 × ligase buffer	50mM Tris HCL pH7.6, 10mM $MgCl_2$, 1mM ATP, 1mM DTT, 5% (w/v) polyethylene glycol-8000
Phosphate buffered salin (PBS)	137mM NaCl, 44mM Kcl, 1.4 mM KH_2PO_4, 8.5 mM Na_2HPO_4
10 × loading buffer	0.45% (w/v) bromophenol blue, 1% (w/v) SDS, 100mM EDTA, 2.5% (w/v) Ficoll 400 in TE
2 x HEPES buffered saline (HBS)	280mM NaCl, 10mM KCl, 1.5mM Na_2-$HPO_4.2H_2O$, 12mM D-glucose, 50mM HEPES
SDS-PAGE Lysis buffer	1M Tris-HCL (pH 6.8), 10% (w/v) SDS, 20% (v/v) glycerol
Lysis buffer	10mM Tris-HCl, 400mM NaCl and 2mM Na_2EDTA, pH 8.2
Tris-glycine electrophoresis buffer	25mM Tris, 250mM glycine and 0.1% (w/v) SDS
2x SDS gel loading buffer	4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol and 100mM Tris, pH6.8
Dephosphorylation buffer	50mM Tris HCl, 0.1mM EDTA. pH 8.0

2.1.4 Cells

CELL LINE	DESCRIPTION
PalF	PalF cells are primary fibroblasts explanted from bovine foetal palate.
PalF transfectants	PalF transfectants were derived from transfection of PalF cells with sub-gnomic fragments of BPV-4 as detailed in Chapter four

2.1.5 Cell Culture Materials

SUPPLIER	MATERIAL
(i).Globerpharm Ltd., Esher, Surrey, England (ii). Harlan Sera-Lab ltd., Crawley Down, England	Foetal Calf Serum Foetal Calf Serum
Gibco Europe Life technologies Ltd., Paisley, Scotland	10% Dulbecco's Modified Eagles Medium 10x F10 (Ham) Medium 200 mM glutamine Geneticin. G418 sulphate MEM amino acids solution (50x) 7.5% sodium bicarbonate 100 mM sodium pyruvate 2.5% Trypsin

2.1.6 Chemicals

Supplier- Amersham International plc, Amersham, Bucks, England

ECL Western detection agent

Supplier- BDH Chemicals Ltd., Poole, Dorset, England.

Calcium chloride

D-glucose

Glycerol

Supplier- Beta Lab., East Mosley, Surrey, England.

Yeast Extract

Supplier- Biogenesis Ltd., Bournemouth, England.

RNAzol B

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

Caesium chloride

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

DNase 1, RNase-free

Protease K

RNase A

Supplier- Difco laboratories, Detroit, Michigan, USA.

Bacto-Agar

Bactotryptone

Supplier- Fisons Scientific Equipment, Loughborough, England.

Acetic acid

Butan-1-ol

Chloroform

di-potasium hydrogen orthophosphate anhydrous

Ethylene diamine tetra acetate (EDTA) disodium salt

Dimethyl sulfoxide (DMSO)

Hydrochloride acid

Magnesium chloride

Magnesium sulphate

Methanol

Potassium chloride

Potassium dihydrogen orthophosphate

Propan-2-ol

Sodium acetate

Sodium carbonate

Sodium chloride

Sodium dodecyl sulphate (SDS)

Sodium hydroxide

Supplier- Fluka AG, Chemisch Fabrik CH-9470 Buchs.

Methocel MC 4000

Supplier- Gibco Europe Life technologies Ltd., Paisley, Scotland.

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

Agarose (ultrapure electrophoresis grade)

Tris

Supplier- James Burrough Ltd., Witham, Essex, England.

Ethanol

Supplier-Severn Biotech Ltd., Kidderminster, Worchester, England.

Acrylaimide

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

Ampicillin

Bicinichonic acid solution

Bovine Serum Albumin

Copper(II) sulphate (pentahydrate 4% (w/v) solution)

Crystal violet

Ethidium bromide

HEPES

Lysozym

Phenol:Chloroform:Isoamyl Alcohol (25:24:1 (v/v))

Salmon tested DNA (sodium salt)

TEMED (N,N,N',N'-tetramethylthylenediamine)

Tween 20 (Polyoxyethylene sorbitan monolaurate)

2.1.7 Equipment and Plasticware

SUPPLIER	EQUIPMENT
Alpha Laboratories Ltd., Eastleigh, Hampshire, England	Microfuge tube
Becton Dickinson Labware, Plymouth, England	Falcon 1059 polypropylene tubes Falcon 2059 polypropylene tubes Falcon 2098 polypropylene tubes Sterile Plastipak syringes 18 gauge sterile syringe noodles 60 and 90 mm tissue culture dishes
Bibby sterilin Ltd., Stone, Staffs, England.	60 and 90 mm bacteriological petri dishes Sterile plastic universal containers
Costar Corporation, High Wycombe, Bucks, England	24 well tissue culture plates 96 well tissue culture plates Disposable Cell scrapers
Dupont Uk Ltd., Stevenage, Hertz, England	Polyallomer ultracentrifuge tubes
Eastman Kodak Co., Rochester, New york, USA	X-ray film (XAR-5)
Gelman Sciences, Northampton, England	Sterile 0.2 μm acrodisc filters
Ilford Ltd., Mobbrrley, Cheshire, England	Ilford PANF 50 black and white film
Nunc, Roskilde, Denmark	T25, 80, and 175 cm^2 tissue culture flasks Cryotubes
Technical Photo Systems., Cumbernauld, Scotland	Fuji RX medical X-ray film
Whatman International Ltd., Maidstone, Kent, England	Whatman 1 filter paper

2.1.8 Kits

SUPPLIER	KIT
Boehringer Mannheim UK Ltd., Lewes, East Sussex, England.	Random Primed DNA Labelling Kit.
Perkin Elmer Cetus, Norwalk, USA.	GeneAmp RNA PCR core kit GeneAmp PCR core kit GeneAmp thinwalled reaction tubes
Promega ltd., Chilworth Research Centre, Southampton, England	Luciferase Assay System Reporter Lysis 5 X Buffer Altered Sites^{R} II invitro Mutagenesis Systems
Qiagen ltd., Dorking, Surrey, England	QLA prep Spin plasmid miniprep kit QLAquick gel extraction kit

2.1.9 Molecular Weight Markers

SUPPLIER	MARKER
Amersham International plc, Amersham, Bucks, England	Rainbow^{TM} coloured protein molecular weight markers (14,300- 200,000Da)
Gibco Europe Life Technologies Ltd., Paisley, Scotland	Bacteriophage λ DNA (EcoR I digested) Bacteriophage λ DNA (HindIII digested) 100bp DNA ladder Low DNA mass ladder

2.1.10 Other Materials

SUPPLIER	MATERIALS
Beatson Institute Central Services	Amphotericin B LB-Medium (Luria-Bertani Medium) Kanamycin Penicillin Sterile distilled water Sterile glycerol Sterile phosphate-buffered saline (PBS) Sterile phosphate-buffered saline + EDTA (PE)
Merck Ltd., Poole, England	Silicone grease
Premier Beverages., Adbaston, Stafford, UK	Marvel (Dried Skimmed milk)

2.1.11 Plasmids

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

pJ4Ω16-E6 was a gift from Dr. L. Crawford (Dept. of Pathology, University of Cambridge). This plasmid construct is a pBR322 derivative. It contains the HPV-16 E6 open reading frame (ORF) cloned into the Bam H1 / EcoR I sites of pJ4Ω downstream of a MoLV LTR promoter (Storey et al., 1988).

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

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

pZipneoE7 contains nucleotides (nts) 652-1250 of the BPV-4 genome cloned into the BamH I site of pZipneo SV (XI) (Pennie et al., 1993). In pZipneoE7 the BPV-4 E7 gene is under the transcriptional control of the Moloney leukaemia virus 5′ long terminal repeat (MoLV LTR).

pZipneoHAE8 contains nts 236-590 of the BPV-4 genome cloned into the Bam HI sites of the vector pZipneo. The sequence coding for the influenza virus haemagglutinin type-1 (HA1) epitope is inserted at the 5′ of the E8 sequences.

pALTER-E8 contains nts 236-590 of the BPV-4 genome cloned into the Bam HI sites of the vector pALTER-1 (Promega). This plasmid construct was a gift from Dr R. Anderson.

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

pZipneoBPV-1 HAE5 contain nts 3879-4012 of the BPV-1 genome cloned into the Bam HI sites of the vector pZipneo. The sequence coding for the influenza virus haemagglutinin type-1 (HA1) epitope is inserted at the 5′ of the E5 sequences. The HAE5 was a gift of Drs R. Schlegel and D. J. Goldestein.

pwt929 is a reporter plasmid for human cyclin A promoter. This plasmid construct was a gift to Dr Vincent O'Brien from Dr William Fahl, University of Wisconsin, USA (Kramer et al., 1996).

2.1.11 Water

Distilled water for the preparation of buffer stocks was obtained from a Millipore MilliRO 15 system, and for protein, enzyme, RNA or recombinant DNA procedures was further purified on a Millipore MilliQ System to 18MΩ/cm. Sterile distilled water for making up tissue culture media was supplied by the Beatson Institute for Cancer Research Technical Service.

2.2 Methods

2.2.1 Molecular biology

2.2.1.1 Oligonucleotide synthesis and purification

Oligonucleotides were synthesised by Beatson Institute technical services staff on an applied Biosystems model 381A DNA Synthesiser or 392 DNA/RNA Synthesiser using the manufactures protocols and Cruachem reagents. The final primers were synthesised with or without trityl group protection. All primers were firstly deprotected after synthesis by incubating in a 55°C water bath overnight.

"Trityl on" primers were detritylated using a Cruachem oligonucleotide purification (COP) cartridge according to manufacturers instructions. Each oligonucleotide was ultimately eluted from COP cartridge minus the trityl group using 1 to 2ml of 20% (v/v) acetonitrile. The acetonitrile was evaporated off and the primer dissolved in 0.5ml sterile distilled water or TE pH 8.0. Primers were stored at -20°C.

"Trityl off" oligonucleotides were provided in ammonia. The oligonucleotides were deprotected by heating to 55°C overnight then purified by precipitation with butan-1-ol. 1ml butan-1-ol was added to 150µl oligonucleotide solution and microcentrifuged at 14000 for 20 minutes at room temperature. Excess butanol was removed by centrifugation under vacuum and the primer dissolved in an appropriate volume of sterile distilled water or TE pH 8.0. Primer concentration was determined as described in section 2.2.1.4.

2.2.1.2 Denaturation of Double-stranded DNA Template

The double stranded DNA (dsDNA) was alkali-denatured by using the following alkaline denaturation reaction. The reaction was carried out in a final volume of 20µl comprising of 0.5pmol of dsDNA template, 0.2M NaOH, 0.2mM EDTA and sterile, deionized water to final volume 20µl. This was incubated for 5 minutes at room temperature

The DNA was precipitated by adding one tenth of 2M ammonium acetate, pH 4.6 and 3.5 volume of ethanol and standing at -70°C for 30 minutes followed by centrifugation (14000 rpm) in a microcentrifuge for 15 minutes at 4°C. The pellet was washed with 70% ethanol before drying under vacuum for 5 minutes to remove all traces of ethanol. The pellet was dissolved in appropriate volume of sterile distilled water and stored at -20°C.

2..2.1.3 DNA Extraction with Organic Solvent and Ethanol Precipitation

DNA samples were purified by extraction with phenol:chloroform in order to remove contaminants, such as residual enzyme activities from a restriction reaction or detergent which might otherwise interfere with subsequent cloning steps. In the first round of extraction the DNA sample was mixed with an equal volume of phenol:chloroform. Phenol:chloroform was freshly prepared from an equal volume of 1M Tris-HCl pH8.0, saturated phenol and chloroform:isoamyl alcohol (24:1 v/v). The aqueous DNA and organic phase were mixed thoroughly by vortexing, then separated by centrifugation in a microcentrifuge at 14000 rpm for 5 minutes at room temperature. The upper aqueous phase was transferred in a clean eppendorf tube, care was taken not to

transfer any of the interphase to the tube, and the extraction process repeated. The aqueous phase was then extracted with an equal volume of chloroform (chloroform:isoamyl alcohol, 24:1 v/v) by vortexing and centrifugation as described above. This was repeated to remove any traces of phenol from the aqueous phase. The aqueous phase was transferred to a fresh eppendorf for ethanol precipitation.

Ethanol precipitation was used to concentrate DNA samples and also to remove solute contaminants such as salt. The aqueous DNA solution was mixed with one tenth volume of 3M sodium acetate pH 5.2 and 2-2.5 volumes of ice cold ethanol. The sample was then mixed well by inversion several times and then stored at -20°C or, alternatively, placed on dry ice for 15-30 minutes to facilitate DNA precipitation. The precipitated DNA was collected by centrifugation in a microcentrifuge at 14000 rpm for 15 minutes at 4°C. The supernatant was discarded, and the pellet was washed with 70 % ethanol to remove any trace of salt, dried under vacuum before resuspension in distilled water at an appropriate concentration. The DNA concentration was determined as described below.

2.2.1.4 Quantitation of nucleic acids

The concentration of nucleic acid in a solution was determined spectrophotometrically in a Beckman DU 650 spectrophotometer. Samples were diluted in TE and transferred to a quartz cuvette with a pathway of 1cm. The spectrophotometer was initially calibrated using TE buffer only as a blank. The optical density reading were obtained at 260nm and 280nm; an O.D. reading of 1

at 260nm (A_{260} = 1) corresponds approximately to a concentration of 50μg/ml of double stranded DNA, for oligonucleotides an A_{260} of 1 was taken to correspond to ~35μg/ml, and for RNA an A_{260} of 1 was taken to correspond to 40μg/ml. The ratio between readings at 260nm and 280nm (A_{260}:A_{280}) provided an estimate of the sample purity; a ratio of ~1.8 indicated that preparations contained essentially no protein or phenol contamination.

2.2.1.5 Restriction enzyme digestion of DNA

Restriction digests were carried out in small reaction volumes using enzymes and their appropriate concentrated buffer solutions according to the manufacturers.

Plasmid DNA was incubated with 5-10 units enzyme/μg DNA in a buffered solution ensuring that the total volume of enzyme added did not exceeded one tenth of the final reaction volume. Small quantities of plasmid DNA (<5μg) were routinely digested in a 20μl reaction volume as specified by the manufacturer for 2 hours at 37°C. Large digests were carried out in proportionally larger reaction volumes. The digestion fragments were analysed by agarose gel electrophoresis as described below.

2.2.1.6 Agarose gel electrophoresis

Horizontal gel cast apparatus from Pharmacia was used. In general, 1% (w/v) agarose gels were used, but smaller fragments (100-400) were separated on 2-4% gels. Low melting point agarose was used at a concentration of 1% (w/v) in order to isolate and purify required DNA restriction fragments. Gel mixes

containing the appropriate amount of agarose were dissolved in 0.5 x TBE buffer by heating the solution in a glass conical flask in a microwave until all the particles of agarose gel had dissolved. The gel was poured when the agarose was hand hot and a comb with the required number and size of teeth placed immediately into the gel to form the sample wells. The gel was submerged in 0.5 x TBE buffer. The samples containing 1 x loading buffer were loaded in each well along with an appropriate size marker (i.e. 100b ladder, 1Kb ladder, DNA mass ladder) into the first and/or last well in the gel and run at 70-100 constant voltage usually until the samples' blue dye front was 1-3 cm from the end of the gel. Once run, the DNA fragments were visualised by staining the gel in running buffer containing 0.5μg/ml ethidium bromide with gentle agitation for 10 minutes at room temperature. The separated DNA was visualised by illumination with short wave (312nm) UV light and photographed through a red filter onto videoprint paper using an Appligene Imager.

2.2.1.7 Isolation and purification of DNA restriction fragment from agarose gel

The DNA fragment to be used for cloning was recovered from low melting point agarose gel and visualised as described in section 2.2.1.6. The fragment was cut out of the gel with a clean scalpel blade and the gel slice placed in an eppendorf tube. Extraction of the DNA fragment from the agarose was achieved using a Qiagen Qiaquick gel extraction kit following the manufacturer's instructions. Alternatively, the DNA fragment was purified from the gel slices using extraction with phenol: chloroform as described in section 2.2.1.3. First the

gel slice was weighed then three volumes of TE buffer were added to the tube containing the gel fragment. The tube was then incubated at 50°C for 10 minutes or until the gel slice had completely dissolved in TE. Extraction with phenol:chloroform was performed as described earlier. Approximately 10% of the extracted DNA was then checked for size and purity by running on a 1% agarose minigel.

2.2.1.8 Ligation of DNA fragments

Both vector DNA and the DNA fragment to be inserted into the vector were separately digested and purified as described above and then isolated by gel electrophoresis as described in section 2.2.1.6.

The vector DNA was dephosphorylated at its termini to prevent religation. After the vector DNA had been linearized by digestion, the reaction mixture was adjusted by adding dephosphorylation buffer and 1 unit of Calf Intestinal Alkaline Phosphatase (CIAP) was added to the reaction mixture and incubated at 37°C for 15 minutes. The reaction was stopped by heating to 50°C for a further 15 minutes. Another 1 unit of CIAP was added to the reaction mixture and incubated at 37°C for 15 minutes. All enzyme activity in the reaction was finally stopped by heating to 50°C for further 15 minutes.

The DNA was phenol:chloroform extracted, ethanol precipitated and then resuspended in appropriate volume of distilled water and stored at -20°C.

The DNA fragment was inserted into dephosphorylated vector (100ng) at a ratio of 3:1 respectively. The vector and inserted DNA were incubated together in a reaction containing 1 × ligase buffer and 1 unit of T4 ligase at 22°C for 1 hour or

overnight. Dilutions of this reaction volume were used to transform competent bacterial cells (section 2.2.1.9).

2.2.1.9 Transformation of bacterial Hosts

All plasmids were propagated in commercially available E. coli JM109 competent cells supplied as frozen stocks (Promega) kept at -70°C until use. Bacteria were transformed following manufacturer's instructions. Competent cells were thawed slowly on ice, and 100 µl of aliquots put into a prechilled polypropylene culture tube (Falcon 2059) and 1-2 ng of the appropriate plasmid DNA added and mixed by gently moving the pipette tip trough the cells while dispensing. The cells were then incubated on ice for 30 min before being heat shocked for 45 seconds at 42°C. The tube was then immediately placed on ice for 2 min. 900 µl of room temperature L-broth (1% w/v Bactotryptone, 0.5% w/v yeast extract, 1% w/v NaCl) was then added to each transformation reaction. The tube was then transferred to a shaking 37°C incubator (approximately 225rpm) for 1 hr to allow expression of the antibiotic resistant marker. Following this, 100µl of cells were spread on an L-agar plate containing the appropriate antibiotic. The plate was inverted and incubated overnight at 37°C to allow colony formation.

2.2.1.10 Glycerol Stocks

Host strains, and their derivatives containing useful plasmids, were stored as glycerol stocks for future retrieval. 850 µl of an overnight culture was mixed gently with 150 µl sterile glycerol in a 1.5 ml Nunc Cryotubes and stored at -70°C. A sterile plastic loop was used to retrieve an aliquot of cells as and when required.

2.2.1.11 Small scale preparation of Plasmid DNA (Miniprep)

Small amounts of plasmid DNA were extracted from transformed bacterial colonies to identify correct clones.

Single colonies of bacteria carrying the required plasmid were picked using a sterile yellow pippet tip and grown in 5 ml culture of L-Broth (1% w/v Bactotryptone, 0.5% w/v yeast extract, 1% w/v NaCl) containing antibiotic (100μg/ml Ampicillin) at 37°C in a shaking incubator (225rpm) overnight. 10 separate colonies were generally picked for screening at any one time. Bacteria were pelleted from 1.5ml of overnight culture by spinning in a microcentrifuge (14000rpm) for 30 seconds at room temperature. DNA was prepared using the QlA prep Spin plasmid miniprep kit following the manufacture's instructions.

2.2.1.12 Large Scale Preparation of Plasmid DNA

Bacteria containing the plasmid of interest were streaked onto an L-agar plate containing the appropriate antibiotic and the plate inverted and incubated overnight at 37°C to allow colony formation. A single colony was picked, using a sterile yellow tip, from this plate and used to inoculate a sterile universal tube containing 5 ml of L-Broth medium and the appropriate antibiotic(100μg/ml Ampicillin) which was then put in a shaking incubator at 225rpm overnight at 37°C. This culture was then added to 500 ml of Superbroth, containing 100μg/ml Ampicillin in a 1 litre glass conical flask (to allow good aeration), then returned to the shaking incubator for 48 hours. Superbroth is composed of two solution, A and B. Solution A consists of 12 g of bactotrypton, 24 g of yeast extract, and 5 ml of glycerol made up to final volume of 900 ml with distilled water. Solution B is

consist of 12.5 g of di-potasium hydrogen orthophosphate (K_2HPO_4) and 3.8 g of potassium dihydrogen orthophosphate (KH_2PO_4) made up to a final volume of 100 ml. Both solution were autoclaved separately and combined just prior to use.

Bacterial cells were pelleted by centrifugation in a sorvall RC-5B centrifuge (Sorvall GS-3 rotor) at 5,000 rpm for 10 min at 4°C and the supernatant was removed. The pellets were resuspended in 18 ml of TEG (50 mM glucose, 10mM Tris-HCl (pH8.0), 10 mM EDTA). Resuspended pellets were pooled into one centrifuge bottle and 2 ml of lysozyme containing 10 mg/ml were added. The suspension was mixed gently and allowed to stand on ice for 10 min. 40 ml of freshly prepared alkaline SDS (0.2 M NaOH, 1% SDS) were added and the suspension mixed by gentle inversion and placed on ice for 5 min. 30 ml of ice cold 5M potassium acetate (490.7 g KAc and 115 ml glacial acetic acid made up to 1 litre in water) were added, the whole solution inverted sharply five times and then returned on ice for 20 min. The flocculate was centrifuged at 8,000 rpm for 5 min at 4°C in a Sorvall GS-3 rotor and the supernatant filtered through gauze into a 250ml measuring cylinder. 0.6 volumes of room temperature propan-2-ol was added, then the whole solution transferred to 250ml centrifuge bottle and mixed by inversion several times and left at room temperature for 10min. The nucleic acid in this cleared lysate was precipitated and pelleted by centrifugation at 8,000 rpm for 5 min at 4°C. The supernatant was removed and the nucleic acid pellet washed with 50ml room temperature 70% ethanol to remove any salt. The solution was centrifuged for a further 5 min at 8,000 rpm at 4°C. After discarding the supernatant, the pellet was allowed to dry at room temperature for 10 min before being resuspended in 9ml of TE.

Ultracentrifugation through a caesium chloride (CsCl) density gradient was then carried out to further purify the plasmid DNA. 10 g of caesium chloride was added to the solution and allowed to dissolve at room temperature. 500µl of a 10mg/ml ethidium bromide solution was added to visualise the DNA band after centrifugation. The refractive index of this solution adjusted to 1.395. The solution was transferred to a sealable centrifuge tube (11.5ml Dupont disposable tube) with a protective metal cap over the top of each tube. Samples were spun in a balanced Beckman ultracentrifuge at 50,000 rpm for 24hr at 20°C in a T1270 rotor. The tube was carefully removed from the centrifuge rotor and placed securely in a clamp on a rotor stand. After centrifugation, any contaminating RNA was found to have a pelleted to the bottom of the tube. Two distinct bands were observed; the upper band contains sheared linear plasmid DNA and residual bacterial chromosomal DNA while the lower band contains closed circular plasmid DNA. An 18 gauge needle was first inserted into the top of the tube to act as an air inlet and the lower band was gently withdrawn by similarly piercing the side of the tube ~ 1cm below the lower band with an 18 gauge needle connected to a syringe. The plasmid DNA band was then transferred to a clean ultracentrifuge tube. The tube was filled with CsCl/TE (RI=1.395) as before and underwent further centrifugation prior to plasmid extraction.

The band removed as described above was transferred to a 5 ml Bijoux tube. Ethidium bromide was removed in the solution by extracting with an equal volume of water saturated butan-2-ol. The solution was mixed and ethedium bromide separated with the upper organic phase which was carefully aspirated off and discarded into appropriate bottles. This extraction process was repeated until

the lower aqueous phase was clear and colourless. The CsCl removed by dialysing the plasmid DNA into a dialysis tube (Collodion Bag) and placed in a large beaker containing 2 litres of TE. Plasmid DNA was dialysed against TE on a magnetic stirrer for 4 hours at room temperature. The TE buffer was changed and dialysis continued for a further 4 hours at room temperature or overnight at 4°C. DNA was then ethanol precipitated and the DNA pellet was finally resuspended in 0.5-1 ml TE (pH 8.0), depending on the size of the pellet. The DNA concentration was determined as described in section 2.2.1.4. The plasmid DNA was aliquoted and stored at -20°C.

2.2.3 Cell Culture and Transfection

2.2.3.1 Cell Culture

All cell culture work was performed following strict aseptic techniques inside a laminar flow hoods (Class II Microbiological safety Cabins; Medical Air Technology Ltd., Manchester, England). Cells were incubated in dry 37°C incubators containing 5% (v/v) CO_2 (Heraeus, Essex, England) and were routinely screened for mycoplasma infection using a fluorescent dye technique (M. Freshney, Beatson Institute, Glasgow).

2.2.3.2 Isolation of Primary Bovine Fibroblasts

The fibroblasts from foetal bovine palate were isolated as described previously by Jaggar et al., (1990). A small section of soft palate tissue was taken from bovine foetuses of less than 5 months gestation obtained from the veterinary post-mortem room at the Glasgow University Veterinary College. The palate

tissue was sterilized by a 30 second wash in 70% ethanol and then dissected into small pieces approximately $2mm^2$ in size using crossed scalpels and placed into a 90mm dish being well spaced apart. The tissue was allowed to adhere to plastic by placing the dishes in a dry 37°C incubator containing 5% (v/v) CO_2 for 5 minutes. Culture medium was then added slowly to each dish so as not to disturb the adherent samples. The samples were fed twice weekly over a period of two weeks in which time fibroblasts and keratinocytes grew out of tissue mass. The medium used for both isolation and subsequent routine growth of PalF cells was DMEM supplemented with 10% foetal calf serum, 2mM glutamine, 1mM pyrovate, 0.375% sodium bicarbonate, 37.5 μg/ml penicillin, 50 μg/ml streptomycin. This medium selectively favoured the outgrowth of fibroblasts and, as expected, keratinocytes died. The newly extracted fibroblasts were trypsinised and reseeded into large (T175 cm^2) flasks. Cells were expanded and stocks of PalF cells were frozen down in liquid nitrogen for further experiments.

2.2.3.3 Maintenance of Primary Bovine Fibroblasts in Culture

Cells were fed twice weekly, old medium was aspirated from sub-confluent flasks and fresh medium added. PalF cells were grown until just sub-confluent whereupon they were passaged approximately 1 in 4. Replating was performed as follows: for T80 cm^2 tissue culture flask medium was aspirated off and the cells washed twice with 5 ml phosphate-buffered saline (PBS). The PBS was removed and 1 ml of trypsin solution (0.25% trypsin in 1x PE buffer; PBS with the addition of EDTA to 1 mM), which had been pre-warmed to 37°C, was added to cells. Flasks were transferred to the 37°C hot room until the cells had

detached from the flasks. Complete medium was added and the cell suspension transferred to a sterile universal tube. The cells pelleted by centrifugation at 1000rpm for 5 mins at room temperature. The pellet was then resuspended in fresh growth medium and the cells reseeded at an appropriate density.

2.2.3.4 Long term cell storage

To freeze cells stocks for storage, confluent cultures were trypsinised, and pelleted as described above (section 2.2.3.3). The pellet was then resuspended at a concentration of approximately 10^6 cells/ml in growth medium containing 10% (v/v) DMSO. The DMSO in the medium acts as a cryoprotectant but all solution must be chilled as DMSO is toxic to cells at room temperature. Suspensions were divided into 1 ml aliquots in 1-2 ml Nunc cryotubes and placed in a polystyrene box and frozen, well insulated, at -70°C overnight to ensure a slow rate of cooling. The ampoules were then transferred to a liquid nitrogen bank containing labelled storage rack until required. Frozen stocks were recovered by removing the ampoules from liquid nitrogen and placed into a small, covered bucket of water at 37°C. Once thawed, the cells were added to 10ml of the appropriate pre-warmed growth medium, centrifuged, resuspended in fresh growth medium and transferred to 80 cm^2 flasks.

2.2.3.5 Transient transfection of Primary Bovine Fibroblasts (PalFs)

Cells were transiently transfected with a range of plasmid DNAs (section 2.1.11) using the standard calcium phosphate-mediated method. Cells (10^5) were plated in each well of 6 well plate, in duplicate, containing 5ml of appropriate

growth medium (DMEM-10) the day before transfection. For each well of cells to be transfected the following mixture was set up.

250µl of a DNA solution (up to 3µg of total plasmid DNA in 0.1x TE pH 8.0 containing 250mM CaCl) was added to 250µl of 2 x HEPES buffered saline (HBS). The addition of the DNA solution to the 2 × HBS must done very gradually with constant but gentle mixing. The mixture was incubated for 20 to 30 minutes at room temperature during which time a fine precipitate formed giving a slight blue/grey colour to the transfection mix. To resuspend the precipitate the mixture was gently pipetted up and down.

This mixture was then added directly into the medium above the cells. The plates was moved slowly to disperse the transfection solution throughout the culture medium and incubated at 37°C for 16-18 hours. After withdrawal of the medium, cells were washed twice with 2ml of pre-warmed PBS and incubated in DMEM medium, which was supplemented with 10% feotal calf serum (FCS), reffered to as DMEM-10 or 0.5% FCS (DMEM-0.5) at 37°C for a further 24 hour before being harvested.

2.2.3.6 Luciferase Assays

Cells were seeded at 20,000 cells/ml in DMEM-10 in each well of 6 well plates, 5ml/well, the day before transfection, and transfection was performed using the standard calcium phosphate method (section 2.2.3.5). After 16-18hr., the cells were washed twice with 2ml. PBS and incubated in DMEM-0.5 or DMEM-10 for a further 24 hr. Cells were then washed twice with PBS, the PBS was completely removed by aspiration and 300µl of 1 × reporter lysis buffer (Promega) added to

each well. following 15 minutes incubation at room temperature, cells were scraped off the culture well and each lysate transferred to a 1.5ml eppendorf. Cell debris was pelleted by spinning lysates at 4°C in a microcentrifuge at 14000 for 5 minutes. The supernatant was transferred to a second eppendorf taking care not to disrupt the cell pellet. The lysate were either assayed for reporter enzyme activity immediately or stored at -20°C.

Luciferase activity was determined using a luminometer with automatic injection (BioOrbit, model 1251). For each sample, 80µl of lysate and 120µl of Luciferase assay buffer (Promega) were used. Luciferase activity was normalised for protein content determined using the BCA assay (Pierce). The reporter plasmid for the human cyclin A promoter, pwt929, was provided by Dr. William Fahl, University of Wisconsin, USA.

2.2.3.7 DNA transfection of Primary Bovine Fibroblasts

PalF cells were transfected with a range of plasmid DNAs (section 2.1.11) using the cationic lipid N-[1-(2, 3-Dioleoyloxy) propyl]-N, N, N-trimethylammoniummethylsulfate (DOTAP; Boehringer Mannheim BCL) following the manufacturer's recommendations. Each reaction contained 5 µg of each relevant plasmid DNA plus 2 µg of a plasmid construct containing the selectable marker gene for neomycin resistance (pZipneo; Chapter 2.1.11).

Transfection classes are described in Chapter four in the Result section. Reaction were made up to 20 µg with sonicated salmon sperm DNA (Sigma). PalF cells were plated at a density of 5×10^5 into an 80 cm^2 flask 24 hr prior to transfection. The cells were fed with 13.5mls of growth medium on the day of

transfection, the transfection mix was as follows; 80 µl of DOTAP was diluted up to 250 µl with complete medium in a separate reaction vial. DNA (20µg) was also diluted up to 250µl with complete medium in a separate reaction vial. Both solution were mixed together and incubated for 10 min at room temperature. This mixture was then slowly added to the flask of cells and incubated at 37°C overnight. After withdrawal of the medium, cells were washed twice in PBS and then fresh complete medium was added to the flask. The next day cells were split at a dilution of 1:2 and allow to settle for 24 hr prior to selection.

2.2.3.8 Selection of transfected cells

Cell were selected in medium containing 500 µg/ml G418 for 21-28 days, being fed twice weekly. After this time, G418-resistant colonies were scored. Where appropriate, several colonies were picked from each transfection class in order to expand them clonally.

2.2.3.9 Isolation of clonal populations

Single neomycin resistant colonies were identified and their position marked using a microscope ring marker attachment. The cells were then washed twice in sterile PBS. A sterile 6 mm stainless steel cloning rings coated with sterile silicon grease (Merck, England) at the base was then placed over the identified colony thus providing a waterproof seal round each isolated colony. A total volume of 100 µl trypsin solution, which had been pre-warmed to 37°C, was pipetted within each cloning ring. After 1-2 min an equal volume of complete medium was added and the cell suspension transferred to a 24-well plate (Costar)

along with 2ml medium and returned to the 37°C incubator for expansion into cell lines.

2.2.3.10 Transformation Assays

2.2.3.10.1 Focus formation assay

200 cells of normal PalF and each clone were mixed with 25,000 normal PalF cells. The mix was plated in each well of 6 well plates, in triplicate. Cells were fed twice weekly. Three weeks later the cells were fixed in methanol and stained with 10% filtered Giemsa.

2.2.3.10.2 Cell population growth in high serum (DMEM-10%) and low serum (DMEM-0.5%) culture (Crystal Violet assay)

Cells were seeded in 96 well tissue culture plates, in triplicate, at 3000 cells/well in 200μl of DMEM-10. One 96 well plate of cells was set up for each day from day 0 to day 7. The outer wells along the edge of the plate were not used and instead 200µl of growth medium was added to each of these wells to reduce evaporation from the inner cell-containing wells. After four hours incubation, during which time the cells attached to the bottom of each well, growth medium was respectively changed to DMEM-10 or DMEM-0.5 in the appropriate wells. The first plate, corresponding to day 0, was treated with 100μl of 0.1% Crystal Violet in 20% Methanol and assayed. Each day thereafter one plate of cells was treated with Crystal Violet to determine the growth characteristics of cells in each well. This was carried out over a period of 7 days (at daily intervals for 7 days).

Treatment of cells with Crystal Violet was performed as follows; Medium from all cells in 96 well plate was aspirated off. Each well was washed with pre-warmed (37°C) PBS before addition of 100µl Crystal Violet. The plate was placed at room temperature overnight. Crystal Violet solution was removed from each well and the well was washed extensively in water then dried at room temperature. Following wash with water, 100 µl of 1% alkaline SDS was added to each well and placed on a horizontal shaking platform for 10 minutes at room temperature. The absorbance of the solubilized dye in each well was determined immediately at 590nm using a automated microwell plate reader (Dynatech MR7000).

2.2.3.10.3 Anchorage independent growth

The ability of cell line to form colonies in semi-solid media is taken as a phenotypic measure of its degree of transformation. The extend of transformation of cell population was assayed by plating cells in Methocel based medium.

Efficiency of methocel colony formation was determined by adding 10^5 cells to 15 ml of 1% methocel including 30% Foetal Calf Serum (FCS).

The mix was plated in bacterial petri dishes. Bacteriological petri dishes were used to discourage cells from adhering to the bottom of the dishes. Cells, tested in duplicate, were left at 37°C for 12 days before being scored and photographed with PanF 50 technical film.

Methocel medium was made up as follows:

3g of Methocel MC 4000 (Fluka) was added to 200 mls of distilled water and autoclaved. The Methocel was left to dissolve with stirring for 2-3 days at

4°C. Following dissolution, 22 mls of 10 × F10-HAM medium (Gibco), 4 mls of 50 × minimum essential amino acids (Gibco), 4 mls of 0.1M sodium pyruvate, 5 mls of 7.5% sodium bicarbonate, 100 mls of foetal calf serum (FCS) and 2% penicillin and streptomycin were added.

2.2.3.10.4 Tumorigenicity Assay in Nude Mice

The ability to form tumours is an indicator of full cellular transformation. The transformed cells were assayed in nude mice. Cells were removed from selection and expanded at identical cell density. The cells were suspended in sterile PBS at a concentration of 10^8 cells/ml. 0.1 ml of this suspension (10^7 cells) were injected subcutaneosly into a four-week old female athymic nude mice, strain MF1 nu/nu (Harlan-Olac, Bicester, England) at a single injection site. Three mice were injected per cell line tested and examined for tumour growth weekly up to 15-20 weeks post injection. If no tumour had developed by then the cells were considered to be non-tumorigenic.

2.2.4 Eukaryotic DNA and RNA analysis

2.2.4.1 DNA extraction from cell lines

Genomic DNA was isolated from cell lines using a modification of the technique of Miller et al (1988). Cells, grown to approximately 80% confluency in a 75 cm^2 tissue culture flask, were washed twice with PBS, trypsinised and pelleted at 1000 rpm at room temperature for 5 minutes in a 20 ml universal tube. The cell pellet was resuspended in 3 mls of lysis buffer and digested overnight at 37°C with 0.5 mls of protease K solution (1mg protease K in 1% SDS and 2mM

Na_2EDTA). After digestion, 1 ml of a saturated NaCl solution was added and the tube shaken vigorously for 15 seconds, followed by centrifugation at 2500 rpm for 15 minutes to precipitate the protein pellet. The supernatant was carefully transferred to a fresh tube and one tenth volume of 3 M sodium acetate, pH 5.2 and 2 volumes of absolute ethanol were added. The sample was mixed by inversion and stored at -20°C overnight or alternatively placed on dry ice for 30 minutes to precipitate the DNA. Precipitated DNA was recovered by centrifugation in microcentrifuge at 14000 rpm for 30 minutes at 4°C. The DNA pellet then was washed with 70% ethanol to remove solute contaminants such as salt, air dried, and resuspended in an appropriate volume of TE. The DNA was allowed to dissolve before quantitation.

2.2.4.2 Total RNA extraction from cell lines

Cells were grown in a $175cm^2$ (T175) flask to approximately 80% confluency following the RNAzol B method of extraction (Biogenesis Ltd, England). Cells washed twice with ice cold PBS and 10mls of RNAzol B was added directly to the flask. The lysate was transferred to a Falcon 2059 polypropylene centrifuge tube and 1 ml of chloroform was added with vigorous pipeting. The top of the tube was then covered with Parafilm (American National Can, USA) and the tube was left on ice for 15 minutes to allow phase separation to take place. The tube was then centrifuged in a sorval RC-5B (HB6 roter) at 10,000rpm for 15 minutes at 4°C. The upper, aqueous phase was transferred to a fresh tube and an equal volume of isopropanol added. The samples were mixed and stored overnight at -20°C to allow precipitation of RNA and the RNA pelleted

by centrifugation as before. The pellet was resuspended in 5 mls of 75% ice cold ethanol (made with diethylpyrocarbonate (DEPC) -treated RNase-free water), and transferred to an eppendorf. The RNA was pelleted in a microfuge at full speed at 4°C for 30min, dried on a speedivac and then resuspended in DEPC-treated RNase free water and the concentration of RNA was measured spectrophotometrically as described in section 2.2.1.4. RNA samples were aliquoted and stored at -70°C.

2.2.4.3 Polymerase chain reaction (PCR)

2.2.4.3.1 Amplification of DNA

All reagent were provided in the Perkine-Elmer Cetus DNA PCR Kit. Primer sequences were designed such that they were complementary to opposite strands and opposite end of the DNA of interest. The forward E8 primer corresponded to the HA epitope and the reverse primer to nucleotide 455-432 of the BPV-4 genome. The forward and reverse E7 primers corresponded to nucleotide 642-661 and nucleotide 812-793 of the viral genome respectively. The forward E5 primer corresponded to the HA epitope and the reverse primer to nucleotide 206-184 of the BPV-1 genome. The forward and reverse Zip primers corresponded to nucleotide 503-527 and nucleotide 5477-5458 of the pZipneo MoLV genome. Primer sequences are described in table 2.1.

Table 2.1 Oligonucleotide PCR primers

Forward E8	5' CCA TAC GAT GTT CCA GAT TAC GCT 3'
Reverse E8	5' CCA TCC ATC TAA CCG AGT AAT AGT 3'
Forward E7	5' CCT TCC AGT CTT AAT TGC AG 3'
Reverse E7	5' CAG TTT CAA TCT CCT CTT CA 3'
Forward E5	5' CCA TAC GAT GTT CCA GAT TAC GCT 3'
Reverse E5	5' GCA TTA AAA GGG CAG ACC TGT AC 3'
Forward Zip	5' GCC TCC GTC TGA ATT TTT GCT TTC G 3'
Reverse Zip	5' GGC TGT TAG TAA CTC TTG TC 3'

All reaction mixture comprising of 200μM of each dATP, dGTP, dCTP and dTTP, 3mM $MgCl_2$, 1 × PCR kit buffer (500mM KCl, 100mM Tris-HCl), 0.15μM of each primer, 2.5 units *Tag* polymerase (a thermolabile DNA polymerase from *Thermus aquaticus*) and 1μg of DNA sample (controls included distilled water, pZipneo HAE8, pZipneo E7 plasmids, and 1μg of DNA from parental PalF cells), was aliquoted into 0.5ml GeneAmp PCR reaction microfuge tubes in a final volume of 50μl. The tubes were placed into the PCR machine (Perkin-Elmer Cetus type 9600) and heated to 95°C for 3 minutes to inactive DNase and ensure all DNA duplexes were melted. The DNA was then amplified (using Perkin-Elmer Cetus type 9600 thermocycler) for 28 cycles at 94°C for 30 seconds, 60°C for 30 seconds, to allow the primers to anneal to the template DNA, followed by 72°C for 30 seconds, to allow extension of the amplimer sequences. After completion of the cycles, the reaction was incubated at 72°C for a further 7 minutes to ensure full extension and then cooled to 4°C. 5μl of each sample was

analysed by agarose gel electrophoresis (as described in section 2.2.1.6) to check the correct product was amplified.

2.2.4.3.2 Amplification from RNA reverse transcriptase-PCR (RT-PCR)

RNA was prepared (see section 2.2.4.2) and used as the template for reverse transcription and PCR amplification of cDNA. Firstly cDNA was synthesised from RNA by reverse transcription using the Perkin-Elmer Cetus RNA PCR kit. The reaction was carried out according to the manufacturers instructions, to the following final concentrations: 5mM $MgCl_2$, 1× PCR kit buffer II (500mM KCl, 100mM Tris-HCl), 1mM of each of dATP, dGTP, dTTP, dCTP, 1 unit RNase inhibitor, ≤1μg RNA, 2.5 units MuLV reverse transcriptase, 0.15μM of each reverse primer, E7, E8 and E5, (section 2.2.4.3.1) and DEPC-treated water to a final volume of 20 μl. Controls included 1μg of RNA treated with DNase, 1μg RNA treated with RNase, nucleic acids from normal parental PalF cells, distilled water, pZipneo HAE8, pZipneo HAE5, and pZipneo E7 plasmids.

All samples were placed in the termocycler and further incubated at 22°C for 10 minutes, 42°C for 15 minutes, 99°C for 5 minutes, and soaked at 4°C for 5 minutes. The above reaction volume was increased to 100μl by adding 2mM $MgCl_2$, 1 × kit PCR buffer II, 0.15μM of forward HA1 E8 primer, 0.15μM of forward HA1 E5 primer, 0.15μM of forward E7 primer and 2.5units of *Tag* polymerase. Amplification proceeded for 35 cycles of 94°C for 30 seconds and 60°C for 1 minutes, plus a 1 second extension per cycle followed by final extension at 60°C for 7 minutes using the Perkin-Elmer Cetus 9600 thermocycler.

The samples were then analysed by agarose gel electrophoresis as described in section 2.2.1.6 to ensure correct amplification.

2.2.5 Protein Analysis

2.2.5.1 Protein preparations from Cells

Cells were lysed by aspirating the culture medium off, washing the cell monolayer twice with ice-cold PBS, the PBS was completely removed by aspiration, adding 1.5ml of ice-cold PBS, scraping the cells off the dish and transferred them to a 1.5 ml microcentrifuge tube. Cells were pelleted by spinning at 4°C in a microcentrifuge at 5,000rpm for 5 minutes. the supernatant was removed and 300µl of boiled lysis buffer was added to the pellet. Cells were lysed following a 5 minutes boiling, and then sonicating the resulting cell suspension using an MSE Soniprep 150 sonicator. Cell debris was pelleted at 14000 rpm and the supernatant transferred to a new microcentrifuge tube.

2.2.5.2 Protein assays

The BCA/CuSO4 Protein assay was used to spectrometrically determine the protein concentration of dilute solutions following the manufacturer's instructions. Protein reduce alkaline Cu(II) to Cu(I) in a concentration-dependent manner. Bicinchoninic acid is a highly specific chromogenic reagent for Cu(I), forming a purple complex with an absorbance maximum at 562nm. 10µl of protein solution was placed in separate wells in 96 well plate. 200µl of developing solution (5ml BCA(Biocinchoninic acid) solution, 100µl of 4% (w/v)

CuSO4 (copper II sulphate pentahydrate solution) was added to the protein samples and incubated at 37°C for 30 minutes.

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

2.2.5.3 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein samples were resolved according to the molecular weight using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). 15cm gels were used, containing gels of varying polyacrylamide content, depending on the molecular weight of the proteins being resolved, but typically 15% polyacrylamide resolving gel was used. For a single 15% gel of 15cm the following solutions were prepared:

30% acrylamide	10ml
Water	4.6ml
1.5 M Tris (pH 8.8)	5.0ml
10% SDS	0.2ml
10% ammonium persulphate (freshly prepared)	0.2ml
TEMED	0.008m

This resolving gel was poured between two glass plates (cleaned with 70% ethanol) sealed on three sides with a gasket, then overlaid with isopropanol and

left to polymerise at room temperature. Once the gel was set, the isopropanol was removed using Whatman 3MM filter paper and the stacking gel consisting of 5% polyacrylamide was poured on top of the resolving gel, a comb inserted and left to polymerise for at least 30 minutes before use. The stacking gel was prepared as follows:

30% acrylamide	0.67ml
Water	2.7ml
1.5 M Tris (pH 8.8)	0.5ml
10% SDS	0.04ml
10% ammonium persulphate (freshly prepared)	0.04ml
TEMED	0.004ml

Once the stacking gel had polymerised the whole gel was transferred to an electrophoresis tank. The tank reservoirs were then filled with Tris-glycine electrophoresis buffer. After the removal of the combs, the wells were flushed with electrophoresis buffer to remove any excess stacking buffer.

Prior to loading, equivalent amount of each protein samples (30-50μg) was mixed with an equal volume of 2x SDS gel loading buffer. The prepared protein samples were then loaded into consecutive wells and 5μl Rainbow^{TM} protein molecular weight marker mix (molecular weight range 14.3KD-200KD) added to the first or last well on the gel. The gel was run by electrophoresis at a constant current ~ 35mA/gel for 2-3 hours. Once the dye front was aproximately 5-10cm from the bottom of the gel, it was removed and used for western analysis.

2.2.5.4 Western blotting

Separated protein samples were transferred to a nitro-cellulose membrane by semi-dry blotting. For this purpose the gel was removed from the electrophoresis tank, then excess gel was cut away. The dimensions of the remaining gel was measured and 2 pieces of Whatman 3MM filter paper were cut to an equal size, as was one piece of nitro-cellulose (ECL-hybond). The transfer of the protein from the gel to the nitro-cellulose was performed as follows:

1. Two sheets of Whatman paper were soaked in transfer buffer (122mM Glycine, 25 mM Tris, 20% Methanol) and placed neatly on the centre of the blotting apparatus, avoiding any air bubbles.

2. Onto these was laid the nitro-cellulose membrane, the gel, then a further 6 sheets of Whatman paper, all soaked in transfer buffer.

3. The stack of sheets was rolled with a glass pipette to eliminate any air bubbles

4. 20 volts was applied across the blot for approximately 30 mins, the time taken for the pre-stained marker proteins to be completely transferred

Once the transfer was completed, the membrane was blocked by shaking for a minimum of 2 hours in100 ml of block buffer (5% Marvel (dried milk) in Tween 20 0.1% (v/v) in PBS) at room temperature. The nitro-cellulose filter was washed in wash buffer (PBS, 0.1% (v/v)Tween 20) for 4 x 10 minutes. The filter was then placed in 50ml blocking buffer containing 12CA5 anti HA mouse monoclonal antibody at a 1/1000 dilution and incubated at room temperature for 1 hour with gentle shaking. The primary antibody solution was removed and the filter rinsed in blocking buffer then washed 4 times, each for 10 minutes, in 100ml

volumes of fresh washing buffer. The filter was then incubated in 50 ml blocking buffer containing a 1/5000 dilution of anti-mouse IgG horse-radish peroxidase for 1 hour at room temperature with gentle shaking. The filter was washed 3 x 10 minutes with washing buffer. Excess surface liquid was removed from the filter by briefly blotting with a piece of Whatman 3MM paper. The detection consisted of incubating the filter in an equal volume of an Amersham Enhanced chemilluminescence (ECL) detection reagents I and 2 for 1 minute at room temperature. The excess detection solution was drained off the nitro-cellulose filter and this was then wrapped in Saran wrap and exposed to Fugi-XR film for 30 second or 20 minutes (depending on the strength of the signal).

2.2.5.5 Immunofluorescence

Cells were grown until 80% confluent on single well glass chamber slides. After removal of tissue culture media the cells were washed once with PBS and fixed in ice cold acetone for 30 mins.

The acetone was removed and 1.5% Marvel PBS blocking solution applied to the cells for 10 mins. After removing the blocking solution 50-100μl of primary antibody (12CA5) was applied at the appropriate concentration and left in a moist chamber at room temperature for 1-2 hours.

The cells were then washed 3 times with the blocking solution and incubated with 50-100μl of secondary FITC-conjugated antibody for 1 hour. The cells received a final wash in blocking solution and were then mounted in 50% glycerol in PBS. Cells were analysed under UV illumination using a Leitz vario orthomate microscope (Confocal microscope).

RESULTS

CHAPTERS THREE - SEVEN

CHAPTER THREE

GENERATION OF MUTANTS

3.1 Introduction

The E8 ORF encodes a small (42 residues) polypeptide that bears some resemblance to the E5 oncoprotein of BPV-1: both are localized to cellular membranes and have two distinct domains: a hydrophobic membrane domain (residue 1-30) with the capacity to form an α-helix and a hydrophilic "tail" region (residue 31-42, E8; 31-44, E5) which is not thought to be membrane localised (Jackson et al., 1996). The structural and functional characteristics of E8 as detailed in chapter one, suggest E8 may transform cells in a way analogous to that of BPV-1 E5.

In an attempt to segregate different transforming functions of the E8 protein and therefore to define its functional domains, several mutations have been introduced in the predicted α helical domain, and in the C terminus, and chimeric molecules between BPV-4 E8 and BPV-1 E5 were constructed (Figures 3.1, 3.2; table 3.1).

Previous studies (Goldstein, et al., 1992a, b) have shown that binding of E5 to ductin requires the N-terminal hydrophobic domain (first 32 residue) and that glutamine at position 17, near the middle of this domain, is important in

protein function. Since the transmembrane domain of BPV-1 E5 appears to mediate its binding to cell proteins it has been hypothesized that it might be possible to determine whether the equivalent residue in the E8 polypeptide of BPV-4 has a similar function to that of BPV-1 E5. Thus N (asparagine) at position 17, chemically similar to the glutamine of BPV-1 E5, crucial for cell transformation and for ductin binding, has been mutated to serine (17S), tyrosine (17Y) or alanine (17A).

A mutant form of E8, E8T, comprising only the first 32 amino acid residues was produced by changing S (serine) at residue 33 to a stop codon, to determine if the membrane domain was sufficient to transform cells. A similar mutation has been introduced in BPV-1 E5 by changing W (tryptophan) at residue 32 to amber stop codon, to create a truncated form of BPV-1 E5 (E5T; residues 1-31).

Two chimeric molecules were also formed between BPV-4 E8 and BPV-1 E5, to define functional regions of the protein and whether these domains were functionally interchangeable between two peptides.

One form, E8N-E5C, retains the N-terminal two third of E8 (E8 body, 1-29) but the carboxyl end is replaced by the last third of BPV-1 E5 (E5 tail, 30-44). The reciprocal chimera, E5N-E8C retains the N-terminal two third of BPV-1 E5 (E5 body, 1-31) but the carboxyl end is replaced by the last third of BPV-4 E8 (E8 tail, 32-42).

To determine whether the postulated α-helix domain of BPV-4 E8 could

have an effect on cell transformation, two mutations were introduced in this portion of E8. Alanine residues at positions 15 and 20 were changed to proline, which has the property of forcing a bend in the main chain and of disrupting an α helix. The same two alanine residues were also mutated to the chemically similar amino acid glycine to control for any effect due to changes in residues rather than in structure.

In the C-terminus hydrophilic tail of E8 there is a possible casein kinase II (CKII) phosphorylation sites (TRLD) at positions 36-39. It is particularly interesting to determine whether the putative phosphorylation of this site can affect cell transformation. To investigate this, mutations were introduced to the putative CKII site. The threonine (Thr) was changed to aspartic acid, alanine, or serine. Aspartic acid is negatively charged, mimicking a phosphorylated amino acid. Alanine can not be phosphorylated, but serine, like threonine, can be phosphorylated. These changes allow us to determine whether incorporation of a negative charge, or the presence of phosphate moieties within the CKII site, is critical for the regulation of cell transformation by BPV-4 E8.

3.2 Site-directed mutagenesis of HAE8

The Altered Sites *In vitro* Mutagenesis System (Promega) was used to construct mutant forms of HAE8. The HAE8 ORF was subcloned into pALTER-1 vector, and DNA was denatured as described in Materials and Methods section 2.2.1.2.

An oligonucleotide, complementary to the single-strand template except for the mutated nucleotide of interest, was synthesized and annealed with the single-strand template DNA. The mutant strand was then synthesized by extending the oligonucleotide with DNA polymerase followed by ligation, to create double-strand DNA.

This *in vitro* mutagenesis system was based on the use of a second mutagenic oligonucleotide to confer ampicillin resistance to the mutant DNA strand, during mutagenesis reaction. The mutant forms of HAE8 were constructed using the oligonucleotides designed with the following mismatches (underlined):

Mutant N17S contained the codon AAT changed to A<u>G</u>T

Mutant N17Y contained the codon AAT changed to <u>T</u>AT

Mutant N17A contained the codon AAT changed to <u>GC</u>T

Mutant E8T S33 contained the codon TCT changed to T<u>GA</u>.

Mutant E5T W32 contained the codon TGG changed to T<u>A</u>G

Mutant E8N-E5C, retains the N-terminal two third of E8 (residues 1-29) but the carboxyl end is replaced by the last third of BPV-1 E5 (residue 30-44).

Mutant E5N-E8C, retains the N-terminal two third of BPV-1 E5 (residues 1-31) but the carboxyl end is replaced by the last third of BPV-4 E8 (residue 32-42).

Mutant A15G-A20G contained the codons GCT- GCA changed to GGT

Mutant A15P-A20P contained the codons GCT-GCA changed to CCC

Mutant ARLD T36 contained the codon ACT changed to GCT (Ala)

Mutant DRLD T36 contained the codon ACT changed to GAT (Asp)

Mutant SRLD T36 contained the codon ACT changed to AGT (Ser)

3.3 Synthesis of mutant strand

The oligonucleotide containing the required mutation was firstly phosphorylated by incubating 100pmol oligonucleotide with 1× kinase buffer, 1mM ATP, 5 units T24 Polynucleotide kinase and sterile deionized water to a final volume of 25 μl. The reaction was then incubated at 37°C for 30 minutes. The reaction was stopped by heating to 70°C for 10 minutes. The reaction products were either stored at -20°C or added directly to the anneal reaction.

1.25pmol phosphorylated mutagenic oligonucleotide was annealed to

0.05pmol of alkaline-denatured dsDNA, 0.25pmol phosphorylated ampicillin repair DNA, 1 × annealing buffer, and sterile deionized water to final volume of 20μl. The reaction was heated at 75°C for 5 minutes to stop it, cooled slowly to 45°C, then placed on ice.

The complementary DNA strand was synthesised by adding 5 units of T4 DNA Polymerase, 3 units of T4 DNA Ligase, 1 × Synthesis buffer and sterile deionized water to final volume of 30μl. The reaction was incubated at 37°C for 90 minutes to perform mutant strand synthesis and ligation. The mutant DNA was then transformed into JM109 as described in section 2.2.1.9., and sequenced as described below to identify plasmid carrying the required mutation.

3.4 DNA Sequencing

To confirm the presence of mutations, sequencing of all new plasmids was carried out using Taq terminator sequencing on an Applied Biosystems 373A automated sequencer which was performed by Beatson Institute technical service staff.

The region to be sequenced was amplified by a 20μl PCR reaction. 0.5μg of template DNA was mixed with 3.2 pmols of the appropriate primer in a total volume of 12μl with RQ grade water and 8 μl of dye terminator cycle sequencing ready reaction premix added to each reaction volume contained in 250μl thin walled PCR tube.

The samples were placed in a PTC-100 programmable thermal controller

(MJ Research) and exposed to 25 cycles of 96°C for 10 seconds, 50°C for 5 second, 60°C for 4 minutes and were then cooled to 15°C. The PCR products were ethanol precipitated as described in materials and methods section 2.2.1.3., washed with 70% ethanol. The pellet was dried in a speedivac for 5 minutes before being given to a member of technical services for loading onto the sequencing gel in 3-4μl of 95% Formamide and Blue Dextran.

3.4.1 Computer analysis of DNA sequence data

Sequence data were aligned and compared to other sequences using the Wisconsin package version 9.1, Genetic Computer Group (GCG), Madison, Wisconsin, U.S.A.

Table 3.1 E8 mutants, E8/E5 chimeras, and E5T

Mutants	Description
N17S	asparagine at 17 mutated to serine
N17Y	asparagine at 17 mutated to tyrosine
N17A	asparagine at 17 mutated to alanine
E8T	serine at 33 mutated to stop codon
E8N-E5C	E8 Body (aa 1-29) - E5 Tail (aa 30-44)
E5N-E8C	E5 Body (aa 1-31) - E8 Tail (aa 32-44)
E5T	tryptophan at 32 mutated to stop codon
A15G-A20G	alanine at 15 and 20 changed to glycine
A15P-A20P	alanine at 15 and 20 changed to proline
ARLD	threonine at 36 changed to alanine
DRLD	threonine at 36 changed to aspartic acid
SRLD	threonine at 36 changed to serine

N17S, N17A, N17Y, and E8T mutants have been made by Dr R. Anderson a previous worker in our laboratory.

E8N-E5C, E5N-E8C, and E5T mutants have been made by Mrs J. Grindlay a present worker in this laboratory. All the other mutants were made by myself.

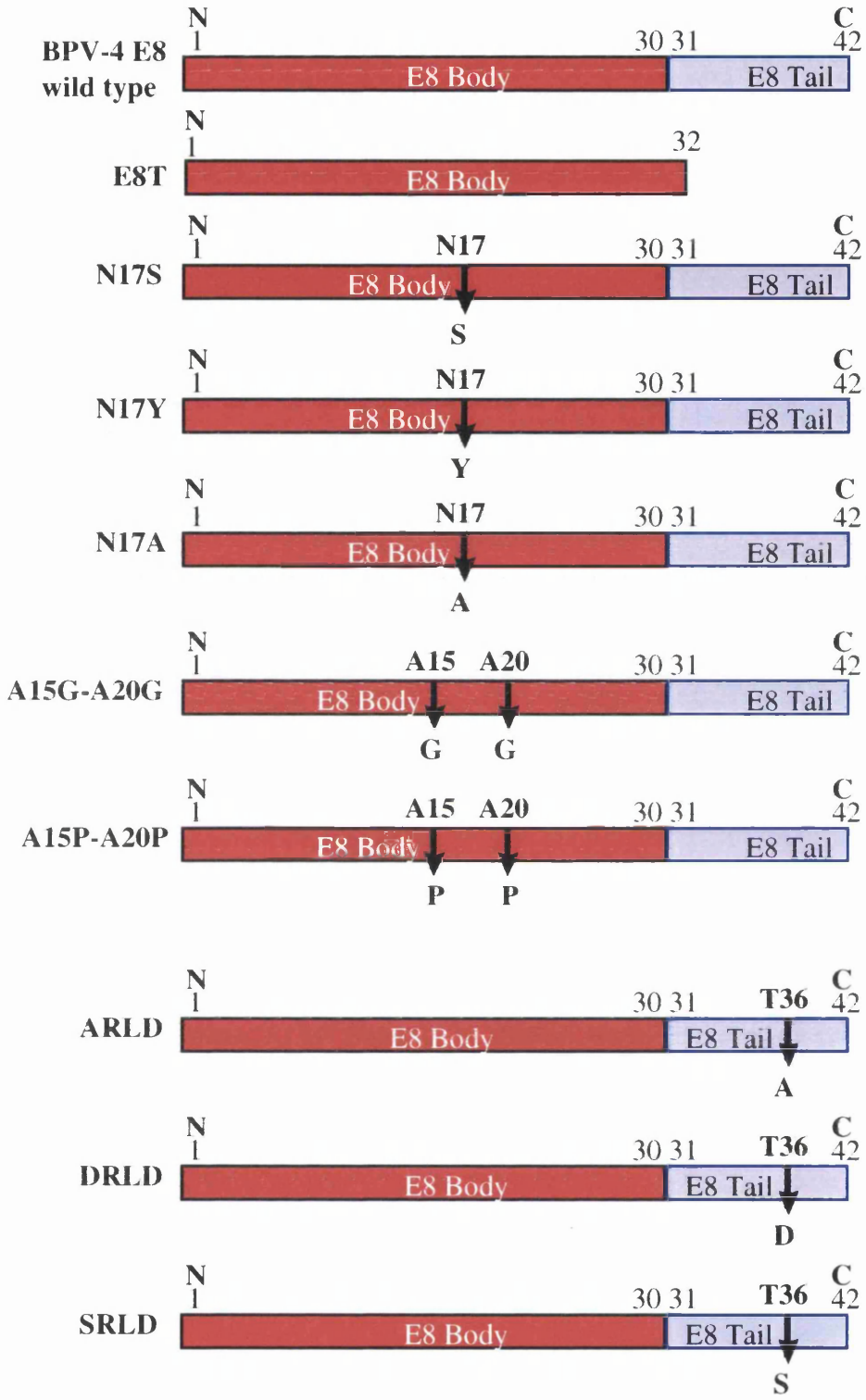


Figure 3.1 Structure of full length, truncated, and point mutations of the BPV-4 E8 protein.

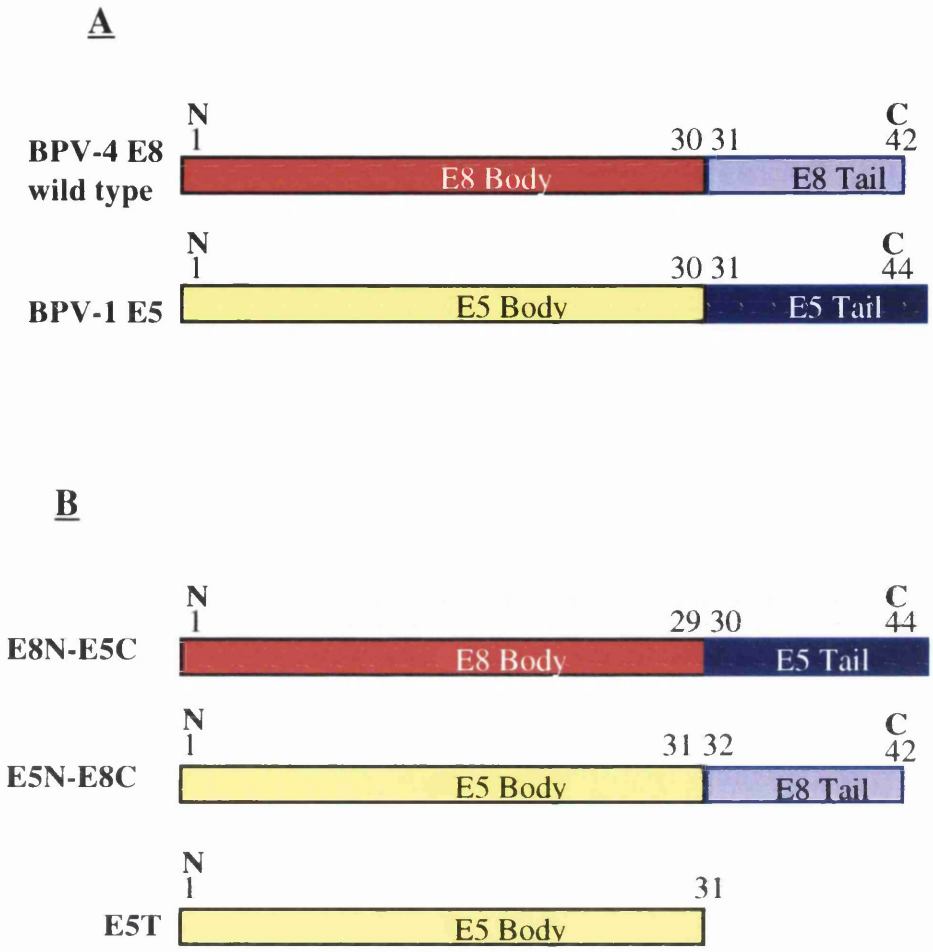


Figure 3.2
A. Structure of full lenght E5 and E8 proteins
B. Structure of truncated E5 and chimeric E5 / E8 proteins

CHAPTER FOUR

CHARACTERISATION OF CELL CLONES

4.1 Transfection classes

PalF cells were isolated and grown in culture medium (DMEM containing 10% serum). Cells were sub-cultured at appropriate intervals to maintain sub-confluent monolayers. Detachment and dispersion of cells was achieved by a brief exposure to a solution of 0.025% trypsin, 0.02% EDTA in PBS. The general tissue culture condition used for PalF cells are described in chapter 2.2.3.2.

For stable transfection, cells were transfected by using a lipofection transfection technique (DOTAP) as detailed in chapter 2.2.3.7. Fourteen transfection classes were performed as in table 4.1. The following plasmids were used, pZipneo-HAE8 wild type and mutants, pZipneo BPV-1 HAE5, pZipneo BPV-4 E7, pT24 (activated *ras*) and pJ4Ω16-E6 (HPV-16 E6).

As described in detail earlier (1.2.3.2), PalF cells can be transformed by BPV-4, or by the E7 and E8 ORFs, only in the precence of an activated *ras* gene (Jaggar et al., 1990; Pennie et al., 1993). Cell immortalization is however achieved only with the addition of the E6 ORF of HPV-16 (Pennie et al., 1993; Scobie et al., 1997). Consequently, the transfection classes contained pZipneo BPV-4 E7, activated *ras* and HPV-16 E6, to generate stable transformants.

Comparison of cells transfected with BPV-1 HAE5 is very useful for the analysis of the functions of chimeric mutants. To investigate the competence of BPV-1 HAE5 to generate immortal transformed PalFs, cells were also transfected with BPV-1 HAE5 + HPV-16 E6 + T24*ras*, BPV-1 HAE5 + T24*ras*, and BPV-1 HAE5 on its own as shown in table 4.1.

Transformation was assessed by using a neomycin resistance assay. Where G418 resistance was not conferred by the plasmid being used, the pZipneo plasmid was co-transfected at a ratio of one to ten of the other constructs.

As detailed in Materials and Methods section 2.2.3.7, each transfection class was performed twice in duplicate; i.e. for each transfection class, two DOTAP/DNA mixes were made, each mix being split 50:50 between two flasks of cells - giving four transfected flasks per class.

Following transfection, cells were selected in medium containing 500 μg/ml G418 for 21-28 days. After this time, G418-resistant colonies were marked. Where appropriate, several colonies were picked from each transfection class (taking representative colonies from all four replicate flasks) and expanded into cell lines for analysis.

Some mutants including A15G-A20G, A15P-A20P, ARLD, DRLD, and SRLD were only used in short term transfection experiments to test their ability to trans-activate the cyclin A promoter but were not used for the generation of stable transfectants because of time limitations.

Table 4.1 Transfection classes

pZipneo HAE8 (Wild type) + pZipneo E7 + T24 *ras* + pJ4Ω16-E6
pZipneo HAE8 Mutant N17S + pZipneo E7 + T24 *ras* + pJ4Ω16-E6
pZipneo HAE8 Mutant N17Y + pZipneo E7 + T24 *ras* + pJ4Ω16-E6
pZipneo HAE8 Mutant N17A + pZipneo E7 + T24 *ras* + pJ4Ω16-E6
pZipneo HAE8T + pZipneo E7 + T24 *ras* + pJ4Ω16-E6
pZipneo HAE8N-E5C + pZipneo E7 + T24 *ras* + pJ4Ω16-E
pZipneo BPV-1 HAE5N-E8C + pZipneo E7 + T24 *ras* + pJ4Ω16-E6
pZipneo BPV-1 HAE5T(E5T) + pZipneo E7 + T24 *ras* + pJ4Ω16-E6
pZipneo BPV-1 HAE5 + pZipneo E7 + T24 *ras* + pJ4Ω16-E6
pZipneo BPV-1 HAE5 + ---------- + T24 *ras* + pJ4Ω16-E6
pZipneo BPV-1 HAE5 + T24 *ras*
---------- pZipneo E7 + T24 *ras* + pJ4Ω16-E6
pZipneo BPV-1 HAE5
pZipneo

4.1.1 Morphological transformation

To determine if expression of BPV-4 E8 wild type and mutant forms of E8 affect PalF cells morphology, cells were fixed in methanol and stained with 10% Giemsa and photographed using PanF 50 film.

4.4.1.1 Results

Non-transfected cells died after about two weeks of selection. Control cells transfected with only empty vector (pZipneo) were not transformed. As showed in table 4.3., a few colonies (6.01%) were observed, but these were not expandable.

Cells expressing BPV-4 E8 and mutants were morphologically transformed (Table 4.2 & 4.3) displaying an irregular morphology than parental PalF cells (Figure 4.1a, 4.1b, & 4.1c).

PalF transfectants (BPV-4 E8, its mutants, and BPV-1 E5) and E7 transfection classes, with no E8, were equivalent with regard to transformation efficiency scores (Table 4.2 & 4.3). This finding agrees with previous studies (Jaggar et al., 1990), that the presence of BPV-4 E7 gene alone (+ neo + ras) is sufficient to morphologically transform PalF cells.

There were a few resistant colonies with BPV-1 E5 + HPV-16 E6 + T24 *ras*, and E5 + *ras*, and E5 on its own (Table 4.2), but none of them was expandable. Therefore, BPV-1 E5 like BPV-4 E8 needs E7 to allow expansion.

Table 4.2 Transformation efficiency of each transfection class

A

Transfected Plasmid	Exp1 F1	Exp1 F2	Exp2 F1	Exp2 F2	Exp3 F1	Exp3 F2	Exp4 F1	Exp4 F2	Exp5 F1	Exp5 F2	Exp6 F1	Exp6 F2	Exp7 F1	Exp7 F2	Average
PalF cells	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
pZipneo	nd	nd	nd	nd	0	2	1	0	3	1	nd	nd	nd	nd	1.2
E7 (no E8)	18	12	27	24	13	18	19	23	19	20	nd	nd	18	16	18.9
E8 wild type	11	24	16	14	nd	nd	nd	nd	23	20	25	20	22	19	19.4
N17S	19	10	nd	nd	nd	nd	nd	nd	nd	nd	17	21	18	24	18.2
N17Y	18	9	nd	nd	nd	nd	nd	nd	nd	nd	19	15	23	19	17.2
N17A	15	13	nd	nd	nd	nd	nd	nd	nd	nd	24	21	17	18	18
E8 T	12	15	nd	nd	nd	nd	nd	nd	nd	nd	21	18	19	17	17
E8N-E5C	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	19	18	19	17	18.2
E5N-E8C	nd	nd	nd	nd	nd	nd	19	15	18	21	22	13	20	19	18.4
BPV-1 E5	nd	nd	nd	nd	nd	nd	13	18	nd	nd	20	24	nd	nd	18.7
E5T	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	17	21	19	16	18.2

All transfectant cell lines (except pZipneo) contain BPV-4 E7, activated *ras* and HPV 16-E6.

nd = not done

Exp1 = experiment 1, **Exp2** = experiment 2 etc.

F1 = flask 1, **F2** = flask 2

Column marked **average** is the numerical average of experiments 1-7.

B

Transfected plasmid	Exp1 F1	Exp1 F2	Exp2 F1	Exp2 F2	Average
BPV-1 E5 [a]	13	18	20	24	18.7
E5 + E6 + ras	3	12	10	8	8.2
E5 + ras	4	6	11	13	8.5
E5 alone	1	1	2	3	1.7

[a] Transfectant cell lines contain BPV-4 E7, activated *ras* and HPV 16-E6.

Table 4.3 Standardized average transformation efficiency of each cell type

A

Cell type	T. E. (%) ± SD
PalF cells	0
pZipneo	6.01 ± 1.16
E7 (no E8)	97.50 ± 4.29
E8 wild type	100 ± 4.52
N17S	93.64 ± 4.70
N17Y	88.48 ± 4.75
N17A	92.78 ± 4
E8 T	87.62 ± 3.16
E8N-E5C	94.07 ± 0.95
E5N-E8C	94.71 ± 3.02
BPV-1 E5	96.64 ± 4.57
E5T	94.07 ± 2.21

All transfectant cell lines (except pZipneo) contain BPV-4 E7, activated *ras* and HPV 16-E6. The above data were obtained by standardizing the average colony numbers (Table 4.1) to the number of colonies obtained with BPV-4 E8 + BPV-4 E7 + T24 *ras* + HPV-16 E6, taken as 100%. T. E. = Transformation efficiency SD = Standard deviation

B

Cell type	T. E. (%) ± SD
PalF cells	0
BPV-1 E5[a]	100 ± 4.57
BPV-1 E5 + 16E6 + T24*ras*	44 ± 3.86
BPV-1 E5 + T24*ras*	45.3 ± 4.20
BPV-1 E5 alone	9.33 ± 0.95

[a] Transfectant cell lines contain BPV-4 E7, activated *ras* and HPV 16-E6.

Numbers were standarized to the number of colonies obtained with BPV-1 E5 + BPV-4 E7 + T24 *ras* + HPV-16 E6 taken as 100%.

Figure 4.1 Morphology of parental PalF cells and examples of morphological transformation of transfected PalF cells

E7 (no E8) cells were transfected with pZipneoE7, pT24 *ras*, and pJ4Ω16-E6, while other cells were transfected additionally with an E8 wild type, mutant forms of E8 or BPV-1 E5 as noted in each panel (Final magnification; X 40).

Figure 4.1a

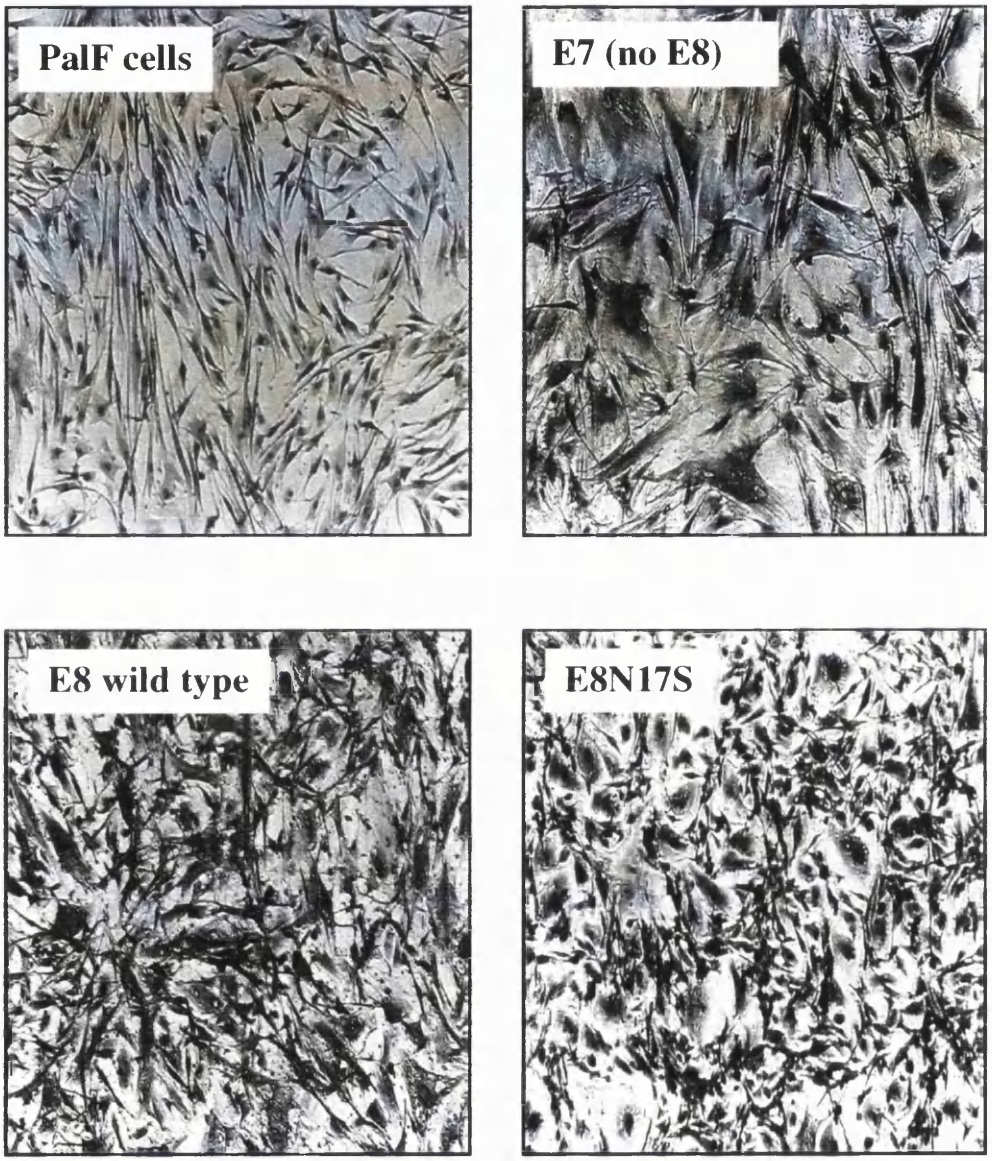


Figure 4.1b

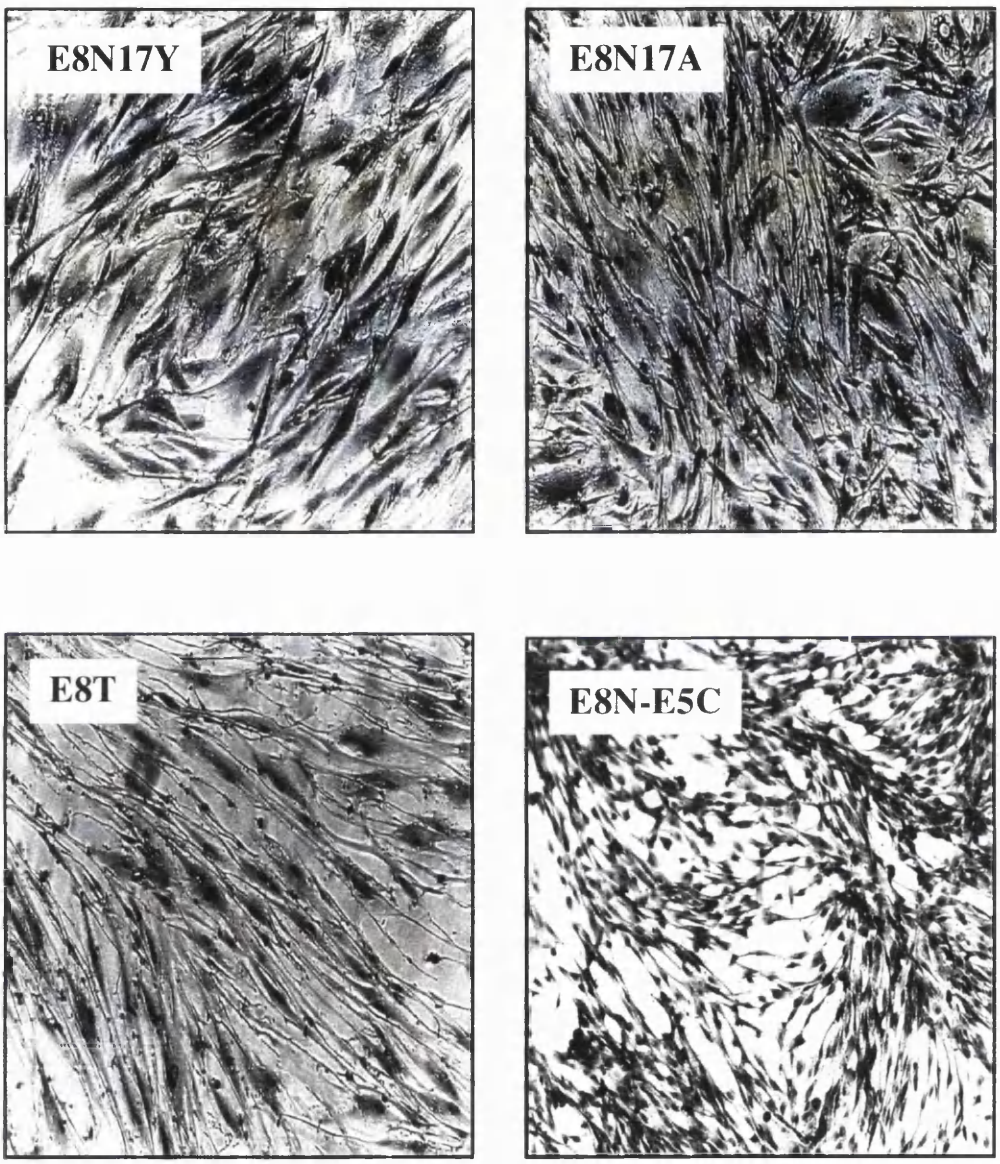
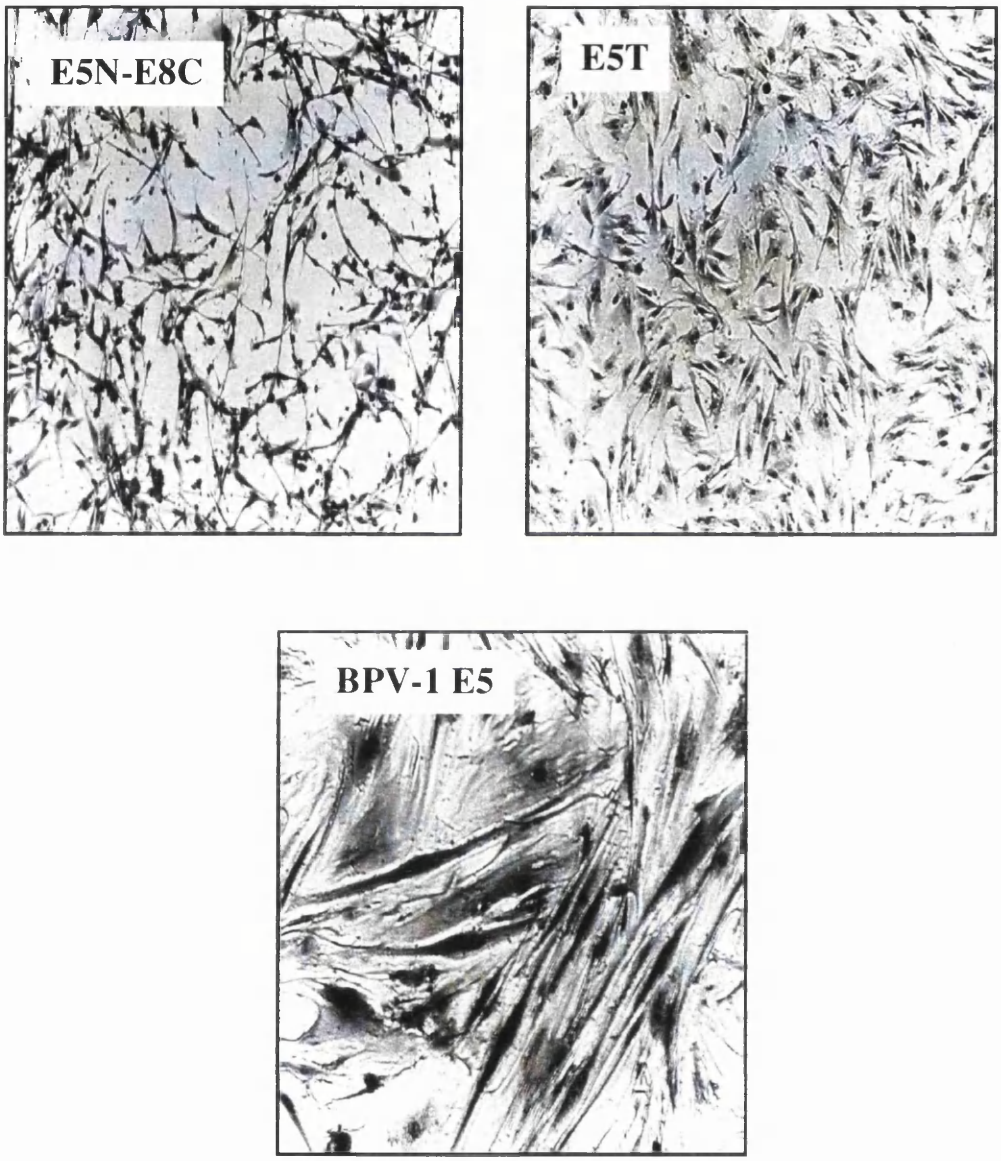


Figure 4.1c



4.2 Detection of viral genes and transcripts in PalF cells

In vitro BPV-4 is not a particularly powerful transforming virus and this in combination with the difficulties of working with primary cells limited the number of clones available for final analysis. Due to the large number of different transfection classes being investigated it was decided to characterise 4-5 clonal lines from each transfection class.

In the event, generally between 2-4 clones were expanded from each class to the stage where they could be fully assayed. By using between 2-4 separate clones, we would expect to find that the results from one clone generally confirmed or supported the results from other clones, thus reinforcing that any effect of BPV-4 E8 wild type or mutants on PalF cells were genuine. In each cases clones from both transfection mixes were represented. Due to cell contamination there were a few instances among the control-treated transfection classes where this target was not met.

The detection and expression of BPV-4 HAE8 wild type, mutants, BPV-4 E7 and BPV-1 HAE5 genes were confirmed by DNA PCR and RT-PCR as described below.

4.2.1 DNA PCR

For the detection of viral DNA, genomic DNA was extracted (chapter 2.2.4.1) from each G418 resistant clone. 1μg of DNA from each clone was used for PCR, applying a Perkin Elmer Kit, together with 0.15μM of Zip primer, described in materials and methods chapter 2.2.4.3.1.

Reactions with control samples which included distilled water, pZipneo HAE8, pZipneo E7 plasmids, pZipneo HAE5 plasmid, and 1μg of DNA from parental PalF cells, were performed at the same time to ensure that the experimental conditions were correct.

To check the correct product was amplified, 5μl of each sample (PCR product) was mixed with DNA loading buffer, and then loaded in each well of a 1.5% (w/v) agarose gel prepared in 0.5% TBE buffer, along with an appropriate size marker (100b DNA ladder) into the first and/or last well in the gel and run at 70-100 constant voltage (using horizontal gel cast apparatus) usually until the samples' blue dye front was 1-3 cm from the end of the gel. Once run, the DNA fragments were visualised by staining the gel in running buffer containing 0.5μg/ml ethidium bromide and then photographing the gel under UV transllumination, as detailed in Chapter 2.2.1.6.

The analysis of DNA PCR showed that there were detectable products which confirmed the presence of transfected viral DNA of interest in the cell lines which co-migrated with the positive control, but, as expected, in the negative controls, there were no detectable bands (Figure 4.2). These results indicated the presence of E8 and E5 genes. The experiment was performed for at least three independent clones for each cell type and a representative result is presented in transfected cell lines with wild type E8, E7 (no E8), and BPV-1 E5 (Figure 4.2).

In transfected cell lines with E7 (no E8) four clones were analysed to confirm the presence of E7, 3/4 showed the presence of E7 DNA. Wild type E8

DNA was found in 3/3 clones; N17S DNA in 3/5; N17Y DNA in 2/4; N17A DNA in 3/5, and E8T DNA 4/5.

E8N-E5C DNA was detected in 3/5 clones and E5N-E8C DNA in 2/3. BPV-1 E5 DNA was found in 2/4 clones and E5T DNA in 2/6. The transfected cell lines in which the presence of viral DNA was confirmed were tested for the expression of viral RNA by RT-PCR.

Figure 4.2 A representative DNA PCR analysis of with wild type E8, E7 (no E8), and BPV-1 E5

The lane marked M is the DNA size marker

pZipneoE8, pZipneoE7, pZipneoE5 = Positive control

H2O/PalFs = Negative control

A = HAE8 wild type transfected sample

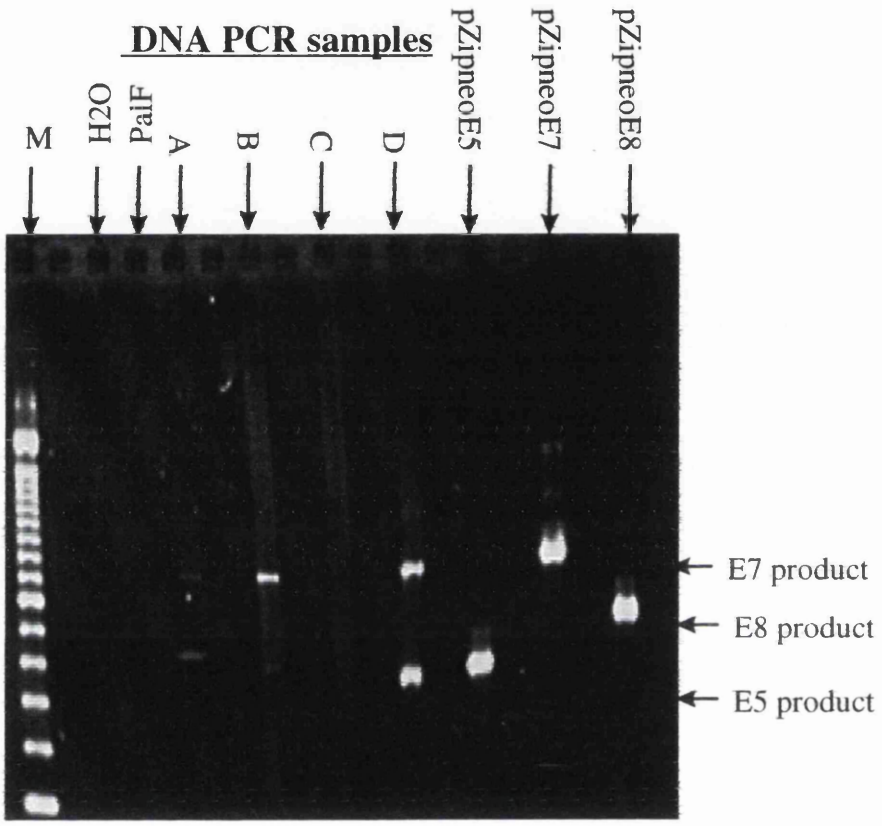
B = E7 (no E8) transfected sample

C = A E5N-E8C transfected sample in which the transfected genes were not detected

D = BPV-1 E5 transfected sample

DNA PCR products were detected and these co-migrate with each specific positive control

Figure 4.2


DNA PCR samples
M
H2O
PalF
A
B
C
D
pZipneoE5
pZipneoE7
pZipneoE8
E7 product
E8 product
E5 product

4.2.2 RT-PCR

To confirm that the plasmid of interest was transcribed in the PalF transfectants, the transfected cells were analysed for the presence of mRNA from each clone by RT-PCR (chapter 2.2.4.3.2). mRNA was selected for during the reverse transcriptase step by using the specific primers (Table 2.1; chapter 2.2.4.3).

Total cell RNA was isolated using RNAzol B method of extraction as detailed in chapter 2.2.4.2. 1μg of RNA was used for RT-PCR using a Perkin Elmer Kit and 0.15μM of the appropriate primers as above.

Controls were carried out using 1μg of RNA treated with DNase to remove DNA, nucleic acids from normal parental PalF cells, DEPC-treated water, pZipneo HAE8, pZipneo HAE5, and pZipneo E7 plasmids, to make sure that the experimental conditions were correct. The samples were then analysed by agarose gel electrophoresis as described above and in section 2.2.1.6. to ensure correct amplification.

The analysis of RT-PCR showed that in the samples which were not treated with DNase, both using MULV reverse transcriptase and with no MULV reverse transcriptase, there were detectable products which confirmed the presence of transfected viral DNA. In the samples treated with DNase using MULV reverse transcriptase a band was observed which co-migrated with the positive control, but, as expected, in the DNase treated samples with no MULV reverse transcriptase, and in the negative control there were no detectable bands (Figure 4.3). These results indicated that the E8 and E5 genes were transcribed. No

differences in the extent of transcription were observed for the different constructs. The experiment was performed for at least three independent clones for each cell type and a representative result is presented in each case (Figure 4.3).

In cell lines transfected with E7 (no E8) three clones were analysed to confirm the expression of E7, all were positive. Wild type E8 RNA was found in 3/3; N17S RNA in 3/3; N17Y RNA 2/2; N17A RNA 3/3, and E8T RNA was found in 3/4 clones, of particular note is the finding that in one cell line although E8T DNA was detected the gene was not expressed (Figure 4.3c, bottom panel).

E8N-E5C RNA was detected in 2/3 clones, again in one clone the E8N-E5C DNA was detected but we could not confirm the presence of its RNA. E5N-E8C RNA was found in 2/2 clones; BPV-1 E5 RNA in 2/2, and E5T RNA in 2/2.

Only the transfected cell lines which transcribed the plasmid of interest were then expanded for further analysis which will be described in following chapters.

Figure 4.3 RT-PCR analysis of transfectant PalF cells

The lane marked M is the DNA size marker

A = not treated with DNase

B = treated with DNase

+ = with MULV reverse transcriptase

- = no MULV reverse transcriptase

HAE8/HAE5 = Positive control

H2O = Negative control

RT-PCR products of 185bp were detected and these co-migrate with the positive control

Figure 4.3a

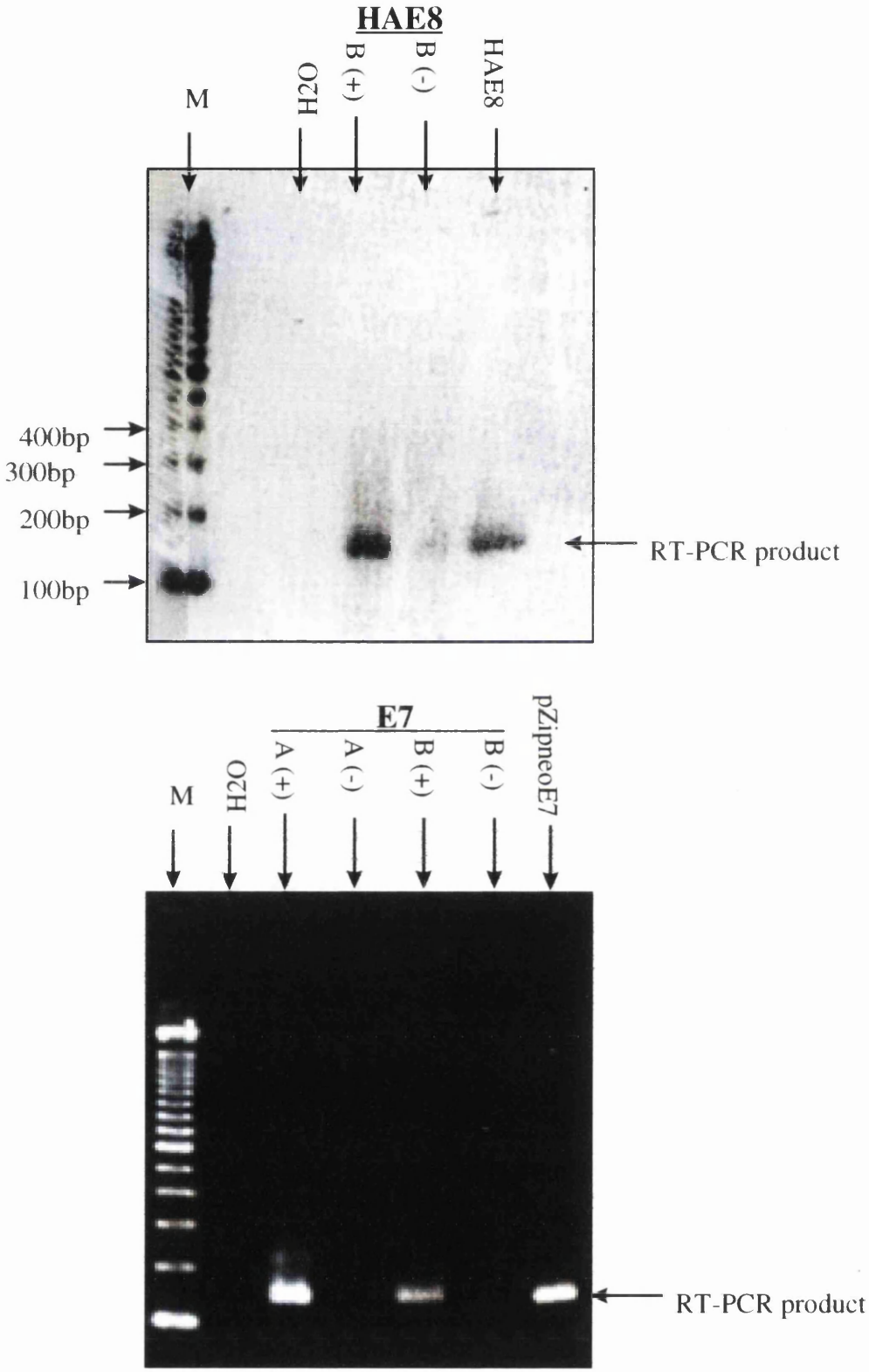

HAE8
M
H2O
B (+)
B (-)
HAE8
400bp
300bp
200bp
100bp
RT-PCR product
E7
M
H2O
A (+)
A (-)
B (+)
B (-)
pZipneoE7
RT-PCR product

Figure 4.3b

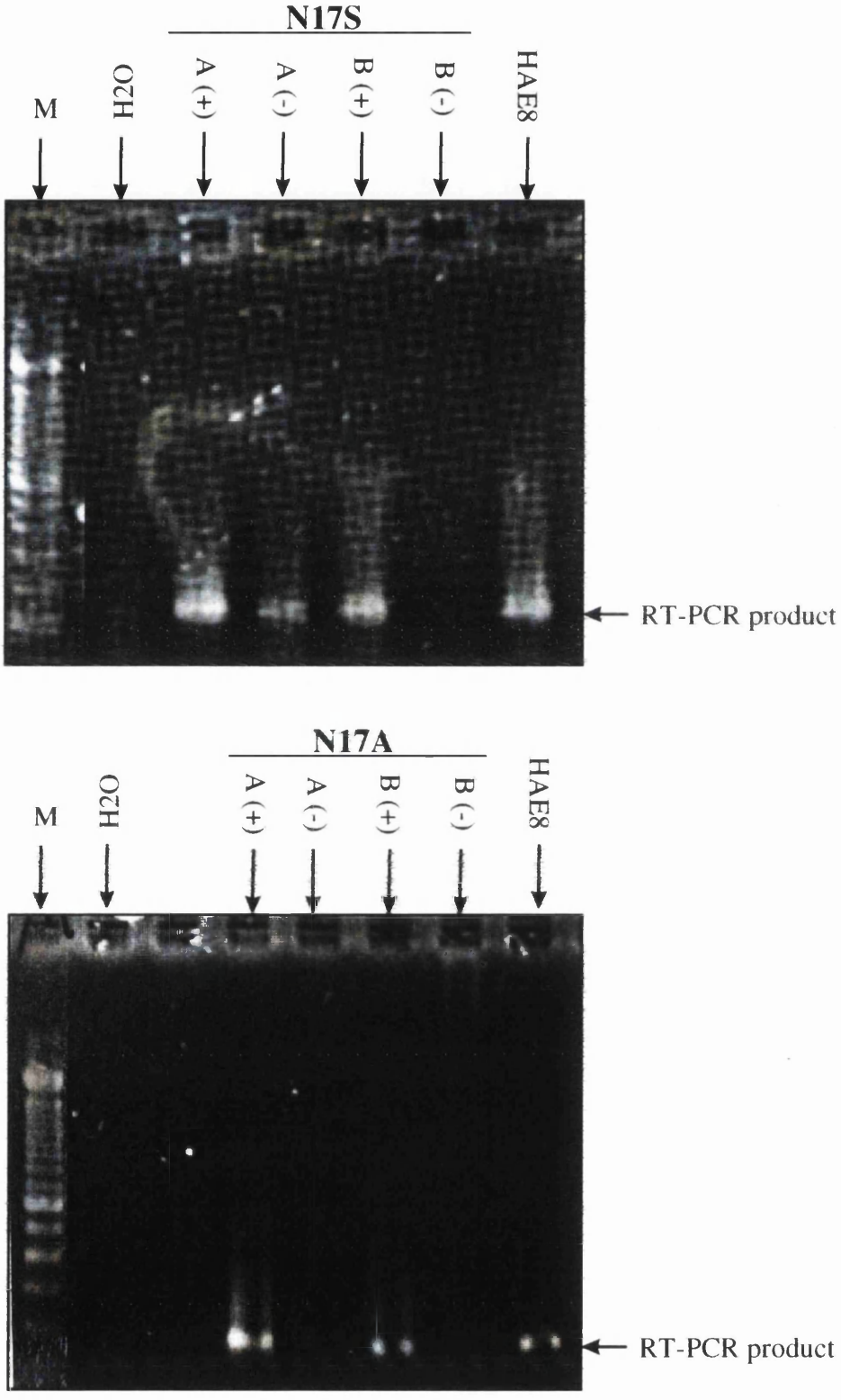

N17S
M
H2O
A (+)
A (-)
B (+)
B (-)
HAE8
RT-PCR product
N17A
M
H2O
A (+)
A (-)
B (+)
B (-)
HAE8
RT-PCR product

Figure 4.3c

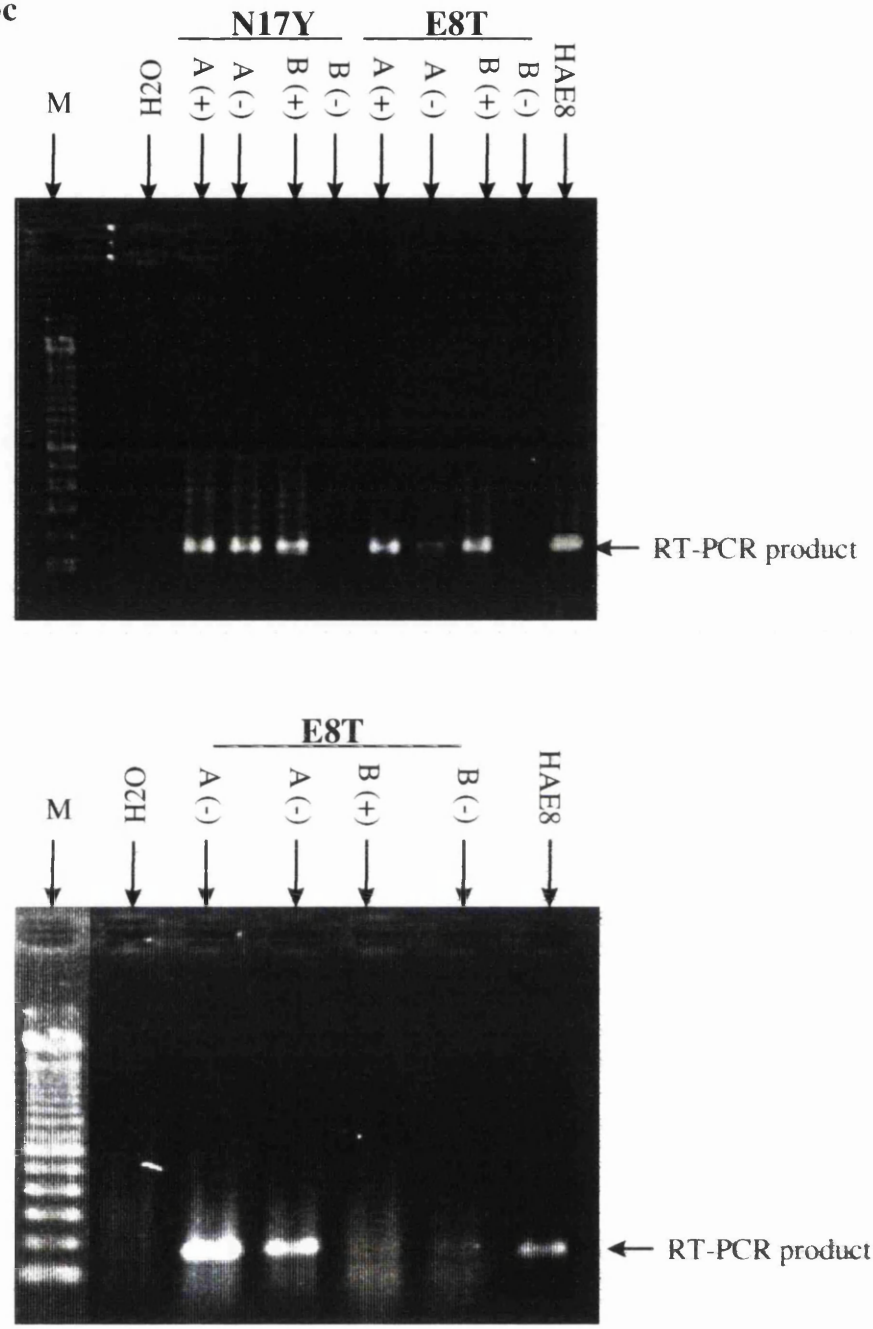

N17Y
E8T
M
H2O
A (+)
A (-)
B (+)
B (-)
A (+)
A (-)
B (+)
B (-)
HAE8
RT-PCR product
E8T
M
H2O
A (-)
A (-)
B (+)
B (-)
HAE8
RT-PCR product

Figure 4.3d

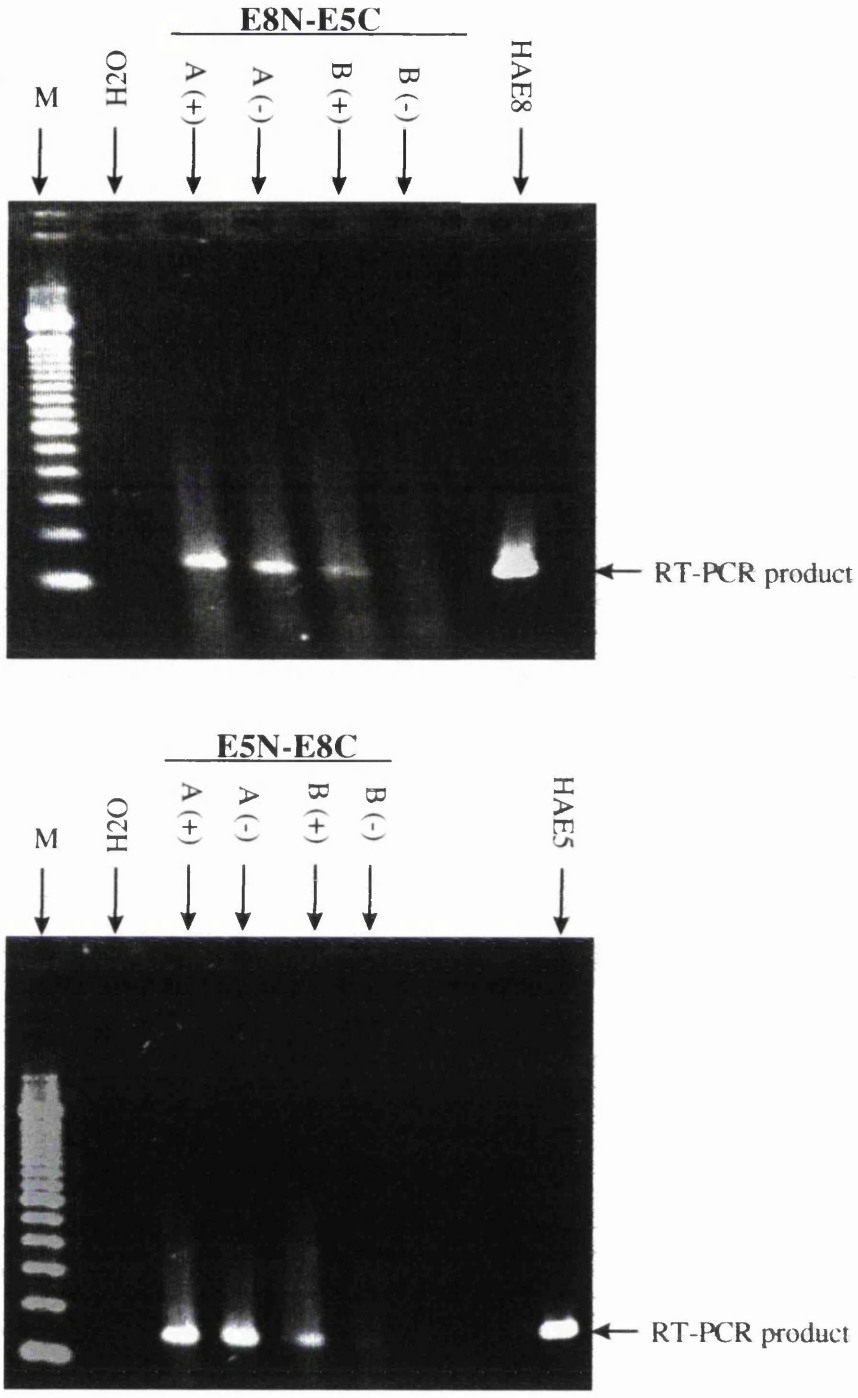
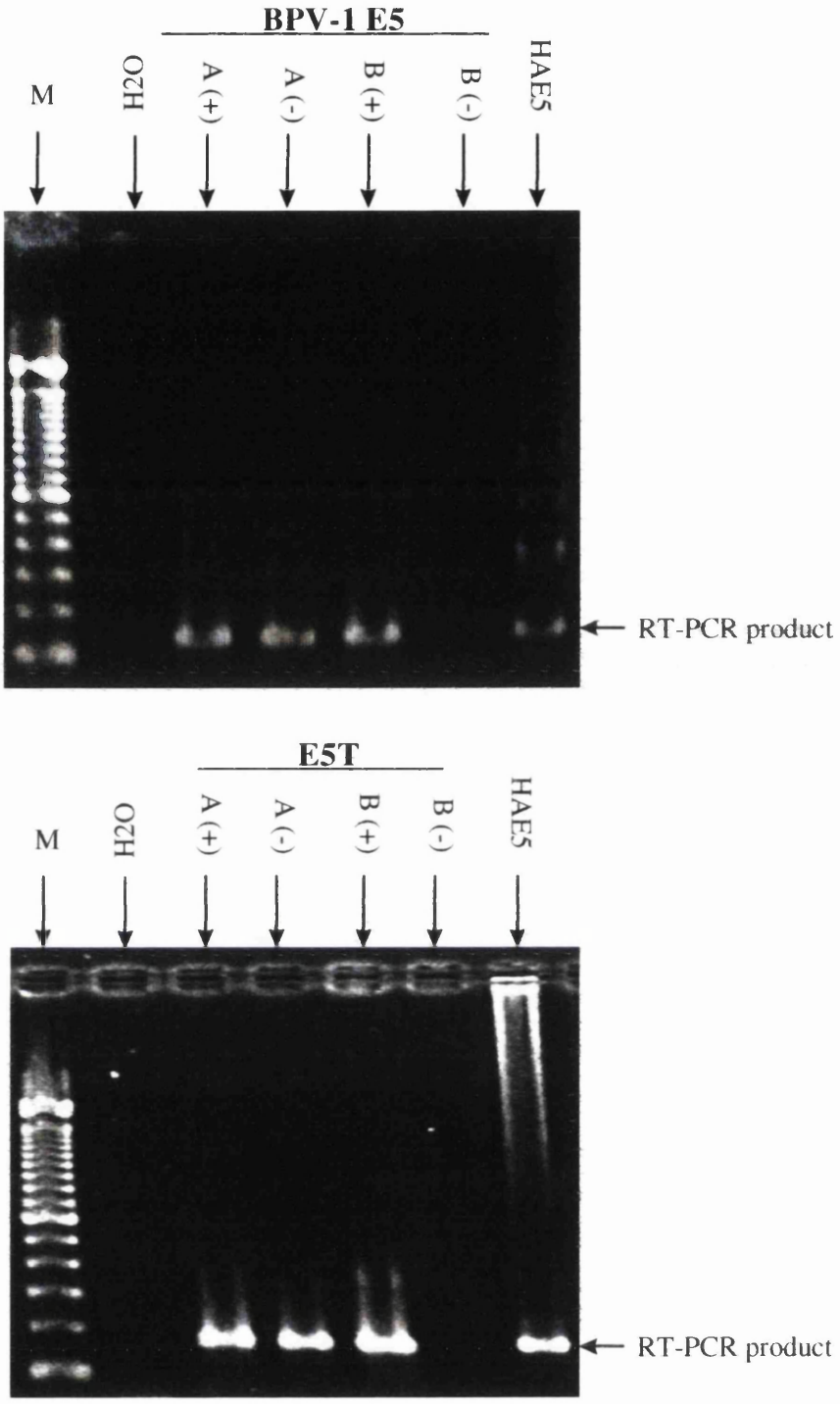

E8N-E5C
M
H2O
A (+)
A (-)
B (+)
B (-)
HAE8
RT-PCR product
E5N-E8C
M
H2O
A (+)
A (-)
B (+)
B (-)
HAE5
RT-PCR product

Figure 4.3e


BPV-1 E5
M
H2O
A (+)
A (-)
B (+)
B (-)
HAE5
RT-PCR product
E5T
M
H2O
A (+)
A (-)
B (+)
B (-)
HAE5
RT-PCR product

4.3 Protein detection and localisation

In order to provide an initial indication of possible interaction between E8 wild type and mutant forms of E8 proteins and cellular structures, we planned to investigate the localisation of HAE8 wild type and mutants proteins by Immunofluorescence and relative expression of wild type and mutants by western blotting, using mouse monoclonal antibody 12CA5 raised against the HA peptide from the influenza virus haemagglutinin tagged at the N-terminus of E8 forms.

Experimental conditions are detailed in chapter 2.2.5.4 and 2.2.5.5. For detection of protein from each transfectant clone total cellular protein was extracted from all cell classes according to the method detailed in chapter 2.2.5.1. Equal amounts of protein (20-30μg) were loaded into separate wells in a 15% SDS-PAGE along with standard protein markers (Rainbow markers) and *in vitro* translated HPV-16 HAE5 protein (a gift from A. Ashby) as a control, separated by electrophoresis and transferred onto nitrocellulose membrane (ECL Hybond) (chapter 2.2.5.3). Membranes were probed using the primary and secondary antibody as described in section 2..2.5.4. Antibody detection was performed using enhanced chemiluminescence (ECL).

A band was observed for the control *in vitro* translated HAE5 (not shown), but we could not detected any protein from PalF transfectants.

For localisation of HAE8 wild type and mutants proteins we used Immunofluorescence technique as detailed in chapter 2.2.5.5. Controls were performed using tubulin antibody (a gift from D. Owens) at the same time to

ensure that the experimental conditions were correct. Tubulin was visualized (not shown) but we could not detect any E8 protein.

Our failure to detect E8 protein may be due to the extreme hydrophobicity of this protein, or low levels of E8 expression.

CHAPTER FIVE

ANALYSIS OF THE EFFECT OF BPV-4 E8 AND MUTANTS ON BEHAVIOUR OF PALF CELLS

5.1 Transformation Assays

The ability to propagate animal cells in culture has allowed the development of *in vitro* assays for the carcinogenic activity of chemicals and viruses. Transformation of cultured cells clearly provides a simpler and more quantitative assay for the biological activity of chemical and viral carcinogens than induction of tumors in experimental animals.

The extent of transformation, in this series experiments, was measured as four separate parameters: anchorage independent growth, focus formation, cell population growth in low serum (0.5% FCS) and high serum (10% FCS), and tumorigenicity in nude mice.

5.1.1 Anchorage independent growth

One property associated with transformation of fibroblasts is the loss of requirement for attachment to the substratum (anchorage independence). The ability of a cell line to form colonies in semi-solid media is taken as a phenotypic measure of its degree of transformation. The extent of transformation of E8 lines was assayed by plating cells in Methocel-based medium.

Efficiency of methocel colony formation was determined by adding 10^5 transfectant cells to 15 ml of 1% methocel including 30% Foetal Calf Serum (FCS) as detailed in Materials and Methods section 2.2.3.10.3. This mix was plated in bacterial petri dishes, in duplicate, and maintained at 37°C for 12 days before scoring.

Each plate was scored for colonies by counting three areas (~ $9mm^2$ per area) from each plate. Averaging the colony numbers of all three areas divided by 9 indicates colony numbers per mm^2, then multiplying by 100 gives colonies per cm^2. To give an estimate of total numbers of colonies, the colonies number in each cm^2 were multiplied by the area of dish ($64cm^2$). The number of colonies was expressed as a percentage of the total number of cells seeded into each plate (10^5) on day 0.

5.1.1.1 Results

Among PalF transfectants only cells expressing BPV-4 E8 wild type, E8N17A, E8N17S, E5N-E8C and BPV-1 E5 showed appreciable anchorage-independent growth, with cells containing E8N17A displaying the highest efficiency of colony formation (Figure 5.1; table 5.1a & 5.1b). The colony formation efficiency of E8N17A is even higher in agar where it reached 25% (not shown). E8N17Y, E8T, E8N-E5C, and E5T were not capable of anchorage independent growth

Thus a single amino acid change at residue 17, from N to Y is capable of abolishing this transforming function of E8, while another from N to A, results in a molecule with a greater ability of inducing anchorage independence than even wild type E8. These results concur with those obtained by other workers for E5 and points to the importance of the residue at position 17 (Goldestein et al., 1992b; Sparkowski et al., 1994; Sparkowski et al., 1996).

Neither E8T or E5T are capable of conferring anchorage independent growth, nor is the E8N-E5C chimera. On the contrary E5N-E8C is equally capable of inducing this transformed phenotype as wild type E8 or wild type E5. The results obtained with E5T concur with those obtained by other workers (Green and Loewenstein, 1987) and point to the importance of an intact hydrophilic tail for the biological activity of both E5 and E8 proteins. The results also show that, while the C-terminal tail of E8 can substitute for the tail of E5 in conferring anchorage independent growth, the reciprocal combination abolishes this ability, and therefore the individual C-terminal domains are not functionally interchangeable.

Together these data suggest that the nature of residue 17 and the hydrophilic tail of the BPV-4 E8 protein are important for anchorage independent growth.

Table 5.1a Anchorage independent growth of each separate clone of PalF transfectants and control cells

Cell line	Description	Number of anchorage Independent colonies in Methocel (%)
PalF	Parental PalFs	0.6 , 1.1
$E7_1$ E7a E7g	Control (no E8)	1 , 1.5 0.8 , 1.31 1.18 , 1.4
E8a (5.5) E8b (1.1)	BPV-4 E8 wild type	12 , 14 9.4 , 11
Mutant 1a Mutant 1c Mutant 1e	N17S	10 , 12 13 , 14.7 13.3 , 14
Mutant 2f Mutant 2g	N17Y	4 , 4.2 1 , 1.2
Mutant 3a Mutant 3b Mutant 3f	N17A	17.7 , 18.9 17.5 , 19.4 19.4 , 20.1
Mutant 4f Mutant 4a Mutant 4_6	E8T	1.2 , 1.9 2.6 , 2.84 2.8 , 3
Mutant 6a Mutant 6e	E8N-E5C	2.1 , 3.3 0.71 , 1.4
Mutant 7b Mutant 7_u	E5N-E8C	12.3 , 14 9.4 , 11.85
$E5_1$ E5d	BPV-1 E5	8 , 10 11 , 12
E5Ta E5Tb	E5T	1.2 , 0.9 1.3 , 1.19

All transfectant cell lines contain BPV-4 E7, T24 *ras* (activated *ras*) and HPV 16-E6.

Colony formation was scored by counting the total number of colonies in each plate of duplicate plates for each cell type. The number of colonies was expressed as a percentage of the total number of cells seeded into each plate (10^5) on day 0.

Table 5.1b Summary of anchorage independent growth

Cell type	Anchorage independent growth	Average no*. of Anchorage independent colonies (%) ± SEM
PalF cells	-	0.9 ± 0.25
Control (no E8)	-	1.2 ± 0.18
E8 wild type	+	11.6 ± 1.36
N17S	+	12.8 ± 1.17
N17Y	-	2.6 ± 1.22
N17A	+	18.8 ± 0.72
E8T	-	2.4 ± 0.49
E8N-E5C	-	1.9 ± 0.78
E5N-E8C	+	11.9 ± 1.34
BPV-1 E5	+	10.3 ± 1.20
E5T	_	1.2 ± 0.12

All transfectant cell lines contain BPV-4 E7, T24 *ras* (activated *ras*) and HPV 16-E6.

* Average calculated from data in table 5.1

SEM = standard error of mean

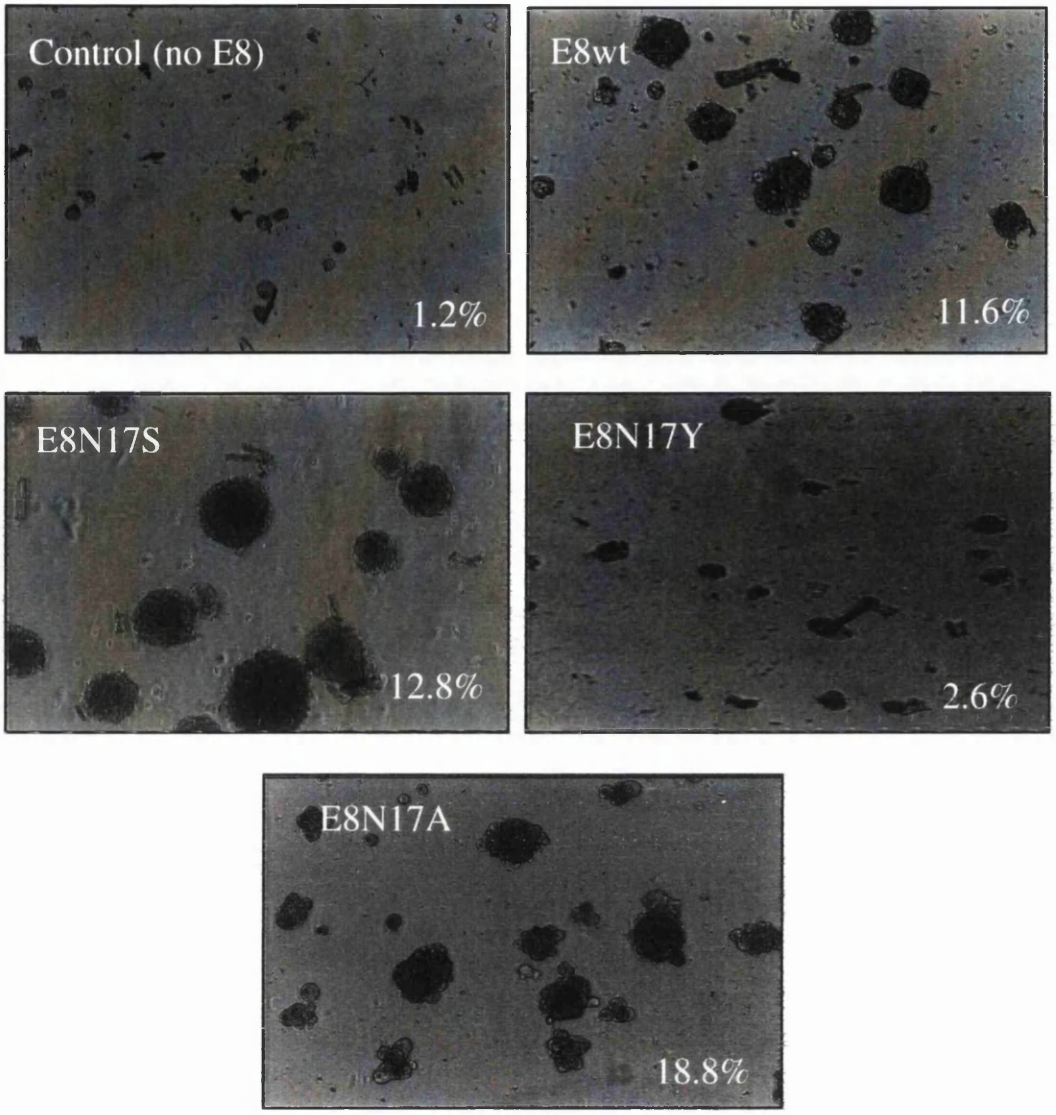


Figure 5.1a Anchorage independent growth of stable PalF transfectants
Control cells (no E8) were transfected with pZipneoE7, pT24 ras, and pJ4Ω16-E6, while other cells were transfected additionally with an E8 wild type or mutant forms of E8 as noted in each panel (E8N17S, E8N17Y, E8N17A).
The average efficiency of growth in methocel is indicated in each panel as percentage of the plated cells (Final magnification; X 40).

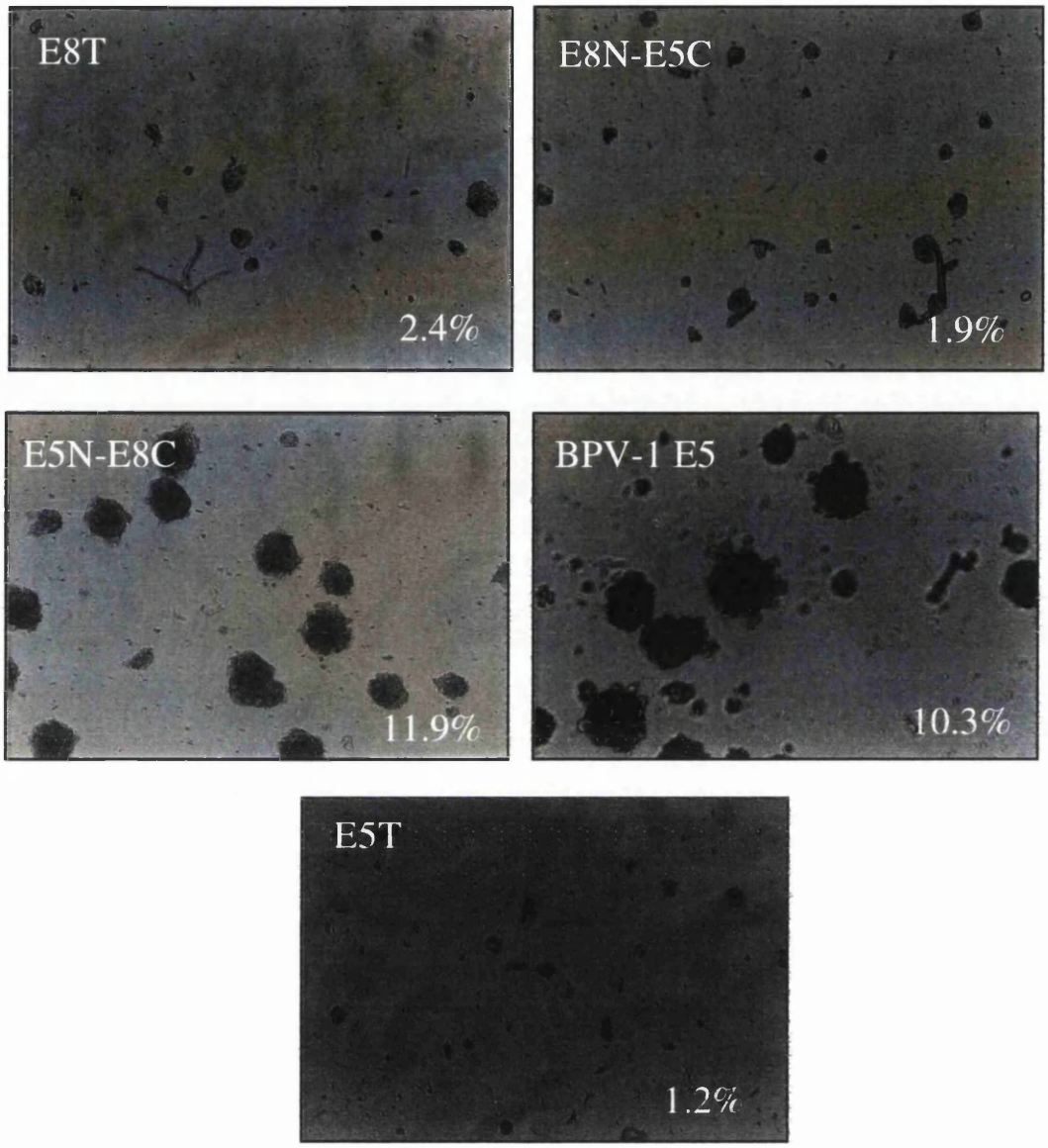


Figure 5.1b Anchorage independent growth of stable PalF transfectants with E8T, E8N-E5C, E5N-E8C, BPV-1 E5, and E5T.
The average efficiency of growth in methocel is indicated in each panel as percentage of the plated cells (Final magnification; X 40).

5.1.2 Focus formation assay

A major characteristic of normal fibroblasts in culture is density-dependent inhibition of growth; once a group of cells grow to completely cover the dish or layer of feeder cells, so that all cells are in contact, cell division ceases (contact inhibition).

To investigate whether the transfectant cells lose contact inhibition when co-cultured with excess normal parental PalF cells, and whether the inhibition correlates with the presence of gap junction intercellular communication (discussed in chapter 7) 200 cells of each PalF transfectant (Table 5.2) were mixed with 25,000 normal PalF cells. The mix was plated in each well of 6 well plate, in triplicate as described in materials and methods section 2.2.3.10.1. Three weeks later the cells were fixed in methanol and stained with 10% Giemsa solution and photographed.

Cells expressing BPV-4 E8 wild type and E8N17A escaped contact inhibition as assessed by focus formation (Figure 5.2). On the contrary, cells expressing all the other E8 mutants were incapable of forming foci. Surprisingly, also the cells expressing BPV-1 E5 were incapable of forming foci. Only E8N17A maintains the transformation potential of E8 wild type, and even BPV-1 E5 can not induce loss of contact inhibition in primary bovine cells. This latter observation suggest that E8 may have additional function to BPV-1 E5 and acts differently from BPV-1 E5.

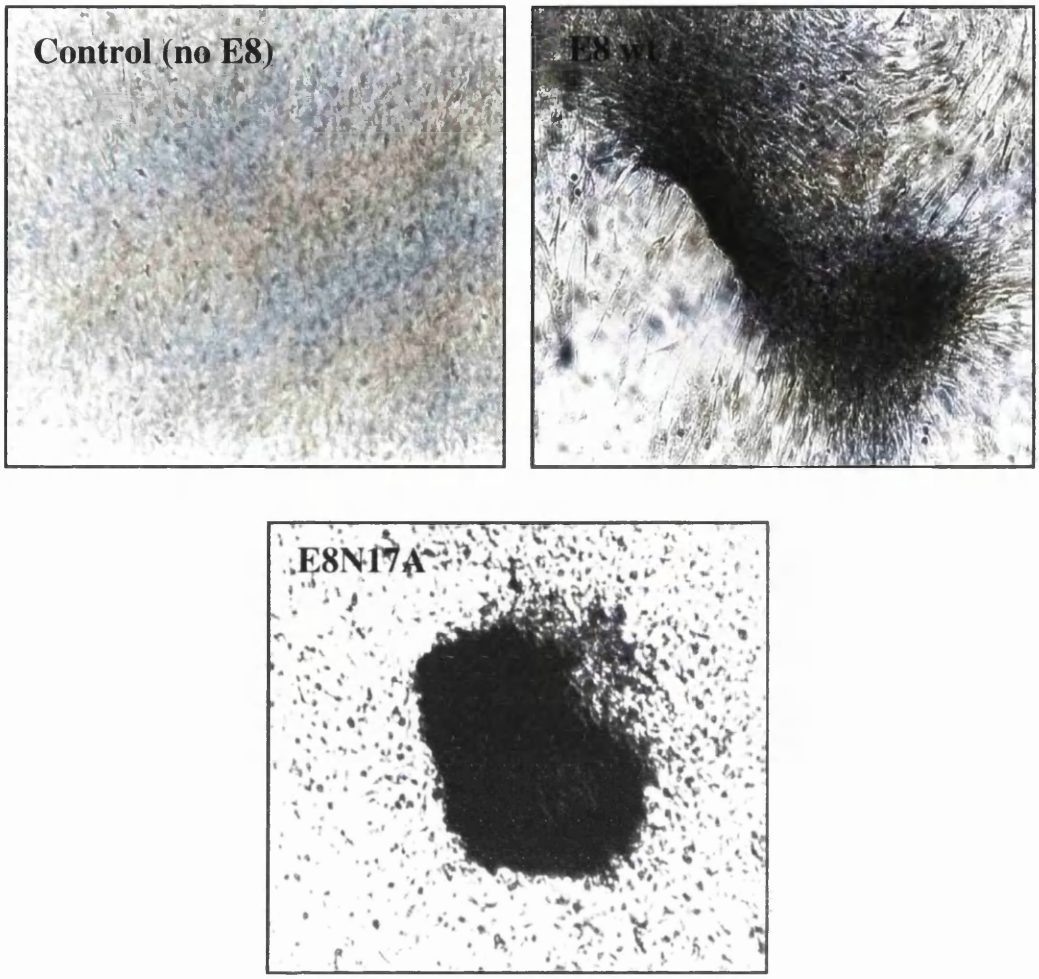


Figure 5.2 Focus formation

Control cells (no E8) were transfected with pZipneo E7, pT24 ras, and pJ4Ω16-E6, while other cells were transfected additionally with an E8 wild type or E8 N17A as noted in each panel

Only cells expressing E8 wild type and E8N17A abolish contact inhibition as assessed by focus formation (Final magnification; X 40).

Table 5.2 Contact inhibition of PalF transfectant cells

Cell type	Contact inhibition
PalF cells	yes
Control (no E8)	yes
E8 wild type	NO
BPV-1 E5	yes
E8 N17S	yes
E8 N17Y	yes
E8 N17A	NO
E8T	yes
E8N-E5C	yes
E5N-E8C	yes

5.1.3 Cell population growth in low (DMEM-0.5%) and high serum (DMEM-10%)

It has been shown that NIH-3T3 cells expressing BPV-4 E8 continue to proliferate in low serum, but do not appear to activate an autocrine mechanism (O'Brien and Campo, 1998).

To determine if the cell lines described here can escape growth arrest in low serum, two separate clones per transfectant class were assayed as follows: Cells (3000 cells/well) were seeded in 96 well tissue culture plates, in triplicate. After four hours incubation, during which time the cells attached to the bottom of each well, growth medium was changed to DMEM containing 10% serum or 0.5% serum. Population growth was determined, at daily intervals for 7 days, by

staining cells with 0.1% Crystal Violet in 20% Methanol. Dye was solubilized and absorbance was determined at 590nm using a automated plate reader (Dynatech MR7000) as described in Materials and Methods, section 2.2.3.10.2. Cell growth is expressed as a growth index representing the fold change in cell population from day 0 (harvested 4hr. after seeding the cells).

5.1.3.1 Results

Transfectants and parental PalF cells have very similar population growth kinetics when maintained in normal growth medium containing 10% serum (Figure 5.3). However, cells expressing E8 wild type, E8N17A or BPV-1 E5 continue to proliferate in low serum, while there is little or no population growth in the control cells (Figure 5.3) or cells with other mutant forms of E8, including E8N17S (Figure 5.3a) and E5N-E8C (Figure 5.3c) which could grow in suspension culture. Some cell lines are therefore both capable of anchorage independent growth and of proliferation in low serum, while others are capable of growing in suspension but cannot proliferate in the absence of serum mitogens. Thus the transformation phenotypes of anchorage independent growth and of growth in low serum can be segregated in cells expressing E8N17S or E5N-E8C.

In addition to this information on E8 transformed cells, this is the first time that the ability of cells expressing BPV-1 E5 to growth in low serum has been demonstrated. A summary of cell population growth in low serum (DMEM-0.5%) is shown in table 5.3.

Table 5.3 Cell growth in low serum

Cell line	Description	Growth in low serum
PalF	Parental PalF cells	no
E7a	Control (no E8)	no
E7b		no
E8a (5.5)	BPV-4 E8 wild type	YES
E8b (1.1)		YES
Mutant 1a	E8 N17S	no
Mutant 1c		no
Mutant 2f	E8 N17Y	no
Mutant 2g		no
Mutant 3b	E8 N17A	YES
Mutant 3f		YES
Mutant 4f	E8T	no
Mutant 4g		no
Mutant 6a	E8N-E5C	no
Mutant 6e		no
Mutant 7b	E5N-E8C	no
Mutant 7_u		no
$E5_1$	BPV-1 E5	YES
E5d		YES
E5Ta	BPV-1 E5 mutant	no
E5Tb	(E5T)	no

Figure 5.3 Cell proliferation growth in high and low serum medium

Two independent clones for each cell type were maintained in high and low serum for 7 days

HS = High serum (10% FCS)

LS = Low serum (0.5% FCS)

Bar = standard deviation

Figure 5.3a

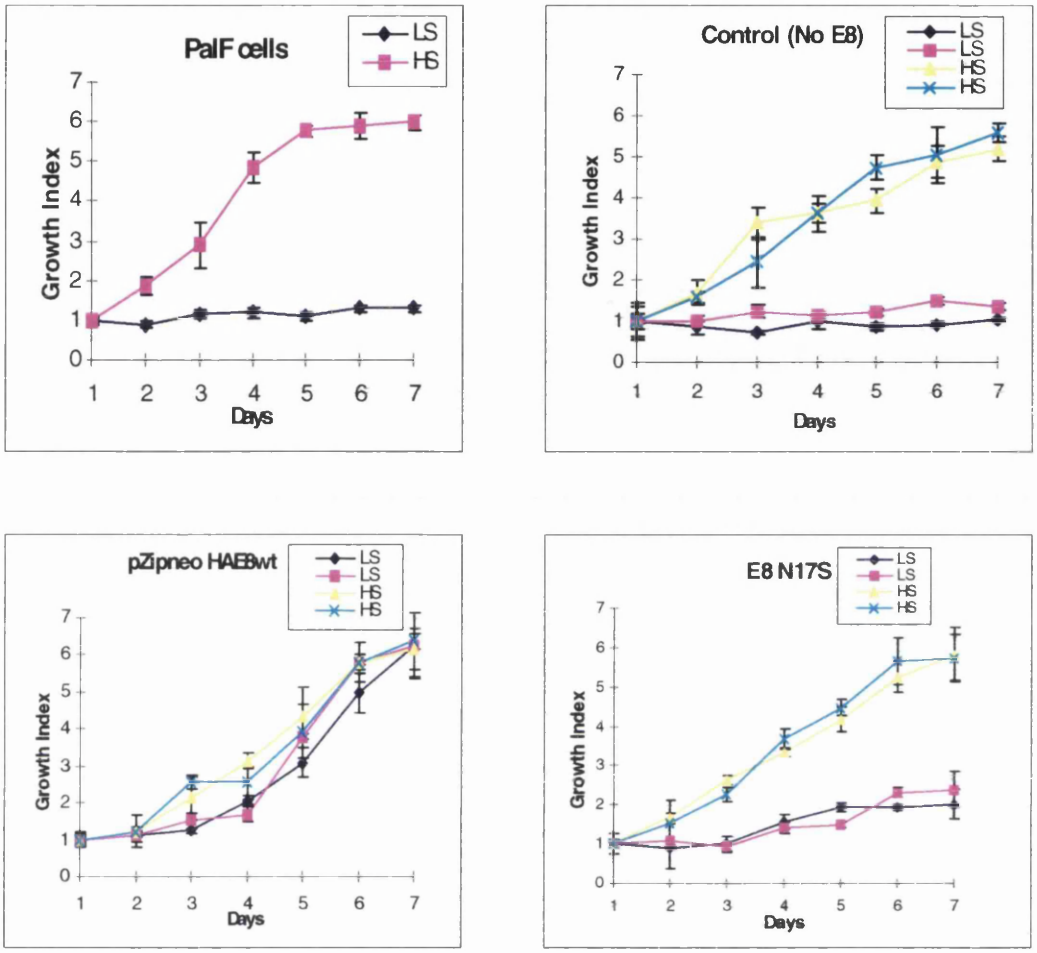

PalF cells
LS
HS
Growth Index
Days
Control (No E8)
LS
LS
HS
HS
Growth Index
Days
pZipneo HAEBwt
LS
LS
HS
HS
Growth Index
Days
E8 N17S
LS
LS
HS
HS
Growth Index
Days

Figure 5.3b

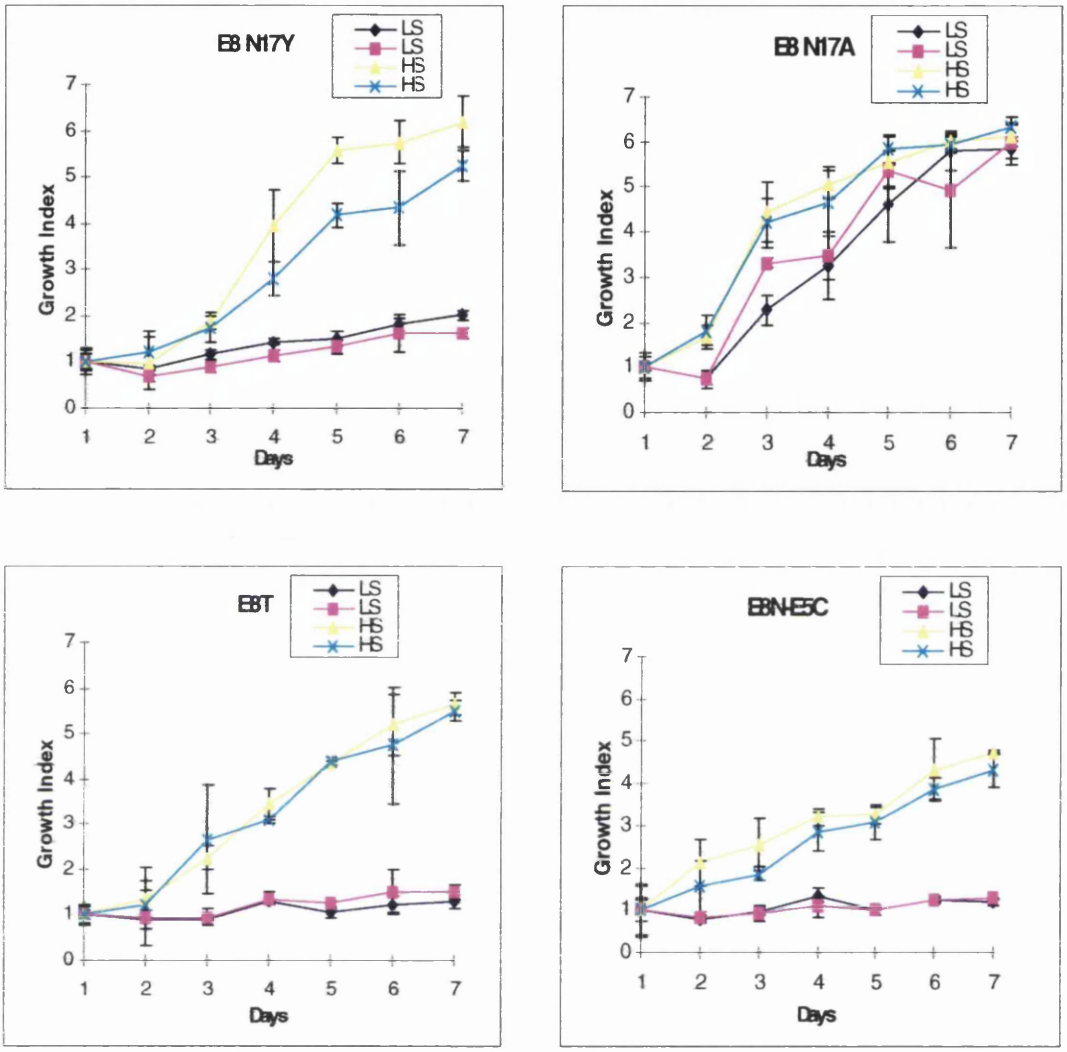
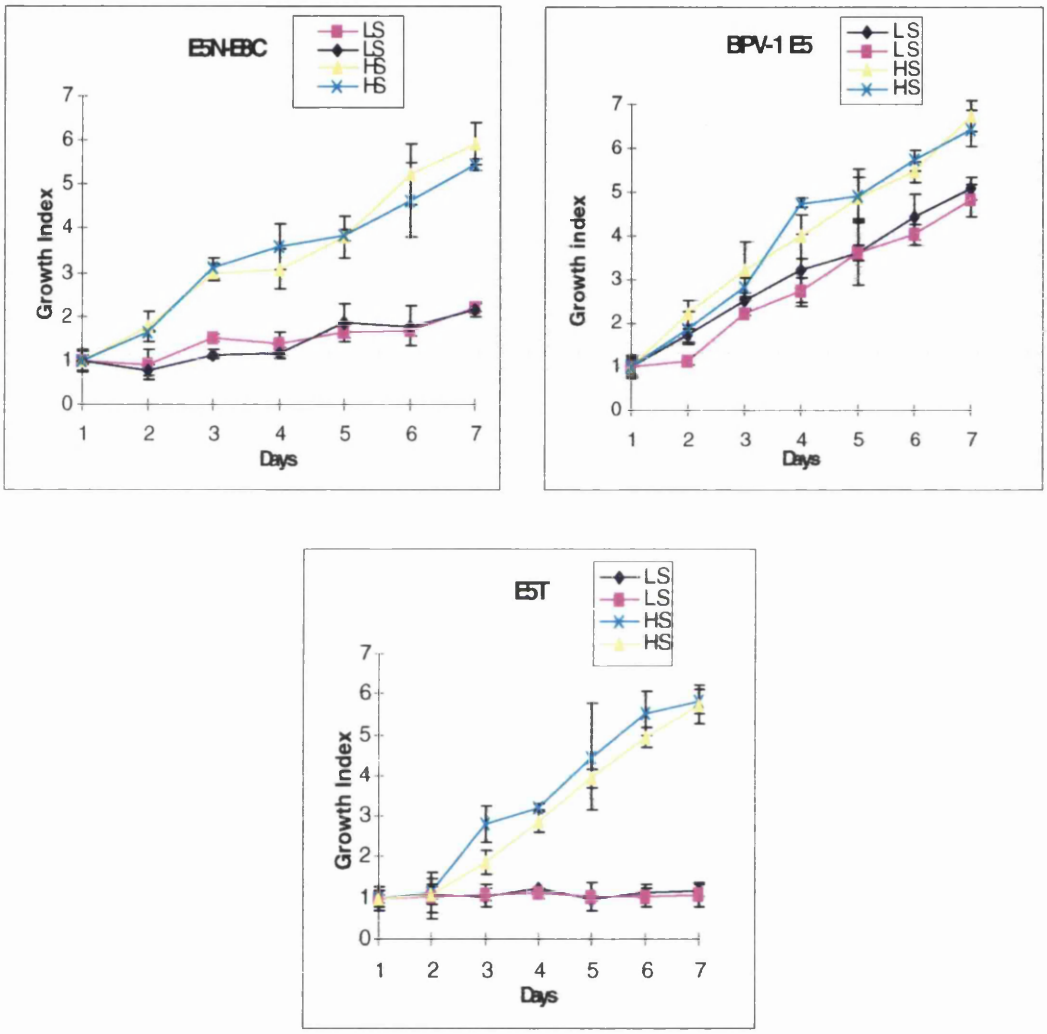

E8 N17Y
E8 N17A
E8T
E8N-E5C
LS
LS
HS
HS
Growth Index
Days
0
1
2
3
4
5
6
7

Figure 5.3c


E5N-EBC
LS
LS
HS
HS
Growth Index
Days
BPV-1 E5
LS
LS
HS
HS
Growth index
Days
E5T
LS
LS
HS
HS
Growth Index
Days

5.1.4 Tumorigenicity Assay in Athymic Mice

The ability to form tumours in experimental animals is an indicator of full cell transformation. An important test system for such *in vivo* assays is the nude mouse, which carries a mutation resulting in thymic deficiency. As a consequence, the thymus-derived (T) lymphocytes do not develop, and the mouse is immunogically incompetent. Inoculation of these mice therefore provides an assay for tumorigenicity that is not complicated by the possibility of immune rejection. This is particularly useful because it allows testing tumorigenicity of cell line in a nonsyngeneic host that would otherwise reject the foreign tumor graft.

To investigate the tumorigenicity of the transfected cells (chapter 4), some transfectant clones, as shown in table 5.4, were suspended in 0.1 ml of sterile PBS at a concentration of 10^8 cells/ml. PFA 15.6 is a tumorigenic cell line transfected with plasmids expressing mutant p53 + BPV-4 + T24 *ras* + HPV-16 E6 (a gift from Dr L. Scobie) and was used as a positive control. Nude mice (three per assay) were each injected subcutaneosly with this suspension (detailed in Materials and Methods, section 2.2.3.10.4), and examined for tumour growth weekly up to 15-20 weeks post injection.

5.1.4.1 Results

Subcutaneous injection of nude mice with the cell lines resulted in the appearance of small nodules which disappeared after three to four weeks. This is

indicative of a reaction at the site of injection and not of tumor formation. Tumorigenicity was detected only in the PFA 15.6 positive clone. Despite showing other evidence of phenotypic transformation none of the injected PalF transfectant cells were tumorigenic. Thus, neither of BPV-4 E8 wild type or its mutant forms cause cells to be tumorigenic. These results concur with the study of Pennie et al (1993) and suggest that additional factors are needed for full transformation of PalF cells. This is similar to the *in vivo* situation where the progression of BPV-4 induced papillomas to carcinomas is co-factor dependent.

Table 5.4 Tumorigenicity of PalF transfectant cell lines in nude mice.

Cell line	**Description**	**Incidence (no. mice positive / No. mice tested)**
PFA 15.6	(mutant p53 + BPV-4 + T24 *ras* + HPV-16 E6)	3/3
E7a	Control (no E8)	0/3
$E7_1$		0/3
E8a (5.5)	BPV-4 E8 wild type	0/3
E8b (1.1)		0/3
Mutant 3a	E8 N17A	0/3
Mutant 3b		0/3
Mutant 3f		0/3

5.1.5 CONCLUSIONS

Together these data suggest that:

Cells expressing E8 are transformed, failing to exit the cell cycle when deprived of serum mitogens, exhibiting loss of contact inhibition and being capable of anchorage independent growth. PalF cells which do not harbour BPV-4 E8 do not show any of these phenotypes, despite the presence of BPV-4 E7, HPV-16 E6 and activated *ras* (Table 5.5). Although E8 plays a pivotal role in PalF transformation, the generation of stable transfectants requires the presence of E7. This contrasts with the transformation of established mouse NIH-3T3 cells by E8 alone (O'Brien and Campo, 1998).

These contradictory observations may be due to inherent differences between established and primary cells. Normal cells in culture have a limit to the number of times they can divide and when this limit is reached the cells irreversibly leave the cell cycle and become senescent. PalF cells are primary cells and, as expected, senesce on continued culture. BPV-4 E7 possesses the two Cys-X-X-Cys motifs and potential p105Rb binding domain (Jaggar et al., 1990). Mutation of either of these domains abolish the protein's transforming ability *in vitro* (Campo et al., 1994b; Jackson et al., 1996). As p105Rb is a tumor suppressor protein which negatively regulates the cell cycle (Sherr, 1994), it could be proposed that BPV-4 E7-mediated disruption of the normal function of p105Rb, and the resulting alterations in cell cycle control, may provide optimal conditions for the action of E8 gene by providing cell survival and clonal expansion.

In established cells one or more immortalizing events have already occurred and any of these may allow the expression of E8 transformation potential. For example, it has been shown that a member of the cell cycle inhibitor family, $p16^{INK4a}$, that induce G_1 arrest (for review see Hunter and Pines, 1994) is lost in the NIH-3T3 fibroblasts (Quelle et al., 1995) therefore the loss of $p16^{INK4a}$ promotes cell proliferation and this event may complement the expression of E8 functions in NIH-3T3 cell.

The asparagine residue at position 17 appears to be critical for the integrity of BPV-4 E8 biological function. Mutation of this single amino acid produces profound changes in the activity of the protein. Mutation to an alanine creates a hypertransforming molecule with increased ability to induce anchorage independent growth, and mutation to a serine residue leads to an intermediate state that allows only the maintenance of the anchorage independent growth.

Another critical domain of BPV-4 E8 is the hydrophilic tail of the protein, required for anchorage independent growth. Neither E8T or E5T are capable of conferring anchorage independent growth, nor is the E8N-E5C chimera. On the contrary E5N-E8C is equally capable of inducing this transformed phenotype as wild type E8 or wild type E5. These results indicate the importance of an intact hydrophilic tail for anchorage independent growth and also demonstrate that the individual C-terminal domains of E8 and E5 are not functionally interchangeable.

The ability of E8 to induce anchorage independent growth can be separated from the ability of forming foci or growing in low serum. Interestingly, cells expressing BPV-1 E5 were incapable of forming foci. Thus, the ability to form

foci, and growth in low serum can be dissociated. The results obtained also suggest that E8 acts differently from BPV-1 E5 to bring about cell transformation.

Table 5.5 Summary of transformation characteristics of cells expressing E8 or its mutants

Cell type	Focus formation	Growth in low serum	A.I. growth
Control (no E8)	-	-	-
E8 wild type	+	+	+
BPV-1 E5	-	+	+
E8 N17S	-	-	+
E8 N17Y	-	-	-
E8 N17A	+	+	++
E8T	-	-	-
E8N-E5C	-	-	-
E5N-E8C	-	-	+
E5T	nd	-	-

All cell lines contain BPV-4 E7, T24 *ras* (activated *ras*) and HPV 16-E6.

A.I. anchorage independent growth

+ represent the extent of expression of the characteristic.

CHAPTER SIX

ANALYSIS OF THE EFFECT OF BPV-4 E8 AND MUTANTS ON TRANS-ACTIVATION OF THE CYCLIN A PROMOTER

6.1 Introduction

The cell cycle is the sequence of events through which a cell duplicates its genome, grows, and divides into two daughter cells. The ability of a cell to duplicate is one of the most fundamental properties that defines life. Most cancers are in essence caused by deregulation of the cell cycle.

The cell cycle is divided into four phases: the G_1 (gap 1) phase before DNA replication, DNA replication itself (DNA synthesis) (S phase), the G_2 (gap 2) phase before cell division, and finally cell division (mitosis) (M phase). The stages outside M phase are collectively known as interphase. Quiescent mammalian cells that are not actively growing reside in G_0, a resting state. In mammalian cells, cell growth control is exerted primarily in the G_1 phase. The factors modulating exit from G_0 and progression through G_1 are critical for determining overall growth rate.

Work in yeast revealed the presence of checkpoints in the cell cycle which are required to ensure that the cell division process is successful. The G_1/S checkpoint ensures that DNA is intact and undamaged before replication proceeds

and the G_2/M checkpoint appears to ensure that DNA replication has successfully completed before cell division occurs (Forsburg and Nurse, 1991; Nasmyth, 1993).

However, some of the controls exerted on progression through the cell cycle are lost when cells become transformed; in particular the controls or check-points at the G_1/S and the G_2/M transitions are less stringent or even absent in cancer cells. It has been shown that some of the most fundamental changes in transformation are in the protein kinase complexes that are thought to regulate cell cycle checkpoints (Pines, 1995). The most intensively studied of these regulators are complexes between members of the cyclin and the cyclin-dependent kinase (CDK) families.

6.1.1 Cyclins and Cyclin-Dependent Kinases (CDKs)

Cyclins are defined as proteins that share homology in a region of approximately 100 amino acid called the cyclin box. The cyclin box binds members of a well-conserved family of protein kinases that have the defining property of requiring a cyclin partner for their activation. Hence their designation as cyclin dependent kinases (Pines, 1995).

In invertebrate oocytes, it was observed that cyclins were synthesised during interphase and destroyed at the end of mitosis. Initially, two cyclins, A and B, were identified. Subsequently, six families of mammalian cyclin genes have been identified; they are classified by the extent of sequence homology and by the

point in the cell cycle at which they function. Cyclins are divided into two functional classes: those that act at the G2/M boundary (cyclins B1 and B2) and those that act at the G_1/S boundary (cyclin C, D, and E). Cyclin A is the exception; it is present and functioning from late G_1 to S through M phase (Pines, 1992; Sherr, 1993).

Progression through the cell cycle in mammalian cells, including anchorage-dependent passage through G_1 (Guadagno and Assoian, 1991), is controlled by the co-ordinate activation of cyclin-dependent kinases (CDKs). CDK activity is regulated at multiple levels including the phosphorylation of specific residues that activate or inhibit the kinase, and the availability of related cyclins; consequently the expression of cyclins is strictly regulated in normal cells.

D-type cyclins (D1, D2, or D3) complexed to CDK4 or CDK6 are required for progression through to early/mid G_1 phase of the cell cycle and in the decision to embark on a new cell cycle or to enter a quiescent state (G_0) after mitosis, linking cell exposure to external cues to entry into the cell cycle (Won et al., 1992; Baldin et al., 1993). Cyclin E is expressed in late G_1 and is also required for entry into S phase in mammalian fibroblasts (Resnitzky et al., 1994; Ohtsubo et al., 1995), while cyclin A is first expressed at the G_1/S transition and is required for the successful completion of S-phase and passage through G_2 (Girard et al., 1991; Pagano et al., 1992). Cyclin B is first expressed in S-phase and its complex with cdc2 is a key regulator of mitosis (Hayles et al., 1994).

Another level of control of cyclin-CDK complexes is via a strong inhibition of activity by two families of small proteins: the INK family, p15, p16, p19 which target cyclin D-CDK4/CDK6 complexes and prevent cyclin binding, and members of the Kip/Cip family composed of p21, p27 and p57, which target cyclin A - and E-CDK2 complexes (Sherr and Roberts, 1995).

Cyclin A gene expression is regulated at the level of transcriptional initiation with expression being repressed in G_1 and strongly induced as cells enter S-phase (Henglein et al., 1994). The absence of degradation of cyclin A might lead to unregulated and premature DNA synthesis and to cell proliferation, thus contributing to cell transformation. Nevertheless, changes in cyclin A expression appear to be rare in most types of cancer. Cyclin E-CDK2 activity has been shown to be required for cyclin A transcription in established murine fibroblast (NIH-3T3) cells (ZerfassThome et al., 1997). Cyclin A expression is down-regulated in NIH-3T3 cells maintained in suspension or in low serum culture conditions; under these conditions $p27^{KIPI}$ is elevated and blocks cyclin E-CDK2 mediated de-repression of the cyclin A promoter (ZerfassThome et al., 1997). Also, cells maintained in low serum (Firpo et al., 1994) or in suspension (Guadagno et al., 1993; Fang et al., 1996) do not express cyclin A and cannot progress into S-phase. Constitutive expression of cyclin A allows NRK fibroblasts to proliferate in suspension (Guadagno et al, 1993), while induction of cyclin A expression is sufficient to promote entry into S-phase of fibroblasts made quiescent by serum withdrawal (Resnitzky et al., 1994).

The intracellular concentration of $p27^{KIPI}$ is a major element of cell cycle progression through the G1 restriction point (Coats et al., 1996) and has emerged as a key target for viral oncoproteins. Both human Adenovirus E1A and HPV-16 E7 proteins can inactivate p27 by forming complexes which make p27 non-functional *in vitro* and *in vivo* (Mal et al., 1996; ZerfassThome et al., 1996). Furthermore, BPV-4 E8, in NIH-3T3 cells inactivates $p27^{KIPI}$ and trans-activates cyclin A gene promoter in both high and low serum conditions (O'Brien and Campo, 1998).

Hence we decided to measure cyclin A promoter activity in our stable transfectant cell lines (chapter 4) to determine if, as in NIH-3T3 cells E8 can de-regulate expression of cyclin A, and to investigate the correlation between promoter activation of cyclin A and cell transformation brought about by the different E8 mutant forms as described in chapter five. We were also interested to test the ability of additional mutants including A15G-A20G, A15P-A20P, ARLD, DRLD, SRLD, to activate the cyclin A promoter in short term transfection experiments.

6.2 Experimental procedure

6.2.1 Cyclin A promoter activity measurement

Growing stable transfectant cells (10^5) (chapter 4) were plated in each well of a 6 well plate, in duplicate, and transiently transfected with calcium phosphate as detailed in chapter 2.2.3.5., using 1 μg of luciferase reporter plasmid

driven by the human cyclin A promoter. Reactions were made up to 3 μg with sonicated salmon sperm DNA. Co-transfection classes are shown in table 6.1. After 16-18hr., cells were washed twice with 2ml. PBS and incubated in low serum or growth medium for a further 24 hr. before being harvested and lysed in reporter lysis buffer. Luciferase activity was determined using a luminometer with automatic injection (chapter 2.2.3.6).

Table 6.1 Co-transfection of stable transfectants

pZipneo E7 (no E8) + 2μg SSD + 1μg luciferase reporter plasmid
pZipneo HAE8 + 2μg SSD + 1μg luciferase reporter plasmid
pZipneo HAE8 N17S + 2μg SSD + 1μg luciferase reporter plasmid
pZipneo HAE8 N17Y + 2μg SSD + 1μg luciferase reporter plasmid
pZipneo HAE8 N17A + 2μg SSD + 1μg luciferase reporter plasmid
pZipneo HAE8 E8T + 2μg SSD + 1μg luciferase reporter plasmid
pZipneo HAE8N-E5C + 2μg SSD + 1μg luciferase reporter plasmid
pZipneo HAE5N-E8C + 2μg SSD + 1μg luciferase reporter plasmid
pZipneo HAE5 + 2μg SSD + 1μg luciferase reporter plasmid

SSD = Salmon sperm DNA

6.2.2 Results and discussion

Under normal growth conditions BPV-4 E8 wild type expression led to at least a 200 fold increase in cyclin A promoter activity when compared with control cells transfected with 2 μg of the empty vector, pZipneo (Figure 6.1a). A similar activation was observed with BPV-1 E5 wild type (at least a 180 fold increase) (Figure 6.1a). Of the E8 mutants, only E8N17A had comparable promoter trans-activation activity with at least a 150 fold increase (Figure 6.1a); low levels of trans-activation were observed with mutant E8N17S (at least a 14 fold increase) and with E5N-E8C (at least a 18 fold increase) (Figure 6.1b). In cells expressing the other mutants including E8N17Y, E8T and E8N-E5C the cyclin A promoter was slightly but significantly transactivated, with at least a 6 fold increase above the control cells containing only pZipneo plasmid. However, the same level of transactivation was observed for PalF transfectants which did not contain E8, but harboured BPV-4 E7, activated *ras* and HPV-16 E6 (Figure 6.1b). Therefore, this 6 fold increase in activity is probably due to the effect of E7 and/or activated ras. It has been reported that activated *ras* induces significant overexpression of cyclin D (Filmus et al., 1994) and that the overexpression of cyclin D leads to p105Rb phosphorylation. The phosphorylated form of pRb loses its affinity for E2F transcription factor, and releases E2F which results in induction of cyclin A promoter (Schulze et al., 1995). Therefore only values of trans-activation higher than those observed in E7 cells can be ascribed to E8. The ability of E8 to trans-activate the cyclin A promoter correlates with its ability to

induce anchorage independent growth: E8 mutants capable of inducing anchorage independent growth can trans-activate the cyclin A promoter, although to different extents.

When the cells were maintained in low serum for 24hr, promoter activity remained high in the case of wild type E8 and E8N17A; low levels of trans-activation were also observed with E5N-E8C, but not with the other mutants or control cells (Figure 6.1a & b). BPV-1 E5 wild type could trans-activate the promoter in low serum , but apparently to a lesser extent than E8 wild type, suggesting a higher degree of growth factor dependence (Figure 6.1a).

Cells expressing E5N-E8C can trans-activate the cyclin A promoter to the same extent in high and low serum but to a lesser degree than either E8 or E5 (Figure 61a and 61b); however these cells fail to grow in low serum. One possible explanation for this apparent discrepancy is that cells expressing E5N-E8C do proliferate in low serum but this is balanced by cell death, resulting in little or no cell growth. This would have to be tested.

These results indicates that trans-activation of the cyclin A promoter in low serum correlates with the ability of the cells to continue growth in these conditions, with the exception of E5N-E8C.

To investigate whether the differences in cyclin A promoter activity between control cells and those expressing BPV-4 E8 wild type or its mutants were due to differences in transfection efficiency between cell lines, the cyclin A

Figures 6.1(a, b), 2, and 3:

Trans-activation of the human cyclin A promoter

To measure of the cyclin A promoter activation, the luciferase reading for each sample (stable and short-term transfection) was corrected according to the following equation:

The fold induction of luciferase activity is given after normalisation for cellular protein content (Sample's luciferase reading - background luciferase reading (Lysis buffer) ÷ protein concentration of related samples) and, in each case, mean values from duplicate samples are given for a representative experiment.

The reading with the empty vector (pZipneo) was set at 1, and the luciferase reading of the samples were normalised to the control reading.

Once normalised, the results from all the separate experiments were amalgamated; the average luciferase reading and the standard error of the mean (S.E.M), were calculated for each transfection class. The experiment performed at least twice.

Bar =standard error of mean

Figure 6.1a Trans-activation of the cyclin A promoter in stable transfectants

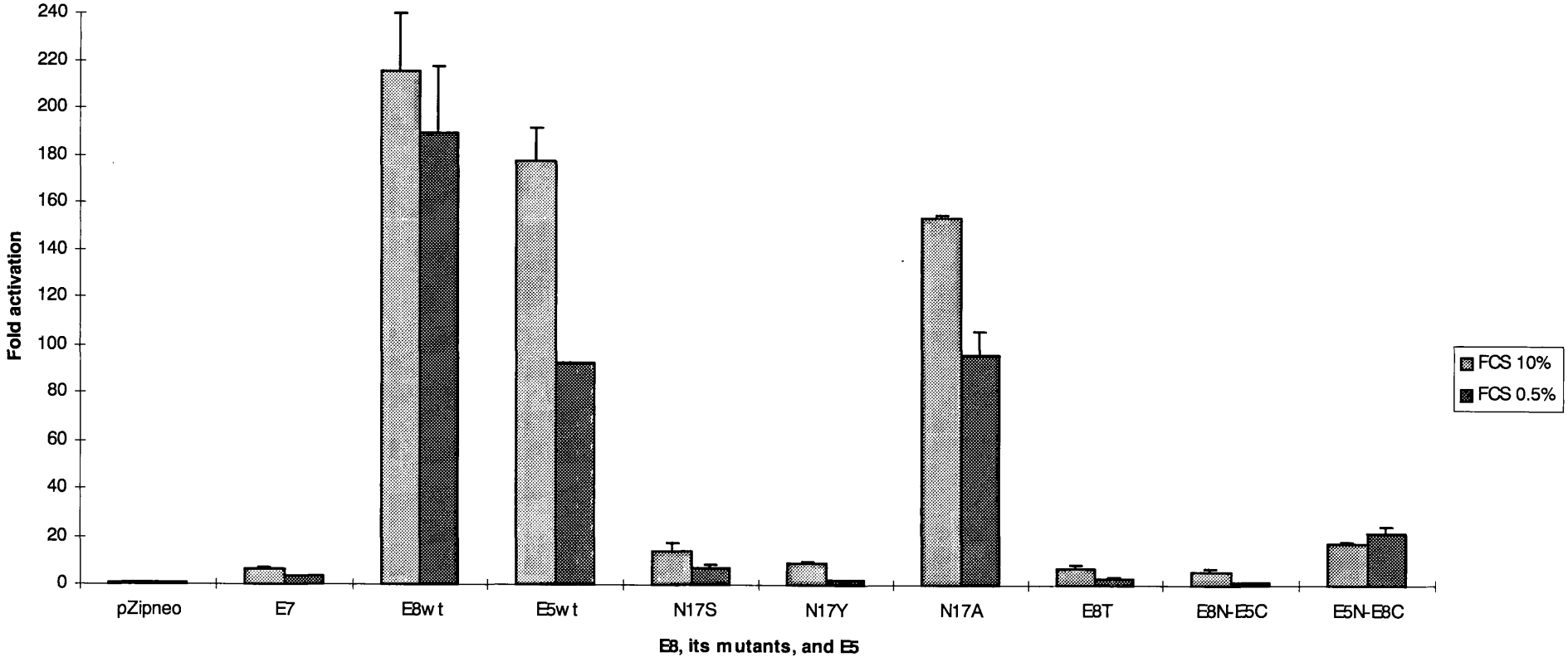
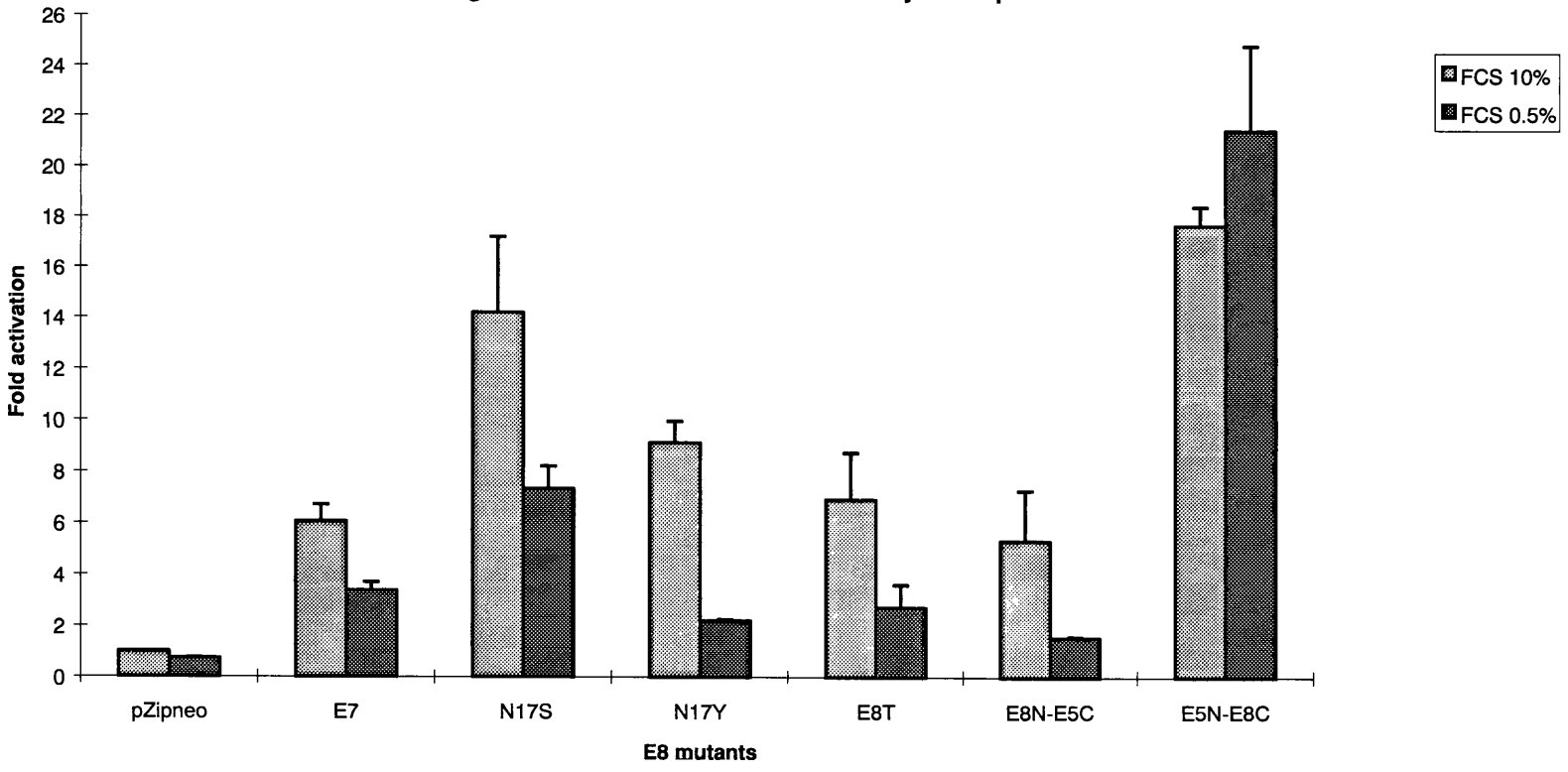


Figure 6.1b Trans-activation of the cyclin A promoter in stable transfectants



reporter gene plasmid (1μg) was also transiently co-transfected with BPV-4 HAE8 wild type and some of its mutants in PalF cells (table 6.2). The procedure of this experiment was explained in section 6.2.1. Control including co-transfected luciferase reporter gene plasmid with pZipneo (empty vector), was performed at the same time to ensure that the experimental conditions were correct.

Qualitatively similar results were obtained in transient transfection experiments to those obtained with stable transformants (Figure 6.2). This demonstrates that the differences in cyclin A promoter activity between control cells and those expressing E8 wild type, its mutants, or E5 were not due to differences in transfection efficiency between cell lines. In addition, a C-terminus truncated forms of BPV-1 E5 (E5T) comprising the membrane-localized region of E5 domain was incapable of trans-activating the cyclin A promoter, as was E8T. The differences in the level of cyclin A promoter activation between transiently and stably transfected cells can be partially attributed to the proportion of cells in each population carrying the transfected DNA.

These results extend those obtained previously with NIH-3T3 cells expressing E8 (O'Brien and Campo, 1998) in which the cyclin A promoter is transactivated by E8, and confirm that cyclin A transactivation correlates with anchorage independent growth and with proliferation in low serum.

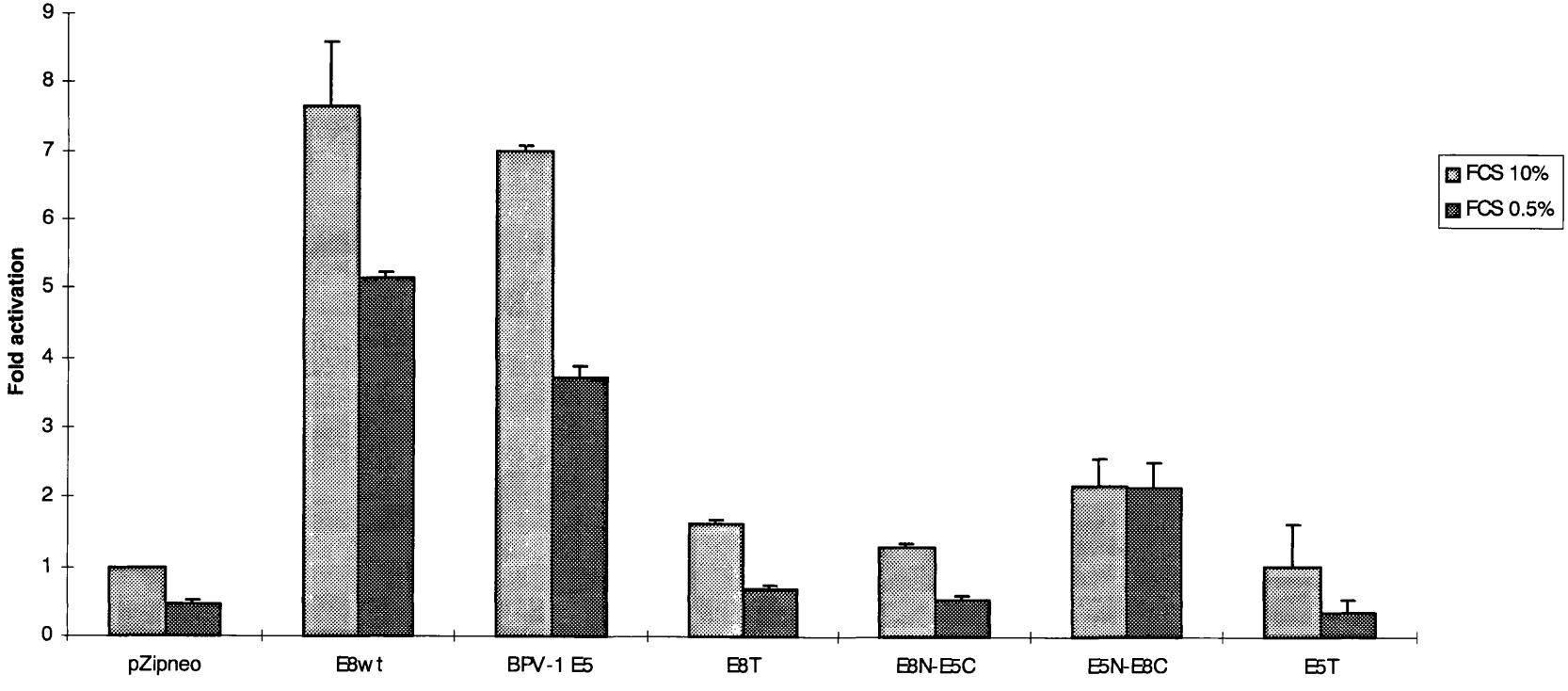
Since E8 has a postulated α-helical domain, we were interested in determining whether this domain is responsible for the biological activity of the protein. Proline has the property of forcing a bend in the main chain and of

Table 6.2 Short-term transfection classes

2 μg pZipneo + 1μg luciferase reporter plasmid
2 μg pZipneo HAE8 + 1μg luciferase reporter plasmid
2 μg pZipneo HAE8T + 1μg luciferase reporter plasmid
2 μg pZipneo E8N-E5C + 1μg luciferase reporter plasmid
2 μg pZipneo E5N-E8C + 1μg luciferase reporter plasmid
2 μg pZipneo HAE5 + 1μg luciferase reporter plasmid
2 μg pZipneo E5T + 1μg luciferase reporter plasmid
2 μg pZipneo HAE8 A15G-A20G + 1μg luciferase reporter plasmid
2 μg pZipneo HAE8 A15P-A20P + 1μg luciferase reporter plasmid
2 μg pZipneo HAE8 ARLD + 1μg luciferase reporter plasmid
2 μg pZipneo HAE8 DRLD + 1μg luciferase reporter plasmid
2 μg pZipneo HAE8 SRLD + 1μg luciferase reporter plasmid

Each transfection class was performed twice

Figure 6.2 Trans-activation of the cyclin A promoter by E8 wild type, mutants, and E5 in short-term assays



disrupting an α helix. Thus alanine residues at positions 15 and 20 were changed to proline (E8 mutant A15P-A20P). The same two alanine residues were also mutated to the chemically similar amino acid glycine (E8 mutant A15G-A20G) to control for any effect due to changes in residues rather than in structure.

These mutants were tested for their ability to trans-activate the cyclin A promoter in transient transfection assays as described for the other mutants.

As shown in figure 6.3., under normal growth conditions PalF cells transiently transfected with E8 wild type led to at least a 7.5 fold increase in cyclin A promoter activity, compared with control cells transfected with empty vector (pZipneo). Mutant A15G-A20G could trans-activate the cyclin A promoter at least 4 fold but to a lesser extent than E8 wild type; cells transiently transfected with E8 mutant A15P-A20P did not transactivate the cyclin A promoter.

When the cells were maintained in low serum conditions for a 24hr. period following transfection, promoter trans-activation was sustained in the case of E8 wild type (at least a 5.2 fold increase). Neither A15G-A20G or A15P-A20P significantly trans-activated the cyclin A promoter in these conditions (Figure 6.3).

Taken together, these results suggest that substitution of these two amino acids with proline could alter the conformation of the hydrophobic domain and that the postulated α-helix form of E8 may have an effect on cell transformation. They also point to the importance of the nature of these residues for the E8

functions, as their mutation to glycine impaired the transactivation ability of E8 although to a lesser extent than the proline substitution.

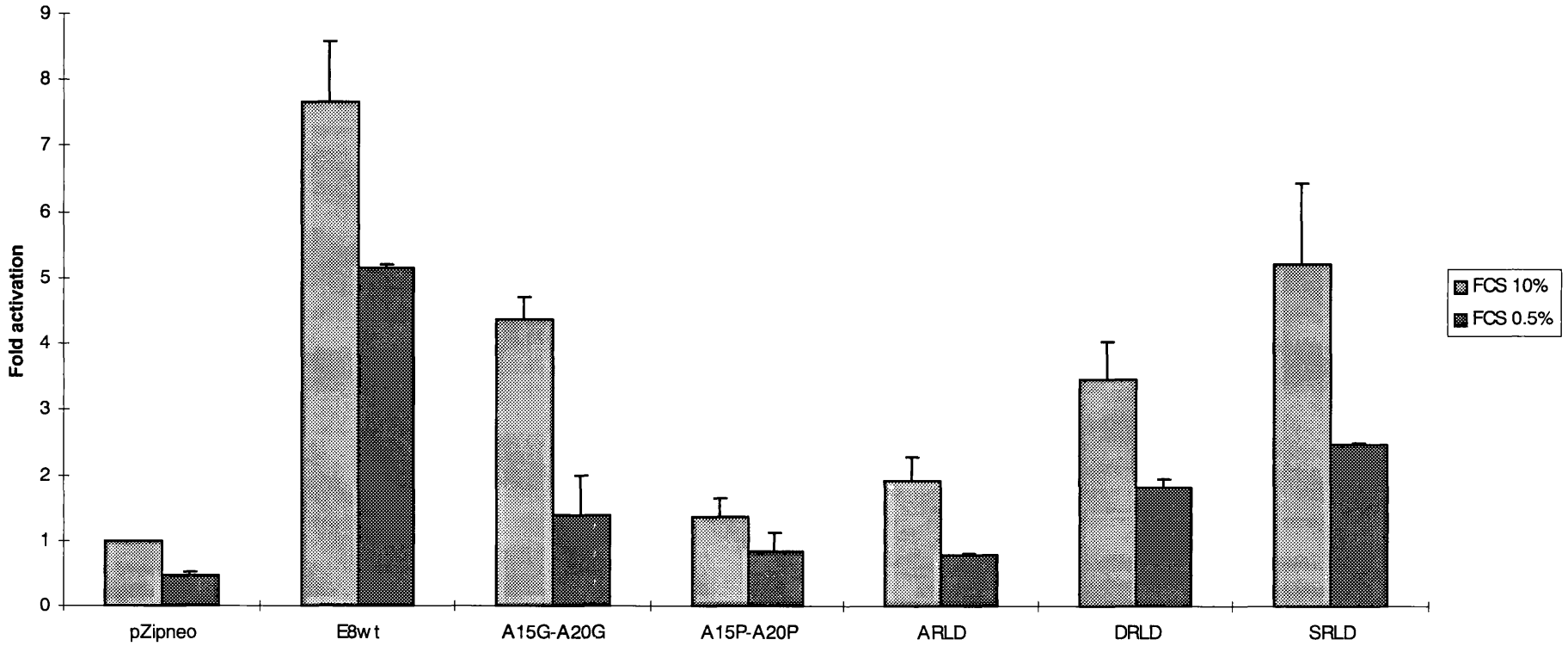
In the C-terminus hydrophilic tail of E8 there is a possible casein kinase II (CKII) phosphorylation sites (TRLD) at positions 36-39. We were interested in determining the possibility that E8 might be phosphorylated by CKII and whether this phosphorylation could affect its biological activities.

To study the effect of phosphorylation by CKII of the putative site on the biological activities of E8, three mutations were generated in this site. The threonine was changed to aspartic acid, alanine, or serine, in mutants DRLD, ARLD and SRLD respectively. Aspartic acid is negatively charged, mimicking a phosphorylated amino acid. Alanine can not be phosphorylated, but serine, like threonine, can be phosphorylated by CKII.

These mutants were tested for their ability to trans-activate the cyclin A promoter in short term transfection experiments.

Under normal growth conditions, PalF cells transiently transfected with E8 wild type led to at least a 7.5 fold increase in cyclin A promoter activity when compared with parental or control cells transfected with empty vector (pZipneo). Cells transiently transfected with mutant ARLD, which has lost the putative CKII phosphorylation site, showed no significant increase in cyclin A promoter activity; those with the mutant DRLD, which is negatively charged, showed about a 3.2 fold increase in cyclin A promoter and those with mutant SRLD, which

Figure 6.3 Trans-activation of the cyclin A promoter by E8 mutants in short term assays



maintains both the putative CKII site, showed about a 5 fold increase in cyclin A promoter activity (Figure 6.3).

When the cells were maintained in low serum conditions for 24 hr. period following transfection, promoter trans-activation was sustained in the case of E8 wild type, with at least a 5.2 fold increase. Mutant SRLD induced a 2.5 fold increase in cyclin A promoter activity; mutant DRLD induced about a 2 fold increase and mutant ARLD was totally incapable of promoter trans-activation (Figure 6.3).

In brief, cells transiently transfected with mutant ARLD, which has lost the putative CKII phosphorylation site, show less cyclin A promoter activitation than those with the negatively charged mutant DRLD or with mutant SRLD in high and low serum conditions. Cells transiently transfected with mutant SRLD, which maintains the putative CKII site, show more cyclin A promoter activity than either mutants DRLD or ARLD in both high and low serum conditions.

These results support the possibility that BPV-4 E8 might be phosphorylated by CKII and that this phosphorylation could have an effect on the biological activities of the protein.

Also, low levels of trans-activation of the cyclin A promoter by mutant SRLD in low serum conditions and mutant DRLD in both high and low serum conditions compared with wild type E8 show that the substitution of a single amino acid in the hydrophylic tail has an effect on the ability of the protein to trans-activate the cyclin A promoter.

Therefore, analysis of the transformation status of established lines containing these mutants (A15G-A20G, A15P-A20P, ARLD, DRLD, SRLD) could be particularly interesting. We would expect the transformation phenotype to be related to the ability of the E8 mutants to trans-activate the cyclin A promoter.

In conclusion, we have demonstrated that in PalF cell, as in NIH-3T3 cells (O'Brien and Campo, 1998), E8 expression promotes transcriptional trans-activation of the cyclin A promoter under conditions where cyclin A transcription is normally diminished or is not detectable in control cells.

Trans-activation of the cyclin A promoter correlates with the ability of the PalF cells to grow in suspension; E8 mutants still capable of conferring these phenotypes can trans-activate the cyclin A promoter, despite doing so to different extents.

We conclude that mutations which selectively abolish the ability of E8 protein to trans-activate the cyclin A promoter cause defects in transforming activity.

CHAPTER SEVEN

GAP JUNCTIONAL INTERCELLULAR COMMUNICATION (GJIC)

7.1 Introduction

7.1.1 Gap junctional intercellular communication (GJIC)

Gap junctional intercellular communication (GJIC) is a characteristic feature of most normal tissues. Apart from a few terminally differentiated cells, such as skeletal muscle, erythrocytes, and circulating lymphocytes, most cells in normal tissues generally communicate via gap junctions. These junctions exist in almost all animals, both vertebrates and invertebrates (for review see Kumar and Gilula, 1996).

The gap junction channel is composed of two hemi-channels termed connexons. Each hemi-channel joins end-to-end with a connexon in the opposing membrane of another cell to provide a direct aqueous pathway between the cytoplasms of the coupled cells for the intercellular exchange of cytoplasmic ions and small molecules (metabolites, cofactors, second messengers, etc.) with molecular weight up to ~1000Da., thus mediating signalling between adjacent cells. The molecular movement through the channels occurs by passive diffusion. Consequently, this type of communication may be an important mechanism for

regulating events between cells during embryogenesis (Warner et al., 1984) and during normal function of organs.

During embryogenesis and tissue differentiation, the specificity of gap junction formation produces complex patterns of communication by establishing well-defined communication compartments, group of cells joined to each other by gap junctions but not to cells in other compartments (Pitts et al., 1988).

GJIC is probably involved in several fundamental processes, e.g. development, differentiation and growth control (Loewenstein, 1979; Caveney, 1985). It has been considered that highly metastatic cells could avoid inhibitory signals from adjacent cells, thus aiding their malignant phenotype, by reducing or completely abolishing their ability for gap-junctional communication. Previous data have indicated that reduction and/or loss of junctional communication may, in some instances, be correlated with the metastatic phenotype (Hamada et al., 1991). The loss of GJIC has also been postulated to be important in carcinogenesis and in maintaining the transformed phenotype of initiated cells (Loewenstein, 1979; Trosko et al., 1990; Yamasaki, 1990). Many tumor cells exhibit aberrant GJIC among themselves or with normal surrounding cells (Mensil et al., 1993; Budunova and Slaga, 1994).

Furthermore a number of tumor promoters, including *12-0*-tetradecanoylphorbol-13-acetate (TPA), have been shown to decrease GJIC in culture (Yotti et al., 1979; Li et al., 1996) and possibly also in the intact tissue *in*

vivo (Sugie et al., 1987; Mensil et al., 1988; Neveu et al., 1990). Thus there are reasons to believe that GJIC may be involved in cancer development. Hence, the components of intercellular communication have been a focus of recent research.

7.1.2 General structure and composition of Gap junction

Gap junctions are simple aggregates of transmembrane channel particles called connexons. Each connexon is composed of 6 subunits (each subunit is composed of 4-α helices), arranged symmetrically around an axial water-filled channel with a diameter of 1-2 nm.

A number of molecules are implicated in cell-cell communication through the formation of intercellular gap junctions, with integrated involvement in cell adhesion. The identity of the subunit components of connexons remains the target of controversy, the debate revolves around two types of proteins, ductin and connexin (Finbow & Pitts, 1993; Finbow, 1997).

Connexins are a family of proteins ranging in size from 26 kDa to 50 kDa and are expressed in a cell specific manner in a variety of mammalian cells (Willecke et al., 1991). Connexins play a part in gap junction structure and/or control of channel permeability (Beyer et al., 1987; Nicholson et al., 1987; Kistler et al., 1988). Although it is argued that these proteins form the gap junction channel, no connexin has been identified in invertebrates despite the successful isolation of gap junction from such organisms, and evidence lends weight to the

argument that gap junctions consist of ductin rather than the more popularly believed connexins (Finbow and Pitts 1993; Finbow 1997).

The ductin polypeptide forms a membrane-membrane channel independent of the connexin. Ductin has been identified as a target for papillomaviral oncoproteins (Finbow et al., 1995). Thus, the study of ductin is of particular interest to our work.

7.1.2.1 Ductin

Ductin is a polypeptide with a molecular mass of 16 kilodalton. It is very hydrophobic and is thought to contain four transmembrane segments arranged as a four α-helical bundle (Finbow et al., 1992), which varies in length between species from 155-165 residues (Finbow and Pitts 1993; Finbow et al., 1995). The mass and primary structure of ductin are therefore highly compatible with the predicted structure of the connexon subunit polypeptide.

It was first found to be the major protein component of a connexon-like channel of gap junctions from both vertebrate and invertebrate sources (Finbow et al., 1993). Antibodies to invertebrate ductin bind to isolated gap junctions (Buultjens et al., 1988) and gap junctional regions in tissue section (Leitch and Finbow 1990). GJIC has been inhibited by the injection of anti-ductin antibodies into mammalian and invertebrate cells (Finbow et al., 1993). Likewise, the lipophilic reagent N,N'-dicyclohexylcarbodiimide, which reacts specifically with a

conserved glutamic acid residue in the fourth transmembrane domain of ductin, also blocks GJIC (Finbow et al., 1992 and 1993). These data show ductin to be involved in gap junctional communication and together with the structural studies (Holzenburg et al., 1993), make ductin a fitting and likely component of the gap junction channel (For a more extensive review see Finbow and Pitts, 1993).

Ductin has also been identified as subunit c of the membrane sector (V_0) of the H+- ATPase (V-ATPase) (Mandel et al., 1988). The V-ATPase is a protein complex made up of ten subunits which consist of two sectors, a cytoplasmic catalytic sector, V_1, that is the site of ATP hydrolysis, and a membrane sector, V_0, which mediates the translocation of protons across the lipid bilayer (for review see Finbow and Harrison, 1997). This V-ATPase is a universal transmembrane proton pump of eukaryotes and is responsible for the acidification of cytoplasmic organelles such as lysosomes, synaptic vesicles and Golgi (Harvey and Nelson, 1992; Nelson, 1992). Ductin provides the pathway for proton translocation in the V_0 sector of the vacuolar H+-ATPase.

7.1.2.1.1 Interaction between viral oncoprotein and ductin

Ductin has been found to complex with a number of viral proteins. The E5 protein of BPV-1 has been shown to interact with the 16-kilodalton ductin via its hydrophobic transmembrane domain (Goldstein et al., 1992a). This interaction is important as it has been shown to be necessary, but not sufficient, for E5

transformation (Kulke et al., 1992; Goldstein et al., 1992b): mutations within the transmembrane region of E5, that maintain the ability of E5 to complex ductin, make the protein non transforming (Sparkowski et al., 1996).

Other viral oncoproteins such as BPV-4 E8, HPV-16 E5, HPV-6 E5 and human T-cell leukaemia virus type 1 $p12^{I}$ also bind to ductin (Conrad et al., 1993; Franchini et al., 1993; Faccini et al., 1996). This suggests that ductin is a common cellular target for these viral oncoproteins, and underlines the important role that ductin plays in the cell.

The binding of E5 or E8 protein to the gap junction form of ductin appears to lead to disruption of gap junctional intercellular communication, as demonstrated for HPV-16, and BPV-4 (Oelze et al., 1995; Faccini et al., 1996).

Complex formation between E5 and the vacuolar H^{+}-ATPase form of ductin is likely to lead to the inhibition of the acidification of endosomes and to the consequent disruption of cellular protein processing and sorting; this would result in the retention and recycling of undegraded EGF receptors from endosomal compartments (Straight et al., 1995), providing a possible explanation for the observed co-operation between HPV-16 and epidermal growth factor receptors in transformation (Banks and Matlashewski, 1993). BPV-1 E5 interacts directly also with growth factor receptors, and different receptors provide a target for E5 interaction depending on cell context (Goldstein et al., 1992a; Cohen et al., 1993; Petti and DiMaio, 1994). Thus the EGF receptor, PDGF receptor and CSF-1

receptor all show stimulation in BPV-1 E5 transformed cells. Therefore, the mitogenic response is potentiated directly by E5 through its interaction with the activation of growth factor receptors, and indirectly by E5 complexing ductin, inhibiting vacuolar H+-ATPase function and/or recruitment to endocytotic vesicles, and so inhibiting receptor down-regulation. It has not yet been determined whether BPV-4 E8 can interact with the ATPase form of ductin or inhibit acidification of vacuoles.

As discussed above, ductin is the major component of a connexon channel of gap junctions, and antibodies that bind to ductin block GJIC (Serras et al., 1988; Finbow and Pitts, 1993). Furthermore, binding of HPV-16 E5, and BPV-4 E8 proteins to ductin, appears to block GJIC (Oelze et al., 1995; Faccini et al., 1996).

To examine the correlation between ductin binding, down regulation of GJIC and cell transformation, the panel of E8 mutants and chimeras described in chapters 3 was analysed for ductin binding, and PalF transformants were assayed for functionality of gap junctions.

7.2 Experimental procedure

7.2.1 Interaction of BPV-1 HAE8, its mutants, and BPV-1 HAE5 with ductin

To examine their interactions with ductin, co-immunoprecipitations of *in vitro* translated E8 wild type, mutants and BPV-1 E5 by monoclonal antibody against HA and a polyclonal antibody raised against 16k ductin were performed. The immunoprecipitated proteins were separated by SDS-PAGE (14% polyacrylamide) and visualized by autoradiography (as described by Faccini et al., 1996). This analysis was conducted by Dr A. M. Faccini.

7.2.2 Gap Junctional Intercellular Communication (GJIC) Measurement

Gap junction mediated cell to cell communication or junctional coupling can be detected and quantified *in vitro* by visualization of cell to cell transfer of a fluorescent probe (such as Lucifer Yellow) microinjected into a single cell. The dye passes through gap junctions but not across the non-junctional membrane (Pitts and Kam, 1985; Pitts et al., 1988).

Dye transfer

In this experiment the level of GJIC was kindly examined by Dr John Pitts, by microinjection of Lucifer yellow CH.

Cells were grown in 60mm tissue culture dishes until 80-100% confluent (in contact). Immediately prior to the dye injection the medium was removed and replaced with fresh culture medium. The cells were then transferred to a

37°C microscope stage and the lid of the dish removed. An individual cell was selected and injected with dye using micro electrodes made from "kwik-fill" thin-wall glass capillaries which were filled with 10μl of 4% Lucifer Yellow CH as described by Pitts and Kam (1985). The cells were injected with dye, and the number of fluorescent cells was counted over a 2 minute period using a current of 10nA in 0.5 second pulses at 1Hz and the process monitored using a Leitz Diavert inverted microscope with UV (epi-illumination) or visible (phase contrast) light sources. The extent of dye spread to the neighbouring cells was recorded (Table7.1) and photographed immediately. Untransfected PalF cells were assayed for the baseline GJIC level.

7.3 Results and Discussion

7.3.1 Mutational analysis of complex formation

Figure 7.1 shows that ductin forms stable complex with HAE8, its mutants including N17S, N17Y, N17A, E8T, and with BPV-1 E5. Ductin also interacts with E8N-E5C (not shown). However, the interaction between the reciprocal chimera E5N-E8C and ductin is still unclear.

It has been shown that binding of BPV-1 E5 to ductin requires the N-terminal hydrophobic domain and that glutamine at position 17 is important for ductin interaction and for the transforming function of the protein (Goldstein et al., 1992a, b; Sparkowski et al., 1994, 1996). We decided to test whether the

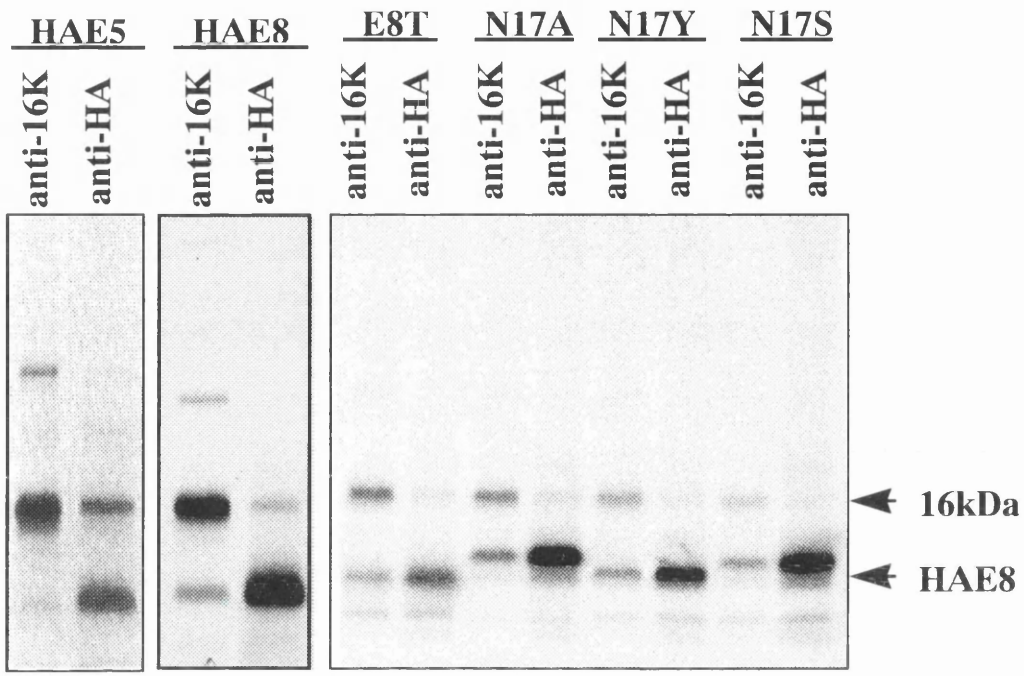


Figure 7.1 ***In vitro*** **association of ductin with E8 wild type and its mutants.**

Co-immunoprecipitation of *in vitro* translated E8/E5 wild type, and examples of E8 mutants by monoclonal antibody against HA (for HA tagged E8/E5) and a polyclonal antibody raised against 16k ductin. The immunoprecipitated proteins were separated by SDS-PAGE (14% polyacrylamide) and visulized by autoradiography (as described by Faccini et al., 1996). This analysis was conducted by Dr A. M. Faccini.

equivalent residue 17, asparagine, in the E8 polypeptide is also crucial for ductin binding and cell transformation.

Results from these experiments show that the C-terminal hydrophilic domain of this protein is not necessary for binding to ductin. Changing asparagine 17, to serine, tyrosine, and alanine did not affect the level of binding to ductin. Therefore, unlike BPV-1 E5, this residue does not appear to be crucial for binding to ductin. As described in chapter five, mutation of this single amino acid to a tyrosine residue abolishes the transforming capacity of E8, while mutation to an alanine residue produces a hypertransforming molecule with increased ability to induce anchorage independent growth, and mutation to a serine residue leads to an intermediate state that allows only the maintenance of the anchorage independent growth.

Taken together, these data suggest that the ability of BPV-4 E8 to bind 16k ductin *in vitro* does not correlate with cell transformation.

7.3.2 Gap Junctional Intercellular Communication (GJIC) and BPV-4 E8 wild type, its mutants, and BPV-1 E5

We have previously shown (Faccini et al., 1996) that there are no detectable differences between E8 and HAE8 regarding binding to ductin and down-regulation of GJIC. Thus it is expected that the presence of the HA epitope in the E8 mutants will not affect their behaviour.

Transfectant cells were compared to non-transfected parental PalF cells and to control PalF cells lacking E8 but harbouring all the other oncogenes (Table 7.1). Untrasfected PalF cells showed extensive dye spread to an average 28.3 cells (Table 7.1; Figure 7.2). PalF cells expressing E8 showed a marked reduction in gap junction intercellular communication in two independent clones (Table 7.1; Figure 7.2). The same was observed with wild type BPV-1 E5 (Figure 7.2), whereas PalF cells not expressing only E8 showed control levels of GJIC (Table 7.1; Figure.7.2).

These results show that BPV-4 E8/BPV-1 E5-expressing PalF cells down-regulate gap junction intercellular communication. To our knowledge the down-regulation of GJIC by BPV-1 E5 has not been reported previously, and this is the first time that loss of GJIC with BPV-1 E5 has been shown.

In contrast, immortalization of PalF cells with HPV-16 E6, BPV-4 E7, and activated *ras* was not accompanied by a disruption of gap junctional communication (Table 7.1) and these cells communicated well. This indicates that sustained loss of junctional communication is not a necessary feature of immortalization of this cell type, and that morphological transformation alone is not sufficient to cause the loss of GJIC observed in E8-expressing PalF (Table 7.2; Figure 7.2).

Mutational analysis of E8 showed that only E8N17A down-regulated GJIC; although all the other mutants bound to ductin *in vitro*, the cells expressing

them showed control levels of GJIC (Table 7.1 & 7.3; Figure 7.2). These data indicate that the ability of BPV-4 E8 to bind 16k ductin is not sufficient for down-regulation of GJIC (Table 7.3). Although the mechanism underlying GJIC down-regulation has thus far not been elucidated, there might be two possibilities: firstly the binding to 16k has been observed only *in vitro*, and it is not known whether any of the E8 proteins bind *in vivo*; secondly the effect of mutant forms of E8 is different from the wild type.

As described earlier in chapter five, cells expressing E8N17S and E5N-E8C showed anchorage independent growth, and, as shown in chapter six, low levels of trans-activation of the cyclin A promoter were observed with these mutants. The respective transfectants displayed normal level of GJIC (Table 7.1; 7.4). Therefore, down-regulation of GJIC, anchorage independent growth and cyclin A promoter activation are independent functions.

As shown in chapter five, PalF cells expressing BPV-4 E8 continue to proliferate in low serum and are capable of forming foci; of the mutants, only E8N17A allowed cells to grow in low serum and form foci; this is the only E8 mutant that can down-regulate gap junction intercellular communication (Table 7.4). These data suggest that down regulation of GJIC correlates with proliferation in low serum, and with focus formation. However it must be pointed out that cells expressing BPV-1 E5 showed reduced GJIC but were unable

to form foci. Thus this relationship could not provide direct evidence that down-regulation of GJIC involved in the forming foci.

7.4 CONCLUSIONS

Mutational analysis of E8 suggests that;

The ability of E8 to bind 16k ductin does not correlate with cell transformation, these results concur with those obtained previously with mutational analysis of BPV-1 E5 in which E5 mutants still capable of complexing ductin had lost the ability to transform cells (Goldstein et al., 1992b; Sparkowski et al., 1996).

PalF cells expressing BPV-4 E8 block gap junction intercellular communication.

Morphological transformation alone is not sufficient to cause the loss of GJIC observed in E8-expressing PalF.

Loss of junctional communication is not a necessary feature of immortalization of this cell type

The ability of BPV-4 E8 to bind 16k ductin is not sufficient for down-regulation of GJIC.

Down-regulation of GJIC, anchorage independent growth, cyclin A promoter activation and focus formation are independent functions.

Down regulation of GJIC correlates with proliferation in low serum.

All these results are summerized in table 7.4

Table 7.1 Levels of gap junctional intercellular communication (GJIC) in control PalFs (untransfected PalF cells) and each separate clone of PalF transfectants

Cell Line	Description	No. of injection	Mean no. cells dye spread to ± S.D.
PalF normal	Untransfeted PalFs	20	28.3 ± 7.40
$E7_1$	Control (no E8)	20	12.8 ± 3.57
E7a		20	34.5 ± 9.31
E7g		20	30.6 ± 10.03
$E5_1$	BPV-1 E5	20	2.8 ± 2.19
$E5_d$		20	3.9 ± 1.25
E8a	BPV-4 E8 wild type	20	4.6 ± 2.32
E8b		20	6.25 ± 4.07
E8b		20	5.15 ± 2.60
Mut. 1a	N17S	20	29.05 ± 8.51
Mut. 1e		20	12.7 ± 5.34
Mut. 1c		20	46.75 ± 5.42
Mut. 2f	N17Y	20	35.4 ± 10.24
Mut. 2g		20	23.05 ± 9.49
Mut. 3a	N17A	20	4.8 ± 1.63
Mut. 3a		20	3.45 ± 1.62
Mut. 3b		20	5.1 ± 1.26
Mut. 3b		20	3.75 ± 1.60
Mut. 3f		20	3.85 ± 1.78
Mut. 4a	E8T	20	16.87 ± 6.13
Mut. 4f		20	46.75 ± 5.42
Mut. 6a	E8N-E5C	20	24.5 ± 6.6
Mut. 6e		20	30.4 ± 9.08
Mut.7b	E5N-E8C	20	37.7 ± 8.7
Mut 7u		20	8.2 ± 3.65
E5Ta	E5T	20	40.1 ± 8.19
E5Tb		20	31.35 ± 7.40

Table 7.2 Summary of the levels of gap junctional intercellular communication (GJIC) in control PalFs and PalF transfectants: Comparison with morphological transformation.

Cell type	Morphological transformation	GJIC mean[a] ± S.D.
PalF (control)[b]	-	28.3 ± 7.40
pZipneoE7	+	25.96 ± 12.45
pZipneoHAE8[c]	+	5.33 ± 3.12
N17S	+	29.5 ± 15.38
N17Y	+	29.225 ± 11.69
N17A	+	4.19 ± 1.73
E8T	+	31.81 ± 16.04
E8N-E5C	+	27.45 ± 15.82
E5N-E8C	+	22.95 ± 15.91
HAE5[d]	+	3.35 ± 1.84
E5T	+	35.72 ± 8.98

+ = positive

All transfectants contained BPV-4 pZipneo E7, activated *ras* and HPV-16 E6

[a] Mean numbers of fluorescent coupled cells ± standard deviations were calculated from table 7.1

[b] Refers to parental PalF cells

[c] Refers to BPV-4 pZipneoHAE8

[d] HAE5 = BPV-1 pZipneoHAE5

Table 7.3 Summary of comparison between GJIC and ductin binding with E8 wild type, mutants and BPV-1 E5.

Cell type[a]	GJIC down regulation	Binding to 16k ductin (*In vitro*)
Control (no E8)	-	-
E8 wild type	+	+
N17S	-	+
N17Y	-	+
N17A	+	+
E8T	-	+
E8N-E5C	-	+
E5N-E8C	-	?
BPV-1 E5	+	+
E5T	-	+

[a] All cell lines contain BPV-4 E7, activated *ras* and HPV 16-E6

+ = Positive

- = Negative for down regulation of GJIC / ductin binding

Table 7.4 Summary of transformation characteristics of cells expressing E8 or its mutants

Cell type	A.I. growth	LS growth	Focus formation	Cyclin A activation HS LS	Binding to 16k ductin (*In vitro*)	GJIC down regulation
Control (no E8)	-	-	-	- -	-	-
E8 wild type	+	+	+	++++/+++	+	+
E8 N17S	+	-	-	+ -	+	-
E8 N17Y	-	-	-	- -	+	-
E8 N17A	++	+	+	+++ ++	+	+
E8T	-	-	-	- -	+	-
E8N-E5C	-	-	-	- -	+	-
E5N-E8C	+	-	-	+ +	?	-
BPV-1 E5	+	+	-	++++ ++	+	+
E5T	-	-	nd	+/- -	+	-

All cell lines contain BPV-4 E7, activated *ras* and HPV 16-E6.

A.I., anchorage independent growth; HS = high (10%) serum; LS = low (0.5%) serum.

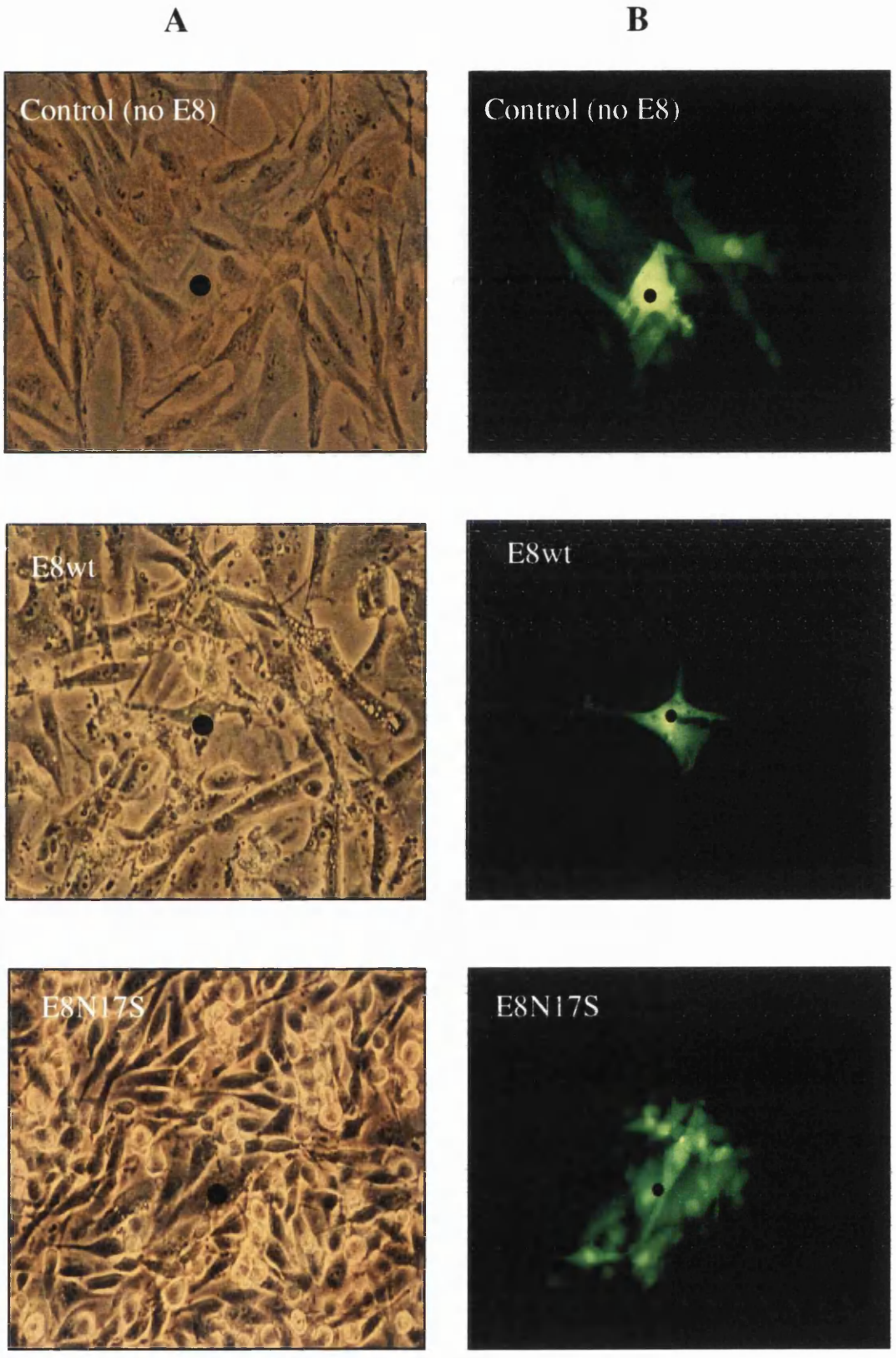
The + represent the extent of expression of the characteristic.

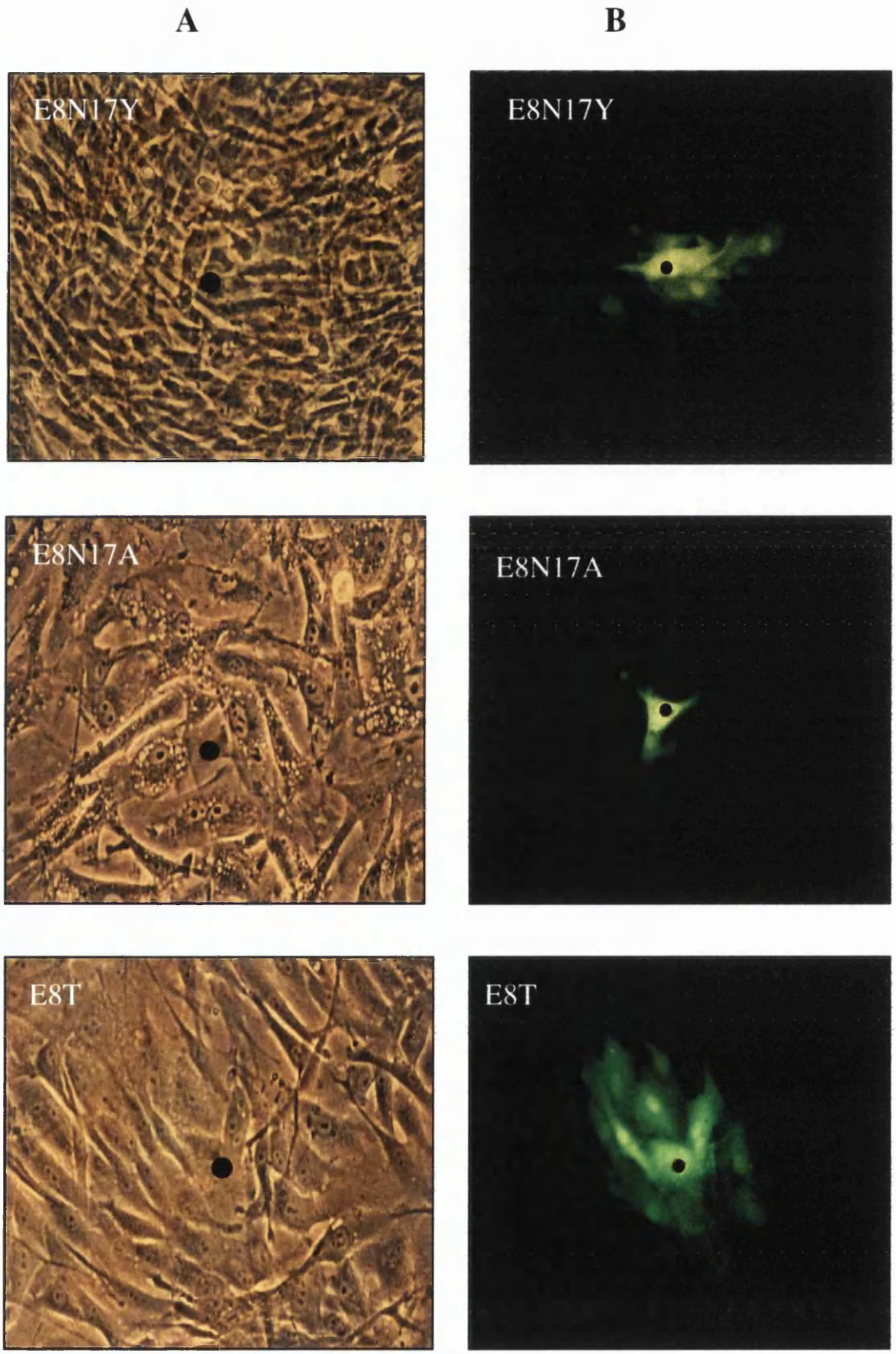
Figure 7.2 Down-regulation of GJIC in virally-PalF transfectants as assayed by dye transfer analysis.

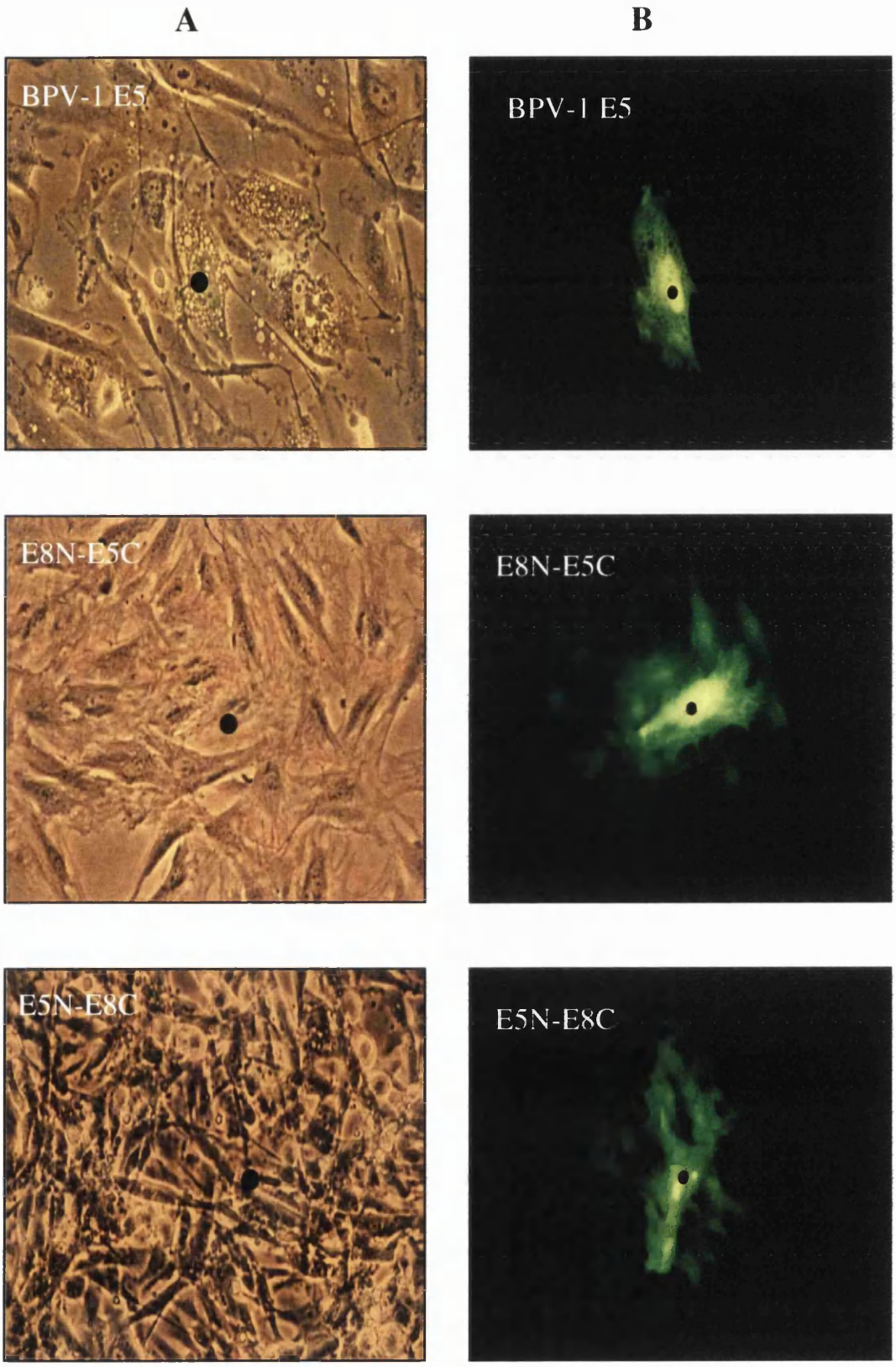
Control cells (no E8) were transfected with pZipneoE7, pT24ras, and pJΩ-16E6, while other cells were transfected additionally with an E8 wild type or mutant forms of E8 as noted in each panel.

The microinjected cell is marked by a black dot.

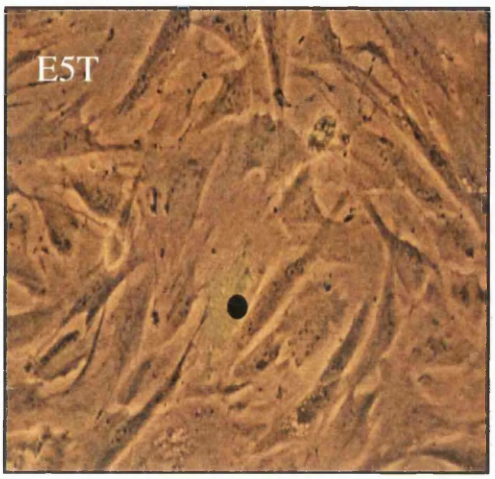
A is phase contrast and B is fluorescence micrographs.


A
B
Control (no E8)
Control (no E8)
E8wt
E8wt
E8N17S
E8N17S

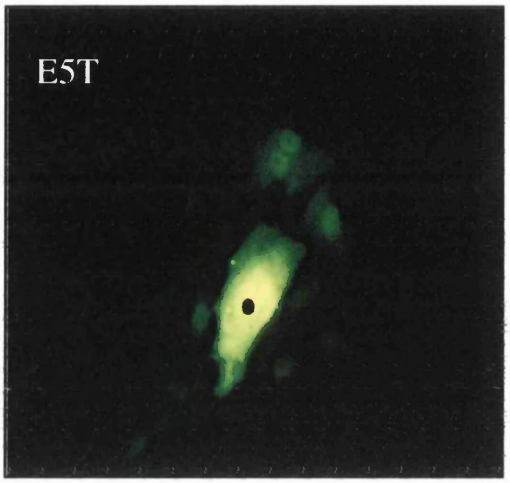

A
B
E8N17Y
E8N17Y
E8N17A
E8N17A
E8T
E8T


A
B
BPV-1 E5
BPV-1 E5
E8N-E5C
E8N-E5C
E5N-E8C
E5N-E8C

A



B



CHAPTER EIGHT

DISCUSSION

8.1 Introduction

Bovine papillomavirus type 4 induces papillomas of the upper alimentary canal of cattle, which can progress to malignancy in animals eating bracken fern (Jarrett et al., 1978; Campo et al., 1980 and 1994a, b). BPV-4 alone, when transfected into PalF cells *in vitro*, is non transforming. The transforming potential of BPV-4 is only realised when BPV-4 DNA is cotransfected into PalF cells with an activated *ras* gene. These partially transformed PalFs have an extended proliferative life span, are capable of anchorage independent growth but are not immortal or tumorigenic in nude mice (Jaggar et al., 1990).

The transforming genes of BPV-4 are the E7 and E8 open reading frames. E7 is one of the transforming gene of BPV-4, in that, in co-operation with activated *ras*, it induces morphological transformation of PalF cells in the absence of other viral genes. Nevertheless, although morphologically transformed, E7 expressing PalF cells are not capable of growing in suspension, thus showing that other viral gene(s) encode function(s) that confer anchorage independent growth. Previous work has shown that this property is dependent on the presence of the E8 ORF (Pennie et al., 1993).

The E8 ORF encodes a small, 42 amino acid long protein that is composed of two domains: a very hydrophobic region, theoretically capable of forming a transmembrane α-helix, encompassing the first 30 amino acids of the protein, and a second region of mainly hydrophilic amino acids comprising the C-terminal 12 residues. It is localised to the cell membranes (Pennie et al., 1993). *In vivo*, E8 expression is limited to the basal and suprabasal layers of early papillomas, i.e., not in areas of vegetative viral DNA replication, with decreased expression in late stage papillomas (Anderson et al., 1997); E8 is therefore a true early protein.

In addition, E8 binds to 16k ductin *in vitro* and down-regulates gap junction intercellular communication (GJIC) in primary bovine fibroblasts (Faccini et al., 1996).

When expressed in an established murine fibroblast cell line (NIH-3T3) E8 is transforming by itself; E8-3T3 cells can grow in low serum and in suspension, and these cellular phenotypes are associated with the ability of E8 to trans-activate the cyclin A gene promoter, to increase cyclin A protein level and cyclin A associated kinase activity and to inhibit the function of the negative regulator of cell cycle, $p27^{Kip1}$ (O'Brien and Campo, 1998).

The work described in this thesis extended the analysis of the functions of BPV-4 E8 in PalF cells and investigated cell behaviour in relation to: morphological transformation, anchorage independent growth, focus formation, cell population growth in low serum, tumorigenicity in nude mice, trans-activation of the cyclin A promoter, binding to ductin and down regulation of GJIC. To

segregate the different transforming functions of E8 and therefore to define critical residues and its functional domains, we also tested a panel of E8 mutants and chimeras constructed with BPV-1 E5 for their transforming ability in PalF cells.

8.2 Cell transformation by the BPV-4 E8, its mutants, and BPV-1 E5 gene *in vitro*

As previously reported (Pennie et al., 1993), results obtained in this study demonstrated that PalF cells expressing BPV-4 E8 can grow in suspension culture (Figure 5.1a), the same was also observed for BPV-1 E5 transformed cells (Figure 5.1b). This demonstrates that E8 overrides the control mechanisms that arrest anchorage-dependent cells in late G_1 phase of cell cycle when maintained in suspension (Guadagno and Assoian, 1991). In addition to anchorage independent growth, PalF cells expressing E8 can grow in low serum and escape contact inhibition. PalF cells which do not express E8 do not show any of these phenotypes, despite the presence of BPV-4 E7, HPV-16 E6 and activated *ras*.

Although PalF cells expressing E8 were capable of anchorage independent growth, these transformed cells were not tumorigenic in nude mice (section 5.1.4). This result concurs with the study of Pennie et al (1993) and suggests that additional factors are needed for full transformation of PalF cells, thus mimicking the natural history of upper alimentary canal carcinoma in cattle, where the progression of BPV-4 induced papillomas to carcinomas is co-factor dependent.

The contribution of E8 to transformation cannot be assessed in isolation as cell lines cannot be derived from cells transfected with E8 in the absence of E7 (Jaggar et al., 1990; Pennie et al., 1993). Our results also show that BPV-1 E5 like E8 needs E7 to allow expansion of PalF cell lines.

Conversely, unlike PalF cells, when E8 is expressed in an established rodent fibroblast cell lines (NIH-3T3) it is transforming (O'Brien and Campo, 1998). Interestingly, previous studies have also shown that BPV-1 E5 is capable of transforming established mouse fibroblasts and keratinocytes in the absence of other viral gene products (Leptak et al., 1991). Also, transformation of established cells with BPV-4 genes does not require co-transfection with activated *ras* (Smith and Campo, 1988). Therefore, the survival of established cells expressing E8 or BPV-1 E5 alone and the requirement of E8/E5 in combination with E7 in PalF cells may be due to inherent differences between established and primary cells.

In established NIH-3T3 fibroblast cells it has been shown that a member of the cell cycle inhibitor family, $p16^{INK4a}$, that induce G_1 arrest (for review see Hunter and Pines, 1994) is lost (Quelle et al., 1995). Loss of $p16^{INK4a}$ promotes cell proliferation and this event results in cell immortalization which may provide optimal conditions for the action of E8 in NIH-3T3 cells.

The absolute requirement for an intact E7 ORF for transformation of PalF cells by E8 is probably due to the inactivation of the tumor suppressor p105 Rb by E7. Although the interaction between BPV-4 E7 and p105 Rb has not been

directly demonstrated, the presence in E7 of a putative pRb binding domain and the observation that deletion or mutation of this domain abrogate cell transformation, strongly suggest that E7 interacts with and inactivate p105 Rb (Jaggar et al., 1990; Pennie et al., 1993; Jackson et al., 1996).

Studies on BPV-1 E5 have been shown that glutamine at position 17 is critical for cell transformation (Sparkowski et al., 1994; Sparkowski et al., 1996). This appears to be the case for the aspargine residue in the same position in BPV-4 E8. Our analysis of E8 mutants show that mutation of this single amino acid produces profound changes in the activity of the protein. Mutation to a tyrosine residue completely abolishes the transforming capacity of E8, while mutation to an alanine causes a hypertransforming molecule with a greater ability of inducing anchorage independent growth (Figure 5.1a), and mutation to a serine residue leads to an intermediate state that allows only the maintenance of the anchorage independent growth.

These results show that the residue is critical for the biological activities of BPV-4 E8 although its role is unknown. It is important to note that substituting serine at position 17 in BPV-1 E5 produces a hypertransforming protein (Sparkowski et al., 1994), while the same substitution in BPV-4 E8 actually decreases the transformation potential of the protein.

Studies with mutant forms of E8 also suggest that the hydrophilic C-terminal tail of the protein is required for the transformating activity of this protein. The truncated E8 mutant (E8T) is transformation defective. Supportive

data that the C-terminal tail of E8 is essential for PalF transformation are provided by the chimera E8N-E5C, containing of the hydrophobic domain of E8 and the hydrophilic tail of E5, which is incapable of inducing a transformed phenotype in PalF cells. Both the membrane domain of E8T and the E8N-E5C chimera may adopt an altered non functional conformation or have a different cellular distribution to wild type E8.

Clearly the C-terminus hydrophilic tail of E8 is necessary for cell transformation and cyclin A promoter activation. This is confirmed by the preliminary studies conducted with forms of E8 mutated in the putative CKII phosphorylation site present in the tail. This will be discussed later in section 8.3.

The transformation defective property of E8N-E5C also points to the non equivalence of the domains of E8 and E5; while the tail of E5 cannot substitute for the tail of E8, the reciprocal exchange leads to a chimeric molecule (E5N-E8C) still retaining the ability to induce anchorage independent growth.

The mutant forms of E8 show that the several transforming functions can be segregated. The ability to induce anchorage independent growth can be separated from the ability of forming foci, or of growth in low serum. Focus formation and growth in low serum still co-segregate in the E8 mutants, but, interestingly, not in BPV-1 E5, which cannot induce escape from contact inhibition, while allowing growth in low serum (Table 7.4).

Altogether, the mutational analysis of E8 indicate that the multiple transforming functions of E8 can be segregated, which probably reflects the

complex interactions of the domains of E8 with different cellular targets such as growth factor receptors.

8.3 The ability to grow in suspension correlates with trans-activation of the cyclin A promoter

It has been shown previously that BPV-4 E8, when expressed in NIH-3T3 cells is capable of transactivating an exogenous (human) cyclin A promoter and can deregulate expression of endogenous cyclin A and associated kinase activities and also inhibit the functions of the negative regulator of cell cycle $p27^{Kip1}$ (O'Brien and Campo, 1998). The authors proposed that deregulated expression of cyclin A and its associated kinase activities may underlie the phenotype of BPV-4 E8 transformed NIH-3T3 cells, as E8-NIH-3T3 cells were capable of growth in low serum and in suspension.

In PalF cells expressing E8, as in NIH-3T3 cells, the cyclin A promoter was trans-activated under conditions (low serum) where cyclin A transcription was normally diminished or was not detectable in control cells. Therefore, the normal mechanisms which restrict cyclin A transcription have been lost in PalF cells expressing E8 (Figures 6.1a, 6.2, and 6.3).

The delineation of the mechanism by which E8 achieves this activation of the cyclin A promoter will require more detailed studies. Repression of cyclin A transcription in cells in low serum or suspension culture has been attributed to $p27^{Kip1}$ via its ability to block cyclin E-CDK2 kinase activity which is required for

de-repression of the cyclin A promoter through a variant E2F site (ZerfassThome et al., 1997). The intracellular concentration of $p27^{KIPI}$ is a major element of cell cycle progression through the G1 restriction point (Coats et al., 1996) and has emerged as a key target for viral oncoproteins. It has recently been reported that both human Adenovirus E1A and HPV-16 E7 proteins can inactivate $p27^{KIPI}$ by forming complexes which make $p27^{KIPI}$ non-functional *in vitro* and *in vivo* (Mal et al., 1996; ZerfassThome et al., 1996). In the case of E1A, the interaction appears direct, at least *in vitro*, while for E7 the presence of a bridging protein is required for complex formation. Such an interaction is unlikely to occur for E8 as its expression is restricted to cellular membranes (Pennie et al., 1993) and may not be available to interact directly with $p27^{KIPI}$ which is located mainly in the cell nucleus (Reynisdottir and Massague, 1997).

The regulation of $p27^{KIPI}$ expression is disrupted by E8 suggesting that $p27^{KIPI}$ is non functional in NIH-3T3 cells expressing BPV-4 E8 as elevated level of $p27^{KIPI}$ do not correlate with cell cycle exit (O'Brien and Campo, 1998).

Building on this information we suggest that in PalF cells, as in NIH-3T3 cells, E8 may act, in part, by abrogating $p27^{KIPI}$ function without promoting degradation of this CDK inhibitor. Further work is required to test this possibility.

We have extended our analysis of BPV-4 E8 functions in primary bovine cells to test for a correlation between promoter activation and cell transformation produced by the different E8 forms as described in chapters five.

Analysis of E8 mutants, indicate that the asparagine residue at position 17 is critical for the ability of E8 to trans-activate the cyclin A promoter, as mutation of this single amino acid produces profound changes in the activity of the protein. Mutation to a tyrosine residue abolishes the cyclin A promoter trans-activation, mutation to a serine residue leads to low promoter activity, but significantly above control, and mutation to an alanine produces promoter trans-activation activity comparable to E8 wild type.

Another critical domain of E8 is its hydrophilic tail which is required for the cyclin A promoter trans-activation and for transformation. Neither E8T or E5T are capable of promoter trans-activation, nor is the E8N-E5C chimera. On the contrary E5N-E8C trans-activates the cyclin A promoter slightly, but significantly above background (Figure 6.2). These results indicate the importance of the intact hydrophilic tail of E8 for cyclin A promoter trans-activation.

In this study, we conclude that the ability of BPV-4 E8 to trans-activate the cyclin A promoter correlates with the ability to induce anchorage independent growth. We also show that mutations which selectively abolish this activity cause a defect in transforming activity. These findings agree with the studies that the ability of E8 to transform NIH-3T3 cells, as assessed by anchorage independent growth, is associated with trans-activation of the cyclin A promoter (O'Brien and Campo, 1998).

The data obtained in co-transfection experiments with cyclin A reporter plasmid and expression plasmid for E8 mutant A15P-A20P showed that changing the alanine residues at positions 15 and 20 to a proline, which has the property of forcing a bend in the main chain and of disrupting an α-helix, abolished cyclin A promoter trans-activation. Mutation of the same alanine residues to glycine, a conservative mutation which should not disrupt an α-helical conformation, in mutant A15G-A20G leads to low promoter activity but still significantly above background (Figure 6.3). These data suggest that substitution of two amino acids could alter the α-helix conformation of the hydrophobic domain with consequences for cyclin A promoter activation and cell transformation and also emphasise the importance of the nature of these residues for the E8 functions.

Results from co-transfection experiments also show that in E8 the putative CKII site appears to be critical for the biological activity of this protein, as mutation of threonine at position 36, in the putative CKII site, produces profound changes in the exogenous cyclin A promoter trans-activation. Thus the E8 mutant with an alanine residue at position 36, which cannot be phosphorylated, shows less cyclin A promoter transactivation than the mutant with the negatively charged asparatic acid residue or with a serine residue in both high and low serum. Mutation to a serine, which maintains the putative CKII site, shows more cyclin A promoter activity than either aspartic acid or alanine (Figure 6.3) in both high and low serum. Therefore, these data point to the possibility that BPV-4 E8 is phosphorylated by CKII and that this phosphorylation has an effect on the

biological activities of this protein as confirmed by loss of transformation by E8T and loss of the cyclin A promoter activation.

8.4 BPV-4 E8 proteins bind ductin and disrupts GJIC in PalF cells but this interaction is not sufficient for down regulation of GJIC

The E8 polypeptide of BPV-4 has homology in length, hydrophobicity, cellular membrane localization and putative α-helix structure with BPV-1 E5 (Burkhardt et al., 1989; Jackson et al., 1991; Pennie et al., 1993). Due to the similarities between the BPV-4 E8 and BPV-1 E5 proteins, it was proposed that these two proteins may share some common function(s).

The E5 polypeptide of BPV-1 has been shown to interact with a number of cellular proteins, including growth factor receptors (Martin et al., 1989; Petti et al., 1991) and a 16 kDa ductin protein (Goldstein and Schlegel, 1990), identified as a component of gap junctions and of the vacuolar ATPase (Finbow et al., 1991; Holzenburg et al., 1993). Other viral oncoprotein such as HPV-16 E5, HPV-6 E5 and human T-cell leukaemia virus type 1 $p12^{I}$ also bind to ductin (Conrad et al., 1993; Franchini et al., 1993). This suggests that ductin is a common cellular target for these viral oncoproteins, and underlines the important role that ductin plays in the cell.

Complex formation between E5 and the vacuolar H+-ATPase form of ductin may result in altered endocytosis pH which favours prolonged ligand-receptor interaction. Thus a growth factor receptor would be actively signalling

for longer and this may contribute to cell transformation. Furthermore, by interfering with 16 k ductin in gap junctions, effective cell-cell communication may be reduced sufficiently for the E5 expressing cell to escape proliferative control signals provided by neighbouring cells (Campo, 1992).

The similarity between BPV-4 E8 and BPV-1 E5 proteins and the proposal that these two oncoproteins may share some common function(s) led to the hypothesis that E8 would bind ductin. Indeed we have shown that this is the case and that *in vitro* E8 interacts directly with ductin (Faccini et al., 1996). Therefore ductin may be a target for E8 protein, *in vivo*, during cell transformation.

Ductin is a structural component of gap junctions. Gap junctions are channels for small molecular weight secondary messengers, important in the homeostatic control in a tissue (Holder et al., 1993): if a transformed cell is released from the control of the surrounding normal cells, it can proliferate freely and give rise to an expanding transformed clone.

Our study shows a marked loss of GJIC in PalF cells transformed by BPV-4 E8 and that this loss depends on the expression of E8 ORF. We found the same for BPV-1 E5, and this is the first time that BPV-1 E5 has been shown to down-regulate GJIC. Transformation is often associated with a loss of GJIC (Mensile and Yamasaki, 1993). However morphological transformation of PalFs by E7 has no significant effect on GJIC (Table 7.1; Figure 7.1). Therefore morphological transformation alone is not sufficient to cause the loss of GJIC observed in E8/E5-expressing PalFs. As loss of coupling is dependent on the expression of E8, a

possible mechanism for the marked reduction in gap junctional communication could be the binding of the E8 polypeptide to ductin. It is interesting that HPV-16 E5, which has been shown to bind to ductin (Conrad et al., 1993), when expressed in an established cell line also inhibits GJIC and does so to an extent similar to that found in this study (Oelze et al., 1995). This implies a common mechanism in that these two proteins, E8 and E5, interfere with GJIC through interaction with ductin.

The lack of GJIC in papillomavirus transformed cells attributable to the viral E5/E8 protein, is probably an early event in transformation. By isolating the newly infected basal and suprabasal cells from the surrounding normal, i.e., non-infected, cells, E8 allows other transformation events to take place. Once the transformed cells have established themselves, continued expression of the oncoprotein is no longer necessary. The expression of E8 only in deep layers of early stage papillomas (Anderson et al., 1997) support this hypothesis.

This proposed model of E8 function would suggest that the E8 protein is critical for the induction of BPV-4 mediated cellular transformation and papilloma development.

Our panel of E8 mutants and chimeras was analysed for their association with ductin and correlation with down regulation of GJIC and cell transformation. Analysis of E8 mutants shows that E8N17Y, E8T, and E8N-E5C maintain the *in vitro* interaction with ductin but are transformation defective. Therefore the ability of E8 to bind 16k ductin does not correlate with cell transformation, in

agreement with results obtained previously with E5. Some mutant forms of E5 maintain the ability to complex with ductin but are non transforming (Sparkowski et al., 1996). This study, therefore, suggests that additional cellular targets might be needed for E8-mediated cell transformation particularly in PalF cells. Activation of growth factor receptors may also be an important aspect of the transformation activity of BPV-4 as increased numbers of EGF receptors are observed in alimentary canal cancer cells (Smith et al., 1987). Further analysis of possible interaction of E8 with other cellular proteins e.g. growth factor receptors would also be of interest. Our previous results led to the hypothesis that the interaction of these viral oncoproteins with ductin causes down regulation of GJIC (Faccini et al., 1996). Thus we investigated the E8 mutants to determine whether 16k ductin binding was sufficient for down regulation of GJIC. Only E8N17A bound 16k ductin and down regulated GJIC; all the other mutants bound to ductin but showed levels of GJIC comparable to control cells (Table 7.1).

These data show that the ability of BPV-4 E8 to bind 16k ductin *in vitro* is not sufficient for down-regulation of GJIC (Table 7.2). The relevance of ductin binding to the *in vivo* studies is still unclear, and also it could be proposed that the effect of mutant forms of E8 is different from the wild type.

Moreover, cells expressing E8N17S and E5N-E8C, capable of anchorage independent growth, displayed normal level of GJIC (Table 7.4). Therefore, these results show that down-regulation of GJIC and anchorage independent growth are independent functions of E8.

Our data suggest a correlation between down regulation of GJIC, and proliferation in low serum: E8N17A cells are capable of both growth in low serum and of down-regulation of gap junction intercellular communication. Cells expressing all the other mutants were incapable of growth in low serum and of focus formation (Table 7.4) and showed normal levels of GJIC. However down-regulation of GJIC can not be correlated with focus formation, at least in the case of BPV-1 E5, as cells that expressed E5 showed reduced GJIC, but were unable to form foci.

8.5 Summary

In this thesis we have further characterised the transforming activities of BPV-4 E8 in PalF cells. In addition to growing in suspension, we show that PalF cells expressing E8 down-regulate GJIC, can grow in low serum and are not contact inhibited. In agreement with the finding in NIH-3T3 cells (O'Brien and Campo, 1998), in PalF cells, E8 expression promotes trans-activation of the cyclin A promoter. Transactivation of the cyclin A promoter correlates with the ability of the PalF cells to grow in suspension. This concurs with the finding of O'Brien and Campo (1998) in that E8 mediated cell transformation is due to the ability of the viral protein to deregulate cyclin A expression and associated kinase activities.

Studies with the mutant forms of E8 show that different transforming function of E8 can be segregated and demonstrate that both the residue at position 17 in the hydrophobic domain and the hydrophilic C-terminal tail of the E8 are

crucial for E8 function. The use of E8 mutants also indicated that the ability of E8 to bind ductin is not sufficient for down regulation of GJIC, and that loss of GJIC and anchorage independent growth are independent functions of E8.

Our co-transfection analysis of additional E8 mutants suggest that substitution of alanine with proline, which is expected to alter the conformation of the hydrophobic domain, may have an effect on cell transformation. Also data obtained in short-term transfection experiments with the other forms of E8 mutants in the putative casein kinase II site support the possibility that BPV-4 E8 might be phosphorylated by CKII and that this phosphorylation could have an effect on the biological activities of this protein.

Despite the overall amino acid, structural, and subcellular localization similarity between E8 and BPV-1 E5 which suggests common functions, there are differences between the two proteins. *In vivo*, E8 is expressed only in the deep layers of papillomas, where little or no viral DNA replication take place (Anderson et al., 1997). This restricted localization in the papillomas is not shared by BPV-1 E5 which is expressed not only in the deep layers but in the differentiated ones as well (Burnett et al., 1992). Therefore, the different location in the papillomas suggests that the two proteins may also have different functions. Also critical amino acids, which are important for dimerization (Horwitz et al., 1988), are either missing, such as the two cysteine residues in the C-terminal domain, or different in E8, such as the presence of possible CKII sites (TRLD) at

positions 36-39 of E8. Thus these distinct amino acid residues in E8 and E5 may contribute to their functional differences.

In agreement, results in this thesis show that there are differences between the two transforming proteins in the PalF system: E8, but not E5, allows cells to escape from contact inhibition, and the same amino acid substitutions have different effects. These observations suggests that these small papillomavirus proteins may have different mechanisms of achieving cell transformation.

8.6 Future work

The results obtained in this thesis show that the ability of E8, its mutants, or BPV-1 E5 to trans-activate the cyclin A promoter in transient transfection experiments was qualitatively similar to those obtained with stable transformants (section 6.2.2). Building on the information obtained from the mutational analysis of E8, we would expect the transformation phenotype to be related to the ability of E8 mutants to trans-activate the cyclin A promoter. Therefore, it would be of particular interest to analyse the transformation status of established lines containing mutants A15G-A20G, A15P-A20P, ARLD, DRLD, and SRLD.

Results obtained by O'Brien and Campo (1998) demonstrated that the ability of E8 to deregulate expression of the cyclin A promoter in NIH-3T3 cells is associated with inactivation of CDK inhibitor $p27^{Kip1}$ as elevated levels of $p27^{Kip1}$ do not correlate with cell cycle exit. It is still unclear whether in PalF cells E8 can do so. We suggest that in PalF cells, as in NIH-3T3 cells, E8 may act by

abrogating $p27^{KIPI}$ function without promoting degradation of this CDK inhibitor. Further work is required to test this possibility.

Normal cells maintained in suspension do not express cyclin A and cannot progress into S phase (Guadango et al., 1993; Fang et al., 1996). Constitutive expression of cyclin A allows NRK fibroblasts to proliferate in suspension (Guadango et al., 1993). It has been shown that E8 in NIH-3T3 cells induces trans-activation of the cyclin A promoter and increases endogenous protein levels in cells maintained in suspension culture (O'Brien and Campo, 1998). It would be interesting to determine whether PalF cells expressing E8 can trans-activate the endogenous cyclin A promoter and give rise to an increase in the level of the endogenous cyclin A protein.

While results in this thesis show that E8 is capable of transforming PalF cells, it is not yet known if these transforming phenotypes are displayed by primary keratinocytes. It would be of great interest to repeat these studies in primary bovine keratinocytes as these are the natural target cell for infection by BPV-4.

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