

Cell transformation by the E5 protein of Bovine Papillomavirus type 4

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Abbreviations

ATP	Adenosine triphosphate
bp	Base pair
BPV	Bovine papillomavirus
BSA	Bovine serum albumin
°C	Degree centigrade
CDK	Cyclin-dependent kinase
CHO	Chinese hamster ovary cells
CIAP	Calf intestinal alkaline phosphatase
CIN	Cervical intraepithelial neoplasia
CKI	cyclin-dependent kinase inhibitor
COS	African green monkey kidney cells
CRPV	Cottontail rabbit papillomavirus
DEPC	Diethyl pyrocarbonate
DM	Dimethyl sulphoxide
DMEM	Dulbecco's modified Eagle's medium
DMEM-10	Dulbecco's modified Eagle's medium plus 10% NBCS or 10% FCS
DMEM-H	DMEM serum free plus 25 mM Hepes (pH 7.5)
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	3' deoxyribonucleoside 5' triphosphate
<i>E.Coli</i>	<i>Escherichia coli</i>
EBV	Epstein-Barr virus
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediamine tetra-acetic acid
EGF	Epidermal growth factor
EGF-R	Epidermal growth factor receptor
EGTA	Ethylene Glycol-bis(β -aminoethyl Ether) N,N,N',N'-tetra-acetic acid
ER	Endoplasmic reticulum
ERK	Extracellular regulated kinases
EtBr	Ethidium bromide
FAK	Focal adhesion kinase
FCS	Foetal calf serum
FITC	Fluorescein-isothiocyanate
g	gram
G418	genetic, G418-sulphate
GFP	Green Fluorescent Protein
GFR	Growth factor receptor
GJIC	Gap junction intercellular communication
GM	Growth medium (10% serum)
HBS	HEPES buffered saline
HBV	hepatitis B virus
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HPV	Human papillomavirus
hr	hour/s
HRP	Horseradish peroxidase
HTLV-1	Human T cell leukaemia virus-1

Ig G	Immunoglobulin G
JNK	Jun N terminal kinase
kb	Kilobase pair
kDa	KiloDalton
l	litre
LCR	Long control region
LS	Low serum (0.5% serum)
M	molar
MAPK	Mitogen activated protein kinase
MEK	MAP kinase kinase/ERK kinase
mg	milligram
min	minute
ml	millilitre
mM	millimolar
MoLV	Moloney murine leukaemia virus
mRNA	Messenger ribonucleic acid
NBCS	New born calf serum
N/D	Not determined
NIH3T3	Swiss mouse embryo fibroblasts
NRK	Normal rat kidney
OD	Optical density (light absorbance)
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PalF	Foetal Bovine Palate Fibroblasts
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD	PD 98059 inhibitor
PDGF β -R	Platelet-derived growth factor β receptor
PE	PBS plus EDTA
PI3-K	Phosphatidyl inositol 3-kinase
<i>ras</i>	refers to the oncogene
Ras	refers to the oncoprotein
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcriptase-polymerase chain reaction
SDS	Sodium dodecyl sulphate
sec	second/s
SV40	Simian virus 40
TBS	Tris-buffered saline
TBST	Tris-buffered saline plus 0.1% Tween
TEMED	N,N,N',N'-tetramethylethylenediamine
TR	Texas red
Tris	Tris (hydroxymethyl) aminomethane
Tween 20	Polyoxyethylene sorbitan monolaurate
U0	U0126 inhibitor
UV	Ultraviolet
V	Volts
v/v	Volume per unit volume
w/v	Weight per unit volume
wt	Wild type

μg	Microgram
μl	Microlitre
μM	Micromolar
%	Percentage
³² P	Phosphorous isotope 32 atom

Single letter DNA nitrogenous base

A	Adenine
C	Cytosine
G	Guanine
T	Thymine

Single letter amino acid code

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

Abstract

The E5 protein of Bovine Papillomavirus type 4 (BPV-4) is, at only 42 amino acids, the smallest transforming protein identified to date. E5 consists of a N-terminal putative α -helical transmembrane body and a hydrophilic C-terminal tail and is localised to cellular membranes. E5 transforms fibroblasts, its expression allows established NIH3T3 fibroblasts to proliferate in suspension and in the absence of mitogens.

Here we analysed the cellular localisation of BPV-4 E5 showing that a form of E5 fused with the green fluorescent protein (GFP-E5) is distributed mainly to the Golgi apparatus and to a lesser extent to the Endoplasmic Reticulum but could not be detected at the plasma membrane.

GFP-E5 was used in transient and stable transfection assays in NIH3T3 cells. These studies revealed that, contrary to E5 wt, GFP-E5 expressing cells are not morphologically transformed, and cannot proliferate in absence of mitogens, however these cells do not exit the cell cycle when maintained in suspension. We concluded that GFP-E5 could segregate the two transforming phenotypes of E5 wt. Here we investigated at what level of the cell cycle machinery the segregation occurs.

Biochemical analysis revealed that GFP-E5 expressing cells do not sustain cyclin D1 and cyclin A expression after serum withdrawal, show an active hypophosphorylated form of pRb and p107 and consequently exit from the cell cycle and become quiescent in these growth conditions. Moreover GFP-E5 ability to promote transcriptional activation of a heterologous cyclin A promoter in normal growth conditions is not maintained in low serum, contrary to E5 wt, and although the cyclin A-associated kinase activity in GFP-E5 cells was 1.5 times higher than the control in low serum, it was clearly not sufficient to sustain cells growth.

However when maintained in suspension GFP-E5 cells, similarly to E5 wt cells, present an up-regulated cyclin D1, can transactivate cyclin A promoter and sustain cyclin A expression level and cyclin A-associated kinase activity. Interestingly only cdc2, but not CDK2, can be detected in cyclin A immunoprecipitates.

The use of inhibitors of the MAPK pathway revealed that in E5 wt cells the level of cyclin D1 and cyclin A expression is independent from this pathway. In GFP-E5 cells, however, cyclin D1 is down-regulated but cyclin A is still elevated after treatment with the inhibitors.

From these data we conclude that GFP-E5 cells ability to grow in suspension correlates with the ability to up-regulate cyclin A expression and cyclin A-associated kinase activity and that the up-regulation is independent from MAPK pathway and cyclin D1 expression level. Moreover, we show here, in both E5 and GFP-E5 cells, that the principal cyclin-dependent kinase associated with cyclin A is cdc2 suggesting that the viral protein may act on the G2/M phase transition of the cell cycle.

CHAPTER 1: INTRODUCTION

1.1 The hallmarks of cancers

After twenty-five years of rapid advances, cancer research has generated a rich and complex body of knowledge, revealing cancer to be a disease involving dynamic changes in the genome (Hanahan and Weinberg, 2000). The observations of human cancer and animal models argue that tumour development proceeds via a process formally analogous to Darwinian evolution, in which a succession of genetic changes, each conferring a type of growth advantage, leads to the progressive conversion of normal human cells into cancer cells (Nowell, 1976; Hanahan and Weinberg, 2000). As the evolution allows the survival of the fittest individual, so too in the case of carcinogenesis, it is those mutations in individual genes which render cells most capable of evading normal homeostatic mechanisms that are selected in cancer cells. Developing cancer cells selected mutations having two basic functions: mutations in genes, called oncogenes, that increase the activity of the proteins they encode for, or mutations which inactivate the function of tumour suppressor genes. A successful cancer cell, predominantly of epithelial origin, is required to have mutations in at least four to seven genes (Cahill et al., 1999). Because the probability of a single cell simultaneously acquiring these mutations is extremely small, this sequential process of acquisition of mutations can only be achieved if cells bearing the initial mutation clonally expand until the population increases to many millions. This process of clonal expansion must then be repeated so that subsequent mutations can be accumulated and cells become progressively better adapted to an independent life. This process is observable clinically, as disease progression is characterised by an increased growth rate, ability to invade neighbouring normal tissue and to metastasise. Hanahan and Weinberg identify six 'hallmark features' of the cancer cell phenotype: disregard of signals to stop proliferating and of signals to differentiate; capacity for sustained proliferation; evasion of apoptosis; invasion; and angiogenesis (Hanahan and Weinberg, 2000).

More than 100 clinically distinct types of cancer have been recognised, each having a set of symptoms and requiring a specific course of therapy. However, most of them can be grouped into four major categories:

Leukaemia: abnormal numbers of white cells are produced by the bone marrow.

Lymphomas: abnormal numbers of lymphocytes are produced by the spleen and lymph nodes.

Sarcomas: solid tumours grown from derivatives of embryonic mesoderm, such as connective tissue, cartilage, bone, muscle and fat.

Carcinomas (the most common form of cancer): solid tumours grown from epithelial tissue. Epithelial tissue comprises the internal and external body surface coverings and their derivatives and thus include skin, glands, nerves, breasts and the linings of the respiratory, gastrointestinal, urinary and genital systems (Pelczar et al., 1986).

1.1.1 How do mutations occur ?

Mutations in the context of carcinogenesis include: point mutations which cause amino acid substitution; frame-shift mutations or mutations to stop codons which truncate the protein product; chromosomal imbalance or instability resulting in amplification, overexpression or inappropriate expression of particular genes; loss of a gene or its fusion with another gene as a result of chromosomal breakage and rearrangement resulting in a chimeric protein with altered function/s; epigenetic modifications to DNA of which the most important is the methylation of cytosine in CpG islands leading to gene silencing (Bertram, 2000).

The vast majority of mutations that give rise to cancer are not inherited, but arise as a consequence of errors in replication, or chemical damage to DNA that may lead to the incorrect reading of the damaged DNA by DNA polymerase (reviewed in Bertram, 2000). The possibility of a mutation to occur and consequently the risk of a cancer to develop are increased by the exposure to external agents that can be grouped in three main categories: chemicals agents (carcinogens and mutagens), physical agents (UV, ionising radiation) and biological agents (infection by pathogens).

Chemical damage occurs as a consequence of exposure to exogenous agents, these agents are defined as mutagens and the mutations induced can act as a molecular fingerprint indicating exposure to these environmental factors; when mutations induce development of cancer these factors are classified as carcinogens.

In 1775 a high incident of tumour in workers exposed to coal tar was reported. It was perhaps the earliest example of an environmental carcinogen and led ultimately to the identification of the polycyclic aromatic hydrocarbon 3,4-benzopyrene and other polycyclic hydrocarbons in coal tar and the discovery of their action as skin carcinogens in laboratory animals.

Since then the realisation that some human cancers have an environmental origin and could be linked directly to chemical exposure grew rapidly and the list of carcinogenic chemicals expanded. The development of rapid *in vitro* assays, principal among these the Ames test conducted in *Salmonella* (Ames, 1984), for the detection of environmental mutagens provided an additional burst of the growing awareness of the presence of potential carcinogens in food and the environment.

Ionising radiation can cause single and double-strand breaks of the DNA helix, and can also induce indirect damage as a consequence of radiolysis of water to yield free radicals (reviewed in Hall and Angele, 1999).

Ultraviolet irradiation is absorbed by DNA bases and is sufficiently energetic to induce chemical reactions. Probably the most important among these occurs between two adjacent thymidines in the DNA helix and results in covalent cross linking to form a cyclobutane-linked thymidine dimer. This becomes an obstacle to DNA polymerase and disturbs normal base pairing and can consequently give rise to mutations. It is not surprising therefore that the 90% of skin cancers arise in sun-exposed areas.

Many different infectious agents have been implicated in various cancers, namely bacteria and viruses. Among these, viruses have been mostly studied in relation to tumour formation. Epstein-Barr virus (EBV), Hepatitis B virus (HBV), Human T cell leukaemia virus-1 (HTLV-1) and several types of papillomaviruses are consistently linked to specific malignancies (zur Hausen, 2001) and are discussed in detail in section 1.3.

1.2 Cancer and cell cycle

In almost all instances deregulation of cell proliferation and suppression of cell death together provide the underlying platform for neoplastic progression. Tumour cells have typically acquired damage to genes that directly regulate their cell

cycle. These are involved in signalling pathways which stimulate proliferation (oncogenes) or act as check-points to cell proliferation or induce cell death (tumour suppressors).

1.2.1 The cell cycle

The cell cycle is divided into four phases: G1, S, G2 and M and it has the duration of about 24 hours in cell cultured *in vitro*. *In vivo*, most mammalian cells are quiescent; few are cycling at any time, this state of quiescence is called G0 state. The decision to divide occurs when cells respond to extracellular signals such as growth factors and cellular adhesion to extracellular matrix (ECM). Growth factors and the ECM bind to and activate specific cellular transmembrane receptors tyrosine kinases (RTKs) and integrins respectively, and induce a series of cytoplasmatic signal transduction cascades.

The best characterised pathways initiated by receptors tyrosine kinases, such as epidermal growth factor receptor (EGF-R) and platelet growth factor receptor (PDGF-R) among others, are the Mitogen Activated Protein Kinase (MAPK) pathway and the phosphatidyl inositol 3'kinase (PI3-K) pathway. The outcome of these kinase cascades is the activation of nuclear transcription factors that leads to an increased expression of the cell cycle machinery components and consequently to the progression of the cell cycle. G1 progression normally relies on stimulation by mitogens and if the growth factors are removed early in this phase, cells will revert to a quiescent state. However, once beyond a specific point in G1, named the Restriction Point (R-point), cells are committed to divide and no longer require extracellular growth factors to complete the cell cycle.

The immediate response of growth factors stimulation is the increased expression of D types cyclins (D1, D2 and D3) in the early-mid G1 phase. These cyclins complex with cyclin-dependent kinases 4 and 6 (CDK4, CDK6) and stimulate their kinase activity. The primary cell cycle target of the cyclin D/CDK cascade is cyclin E (Geng et al., 1999). In combination with another cyclin-dependent kinase, CDK2, cyclin E is responsible for the progression from G1 to S phase. The activity of cyclin D/CDK and cyclin E/CDK2 allows cells to pass through the R-point. Early on in S phase, cyclin D and E are targeted by ubiquitination to be degraded by proteosomes. At this time, cyclin A levels raise activating CDK2 and enabling S phase progression (Chapter 5). Finally cyclin A and cyclin B associate with cdc2 to

promote entry to mitosis and are degraded at G2-M transition and at the end of the M phase respectively.

The fundamental role of the cyclin/CDK complexes, defined as the “cell cycle engines”, is the phosphorylation of crucial proteins responsible for carrying out the cellular changes leading to cell division. Active cyclin D/CDK complexes phosphorylate the retinoblastoma protein (pRb). pRb is a tumour suppressor protein that, in its active state, binds and inhibits transcription factors, especially E2F family. When pRb is phosphorylated it releases the E2F transcription factors, enabling the expression of numerous genes whose products are important for S phase entry (Weinberg, 1995). Cyclin E/CDK2 complex continues the phosphorylation of pRb and pRb-related proteins (p107 and p130). Because cyclin E is itself E2F responsive, cyclin E/CDK2 acts through positive feedback freeing factors required for its own transcription. The cyclin/CDK complexes are tightly regulated via phosphorylation events. An activating phosphorylation of CDK, by the CDK-activating kinase (CAK), stabilises the cyclin/CDK complexes and increases their activity, however two negative phosphorylation events can repress the kinase activity of the complexes occupying the ATP-binding region of CDKs. A family of phosphatases (cdc25A, B and C) act to remove the inhibitory phosphorylations and enhance cyclin/CDK complexes activity.

An additional control on cyclin/CDKs activity is played by the CDK inhibitors (CKIs). There are two families of CKI: the INK4 family, inhibitors of CDK4 and CDK6 that can directly block cyclin D/CDK kinase activity and cause G1 phase arrest (four INK4 inhibitors are known: p16, p15, p18 and p19); and the CKI p21, p27 and p57 that regulate both cyclin/CDK4-6 and cyclin/CDK2 complexes (Sherr and Roberts, 1995; Sherr, 1996).

1.2.2 Alteration of the cell cycle components in cancer

Disruption of normal cell cycle control is associated with oncogenesis suggesting that cell cycle regulatory proteins, such as cyclins and CDKs, may play a direct role in the transformation process by functioning as oncogenes.

As mentioned above, D-type cyclins act as growth factor sensors. The deregulation of cyclin D synthesis makes cell cycle progression less dependent on growth factors and can consequently contribute to oncogenesis. Indeed cyclin D1 is overexpressed in many human cancers as a result of gene amplification or

translocations targeting the D1 locus (Hunter and Pines, 1994). Amplification of chromosome 11q13 detected using *in situ* fluorescence hybridisation (FISH) (region where the cyclin D1 gene maps) is frequent in a broad spectrum of common adult cancer, including squamous cell carcinoma of head and neck (43% of cases on average), oesophageal carcinomas (30%), bladder cancer (15%), primary breast carcinoma (13%) (reviewed in Donnellan and Chetty, 1998). In oesophageal, hepatic and head and neck cancers D1 gene amplification is correlated with protein over-expression, however in cancers (breast, colorectal tumour and melanomas) where gene amplification is a rare event, aberrant over-expression of cyclin D1 protein has also been seen (Hall and Peters, 1996). That cyclin D1 can directly contribute to oncogenesis is supported by studies with transgenic mice, in which targeted overexpression of D1 in mammary epithelial cells leads to cell hyperproliferation and eventually tumour formation (Wang et al., 1994; Geng et al., 1999).

In human cancers the alteration of cyclin E and cyclin A is a rare event. Few cases of cyclin E amplification have been reported in established tumour cell lines, however the protein is overexpressed in carcinomas of breast and stomach among others (Kim et al., 2001). In studies on a human hepatocellular carcinoma, it was found that the hepatitis B virus was integrated into the cyclin A locus with the disruption of the region encoding the N-terminal portion of the protein, comprising the destruction box. This integration resulted in the production of a non-degradable HBV preS-cyclin A chimeric protein, overexpression of which was thought to contribute to transformation of liver cells (Wang et al., 1992). Mutations in cyclin A have not been documented in other tumours or cell lines. Moreover transgenic mice expressing a mutated non-degradable form of cyclin A do not show tumour development, although present abnormalities that may be preneoplastic in mammary glands (Bortner and Rosenberg, 1995).

Another controller of the G1 restriction point is pRb (together with the other pocket proteins). Mutation in pRb is found in 100% of retinoblastoma cancers and pRb is inactivated by mutations or by interaction with viral protein (like HPVs E7, see section 1.4.2) in other types of cancers (reviewed in DiCiommo et al., 2000). As expected also mutations that involve the CDK inhibitors, such as p16, p15 and p19 have been frequently found in human cancers: in biliary track carcinoma, oesophageal carcinoma and nasopharyngeal carcinoma among others. Reciprocally a

mutation in CDK4 that prevents its interaction with p16 has been found in melanoma (Wolfel et al., 1995). A loss or decrease in the level of p27 protein is seen in many human cancers and low p27 levels correlate with aggressive tumour growth (reviewed in Slingerland and Pagano, 2000).

The detection of the deregulation of the cell cycle components may have a prognostic potential in cancers. Moreover, the observations that the majority of human neoplasia has an aberrant pRb pathway involving pRb deletion and/or CDK and Cyclin D hyperactivation lead to the hypothesis that modulators of CDK may have a role in the treatment of human malignancies.

1.3 Viruses and cancer

Oncogenic activity has been described for several human viruses and worldwide viral infection is thought to contribute up to 20% of all human cancers. However, although associated with cancers, oncogenic progression is not part of the normal virus cell cycle.

Small DNA viruses, being dependent on their host replicative machinery, need to induce cell DNA synthesis in infected cells. Moreover virally encoded proteins can bypass check-points in cell cycle progression, playing therefore an important role in the normal viral life cycle. However under unusual circumstances these proteins can also contribute to viral oncogenesis (Vousden, 1995).

Viruses may contribute to the development of human tumours by different mechanisms: altering the expression of host cell proteins at the site of viral DNA integration, by interacting directly with proteins involved in the control of cell cycle check-point (tumour suppressors) or in signalling pathways.

Within the pathogenesis of virus-associated cancers, the role played by viral oncoproteins is conceptually similar to the role of a genetic predisposing mutation which will favour the appearance of a tumour in a specific organ, consequently the ability of viruses to alter cells in culture has been critical to identifying oncogenes and tumour suppressors.

Examples of viruses associated with tumours are: Hepatitis B virus (HBV) that has been associated with hepatocellular carcinoma (reviewed in Arbuthnot and Kew, 2001); Epstein-Barr virus (belonging to the Herpes virus family) is the

etiological agent of infectious mononucleosis and has been associated with the genesis of Burkitt's lymphoma and nasopharyngeal carcinoma (reviewed in Niedobitek et al., 2001); human T cell leukaemia virus-1 (HTLV-1) is a retrovirus that causes adult T cell leukaemia (ATL) (reviewed in Yoshida, 2001). Finally, papillomaviruses have been linked to a variety of anogenital cancers, particularly cervical carcinoma as elucidated in the following section.

None of these virus infections per se is sufficient to induce cancer. Long latency periods, often lasting several decades, the low number of infected individuals who eventually develop particular type of cancer and in some instances interactions which chemical or physical factors point to requirement for additional genetical modifications in cancer development after viral infection (zur Hausen, 1991b).

1.3.1 Papillomaviruses

The first papillomavirus was described in 1933 when Richard Shope (Shope and Hurst, 1933) recognised the cottontail rabbit papillomavirus (CRPV) as the etiologic agent responsible for cutaneous papillomatosis in the cottontail rabbit. Since then papillomaviruses have been characterised from humans, cattle, rabbits, horses, dogs, and a variety of other higher vertebrates and different virus genotypes exist for each host, for example six Bovine papillomaviruses (BPV) and 85 Human papillomaviruses have been identified so far (Jarrett et al., 1984; zur Hausen, 2000). The papillomaviruses have therefore proved to be the most complex group of pathogenic viruses (zur Hausen, 1999).

Papillomaviruses are small nonenveloped icosahedral DNA viruses that replicate in the nucleus of infected cells. All human and most animal papillomaviruses are associated with purely squamous epithelial proliferative lesions (warts) which can be cutaneous or can involve the mucosal squamous epithelium from the oropharynx, the larynx, the oesophagus, or the ano-genital tract. There is a group of animal papillomaviruses that induce benign fibropapillomas, which present a proliferative dermal fibroblastic component as well as a proliferative squamous epithelial component (Howley, 1996).

The genome of the papillomaviruses consist of a single molecule of a double-stranded circular DNA approximately 8000 bp in size, contained within a spherical protein coat, or capsid, composed of 72 capsomeres. The capsid consists of two

structural proteins: the major capsid protein (L1) that has a molecular weight of approximately 55 kDa and represents approximately 80% of the total viral protein and the minor protein (L2) with a molecular size of approximately 70 kDa.

The Open Reading Frames (ORFs) are located on one strand of the viral DNA, indicating that only one strand serves as a template for transcription (Chen et al., 1982). The coding strand for each of the papillomaviruses contains the early (E) region which comprises approximately two-thirds of the genome and encodes proteins that control the replication and transcription of viral DNA. The remaining third of the genome comprises the late region that encodes the viral capsid proteins and finally a non coding region defined as Long Control Region (LCR) containing the origin of DNA replication, transcription enhancers and promoters.

During infection the virus enters the epithelial cells; upon cell division, one daughter cell will remain part of the basal epithelium, while the other daughter cell will migrate up to the next layer and start to differentiate. At this stage the viral DNA will segregate with the two daughter cells and replicate itself to maintain a certain number per cell. The life cycle of the virus is tightly coupled with the differentiation process of the epithelial cell: the virus infects the basal and suprabasal layers, replicates its genome in the differentiating spinous and granular layers, expresses its structural genes and packages its DNA in the squamous layers, and new infectious virus is finally released with the keratinised squames (Campo, 1995).

While warts are usually benign and regress spontaneously, papillomaviruses can also cause a number of more life-threatening diseases (zur Hausen, 1999). Papillomaviruses are implicated in the aetiology of squamous cell carcinomas both in humans and in animals and the viruses associated with naturally occurring cancers are several HPVs (zur Hausen, 1987), the cottontail rabbit papillomavirus (CRPV) (Rous and Beard, 1935) and the Bovine papillomavirus (BPV) type 4 (Campo et al., 1980). The characteristics of HPVs and BPVs will be discussed below.

1.3.2 HPVs

HPVs are the most diverse group of DNA viruses involved in human disease with 85 different types identified so far. They have traditionally been differentiated by sequence divergence; based on criteria adopted by the Papillomavirus Nomenclature Committee, the nucleotide sequences of the E6, E7 and L1 ORFs of a

new type should not exceed 90% homology of the corresponding sequence of the genomes of known HPVs (Howley, 1996). HPVs fall into two major groups: those that infect cutaneous epithelia and those that infect mucosal surface.

While the viruses often cause benign proliferative lesions or warts, there is a subgroup of viruses which cause premalignant and malignant lesions including skin cancer (Bunney et al., 1987; Pfister, 1992), upper respiratory tract cancer (Syrjanen et al., 1987) and anogenital carcinoma (zur Hausen, 1991a) with carcinoma of the cervix being by far the most common cancer.

In 1983 and 1984, respectively two new HPVs, HPV-16 and HPV-18 were identified, and their DNAs were molecularly cloned from two cervical carcinoma biopsies (Durst et al., 1983; Boshart et al., 1984). As a consequence of this discovery, samples worldwide were then assayed and HPV DNA was found regularly in tumour biopsy specimens (Munoz, 2000). At the present, after two decades of epidemiological work with human specimens, work with animal model and experimental work in many laboratories, overwhelming evidence exists to show that papillomaviruses are causative agents of squamous cell carcinomas. A further indirect evidence for the association between papillomavirus and genital cancer is the observation that in adults cervical squamous cell carcinoma has all the characteristics of a sexually transmitted disease as observed in 1842 by Rigoni-Stern who reported that cervical cancer was extremely rare in virgins and predominant among prostitutes. Numerous subsequent studies have clearly established that sexual history, especially the number of sexual partners, is the predominant risk factor for cervical cancer (Broker and Botchan, 1986).

HPVs cause similar lesions in both males and females (Schiffman and Brinton, 1995), however, cancer of the cervix is more common than cancer at other sites and, with approximately 471000 new cases diagnosed annually (Lazo, 1999), is the second most common type of cancer of women world-wide, after breast cancer. The higher incidence of cervical cancer seems due to the nature of the tissue located between the mature epithelium of the exocervix and the columnar epithelium of the endocervical canal which is immature, metaplastic and hormonally responsive. This zone is more susceptible to external insults such as viral infection resulting in the production of premalignant and possibly malignant cells (McMurray et al., 2001).

Invasive cervical cancer is preceded by a progressive spectrum of abnormalities of cervical epithelium. Preinvasive lesions are classified as cervical

intraepithelial neoplasia (CIN) grades 1, 2, and 3; or as mild dysplasia, moderate dysplasia and carcinoma *in situ* (CIS). The severity of the lesions is graded by the extent to which the non-differentiating basal-like cells replace the normally differentiating cervical epithelium. In invasive cervical carcinoma, the abnormal cells breach the basement membrane, invade the stromal tissue, and eventually metastasise to lymph nodes and to other sites in the body. The time interval between the early cervical abnormalities and invasive carcinoma may span several decades (Shah and Howley, 1996).

HPVs are found in a large majority of the lesions spanning the entire spectrum of cytological abnormalities, but the distribution of HPV types changes markedly with increasing severity disease (Bergeron et al., 1992). HPVs rarely found in invasive cancers are characterised as low-risk HPVs, in this group HPV types 6, 11 are the most studied. High-risk HPV types, mostly 16, 18, 31, 33 and others are found frequently in invasive cancers. The severity of HPV-16 and HPV-18 infection is demonstrated also by their ability to immortalise human keratinocytes in cell culture while low-risk HPVs do not (Woodworth et al., 1989).

The relationship of HPV genome to the chromosomal DNA may change during progression from premalignant to malignant phase of the disease. Most of the metastatic cells have integrated HPV sequences (~70%) while the rest have predominantly episomal copies (McCance et al., 1985). Viral integration occurs randomly in cellular chromosomes, but all viral DNA integration events associated with malignant disease, allow for the expression of E6 and E7 proteins. The integration often results in the disruption of the E2 gene, which leads to increased transcription of E6 and E7 genes (Sang and Barbosa, 1992a). Finally, these proteins have been shown to contribute to keratinocytes immortalisation *in vitro* (Hawley-Nelson et al., 1989; Munger et al., 1989a; Barbosa and Schlegel, 1989) and are consequently considered the two major oncoproteins of HPVs. Another viral protein, E5, has been found to be weakly oncogenic and co-operate with E7 in cell transformation (Leptak et al., 1991). The role of E6, E7 and E5 in HPV-associated transformation is detailed in section 1.4.

1.3.3 BPVs

As reported in the previous section HPVs are closely linked with human cancers, however due to the lack of suitable system for propagation of HPVs and the

absence of animal models in which a sexual transmitted papillomavirus produces cervical cancer, the most extensively studied papillomaviruses are BPVs. These papillomaviruses offer the distinct advantage to study the interaction of the virus with the natural host, providing the opportunity to study carcinogenesis in a natural system (Campo and Spandidos, 1983).

The BPVs are classified in two main groups: subgroup A comprises the fibropapillomaviruses (type 1, 2, 5) and subgroup B the epitheliotrophic papillomaviruses: BPVs type 3, 4, and 6 (Jarrett et al., 1984).

The members of subgroup A infect subepithelial fibroblasts as well as epithelial cells and induce fibropapillomas with a proliferative dermal fibroblastic component and a proliferative squamous epithelial component. The viruses belonging to subgroup B infect exclusively the epithelium and induce papillomas without fibroblast involvement (Campo et al., 1981; Jarrett et al., 1984). A further feature of the subgroup B is the lack of the E6 ORF, present in all other papillomaviruses and apparently replaced in this group by an ORF previously designed as E8. It has been speculated that the absence of the E6 ORF in subgroup B of BPVs is the result of a deletion of this ORF (Jackson et al., 1991) as a consequence of the E8 ORF translocation in the previous E6 ORF position. Because of the similarities in function between BPV-1 E5 and BPV-4 E8 (see section 1.4.3); the latter has recently been renamed BPV-4 E5 (Morgan and Campo, 2000) and will be referred to as E5 hereafter.

BPV-1 causes teat frond and penile fibropapillomas; thanks to its ability to transform establish murine fibroblasts most of the early research on papillomaviruses focused on this virus.

BPV-2 is the agent of common cutaneous warts.

BPV-5 causes rice grain fibropapillomas of the udder.

BPVs 3, 4 and 6 were isolated from cutaneous, alimentary and teat frond papillomas, respectively.

BPV-2 and BPV-4 have been associated with malignant progression of papillomas in cattle. BPV-4 has been identified as the etiologically agent of upper alimentary canal cancer in cattle, (see sections 1.3.3.1, 1.3.3.2 and Campo et al., 1980). Although necessary in the initial stages of tumour formation in cattle BPV-4 is not required for maintenance of these malignancies and viral DNA cannot be

detected in the cancer (Campo et al., 1985). It has been speculated that the virus is involved in one of the early steps of cell transformation and that other factors contribute to the establishment and the maintenance of the malignant phenotype (Smith and Campo, 1988; see below).

1.3.3.1 BPV-4

In 1980 Campo and co-workers discovered a new bovine papillomavirus associated with alimentary cancer in cattle: Bovine papillomavirus type 4 (BPV-4) (Campo et al., 1980).

BPV-4 belongs to the subgroup B of bovine papillomavirus (epitheliotrophic papillomaviruses), thus its tissue specificity resembles that of HPVs. For this reason and because, as the high-risk HPVs, it is associated with naturally occurring cancer in its host, BPV-4 represents one of the best models to clarify the relationship between viral infection and carcinoma progression.

The genomic organisation of each papillomavirus is remarkably similar and also BPV-4 presents features common to the group.

The genome consists of a single molecule of double-stranded circular DNA of 7265 bp contained within a spherical capsid composed of 72 capsomeres. The genome is divided in three regions: Long Control Region (LCR), a region encoding the early products (E) and one encoding the late (L) products.

In the BPV-4 LCR, a region of one kilobase in size, the two major viral transcriptional promoters have been mapped: P_L, promoter for the late genes (at nucleotide 777), and P_E, promoter for the early genes (at nucleotide 283) (Stamps and Campo, 1988; Jackson and Campo, 1995). Viral transcripts are obtained from one strand of the genome and seven transcripts have been identified in tumours induced by BPV-4 (Smith et al., 1986).

The E1 ORF is transcribed into a series of mRNAs and it is not known which of them, if any, encodes a functional E1 protein. The role/s of E1 has not been established for BPV-4, but in BPV-1 it is a nuclear phosphoprotein with ATPase activity (MacPherson et al., 1994), which binds to the viral origin of replication (Ustav et al., 1991).

The E2 protein has three functional domains: a central region that joins the transactivation domain at the N-terminus with the DNA binding domain at the C-

terminus. E2 acts as a dimer and the dimerisation is mediated by the C-terminal region. In HPVs and BPV-1, as well as regulating transcription, E2 is involved in viral replication. In BPV-4 the transcriptional activity of E2 has been characterised: the protein binds to the three E2 binding sites of the LCR and induces promoter activity, disruption of these sites causes a dramatic reduction in E2 promoter activation (Jackson and Campo, 1991).

The E4 ORF is transcribed into several RNA species (Stamps and Campo, 1988). The exact function of the E4 protein has not been identified in BPV-4, however, in HPVs, E4 interferes with cytokeratin assembly (Doorbar et al., 1991; Roberts et al., 1993) and consequently with the differentiation programme of the cell, favouring the production of viral progeny instead. Like HPV-1 E4 (Breitburd et al., 1987), BPV-4 E4 was observed only in differentiated layers of the papillomas (Anderson et al., 1997) coincident in time and in location with the vegetative replication of viral DNA (Campo et al., 1994a), it is therefore reasonable to assume that BPV-4 E4 shares similar functions as well as similar localisation within the papillomas with HPV-1 E4.

The E7 and E5 ORFs are the transforming proteins of the BPV-4 (Pennie et al., 1993) and they are described in detail in section 1.4

Other early ORFs have no ATG codon and are assumed to have no function.

The L1 and L2 ORFs encode the structural proteins of the virion; they are transcribed into a 2.8 kb RNA capable of encoding L1, and a 4.2 kb RNA which has the capacity to encode both L1 and L2. These RNAs are found only in papillomas, in agreement with the lack of structural proteins in transformed cells (Campo et al., 1994a).

The short L3 and L4 ORFs do have ATG start codons but their function, if any, is not known.

1.3.3.2 BPV-4 and Cancer

As mentioned above, in healthy cattle, BPV-4 induces benign papillomas that regress spontaneously after approximately a year (Jarrett, 1985). However, in cattle grazing on bracken fern the papillomas can progress to squamous cell cancer (Campo and Jarrett, 1986). The progression of papillomas to carcinomas has been experimentally reproduced by infecting cattle with BPV-4 and feeding them on a diet of bracken, thereby confirming the viral-environmental nature of the disease (Campo

et al., 1994b). Although the connection between virus infection and the development of cancer has been established, BPV-4 presence it is not required for the progression or the maintenance of the malignant state (Campo et al., 1985). When 70 cases of cancers in cattle, including premalignant lesions and primary cancers of alimentary canal and secondary metastatic deposits, were analysed for the presence of BPV-4 DNA by Southern blot, the viral genome was detected only in two cases (transforming papilloma of the oesophagus and carcinoma of the tongue) (Campo et al., 1985). On the contrary in young productive papillomas the virus was detected in 91% of the cases. Similar situation is observed *in vitro*.

In vitro BPV-4 can transform established murine fibroblasts (NIH3T3 and C127) (Campo and Spandidos, 1983; Smith et al., 1987). NIH3T3 cells transformed by the whole genome of BPV-4 cloned in pAT153, were anchorage-independent, grew in low serum, lost contact inhibition and were tumourigenic in nude mice. Conversely, complete morphological transformation of C127 mouse fibroblasts requires cooperation between the viral DNA and the tumour promoter 12-o-tetradecanoylphorbol-13-acetate (TPA) (Smith and Campo, 1988). Moreover when cell lines were analysed for the presence of the BPV-4 genome only 9 out of 60 harboured BPV-4 DNA and no relationship was observed between the presence of the viral genome and the transformed phenotype (Smith and Campo, 1988).

On the basis of these observations the authors proposed for BPV-4 an “hit-and-run” mechanism: viral infection would induce permanent changes in the infected cells, which would be susceptible to environmental carcinogens, and would be able to escape immunosurveillance in immunosuppressed animals (Smith and Campo, 1988).

BPV-4 ability to transform primary bovine cells has also been assessed (Jaggar et al., 1990). Primary bovine fibroblasts (PalF) from the foetal palate were chosen for these studies thanks to their characteristics: being non-established cells, belonging to the natural host of BPV-4 and deriving from one of the sites of virus infection. Contrary to fibropapillomaviruses (BPV-1 and BPV-2), able to fully transform primary bovine fibroblasts, BPV-4 did not induce any modification in PalF cells. However the transfection of the BPV-4 genome together with an activated *ras* caused morphological transformation, anchorage-independent growth and extended the cell life-spans (Jaggar et al., 1990); nevertheless these cells were neither immortal nor tumourigenic (Jaggar et al., 1990; Pennie et al., 1993). BPV-4 transformed cells

achieved immortalisation only when transfected with HPV-16 E6 (Pennie et al., 1993) and tumourigenic status when a mutant p53 was added to the transfection (Scobie et al., 1997).

The data collected from *in vitro* experiments point to the conclusion that the BPV-4 genome does not contain all the necessary information to fully transform primary cells and reinforce the early *in vivo* observations of a necessary cooperation between the virus and cofactors (bracken fern) for the progression of benign papillomas to premalignant and malignant stage.

Further analyses *in vitro* were carried out to better define the molecular mechanisms of the synergism between virus and chemicals. The first candidate in these investigations was one of the most potent mutagens in bracken fern: 5,7,3',4'-tetrahydroxyflavone quercetin (Evans et al., 1982). Primary bovine fibroblasts transfected with BPV-4 genome and an activated *ras* and treated with quercetin (20 or 45 μ M) induced tumours in nude mice approximately four weeks after injection (Pennie and Campo, 1992). Moreover, in E7-transformed PalFs cells quercetin substitutes for HPV-16 E6 in cell immortalisation, for BPV-4 E5 in conferring anchorage-independent from substratum and for mutated p53 for inducing tumourigenity (Cairney and Campo, 1995). Other studies showed that BPV-4 can synergise also with both TPA and the tumour initiation 7,12-dimethylbez[a]anthracene (DMBA) with enhanced production of tumours (Gaukroger et al., 1993).

It has been shown that, in both primary and established murine fibroblasts, the transfection of a subgenomic fragment of BPV-4 genome was sufficient to induce the morphological phenotypes conferred by the full BPV-4 genome (Smith and Campo, 1989; Pennie et al., 1993). This region encodes the E5 and E7 ORFs. BPV-4 DNA digested with enzymes that cut within the E7 ORF (Smith and Campo, 1989) and mutant constructs lacking the 3'third of the E7 ORF (Jaggar et al., 1990) abolish BPV-4 ability of transformation. Moreover E7 ORF does not need any other viral gene to morphologically transform PalFs (Pennie et al., 1993). Although morphologically transformed E7 expressing PalFs are incapable of anchorage-independent growth. The ability to grow independently from substratum is conferred when E5 is co-transfected in E7-cells, leading to the conclusion that this phenotype is provided by E5. In primary bovine fibroblasts it has not been possible to evaluate if

the sole E5 expression and an activated Ras can induce anchorage-independent growth because the transfected cells did not survive selection.

Recently, however, O'Brien and Campo (1998) showed that in NIH3T3 cells the sole E5 expression confers to the fibroblasts the ability to grow in low serum and independently from substrate, providing a system for the evaluation of the viral protein effects on the cell proliferation machinery, without the confounding influence of other viral oncogenes (O'Brien and Campo, 1998).

The transforming mechanisms of E7 and E5 are detailed in section 1.4

1.4 Transforming proteins of the Papillomaviruses

In the study of papillomaviruses, the transforming proteins have received the most attention in the attempt to determine the mechanisms by which they override the normal cellular control on proliferation.

The transforming properties of the onco-papillomaviruses have been mapped in the early region, in particular in the E6, E7 and E5 genes. These proteins contribute to the transformation process to different extent in different papillomaviruses: E6 and E7 are the major transforming protein of HPVs while in BPV-4, which lacks the E6 ORF, E7 and E5 play a fundamental role in transformation and in BPV-1 E5 is the major transforming protein.

The characteristics of these proteins are discussed in detail below.

1.4.1 E6

The E6 proteins of HPVs are approximately 150 amino acid long polypeptides that have an apparent molecular mass of 18 kDa. All the HPV E6 proteins present four Cys-X-X-Cys motifs that permit the formation of two zinc fingers (Barbosa et al., 1989; Grossman and Laimins, 1989). The disruption of these motifs results in a defective E6 protein (Sherman and Schlegel, 1996).

The E6 genes of various HPV types were shown to immortalise human mammary epithelial cells (HMEC) (Wazer et al., 1995; Liu et al., 1999), and, in cooperation with E7, immortalize human foreskin keratinocytes (HFK) *in vitro* (Munger et al., 1989a; Hawley-Nelson et al., 1989). Moreover, recently HPV-16 E6 has been shown to induce the formation of malign tumour in skin of transgenic mice (Song et al., 1999). E6 is one of the earliest expressed proteins during infection and is

believed to provide several functions that alter the cellular environment, to favour the viral replication. Different studies have provided evidence to sustain this hypothesis: E6 blocks apoptosis through both p53 degradation and in a p53-independent way, alters transcription of cellular genes, interacting with transcriptional coactivators and increases cellular life-span through increased telomerase activity.

E6 encoded by high-risk HPV (type 16 and 18) was shown to bind to p53 *in vitro* (Werness et al., 1990), to induce its degradation (Scheffner et al., 1990) via binding to an ubiquitin ligase called E6-associated protein (E6AP) (Huibregtse et al., 1991; Scheffner et al., 1993). Interestingly E6 from low-risk HPVs showed a reduced ability to bind to p53, could not induce detectable degradation of the protein and presented a lower efficiency in the modulation of p53 transcriptional control than E6 proteins from high risk HPVs (Crook et al., 1991; Crook et al., 1994).

Further evidence for the ability of HPV-16 E6 to inactivate p53 function was obtained *in vivo* by the observation that expression of E6 in the eyes of transgenic mice suppresses apoptosis during lens development (Pan and Griep, 1994). These same studies however showed that E6 mediated apoptosis could occur in both p53-dependent and -independent way (Pan and Griep, 1994; Pan and Griep, 1995). More recently Jackson and Storey (2000) demonstrated that E6 genes from mucosal and cutaneous subtypes of HPV were all capable of blocking apoptosis induced by ultraviolet radiation but only the HPV-16 E6 induced p53 degradation (Jackson and Storey, 2000). These data suggested that the modulation of p53 plays some role in the inhibition of apoptosis by E6, but does not account for all.

Evidences that E6 may interfere with apoptosis via a p53-independent pathway came when Thomas and Banks (1998) showed that HPV-16 and -18 E6 can interact with Bak, a pro-apoptotic protein expressed at high levels in the upper layers of differentiating epithelium (Thomas and Banks, 1998). This interaction leads to degradation of Bak by ubiquitination, again through interaction with E6AP, and consequently reduction of apoptosis. E6 from low-risk HPV types (like HPV-11) shows a weaker interaction with Bak than HPV-16 and -18 E6.

Another E6 cellular target, only recently emerged, is CBP/p300 transcriptional co-activator (Patel et al., 1999; Zimmermann et al., 1999). Interestingly CBP/p300 is a p53 transcriptional coactivator and the interaction with E6, mediated by the C-terminal zinc finger of the viral protein, correlates with down-regulation of p53 transcriptional activity (Zimmermann et al., 1999).

Among other functions, E6 can up-regulate the activity of the cellular telomerase complex (Klingelhutz et al., 1996) in human keratinocytes and mammary epithelial cells. Telomerase is an enzyme that controls the telomeric DNA at the ends of linear chromosomes and is inactive in normal cells (with the exclusion of stem cell) where telomeres shorten with each cell division until they reach a critical short length beyond which cells senesce. It has been shown that E6 mutations that do not increase telomerase map to the internal portion of protein in the zinc-finger domains and central region, suggesting that these regions of E6 are necessary for telomerase activation. Despite the increased activity of the telomerase, however, telomere length is not increased and HFK cells are not immortalised by the sole expression of E6 (Klingelhutz et al., 1996). These data question the relevance of telomerase activation.

E6 reveals a remarkable pleiotropism in binding further host-cell proteins. It interacts with the calcium-binding protein ERC 55 (Chen et al., 1995) with paxillin (Tong and Howley, 1997) and with the mammalian homologue of *Drosophila* disc large tumour suppressor protein (hDLG) (Lee S.S. et al., 1997) and targets the latter for proteasome-mediated degradation (Gardioli et al., 1999). The interaction between hDLG and E6 is mediated by the C-terminus of the viral protein and at least one PDZ domain of hDLG; interestingly HPV-11 (a low risk HPV) fails to induce hDLG degradation (Lee S.S. et al., 1997; Gardioli et al., 1999). As for hDLG, a correlation between the ability to bind paxillin and HPVs transformation ability exists (Tong and Howley, 1997). Although these interactions may lead to substantial functional consequences for E6 expressing cells, they are, at present, not fully understood (zur Hausen, 2000).

The two most prominent functions of E6 can be summarized as follows: it induces the overcome of the G1/S check-point control in DNA damaged cells by binding to p53, in addition it leads to immortalisation that may result from a large number of modifications, primarily the alteration of the telomerase enzyme.

BPV-1 E6 is a 137 amino acids long protein with two zinc finger motifs (Barbosa et al., 1989). The expression of BPV-1 E6 is sufficient to induce anchorage-independent growth and focus formation in C127 cells (Schiller et al., 1984; Neary and DiMaio, 1989). The mechanism of BPV-1 E6 induced cell transformation is unknown. Recently an interaction between the BPV-1 E6 protein and the focal

adhesion associated protein paxillin, the trans-Golgi network-specific clathrin adaptor complex and CBP/p300 have been described (Tong and Howley, 1997; Tong et al., 1998; Zimmermann et al., 2000) although the biological relevance of these interactions remains to be established.

BPV-4, as all bovine papillomaviruses of subgroup B, lacks the E6 ORF.

1.4.2 E7

The E7 proteins of HPVs are primarily nuclear proteins of approximately 18 kDa in size (Howley, 1996); they present amino acid sequence similarities to adenovirus E1A protein and based upon this homology they have been divided into domains. In HPV-16 E7 these domains are the CR1, from amino acid 1 to 20, CR2, containing residues 21-39 and CR3 composed of amino acids 40-98 (Phelps et al., 1988; Phelps et al., 1992). HPV-16 E7 has two casein kinase II phosphorylation site at serine 31 and 32 (Barbosa et al., 1990) and two Cys-X-X-Cys zinc binding domains in the CR3 (Barbosa et al., 1989) that mediate protein dimerisation.

The major function of E7 during HPV infection is the stimulation of cell cycle progression by favouring the exit of quiescent cells from G0 and entry into S phase. To achieve its target E7 interacts with numerous cellular proteins mainly involved in the regulation of cell growth such as pRb and the pocket proteins, Ap-1 transcription factors, cyclins and CDKs inhibitors (CDIs).

HPV E7 induces transformation of rodent cell lines (Phelps et al., 1988), can cooperate with Ras to transform primary rodent fibroblasts (Matlashewski et al., 1987; Phelps et al., 1988) and in combination with E6 extend the life span of primary human cells (Munger et al., 1989a; Hawley-Nelson et al., 1989).

Two groups of regulatory interactions mainly contribute to the ability of HPV-16 E7 to over-ride cell cycle control at the G1/S boundary: E7-driven activation of cellular E2F-dependent genes and the direct interaction of the viral protein with the inhibitors of the cyclin-dependent kinases.

Certainly the best characterised function of E7 is its ability to bind the pRb protein and pRb related pocket proteins (Munger et al., 1989b). pRb and the pocket proteins operate as signal transducers connecting the cell cycle with the transcription machinery (Weinberg, 1995). They bind to transcription factors, mainly the E2F family, and prevent expression of genes important for progress through the cell cycle. When the cell is ready to enter the cell cycle, cyclin-dependent kinases inactivate

pRb, p107 and p130 by sequential phosphorylation of their specific sites, free the transcription factors and induce cell cycle progression. E7 interferes with this equilibrium binding to pRb, p107 and p130 and mimicking the phosphorylation event. The transcription factors are consequently available to promote the transcription of genes required for S phase progression with the result of a premature cell stimulation into S phase.

Genetic studies of HPV-16 E7 revealed the presence of a pRb binding site within the CR2 domain (Barbosa et al., 1990); this region presents the LXCXE motif, that has been found in other viral proteins able to interact with pRb (E1A and SV40T). The same region is responsible for the E7 interaction with p107 and p130. Moreover, it has been shown that E7 does not only interact with pRb but it triggers its proteolytic degradation via ubiquitin-proteasome pathway (Boyer et al., 1996) resulting in an increase level of free transcriptionally active E2F.

Interestingly the E7 proteins from low-risk HPV types 6b and 11 bind pRb with about ten fold lower efficiency, on average, than the E7 proteins of HPV type 16 and 18 (Munger et al., 1989b). A single difference in the sequences next to their respective LXCXE motif is though to be responsible for the difference in binding efficiency: HPV-6 E7 contains a glycine at amino acid 22, while HPV-16 E7 possesses an aspartic acid at equivalent position (Sang and Barbosa, 1992b). The substitution of the glycine at position 22 with an aspartic acid conferred to HPV-6 E7 higher affinity for pRb and the ability to transform NIH3T3 cells to the same degree as HPV-16 E7 (Sang and Barbosa, 1992b). In addition to the motif within the CR2 domain, a region in the CR3 seems also to be required for the E7/pRb interaction (Patrick et al., 1994).

Beside modulation of the pocket proteins/E2F pathway E7 has been shown to interact with other proteins involved in gene transcription control, principally members of the AP-1 family of transcription factors, that includes c-Jun, JunB, JunD and c-Fos. Specific mutation analysis and binding data to c-Jun indicate that E7 zinc finger motif, but not pRb binding domain, is involved in this interaction (Antinore et al., 1996).

As described above HPV-16 E7 can override cell cycle control at the G1/S boundary, these observations pointed to the possibility that E7 can neutralise or bypass the inhibitory effects of the CDK inhibitors: p21 and p27. Studies revealed

that E7 blocks the ability of CKIs to inhibit CDK activity through the direct binding and sequestration of p21 and p27 (Zerfass-Thome et al., 1996; Funk et al., 1997).

Cells expressing E7 show increase kinase activity associated with cyclin E and cyclin A and this activity is sustained even in the presence of growth arrest signals such as serum deprivation and anchorage-independent growth (Zerfass et al., 1995; Schulze et al., 1998; Ruesch and Laimins, 1998). E7-induced S phase entry is not accompanied by cyclin D activation (Schulze et al., 1998); these observations are consistent with the observation that cyclin D is not required for S phase entry of cells expressing pRb-inactivating viral protein. The effects of E7 on de-regulation of Cyclin E/CDKs and cyclin A/CDK2 may contribute to extending the duration of 'replicative competence' in host cell (McMurray et al., 2001).

In contrast to HPV E7 little is known about BPV-1 E7. The protein has been detected in BPV-1 transformed cells and it presents an apparent molecular mass of 15 kDa (Jareborg et al., 1992). No independent transformation activity has been detected for BPV-1 E7. A recent report showed that C127 expressing BPV-1 E7 are more sensitive to Tumour Necrosis Factor α -induced apoptosis in a pRb-independent way (Liu et al., 2000).

The transforming functions of BPV-4 have been mapped to a genome region that encodes the E5 and E7 genes (Jaggar et al., 1990). The E7 protein of BPV-4 is 98 amino acids long and, similarly to HPV-16 E7, has a putative pRb binding domain as well as two Cys-X-X-Cys zinc binding domains, however BPV-4 E7 lacks the casein kinase II phosphorylation site (serine 31 and 32 in HPV-16 E7). It is not known if BPV-4 E7 binds to pRb but deletions of the second zinc binding domain or mutations in the putative pRb binding domain abolish cell transformation by E7 or by the complete BPV-4 genome, demonstrating that these sites are crucial for the role of E7 in cell transformation (Jaggar et al., 1990). *In vivo* the E7 protein was detected from the basal to the squamous layers of papillomas, it was consistently present in the nucleus of the basal and suprabasal cells and in the cytoplasm of the spinous and squamous layers (Anderson et al., 1997). *In vitro*, in transient expression experiments, BPV-4 E7 was localised mainly in the cytoplasm, and only some cells showed nuclear localisation (Pennie et al., 1993). The sole expression of BPV-4 E7 can induce growth and morphological changes in PalF cells expressing an activated Ras. For this reason the protein has been considered the major transforming protein

of BPV-4. However, E7 expressing cells do not grow in suspension or in absence of mitogens, and these phenotypes are the results of the additional expression of BPV-4 E5 (Pennie et al., 1993; see below).

1.4.3 PVs E5

As presented above the transforming and immortalising activities of high-risk HPVs are attributed to E6 and E7 proteins, however another viral protein, E5, has been found to be weakly oncogenic and to cooperate with E7 to transform cells (Leptak et al., 1991; Pim et al., 1992; Leechanachai et al., 1992; Bouvard et al., 1994; Faulkner Valle and Banks, 1995). On the contrary, in BPVs, E5 is the major transforming protein.

The amino acid sequence of E5 proteins is not well conserved between HPVs or animal viruses although these proteins present homologies in the physico-chemical properties: they are generally small, highly hydrophobic and membrane localised (Figure 1.1).

BPV-1 E5 has been the most extensively characterised among the members of the E5 protein family and is consequently considered the prototype of the group. The structural similarity of the HPVs and BPV-4 E5 proteins to BPV-1 E5 has motivated studies on their potential transforming activities.

HPVs E5 are discussed in detail in the section below followed by BPV-1 E5 and BPV-4 E5 (Figure 1.1).

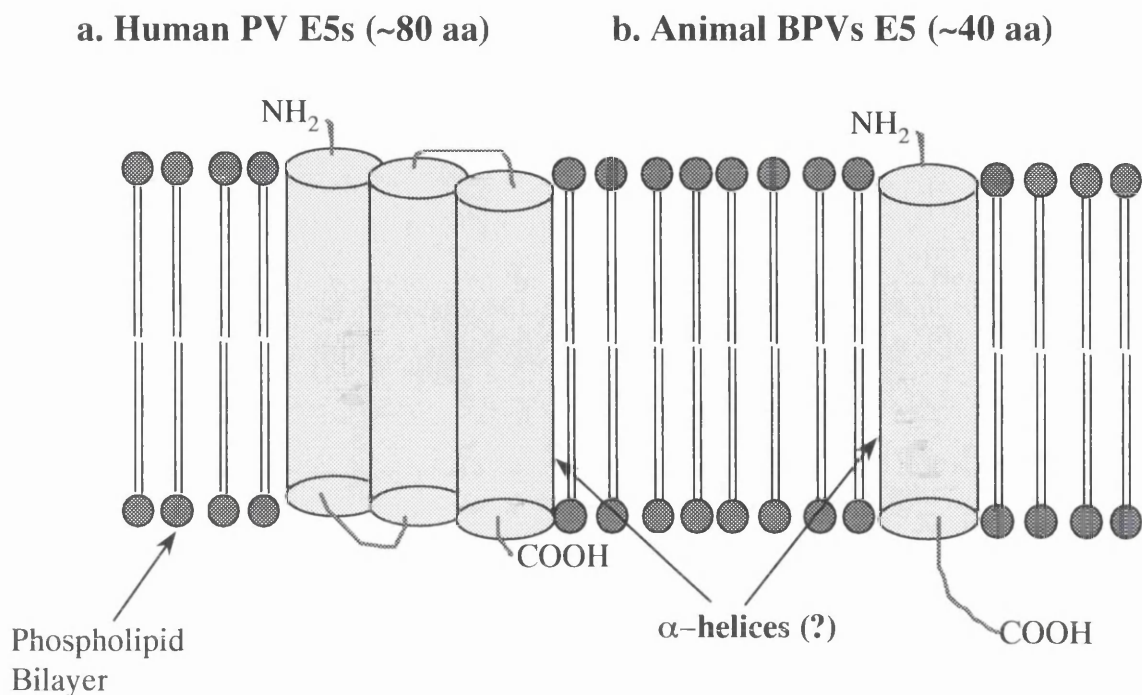


Figure 1.1 The E5 family members are small membrane-localised peptides

Highly schematic representation of human and animal PVs E5 proteins. The cylinders represent α -helices transmembrane segments, established in BPV-1 E5 and postulated in other E5s.

1.4.3.1 HPVs E5

The E5 ORF of both high-risk and low-risk HPVs encodes a hydrophobic peptide that is poorly conserved between different virus types. Most HPV E5s are ~80 residues, with the exception of HPV-83 E5 which is only 47 residues (Brown et al., 1999). The best studied E5, from HPV-16, is an 83 residue hydrophobic protein with three hydrophobic peaks and less hydrophobic troughs and consequently likely to form three transmembrane α -helices (Bubb et al., 1988). Further biochemical analysis of the HPV-16 E5 protein containing a N-terminal epitope tag expressed in transiently transfected COS cells revealed that HPV E5s exist as predominantly ER-localised, monomeric membrane proteins (Conrad et al., 1993).

The protein is expressed in productive infections and is thought to play a critical function in the early expansion of an infected cell clone. The ORF encoding E5 is frequently deleted in cervical carcinoma cells (Schwarz et al., 1985) suggesting that E5 does not play an essential role in maintaining the malignant phenotype of

cervical carcinoma cells (zur Hausen, 1994). *In vitro* HPV E5 proteins cannot immortalise primary mouse or human cells, although both HPV-6 and HPV-16 E5s stimulate mitogenesis in primary keratinocytes and induce growth stimulation of primary rat kidney epithelial cells in cooperation with HPV-16 E7 (Straight et al., 1993; Bouvard et al., 1994). HPV-16 E5 can cause tumourigenic transformation of mouse keratinocytes (Leptak et al., 1991) and fibroblasts (Leechanachai et al., 1992; Pim et al., 1992).

Unlike E6 and E7 proteins which abrogate the effects of negative cellular regulators, the major biochemical function of E5 proteins appears to be through cellular tyrosine kinase growth factors receptors. Human keratinocytes expressing HPV-16 E5 display elevated levels of epidermal growth factor receptor (EGF-R) on their surface, apparently due to increased receptor recycling. In these cells the receptor displays increased tyrosine phosphorylation in response to epidermal growth factor (EGF) treatment (Straight et al., 1993; Straight et al., 1995; Crusius et al., 1998). The expression of HPV-16 E5 in mouse fibroblasts (3T3-A31) leads to anchorage-independent growth and the efficiency of colony formation is increased by EGF treatment (Pim et al., 1992), in NIH3T3 the expression of human EGF-R is required to induce anchorage-independent growth transformation by HPV-16 E5 (Leechanachai et al., 1992). Also E5s of low risk HPVs (HPV-6, -11 E5a) can cooperate with EGF in the transformation of rodent fibroblasts (Straight et al., 1993; Crusius et al., 1997). The exact mechanisms of receptor activation by E5s are unknown. Different results have been reported on the ability of HPV-16 E5 to physically interact with growth factor receptors. Conrad and co-workers showed that, when overexpressed in COS-7 cells, a tagged HPV-6 E5 associated with a number of growth factor receptors included EGF-R, whereas HPV-16 E5 could not interact with any (Conrad et al., 1994); Hwang and co-workers however showed a direct binding of HPV-16 E5 to EGF-R in the same cell system (Hwang et al., 1995). In the former studies, both E5s were epitope tagged, and anti-tag antibodies were used to coimmunoprecipitate the proteins, while in the latter HPV-16 E5 was not tagged and anti-E5 peptide antibodies were used. It is possible therefore that the hydrophilic tag may have interfered with the immunoprecipitation. However, HPV-16 E5 does not appear to promote ligand-independent activation (autophosphorylation) of the EGF-R (Straight et al., 1993) and association between E5 and EGF-R has not yet been shown in human keratinocytes.

The E5 protein from HPV-16 binds to the 16 kDa protein (Conrad et al., 1993; Faulkner Valle and Banks, 1995). This protein is the subunit c of the V0 sector of the vacuolar ATPase (V-H⁺-ATPase), and a component of the Gap Junctions (GJ). The V-H⁺-ATPase proton pump is a multi-subunit enzyme responsible for the acidification of many endomembrane compartments including Golgi apparatus, secretory vesicles, endosome and lysosomes (Nelson, 1989; Finbow et al., 1991). The GJ provide sites for the movement of low molecular mass solutes from cell-to-cell; as component of the GJ the 16 kDa protein is defined as ductin. We will be referring to it as 16K hereafter.

It has been shown that in E5-expressing keratinocytes there is a delay in the acidification of the endosomes and the pH of the endosomal compartment does not drop below 6 (Straight et al., 1995). This inhibition of endosomal pH acidification may not be sufficient to prevent EGF-EGF-R dissociation but does allow increased recycling of the unoccupied receptor and this may account for the increased EGF-R numbers observed in E5 cells (Straight et al., 1993; Straight et al., 1995) and the enhanced mitogenic signalling from occupied EGF-R. Although binding of E5 to 16K has not been shown in human keratinocytes, the co-expression of E5 and the bovine vacuolar ATPase subunit c in COS-1 cells has shown a complex containing both proteins (Conrad et al., 1993). Recent studies dissociate the binding of HPVs E5 to 16K from its effects on EGF-R activation (Rodriguez et al., 2000). Mutants of E5 which did not interact with 16K efficiently activated the EGF-R receptor in keratinocytes stably expressing these forms of E5. Other reports have questioned the functional significance of E5/16K interactions (Adam et al., 2000; Ashby et al., 2001; Briggs et al., 2001). Using a panel of HPV-16 E5 mutant forms, Adam et al (2000) showed that the interaction of E5 and its mutants with 16K, tested in COS-1 cells, is not sufficient to inhibit V-H⁺-ATPase activity in *Saccharomyces cerevisiae* model system (Adam et al., 2000); recent data from the same group reinforced these observations demonstrating that the binding between HPV-16 E5 and 16K has to result in the disruption of the stability and assembly of the V-H⁺-ATPase to be effective (Briggs et al., 2001). However, HPV-16 E5, BPV-1 E5 and BPV-4 E5 did not perturb V-H⁺-ATPase activity in any detectable way in *S. pombe* (Ashby et al., 2001), leaving the importance of E5s/16K interaction still an open question.

The interaction between E5 and 16K may have other biological consequences: 16K has been reported to be a component of gap junctions (Finbow et al., 1991) and human epithelial cells expressing HPV-16 E5 show a reduced capacity for GJ Inter cellular Communications (GJIC) (Oelze et al., 1995). Loss of communication is a common feature of a variety of transformed and tumour cells and it has been proposed that oncogenes could operate, in part, by blocking gap junctions and allowing cells to escape the growth regulation imposed by surrounding normal cells (Yamasaki, 1990).

There are downstream effects of E5 expression in both rodent fibroblasts and human keratinocytes. Ligand-dependent activation of EGF-R leads to up-regulation of c-Fos and c-Jun mRNA and cells expressing HPV-16 E5 have supraphysiological levels of these AP-1 component mRNAs in response to serum and EGF but also in response to PDGF, although there are small increase in both mRNAs in serum free media (Leechanachai et al., 1992; Bouvard et al., 1994). There is an enhanced activation of MAP kinases ERK 1 and ERK 2 in cells expressing HPV-16 E5 following EGF stimulation, and this activation is prolonged by E5 expression (Gu and Matlashewski, 1995; Crusius et al., 1997). Additionally, HPV-16 E5 can mediate an EGF-R-independent activation of MAPKs in response to some forms of osmotic shock (Crusius et al., 2000). Therefore it appears that the various pathways activated by interaction of EGF with the EGF receptor are enhanced and prolonged in the presence of HPV-16 E5.

1.4.3.2 BPV-1 E5

BPV-1 induces fibropapillomas, tumours with both a fibroblastic and an epithelial component. The precise role of E5 in the virus life cycle is not known however, it is hypothesised that in basal keratinocytes E5 is involved in the initiation and maintenance of the transformed state of these cells and it may contribute to viral maturation, through the stimulation of DNA synthesis in differentiated cells during vegetative replication (Burnett et al., 1992). *In vitro* E5 can transform murine fibroblast lines (NIH3T3, C127), mouse keratinocytes cells p117 (Leptak et al., 1991) and human dermal fibroblasts (HDFs) (Petti and Ray, 2000). The expression of E5 in fibroblasts and keratinocytes leads to induction of DNA synthesis, causes

malignant transformation and confers the ability to grow in suspension, in the absence of mitogens, and tumorigenicity in nude mice.

The E5 gene encodes a 44 residues peptide with an extremely hydrophobic amino terminal region (two-third of the protein) and a predominantly hydrophilic C-terminus. Immunoprecipitation studies using an antibody against the carboxyl-terminus of E5 showed that E5 ORF encodes a 7 kDa polypeptide, which fractionates exclusively with cellular membranes and, in non-reducing condition, migrates as a 14 kDa dimer (Schlegel et al., 1986). The hydrophobic N-terminus region of E5 forms a α -helical secondary structure (Surti et al., 1998) and acts as a membrane anchor, while E5 dimerisation is thought to be mediated by cysteine residues in the hydrophilic C-terminus via disulphide bond formation (Horwitz et al., 1988). Recent data revealed that residues within the transmembrane domain also play a role in protein dimerisation (Adduci and Schlegel, 1999). Immunocytochemistry and immunoelectron microscopy studies revealed that E5 is located within both the Endoplasmic Reticulum (ER), the Golgi apparatus and, to a lesser degree, at the cell surface, and revealed that the protein is a type II transmembrane protein, i.e. with its C-terminus oriented intraluminally in endomembranes (Burkhardt et al., 1989).

The small size of E5 and its ability to tolerate mutations led to the hypothesis that it acted by modifying the activity of cellular proteins, rather than through intrinsic enzymatic activity. In 1989 Martin *et al.* showed that E5 affected the function of the EGF-R in NIH3T3 cells, providing the first demonstration that E5 could modulate the activity of cellular proteins involved in growth control (Martin et al., 1989). However, a direct interaction of E5 with EGF-R has never been demonstrated. On the contrary the interaction between BPV-1 E5 and the platelet-derived growth factor β receptor (PDGF β -R) is well established (Petti et al., 1991; Drummond-Barbosa et al., 1995). In rodent fibroblasts (C127 and FR3T3), stably transformed by E5, the mature and immature forms of PDGF β -R are constitutively phosphorylated on tyrosine residues and in these cells both mature and immature precursor forms exist in stable complexes with E5 (Petti et al., 1991). Several studies have confirmed the requirement of E5-PDGF β -R interaction and receptor activation for E5 transforming activity (Nilson and DiMaio, 1993; Petti and DiMaio, 1994; Goldstein et al., 1994; Drummond-Barbosa et al., 1995; Klein et al., 1998). Cells that normally do not express PDGF β -R e.g. normal mouse mammary gland (NMuMG)

epithelial cells, lymphoid Ba/F3 and 32D cells, cannot be transformed by E5, but can be made susceptible to E5-induced transformation by the introduction of a gene encoding the PDGF β -R, but not genes encoding other growth factor receptors (GFRs) (Nilson and DiMaio, 1993; Goldstein et al., 1994). In Ba/F3 and 32D cells only the co-expression of human PDGF β -R and E5 generates a mitogenic signal which allows the lymphoid cells to grow in an IL-3-independent manner and 32D cells become tumourigenic (Goldstein et al., 1994). As corollary, cells selected for their resistance to mitogenic stimulation by PDGF are also resistant to transformation by E5 (Riese and DiMaio, 1995). Moreover treatment of E5-transformed C127 cells with a specific inhibitor of the PDGF β -R tyrosine kinase activity (AG1295) results in a loss of constitutive tyrosine phosphorylation of the PDGF β -R and a reversion of morphological transformation that was completely reversible upon removal of the inhibitor (Klein et al., 1998). Treatment with AG1295 prevents IL-3-independence of Ba/F3 cells co-expressing the PDGF β -R and E5 (Klein et al., 1998). Like PDGF and its viral oncogenic form *v-sis* (Devare et al., 1984), E5 induces dimerisation and trans-phosphorylation of the receptor (Lai et al., 1998; Lai et al., 2000). When complexed with E5, the PDGF β -R associates with c-Src homology domain 2 (SH2)-containing proteins. The p85 subunit of phosphatidylinositol 3'kinase (PI3-K), phospholipase C γ (PLC γ) and Ras-GTPase activating protein, all associate with E5/PDGF β -R complexes (Lai et al., 2000).

The binding of E5 and the PDGF β -R is mediated entirely by transmembrane (TM) hydrophobic domain of the viral protein (Goldstein et al., 1992a; Adduci and Schlegel, 1999). Mutants in alanine residue at position 17, 21, and 24 inhibit E5 association with PDGF β -R suggesting that these residues are crucial for the association with PDGF β -R. Moreover, E5 TM contains functional domains that separately regulate homologous (E5/E5) interaction and heterologous (E5/PDGF β -R) interaction (Adduci and Schlegel, 1999).

PDGF β -R participates in complex formation with E5 via transmembrane (Thr 513) and juxtamembrane (Lys 499) residues (Petti et al., 1997). In light of these data, DiMaio and co-workers proposed that the E5/PDGF β -R complex may be stabilised largely by an electrostatic interaction between the opposite charged juxtamembrane lysine 499 of PDGF β -R and aspartic acid 33 of E5 and by a hydrogen-bond or packing interaction between the transmembrane threonine 513 of PDGF β -R and glutamine 17 of E5 (Meyer et al., 1994; Petti et al., 1997; Klein et al., 1998; Klein et

al., 1999). Surti and co-workers (1998) suggested that E5 dimer might serve as molecular scaffold for dimerisation and ligand-independent activation of PDGF β -R (Surti et al., 1998). The model predicts that on each face of the dimer aspartic acid is contributed by one E5 monomer and glutamine by the other and this could explain why dimerisation-defective E5 mutants could not induce receptor dimerisation and activation.

From the above, it could be concluded that the interaction and activation of PDGF β -R is the key element and probably the sole requirement for the E5 transforming pathway but other reports question the validity of this conclusion. E5 can transform cultured epidermal keratinocytes p117 (Leptak et al., 1991), even though they do not appear to express PDGF β -R. Recently, Adduci and co-workers analysed E5 mutants that were defective for binding and/or activating PDGF β -R but could still transform cells (Adduci and Schlegel, 1999). Interestingly, some transforming mutants exist as stable E5 tetramers and bind the PDGF β -R but cannot induce its phosphorylation. These studies show that E5 tetramers may have transforming activity and point to the existence of an E5 transforming mechanism independent from the PDGF β -R or, at least, independent from its phosphorylation. In NIH3T3 cells E5 mutants defective for PDGF β -R activation, induce the phosphorylation and activation of PI3-K in a PDGF β -R independent way as shown by the use of a specific inhibitor of PDGF β -R phosphorylation (AG1296). These mutants can still form foci and sustain growth in suspension cells but are unable to support growth factor-independent proliferation of NIH3T3 cells (Suprynowicz et al., 2000). In cells expressing E5 wild type (wt) the drug treatment diminished PI3-K phosphorylation by 70%, but did not completely abolish it, leading to the conclusion that the residual drug-resistant activation of PI3-K may depend on a similar PDGF β -R-alternative pathway hypothesised for the mutants. Some of the discrepancies from *in vitro* cell transformation studies may be cell type specific. For example one of the mutants (Q17S) that Suprynowicz *et al.* (2000) reported unable to activate the PDGF β -R, behaves differently in a different system, C127 cells, where it increases PDGF β -R phosphorylation 7-fold (Klein et al., 1998). For these reasons the data should be cautiously interpreted.

In 1990 Goldstein and Schlegel found that the 16K (see section 1.4.3.1) specifically co-precipitated with the E5 protein in COS-1 and NIH3T3 cells

(Goldstein and Schlegel, 1990). Mutational analysis revealed that the binding of E5 protein to 16K is mediated entirely by the intramembrane hydrophobic domain, the same region that is responsible for the E5/PDGF β -R interaction (Goldstein et al., 1992a). Confirmation of this came from the fact that none of the missense mutations in the hydrophilic C-terminus of the E5 protein affected binding with the 16K protein, including a number of mutations that severely inhibit transformation and/or block dimerisation. In particular, the association between E5 and the 16K is mediated by an interaction between Q17 in E5 and glutamic acid (Glu 143) in the fourth TM domain of 16K (Andresson et al., 1995). Mutational analysis revealed that a truncated form of 16K, lacking the fourth α -helix, and a point mutation, with glutamic acid 143 to arginine substitution, allowed NIH3T3 cells to grow in soft agar, although with lower efficiency than E5 wt. This might indicate that mutations within the fourth TM domain or the interaction of E5 with the 16K affect the normal function of V-H⁺-ATPase and lead to cell transformation (Andresson et al., 1995).

The interaction between E5 and 16K might impair the normal activity of the V-H⁺-ATPase in the acidification of endomembrane compartments. Shapiro and co-workers looked at the pH of the Golgi apparatus in NIH3T3 expressing E5. They reported that E5 cells display an elevated pH in the Golgi lumen when compared to control cells as a consequence of the possible inhibition of V-H⁺-ATPase activity by E5 (Schapiro et al., 2000).

As reported before the 16K protein is a component of the Gap Junctions (GJ). Similarly to what shown for HPV-16 E5 expressing cells, primary bovine fibroblasts (PalF) expressing BPV-1 E5 show closure of the GJIC (Ashrafi et al., 2000).

As with the role of the PDGF β -R in E5-mediated transformation pathway, there are discordant opinions about the importance of the interaction of E5 with 16K. Some studies found that the interaction with 16K may be required for transforming activity but it is not sufficient since transformation-defective mutants can still bind to 16K (Goldstein et al., 1992b). Similarly, Sparkowski *et al* (1996) showed that E5 transforming activity can be dissociated from the interaction since mutants that do not associate with 16K protein are still transforming (Sparkowski et al., 1996).

The demonstration that E5 activates immature PDGF β -R forms that are localised in the Golgi apparatus led to the hypothesis that this organelle could be a critical location for E5 during cell transformation. To test this a form of E5 bearing a

KDEL (E5/KDEL) retrieval signal for the ER was used in transformation studies in NIH3T3 and C127 cells. The results showed that the Golgi location is required for E5 transforming activity since E5/KDEL cannot induce focus formation in mouse fibroblasts (Sparkowski et al., 1995). Surprisingly this form of E5 can still bind to 16K protein and to the immature form of PDGF β -R and induce phosphorylation of both mature and immature forms of the receptor. The transformation deficiency of the E5/KDEL form is thought to depend on the sequestration of the PDGF β -R complexed with E5/KDEL in the ER where the PDGF β -R cannot transmit a mitogenic signal (Sparkowski et al., 1995). The wild type form of the viral protein, however, could activate PDGF β -R from the Golgi apparatus, without necessarily moving to the plasma membrane, or proceed together with it to the cell surface

Although necessary, the Golgi location is not sufficient for E5 transformation since mutants unable to transform are still localised in this subcellular compartment (Klein et al., 1999; Adduci and Schlegel, 1999; Schapiro et al., 2000). The importance of the E5 Golgi location has been stressed by the finding that NIH3T3 E5 expressing cells present a more alkalinised Golgi compartment but not gross morphological alterations to this organelle (Schapiro et al., 2000). In this work the authors studied how Golgi alkalinisation is associated with the transforming ability of E5 using the following mutants: (I) those non-transforming and cable of PDGF β -R phosphorylation and not localised in the Golgi, (E5/KDEL); (II) those non-transforming, and incapable of PDGF β -R activation but localised in the Golgi and (III) those that are transforming, Golgi localised and either capable or incapable of PDGF β -R activation. When the Golgi pH was measured in NIH3T3 stably expressing the mutants the authors found a perfect correlation between the ability of the mutants to induce Golgi alkalinisation and to transform cells, suggesting an E5 transforming mechanism independent of PDGF β -R activation. Schlegel and co-workers proposed that inactivation of the V-H⁺-ATPase and alkalinisation of the Golgi apparatus could be a crucial activity of E5 (Schapiro et al., 2000). The acidification of the Golgi apparatus may regulate the transport/processing of itinerant proteins and lipids and have pleiotropic effects on cellular processes.

1.4.3.3 BPV-4 E5

The BPV-4 E5 protein shares some structural features with BPV-1 E5 (Figure 1.2). It is only 42 residues with a putative transmembrane domain, the hydrophobic

N-terminal region (residues 1-30) and a hydrophilic C-terminal “tail” of 12 residues (Jackson et al., 1991). BPV-4 E5 is expressed *in vivo* only in the basal and suprabasal layers of early papillomas and its expression decreases in late stage papillomas (Anderson et al., 1997). *In vitro* E5 is transforming in NIH3T3 cells. Established murine fibroblasts expressing E5 grow in low serum concentration and in suspension (O'Brien and Campo, 1998). Moreover, E5 confers ability to grow in low serum, to escape contact inhibition and to grow in suspension to primary bovine fibroblasts co-expressing BPV-4 E7, HPV-16 E6 and an activated Ras (Ashrafi, 1998; O'Brien et al., 1999).

E5 has been subjected to a limited genetic analysis in the attempt to identify important functional domains and segregate transforming functions of the protein. These studies revealed that the hydrophilic “tail” (31-42 amino acids) is critical since neither a form of E5 lacking the last 12 amino acids or the chimera comprising the BPV-4 E5 body and the BPV-1 E5 “tail” are capable of promoting anchorage-independent growth, although the reciprocal chimera is still transforming (Ashrafi, 1998; O'Brien et al., 1999). The truncated form of E5 is non-transforming both in PalFs and NIH3T3 cells (Ashrafi, 1998; O'Brien and Campo, 1998; O'Brien et al., 1999). Mutation of the asparagine residue in position 17, potentially functionally homologous to glutamine 17 in BPV-1 E5, to serine, tyrosine or alanine produce forms of BPV-4 E5 partially transforming, non transforming and hypertransforming respectively, pointing to the importance of this residue for E5 transforming ability in PalF cells (Ashrafi, 1998; O'Brien et al., 1999). The substitution of two amino acids that could alter the potential α -helix conformation of the hydrophobic domain emphasise the importance of the nature of these residues for E5 activity (Ashrafi, 1998); (see Chapter 3 and 6).

It is not known if E5 can interact with growth factor receptors to promote their activation, but it can bind with the 16K protein *in vitro* (Faccini et al., 1996), induces down-regulation of GJIC in PalF cells (Ashrafi et al., 2000) and alkalinises the Golgi apparatus (R. Schlegel, personal communication) in NIH3T3 cells. It has been shown, however, that binding to 16K may be necessary but not sufficient for E5-induced transformation or GJIC down-regulation since non transforming mutants can still bind to 16K but do not inhibit GJIC (Ashrafi et al., 2000). Moreover asparagine in position 17 is not crucial for E5/16K association contrary to glutamine

17 for BPV-1, and the hydrophilic domain is dispensable (Ashrafi et al., 2000). Even if not necessary the inhibition of GJIC may be important for fibroblasts transformation *in vitro* and for the establishment of transformed cells *in vivo*: E5 may interfere with the GJIC activity in infected tissue and affect basal and suprabasal cell transformation by removing them from homeostatic control exerted by surrounding non-transfected cells (Jackson et al., 1996).

In transiently transfected PalFs E5 is present in the perinuclear membranes, the endoplasmic reticulum and the Golgi apparatus (Pennie et al., 1993). The importance of BPV-4 E5 Golgi location has been underlined by recent observations. Ashrafi and co-workers have shown that PalF and NIH3T3 cells expressing E5 present a down-regulation of surface MHC I (histocompatibility complex class I) expression (Ashrafi et al., 2002). MHC I is synthesised in the ER, post-translationally modified in the Golgi and transported in the plasma membrane (Cresswell et al., 1999). This process appears inhibited in cell expressing E5, it is still unclear if this is due to a direct effect of E5 on MHC I (i.e. E5/MHC I interaction) or is a consequence of the lack of Golgi acidification observed in E5 expressing cells. In PalF cells a down-regulation of the MHC I protein level and transcripts have been also observed (Ashrafi et al., 2002). In light of these data the authors proposed a model by which E5 produced early in the infection process, would directly affect immune recognitions and clearance of virally-infected cells favouring viral persistence and subsequently the development of the malignancy.

In NIH3T3 cells transformation by E5 is associated with up-regulation of cyclin A expression and associated kinase activity and, surprisingly, with the up-regulation of the cyclin-dependent kinase inhibitor p27 level in all growth condition tested (O'Brien and Campo, 1998). In these cells E5 does not increase cyclin E expression level or the cyclin E-CDK2 kinase activity. Recently O'Brien *et al.*, demonstrated that in NIH3T3 E5 expressing cells p27 is prevented in its function as CDK2 inhibitor because it is sequestered by an elevated pool of Cyclin D1/CDK4 complexes that retain their kinase activity in normal growth conditions or in absence of mitogens (O'Brien et al., 2001). Consequently the sequestration of p27, the inactivation of pRb and the increased expression of cyclin A and its associated activity provide all the requirements to allow mitogen-independent proliferation of NIH3T3 E5 transformed cells.

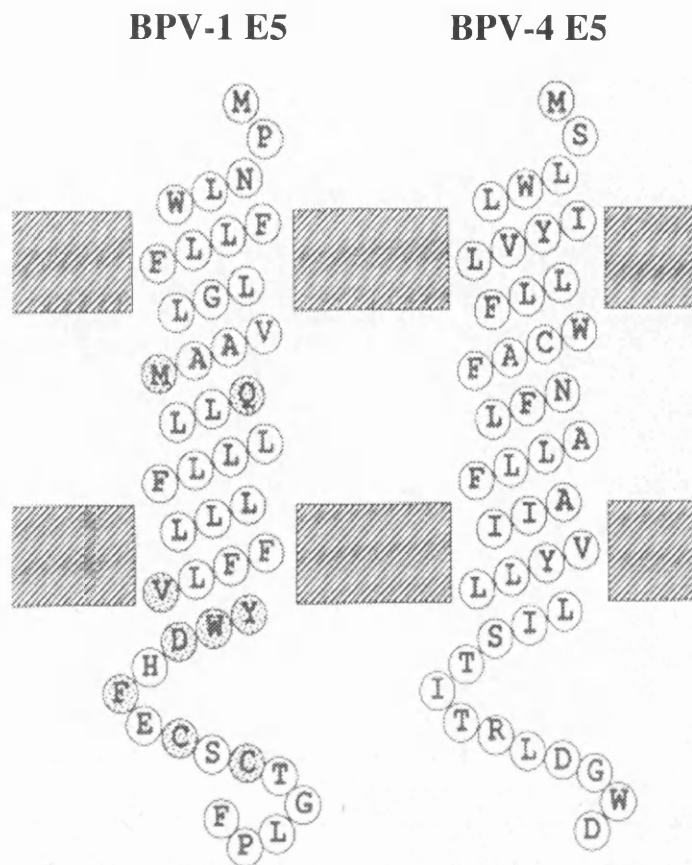


Figure 1.2 BPV-1 E5 and BPV-4 E5

Amino acids sequence and transmembrane configuration of BPV-1 E5 and BPV-4 E5. The amino acids that have been shown critical for cell transformation are shaded in BPV-1 E5 (Horwitz et al., 1988; Horwitz et al., 1989).

1.5 Project aims

How BPV-4 E5 is able to induce cell transformation is poorly understood; its small size has led to the hypothesis that E5 may act by modifying the activity of cellular proteins as it has been shown for BPV-1 E5, rather than through a direct enzymatic activity.

Thus the initial aim of this thesis was to define whether E5 cellular location is crucial for its transforming activity. Consequently a routine detection system had to be identified to allow cellular localisation of the protein. All previous attempts to visualise E5 within the cells failed, with the exception of one case (Pennie *et al.*, 1993), either using anti-E5 antibodies or antibodies against small tag-epitopes at the N-terminus of E5 (e.i. HA). To overcome these problems we decided to construct a fusion form of E5 with the Green Fluorescence Protein (GFP). GFP is a naturally fluorescence protein from the jellyfish *Aequorea victoria* and has become an important molecular biology tool; when used as a tag on other proteins GFP allows study of their expression and localisation in live, as well as in fixed cells. We used GFP-E5 in localisation studies.

Additionally, studies of GFP-E5 transforming ability in NIH3T3 cells were to be undertaken to confirm that the transforming abilities of E5 had been maintained in its GFP fusion form and in the attempt to identify new cellular targets of E5. NIH3T3 GFP-E5 expressing cells were analysed for their ability to grow in absence of mitogens and in semi-solid media. Biochemical studies to define the changes on the cell cycle machinery due to GFP-E5 expression were carried out in different growth conditions.

In the attempt to segregate the transforming functions of E5 protein and to define its possible functional domains a panel of mutants have been generated and tested for transformation in primary bovine fibroblasts (PalFs) co-expressing BPV-4 E7, HPV-16 E6 and an activated Ras (Ashrafi, 1998; O'Brien *et al.*, 1999). Here, in the attempt to expand our information on E5 transformation abilities, we wanted to carry out transformation studies of E5 mutant forms in NIH3T3 cells.

In PalFs it has been shown that the altered phenotypes observed for E5 mutants compared with the wt protein do not depend on the expression level of the proteins (O'Brien et al., 1999). To establish if a mis-location of the mutants may be an explanation for their different transforming ability, we generated fusion forms of these peptides with GFP and analysed their cellular location.

CHAPTER 2: MATERIAL AND METHODS

2.1 Material

2.1.1 Antibodies

Babco Covance Research Product, Richmond, USA	Anti-GFP (B34N) mouse monoclonal
Bioquote Limited, Yorkshire, UK	Anti Protein Disulphide Isomerase (PDI) mouse monoclonal
Cell Signaling technology, Beverly , USA	Anti phospho-S780 pRb rabbit polyclonal
Jackson ImmunoResearch Laboratories, West Grove, PA, USA	Texas Red dye -conjugated AffiniPure Sheep Anti-Mouse IgG (H+L) Texas Red dye -conjugated AffiniPure Goat Anti-Rabbit IgG (H+L) Fluorescein (FITC)-conjugated AffiniPure Goat Anti-Rabbit IgG (H+L) Fluorescein (FITC)-conjugated AffiniPure Sheep Anti-Mouse IgG (H+L)
MBL Medical & Biological Laboratories, Nagaya, Japan	Anti-GFP rabbit polyclonal
New England Biolabs, Hertfordshire, UK	Anti-Mouse IgG, HRP-linked Antibody Anti-Rabbit IgG, HPR-linked Antibody
PharMingen, San Diego, USA	Anti-Human pRb (G3-245) mouse monoclonal
Santa Cruz Biotechnologies, SantaCruz, USA	Anti-cyclin A rabbit polyclonal, Anti-cyclin E rabbit polyclonal Anti-cdc2 mouse monoclonal Anti-CDK2 (M2) rabbit polyclonal Anti-p27 rabbit polyclonal Anti-cyclin D1 mouse polyclonal Anti-p130 rabbit polyclonal Anti-p107 rabbit polyclonal
Sigma Chemical Co., Ltd., Dorset, UK	Anti- α -tubulin mouse monoclonal Anti-total ERK 1/2

	Anti-phospho ERK 1/2
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2.1.2 2.1.2 Bacterial Hosts

Invitrogen Life Technologies, Ltd., Paisley, UK	<i>E.coli</i> DH5α competent cells
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2.1.3 Buffers

2X HEPES buffered saline (HBS)	280 mM NaCl, 10 mM KCl, 1.5 mM Na ₂ -HPO ₄ .2H ₂ O, 50 mM HEPES
Kinase buffer	50 mM HEPES, 50 mM NaCl, 10 mM MgCl ₂ , 1 mM DTT
Kinase lysis buffer	50 mM HEPES pH 7.5, 150 mM NaCl, 1% (v/v) NP40 detergent, 2.5 mM EGTA pH 8, 10 mM NaF, 10 mM, β-glycerolophosphate, 1 mM Sodium Orthovanadate, 1 mM DTT, Mini Proteinase inhibitor cocktail (Roche)
Kinase reaction Buffer	0.05 µg/µl Histone H1, 20 µM ATP, 10 µCi γ- ³² P-ATP, 1X Kinase Buffer
10X loading buffer for PCR	0.45% (w/v) bromophenol blue, 1% (w/v) sodium dodecyl sulphate (SDS), 100 mM EDTA, 2.5% (w/v) Ficoll 400 in TE
Phosphate buffered saline (PBS)	137 mM NaCl, 44 mM KCl, 1.4 mM KH ₂ PO ₄ , 8.5 mM Na ₂ HPO ₄
SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)-Lysis buffer	100 mM Tris-base pH 6.8, 2% (w/v) SDS, 20% (v/v) glycerol
2X SDS gel loading buffer	4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol and 100 mM Tris, pH6.8, 200 mM DTT
5X TBE buffer	40 mM Tris-base, 16 mM acetic acid, 1 mM EDTA, pH8.0
TBS	0.2 M Tris-base, 1.5 M NaCl, pH 7.6

TE	10 mM Tris-HCl, 1 mM EDTA pH 8.0
Transfer buffer	39 mM glycine, 48 mM Tris-base, 0.037% (w/v) SDS, 20% methanol
Tris-glycine electrophoresis buffer	25 mM Tris-base, 250 mM glycine, 0.1% (w/v) SDS

2.1.4 Cells

CELLS TYPES	DESCRIPTION	GROWTH MEDIUM
NIH3T3	Swiss mouse embryo fibroblasts	Dulbecco's Modified Eagle's Medium, 10% New Born Calf Serum 1 mM sodium pyruvate 0.375% sodium carbonate 2 mM glutamine
COS-7	Cells expressing SV-40 T Ag, derived from the African green monkey kidney cell line CV1	Dulbecco's Modified Eagle's Medium, 10% Foetal Calf Serum 1 mM sodium pyruvate 0.375% sodium carbonate 2 mM glutamine
CHO-1K (CHO)	Chinese hamster ovary cells	Special Liquid Medium 10% Foetal Calf Serum 1 mM sodium pyruvate 0.375% sodium carbonate 2 mM glutamine

2.1.5 Cell Culture Materials

SUPPLIER	MATERIAL
Harlan Sera-Lab Ltd., Crawley Down, UK	Foetal Calf Serum

Invitrogen Life technologies Ltd., Paisley, UK	New born Calf Serum 1X Dulbecco's Modified Eagles Medium 200 mM glutamine Geneticin, G418 sulphate 7.5% sodium bicarbonate 100 mM sodium pyruvate 2.5% Trypsin
Sigma Chemical Co., Ltd., Poole, Dorset, UK	10X Dulbecco's Modified Eagles Medium

2.1.6 Chemicals, enzymes & kits

SUPPLIER	CHEMICALS AND ENZYMES
Ambion the RNA company™, Oxon, UK	DNA-free™
Amersham Pharmacia Biotech plc, Amersham, Bucks, UK	ECL Western detection agent Hybond ECL, nitrocellulose membrane [$\gamma^{32}\text{P}$] dATP Rainbow™ coloured protein molecular weight markers (220-14 KDa; 45-2.5 KDa)
Becton Dickinson, Michingam, USA	Bacto-Agar
Biogenesis Ltd., Bournemouth, UK	RNAzol B
Bioline, London, UK	X-gal, 5-bromo-4-chloro-3-indolyl- β -D- galactopyranoside
Calbiochem San Diego California	MAPK inhibitor PD98059

Fisher Scientific Equipment, Loughborough, UK	Acetic acid Butan-1-ol Chloroform di-potassium 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
Invitrogen Life technologies Ltd., Paisley, UK	All DNA restriction enzymes and appropriate buffer concentrates (unless otherwise stated) Agarose (ultrapure electrophoresis grade) Tris-base 100 bp DNA ladder 1 Kb DNA ladder Low DNA mass ladder
James Burrough Ltd., Witham, Essex, UK.	Ethanol
Merck Eurolab Ltd., Dorset, UK	Calcium chloride Glycerol

Molecular Probes, Inc. Oregon, USA	<i>N</i> -((4-(4,4-difluoro-5-(2-thienyl)-4 bora-3a,4a-diaza- <i>s</i> -indacene-3-yl)phenoxy)acetyl)sphingosine) BODIPY [®] TR ceramide
Perkin Elmer Cetus, Norwalk, USA	GeneAmp RNA PCR core kit GeneAmp PCR core kit
Promega Ltd., Chilworth Research Centre, Southampton, UK	Luciferase Assay System Reporter Lysis 5 X Buffer MAPK inhibitor U0126
Qiagen Ltd., Dorking, Surrey, UK	QIAprep Miniprep kit QIAprep Maxiprep kit QIAquick gel extraction kit QIAquick PCR purification kit Effectene Transfection Reagent
Roche UK, Lewes, East Sussex, UK	Complete [™] Mini, protease inhibitor cocktail tablets
Severn Biotech Ltd., Kidderminster, Worcester, UK	Acrylamide
Sigma Chemical Co., Ltd., Poole, Dorset, UK	Ampicillin Bicinichonic acid solution Bovine Serum Albumin Copper(II) sulphate (pentahydrate 4% (w/v) solution) Crystal violet DL-Dithiothreitol Ethidium bromide Kanamycin HEPES Methocel MC 4000 Paraformaldehyde Phenol:Chloroform:Isoamyl Alcohol (25:24:1 (v/v))

	Penicillin Protein-A sepharose Saponin Sepharose CL 4B TEMED Tween 20 (Polyoxyethylene sorbitan monolaurate)
Stratagene Ltd, Cambridge, UK	Cloned <i>Pfu</i> DNA polymerase 10X cloned <i>Pfu</i> buffer
Transgenomic, Northumberland, UK	Calf Intestinal Alkaline Phosphatase (CIAP) RNase A T4 DNA ligase
Upstate biotechnology New York , USA	Histone H1
Vector Laboratories, Burlingame, USA	VECTASHIELD [®] Mounting Medium H-1000

2.1.7 Equipment and Plasticware

SUPPLIER	EQUIPMENT
Becton Dickinson Labware, Plymouth, UK	Falcon 1059 polypropylene tubes Falcon 2059 polypropylene tubes Falcon 2098 polypropylene tubes Sterile Plastic pack syringes 18 gauge sterile syringe needles 60, 90 and 150 mm tissue culture dishes cell strainers 40µm Nylon
Bibby sterilin Ltd., Stone, Staffs, UK	60, 90 and 150 mm bacteriological petri dishes Sterile plastic universal containers
Costar Corporation, High Wycombe, Bucks, UK	24 well tissue culture plates 96 well tissue culture plates Disposable Cell scrapers

Elkay Laboratory products UK Ltd., Eastleigh, Hampshire, UK	Microcentrifuge tube
Gelman Sciences, Northampton, UK	Sterile 0.2 µm acrodisc filters
Ilford Ltd., Mobbrrley, Cheshire, UK	Ilford PANF 50 black and white film
Nunc, Roskilde, Denmark	T25, 80, and 175 cm ² tissue culture flasks Cryotubes
Technical Photo Systems., Cumbernauld, UK	Fuji RX medical X-ray film
Whatman International Ltd., Maidstone, Kent, UK	Whatman 1 filter paper

2.1.8 Other Materials

SUPPLIER	MATERIALS
Beatson Institute Central Services	LB-Medium (Luria-Bertani Medium) Streptomycin Sterile distilled water Sterile glycerol Sterile phosphate-buffered saline (PBS) Sterile phosphate-buffered saline plus EDTA (PE)
Merck Ltd., Poole, UK	Silicone grease
Premier Brands, Adbaston, Stafford, UK	Marvel (Dried Skimmed milk)

2.1.9 Plasmids

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 BamHI 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).

pZipneoHAE5 contains nucleotides 236-590 of the BPV-4 genome cloned into the BamHI sites of the vector pZipneo. The sequence coding for the influenza virus haemagglutinin type-1 (HA1) epitope is inserted at the 5' of the BPV-4 E5 sequences.

pwt929 is a luciferase reporter plasmid for the human cyclin A promoter. The plasmid carries a fragment of the human cyclin A promoter (region between -754 bp and +175 bp). This plasmid construct was a gift to Dr Vincent O'Brien from Dr William Fahl, University of Wisconsin, USA (Kramer et al., 1996).

pEGFPC3 is an eukaryotic expression plasmid that encodes the enhanced green fluorescence protein (EGFP) (which contains the double amino-acid substitution of Phe-64 to Leu and Ser-65 to Thr, to increase the fluorescence of 35 times). The EGFP expression is driven by the CMV promoter and the multiple cloning site is between the EGFP sequences and SV40 poly A site.

pEGFPN2 is an eukaryotic expression plasmid that encodes the enhanced green fluorescence protein (EGFP) (which contains the double amino-acid substitution of Phe-64 to Leu and Ser-65 to Thr). The multiple cloning site is between the immediate early promoter of CMV and the EGFP sequence.

pcPEP-GFP expresses the signal peptide/membrane anchoring domain of the rabbit neutral endopeptidase fused with GFP. The fusion protein is inserted in the multiple cloning site of pcDNA3 vector (Invitrogen). The plasmid is a kind gift of Dr. Maria Simonova, Center for Molecular Imaging Research, Massachusetts General Hospital and Harvard Medical School (Simonova et al., 1999).

2.1.10 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 18 M Ω /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 Oligonucleotides synthesis and purification

Oligonucleotides were synthesised by the Beatson Institute technical services staff on an applied Biosystems model 381A DNA Synthesiser or 392 DNA/RNA Synthesiser using the manufacturer's protocols and Cruachem reagents.

The oligonucleotides were purified by precipitation with butan-1-ol. One ml butan-1-ol was added to 150 µl oligonucleotide solution and microcentrifuged at 14000 rpm 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 2.2.1.12.

2.2.1.2 Amplification of DNA by polymerase chain reaction (PCR)

All the reagents were provided from Stratagene with the exception of dNTPs which were obtained from Perkin-Elmer Core DNA PCR Kit. Primer sequences were designed such that they were complementary to opposite strands and opposite end of the DNA of interest.

For the cloning of BPV-4 E5 in pEGFPC3 and pEGFPN2 (2.1.9), at the C and N terminus of the green fluorescent protein respectively, the forward E5 primer correspond to the first 11 amino acids of the influenza virus haemagglutinin (HA) and the reverse primer to nucleotide 455-434 of the BPV-4 genome. The primers were designed to carry the specific restriction sites as required from the cloning strategy (see table 2.1). The constructs obtained have been named GFP-E5 and E5-GFP respectively. For the cloning of GFP in pZipneo the forward primer corresponded to the first 21 nucleotide of the GFP protein and the reverse primer to the last 21 of GFP sequence. For the cloning of GFP-E5 in pZipneo the forward primer corresponded to the first 21 nucleotide of GFP and the reverse primer to nucleotide 455-434 of the BPV-4 genome.

Table 2.1 Oligonucleotides PCR primers

Vector	primer name	primer sequence
pEGFPC3	E5C3 forward	5'tta cgt tgc aag ctt atg tac cca tac gat gtt cca 3' (HindIII)
	E5C3 reverse	5'atc ttc tac ctg cag tca atc cca tcc atc taa ccg 3' (PstI)
pEGFPN2	E5N2 forward	5'tta cgt tgc aag ctt atg tac cca tac gat gtt cca 3' (HindIII)
	E5N2 reverse	5'atc ttg gat ccc atc cca tcc atc taa ccg agt aat 3' (BamHI)
pZipneo	GFP forward	5'tta cgt gga tcc gag aca tgg tga gca agg gcg 3' (BamHI)
	GFP reverse	5'atc tac gga tcc tca ctt gta cag ctc gtc cat 3' (BamHI)
pZipneo	GFP-E5 forward	5'tta cgt gga tcc gag aca tgg tga gca agg gcg 3' (BamHI)
	GFP-E5 reverse	5'atc tac gga tcc tca atc cca tcc atc taa ccg 3' (BamHI)
pEGFPC3	E5C3 forward	5'tta cgt tgc aag ctt atg tac cca tac gat gtt cca 3' (HindIII)
	E5KDEL reverse	5'tgc ctg cag tca caa ctc gtc ctt ctc aga atc cca tcc atc taa 3' (PstI)
pEGFPC3	E5C3 forward	5'tta cgt tgc aag ctt atg tac cca tac gat gtt cca 3' (HindIII)
	E5GGEV reverse	5'tgc ctg cag tca aac ctc tcc tcc tcc aga atc cca tcc atc taa 3' (PstI)

NB: in bold is the restriction site sequence; in brackets is the name of the restriction enzyme.

All reaction mixtures comprised 200 μ M of each dATP, dGTP, dCTP and dTTP, 1X cloned *Pfu* buffer (200 mM Tris-HCl pH 8.8, 20 mM MgSO₄, 100 mM KCl, 100 mM (NH₄)₂SO₄, 1% Triton X-100, 1 mg/ml nuclease free BSA), 40 pmoles of each primer, 2.5 units *Pfu* polymerase (a thermolabile DNA polymerase from *pyrococcus furiosus*) and 1 μ g of DNA. The mixtures were aliquoted into 0.5 ml GeneAmp PCR reaction microfuge tubes in a final volume of 100 μ l.

The tubes were placed into the PCR machine (Perkin-Elmer Cetus type 9600 thermocycler) and heated to 95°C for 5 minutes to inactivate DNase and ensure all DNA duplexes were melted, the DNA was then amplified for 35 cycles at 94°C for 30 seconds, 55°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. 5 μ l of each sample was

analysed by agarose gel electrophoresis (as described in section 2.2.1.5) to check the correct product had been amplified.

2.2.1.3 PCR products purification

The PCR products were cleaned from excess dNTPs, enzyme, and salt using the QIAquick PCR purification kit from Qiagen following the manufacturer's recommendations.

2.2.1.4 Restriction enzyme digestion of DNA

Restriction digests were carried out using appropriate enzymes and their concentrated buffer solutions according to the manufacturer's recommendation.

PCR products were incubated with 5-10 units enzyme in a buffered solution ensuring that the total volume of enzyme added did not exceed one tenth of the final reaction volume. PCR products were digested in 100 µl final volume overnight at 37°C.

Small quantities of plasmid DNA (<5 µg) were routinely digested in a 20 µl reaction volume for 2 hours at 37°C using 1-2 units enzyme. The digestion fragments were analysed by agarose gel electrophoresis as described below.

2.2.1.5 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 bp) were separated on 2-4% gels. Gel mixes containing the appropriate amount of agarose were dissolved in 0.5X 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 0.5X TBE buffer. The samples containing 1X loading buffer were loaded in each well along with an appropriate size marker (i.e. 100 bp ladder, 1 Kb 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 0.5 µg/ml ethidium bromide with gentle agitation for 10 minutes at

room temperature. The separated DNA was visualised by illumination with short wave (312 nm) UV light and photographed through a red filter onto videoprint paper using an Appligene Imager.

2.2.1.6 Isolation and purification of DNA fragments and vectors

All DNA to be used for cloning was purified from contaminants, such as residual enzymes from the restriction reaction, which might otherwise interfere with subsequent cloning steps. The PCR products were 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.

For vectors bigger than 5 Kb the DNA were purified using extraction with phenol: chloroform as follows. In the first round of extraction the DNA sample was mixed with an equal volume of phenol:chloroform. 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 transferred to a fresh Eppendorf tube 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 3 M 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.

2.2.1.7 Ligation of DNA fragments

The vector, to be used in the ligation step, was dephosphorylated at its termini to prevent its religation. After the vector DNA had been linearised 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 55°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 55°C for further 15 minutes. The DNA was phenol:chloroform extracted, ethanol precipitated and then resuspended in an appropriate volume of distilled water.

The size and the concentration of both PCR products and plasmid were checked by running few µl of each on a 1% agarose minigel with 2 µl of Low Mass DNA ladder. The DNA fragment was inserted into dephosphorylated vector (100 ng) at a ratio of 3:1 respectively. The vector and inserted DNA were incubated together in a reaction of 10 µl final volume, containing 1X ligase buffer and 1 unit of T4 ligase at 15°C overnight. Ligases reactions were then diluted 1:5 adding 40 µl of distilled water and 1 to 5 µl of the reaction were used to transform competent bacterial cells (section 2.2.1.8).

2.2.1.8 Transformation of bacterial host

All plasmids were propagated in commercially available DH5α *E. coli* competent cells supplied as frozen stocks kept at -70°C until use. Competent cells were thawed slowly on ice, and 100 µl aliquots put into a prechilled polypropylene culture tube and 1-5 µl of appropriate diluted ligase reaction added and mixed by gently moving the pipette tip through 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 were then immediately placed on ice for 2 min. 900 µl of L-Broth (1% w/v Bactotryptone, 0.5% w/v yeast extract, 1% w/v NaCl) was then added to each transformation reaction at room temperature. The tubes were then transferred to a shaking 37°C incubator (approximately 225 rpm) for 1 hr to allow expression of the antibiotic resistant marker. Following this, 100 µl 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.

2.2.1.9 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 tip and grown in 5 ml culture of L-Broth containing antibiotic (100 µg/ml Ampicillin and 50 µg/ml Kanamycin) at 37°C in a shaking incubator (225 rpm) overnight. 10 separate colonies were generally picked for screening. Bacteria were pelleted from 1.5 ml of overnight culture by spinning in a microcentrifuge (14000 rpm) for 30 seconds at room temperature. DNA was prepared using the QIAprep miniprep kit following the manufacturer's instructions.

2.2.1.10 DNA sequencing

The sequences of the new plasmids were checked using Tag terminator sequencing on an Applied Biosystems 373A automated DNA sequencer which was performed by the Beatson Institute technical service staff.

The region to be sequenced (the insert) first underwent PCR amplification. 0.5 µg of template DNA (around 1 µl of miniprep preparation) was added to 12 µl RQ grade H₂O plus 3.2 pmoles of the appropriate primer. 8 µl of dye terminator cycle sequencing ready reaction premix (Perkin Elmer) was added to each reaction contained in 250 µl thin walled Eppendorf tubes. The samples were placed in a PTC-100 programmable thermal controller (Genetic Research Instrumentation Ltd) and exposed to 25 cycles of 95°C for 30 seconds , 50°C for 15 seconds and 60°C for 4 minutes. The PCR products were ethanol precipitated as detailed in section 2.2.1.6 washed with 70% ethanol and finally dried under vacuum before being given to a member of the technical services for loading onto the sequencing gel.

2.2.1.11 Large scale preparation of plasmid DNA (Maxiprep)

The plasmid caring the insert in the correct orientation was used to transform 25 µl *E. coli* DH5α competent cells as described in section 2.2.1.8. Bacteria were plated 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 from this plate using a sterile yellow tip, and used to inoculate a sterile universal tube containing 5 ml of L-Broth medium with the appropriate antibiotic. The tube was put in a shaking incubator at 225 rpm for 6 hr at 37°C and consequently the culture was added to 250 ml (for high copy plasmid) or 500 ml (for low copy plasmid) of L-Broth containing appropriate antibiotic and returned to the shaking incubator overnight.

The large scale preparation of plasmid was carried using the Qiagen maxiprep kit following the manufacturer's recommendations. The DNA concentration was determined as described below and the plasmid DNA was aliquoted and stored at minus 20°C.

2.2.1.12 Quantitation of nucleic acids

The concentration of nucleic acid was determined spectrophotometrically in a Beckman DU 650 spectrophotometer. Samples were diluted in TE and transferred to a quartz cuvette with a pathway of 1 cm. The spectrophotometer was initially calibrated using TE buffer only as a blank. The optical density reading were obtained at 260 nm and 280 nm; an OD reading of 1 at 260 nm ($A_{260} = 1$) corresponds approximately to a concentration of 50 µg/ml of double stranded DNA, for oligonucleotides an A_{260} of 1 was taken to correspond to 35 µg/ml and for RNA an A_{260} of 1 was taken to correspond to 40 µg/ml. The ratio between readings at 260 nm and 280 nm ($A_{260}:A_{280}$) provided an estimate of the sample purity; a ratio of ~1.8 indicated that preparations contained essentially no protein or phenol contamination.

2.2.2 Cell Culture

2.2.2.1 Cell culture

All cell culture work was performed following strict aseptic techniques inside a laminar flow hoods (Class II Microbiological safety Cabins). Cells were incubated in dry or humid incubators containing 5% (v/v) CO₂ at 37°C and were routinely screened for mycoplasma infection using a fluorescent dye technique.

2.2.2.2 Maintenance of cells in culture

Cells were passaged approximately 1 in 5, just before reaching confluence as follows: the medium was removed by aspiration from a T175 flask, cells washed with 10 ml of phosphate-buffered saline (PBS). After the PBS was aspirated, 5 ml of 0.25% trypsin in PE were added to the cells for two-three minutes and then removed. Trypsin was inhibited by the addition of complete medium and cells were detached from the flask. The cell suspension was pelleted by centrifugation at 1000 rpm for 5 minutes at room temperature, resuspended and seeded at the wanted density.

2.2.2.3 Long term cell storage

To freeze cells stocks for storage, confluent cultures were trypsinised, and pelleted as described above. The pellet was then resuspended at a concentration of approximately 10^6 cells/ml in medium containing 50% serum and 10% (v/v) DMSO. The DMSO in the medium acts as a cryoprotectant but all solutions must be chilled as DMSO is toxic to cells at room temperature. Suspensions were divided into 1 ml aliquots in 1 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 10 ml of the appropriate pre-warmed growth medium, centrifuged, resuspended in fresh growth medium and transferred to the flasks.

2.2.2.4 Transient transfection of NIH3T3

Cells were transfected with pwt 929 luciferase reporter plasmid ($1\mu\text{g}$) and the plasmid expressing appropriated sequences of E5 or E5 mutant forms ($2\mu\text{g}$), using the calcium phosphate method. Cells (2×10^5) were plated in each well of 6 well plate, in 5 ml of DMEM medium containing: 1 mM sodium pyruvate, 0.375% sodium carbonate, 10% New Born Calf Serum (NBCS) and 2mM glutamine (DMEM-10) the day before transfection. For each well the following mixture was set up: 250 μl of a DNA solution (up to 3 μg of total plasmid DNA in H_2O containing 250 mM CaCl_2) was added to 250 μl of 2X HEPES buffered saline (HBS). After 16-18 hours precipitates were washed off with 2ml of pre-warmed PBS and incubated in low serum (0.5%) or growth serum (10%) for further 24 hours before being analysed.

2.2.2.5 Luciferase Assays

Cells were seeded at 2×10^5 cells/ml in DMEM-10 in each well of 6 well plates the day before transfection, and transfection was performed using the Effectene transfection reagents (Qiagen) following the manufacturer's recommendations. After 16-18hr, the cells were washed twice with 2 ml PBS and incubated in DMEM-10 or

0.5% serum (DMEM-0.5) for a further 24 hr. Cells were then washed twice with PBS, the PBS was completely removed by aspiration and 300 µl of 1X reporter lysis buffer added to each well. Following 15 minutes incubation at room temperature, cells were scraped off the culture well and each lysate transferred to a 1.5 ml Eppendorf tube. Cell debris was pelleted by spinning lysates at 4°C in a microcentrifuge at 14000 rpm for 5 minutes. The supernatant was transferred to a second Eppendorf tube taking care not to disrupt the cell pellet. The lysate were either assayed for reporter enzyme activity immediately or stored at -70°C.

Luciferase activity was determined using a microplate luminometer TR717 (Tropix), for each sample, 80 µl of lysate and 120 µl of luciferase assay buffer were used. Luciferase activity was normalised for protein content determined using the bicinichonic acid solution assay (BCA) as described in section 2.2.5.2.

2.2.2.6 Production of stable transfectants

NIH3T3 and COS-7 cells were transfected with a range of plasmids using the standard calcium phosphate-mediated precipitation. Cells were plated at a density of 0.5×10^6 in a 90 mm tissue culture dish the day before transfection. The cells were fed with 10 ml of DMEM-10 (10% NBCS for NIH3T3 cells; 10% FCS for COS-7 cells) on the day of transfection. For each plate the following mixture was set up: 500 µl of a DNA solution (up to 5-10 µg of total plasmid DNA in H₂O containing 250 mM CaCl₂) was added to 500 µl of 2X HEPES buffered saline (HBS). The addition of the DNA solution to the 2X HBS must be 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 slowly added to the medium and cells were 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 dish. The next day cells were selected in medium containing 500 µg/ml G418 for 21-28 days, being fed twice weekly. After this time, for NIH3T3 cells, G418-resistant colonies were scored and picked from each transfection class in order to expand them clonally. COS-7 cells stably transfected were pooled together and expanded.

2.2.2.7 Isolation of clonal populations

Single G418 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 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 along with 2 ml medium and returned to the 37°C incubator for expansion into cell lines.

2.2.2.8 Total RNA extraction from cell lines

Cells were grown in a 175 cm² (T175) flask to approximately 80% confluence following the RNazol B method of extraction (Biogenesis Ltd, UK). Cells were washed twice with ice cold PBS and 10 ml 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 pipetting. The top of the tube was then covered with parafilm 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 rotor) at 10000 rpm 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 ml of 75% ice cold ethanol (made with diethylpyrocarbonate (DEPC) -treated RNase-free water), and transferred to an Eppendorf tube. The RNA was pelleted in a microcentrifuge at full speed at 4°C for 30 min, dried on a speed-vacum centrifuge and then resuspended in DEPC-treated RNase free water. Samples were treated with DNA-free (Ambion), following the manufacturer's recommendations, to eliminate any possible residual of genomic DNA. Finally the concentration of RNA was measured spectrophotometrically as described in section 2.2.1.12. RNA samples were aliquoted and stored at -70°C.

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

RNA was prepared as described above and used as 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: 2.5 mM MgCl₂, 1X PCR kit buffer II (500 mM KCl, 100 mM Tris-HCl), 1 mM of each of dATP, dGTP, dTTP, dCTP, 1 unit RNase inhibitor, ≤ 1 μ g RNA, 2.5 units MuLV reverse transcriptase, 0.15 μ M of appropriate reverse primer and DEPC-treated water to a final volume of 20 μ l. As control, for DNA contamination, an identical reaction mix was prepared for each sample excluding the addition of reverse transcriptase. To verify that the products obtained had the correct dimension a RT-PCR reaction was performed with pZipneoHAE5 plasmid as template. Finally, to check the reaction parameters and the quality of the RNA parallel mixes were set up utilising the RNA, samples with a reverse for actin mRNA.

All samples were placed in the thermocycler 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 100 μ l by adding 2 mM MgCl₂, 1X kit PCR buffer II, 0.15 μ M of the appropriate forward primer and 2.5 units of *Taq* polymerase. Amplification proceeded for 2 min at 95°C and then 35 cycles of 94°C for 30 seconds and 60°C for 30 seconds followed by final extension at 72°C for 10 minutes using the Perkin-Elmer Cetus 9600 thermocycler. The samples were then analysed by agarose gel electrophoresis as described in section 2.2.1.5 to ensure correct amplification.

2.2.3 Transformation assays

2.2.3.1 Cell population growth in high and low serum culture (Crystal Violet assay)

Cells were seeded in 96 well tissue culture plates, in triplicate, at 5×10^3 cells/well in 200 μ l of DMEM-10. One 96 well plate of cells was set up for each day from day 0 to day 6. The outer wells along the edge of the plate were not used and instead 200 μ l 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 containing different serum concentrations (10%; 1%; 0.5%; 0.2%) in the appropriate wells.

The first plate, corresponding to day 0, was treated with 100 μ l 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 at daily intervals for 6 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 100 μ l 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 μ l of 1% alkaline SDS and 0.2 M NaOH 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 590 nm using a automated microwell plate reader (Dynatech MR7000).

2.2.3.2 Flow cytometry analysis (FACS)

Flow cytometry analysis (FACS) was used to analyse the cell cycle profile of cell lines in different growth conditions.

Cells were seeded at the density of 1×10^6 in a 90 mm dish and grown in DMEM-10 or DMEM-0.2 for 24 or 48 hr. Cells were harvested by trypsinisation and pelleted at 4°C in PBS. Each pellet was washed twice with ice cold PBS, by centrifugation. PBS was aspirated off and the pellet resuspended in a small volume of PBS (500 μ l) and cells were fixed by slow addition of 4.5 ml of ice cold ethanol. At this point samples were store at -20°C or used immediately for analysis as follows.

After one hour on ice, cells were pelleted at 4°C for 5min at 2000 rpm. Ethanol was aspirated off and each cell pellet was washed once with ice cold PBS to remove any residual ethanol. Cells were then again pelleted at 4°C for 5 min at 2000 rpm then resuspended in 500 μ l of staining solution (PBS containing 250 μ g/ml of RNase, 20 μ g/ml propidium iodide). Samples were left covered at 4°C for at least 3 hr before analysis.

Stained cell samples were filtered through 40 µm nylon cell strainer into polystyrene round bottom tubes. Samples were assayed using a Becton Dickinson FACScan machine and analysed using the “Modfit v2.0” software package.

2.2.3.3 Anchorage-independent growth

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 cell lines was assayed by plating cells in methyl cellulose based medium (methocel).

Methocel medium was made up as follows: 6.4 g of Methocel MC 4000 (Fluka) was added to 400 ml of distilled water and autoclaved. The methocel was left to dissolve with stirring overnight at 4°C. The final working medium was obtained mixing an equal volume of the 1.6% methocel and 2xDMEM containing 2 mM sodium pyruvate, 0.75% sodium carbonate, 4 mM glutamine and 40% Foetal Calf Serum (FCS).

Efficiency of methocel colony formation was determined by adding $2.5-5 \times 10^4$ cells to 15 ml of 0.8% methocel medium. The 15 ml mix was plated in two 60 mm bacterial petri dishes. Bacteriological petri dishes were used to discourage cells from adhering to the bottom of the dishes. Cells were left at 37°C for 2-3 weeks before being scored and photographed with PanF 50 technical film.

2.2.3.4 Luciferase assay in stable transfectants

Stable transfectants were seeded at 2×10^5 cells/well in a 6 well plate the day before transfection. 0.2 µg of pwt929, luciferase gene reporter plasmid, was transfected on the cells using Effectene transfection agent (Qiagen) following the manufacturer’s recommendations. Cells were incubated with the DNA at 37°C for 16-18 hr. After withdrawal of the medium, cells were washed twice with pre-warmed PBS and incubated in DMEM-10 or DMEM-0.5 at 37°C for further 24 hr before being harvested and assayed as described in section 2.2.2.5.

For the assay in suspension cells were transfected as described above. After an overnight incubation cells were washed with PBS, trypsinised and transferred on a 60 mm bacterial petri dish in 7 ml of 0.8% methocel medium (see section 2.2.3.3) with 10% NBCS and incubated in a humid incubator for 24 hr. The following day cells were pelleted as follows: 5 ml of warmed PBS were added to the dish in order to dilute the methocel, the content of the dish was then transferred in a 15 ml Falcon

tube and cells were pelleted for 5 min at 1000 rpm. The pellet was washed twice with warm PBS to eliminate any trace of medium. The PBS was then completely removed and the pellet was resuspended in 300 µl of 1X reporter lysis buffer and left at room temperature for few minutes before being assayed as described in section 2.2.2.5.

2.2.3.5 *In situ* staining for β-galactosidase

The plasmid pHSV β-gal expressing the β-galactosidase gene was used as control for the transformation efficiency of the stable clones.

For the staining 2×10^5 cells for each cell line were seeded in a six well plate and transfected with pHSV β-gal plasmid using Effectene transfection reagent and following the same protocol used for the pwt929 plasmid (see section 2.2.3.4). Cells were left at 37°C overnight. The following morning cells were washed with PBS and fixed in 2% formaldehyde, 0.2% glutaraldehyde in PBS for 5 min at 4°C.

Cells were further washed with PBS and then incubated overnight at 37°C with 4 ml of the reaction mix consisting of 1 mg/ml of X-galactopyranoside, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM magnesium chloride in PBS. During the incubation the plates were left in the dark.

β-galactosidase can catalytically convert X-Galactopyranoside and produce an intense blue precipitate, consequently it is possible to distinguish the cells expressing the β-galactosidase gene from their blue colour. The number of coloured cells scored in each plate is an indication of the transfection efficiency of each cell line.

2.2.4 Immunocytochemistry

2.2.4.1 Cell transfection

Cells were transiently transfected using the standard calcium phosphate-mediated precipitation. COS-7 cells were plated in a 8 wells chamber slide, 2×10^4 cells per well in 500 µl of DMEM-10 (10%FCS), and left overnight at 37° C in a humid incubator. The following day the medium was removed three hours before transfection and replaced with 250 µl of fresh medium. For each well of cells to be transfected the following mixture was set up: 12.5 µl of DNA solution containing 0.1 µg of appropriate plasmid, 250 mM CaCl₂ and water, once ready the DNA solution was added drop by drop to 12.5 µl of 2x HBS, mixing continuously. The final solution of 25 µl was left 20-30 min at room temperature and then gently added to

the medium while the chambers were moved gently to disperse the solution throughout the medium. The cells were returned at 37°C for a further 3 hours for COS-7 cells then washed twice with PBS and incubated with 500 µl. Precipitates were washed off 16 hours after transfection for NIH3T3 and CHO cells. Twenty-four or forty-eight hours after the last wash, cells were fixed in 3% paraformaldehyde, mounted and visualised using the confocal microscope

2.2.4.2 Immunofluorescence

Cells, transfected as described above, were washed twice with PBS and fixed with a solution of 3% paraformaldehyde in PBS pH 7.4 (250 µl each well) for 15 minutes. The paraformaldehyde was removed and each chamber was washed three times with 100 mM glycine in PBS and two times with PBS. Cells were either mounted at this point (as described below in this section) or permeabilized with permeabilising solution (250 µl of 0.1% saponin, 10% FCS, and 20 mM glycine in PBS) for 20 min and then blocked in 0.1% saponin, 10% FCS in PBS for 1 hr. After removing the blocking solution 100-200 µl of primary antibody, at the appropriate dilution, was applied to each well for 1 hr.

After the incubation, the cells were washed 3 times for 5 min with the blocking solution and incubated with 200 µl of secondary antibody at the recommended dilution for one hr. Cells were finally washed three times with the blocking solution and mounted using Vectashield™ solution. All operations were carried out at room temperature and during the incubations cells were kept in the dark. Cells were analysed at the confocal microscope (Biorad Nikon Diaphot inverted microscope) using the appropriate filter.

For the BODIPY® TR ceramide staining, prior to fixation, cells were washed twice with 25 mM Hepes (pH 7.5) in DMEM serum free (H-DMEM) and incubated with 5 µM of BODIPY-TR in H-DMEM for 30 min at 4°C. Subsequently the cells were washed two times with H-DMEM and incubated at 37°C for 30 min. At the end of incubation cells were further washed with H-DMEM, then fixed, mounted and analysed as described above.

2.2.5 Protein Analysis

2.2.5.1 Protein preparation and Protein Concentration measurement for Western Blot Analysis

For western blot 1×10^6 cells were seeded in a 90 mm dish (two for each cell lines). The following day the medium was aspirated from the plates and cells incubated in 10% or 0.5% medium for further 24 hr. Cells were then washed twice with ice cold PBS, the PBS was completely removed by aspiration and a 100 μ l of boiled SDS-PAGE lysis buffer (2.1.3) was added at the cell monolayer. Cells were consequently scraped and transferred in a 1.5 ml microcentrifuge tube and boiled for five minutes. Consequentially cells were sonicated using an MSE Soniprep 150 sonicator. Cell debris was pelleted at 14000 rpm for 10 min at 4°C and the supernatant transferred to a new microcentrifuge tube. To measure protein concentration 10 μ l of total extract were diluted in 490 μ l of water and transferred to a 500 μ l quartz cuvette with a pathway of 1 cm, 10 μ l of lysis buffer in 490 μ l of water were used as blank. The absorbance of each sample was read using a Beckman DU 650 spectrophotometer at the fix wavelength of 280 nm. The concentration of each sample was calculated knowing that the absorbance of 1.0 OD at 280 nm corresponds approximately to a concentration of 1 mg/ml protein. Aliquots of 30 μ g to 150 μ g were made up in lysis buffer and prepared for loading with equal volume of 2X SDS loading buffer. Samples were boiled for 5 min and protein separated by SDS-PAGE as described in section 2.2.5.4.

For the assay in suspension 5×10^6 cells were resuspended in 30 ml of 0.8% methocel DMEM-10 and transferred on a 150 mm bacterial petri dish and incubated in a humid incubator for 24 hr or 48 hr. At the end of the incubation time the cells were pelleted as follows: 20 ml of ice cold PBS was added to the dish in order to dilute the methocel, the content of the dish was then transferred in a 50 ml Falcon tube and cells were pelleted for 5 min at 1000 rpm at 4°C. The pellet was washed twice with cold PBS to eliminate any trace of medium, before the last wash cells were moved to a microcentrifuge tube and pelleted as above. The PBS was then completely removed and the pellet was resuspended in 100 μ l of boiled SDS-PAGE lysis buffer, boiled for 5 min and then cooled down on ice. Samples were stored at -70 or assayed for protein concentration as described above. Protein extracts (30-100 μ g) were then prepared for loading with equal volume of 2X SDS loading buffer.

2.2.5.2 Protein preparation and Protein Concentration measurement for Kinases assays

Cells were seeded in 150 mm tissue culture dishes at 3×10^6 cells for dish in 25 ml of DMEM-10 and left at 37°C overnight. The following day the medium was changed and cells left in DMEM-10 or DMEM-0.5 for further 24 hr. The plates were then put on a ice bath for 10 min and then washed twice with ice cold PBS. After the PBS was completely removed, 500 μl of kinase lysis buffer (2.1.3) were added to the cells and carefully distributed on the entire surface of the plate. Cells were frozen by placing the plates on methanol/dry ice bath for 30 sec, then thawed on ice and transferred in a microcentrifuge tube and frozen in methanol/dry ice a second time or store at -70°C .

For the assay in suspension 5×10^6 cells were resuspended in 30 ml of 0.8% methocel DMEM-10 and transferred on a 150 mm bacteria petri dish (three dishes were prepared for each cell line) and incubated in a humid incubator for 24 or 48 hr. At the end of the incubation time the cells were pelleted as follows: 20 ml of ice cold PBS was added to the dish in order to dilute the methocel, the content of the dish was then transferred in a 50 ml Falcon tube and cells were pelleted for 5 min at 1000 rpm. The pellet was washed twice with cold PBS to eliminate any trace of medium, before the last wash cells were move to a microcentrifuge tube. The PBS was then completely removed and the pellet was resuspended in 500 μl of ice cold kinase lysis buffer and frozen by placing the tubes on methanol/dry ice for 30 sec, subsequently cells were thawed on ice and frozen in methanol/dry ice a second time or stored at -70°C .

Lysates were spun at 14000 rpm for 10 min at 4°C and supernatant transferred in a fresh microcentrifuge tube and protein measured using BCA/ CuSO_4 as described below.

10 μl of protein solution was placed in a 96 well plate in duplicate, 200 μl of developing solution consisting of 5 ml BCA and 100 μl of 4% (w/v) CuSO_4 (copper II sulphate pentahydrate solution) was added to the protein samples and the plate incubated at 37°C for 30 minutes. The assays is based on the ability of proteins to reduce alkaline Cu(II) to Cu(I) in a concentration-dependent manner. BCA is a highly specific chromogenic reagent for Cu(I) , forming a purple complex with an

absorbance maximum at 562 nm. After the incubation the absorbance of each sample was read at 590 nm using a Dynatech MR7000 automatic plate reader.

The absorbance reading was converted to concentration in $\mu\text{g/ml}$ 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

Sodium dodecyl sulphate (SDS) gels containing different concentration of polyacrylamide (i.e. 7.5%, 10%, 15%) were used to resolve proteins according to their molecular weight. For a single 1 mm, 10% polyacrilamide gel, for example, the solution was prepared as follows: 7.8 ml of water, 6.7 ml of 30% acrylamide, 5 ml of 1.5 M Tris-HCl pH 8.8, 0.2 ml 10% SDS, 0.2 ml 10% ammonium persulphate (APS) and 8 μl of TEMED.

The resolving gel was poured between two glass plates, previously washed with 1% decon, carefully rinsed in water and finally cleaned with 70% ethanol. A thin layer of water saturated butan-1-ol was added at the top of the gel and the solution left to polymerise at room temperature. The layer of water saturated butan-1-ol was washed away at completed polymerisation and a 5% stacking gel was poured at the top of the resolving gel.

The stacking gel was prepared as follows: 6.8 ml of water, 1.7 ml of 30% acrylamide, 1.25 ml of 1M Tris-HCl pH 6.8, 0.1 ml of 10% SDS, 0.1 ml of 10% APS and 10 μl of TEMED.

Once ready, the gel was transferred to an electrophoresis tank filled with running buffer, the comb was removed from the stacking gel and each well carefully washed. The RainbowTM protein markers (high molecular weight range 200-14 kDa and low molecular weight range 45-14 kDa) and the samples solutions were prepared for loading as follows: 50-100 μg of protein extract or 5 μl of the markers were mixed with equal volume of 2X loading buffer, boiled for 5 min and then cooled down on ice. The samples were loaded into consecutive wells between the high molecular weight marker on the left and the low molecular weight marker on the right.

The gel was run at 20 mA for approximately 20 min to allow the samples to enter in the stacking gel, and at 40 mA constant current for ~2 hr.

2.2.5.4 Western Blotting

As soon as the blue dye had run off the bottom the gel run was stopped and the protein transferred to a nitro-cellulose membrane by semi-dry blotting. The gel was removed from the glass plates and soaked for 10 min in transfer buffer (2.3.1), while 8 pieces of Whatman 3MM paper and a piece of nitro-cellulose membrane (ECL-hybond) of the same dimension of the gel, were wetted in transfer buffer.

Four sheets of the Whatman 3MM paper were placed on the transfer apparatus and the nitro-cellulose put on the top of them followed by the gel. Any air bubbles between the gel and the membrane were eliminated by gently rolling a plastic pipette over the gel. Finally the four remaining Whatman 3MM sheets were placed on the top of the gel, the apparatus closed and a voltage of 20V applied for 1 h.

Once the transfer was completed the membrane was blocked for 1 hr in 5% Marvel (dry milk) in TBST (TBS plus 0.1% Tween) at room temperature. Subsequently the membrane was washed for 5 min in TBS and incubated with the appropriate primary antibody (diluted following supplier's recommendation in 3%BSA, 0.02% NaAzide in TBS) and left shaking at 4°C overnight (Table 2.2).

The membrane was then washed three times for 10 min each with TBST and incubated in 50 ml of blocking solution containing 1/5000 dilution of the appropriate secondary antibody linked to horse-radish peroxidase and left shaking for 1 hr at room temperature. After three further washes with blocking solution of 10 min each the blot was rinsed in TBST. The membrane was then placed on cling film and covered with a detection solution consisting of an equal volume of Amersham Enhanced chemiluminescence (ECL) reagents 1 and 2 for 1 min. The liquid was then removed from the nitro-cellulose membrane and this was wrapped in cling film and placed in a film cassette to be exposed to a Fuji-XR film at appropriated time depending on the strength of the signal.

2.2.5.5 Immunoprecipitation and Kinase reaction

Aliquots of 500 µg protein were made up to 500 µl in lysis buffer. 2 µl of the appropriate antibody were added to each tubes and the samples placed in rollers at 4°C for 1.5 hr. Protein-A sepharose was pre-swollen in PBS for 1 hr at 4°C and then washed three times with PBS and three times with kinase lysis buffer and finally

resuspended in kinase lysis buffer to a 50% slurry solution. After the incubation with the antibody 12.5 μ l of 50% slurry protein A-sepharose were added to each tube then placed back on rollers for 1.5 hr. After the incubation 12.5 μ l of sepharose CL 4B, prepared as a 50% slurry solution in lysis buffer was added to each tube as carrier. The samples were spun down for 30 sec at maximum speed at 4°C and the supernatant discarded, the sepharose beads were washed three times in ice cold kinase lysis buffer and two times in ice cold kinase buffer. After the last wash the supernatant was removed and 20 μ l of reaction buffer (0.05 μ g/ μ l Histone H1, 20 μ M ATP, 10 μ Ci γ -³²P-ATP, 50 mM HEPES, 50 mM NaCl, 10 mM MgCl₂, 1 mM DTT) were added to each tubes and the samples incubated at 30°C for 30 min. The reaction was stopped by adding 20 μ l of 2X loading buffers and boiling for 5 min. The samples were loaded in a 10% acrylamide gel.

At the end of the run the gel was washed in fixing solution (50% methanol and 10% acetic acid) for an hour, during which the solution was changed four times, and then in 10% glycerol for a following hour. The gel was finally dried for 2 hr at 80°C in an Appligen Slab gel dryer and exposed to X-ray film for the appropriated time and analysed at the phosphorimager.

2.2.6 MAPK inhibitors

The effects of two MAPK inhibitors (PD98059 and U0126) to the cells maintained in suspension culture were determined.

For the assay in suspension cells (3×10^6) were resuspended in 10 ml of 0.8% methocel DMEM-10 containing PD98059 or U0126 inhibitors (final concentration 50 μ M and 20 μ M in DMSO respectively). As control cells treated with only DMSO were prepared. Plates were incubated in a humid incubator at 37°C for 48 or 72 hours.

At the end of the incubation time cells were harvested and total protein extracted as described in section 2.2.5.1. Protein concentration was measured and aliquots of 30 μ g were made up in lysis buffer and prepared for western blot analysis as described in section 2.2.5.5. PD98059 inhibitor blocks the activation of MEK while U0126 specifically inhibits MEK in both active and inactive forms.

Table 2.2 Summary of antibodies

Antibodies	Supplier-Cat. Number	Application-Dilution
Cyclin A	Santa Cruz sc-596	WB 1:500
Cyclin A	Santa Cruz sc-751	IP 1:250
Cyclin D1	Santa Cruz sc-450	WB 1:1000
Cyclin E	Santa Cruz sc 481	WB 1:500 IP 1:250
CDK2	Santa Cruz sc 163	WB 1:500 IP 1:250
CDC2	Santa Cruz sc-54	WB 1:500
pRb Total	Pharmigen-14001A	WB 1:1000
pRb Ser-780	NEBiolab-9307S	WB 1:1000
p107	Santa Cruz sc-318	WB 1:5000
p130	Santa Cruz sc-317	WB 1:5000
p27	Santa Cruz sc-528	WB 1:500
Total ERK 1,2	Sigma M5670	WB 1:1000
Phospho ERK 1,2	Sigma M8159	WB 1:1000
GFP	Babco MMS-118	WB 1:1000
α -Tubulin	Sigma T9026	WB 1:1000

WB: western blotting

IP: immunoprecipitation

All solutions were made up in 3% BSA in TBS

CHAPTER 3: DETECTION AND LOCALISATION OF THE BOVINE PAPILLOMAVIRUS TYPE 4 E5 PROTEIN

3.1 The E5 proteins of papillomaviruses

The E5 protein family is a group of small membrane-localised peptides comprising E5 proteins from animal and human papillomavirus (HPVs). The gene is not well conserved at the DNA level between HPVs, or animal viruses although there is a conservation of the physico-chemical properties in that the proteins are all highly hydrophobic and membrane bound (Figure 1.2), (Burkhardt et al., 1989; Conrad et al., 1993).

It is notoriously difficult to detect the expression and localisation of this group of proteins. Like most viral proteins, they are low-abundance proteins expressed at specific stage of the viral cell cycle, moreover their hydrophobic nature have rendered their purification extremely difficult because the proteins are insoluble. Antibody reagents directed against E5s are consequently limited and not very efficient.

In order to overcome the limitations in the detection of E5 proteins the addition of peptides as tags to the N-terminus of the proteins has been used (Schlegel et al., 1986; Conrad et al., 1993). Moreover, for most of the studies the E5 ORFs have been inserted into vectors where their expression is driven by heterologous, strong promoters (Schlegel et al., 1986; Burkhardt et al., 1989; Leechanachai et al., 1992).

In cells transiently or stably transfected with E5s, the proteins have been mostly detected by immunoprecipitation after metabolic labelling of cellular proteins with different radioactive amino acids ($[^{35}\text{S}]$ -methionine, $[^{35}\text{S}]$ -cysteine and $[^3\text{H}]$ -leucine) (Schlegel et al., 1986; Conrad et al., 1993; O'Brien et al., 2001).

3.1.1 Detection of the Bovine Papillomavirus type 4 (BPV-4) E5 protein

The BPV-4 E5 ORF could not be expressed in bacteria, probably because of the high hydrophobicity of the peptide (Jackson et al., 1991). In 1993 Pennie and co-

workers used an antiserum raised in rabbits against a synthetic oligopeptide corresponding to C-terminal 10 amino acids of E5. The cellular localisation of the viral protein was investigated by immunostaining primary bovine fibroblasts (PalF) transiently transfected with a vector expressing the E5 gene. E5 showed a clear membrane localisation: it was found in the nuclear membrane, in the endoplasmic reticulum (ER), in the Golgi apparatus and occasionally on the cell surface (Pennie et al., 1993).

In vitro synthesis and characterisation of BPV-4 E5 was also performed (Faccini et al., 1996). BPV-4 E5, tagged with the 11 amino acid sequence from the influenza virus haemagglutinin (HA), was *in vitro* transcribed and translated in the presence of microsomes and labelled with [³⁵S]-methionine. A specific band of ~7 kDa was detected by immunoprecipitation with a monoclonal antibody against HA. This is the predicted size of E5 as deduced from its amino acids sequence.

In more recent studies the HA tagged protein was detected in stably transformed PalFs by dot blot analysis using the anti-HA antibody (O'Brien et al., 1999).

Finally BPV-4 E5 was detected in clonal populations of NIH3T3 cells. BPV-4 E5 was immunoprecipitated with an antiserum raised against the last 10 amino acids of the protein after cells had been metabolically labelled with [³H]-leucine. Polypeptides of different molecular weights (14, 40 and 46 kDa) could be visualised by autoradiography. The authors proposed that the aberrant migration of these proteins could be the result of dimers or higher-order oligomer formation by E5 (O'Brien et al., 2001).

3.2 The Green Fluorescent Protein

3.2.1 Discovery and structure

The green fluorescence protein (GFP) was discovered by Shimomura *et al.*, in 1962, as a companion protein to aequorin, a chemiluminescent protein from the jellyfish *Aequorea Victoria* (Shimomura et al., 1962). The protein was subsequently purified and crystallised, its absorbance spectrum measured and fluorescence quantum determined (Morise et al., 1974). Prendergast & Mann (1978) estimated its molecular weight at 27 kDa (Prendergast and Mann, 1978). The crucial breakthroughs in the use of GFP came, only years later, with the cloning of the gene

(Prasher et al., 1992) and the demonstration that its expression in other organisms creates fluorescence. This revealed that the gene contains all the information necessary for the post-translation synthesis of the chromophore and jellyfish-specific enzymes are not needed (Chalfie et al., 1994; Inouye and Tsuji, 1994).

The natural fluorescence of GFP depends on a chromophore formed from residues 65-67 which are serine-tyrosine-glycine in the native protein (Heim et al., 1994). Its three dimensional structure consists of eleven β -strands forming a hollow cylinder through which is threaded a helix running up the axis of the cylinder. The chromophore is attached to the α -helix and is buried in the centre of the cylinder (Yang et al., 1996).

3.2.2 Application of the green fluorescent protein

GFP has become a versatile reporter protein for monitoring gene expression and protein localisation in a variety of cells and organisms. GFP fluorescence is stable, species-independent, and can be monitored non-invasively in living cells by both fluorescence microscopy and flow cytometry. Moreover, a variety of N- and C-terminal proteins fused with GFP have been constructed and shown to maintain both the fluorescence properties of native GFP and the biological function of the fusion partners (Kain et al., 1995). In order to overcome some undesirable properties of the wild type GFP, including low fluorescent intensity when excited by blue light at 470 nm, a variant form has been produced. A mutant of GFP that fluoresces 35 times stronger than the wild type GFP (Cormack et al., 1996) has been combined with a re-engineered GFP gene sequences containing codons preferentially found in highly expressed human proteins. The mutant has been called EGFP (Enhanced GFP) and bears a serine to threonine mutation at codon 65 and a phenylalanine to leucine at position 64. It presents a single peak of excitation at 490 nm and emits at 510 nm. (I will be referring to EGFP as GFP hereafter).

3.2.2.1 Proteins tagged with GFP

There are various cases in the literature where viral proteins have been tagged to GFP without any effect on their biological functions.

To detect HPV 11 E5a expression in eukaryotic cells, GFP was fused to the 3' of the E5a gene (Brown et al., 1998). GFP has been inserted into human

immunodeficiency virus type 1 (HIV-1) genome by replacing the *nef* gene. The GFP-expressing HIV-1 replicated efficiently in established human T cells and GFP was maintained in the viral genome during prolonged passages (Lee A.H. et al., 1997). GFP was inserted also in a Herpes simplex virus type 1 (HSV-1) genome in frame with the capsid protein VP26. The engineered virus was shown to grow as well as the wild type in cell culture and the fusion protein was incorporated into intranuclear capsids and mature virions (Desai and Person, 1998).

GFP has been used to tag a large number of transmembrane proteins (type I or type II) and it does not interfere with the assembly, insertion in lipids bilayer or transport of these proteins. TGN38, for example, is a type I integral membrane protein which cycles between the trans-Golgi network (TGN) and the cell surface, forms of TGN38 fused at the N- or C-terminus of GFP showed identical response to drugs and temperature blocks known to perturb intracellular morphology and membrane traffic pathways. Moreover GFP did not interfere with the transport of TGN38 along the secretory pathway or its retention in TGN (Girotti and Banting, 1996).

From the properties listed above it appeared that the use of GFP could overcome the difficulties encountered so far in the detection of E5. For the nature of the research we wanted to pursue, it was necessary to have a reliable and consistent system to visualised E5 routinely. For these reasons two fusion forms of E5 with GFP were created in the attempt to visualise the location of the viral protein within the cell. Moreover, the GFP tagged form of E5 has been utilised in transformation assays (growth in absence of mitogens and substratum), to verify that E5 transforming abilities had been maintained (Chapter 4).

3.3 Experimental work

3.3.1 Construction and detection of BPV-4 E5 fusion forms with GFP

3.3.1.1 Cloning and DNA sequencing

In order to obtain two fusion proteins of BPV-4 E5 with GFP the E5 sequence was cloned in pEGFPC3 and pEGFPN2 vectors, at the C-terminus and N-terminus of GFP respectively using a PCR cloning strategy (section 2.2.1). The constructs were

consequently sequenced to confirm their identity and called GFP-E5 and E5-GFP (Figure 3.1).

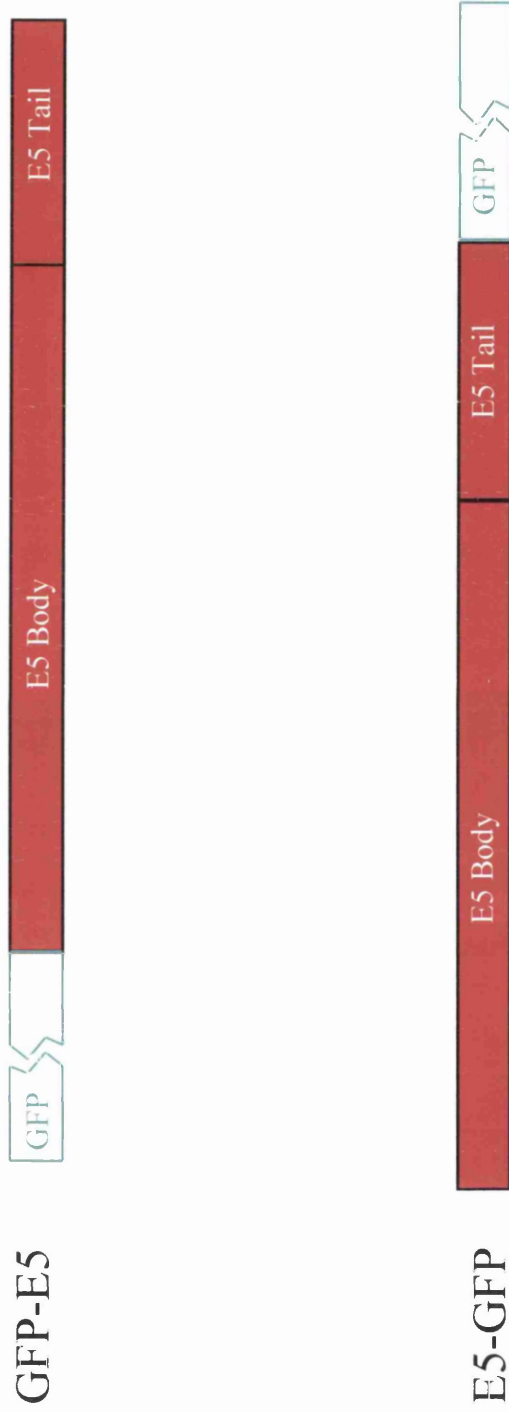


Figure 3.1 Structure of BPV-4 E5 full length protein fused at the C-terminus and at the N-terminus of GFP

3.3.1.2 Visualisation of GFP-E5 and E5-GFP in transient transfectants

COS-7, NIH3T3 and CHO cells were transiently transfected with plasmids expressing GFP-E5 or E5-GFP and the vector expressing GFP alone, using a calcium phosphate precipitation method (section 2.2.4.1). Precipitates were washed off three hours after transfection for COS-7 cells and 16 hours for NIH3T3 and CHO cells. Twenty-four or forty-eight hours after the last wash, cells were fixed in 3% paraformaldehyde, mounted and visualised using the confocal microscope as described in section 2.2.4.2.

In all three cell types used, GFP transfected cells showed a green fluorescence uniformly distributed in the cytoplasm and nucleus, while both GFP-E5 and E5-GFP expressing cells presented a well-delineated area of fluorescence adjacent to the nucleus (Figure 3.2).

The fact that both fusion forms of E5 presented the same perinuclear localisation despite having GFP at the N or C terminus suggested that the cellular localisation of the fusion proteins was exclusively due to E5 sequence. However it has not been possible to rule out completely that the perinuclear fluorescence of GFP-E5 and E5-GFP transfected cells was due to mis-localisation caused by the GFP epitope because any attempt to detect BPV-4 E5, or the HA tagged form of E5, with an anti E5 antibody or antibodies against the HA epitope failed. However, E5 has been shown to have an ER and Golgi location on one previous occasion (Pennie et al., 1993).

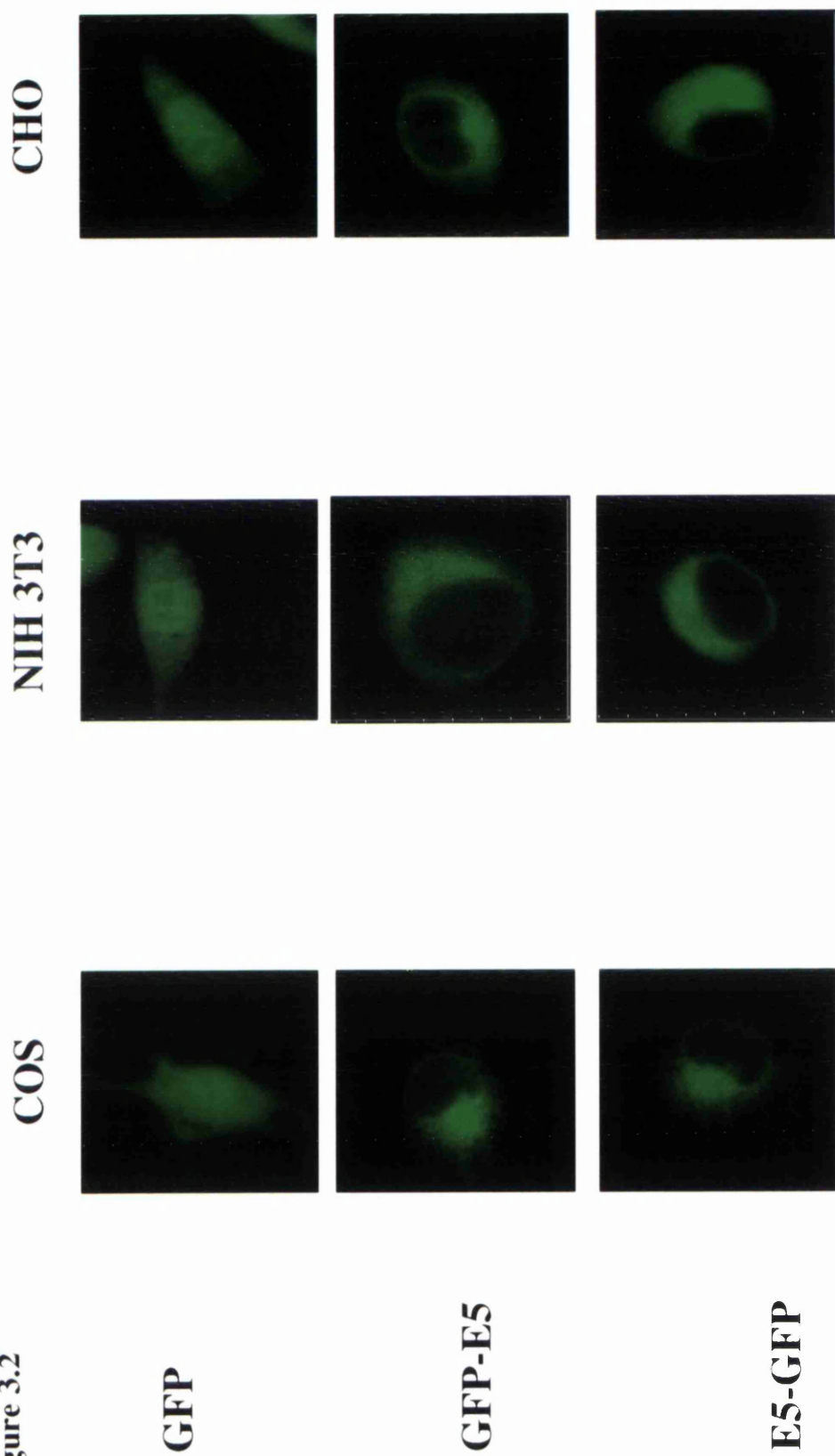
All cells even with the weakest fluorescence presented a similar distribution of GFP-E5, and this excluded the possibility that the cellular location of the GFP-E5 was due to its over-expression (data not shown). Taken all together these data sustain the hypothesis that GFP-E5 and E5-GFP location in the cell is likely to represent BPV-4 E5 natural localisation.

Because the two fusion proteins showed an identical cellular localisation in all cell types, only GFP-E5 form was utilised in subsequent experiments and all transient expression experiments were carried out in COS-7 cells.

Figure 3.2 Confocal images of GFP, GFP-E5 and E5-GFP expressed in COS-7, NIH3T3 and CHO cells

Cells were transfected with 0.1 µg of DNA, fixed after 24 hr with 3% paraformaldehyde and visualised at the confocal microscope. All images have been captured with 60X objective lens, images have been further magnified in Microsoft Powerpoint.

Figure 3.2



3.3.1.3 Visualisation of GFP-E5 in stable transfectants

COS-7 and NIH3T3 cells stably expressing GFP and GFP-E5 were produced to confirm that cells could sustain long term expression of the fusion protein and that GFP-E5 localises in the same sub-cellular compartment when stably expressed.

GFP and GFP-E5 constructs were transfected in COS-7 and NIH3T3 cells using the calcium phosphate-precipitation method as described in section 2.2.2.6. After 21-28 days of G418 selection, resistant colonies from COS-7 cells were pooled together and expanded. From NIH3T3 plates single, resistant colonies were picked in order to expand them clonally as described in Chapter 4.

When analysed at the confocal microscope the cellular distribution of GFP and GFP-E5 confirmed the distribution observed in transient expression experiments (Figure 3.3, 4.2).

3.3.1.4 Detection of GFP-E5 protein expression in stable transfectants

As mentioned before an important advantage in the use of GFP fusion proteins is the possibility to detect expression of otherwise not easily detectable proteins, like BPV-4 E5.

In order to visualise protein expression in western blot, whole cell extracts (100 µg) from GFP and GFP-E5 expressing cells were prepared and fractionated by SDS-PAGE, transferred to a nitrocellulose membrane and probed with an anti GFP antibody (Babco). In COS-7-GFP cells a specific band with an apparent molecular weight of 27 kDa, corresponding to the reported electrophoretic mobility of GFP, was detected. The GFP-E5 expressing cells showed a specific band of ~34 kDa. This is the predicted size of the fusion protein resulting from the apparent 7 kDa molecular weight of E5 and the 27 kDa of GFP (Figure 3.3).

Here we demonstrated that in the GFP fusion form BPV-4 E5 can be easily visualised and detected, as predicted, and for the first time its expression could be observed in western blot.

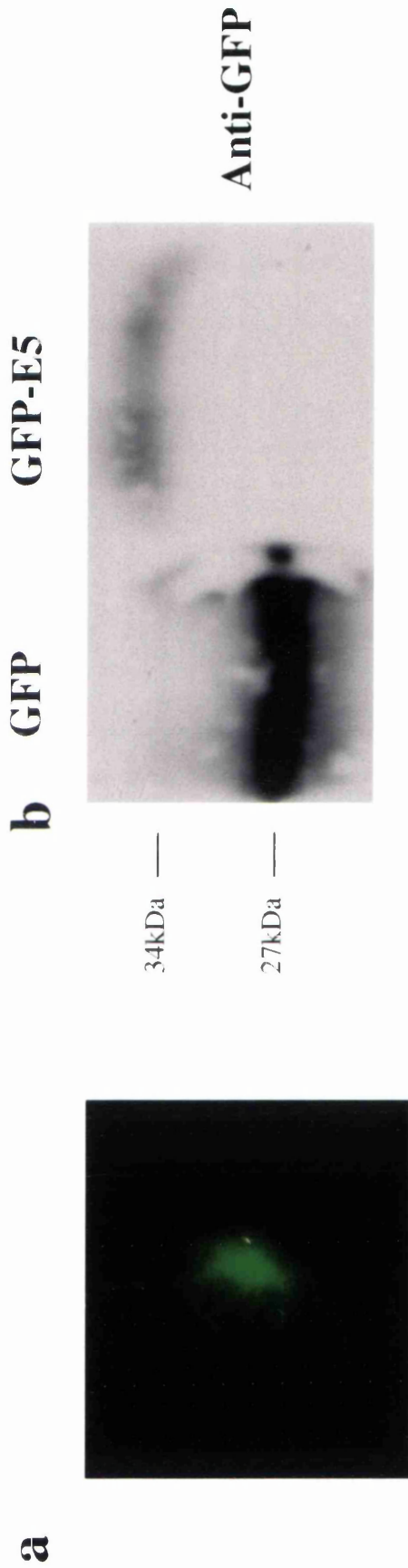


Figure 3.3 a, b Visualisation and detection of GFP-E5 in COS-7 cells

- a) Confocal image of COS-7 cells stably transfected with GFP-E5;
 b) Western Blot analysis of GFP and GFP-E5 expression in COS-7 stable transfectants; 100 μ g of whole cell extracts were fractionated in SDS-PAGE (10% polyacrylamide) and the membrane was probed with anti-GFP antibody (1:1000)

3.3.1.5 Colocalisation of GFP-E5 with ER and Golgi apparatus markers

The perinuclear fluorescence shown by GFP-E5 and E5-GFP suggested that the signal was coming from the two principal perinuclear cellular organelles: the ER and the Golgi apparatus.

In order to define better the exact sub-cellular location of GFP-E5, colocalisation studies with an ER or a Golgi apparatus marker were performed using the confocal microscope. The ER marker is an antibody against the Protein Disulphide Isomerase (PDI) (Vaux et al., 1990), an enzyme that catalyses the rearrangement of disulphide bonds of itinerant and resident ER proteins. This protein is characterised by a KDEL motif and its presence in the ER is achieved by a retrieval system (see section 3.3.2). As a Golgi marker we used a Texas-red conjugated ceramide homologue (BODIPY[®] TR ceramide) from Molecular Probes that stains the Golgi apparatus through the interaction with endogenous lipids and cholesterol (Pagano et al., 1991).

COS-7 cells, transfected with 0.1 µg of GFP-E5, were fixed in paraformaldehyde and permeabilised with 0.1% saponin in PBS. After one hour incubation with the blocking solution cells were stained with anti-PDI antibody (dilution 1:200 in blocking solution) for one hour at room temperature. The primary antibody was washed off with blocking solution and the secondary antibody (anti-mouse antibody texas-red conjugated) added for one hour. Cells were finally washed, mounted and visualised at the confocal microscopy (section 2.2.4). For the staining of the Golgi apparatus live transfected COS-7 cells were incubated with 5 µM of BODIPY-TR ceramide and left at 4°C for 30 min to allow the marker to accumulate on the plasma membrane. The cell metabolism was switched back on with a 30 min incubation at 37°C so that the marker was transported and accumulated in the Golgi apparatus. Cells were then fixed and mounted as described in section 2.2.4.2.

Cells were visualised at the confocal microscope to investigate the relative spatial localisation of the organelles markers and GFP-E5. Appropriate wavelengths were used to excite the chromophores (~596 nm for texas red, ~490 nm for GFP) and the emission spectra (615 nm for texas red and 510 nm for GFP) were captured with different filters. Signals were captured separately then the pictures from the two channels were overlapped and the result analysed. Colocalisation studies are based on

the principle that red and green dyes form a yellow image from spectral summation when the signals colocalise.

GFP-E5 is partially localised in the ER. When the red signal, coming from the ER marker was overlapped with the green of GFP-E5 the two fluorescence could still be distinguished and the region of colocalisation, characterised by the yellow fluorescence, was only minimal (Figure 3.4 a) maybe due to the partially distribution of the ER marker in the Golgi. On the contrary when the Golgi apparatus marker was used, most of the green fluorescence of GFP-E5 was converted to yellow showing that GFP-E5 is mainly localised in the Golgi (Figure 3.4 b).

The observations that GFP-E5 is located mainly in the Golgi apparatus supports what previously reported by Pennie *et al* (1993), and concords with the BPV-1 E5 and HPVs E5 subcellular localisation (Burkhardt et al., 1989; Conrad et al., 1993).

Figure 3.4 a, b GFP-E5 is mainly located in the Golgi apparatus

a) On the left COS-7 cells transiently expressing GFP-E5 and stained with the ER marker (anti PDI antibody), on the right the result of the merge between the two channels.

b) On the left COS-7 cells transiently expressing GFP-E5 and stained with the Golgi marker (BODIPY-TR ceramide), on the right the result of the merge between the two channels.

In both a and b yellow colour indicates colocalisation. Images have been captured with a 60X objective lens and colour pictures have been magnified in Microsoft powerpoint.

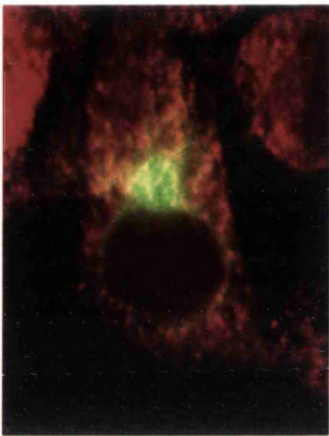
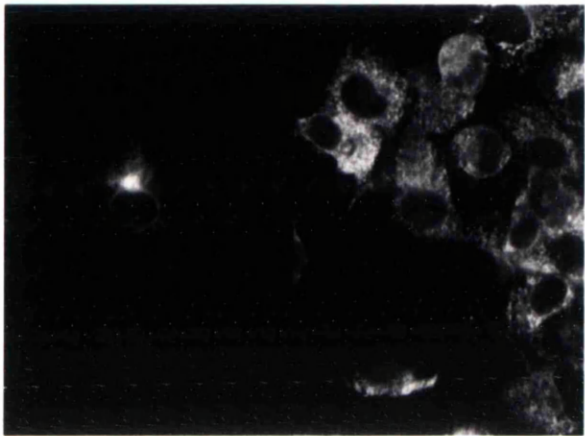
Figure 3.4 a, b

a

GFP-E5

ER Staining

Merge

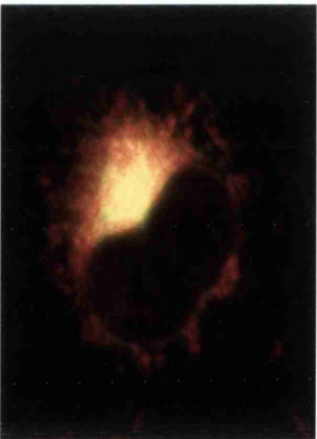
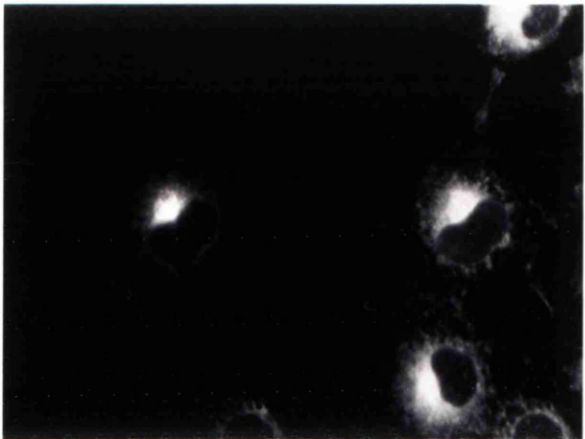


b

GFP-E5

GOLGI Staining

Merge



3.3.2 The importance of a Golgi location for BPV-4 E5 protein

3.3.2.1 Introduction

Immunocytochemistry and immunoelectron microscopy studies revealed that BPV-1 E5 is located within both ER and Golgi apparatus and to a lesser degree at the cell surface. The demonstration that BPV-1 E5 activated immature forms of platelet-derived growth factor receptor type β (PDGF β -R) that are in transit through the Golgi led to the speculation that this organelle was a critical location for E5 during cell transformation (see Chapter 1). To test this hypothesis a form of BPV-1 E5 bearing a SEKDEL (BPV-1 E5/KDEL) retrieval signal for the ER (Townesley et al., 1993) was used in transformation studies (Sparkowski et al., 1995). It is known that proteins bearing the KDEL amino acids are resident in the ER thanks to a retrieval system that involves a receptor protein that normally accumulates in the Golgi and is redistributed in the ER in the presence of ligands after it has picked up its cargo. BPV-1 E5/KDEL was localised in the ER and it was deficient for transformation in mouse fibroblasts (Sparkowski et al., 1995). These results led to the conclusion that the Golgi location is necessary for BPV-1 E5 transforming activity.

In the attempt to test if also BPV-4 E5 required to reside in the Golgi compartment or to travel through it in order to transform NIH3T3 cells, a mutant form of the viral protein bearing the SEKDEL sequence was generated (O'Brien and Campo, 1998). This form of E5 maintained the ability to transactivate the cyclin A promoter under normal growth conditions but was not capable of transactivating the cyclin A promoter in suspension cells (O'Brien and Campo, 1998); (for the relation between transformation activity of E5 and its mutant forms and their ability to transactivate cyclin A promoter see Chapter 6). These data suggest that the cellular location of E5 is crucial for its ability to transactivate cyclin A promoter and that at least a portion of E5 molecules adopt an orientation in which the C-terminus is luminal in membranes compartments. In these studies, however, it has not been possible to show where BPV-4 E5/KDEL was localised within the cell.

3.3.2.2 Experimental results and discussion

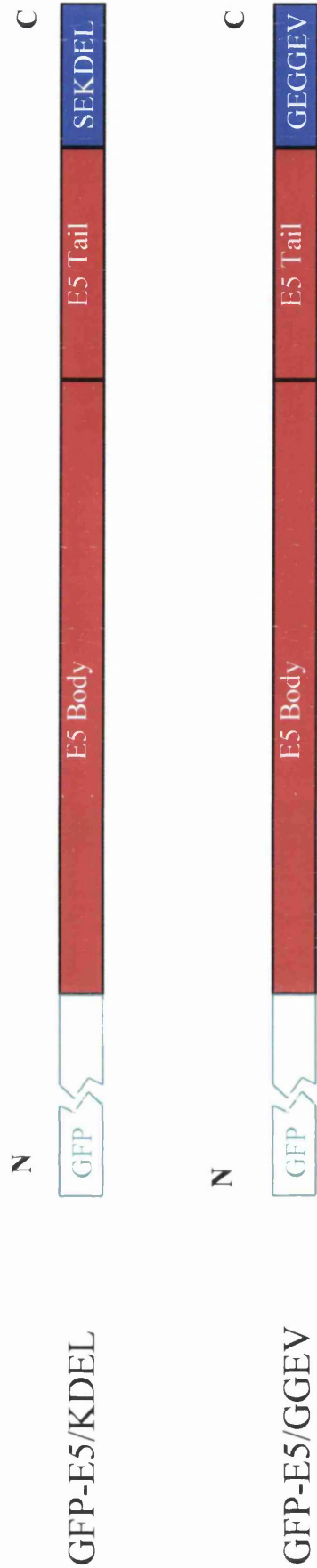
To confirm that the introduction of the SEKDEL sequence at the C-terminus of E5 leads to the retention of the viral protein in the ER, localisation studies were performed with a fusion form of the E5 mutant with GFP. As control we used a

second mutant in which the C-terminus was extended by six amino acids (SGGGEV) that have been shown not to influence BPV-1 E5 localisation (Sparkowski et al., 1995) and BPV-4 E5 ability to transactivate cyclin A promoter both in normal condition and suspension culture (O'Brien and Campo, 1998). Also this control mutant was expressed as a fusion form with GFP.

The cloning was performed using a PCR strategy utilising the E5C3 forward and the E5/KDEL or E5/GGEV reverse primers (section 2.2.1; Table 2.1). The constructs obtained are illustrated in Figure 3.5. COS-7 cells were transiently transfected with GFP-E5/KDEL, GFP-E5/GGEV and GFP-E5 using the calcium phosphate method, precipitates were washed three hours after transfection and cells visualised after 16 hr, 24 hr and 48 hr by confocal microscopy.

GFP-E5/KDEL shows a perinuclear localisation comparable to GFP-E5/GGEV and GFP-E5 at all different times tested (Figure 3.6 shows staining after 24 hr incubation). These data demonstrate that the retrieval signal was not sufficient to relocate E5 to the ER.

a



b

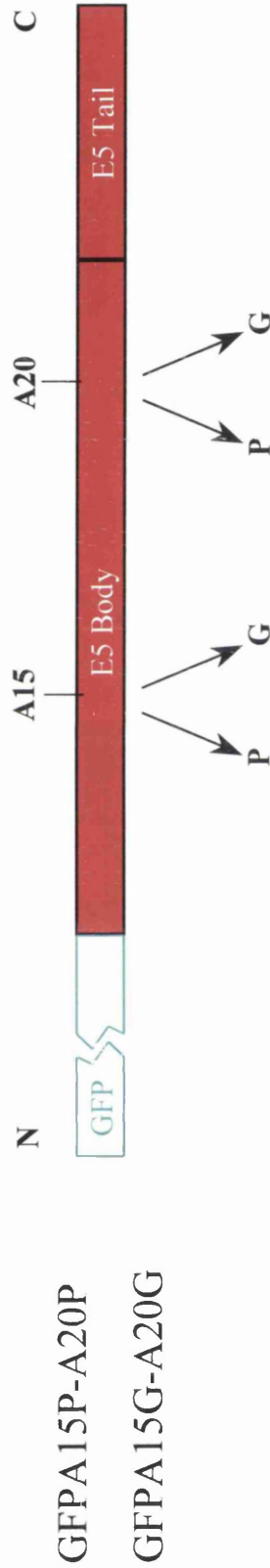
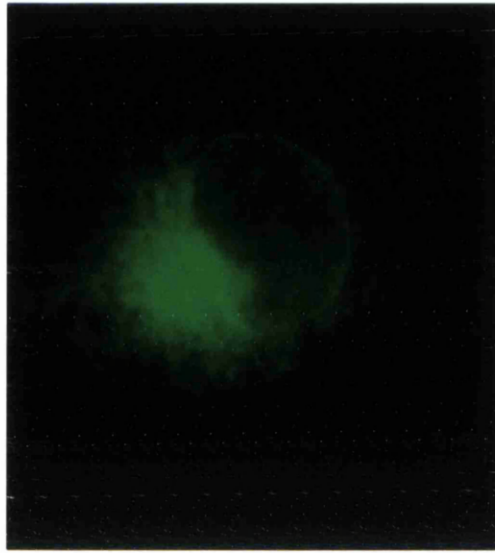


Figure 3.5 a, b E5 mutant forms fused with GFP

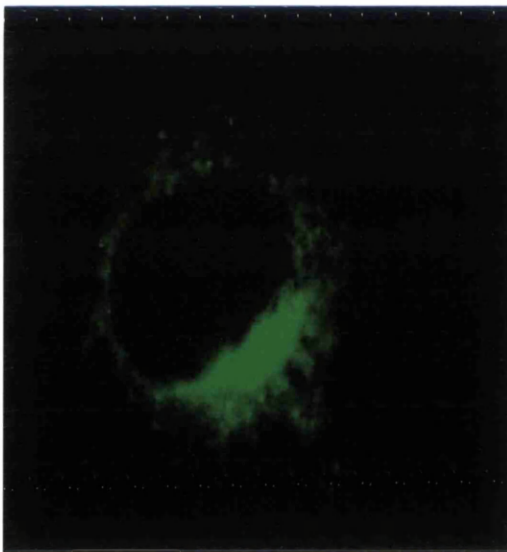
a) Structure of BPV-4 E5 KDEL, GGEV forms fused at the C-terminus of GFP

b) Structure of BPV-4 E5 double point mutants fused at the C-terminus of GFP

GFP-E5



GFP-E5/KDEL



GFP-E5/GGEV



Figure 3.6 GFP-E5/KDEL and GFP-E5/GGEV mutants show a subcellular localisation similar to GFP-E5

COS-7 cells were transiently transfected with 0.1 μ g of DNA of specific plasmid, fixed in 3% paraformaldehyde and visualised at the confocal microscope 24 hr after transfection.

3.3.3 Role of the amino acids secondary structure of BPV-4 E5 in the protein cellular location

3.3.3.1 Introduction

The N-terminus, the first 30 amino acids, of BPV-4 E5 comprises a very hydrophobic region, theoretically cable of forming a transmembrane α -helix (Figure 1.2). It has been shown that the secondary structure of the E5 hydrophobic region is important for the protein ability to transactivate the cyclin A promoter. Two mutants of E5 were tested: A15P-A20P mutant, where the alanine residues at position 15 and 20 had been converted to a proline, which has the property of forcing a bend in the main chain and disrupting an α -helix; A15G-A20G mutant, where the same alanine were mutated to glycine residue, a conservative mutation which should not disrupt an α -helical conformation. While A15G-A20G shows a low promoter trans-activation activity but still significantly above background, the introduction of proline residues abolished cyclin A promoter transactivation (Ashrafi, 1998).

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 also emphasise the importance of the nature of these residues for the E5 functions.

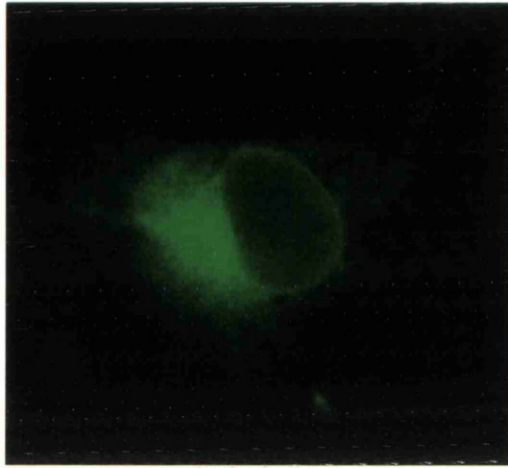
3.3.3.2 Experimental results and discussion

To determine whether the disruption of the postulated α -helix domain of BPV-4 E5 has an effect on protein localisation the E5 mutant forms A15P-A20P and A15G-A20G have been used in localisation studies.

A15P-A20P and A15G-A20G were cloned in pEGFPC3 vector, at the C-terminus of GFP by PCR using E5C3 forward and E5C3 reverse primers (section 2.2.1). The constructs obtained were called GFPA15P-A20P and GFPA15G-A20G (Figure 3.5). The plasmids were transiently expressed in COS-7 cells for 24 hours, cells were then fixed, mounted and analysed at the confocal microscope.

Both mutants show a perinuclear location, similar to E5 wild type (Figure 3.7).

GFPA15P-A20P



GFPA15G-A20G

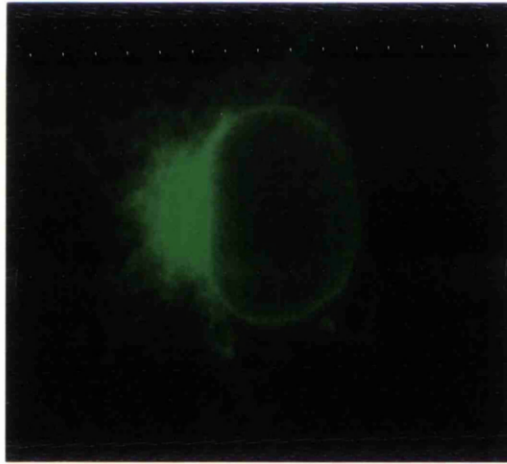


Figure 3.7 A double point mutation within the hydrophobic body of BPV-4 E5 does not influence its cellular localisation

COS-7 cells were transiently transfected with 0.1 μ g of DNA plasmid, fixed and visualised at the confocal microscope 24 hr after transfection.

3.3.4 Is E5 expressed on the plasma membrane?

3.3.4.1 Introduction

It has been previously reported that BPV-4 E5 is occasionally detected in the plasma membrane (Pennie et al., 1993); in the same way BPV-1 E5 has been shown to have a predominantly ER and Golgi location and been only in low concentration on the surface. While it has been established that the Golgi location is required for BPV-1 E5 transforming activity (Sparkowski et al., 1995; Schapiro et al., 2000), the relation between the cell surface location of the protein and its transformation ability has not been clarified.

3.3.4.2 Experimental results and discussion

In an attempt to define if BPV-4 E5 is present on the cell surface high-magnification (100X objective; zoom 6), high-resolution images of COS-7 cells transiently expressing (24 hr) GFP-E5 were taken (Figure 3.8). These images revealed a distinct localisation of GFP-E5 on individual ER canaliculi extending to the periphery of the cell but no fluorescence was detectable on the plasma membrane.

To further analyse the absence of GFP-E5 and E5-GFP from the cell surface we performed immunostaining experiments using an anti GFP antibody (MBL) on non permeabilised COS-7 cells transiently expressing (24 and 48 hr) GFP-E5 and E5-GFP. As a control for these experiments we used the pcPEP-GFP expression vector (kind gift from Dr. Maria Simonova, Harvard Medical School). This vector contains GFP cDNA fused with the fragment encoding the 27 amino acids residues of the positively charged N-terminal cytoplasmatic domain and the 23 residues hydrophobic signal peptide/membrane anchoring domain of the rabbit neutral endopeptidase 24.11, a type II transmembrane protein PEP-GFP (Figure 3.9 b). The authors showed by immunostaining and flow cytometry analysis that the N-terminal fragment of endopeptidase can target the fusion protein PEP-GFP to the cell surface of COS-1 cells transiently expressing the construct (Simonova et al., 1999).

PEP-GFP, GFP-E5 and E5-GFP were transiently expressed in COS-7 cells for 24 and 48 hr. Cells were fixed and stained with an anti-GFP antibody at 1:100 dilution for one hour at room temperature. Cells were then washed and incubated with an anti-rabbit antibody texas red conjugated for a further hour. Finally cells were

mounted and visualised at the confocal microscope. As graphically explained in Figure 3.9 a, the use of the two fusion forms of E5, with the GFP at the N-terminus or the C-terminus of the viral protein, would have allowed the detection of their expression at the cell surface independently from the orientation of E5 in the membrane bilayer.

As shown in Figure 3.10 a, PEP-GFP could be detected on the cell surface after 24 hr and 48 hr expression (data not shown) while neither GFP-E5 nor E5-GFP gave surface signal when expressed for 24 hours (Figure 3.10 b) or for 48 hours (data not shown). GFP does not affect the PEP trafficking through cell membranes despite the difference in size between the two proteins indicating that similarly GFP should not influence E5 location. These results show that it is not possible to detect BPV-4 E5 fusion forms on the cell surface and would suggest that GFP-E5 and E5-GFP are not expressed on the plasma membrane (Figure 3.10 b). It is however possible that the immunostaining approach we have taken is not sensitive enough to detect small amount of fusion proteins at the cells surface.

It will be extremely important to clarify whether or not E5 is present on the plasma membrane in order to better define a model of possible transforming mechanisms by E5. More information could come from studies with the electron microscope.

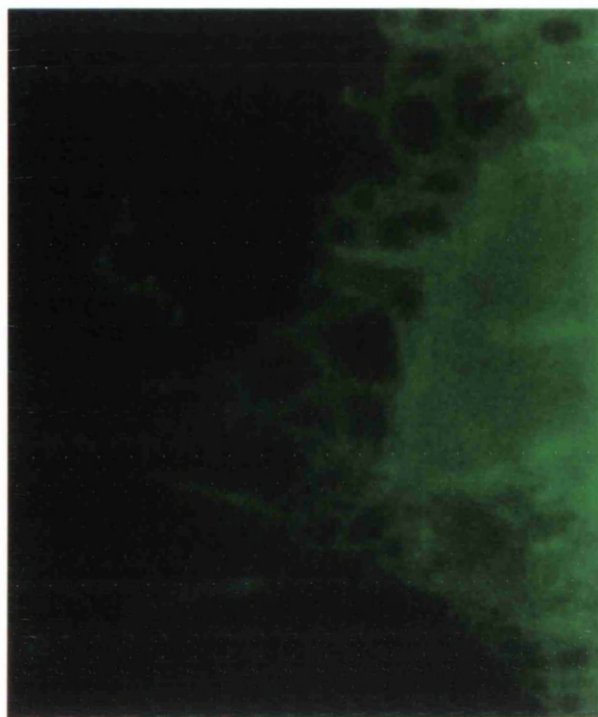
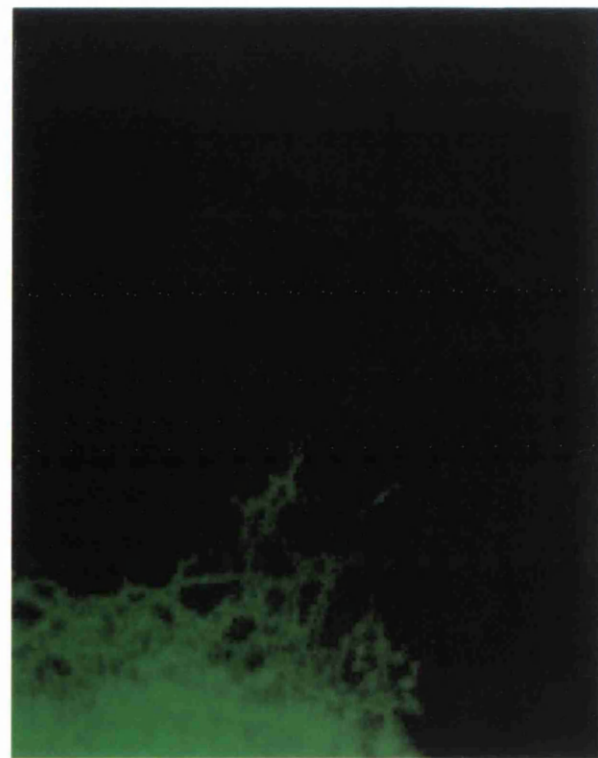


Figure 3.8 High power images of COS-7 cells transiently expressing GFP-E5

The images revealed a distinct localisation of GFP-E5 on individual ER canaliculi extending to the periphery of the cell but no fluorescence was detectable on the plasma membrane. The images have been captured with a 100X objective lens, zoom 6.

Figure 3.9 a, b Graphic representation of GFP-E5, E5-GFP and PEP-GFP

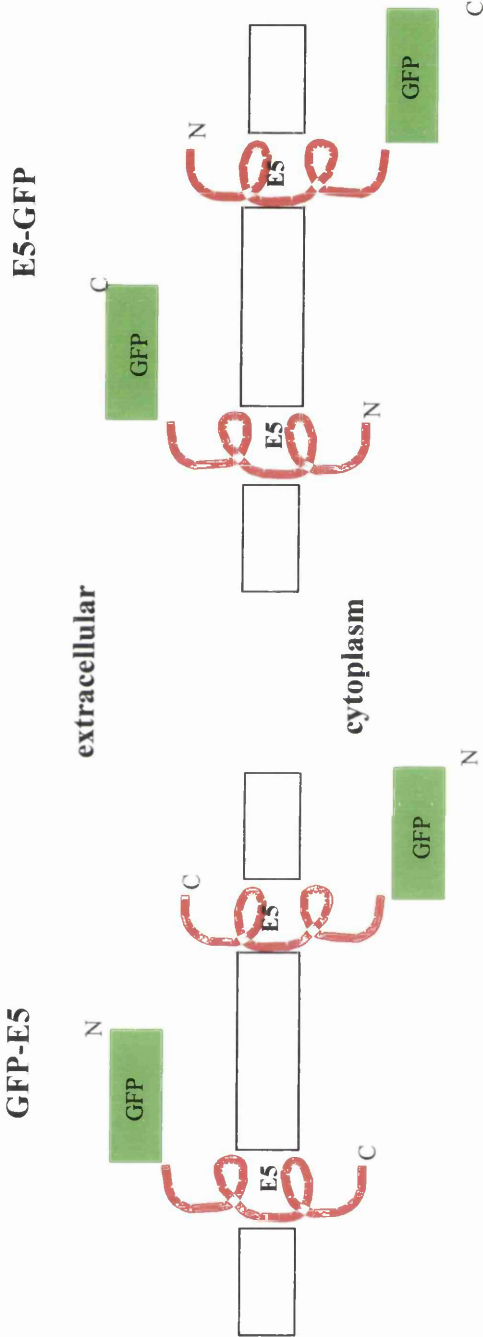
a) Possible orientation of GFP fusion forms of E5.

The diagram shows the two possible orientations of GFP-E5 and E5-GFP in the membrane bilayer. The use of the two fusion forms of E5, with the GFP at the N-terminus or the C-terminus of the viral protein, would have allowed the detection of their expression at the cell surface independently from their orientation.

b) The 50 amino acid signal peptide/membrane anchoring domain of the rabbit Neutral endopeptidase has been fused at the N-terminus of GFP. PEP is a type II transmembrane protein. When expressed at the plasma membrane it has an extracellular C-terminus and the N-terminus is orientated towards the cytoplasm.

Figure 3.9 a, b

a



b

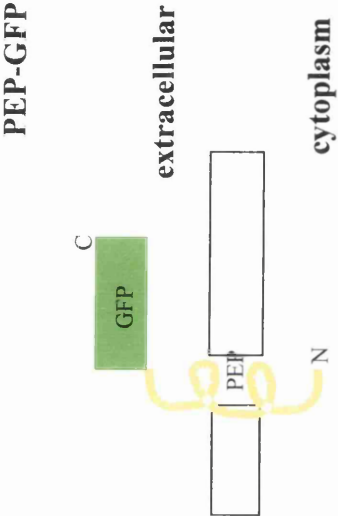


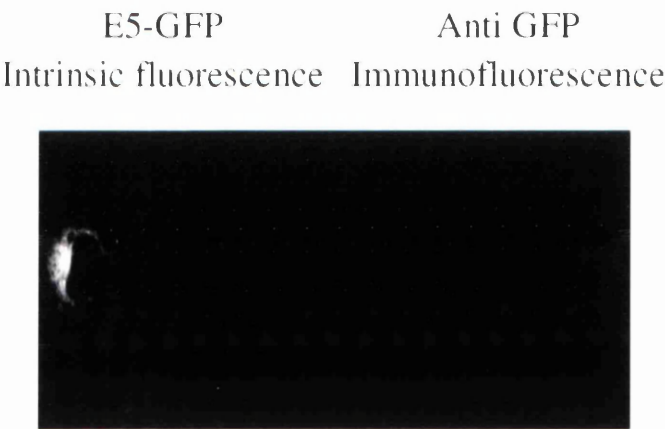
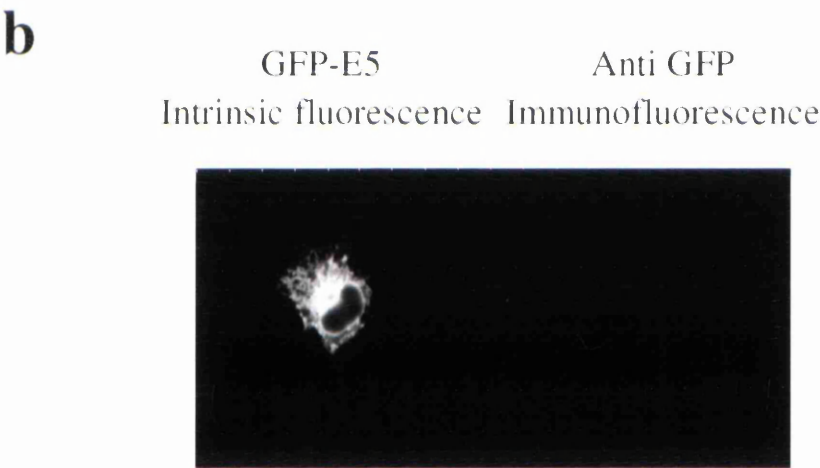
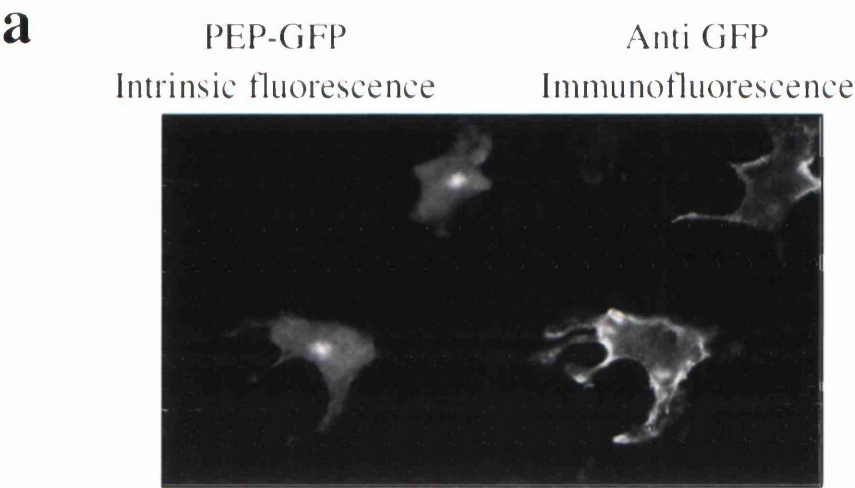
Figure 3.10 a, b GFP-E5 and E5-GFP could not be detected on cell surface

a) PEP-GFP transfected in COS-7 cells and stained with anti-GFP antibody;

b) GFP-E5 and E5-GFP transiently expressed in COS-7 cells and stained with anti-GFP antibody.

Cells were fixed 24 hr after transfection, stained with GFP antibody (MBL) and visualised at the confocal microscope. Each image, starting from the top panel, shows, on the left, the fluorescence coming from PEP-GFP, GFP-E5 and E5-GFP respectively and on the right the fluorescence coming from the anti-GFP antibody.

Figure 3.10 a, b



3.4 Conclusions

The use of GFP has provided an efficient detection system for routinely visualising E5. The two fusion forms of the viral protein with GFP (GFP-E5 and E5-GFP) show a perinuclear localisation that co-localisation studies have identified as Golgi apparatus (Figures 3.2 and 3.4). Similarly BPV-1 E5 and HPV E5s are mainly localised in the Golgi (Burkhardt et al., 1989; Conrad et al., 1993;).

The BPV-4 E5 mutant A15P-A20P also localises in the Golgi. This result would suggest that the high hydrophobicity of the first 30 residues is sufficient to target E5 to the Golgi apparatus and an intact secondary structure of its N-terminus is not required to localise the protein to the membrane bilayers. It is possible, however, that the E5 secondary structure is not affected by the introduction of proline residues and the abolition of A15P-A20P ability to transactivate cyclin A promoter is the result of other modifications in E5 activity such as interactions with different cellular targets due to residue substitutions. Moreover a form of E5 bearing the ER retrieval signal is still mainly localised in the Golgi. There could be various reasons for why GFP-E5/KDEL is still localised mainly in the Golgi compartment: (i) it is known that proteins bearing the KDEL amino acids sequence although resident in the ER can yet slip out of it, since they are able to acquire Golgi specific carbohydrate modifications. They are in fact retrieved from Golgi in a sorting process that returns them to the ER. It is therefore possible that over expression of ligands causes a saturation of the retrograde mechanism and allows protein to escape the control point and move towards the Golgi compartment; (ii) BPV-4 E5 could be a type I transmembrane protein with a cytoplasmic C-terminus. This means that the KDEL sequence is not facing the endomembranes lumen and cannot be recognised by the KDEL-receptors which reside in the ER-Golgi intermediate compartment.

The influence of the GFP tag in the retention of E5 in the Golgi could not be definitely excluded, however it could be considered unlikely. Using a fragment (PEP; 50 amino acids) of the rabbit neutral endopeptidase 24.11, that presents similar size to BPV-4 E5, fused with GFP, we demonstrated that GFP does not interfere with PEP trafficking through cell compartments despite the difference in size between the two proteins (Figure 3.10). We conclude that it is unlikely that GFP affects BPV-4 E5 cellular location. The possible mechanisms of how GFP-E5 could be retained in the Golgi will be discussed in Chapter 7.

CHAPTER 4: CHARACTERISATION OF GFP-E5 EXPRESSING CELLS

4.1 Introduction

4.1.1 Bovine papillomavirus type 4 and cell transformation

BPV-4 is a weakly transforming papillomavirus, it induces papillomas in the mucosal epithelium of upper gastrointestinal tract of cattle; the papillomas are, in general, benign and eventually regress spontaneously (Jarrett, 1985), but lesions can progress to carcinoma in animals feeding on bracken fern (Campo et al., 1994b).

The transforming functions of BPV-4, in established murine fibroblasts (NIH3T3 and C127 cells) have been mapped to the E5 and E7 open reading frames (Campo and Spandidos, 1983; Smith and Campo, 1988). The importance of the two viral proteins has been confirmed in studies with primary bovine fibroblasts (PalFs) (Jaggar et al., 1990). The expression of E5 and E7 ORFs, under the transcriptional control of the BPV-4 long control region (LCR) in pSVneo vector or of moloney leukaemia virus, MoLV, 5' long terminal repeat (LTR) in pZipneo vector, is sufficient, in co-operation with *ras*, to drive cells to morphological transformation with an anchorage-independent and non-contact inhibited phenotype (Jaggar et al., 1990). The immortalisation of these cells is achieved only with the addition of HPV-16 E6 ORF (Pennie et al., 1993).

An intact E7 ORF is crucial for transformation, as deletion of its 3' third resulted in loss of transformation (Jaggar et al., 1990). E7 expression in co-operation with *ras* induces morphological transformation of PalFs (Pennie et al., 1993), however the expression of E5 is required for anchorage-independent growth.

4.1.2 BPV-4 E5 and cell transformation

When expressed in PalFs with an activated Ras, but in the absence of other BPV-4 genes, E5 did not display transforming activity (Jaggar et al., 1990). However, PalFs transformed by BPV-4 E5, BPV-4 E7, HPV-16 E6 and an activated Ras displayed loss of contact inhibition, ability to grow in absence of mitogens and in suspension (Pennie et al., 1993; O'Brien et al., 1999) while PalFs expressing BPV-4

E7, HPV-16 E6 and Ras failed to grow under the above conditions (Jaggar et al., 1990; Pennie et al., 1993) confirming that these phenotypes are dependent on E5 expression.

Recently it has been demonstrated that the expression of E5 by itself is capable of transforming NIH3T3 cells (O'Brien and Campo, 1998), providing a system where it is possible to analyse the transforming activities of E5 and its mutants avoiding the influence of other viral or cellular oncogenes. NIH3T3 E5 expressing cells exhibit a transformed morphology, do not exit the cell cycle after serum withdrawal and form colonies when maintained in suspension culture (O'Brien and Campo, 1998).

In the previous Chapter we have shown that the use of a GFP tagged form of E5 has overcome the difficulties in the detection of the viral protein. GFP-E5 could be readily observed by immunostaining and routinely detected in western blot. Here we want to investigate if E5 could be stably expressed and easily detected in NIH3T3 and if the viral protein had fully maintained its transforming ability in its fusion form with GFP. Because of unforeseen problems only GFP-E5 form was utilised in these studies, however we are aware of the importance to test also E5-GFP in the future.

4.2 Experimental Results

4.2.1 Generation of cell lines

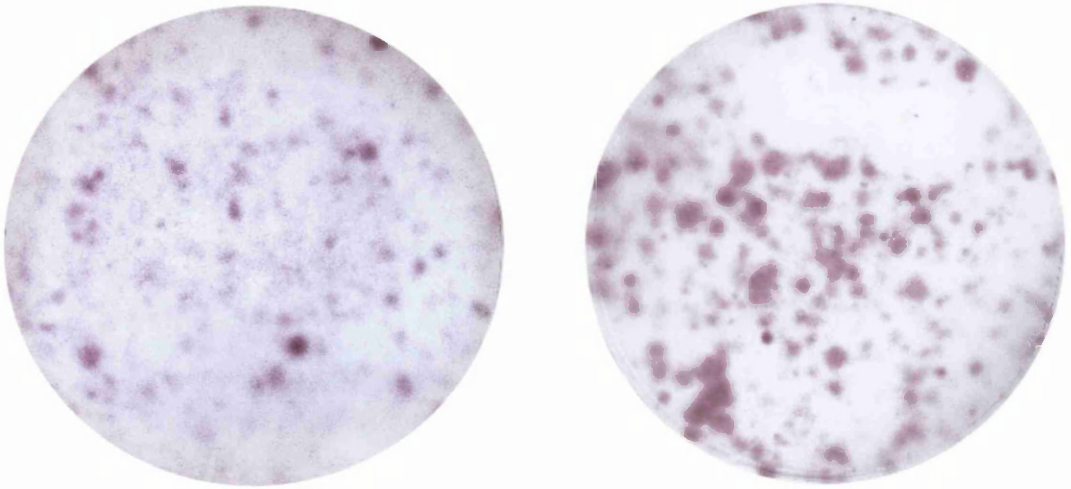
Before proceeding with the production of stable NIH3T3 transfectants, GFP and GFP-E5 were subcloned into the pZipneo vector (section 2.1.9) to be consistent with all the previous E5 transformation studies where the same expression system had been used (O'Brien and Campo, 1998; O'Brien et al., 1999). The subcloning was performed with a PCR strategy and the constructs were subsequently sequenced to confirm their identity (section 2.2.1.10).

NIH3T3 cells were seeded at 0.5×10^6 in a 90 mm tissue culture dish the day before transfection, three dishes for each transfection class. Cells were transfected with 10 μ g of GFP or GFP-E5 plasmids by using the calcium phosphate method as detailed in section 2.2.2.6. After 21-28 days of drug selection, with medium containing 500 μ g/ml of G418, resistant colonies were marked, picked and expanded into cell lines for analysis.

In parallel experiments two plates for each class of transfection were fixed and stained with 0.1% crystal violet in 20% methanol (Figure 4.1).

The results obtained from duplicate plates from three independent experiments show that GFP-E5 gives more colonies (up to 10 times) than the GFP control, implying that the fusion protein confers some survival and/or growth advantage to the fibroblasts. It has not been established if this phenotype is the result of an anti-apoptotic effect of the fusion protein or a growth advantage conferred by GFP-E5.

GFP-E5



GFP

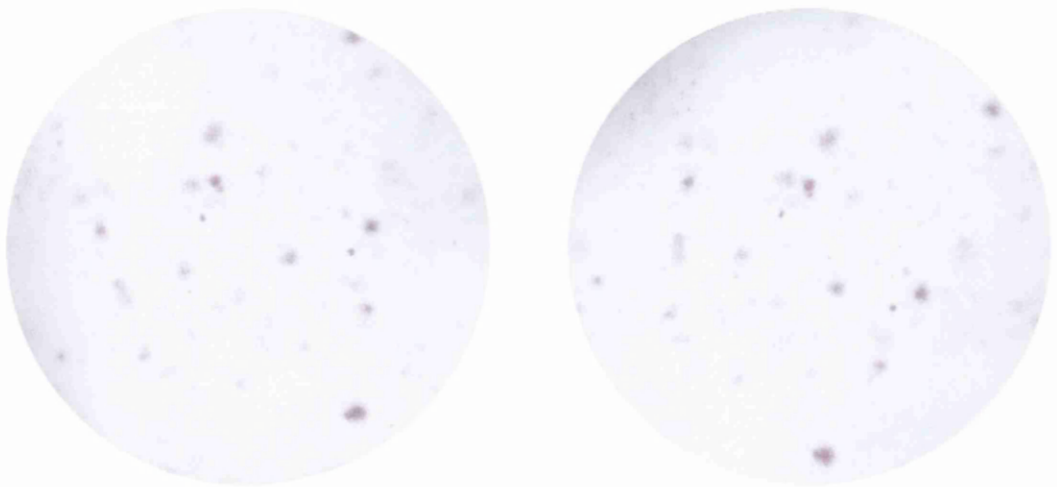


Figure 4.1 Colony formation efficiency

NIH3T3 cells transfected with GFP and GFP-E5, fixed and stained after 21-28 days of G418 selection (0.5 mg/ml). GFP-E5 shows an ability to form colonies up to 10 times the GFP control. The experiment has been repeated at least three times with the same outcome.

4.2.2 Detection and visualisation of GFP-E5

4.2.2.1 Confocal Microscopy

Being naturally fluorescent GFP allows the study of protein expression by direct visualisation of live or fixed cells using fluorescence or confocal microscopy. To determine if NIH3T3 were expressing the GFP and GFP-E5 constructs, stable transfectants were analysed by confocal microscopy (section 2.2.4.2). Protein expression was tested in two GFP clones (GFP n 3 and n 10) and in six GFP-E5 clones (GFP-E5 n 4, 7, 11, 15, 16, 17) derived from two different transfection experiments.

Details of confocal microscopy are described in section 2.2.4. In brief, 2×10^4 cells were seeded in an eight wells chamber slide in 500 μ l normal growing medium (DMEM 10% NBCS). The following day, cells were fixed in 3% paraformaldehyde for 15 min at room temperature, mounted using Vectashield™ and then visualised at the confocal microscope.

As shown in Figure 4.2, the transfection and the following selection had been successful since NIH3T3 cells were expressing GFP and GFP-E5. The cells showed a fluorescence distribution that resembled the one observed in transient expression experiments (Figure 3.2): GFP clones presented a uniform cytoplasmic and nuclear fluorescence while GFP-E5 cell showed a perinuclear fluorescence (Figure 4.2 a).

4.2.2.2 Western blotting

GFP-E5 and GFP expression was also confirmed through immunoblotting. Whole cell extracts (100 μ g cellular protein) from two GFP control clones, six GFP-E5 clones (the same clones tested above) and from NIH3T3 parental cells, growing in normal conditions, were prepared and fractionated by SDS-PAGE (15% polyacrylamide gel), transferred to a nitrocellulose membrane and probed with an anti GFP antibody (Babco).

Both GFP clones gave a specific band with an apparent molecular weight of 27 kDa, corresponding to electrophoretic mobility of GFP, while the GFP-E5 clones showed a specific band of 34 kDa corresponding to the predicted size of the fusion

protein (Figure 4.2 b). Cellular extracts from untransfected NIH3T3 cells gave no signal, confirming the specificity of these bands (Figure 4.2 b).

These results indicate that GFP and GFP-E5 cells express the correct proteins and these proteins are not subjected to degradation. For these reasons we considered all clones at our disposal suitable for further analysis.

Because all clones showed protein expression and no apparent difference was observed among GFP clones or GFP-E5 clones, most of the further experiments were carried out with two GFP-E5 clones (mainly n 4 and n 11) and two GFP clones (n 3 and n 10).

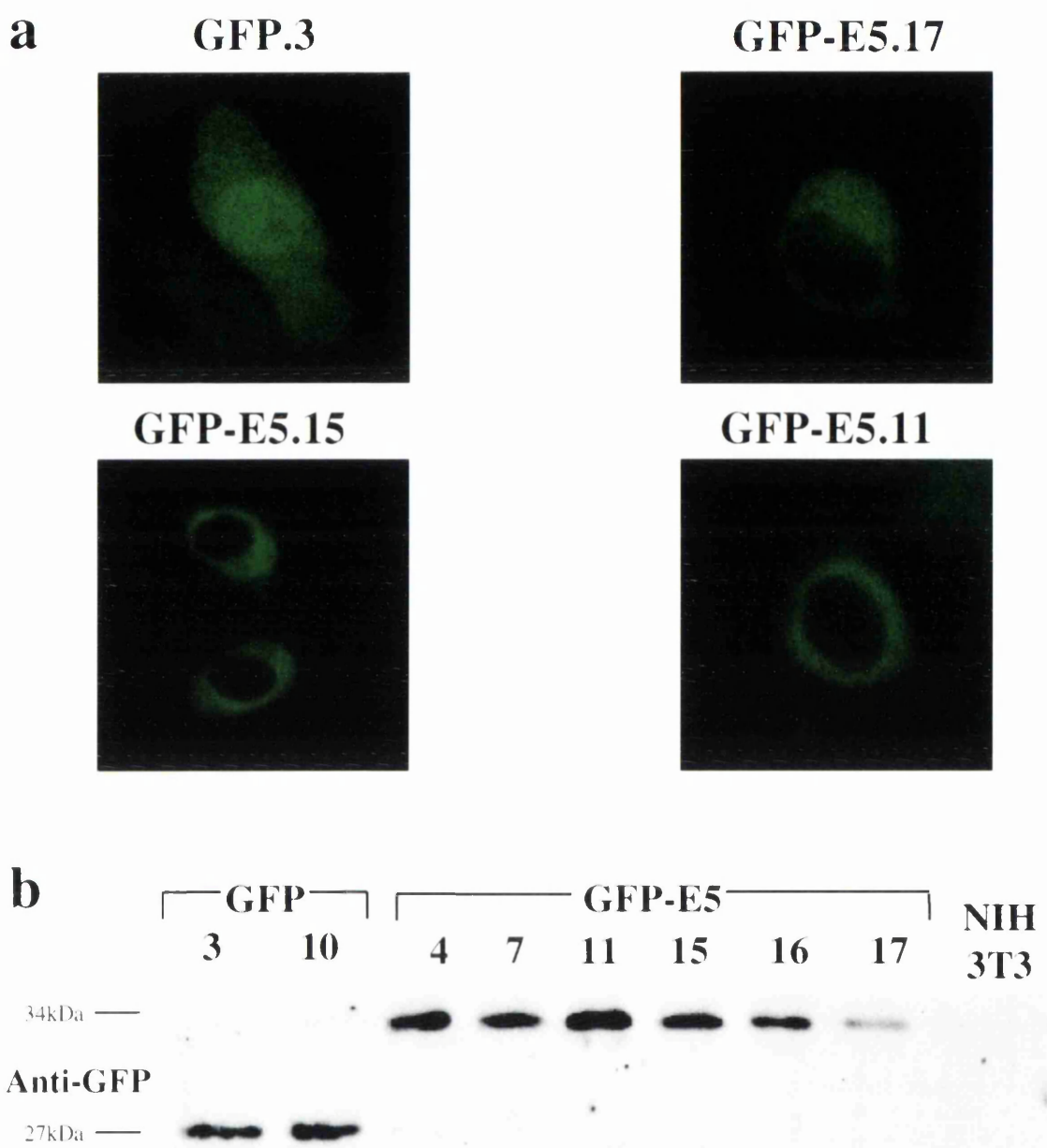


Figure 4.2 a, b Visualisation and detection of GFP-E5 in NIH3T3 cells

a) Confocal images of NIH3T3 clonal populations expressing GFP and GFP-E5

b) Detection of GFP and GFP-E5 expression in NIH3T3 clonal populations; 100 µg of whole cell extracts were fractionated in SDS-PAGE (15% polyacrylamide) and the membrane was probed with anti-GFP antibody (1:1000).

All images have been captured with 60X objective lens, GFP-E5 images have been further magnified in Microsoft powerpoint.

NIH3T3 cells were used as control.

4.2.3 Transformation assays

Growth factors and anchorage to substratum are two requirements of fibroblast cell division *in vitro*. When nutrients are removed from the growth medium or cells are deprived of substrate, fibroblasts exit the cell cycle. Fibroblasts transformation often leads to a decreased demand of serum factors and loss of anchorage requirement for cell proliferation (Pardee, 1989).

NIH3T3 cells expressing E5 exhibit a transformed morphology, do not exit the cell cycle after serum withdrawal and overcome the block in G1 when grown in suspension (O'Brien and Campo, 1998). To evaluate if GFP-E5 cells were transformed and the extent of their transformation, GFP-E5 clones were assessed for morphological transformation, their ability to grow in low serum conditions and independently of substratum.

4.2.3.1 Morphological transformation

To determine if the expression of GFP-E5 affected NIH3T3 cells morphology live cells were photographed using a Fuji digital camera (Figure 4.3). For completion, pictures of NIH3T3 expressing pZipneo empty vector (Z3T3) and E5 were taken. As shown before by O'Brien and Campo (1998), E5 cells show a less elongated shape compared with Z3T3 cells (Figure 4.3). In contrast, NIH3T3 cells expressing GFP-E5 do not show an obvious morphological difference from GFP and Z3T3 controls (Figure 4.3).

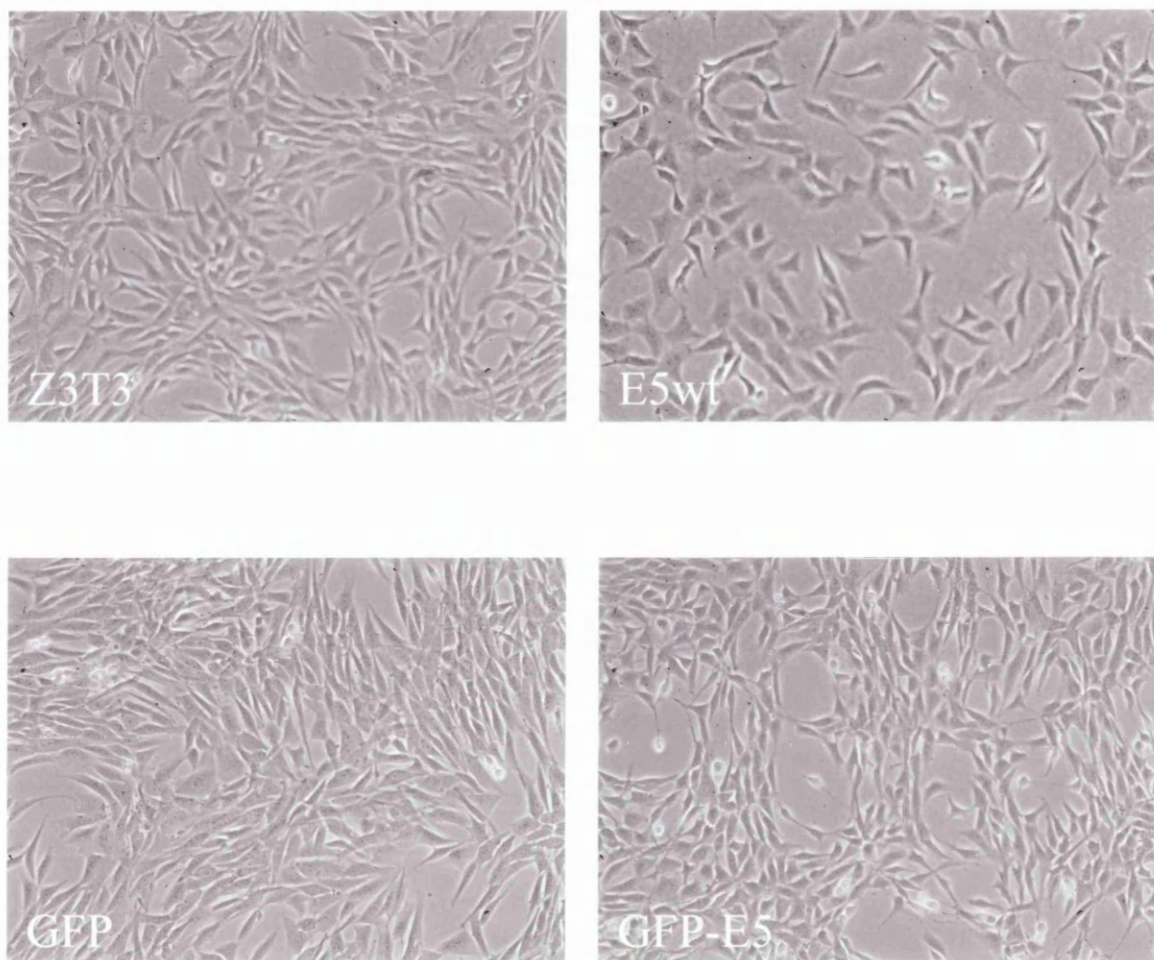


Figure 4.3 Morphology of Z3T3, E5 wt, GFP and GFP-E5 stable transformants

Phase contrast images illustrate that cells expressing BPV-4 E5 show a modified shape then the Z3T3 controls. GFP-E5 cells do not show an obvious morphological modification compared to GFP cells.

Images have been captured with a 10X objectives lens.

4.2.3.2 Cell population growth in low and high serum

It has been shown that NIH3T3 E5 expressing cells do not exit the cell cycle after serum withdrawal, but do not appear to activate autocrine mechanism (O'Brien and Campo, 1998). To determine if E5, in its fusion form with GFP, could still confer to NIH3T3 cells the ability to escape growth arrest in low serum, GFP-E5 and GFP cells were tested in short term and long term growth assays.

4.2.3.2.1 Cell cycle analysis

The full methodology of cell cycle analysis is described in section 2.2.3.2. In brief, cells (1×10^6) were plated in 90 mm culture dishes, in duplicate for each cell line. The following day fresh medium, 10% serum, was added to one dish for each cell line, while the other was switched to medium containing 0.2% serum and incubated at 37°C for a further 24 hr or 48 hr. Cells were then harvested, fixed in ethanol and stained with propidium iodide (PI) for DNA content. The samples were analysed by FACS scan to determine the percentage of the cells in each phase of the cell cycle. Sample data were then modelled on Modfit v2.0 software, to determine cell cycle distribution. As controls Z3T3 and E5 cells were tested under the same growth conditions.

The profiles for both GFP and GFP-E5 were the same or very similar under normal growth conditions. NIH3T3 expressing E5 wt had the highest percentage of cells in S phase (35%).

GFP-E5 cells left in 0.2% serum for 24 hr presented a reduced percentage of cells in S phase (Table 4.1) and showed an increased accumulation of cells in G0/G1 (~89%). GFP clones and Z3T3 cells showed a similar behaviour. This profile is characteristic of cells that exit from the cell cycle after serum withdrawal. E5 expressing cells had a high percentage (21%) of cells in S phase (Table 4.1 and Figure 4.4) confirming their ability to cycle in low serum conditions (O'Brien and Campo, 1998). Similar results were obtained when cells were treated with 0.5% serum and incubated for 48 hr (not shown).

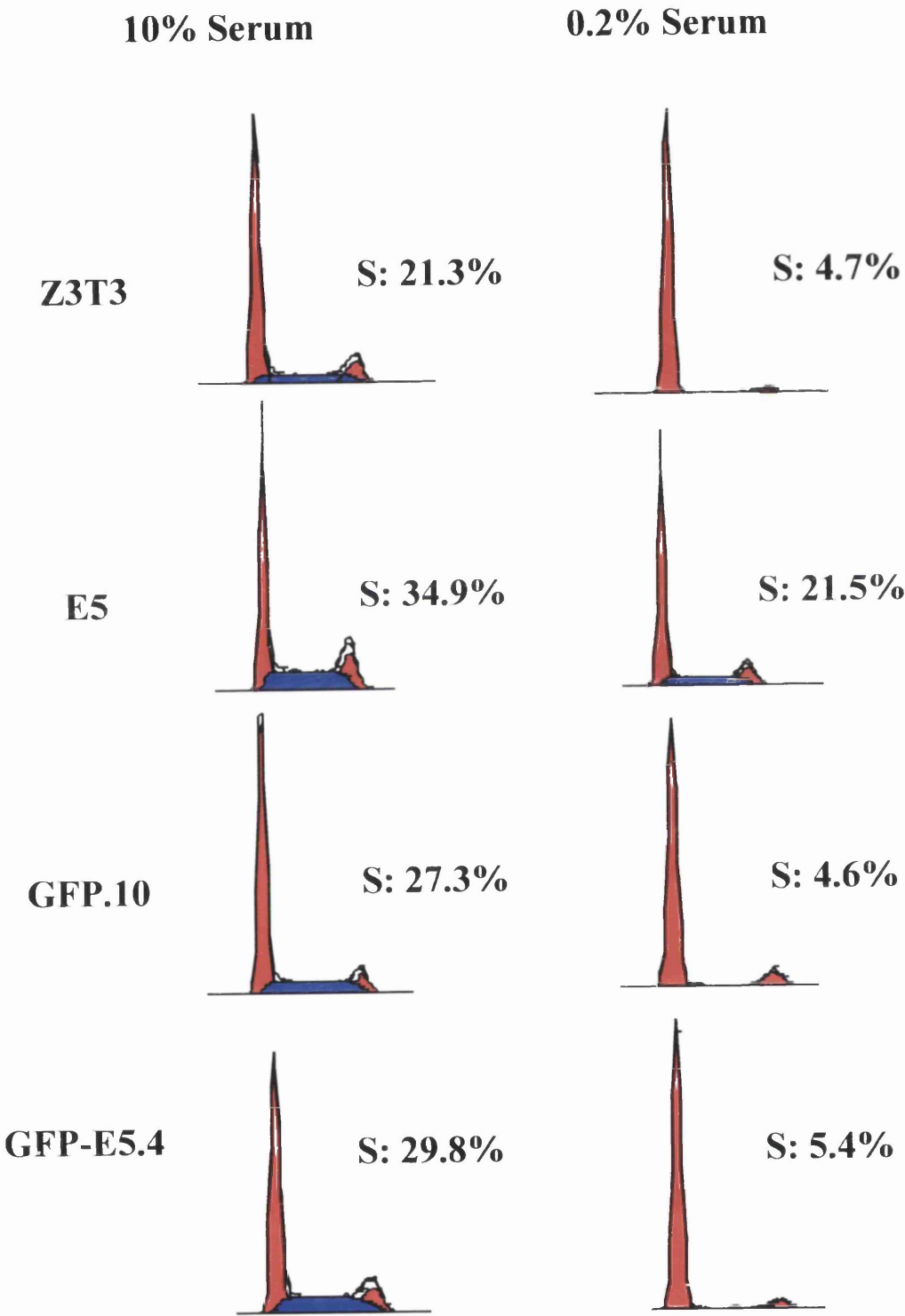
Table 4.1 Summary of the FACS analysis

Cell line	Serum Concentration	G0/G1 phase	S phase	G2/M phase
Z3T3	10%	65.37%	21.3%	13.33%
	0.2%	92.07%	4.7%	3.23%
E5	10%	50.59%	34.99%	14.42%
	0.2%	68.38%	21.55%	10.07%
GFP.10	10%	63.95%	27.32%	8.73%
	0.2%	85.67%	4.6%	9.73%
GFP-E5.4	10%	58.33%	29.81%	11.86%
	0.2%	88.98%	5.35%	5.67%

Figure 4.4 Cells cycle analysis by FACS

GFP, GFP-E5, Z3T3 and E5 cells (1×10^6) were tested for the ability to grow in growth medium (10% serum) and low serum (0.2% serum) for 24 hr. Cells were analysed for DNA content by flow cytometry to identify the percentage of cells in S phase. Control cells (GFP and Z3T3) and GFP-E5 clone show an arrest in G0/G1 phase when kept in 0.2% serum (Table 4.1).

Figure 4.4



4.2.3.2.2 *Long term studies of growth in low serum*

To further analyse the behaviour GFP and GFP-E5 cells in low serum, we extended cell growth studies over a 6 days period.

Cells (5×10^3 cells/well) were seeded in 96 well plates in triplicate, one plate was set up for each day from day 0 to day 6. After cells had attached to the bottom of each well, the growth medium was changed to DMEM containing 10% serum or 0.5% serum. Population growth was assayed as follows: one plate for each day was stained with 0.1% crystal violet in 20% methanol, dye was solubilised and absorbance determined at 590 nm by an automatic plate reader (section 2.2.3.1).

All cell tested showed very similar population growth kinetics when maintained in normal growth medium containing 10% serum (Figure 4.5), with GFP-E5 clones growing faster than GFP, Z3T3 and E5 cells. In concordance with the cell cycle analysis data (section 4.2.3.2.1) Z3T3 cells, GFP and GFP-E5 clones did not grow in 0.5% serum (or in 1% or 0.2%, data no shown), while E5 cells continued to proliferate in LS at the same rate shown in 10% serum.

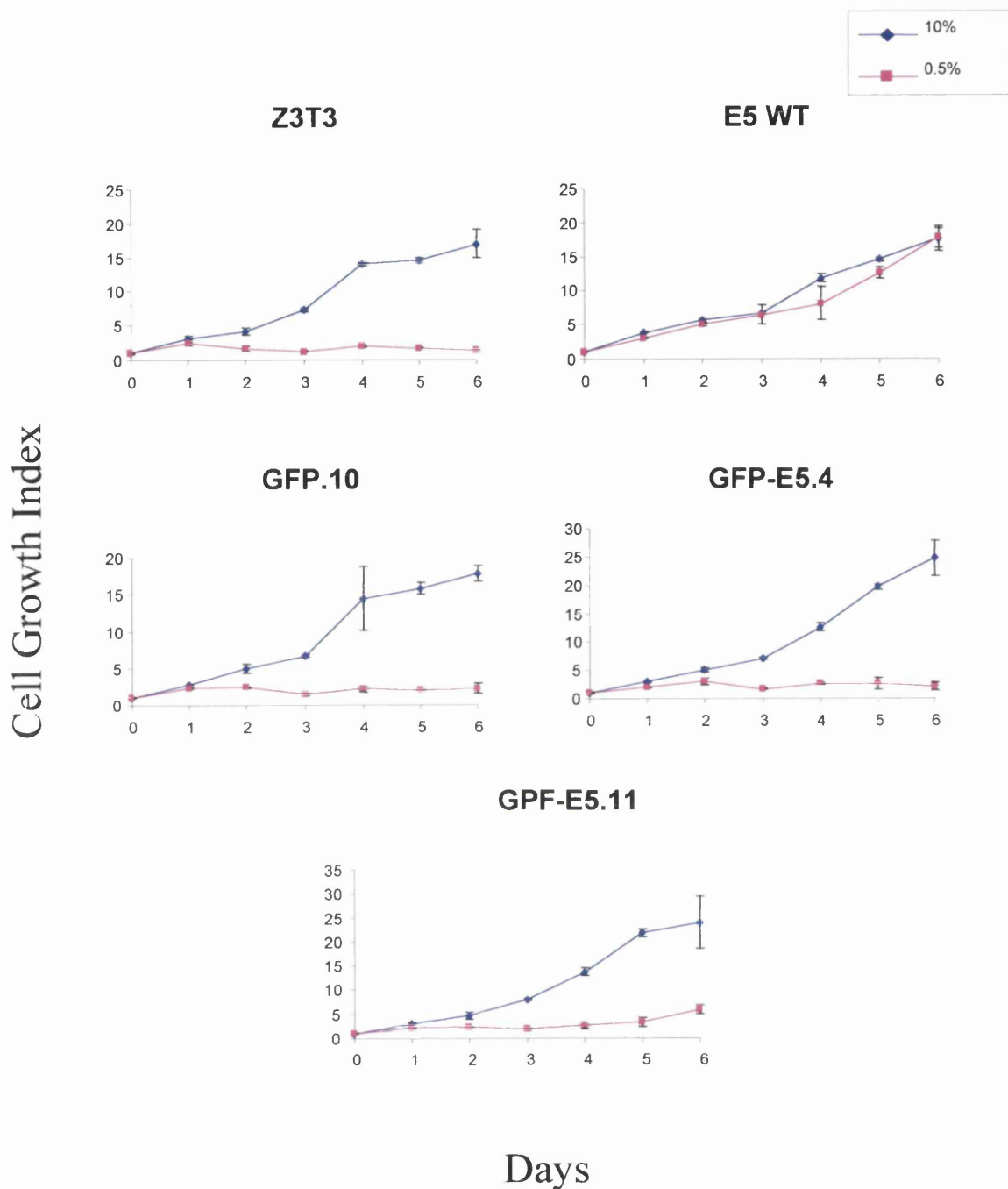


Figure 4.5 Cell growth curves

Growth curves representative of cell proliferation in high and low serum medium. Cell growth was determined by dye staining (section 2.2.3.1) and is expressed as a growth index (Y axis) representing the fold change in cell population from day one (X axis); the bars represent the standard deviation.

4.2.4 Anchorage-independent growth

The ability of GFP, GFP-E5, Z3T3, and E5 clonal populations to form colonies in semi-solid media was assayed by plating cells in methocel-based medium. Experimental details are explained in section 2.2.3.3. In brief, 1.25×10^4 - 2.5×10^4 cells were added to 7 ml of 0.8% methocel medium including 20% Foetal Calf Serum (FCS) and plated in 60 mm petri dish. Cells were left at 37°C for 2-3 weeks before being scored.

Colony formation efficiency was scored by counting the number of colonies, with a diameter greater than 0.1 mm, in three different areas ($\sim 16 \text{ mm}^2$) of each plate and estimating the total number of colonies in the whole dish (the average number of colonies in the three areas was divided by 16 to give the number of colonies per mm^2 and then multiplied by the total area of the dish, 2827 mm^2). The number of colonies was expressed as a percentage of the total number of cells seeded on day 0 (Table 4.2).

Table 4.2 Summary of anchorage-independent growth assay

Cell line	Number of colonies in methocel (%) \pm SE			
	Exp.1 *	Exp. 2 *	Exp. 3 #	Exp. 4 #
Z3T3	2.9% \pm 0.19	3.6% \pm 0.39	N/D	N/D
E5	18.2% \pm 0.98	20.4% \pm 0.52	N/D	N/D
GFP.3	3.1% \pm 0.13	3.3% \pm 0.36	2.4% \pm 0.72	2.4% \pm 0.72
GFP.10	N/D	N/D	3.3% \pm 0.27	2.4% \pm 0.72
GFP-E5.4	12.4% \pm 1.45	11.9% \pm 0.59	14% \pm 0.47	13.3% \pm 0.89
GFP-E5.7	N/D	N/D	10.4% \pm 0.89	9.5% \pm 0.27
GFP-E5.11	10.9% \pm 0.89	6.9% \pm 0.36	11.4% \pm 1.42	9% \pm 1.52

* 2.5×10^4 cells seeded

1.25×10^4 cells seeded

N/D= not determined

Figure 4.6 Anchorage-independent growth

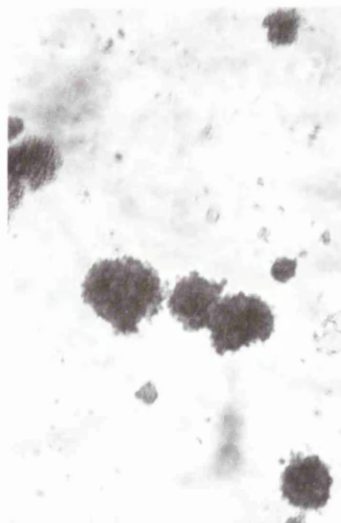
Z3T3, E5, GFP and GFP-E5 cells (2.5×10^4) were plated in 0.8% methocel-based medium. The efficiency of colony formation was determined after 3 weeks scoring colonies with a diameter greater than 0.1 mm.

Only E5 and GFP-E5 cells are capable of anchorage-independent growth (Table 4.2).

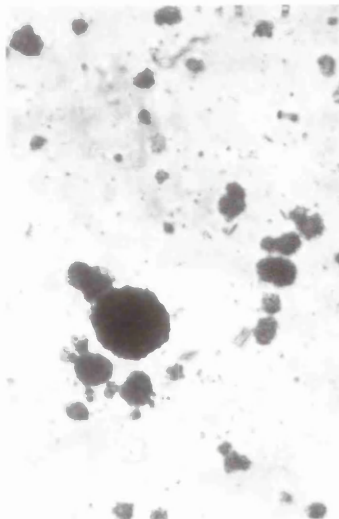
Phase contrast images at 40X final magnification.

Figure 4.6

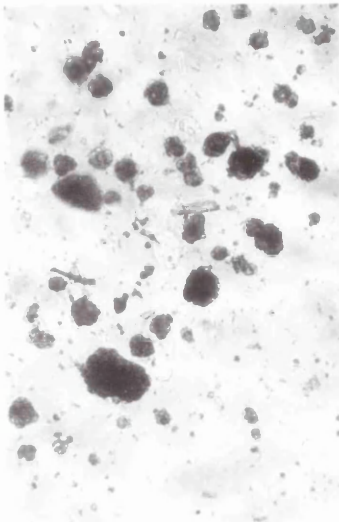
E5 wt



GFP-E5.4



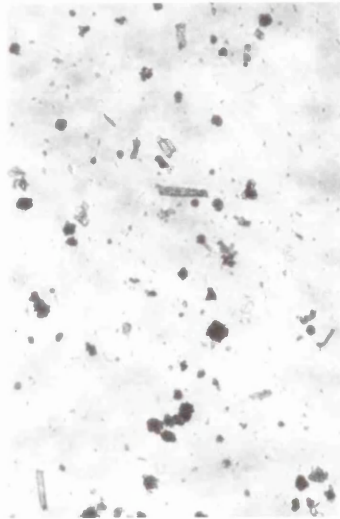
GFP-E5.11



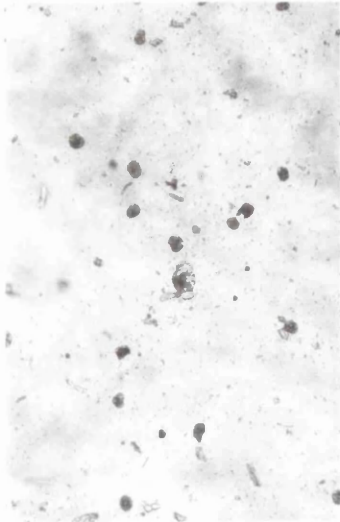
Z3T3



GFP.3



GFP.10



In four independent experiments, two carried out using 2.5×10^4 cells per plate and two with 1.25×10^4 cells per plate (Table 4.2), GFP-E5 clones showed an appreciable anchorage-independent growth, with clone n. 4 displaying the highest efficiency of colony formation 14% (Table 4.2, Figure 4.6). GFP-E5 clones showed an efficiency of ~ 3.9 times on average higher than GFP clones, moreover colonies larger than 0.25 mm were found only in GFP-E5 plates. It has been shown previously that E5 expressing cells showed an efficiency of colony formation approximately 3.5 times than Z3T3 control cells (O'Brien and Campo, 1998). E5 cells behaviour has been confirmed in these experiments where these cells present a colony formation efficiency of $\sim 20\%$.

4.3 Conclusions

The results presented here show that it is possible to produce stable cell lines expressing GFP-E5 fusion protein and GFP protein. In these cells proteins could be visualised and detected and there was no evidence of protein degradation (Figure 4.2).

In three independent experiments the number of drug-resistant colonies obtained from NIH3T3 cells transfected with GFP-E5 was higher than in the GFP plates (Figure 4.1). This implies that GFP-E5 confers some survival and/or growth advantage to the fibroblasts. Moreover cells expressing GFP-E5, like E5 wt, are capable of anchorage-independent growth, a phenotype that is believed to correlate with tumour growth *in vivo*. Surprisingly GFP-E5 cells exit the cell cycle when deprived of serum mitogens, in disagreement with E5 cells behaviour (O'Brien and Campo, 1998).

It has been shown previously that PalFs cells expressing an E5 mutant form, N17S (Chapter 6) and a chimera that retains the first 31 residues of BPV-1 E5 fused with the last 10 amino acids of BPV-4 E5 (1E5N-4E5C) can grow in suspension but not in low serum, showing that the transformation phenotypes of anchorage-independent growth and of growth in low serum can be segregated (Ashrafi, 1998; O'Brien et al., 1999).

GFP-E5 resembles the characteristics of the above mutants when expressed in NIH3T3 cells. This fusion form of E5 is therefore an important tool with which to investigate how the segregation of the two phenotypes occurs in NIH3T3, where the

sole requirement for cell transformation is the introduction of E5. Moreover in this system the analysis can be expanded at the biochemical level thanks to the commercial availability of the required reagents. Our initial analysis of biochemical changes to key components of the cell cycle machinery will be discussed in Chapter 5.

CHAPTER 5: THE EFFECTS OF GFP-E5 ON THE REGULATION OF THE CELL CYCLE

5.1 Introduction

5.1.1 The cell cycle

The cell cycle is a series of events that culminates with cell division and production of two daughter cells. This process consists of four phases; the S phase in which DNA replication occurs; M phase or mitosis when the DNA is segregated and the cell actually divides; and two gap phases: G1 (before DNA synthesis) and G2 (before mitosis). Cell cycle progression is regulated by both intracellular and extracellular control mechanisms. Intracellular controls ensure that cell division proceeds successfully and responds to irregularities such as DNA damage (G1/S check-point) or incomplete DNA replication (G2/M check-point) (Forsburg and Nurse, 1991). The extracellular controls, both cell adhesion and the presence of growth factors, may determine cell fate such as proliferation, differentiation or programmed cell death. The regulation of cell proliferation by growth factors of normal mammalian cells occurs at two levels in the cell cycle: during transition from quiescent G0 state to the G1 phase, and during progression through the different subphases of G1. Fibroblasts leave the cell cycle and enter a quiescent, G0 state, when growth factors are removed (Pardee, 1989). Adhesion to substratum is also required for cell cycle progression, with the exception of some cells in the haematopoietic lineage (Zhu et al., 1996). In fibroblasts, cell cycle control by anchorage to substratum has been mapped at the transit through G1 into S phase (Guadagno and Assoian, 1991).

In cancer cells the intracellular and extracellular check-points are lost and growth control is deranged. In particular the G1/S check-points and the G2/M check-points are less stringent or even absent. Moreover fibroblast transformation often leads to loss of growth factor and anchorage requirements for cell proliferation and anchorage-independent growth *in vitro* is closely correlated with tumourigenicity and tumour metastasis *in vivo* (Shin et al., 1975).

5.1.2 The cell cycle regulators

5.1.2.1 Cyclins and cyclin-dependent kinases

Proper cell cycle progression is accurately regulated by a family of protein kinases termed cyclin-dependent kinases (CDKs). CDKs are serine/threonine protein kinases that control the transition between successive phases of the cell cycle and require binding of regulatory subunits, named cyclins, as an initial step in their activation process. In mammalian cells at least nine CDKs (CDK1-CDK9) have been described. Their positive regulatory subunits, the cyclins, also constitute a large family, so far 12 have been characterised. Cyclins can form catalytically active complexes with different types of CDKs in mammalian cells, and different cyclin/CDKs complexes are assembled and activated at specific points in the cell cycle.

The first cyclin/CDK holoenzyme, which is activated during the G1 phase, is composed of a D-type cyclin (D1, D2, or D3) in association with CDK4 or CDK6 (cyclin D/CDK4-6). The expression of cyclin D-types and their assembly with their catalytic partners depends on stimulation by growth factors. (Diehl et al., 1997).

As cells progress through the G1 phase, cyclin E is synthesised with a peak middle/late G1. In combination with another cyclin-dependent kinase, CDK2, cyclin E is responsible for the progression from G1 to S phase (Resnitzky et al., 1994; Ohtsubo et al., 1995). At the transition point from G1 to S phase cyclin E is degraded and CDK2 then associates with cyclin A.

Cyclin A gene expression is regulated at the level of transcription initiation with expression being repressed in G1 and strongly induced as the cell enters S phase (Henglein et al., 1994). 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).

Finally, both cyclin A and B type cyclins associates with cdc2 to promote entry into mitosis; cyclin A binds to cdc2 with a peak of activity in G2 and is then suddenly degraded, whereas entry into mitosis is triggered by cyclin B/cdc2. Cyclin B destruction is required for exit from mitosis.

It has been shown that de-regulation of cell cycle regulatory proteins, such as cyclins, cyclin dependent kinases, and their inhibitors (CKIs) is a hallmark for cell transformation (Chapter 1).

5.1.2.2 Cyclin Dependent Kinase Inhibitors (CKIs)

Another level of control of cyclin-CDK complexes is via a strong inhibitory activity by two families of small proteins: the Kip/Cip family composed of p21, p27 and p57, which target cyclins/CDK2 and cyclins/CDK4-6 complexes and the INK family, p15, p16, p19 which target cyclin D/CDK4-6 complexes (Sherr and Roberts, 1995).

Expression of p21 can be induced by the tumour suppressor p53 in response to DNA damage. Up-regulated p21 arrests the cell cycle not only by inhibiting cyclin/CDK complexes but also by binding to the DNA polymerase- δ subunit PCNA, an important player in replication and repair (Xiong et al., 1992).

Another CDK inhibitor that is structurally related to p21 is p27 (Toyoshima and Hunter, 1994). The gene of p27 is transcribed both in proliferating and in differentiated cells. The expression of p27 does not appear to be regulated at the transcriptional level (Hengst and Reed, 1996) but protein levels seem to change during the cell cycle due to translational and post-translational regulation. p27 is expressed at high levels in quiescent cells and is rapidly down-regulated upon growth factor stimulation. In proliferative cells p27 is present at lower level and apparently is sequestered by cyclinD/CDK4-6 complexes which need to overcome a p27 threshold to become active (Sherr and Roberts, 1995). Since p27 can interact with multiple cyclin/CDK complexes, its availability for inhibition of cyclins/CDK2 complexes also depends on the abundance of cyclin D/CDKs (LaBaer et al., 1997).

5.1.2.3 Pocket proteins

The 'pocket protein' family includes the retinoblastoma tumour suppressor protein (pRb) and two related proteins, p107 and p130 (Weinberg, 1995). The term 'pocket' was originally used to describe a conserved structural and functional domain of pRB and p107 that binds to transforming viral oncoproteins and cellular proteins. The three members of the pRb family are phospho-proteins and their phosphorylation status is modulated during the cell cycle as well as at the cell cycle entry and exit transitions in mammalian cells. The growth suppressive activity of pocket proteins is mediated through the association with a number of cellular proteins, mainly the members of the E2F family of transcription factors, which regulate the expression of a number of genes required for cell cycle progression (reviewed in Dyson, 1998).

Phosphorylation of the pocket proteins in mid-to-late G1 phase by cyclin/CDKs frees the transcription factors, reactivates transcription and induces cell cycle progression.

From recent studies it appears that the pocket proteins, pRb in particular, are progressively inactivated by multiple cyclin/CDK complexes and phosphorylation at different sites is a prerequisite for distinct molecular functions (Adams, 2001).

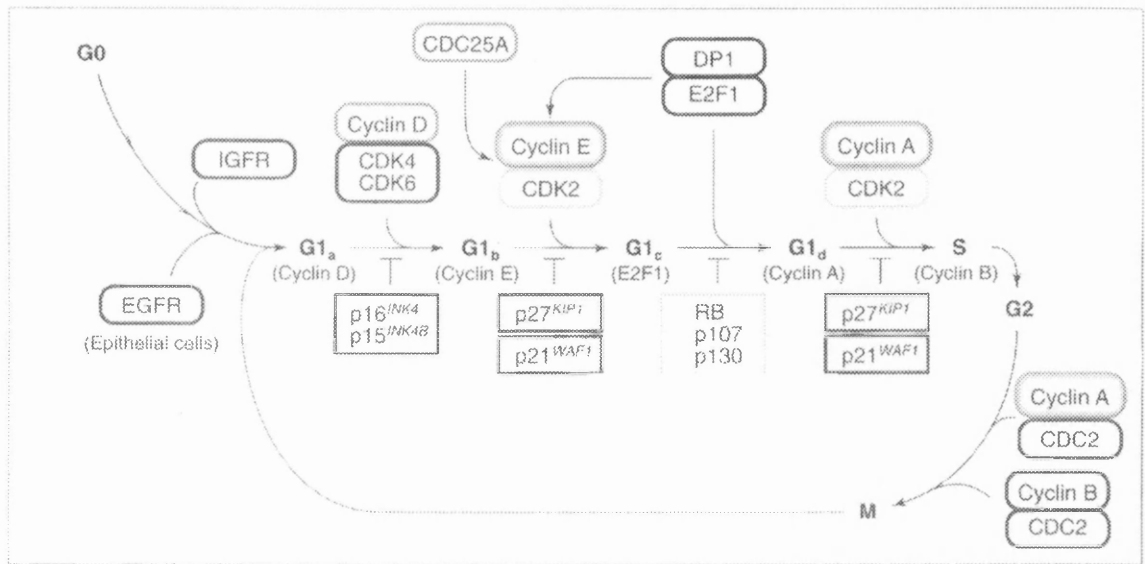


Figure 5.1 The key regulators of the cell cycle progression

Exit of resting mammalian cells from the quiescent state (G0) is triggered by growth factors and their specific receptors. The immediate response to growth factors stimulation is the increased expression of D type cyclins in early G1. These cyclins bind and activate their associated kinases (CDK4 or CDK6). As cells progress, cyclin E and cyclin A are synthesised. Cyclin E, in combination with CDK2, is responsible for the progression through the G1 phase while cyclin A/CDK2 complex enables S phase progression. Finally cyclin A and cyclin B associate with cdc2 to promote entry into mitosis. The pocket proteins (pRb, p107 and p130) block cell cycle progression by sequestering transcription factors (E2F/DP). Pocket proteins are inactivated by multiple phosphorylations by CDKs, resulting in release of transcriptional active E2F/DP heterodimers. The CDK inhibitors (p15, p16; p21 and p27) arrest cells in G1 by preventing phosphorylation of the pocket proteins; modified from Jansen-Durr, 1996.

5.2 Analysis of the cell cycle components in different growth conditions in NIH3T3 cells expressing GFP-E5

In NIH3T3 cells, E5 transformation is accompanied by the up-regulation of cyclin D and cyclin A expression and associated kinase activity under conditions where the expression of these cyclins is diminished or not detectable in control cells (O'Brien and Campo, 1998; O'Brien et al., 2001).

Differently from E5 wt, GFP-E5 expressing cells grow in suspension culture but exit the cell cycle after serum withdrawal (Chapter 4). Hence we decided to investigate at what level of cell cycle progression the segregation of the two phenotypes occurs, by the analysis of the cell cycle components in GFP-E5 cells in different growth conditions.

5.2.1 Experimental procedures

5.2.1.1 Western blotting

Cells (1×10^6) were seeded in a 90 mm dish, once attached they were switched to growth medium (GM; 10% serum) or low serum (LS; 0.5% serum) for 24 hours before being harvested and prepared for western blotting analysis as explained in section 2.2.5.1.

For the assay in suspension 5×10^6 cells were resuspended in 30 ml of 0.8% methocel in DMEM-10 and transferred on a 150 mm bacteria petri dish and incubated in a humid incubator for 24 hr or 48 hr. At the end of the incubation time, the cells were pelleted as described in section 2.2.5.1.

Samples were loaded in a 10% SDS-polyacrylamide gel and run at 40 mA for 1.5 hr. The gel was then transferred to a nitrocellulose membrane and the membrane probed with specific antibodies (Table 2.2).

5.2.1.2 Kinase assays

For the kinase assays experimental details are described in section 2.2.5.5. In brief, from 500 μ g aliquots of lysates, obtained from adherent or suspension culture (section 2.2.5.2) cyclin A, cyclin E and CDK2 complexes were immunoprecipitated

using specific antibodies (Table 2.2). Samples were incubated with Histone one (H1) substrate and [γ^{32} -P] ATP. The reaction was incubated for 30 min at 30°C and then stopped by adding 20 μ l of 2X loading buffer. Samples were boiled for 5 min and loaded on a 10% polyacrylamide gel. The gel was dried and the intensity of the bands was analysed with a phosphorimager.

5.2.1.3 Cyclin A promoter activity measurement

Growing stable transformants cells (2×10^5) were plated in 6 well plates, in triplicate. The following day cells were transfected with 0.2 μ g of luciferase reporter plasmid, (luciferase reporter gene under the transcriptional control of the human cyclin A promoter, region between -754 bp and +175 bp; section 2.1.9) using Effectene transfection reagent. After 16-18 hr, precipitates were washed off with 2 ml of PBS and cells incubated in LS or GM for further 24 hr.

For the assay in suspension, cells (2×10^5) were seeded in 6 well plates, in quadruplicates and transfected as described above. The following day cells were washed with PBS and two wells, for each cell lines, were incubated with GM and two were trypsinised and transferred on a 60 mm bacteria dish with 7 ml of methocel medium (section 2.2.3.3) containing 10% of NBCS. Cells were incubated in a humid incubator for a further 24 hr before being harvested and assessed as described in section 2.2.3.4.

Luciferase activity was determined using a microplate luminometer TR717 (section 2.2.3.4).

5.2.1.4 MAPK inhibitors

Experimental details are described in section 2.2.6. Briefly 3×10^6 cells were seeded in 10 ml of 0.8% methocel 10% NBCS with the addition of PD98059 (Calbiochem) or U0126 (Promega) inhibitor (final concentration 50 μ M and 20 μ M in DMSO respectively) as control cells were treated in parallel with an equal amount of DMSO. Cells were harvested 48 hr and 72 hr later, protein concentration assayed and 30 μ g of this analysed by western blotting.

5.2.2 Cell growth in normal and low serum conditions

5.2.2.1 Cyclin D1 and the pocket proteins

Cyclin D1 is the primary D-type cyclin in several cell types. It is known that cyclin D1 acts as a growth factor sensor: it is rapidly induced by mitogens and rapidly degraded in cells deprived of growth factors (Diehl et al., 1997). Cyclin D1/CDK4-6 complexes are responsible for the phosphorylation of the pocket proteins. This phosphorylation, during G1, leads to inactivation of pocket proteins as transcriptional repressors of fundamental genes required in S phase and allows the bypass of the restriction point at the G1/S transition and the progression of the cell cycle.

p130 is modulated slightly differently and is accumulated in G0 and mid-G1 whereas its level is low or not detectable in S phase and G2 phase (Grana et al., 1998).

Phosphorylated forms of the pocket proteins can be resolved by SDS-PAGE thanks to differences in their electrophoretic mobility, with the hyperphosphorylated forms running slower than the hypophosphorylated forms. Two different forms of p107 are detectable in most cell types, while in the case of p130 four forms are detectable which are defined as unphosphorylated p130 and phosphorylated forms 1, 2 and 3 (Grana et al., 1998). Moreover antibodies raised against specific phosphorylation sites of pRb exist (see below).

Given the crucial role of cyclin D1 in the integration of mitogen signal with the cell cycle progression we investigated the state of this cyclin in our stable transformants, moreover we analysed the state of pRb, p107 and p130 proteins in different growth conditions in GFP-E5, GFP, E5 and Z3T3.

As previously shown, NIH3T3 cells expressing E5 sustained the expression of cyclin D1 in the absence of mitogens (O'Brien et al., 2001), (Figure 5.2 a). GFP-E5 clones showed an increased in the basal level of cyclin D1 compared to GFP control, however in both GFP-E5 and control cells the expression of this cyclin decreased in cells cultured in low serum (Figure 5.2 a). The data show that cyclin D1 is still largely mitogen-dependent in these cells.

Cells growing in 10% serum clearly presented hyperphosphorylated forms of pRb and p107 and a low level or absence of p130. Moreover a pRb form specifically phosphorylated at site 780 (serine) could be detected using a phospho-specific

antibody (Figure 5.2 b). GFP-E5, GFP and Z3T3 cells left in 0.5% serum presented changes in the phosphorylation state and/or abundance of pRb, p107 and p130. In these conditions, in agreement with the expression pattern of cyclin D1, pRb and p107 were found mainly in their hypophosphorylated state, whereas p130 accumulated, as is typical of quiescent cells. On the contrary, in E5 expressing cells grown in the absence of mitogens, pRb and p107 are largely hyperphosphorylated while p130 levels are significantly diminished (O'Brien et al., 2001), (Figure 5.2 b). In these cells the detection of pRb780 was still possible. Both cyclin D1/CDK4 and cyclin E/CDK2 complexes phosphorylate this site. It has been shown that in E5 cells cyclin E/CDK2 kinase activity is not elevated in absence of mitogens, leading to the conclusion that cyclin D/CDK4 complexes are the principal responsible for pRb phosphorylation (O'Brien and Campo, 1998; O'Brien et al., 2001). The failure to detect pRb phosphorylation in GFP-E5 cells demonstrated that both cyclin D/CDK4 and cyclin E/CDK2 were inactive in low serum. These observations are consistent with the inability of GFP-E5 cells but not E5 cells, to proliferate in low serum (Chapter 4).

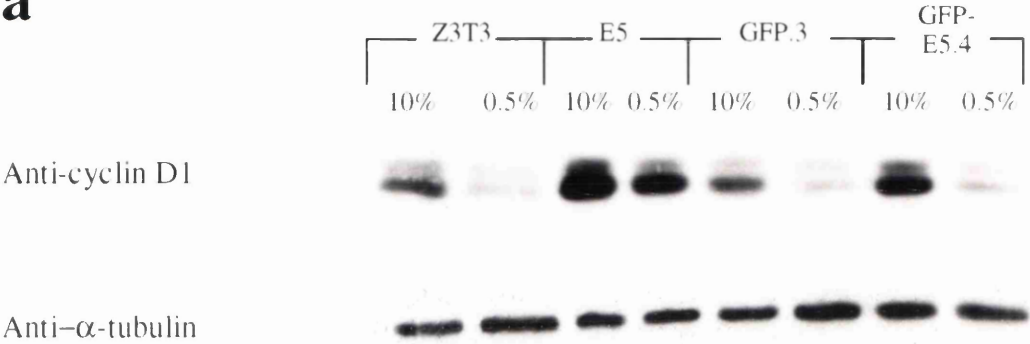
Figure 5.2 a, b Analysis of the cyclin D1 and the pocket proteins expression in normal (10%; GM) and low serum (0.5%; LS) conditions

a) Cells were incubated in 10% or 0.5% serum for 24 hr. After treatment, 50 µg of total cell extracts were separated in 10% polyacrylamide gel and membranes were probed with indicated antibodies. In 10% serum Cyclin D1 is elevated in E5 and GFP-E5 cells, while in LS is sustained only in E5. Levels of α -tubulin serve as a loading control.

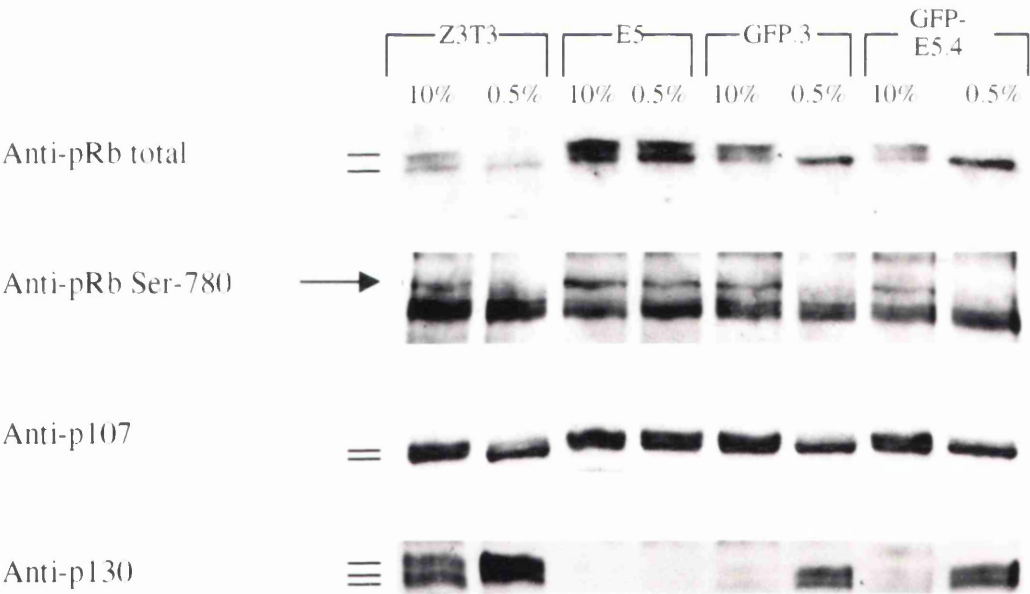
b) After treatment, as above, 100 µg of total cell extracts were separated in 7.5% polyacrylamide gel. Membranes were probed with antibodies to total pRb, Ser-780 pRb phosphorylation site, p107 and p130. The different species of the pocket proteins are marked. Hyperphosphorylated forms of pRb and p107 were detected in all cell lines grown in GM. Specifically the pRb-Ser780 form was detected. After serum withdrawal the active forms of the pocket proteins were observed only in E5 expressing cells but disappeared in GFP-E5 and control cells.

Figure 5.2 a, b

a



b



5.2.2.2 Cyclin A promoter transactivation

In NIH3T3 cells and primary bovine fibroblasts the expression of BPV-4 E5 can promote transcriptional activation of a heterologous (human) cyclin A promoter and in both cells types this activity correlates with cell transformation by E5 (Ashrafi, 1998; O'Brien and Campo, 1998; O'Brien et al., 1999). We decided to measure cyclin A promoter activity in our stably transformed cell lines.

Under normal growth conditions GFP-E5 clonal lines show a 3 to 7 fold increase in cyclin A promoter activity compared to GFP clone (Figure 5.3 a and 5.9). E5 wt shows a 8-15 fold activation of cyclin A promoter activity at the same conditions. When cells were maintained in low serum for 24 hr, promoter activity remained high in wild type E5 cells but not in GFP-E5 or control cells (Figure 5.3 a). Similar results were seen in transient transfection experiments in which cyclin A reporter plasmid was co-transfected with pZipneo expressing GFP, GFP-E5, E5 or pZipneo empty vector in NIH3T3 cells (Figure 5.3 b).

To investigate whether the differences in cyclin A promoter activity between GFP control cells and those expressing GFP-E5 were due to differences in transfection efficiency between cell lines, GFP and GFP-E5 cells were transfected with the pHSV vector expressing β -galactosidase, using Effectene transfection agent. Cells were stained *in situ* with developing solution for β -galactosidase and photographed after an overnight incubation at 37°C (section 2.2.3.5). The number of blue cells, used as an indicator of transfection efficiency (~ 10%), did not differ between cell lines (data not shown). This demonstrates that the differences in cyclin A promoter activity between control cells and those expressing GFP-E5 were not due to differences in transfection efficiency between cell lines.

5.2.2.3 Cyclin A protein expression

NIH3T3 cells expressing BPV-4 E5 wt show an increased expression of endogenous cyclin A protein under conditions where cyclin A expression is normally diminished or is not detectable in control cells (O'Brien and Campo, 1998). As shown above, GFP-E5 can promote transcriptional activation of a heterologous cyclin A promoter in normal growth conditions, thus we examined whether GFP-E5 has an effect on the transcriptional activation of the homologous promoter by looking

at the expression level of the endogenous cyclin A protein. GFP-E5, GFP, E5 and Z3T3 cells were utilised in this investigation.

The results showed that, as previously reported by O'Brien and Campo (1998), cyclin A expression is up-regulated in E5 expressing cells compared to control cells in growth medium and low serum culture (Figure 5.4 a). In normal growth conditions GFP-E5 cells do not show an elevation in cyclin A expression compared to GFP control clones. As expected from the transformation assays and the data from the cyclin A promoter trans-activation, GFP-E5 clones show a decreased cyclin A expression level in low serum, similarly to GFP clones (Figure 5.4 a).

5.2.2.4 Cyclin A kinase activity

To test if the de-regulation of cyclin A level in GFP-E5 cells was accompanied by a variation in cyclin A-associated kinase activity, cyclin A-associated complexes were immunoprecipitated from lysates of GFP-E5, GFP, E5 and Z3T3 cells and the associated kinase activity was assayed with Histone one (H1) as a substrate.

A higher level of cyclin A-associated kinase activity was observed in GFP-E5 clones, up to 2.5 times the GFP control clones kept in normal growth conditions. However, little or no difference was observed in GFP-E5 cells compared to control under low serum growth conditions (Figure 5.4 b). E5 expressing cells confirmed what was previously reported: the cyclin A-associated kinase activity was sustained in cells kept in normal and low serum (Figure 5.6), (O'Brien and Campo, 1998). It is interesting to note that there was a very good agreement between the cyclin A expression level shown by western blotting in normal and low serum and the amount immunoprecipitated with a specific antibody (compare Figure 5.4 a and 5.6).

Figure 5.3 a, b Trans-activation of the human cyclin A promoter

Measurement of the cyclin A promoter activation after 24 hr incubation in 10% or 0.5% serum. The luciferase reading for each sample (stable and short term transfection) was corrected according to the following equations: 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]. The reading obtained with control cells (GFP clone or Z3T3 cells for stable transfectants or pZipneo empty vector or pZipGFP for short term transfectants) was set at 1, and the luciferase readings of the samples were normalised to the control reading.

The mean values from triplicate samples are given for a representative experiment and standard deviation (bars) was calculated for each transfection class.

Figure 5.3 a

Transactivation of the cyclin A gene promoter in stable transformants

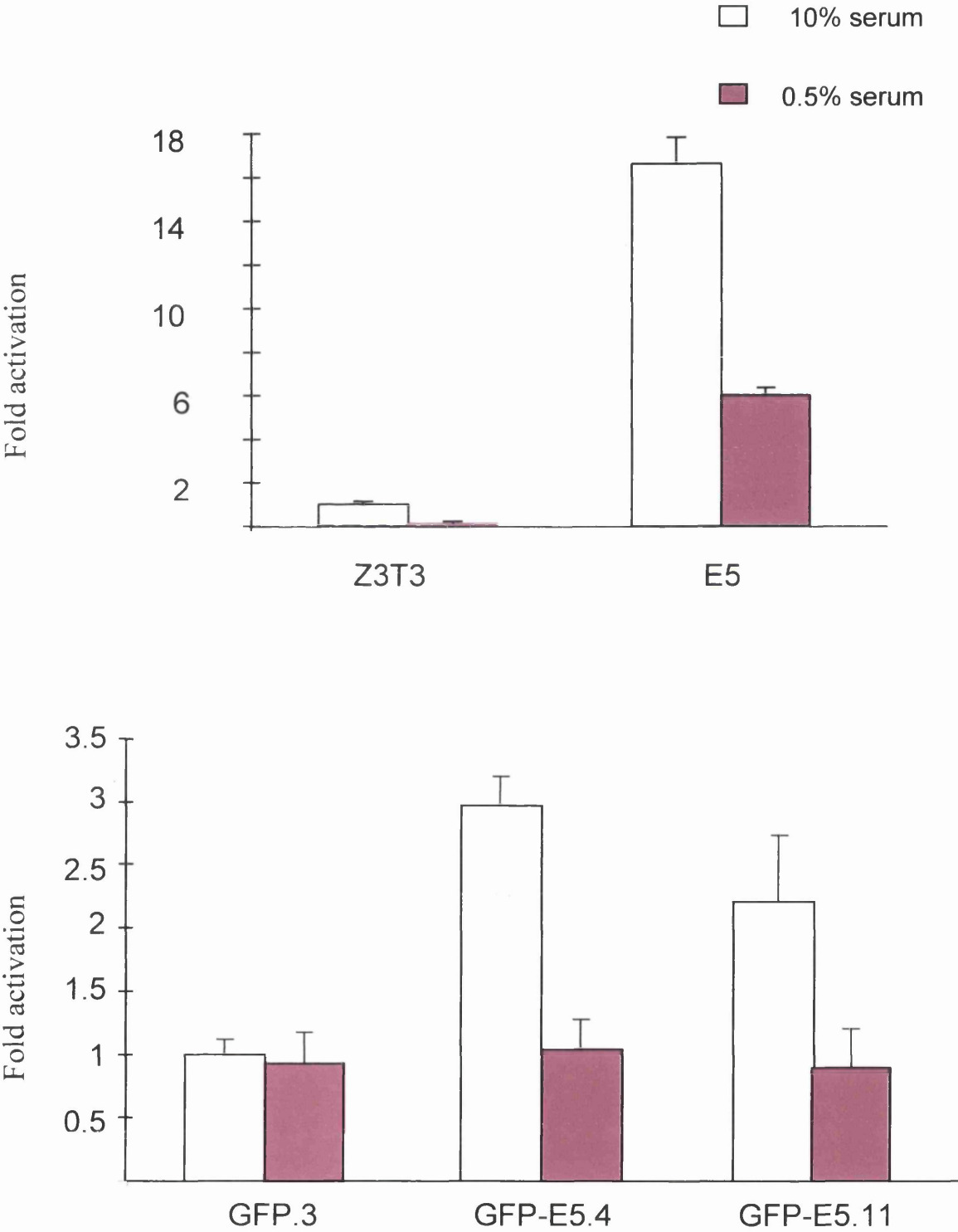


Figure 5.3 b

Transactivation of the cyclin A gene promoter in transient transfectants

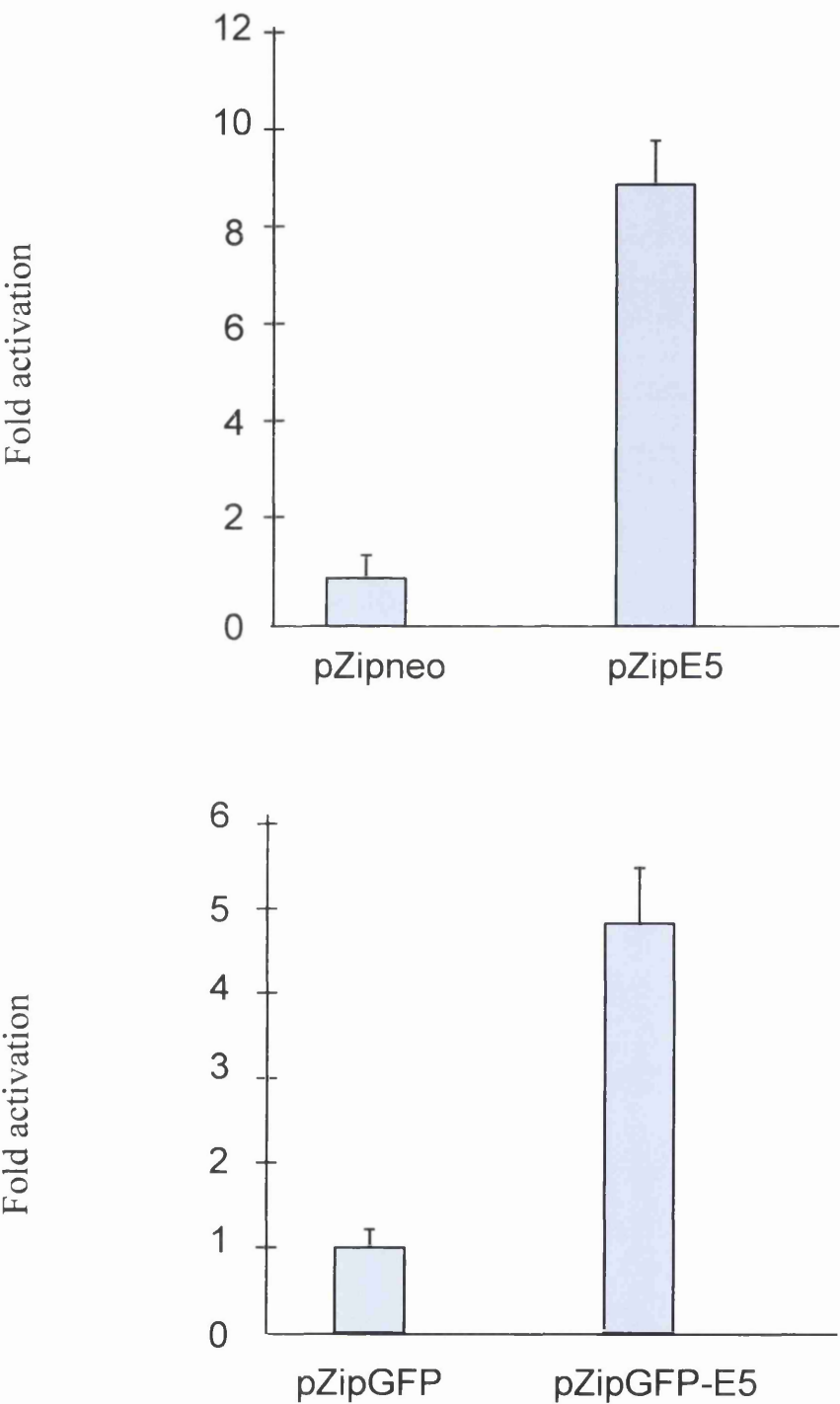


Figure 5.4 a, b Cyclin A expression level and associated kinase activity in 10% and 0.5% serum

a) Cells extracts (50 µg) were separated in SDS-PAGE (10% polyacrylamide) and membranes were probed with indicated antibodies.

Cyclin A is down-regulated in control and GFP-E5 cells after 24 hr in 0.5% serum but is sustained in E5 cells. Levels of α -tubulin serve as a loading control.

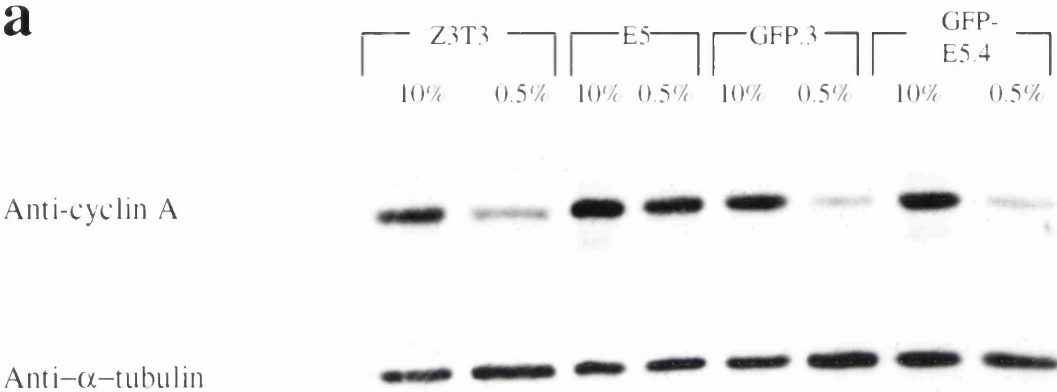
b) Cyclin A/CDK complexes were immunoprecipitated from 500 µg of total cell extracts using a cyclin A polyclonal antiserum (1:250). Cyclin A-associated kinase was assayed using Histone H1 as substrate.

The amount of incorporated radioactivity was determined by Phosphorimager. The reading obtained with control cells was set at 1, and the activity of the samples were normalised to the control reading.

Cyclin A-associated activity is elevated up to 2.5 fold in GFP-E5 cells cultured in 10% serum compared to the control cells.

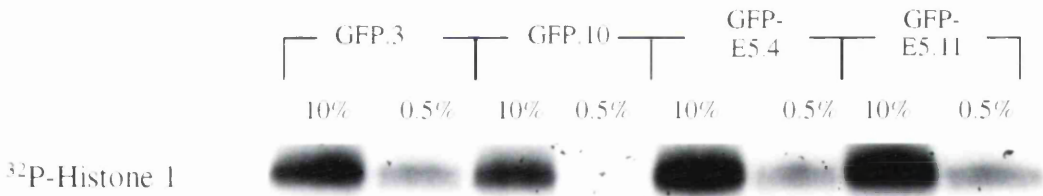
Figure 5.4 a, b

a

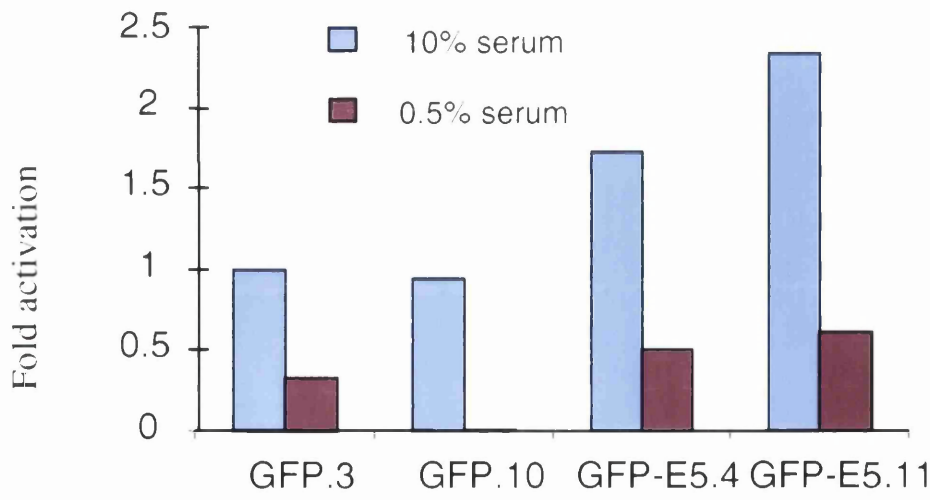


b

Cyclin A-associated kinase activity



Phosphorimager Analysis



5.2.2.5 Cyclin dependent kinase inhibitor (CKI) p27 expression

Active cycling fibroblasts down-regulate the CKI p27. Surprisingly, in NIH3T3 E5 cells, transformation is associated with up-regulation of the cyclin kinase inhibitor p27 in all growth conditions tested (O'Brien and Campo, 1998). Recent studies have shown that high levels of nuclear p27 are tolerated because the inhibitor is sequestered by an elevated pool of cyclin D1/CDK4 complexes (O'Brien et al., 2001). In light of these observations we investigated the state of p27 in GFP-E5 expressing cells.

In GFP-E5 expressing cells p27 expression is not significantly altered in cells grown in normal serum. When cells were kept at 0.5 % serum for 24 hours the p27 protein level increased in both GFP-E5 and GFP control cells (Figure 5.5). Moreover p27 was associated with cyclin A/CDK complexes immunoprecipitated from GFP-E5, GFP and Z3T3 cells, kept in low serum. The smaller amount of p27 associated with cyclin A in GFP-E5 cells could explain the residual kinase activity observed in these cells (Figure 5.6).

These results are concordant with the inability of GFP and GFP-E5 cells to sustain cell proliferation in LS (Chapter 4).

Figure 5.5 State of the CDKI p27 in 10% and 0.5% serum

Cells were cultured in 10% or 0.5% serum for 24 hr. Cells extracts (50 μ g) were separated in SDS-PAGE (10% polyacrylamide) and membranes were probed with indicated antibodies.

The expression of p27 is deregulated in E5 cells in all conditions tested compared to Z3T3 control cells. In GFP and GFP-E5 clones p27 is down-regulated in GM and up-regulated in LS. Levels of α -tubulin serve as a loading control.

Figure 5.6 Detection of Cyclin A and p27 protein in immunoprecipitated complexes

Samples of the cyclin A immunoprecipitates, from 250 μ g of total cell extracts, were fractionated in 10% polyacrylamide gel. The amount of immunoprecipitated cyclin A and associated p27 were determined by immunoblotting. Cyclin A-associated H1 kinase activity was determined in immunoprecipitated complexes.

Figure 5.5

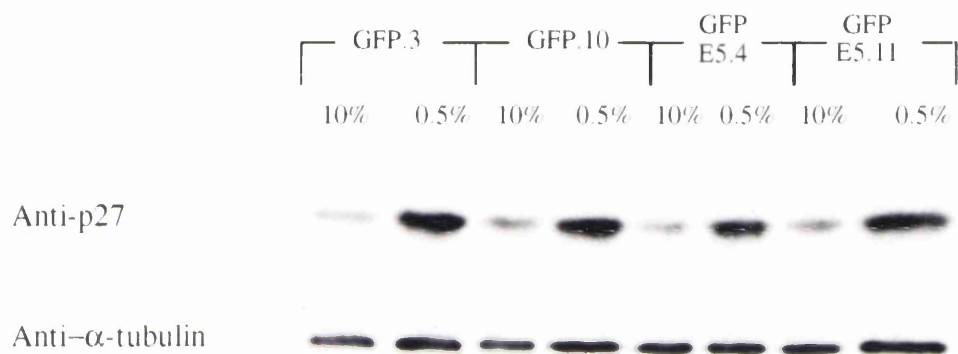
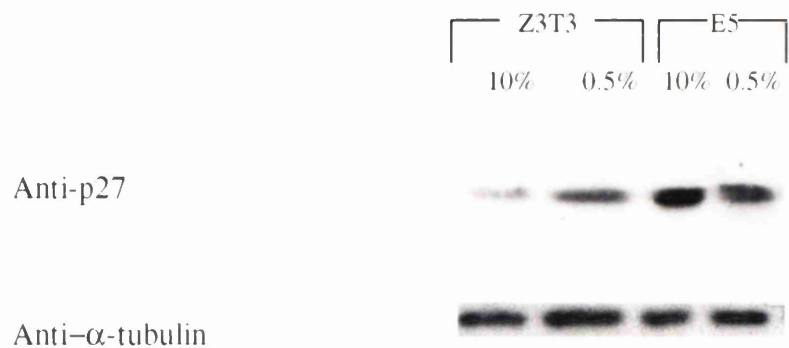
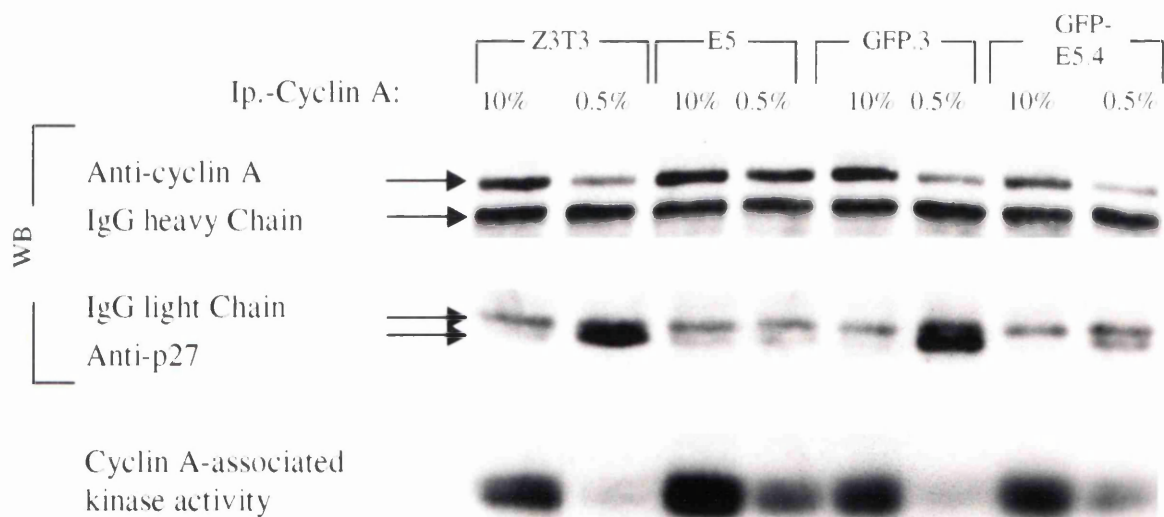


Figure 5.6



5.2.3 Cell growth in semi solid media

5.2.3.1 Cyclin D1 and the pocket proteins

The extracellular matrix proteins (ECM) are jointly required with mitogens to induce cyclin D1 expression: in NIH3T3 cells cyclin D1 mRNA and protein are not induced if quiescent cells are stimulated with mitogens in absence of substratum (Zhu et al., 1996).

It has been shown that in both NIH3T3 cells and human fibroblasts the phosphorylation of pRb protein during the G1 phase of cell cycle requires information provided by both mitogens and the extracellular matrix and neither signal alone is sufficient for pRb phosphorylation. However, enforced expression of cyclin D1 protein in late G1 rescues pRb phosphorylation and entry in S phase in non adherent cells (Zhu et al., 1996).

NIH3T3 expressing E5 wt and GFP-E5 grow in suspension culture (O'Brien and Campo, 1998), (Chapter 4); therefore we investigated the state of cyclin D1 and the pocket proteins in these growth conditions.

In E5 and GFP-E5 cells cultured in suspension for 48 hr cyclin D1 expression is significantly up-regulated compared to their respective control cells in the same growth conditions (Figure 5.7 a). In E5 and GFP-E5 cells the state of cyclin D1 is consistent with the ability of these cells to proliferate in semi-solid medium.

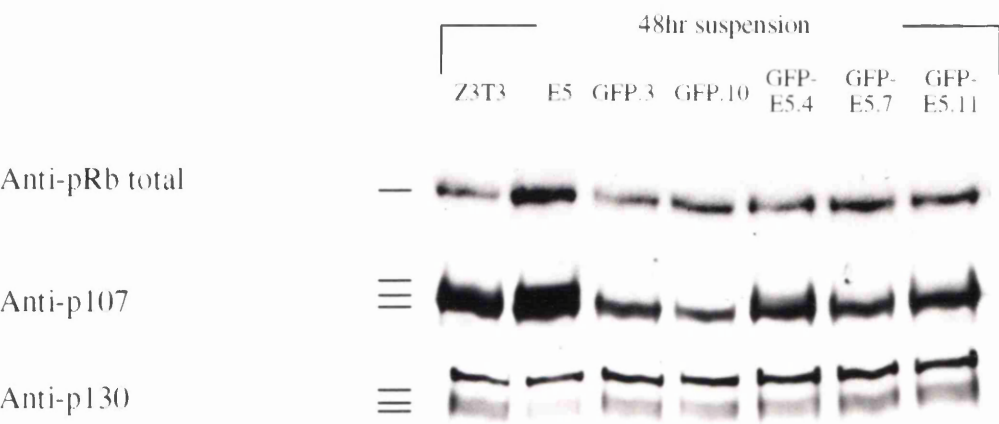
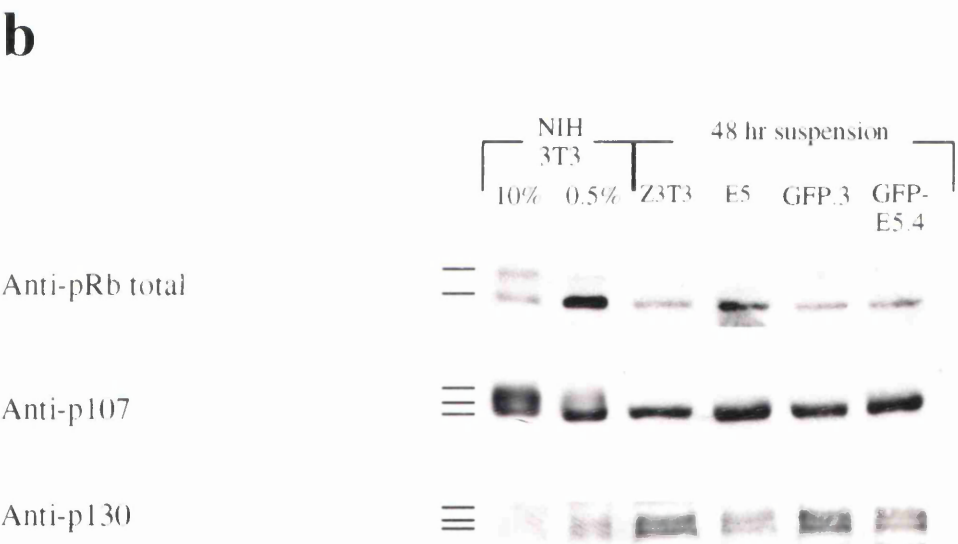
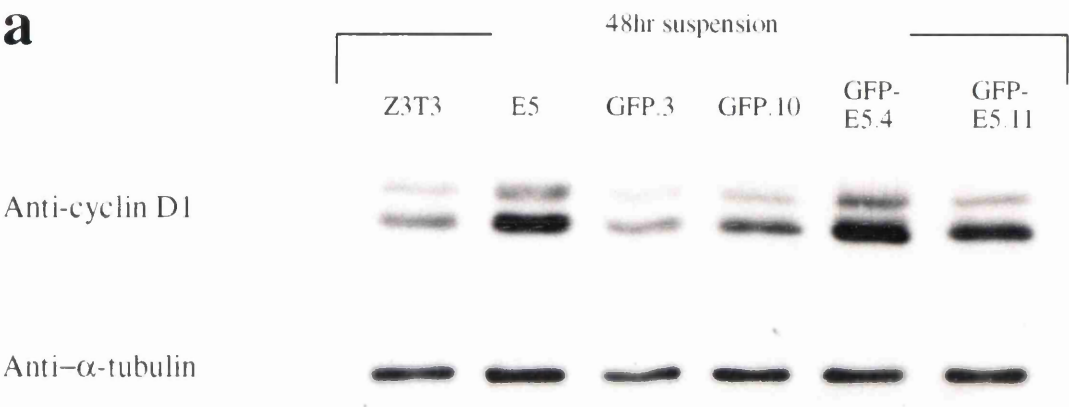
In cells grown in suspension only the hypophosphorylated form of pRb and p107 could be detected in control cells (GFP and Z3T3) while a form of p107 with a different electrophoretic mobility, probably due to protein phosphorylation, was observed only in GFP-E5 and E5 cells. Surprisingly it was not possible to detect the hyperphosphorylated forms of pRb in GFP-E5 and E5, either with an antibody against total pRb (Figure 5.7 b) or with the phospho-specific antibody against serine 780 (data not shown), despite the good separation between hyper and hypophosphorylated forms as shown for adherent cells (Figure 5.7 b). Interestingly pRb level increased in E5 expressing cells. p130 level was similar in control and GFP-E5 cells and slightly lower in E5 cells (Figure 5.7 b). Because p130 accumulates in quiescent cells the level of the protein detected is more representative of the percentage of quiescent than the proliferating cells in each cell lines.

Figure 5.7 a, b Analysis of the cyclin D1 and the pocket proteins expression in suspension culture

a) Cells were cultured in suspension for 48 hr. Cells extracts (50 μ g) were separated in SDS-PAGE (10% polyacrylamide) and membranes were probed with indicated antibodies. GFP-E5 and E5 cells show up-regulated expression of cyclin D1. (Similar results were obtained after 24 hr incubation data not shown). Levels of α -tubulin serve as a loading control.

b) Cells were cultured as above. Cells extracts (100 μ g) were separated in SDS-PAGE (7.5% polyacrylamide) and membranes were probed with indicated antibodies. The inactive form of pRb was not detected in E5 or GFP-E5 cells, surprisingly E5 cells show up-regulation of pRb. p107 was up-regulated and possibly phosphorylated in E5 and GFP-E5 cells. p130 protein level is only slightly down regulated in E5 and GFP-E5 cells. The results are representative of two independent experiments.

Figure 5.7 a, b



5.2.3.2 Cyclin E expression and associated kinase activity

The expression level of cyclin E and its catalytic subunit CDK2 is anchorage-independent in NIH3T3 cells (Guadagno et al., 1993; Zhu et al., 1996), however the cyclin E/CDK2 kinase activity is strongly down-regulated in these conditions. We determined the level of Cyclin E expression and its associated kinase activity in stable transfectants.

The protein level of cyclin E and CDK2 is similar in Z3T3, GFP-E5 and GFP cells and only moderately up-regulated in E5 cells (Figure 5.8 a). The cyclin E/CDK2 activity is only slightly up-regulated in E5 and GFP-E5 expressing cells compared with the respective controls (Figure 5.8 b). Nevertheless the cyclin E appears to be associated mainly with the active form of CDK2 (Figure 5.12).

When the kinase assay was performed immunoprecipitating CDK2, no kinase activity could be detected (Figure 5.8 b). As shown by western blot analysis of the immunoprecipitates, the absence of appreciable CDK2 kinase activity is due to the inability of the anti-CDK2 antibody to efficiently immunoprecipitate the active form of CDK2 (Figure 5.12).

5.2.3.3 Cyclin A promoter transactivation in suspension culture

E5 expressing cells can transactivate the cyclin A promoter when maintained in suspension culture (O'Brien and Campo, 1998). Attachment-dependent expression of the cyclin A mRNA has been shown in NIH3T3 cells (Guadagno et al., 1993). We measured cyclin A promoter activity in our cells lines in suspension culture.

As shown in Figure 5.9 the promoter activity was relatively high in GFP-E5 cells kept in suspension, up to 5 times the control cells. In the same conditions E5 wt expression in NIH3T3 led to ~ 8 fold increase in cyclin A promoter activity (O'Brien and Campo, 1998), (Figure 5.9).

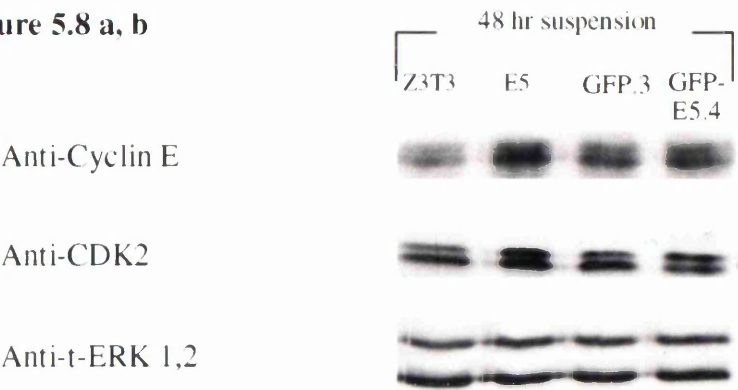
Figure 5.8 a, b Cyclin E and CDK2 expression level and associated kinase activity in suspension culture

a) Cells were cultured in suspension for 48 hr. Cells extracts (50 µg) were separated in SDS-PAGE (10% polyacrylamide) and membranes were probed with indicated antibodies. Cyclin E and CDK2 expression level remains similar in E5, GFP-E5 and control cells. Levels of total ERK 1/2 (t-ERK 1/2) serve as a loading control.

b) Cyclin E/CDK2 and cyclins/CDK2 complexes were immunoprecipitated from 500 µg of total cell extracts using cyclin E and CDK2 polyclonal antisera (1:250). CDK2 and cyclin E/CDK2 associated kinase was assayed using Histone H1 as substrate. The amount of incorporated radioactivity was determined by Phosphorimager. The reading obtained with control cells was set at 1, and the activity of the samples were normalised to the control reading.

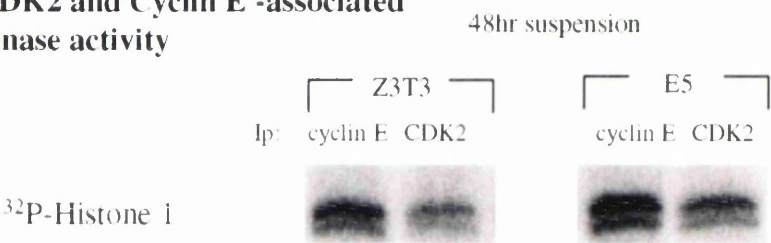
Figure 5.8 a, b

a

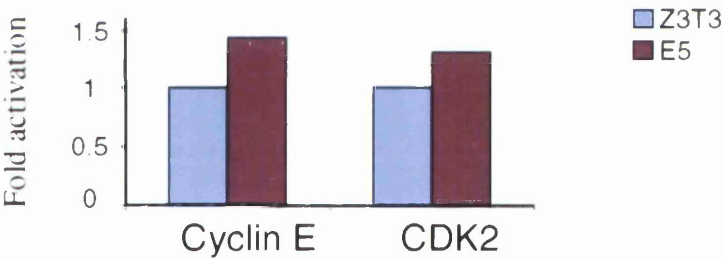


b

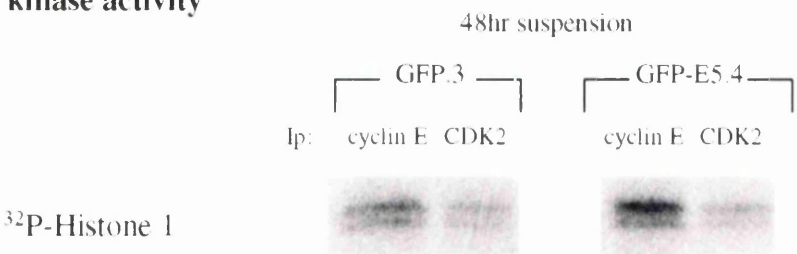
**CDK2 and Cyclin E -associated
kinase activity**



Phosphorimager Analysis



**CDK2 and Cyclin E-associated
kinase activity**



Phosphorimager Analysis

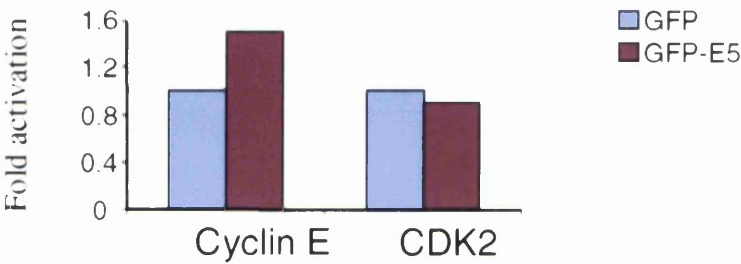
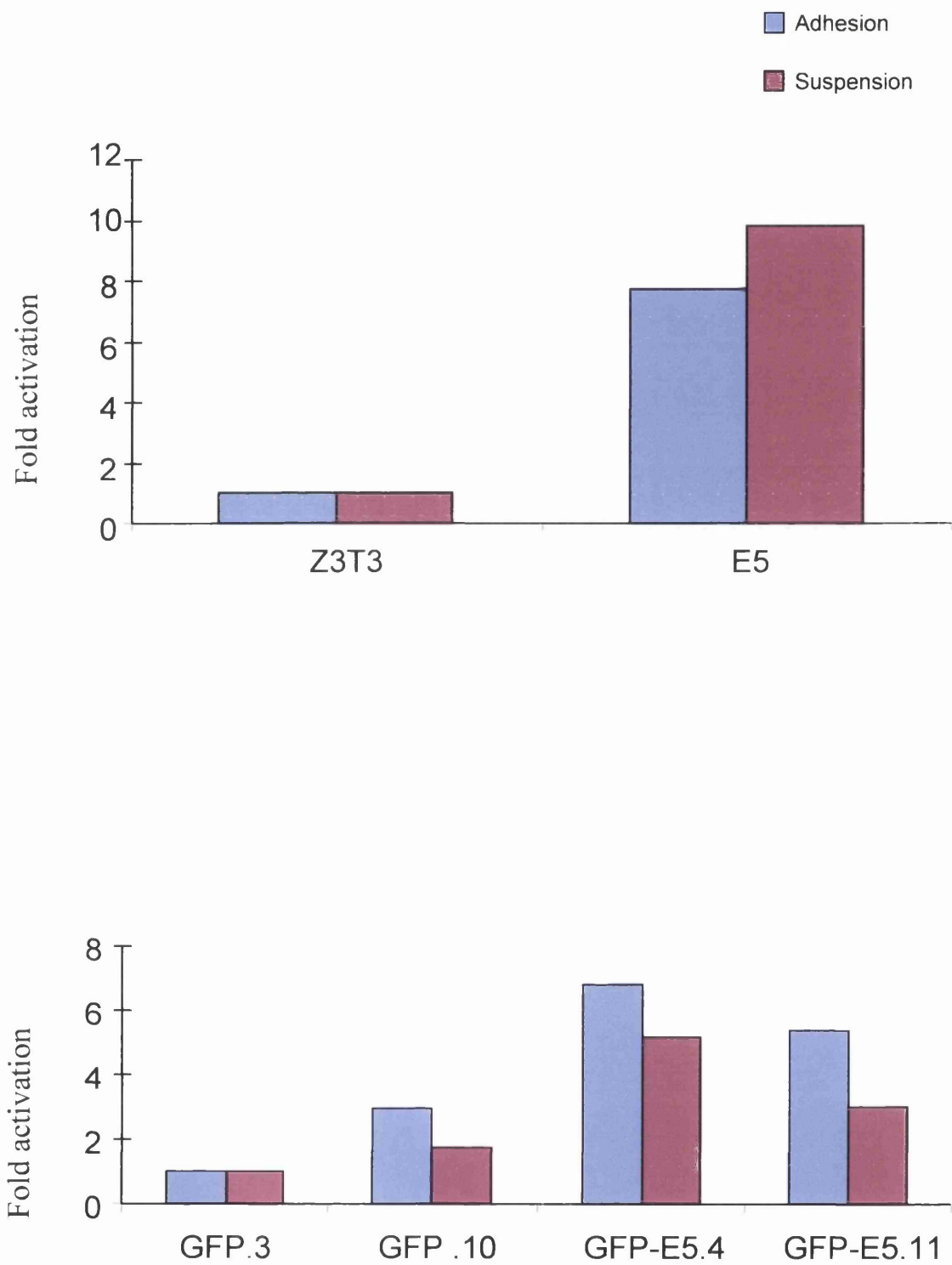


Figure 5.9 Trans-activation of the human cyclin A promoter in suspension culture

The measure of the cyclin A promoter activation has been performed as reported in Figure 5.3. The reading obtained with control cells (GFP or Z3T3 cells) in adhesion and suspension was set at 1, and the luciferase readings of the samples were normalised to the control reading. The mean values were obtained from duplicate samples. These results are representative of three independent experiments.

Figure 5.9



5.2.3.4 Cyclin A expression and associated kinase activity

Cyclin E expression level and activity are required for elevated cyclin A expression in NIH3T3 cells in adhesion conditions (Zerfass-Thome et al., 1996), E5 expressing cells however have been shown to bypass this requirement (O'Brien and Campo, 1998). It is known that in suspension ectopic expression of cyclin A but not cyclin E or CDK2 allows proliferation of NRK cells (Guadagno et al., 1993). Thus we investigated the expression level and the associated kinase activity of cyclin A in these growth conditions.

In GFP-E5 cells the cyclin A level was significantly elevated compared to GFP control clones (Figure 5.10 a). Again these results were consistent with the transcriptional activation of the cyclin A promoter in suspension and the anchorage-independent growth displayed by GFP-E5 expressing cells (Chapter 4).

Moreover, in non-adherent growth conditions, E5 and GFP-E5 expressing cells sustained cyclin A-associated kinase activity up to 5 and 6 times compared to the respective control cells (Figure 5.10 b).

Cyclin A is companion of two cyclin dependent kinases, CDK2 and cdc2, at different stages the cell cycle. It complexes with the CDK2 at the G1/S phase boundary and the complex-associated kinase activity is required through S phase, while cdc2-associated kinase activity is necessary for passage through G2 and mitosis (Pagano et al., 1992). Our investigations reveal that cyclin A is mainly, if not exclusively associated with the cdc2 (Figure 5.12). We therefore concluded that the sustained cyclin A-associated kinase activity detected in suspension culture is due mainly to cyclin A/cdc2 complexes.

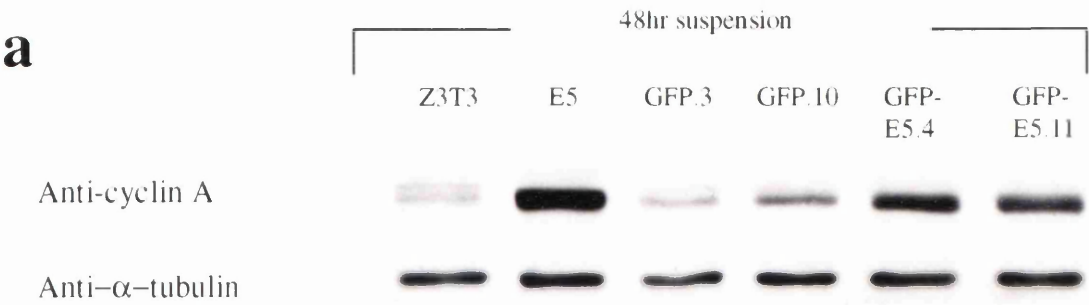
Figure 5.10 a, b Cyclin A expression level and associated kinase activity in suspension culture

a) Cells were cultured in suspension for 48 hr. Cells extracts (50 μ g) were separated in SDS-PAGE (10% polyacrylamide) and membranes were probed with indicated antibodies. Only GFP-E5 and E5 cells show a sustained expression of cyclin A protein following 48 hr incubation in semi-solid medium. Levels of α -tubulin serve as a loading control.

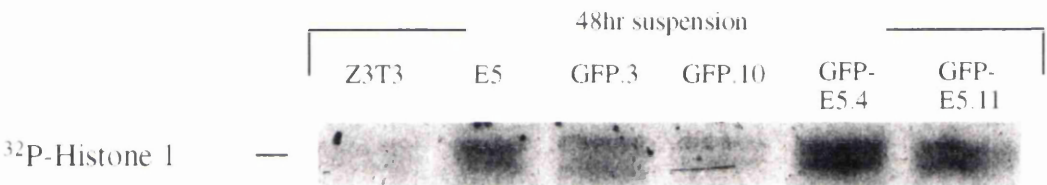
b) Cyclin A/CDK complexes were immunoprecipitated from 500 μ g of total cell extracts using a cyclin A polyclonal antiserum (1:250). Cyclin A-associated kinase was assayed using Histone H1 as substrate.

In suspension GFP-E5 cells sustained cyclin A-kinase activity up to 6 times the control cell. E5 cells show 5 fold activation in cyclin A-associated kinase activity compared to Z3T3 cells.

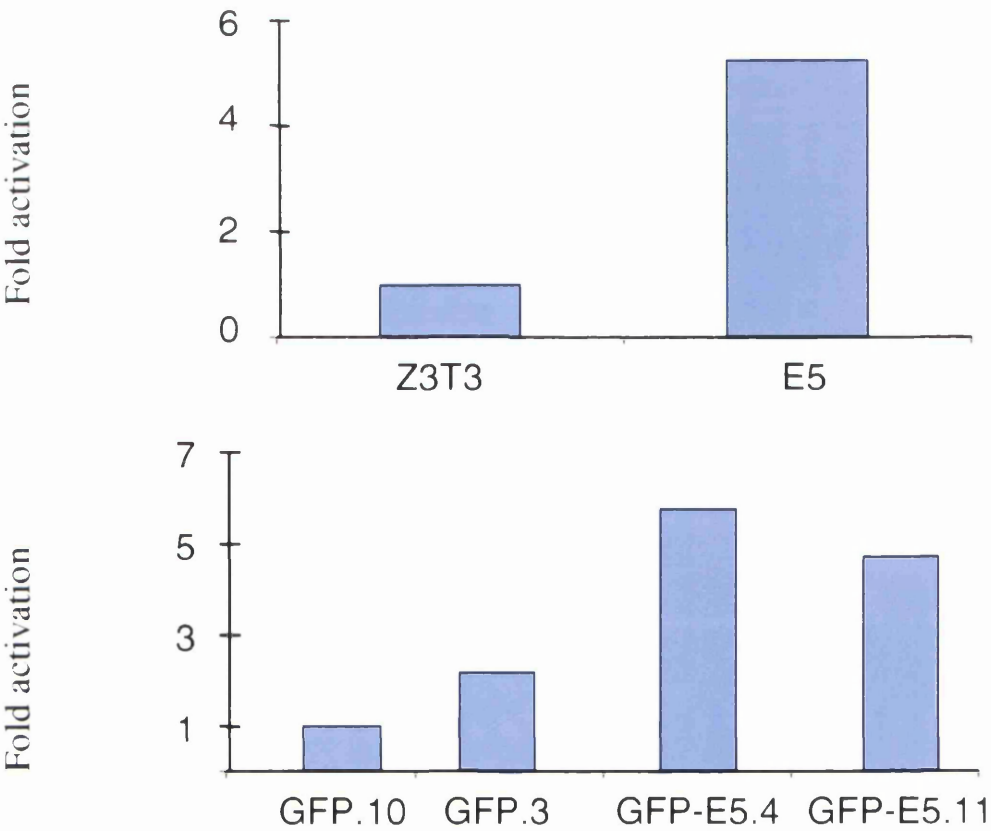
Figure 5.10 a, b



b Cyclin A-associated kinase activity



Phosphorimager Analysis



5.2.3.5 Cyclin dependent kinase inhibitor (CKI) p27 expression

In suspension GFP and GFP-E5 cells showed the same level of p27 expression although GFP-E5 cells still cycled in these conditions (Figure 5.11). A possible explanation for this event is the contribution of cells arrested in G1 present in both GFP-E5 and GFP plates in substratum-independent growth conditions (87% and 97.2% of no cycling cells respectively, Table 4.2). In these studies E5 expressing cells confirm what previously reported, p27 expression level was elevated under these growth conditions (O'Brien and Campo, 1998), (Figure 5.11).

Figure 5.11 Analysis of p27 expression level in suspension culture

Cells were cultured in suspension for 48 hr. Cells extracts (50 μ g) were separated in SDS-PAGE (10% polyacrylamide) and membranes were probed with indicated antibodies. The expression of p27 is sustained in E5 cells, while no gross difference is observed in the CKI level between GFP and GFP-E5 clones. Levels of α -tubulin serve as a loading control.

Figure 5.12 Cyclin A does not associates with CDK2

Samples of cyclin E, CDK2 and cyclin A immunoprecipitates (E, CDK2, A) were fractionated by SDS-PAGE and analysed by western blot using the antibodies indicated. As expected from previous observations on cyclin A in total cells extracts (Figure 5.10), the amount of immunoprecipitated cyclin A is higher in E5 and GFP-E5 cells. The active form of CDK2 is predominantly associated with cyclin E but cannot be detected associated with cyclin A. On the contrary, cdc2 is predominantly associated with cyclin A in E5 and GFP-E5 cells. The inactive form of CDK2 was predominantly immunoprecipitated by CDK2 antiserum. Finally p27 is associated with cyclin E, CDK2 and cyclin A complexes in similar amount for Z3T3, GFP, and GFP-E5 cells but is more abundant in E5 cells were is mainly associated with cyclin E/CDK2.

Figure 5.11

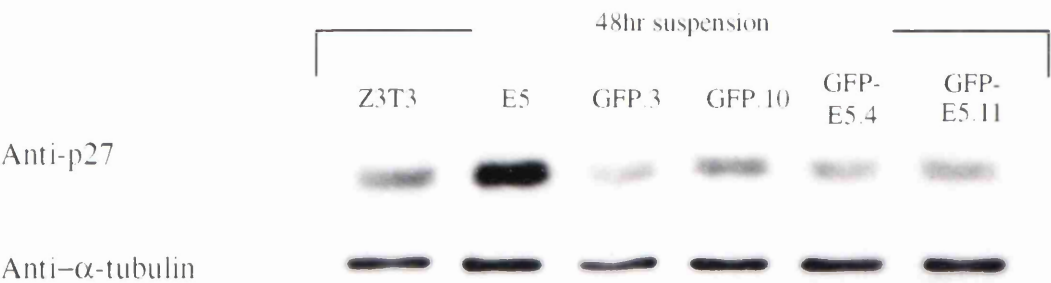
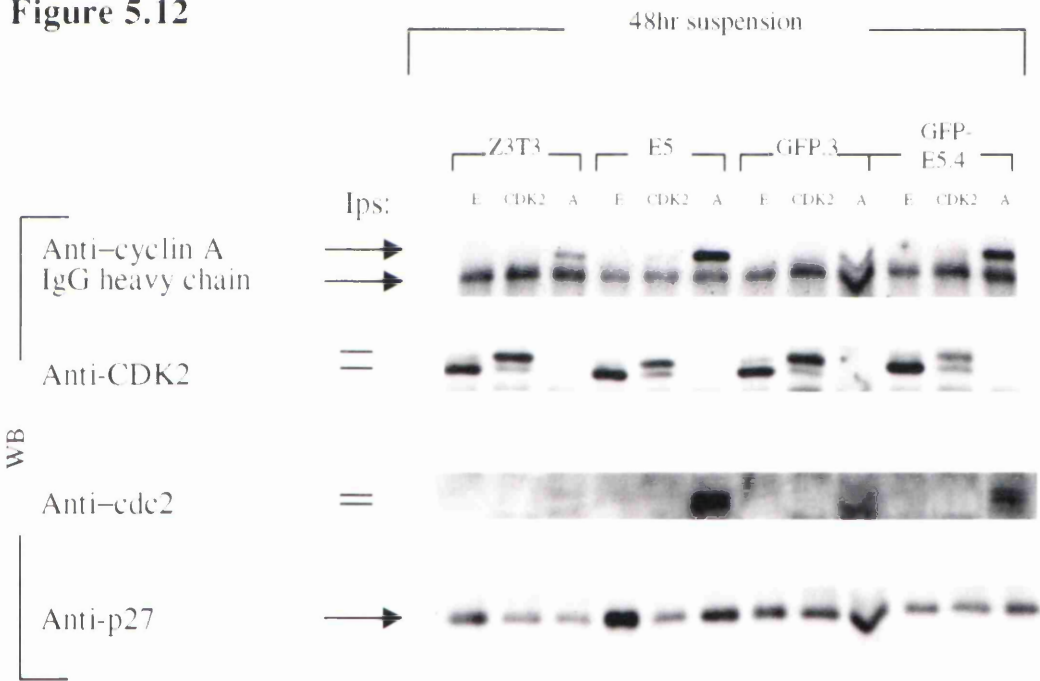


Figure 5.12



5.2.4 MAPK inhibitors

It has been shown that anchorage-independent conditions do not prevent cells from responding to mitogens and entering the cell cycle (Guadagno and Assoian, 1991); G0/G1 transition and early G1 events can be stimulated by growth factors even when cell division is precluded. Moreover, these studies reported that the block in anchorage-independent growth leads to selective inhibition of G1/S cell cycle gene expression (Guadagno and Assoian, 1991). Growth factors activate a series of signal transduction cascades among which activation of the mitogen-activated protein kinase cascade, Ras-Raf-MEK-ERK is the best characterised. Most studies indicate that these signals eventually control G1 phase progression by regulating cyclin D1 expression.

In GFP-E5 and E5 expressing cells grown in suspension the G0/G1 transition events, such as cyclin D1 expression, occur and the cells progress throughout the cell cycle as shown by cyclin A up-regulation.

We wanted to determine if the transduction cascade activated by mitogens and ultimately the expression of cyclin D1 were a necessary prerequisite for cell progression in suspension. To achieve this we utilised inhibitors of the MAPK pathways to block the signal cascade and analysed the state of the cell cycle components after 48 hr treatment. PD98059 inhibitor blocks the activation of MEK while U0126 specifically inhibits MEK in both active and inactive forms.

As shown in Figure 5.13 (top panel) the treatment with the PD98059 inhibitor blocked the phosphorylation of ERK 1/2 (p-ERK 1/2), while the level of total ERK 1/2 (t-ERK 1/2) was not affected. The inhibition of the MAPK pathway induced a down-regulation of cyclin D1 level in Z3T3, GFP-E5 and GFP cells but not in E5 cells. Interestingly cyclin A level did not change in E5 and is minimally effected in GFP-E5 contrary to their control cells. Similar results were obtained in Z3T3 and E5 cells when the treatment was prolonged to 72 hr (middle panel). Surprisingly no p-ERK 1/2 down-regulation could be detected in control cells. As mentioned above PD98059 inhibits the activation of MEK, thus the phosphorylated ERK 1/2 could result from residual activated MEK.

In GFP-E5 and GFP cells the use of the U0126 inhibitor (bottom panel) confirmed the independence of cyclin A expression from MAPK pathway and cyclin D1 expression.

Figure 5.13 Cyclin A expression is independent from the MAPK pathway

Z3T3, E5 cells were treated with PD98059 inhibitor for 48 or 72 hr in suspension culture (top and middle panels). GFP and GFP-E5 cells were treated also with PD98059 for 48 hr and with U0126 inhibitor for 72 hr (top and bottom panel). The activation state of ERK 1/2 kinase after the treatment was assessed by using a phosphospecific antiserum (p-ERK 1/2), cyclin D1 and cyclin A expression levels were also analysed. Total amount of ERK 1/2 was not influence by the treatment (t-ERK 1/2). In Z3T3, GFP and GFP-E5 cells cyclin D1 is down-regulated by the inhibitors while in E5 cells cyclin D1 level is independent from the MAPK kinase pathway. Cyclin A shows a different pattern: the protein level is sustained after inhibitors treatment in both GFP-E5 and E5 cells.

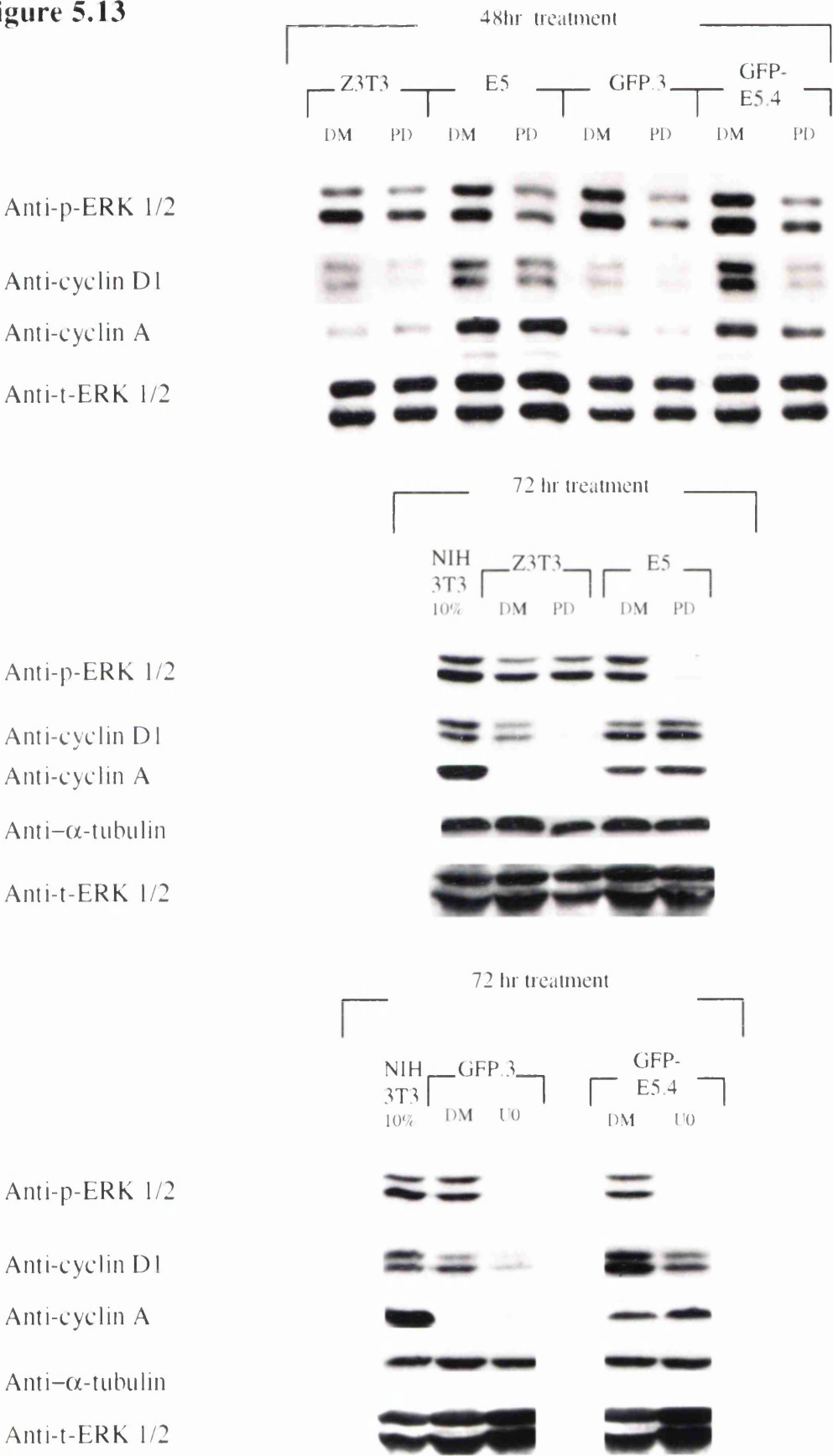
DM= cells treated with DMSO as control

PD= cells treated with 50 μ M of PD98059 inhibitor

U0= cells treated with 20 μ M of U0126 inhibitor

NIH3T3 10%= parental NIH3T3 cells grown at 10% serum in adhesion

Figure 5.13



5.3 Conclusions

GFP-E5 cells did not cycle in low serum but could proliferate in suspension. Cyclin D1 was down-regulated in GFP-E5 grown in LS and consequently only the hypophosphorylated, repressing forms of pRb and p107 were detected and p130 was accumulated. However, in suspension culture, where GFP-E5 cells continued to proliferate (Chapter 4), cyclin D1 expression was up-regulated and p107 was phosphorylated. Surprisingly the hyperphosphorylated form of pRb was not detected. Overall the state of these cell cycle components was consistent with the proliferative profile of GFP-E5 cells in the different growth conditions.

Previous studies have demonstrated a correlation between BPV-4 E5 transformation ability and trans-activation of cyclin A promoter. In NIH3T3 and PalF cells only E5 wt and its transforming mutants can trans-activate the cyclin A promoter. In addition mutants that transactivate the cyclin A promoter confer to PalFs the ability to grow in suspension, and mutants that strongly trans-activate the promoter permit cells growth in low serum (Ashrafi, 1998; O'Brien and Campo, 1998; O'Brien et al., 1999).

GFP-E5 expressing cells follow the pattern described above: a heterologous cyclin A promoter was trans-activated in normal growth conditions but not in low serum, conditions that induced cells to withdraw from the cell cycle (Chapter 4). However, in semi-solid medium GFP-E5 clones were able to trans-activate the cyclin A promoter up to 5 times the control cells. These data, together with the previous studies on E5 wt and its mutants, support the hypothesis that an unknown threshold of cyclin A promoter trans-activation correlates with cell transformation, as judged by growth in suspension.

Accordingly GFP-E5 cells showed a sustained cyclin A expression and associated kinase activity when cultured in suspension but down-regulated cyclin A expression and its associated kinase activity in LS.

Moreover, we showed that the cyclin A-associated kinase activity in E5 and GFP-E5 cells grown in suspension is mainly due to cyclin A/cdc2 complexes (Chapter 7).

The data obtained with the MAPK inhibitors suggest that in E5 cells the expression of cyclin D1 and cyclin A was not affected by the inhibition of the MAPK pathway. The MAPK cascade was required to sustain cyclin D1 expression in GFP-E5 cells, however cyclin A level was not effected by the inhibition of this pathway. From these results it was possible to hypothesise that adherent GFP-E5 cells require growth factors to sustain cyclin D1 expression and its kinase activity and to reach a sufficient level of cyclin A and cyclin A-associated kinase activity to allow cell cycle progression. In contrast the ability of GFP-E5 cells to grow in suspension was determined by the ability to sustain cyclin A expression and associated kinase activity independently from cyclin D1 expression and cyclin E/CDK2 activity.

We conclude therefore that the ability of GFP-E5 expressing cells to overcome the G1 arrest induced by loss of cell adhesion correlates with the status of cyclin A expression and of its cdc2 associated kinase activity and reinforce the hypothesis that E5 transforming ability is at least in part, due to the ability of the viral protein to deregulate cyclin A expression (Ashrafi, 1998; O'Brien and Campo, 1998; O'Brien et al., 1999).

CHAPTER 6: MUTANT FORMS OF BPV-4 E5

6.1 Introduction

BPV-4 E5 transforms established murine fibroblasts conferring anchorage-independent growth and ability to grow in absence of mitogens. When expressed in PalFs in collaboration with BPV-4 E7, HPV-16 E6 and an activated Ras, E5 expression induces, in addition to the phenotypes mentioned above, loss of contact inhibition. In an attempt to segregate different transforming functions of E5 and therefore to define its functional domains, a panel of E5 mutants and chimeras between BPV-4 E5 and BPV-1 E5 were made and tested in PalFs (Ashrafi, 1998; O'Brien et al., 1999).

BPV-4 E5 shares some structural and functional homology with BPV-1 E5 (Figure 1.2) suggestive of a similar mechanism of cell transformation. The extensive genetic analysis of BPV-1 E5 revealed the presence of critical amino acids in the hydrophobic body (Horwitz et al., 1988; Kulke et al., 1992) and the requirement of the hydrophilic C-terminus (tail) for transformation activity (Settleman et al., 1989; Horwitz et al., 1989). Other studies have shown that binding of BPV-1 E5 to ductin requires the N-terminal hydrophobic domain and specifically a glutamine at position 17 is important in this protein function (Goldstein et al., 1992a; Goldstein et al., 1992b). In the hydrophobic body of BPV-4 E5 the residue of position 17 is an asparagine (N), potentially functionally homologous to glutamine 17 in BPV-1 E5. To test this hypothesis asparagine 17 was mutated to serine, tyrosine, or alanine. The mutation to alanine (N17A) produced a hypertransforming protein with great ability of inducing anchorage-independent growth, cell growth in low serum and escape from contact inhibition; the introduction of tyrosine (N17Y) at the same position completely abolished the transforming capacity of E5, whereas mutation to a serine (N17S) made cells capable of anchorage-independent growth but not in low serum (O'Brien et al., 1999).

Further investigations revealed that the 12 hydrophilic amino acids (tail) at the BPV-4 E5 C-terminus are critical since neither a form of BPV-4 E5 lacking this region (E5T) nor the chimera comprising the BPV-4 E5 body and the BPV-1 E5 "tail" (4E5N-1E5C) are capable of promoting anchorage-independent growth, growth in low serum or loss of contact inhibition, although the reciprocal chimera (1E5N-4E5C) still allows PalFs to grow in suspension (Ashrafi, 1998; O'Brien et al., 1999).

To test the importance of the secondary amino acid structure of the E5, two mutants, carrying a double modification in the hydrophobic N-terminus hypothesised to be a transmembrane α -helix (Figure 1.2), were produced and tested in PalFs. A15P-A20P mutant, where the alanine residues at position 15 and 20 had been converted to a proline (which has the property of forcing a bend in the main chain and disrupting an α -helix) and A15G-A20G mutant, where the same alanine residues were mutated to glycine residues, a conservative mutation which should not disrupt an α -helical conformation. While A15G-A20G showed low trans-activation of the cyclin A promoter but still significantly above background, A15P-A20P failed to activate the cyclin A promoter (Ashrafi, 1998). 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 also emphasise the importance of the nature of these residues for E5 functions.

The use of mutant forms of BPV-4 E5 has allowed the segregation of its several transforming functions. Thus the ability to induce substrate-independent growth can be separated from the ability to form foci or to prevent cell cycle exit in low serum while focus formation and growth in low serum still co-segregate. Moreover these studies showed that the different transforming phenotypes conferred by the E5 mutants correlate with their ability to transactivate the cyclin A promoter: E5 mutants that transactivate cyclin A promoter confer to PalF cells the ability to grow in suspension and mutants that strongly transactivate the promoter permit cell growth in low serum (Ashrafi, 1998; O'Brien et al., 1999).

The transforming activities of E5T mutant have also been analysed in NIH3T3 cells. Contrary to E5 wild type, E5T is incapable of transforming NIH3T3 cells, active transcription of the cyclin A promoter or de-regulates expression of cyclin A, underlying once more the existing correlation between transformation and cyclin A de-regulation (O'Brien and Campo, 1998).

As detailed in Chapter 1, BPV-4 E5 transforms primary bovine fibroblasts in collaboration with BPV-4 E7, HPV-16 E6 and an activated Ras. On the contrary in NIH3T3 cells E5 expression is sufficient for cell transformation, consequently we wanted to analyse E5 mutants in established murine fibroblasts to expand the information on the transformation ability of this viral protein and its correlation with the regulation of cyclin A expression.

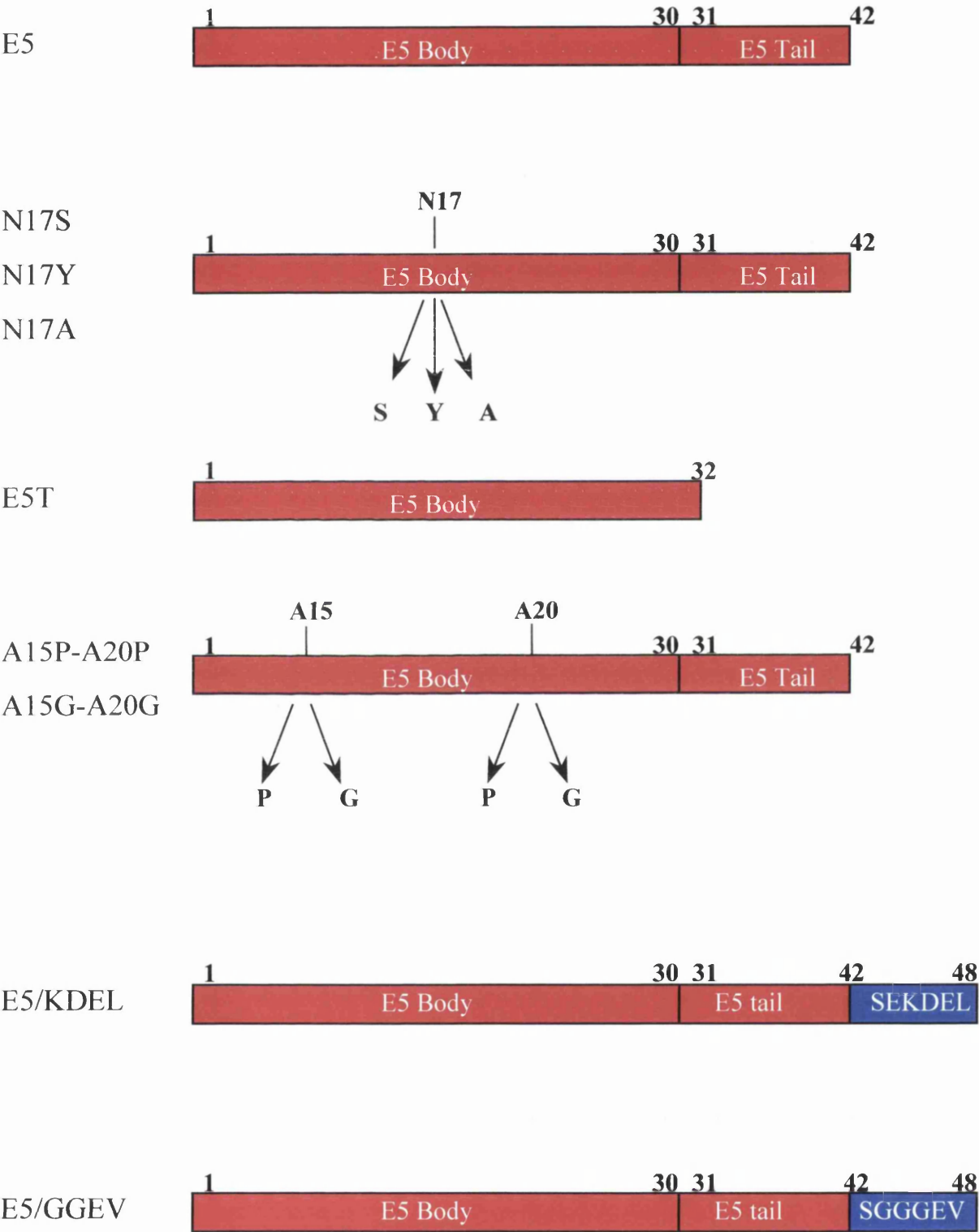
It has been shown that in the PalFs cells the altered phenotypes observed for E5 mutants do not depend on the expression level or stability of the proteins and there is no correlation between amount of protein and cell transformation (O'Brien et al., 1999).

In order to establish if a mis-location of the mutants may be an explanation for their different transforming ability, we generated fusion forms of these peptides with GFP. (A panel summarising the mutant forms of E5 is shown in Figure 6.1).

Figure 6.1 E5 wt and its mutant forms

N17S, N17A, N17Y and E5T mutants have been made by Dr. R. Anderson. A15P-A20P and A15G-A20G mutants have been made by Dr. H. Ashrafy. E5/KDEL and E5/GGEV have been made by Dr. Vincent O'Brien. All constructs are HA tagged.

Figure 6.1



6.2 Experimental results and discussion

6.2.1 Localisation of GFP-E5 mutants in transient transfectants

In order to obtain fusion forms of BPV-4 E5 mutants their sequences were cloned into pEGFPC3, at the 3' end of the GFP gene using a PCR cloning strategy (section 2.2.1). The constructs were sequenced to confirm their identity and called GFP-N17S, GFP-N17Y, GFP-N17A and GFP-E5T (Figure 6.2).

The constructs were transfected into COS-7 cells using the calcium phosphate method. Cells were fixed, mounted and visualised at the confocal microscope (section 2.2.4). Colocalisation studies were performed using an ER marker and a Golgi marker, as described in section 3.3.1.5.

All three point mutants at position 17 and the truncated form of E5 show the same perinuclear fluorescent distribution observed for the wild type protein (Figure 6.3). Similarly to GFP-E5, when the location of the mutants were further investigated by colocalisation studies, GFP-N17S, GFP-N17Y, GFP-N17A and GFP-E5T were localised mainly in the Golgi apparatus and partially in the ER (Figure 6.4 a, b).

These studies show that all E5 mutants have a similar cellular location and consequently seem to exclude gross mis-location as a potential reason for their different transformation abilities.

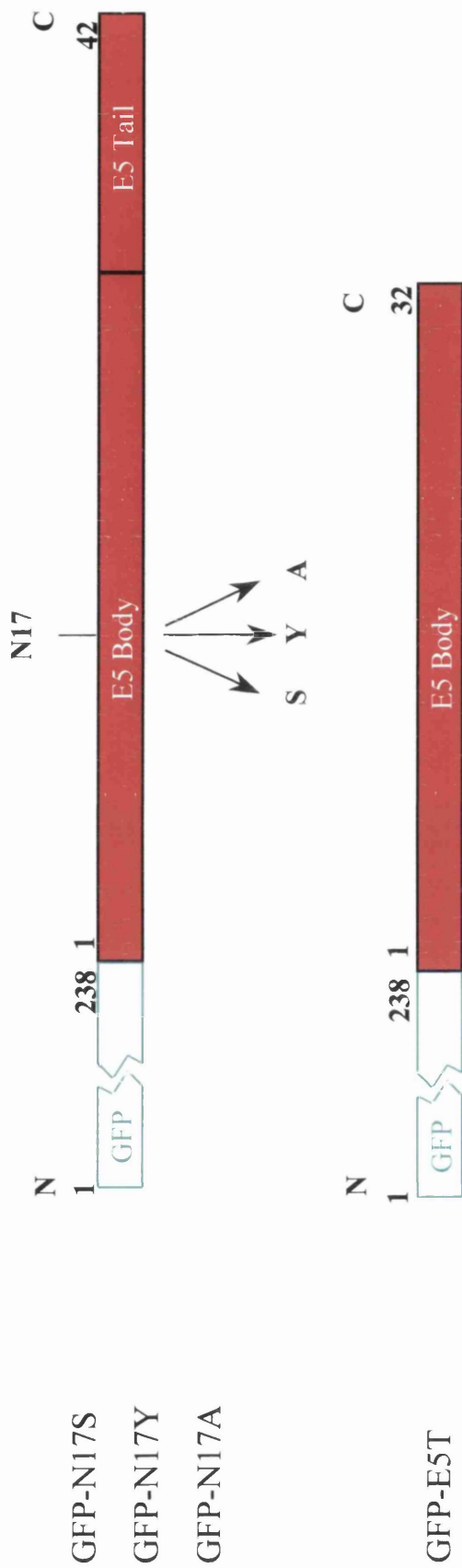


Figure 6.2 Structure of mutant forms of E5 fused with GFP

Figure 6.3 Confocal images of GFP-E5, GFP-N17S, GFP-N17Y, GFP-N17A and GFP-E5T

COS-7 cells were transfected with 0.1 µg of DNA, fixed with 3% paraformaldehyde and visualised at the confocal microscope 24 hr after transfection.

Figure 6.3

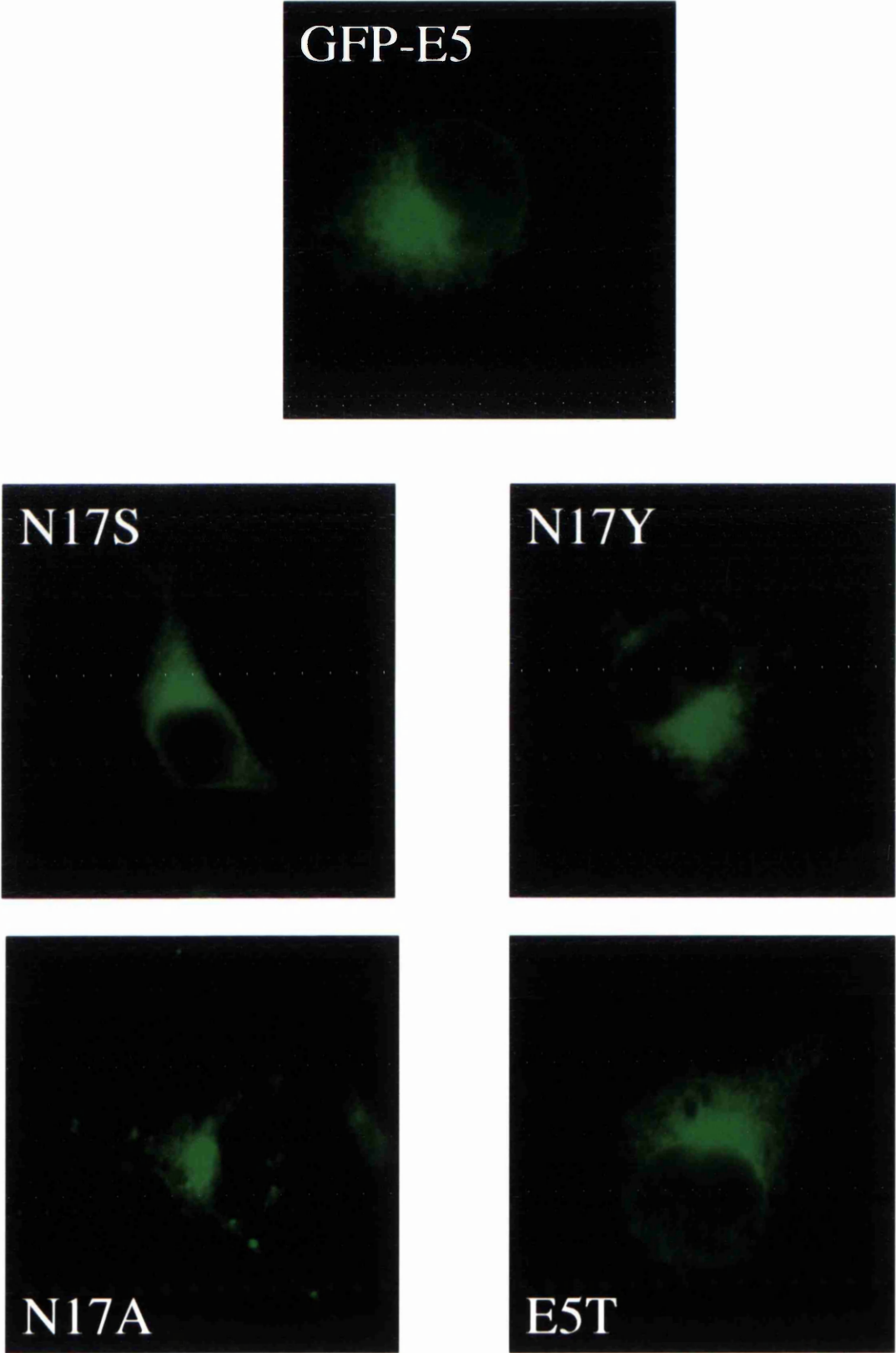


Figure 6.4 a, b Colocalisation studies with GFP-N17S, GFP-N17Y, GFP-N17A and GFP-E5T

COS-7 transiently expressing GFP fusion mutants were stained with an ER marker (anti PDI antibody) or a Golgi marker (BODIPY-TR ceramide) (black and white pictures). Pictures were captured with a 60X objective lens. Coloured pictures were magnified further in Microsoft Powerpoint. They present the result of the merge between the green and red channel, yellow colour indicates colocalisation.

Figure 6.4 a

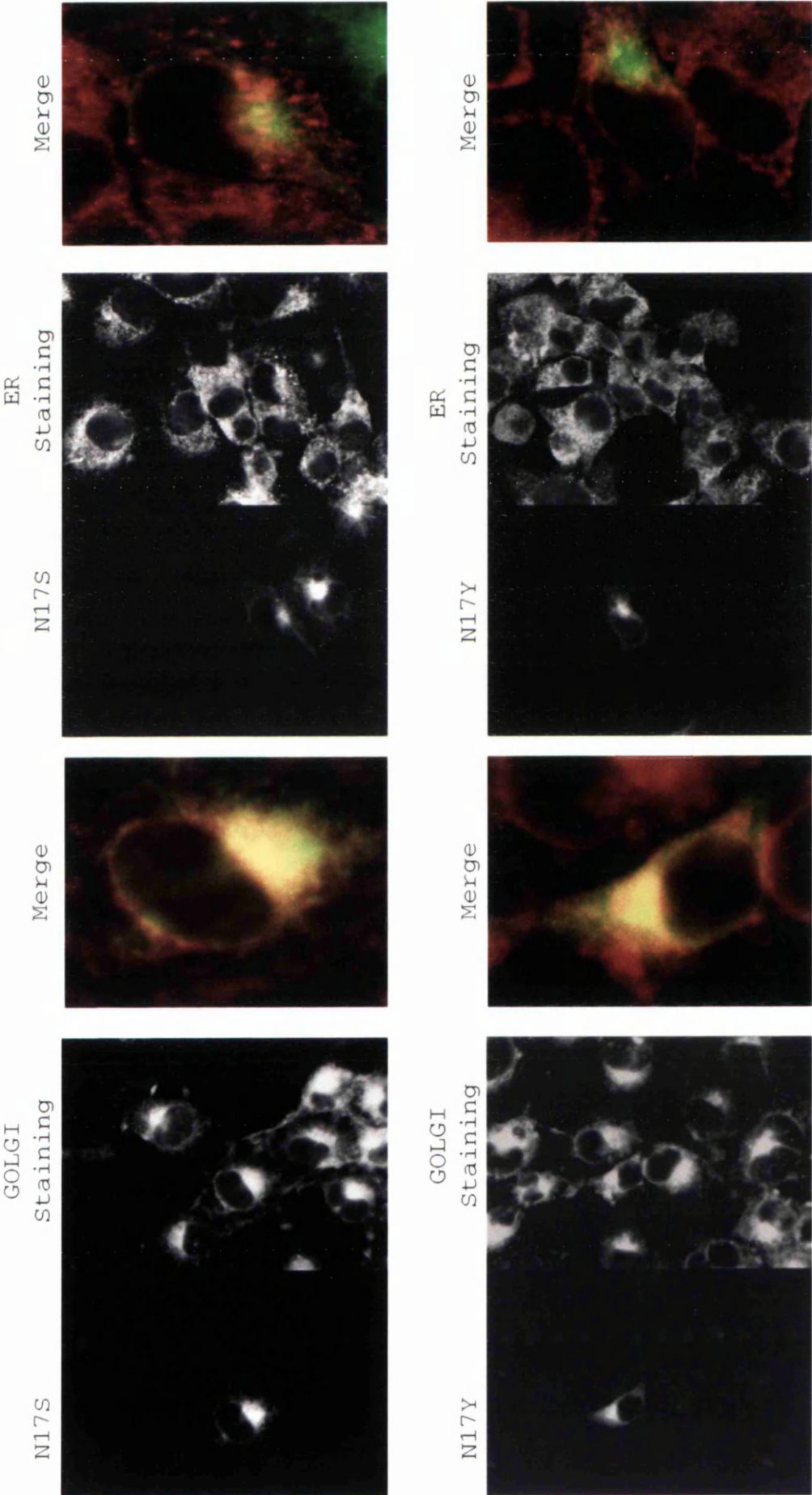
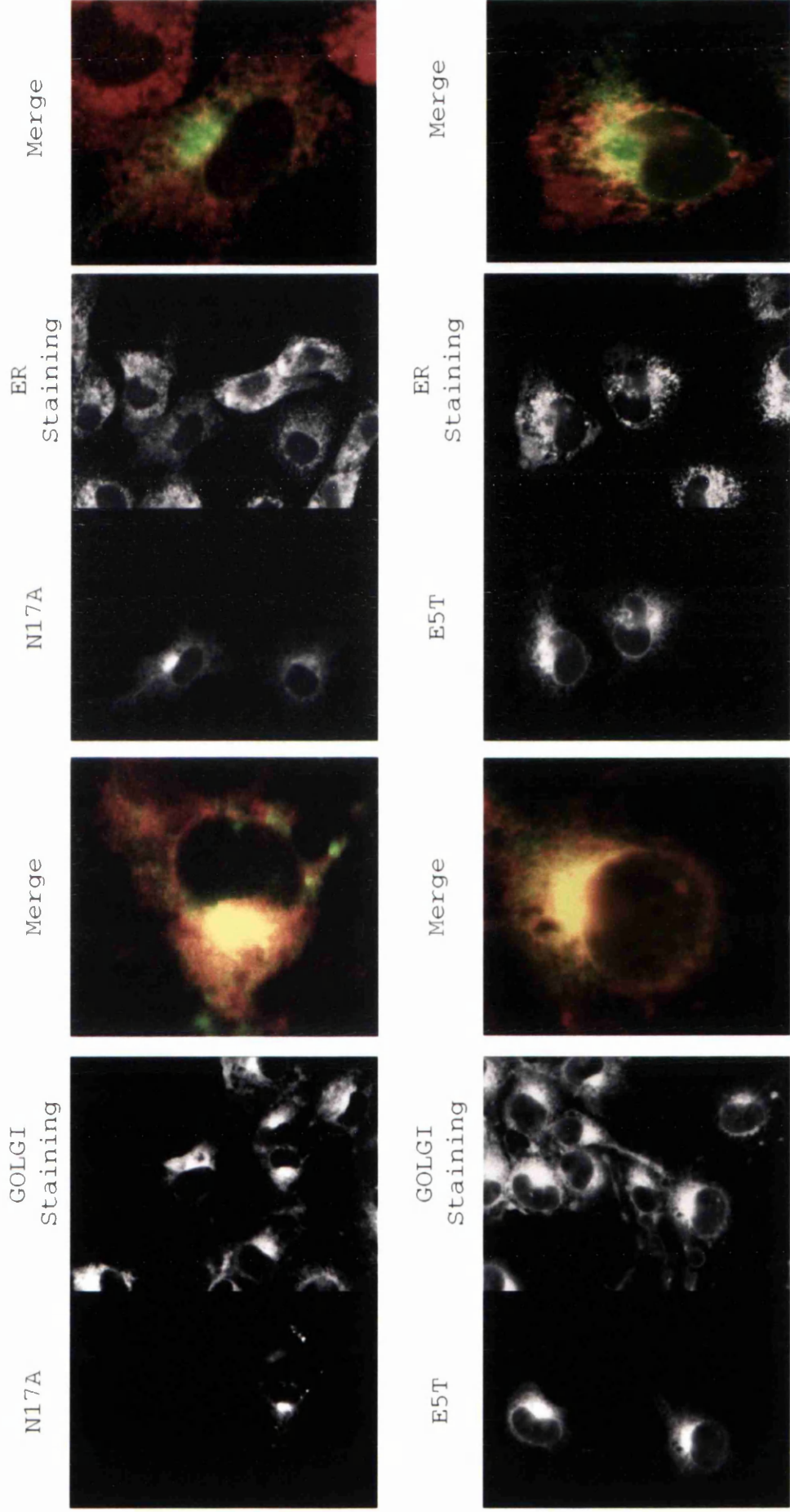


Figure 6.4 b



6.2.2 Detection of protein expression of GFP-E5 mutants in stable transformants

NIH3T3 cells stably expressing GFP-E5 mutants fused with GFP were produced in order to investigate the long-term expression of the fusion proteins. For each transfection class two 90 mm tissue culture dishes of 0.5×10^6 NIH3T3 cells were prepared. Cells were transfected with 10 μ g of each plasmids using the calcium phosphate method (section 2.2.2.6). After 21-28 days of G418 selection the resistant colonies were pooled together and expanded for further analysis. As control, NIH3T3 expressing pEGFPC3 vector were produced and included in the studies.

Total protein extracts (100 μ g) from the pooled population of GFP, GFP-E5, GFP-N17S, GFP-N17Y, GFP-N17A and GFP-E5T were subjected to western blot analysis as described in section 2.2.5. Lysates were fractionated by SDS-PAGE (10% polyacrylamide gel), transferred to a nitrocellulose membrane and probed with an anti-GFP antibody (Babco).

The results (Figure 6.5) demonstrate that E5 and its mutants are all expressed in NIH3T3, although at somewhat different levels. As previously demonstrated in PalFs (O'Brien et al., 1999), E5 wild type and its hypertransforming mutants (N17A) are expressed at lower level than non transforming mutants. Interestingly single point mutations in E5 hydrophobic body influence the protein electrophoretic mobility; similar observations were obtained when mutants were translated *in vitro* in the presence of microsomes and immunoprecipitated with an anti-HA monoclonal antibody (Ashrafi et al., 2000).

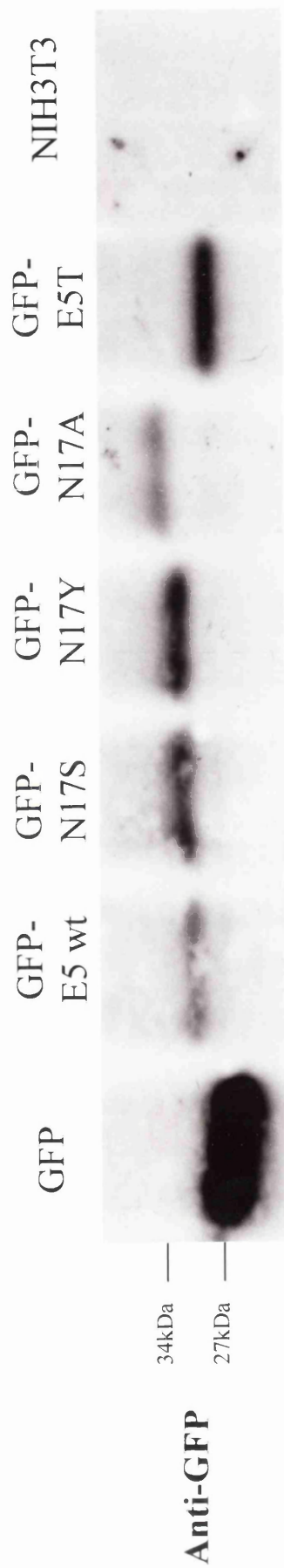


Figure 6.5 Detection of GFP-E5 mutants protein expression in NIH3T3

Western Blot analysis of GFP-E5 wt and mutants expression in NIH3T3 stable transformants; 100 µg of whole cell extracts were fractionated in SDS-PAGE (10% polyacrylamide) and the membrane was probed with anti-GFP antibody (1:1000). Untransfected NIH-3T3 were used as control

6.2.3 Transformation studies with mutant forms of E5

During our investigations we have shown that BPV-4 E5 does not fully maintain its transforming ability when fused with GFP (Chapter 4 and 5). Consequently we decided not to use GFP fusion forms of E5 mutants for transformation studies in NIH3T3. Instead we carried out further investigations using HA-tagged E5 wt and mutants expressed in pZipneo vector system. These same constructs have been used previously for transformation studies in PalF cells (Ashrafi, 1998; O'Brien et al., 1999) and E5 wt and E5T have been also tested in NIH3T3 cells (O'Brien and Campo, 1998). Transient transfection experiments were performed first and NIH3T3 stable transformants were derived later.

6.2.3.1 Transactivation of the cyclin A promoter

NIH3T3 fibroblasts (2×10^5) were seeded in a six well plate. The following day cells were transfected with 1 μg of luciferase reporter plasmid for the human cyclin a promoter (ptw929) and 2 μg of E5 wt and its mutants using calcium phosphate method. One plate was used for each transfection class (Table 6.1). After 16-18 hr, precipitates were washed off with 2 ml of PBS and cells incubated in low serum (LS; 0.5%) or growth serum (GM; 10%) medium for further 24 hr. Luciferase activity was determined using a microplate luminometer TR717.

Table 6.1 Short-term transfection classes

2 μg pZipneo + 1 μg luciferase reporter plasmid
2 μg pZipneo E5 + 1 μg luciferase reporter plasmid
2 μg pZipneo N17A + 1 μg luciferase reporter plasmid
2 μg pZipneo E5T + 1 μg luciferase reporter plasmid
2 μg pZipneo A15G-A20G + 1 μg luciferase reporter plasmid
2 μg pZipneo A15P-A20P + 1 μg luciferase reporter plasmid

All constructs are HA tagged; each transfection class was performed twice

Under normal growth conditions BPV-4 E5 wild type expression led to at least a 9 fold increase in cyclin A promoter activity when compared to control cells transfected with pZipneo empty vector (Figure 6.6 top panel). Among the E5

mutants, N17A showed higher transactivation ability than the wild type (40 fold the control cells; Figure 6.6 bottom panel), while A15G-A20G has a promoter transactivation comparable to the wild type. In cells transfected with E5T and A15P-A20P the promoter was transactivated up to 5 times (Figure 6.6 bottom and middle panel). When cells were maintained in low serum, E5 wild type, N17A and A15G-A20G show a promoter activity 5-6 times higher than the control, while E5T and A15P-A20P show a lower level of transactivation, less than 2 fold, compared to control cells (Figure 6.6).

These results are in concordance with what previously shown for E5 wild type and E5T in NIH3T3 and PalF cells (O'Brien and Campo, 1998; O'Brien et al., 1999) and for N17A, A15G-A20G and A15P-A20P in PalFs (Ashrafi, 1998; O'Brien et al., 1999). In these cell types the different level of cyclin A promoter transactivation correlated with different transforming phenotypes (O'Brien and Campo, 1998; O'Brien et al., 1999). To test if this correlation held for E5 mutants expressed in NIH3T3, stable murine fibroblasts expressing E5 mutant forms were derived.

Table 6.2 Summering of cyclin A promoter transactivation in transient transfectants

Transient-transfection classes	Cyclin A promoter transactivation	
	GM	LS
pZipneo	-	-
BPV-4 E5 wt	+++	+++
E5 N17A	+++	+
E5T	+	-
A15G-A20G	++	+
A15P-A20P	+	-

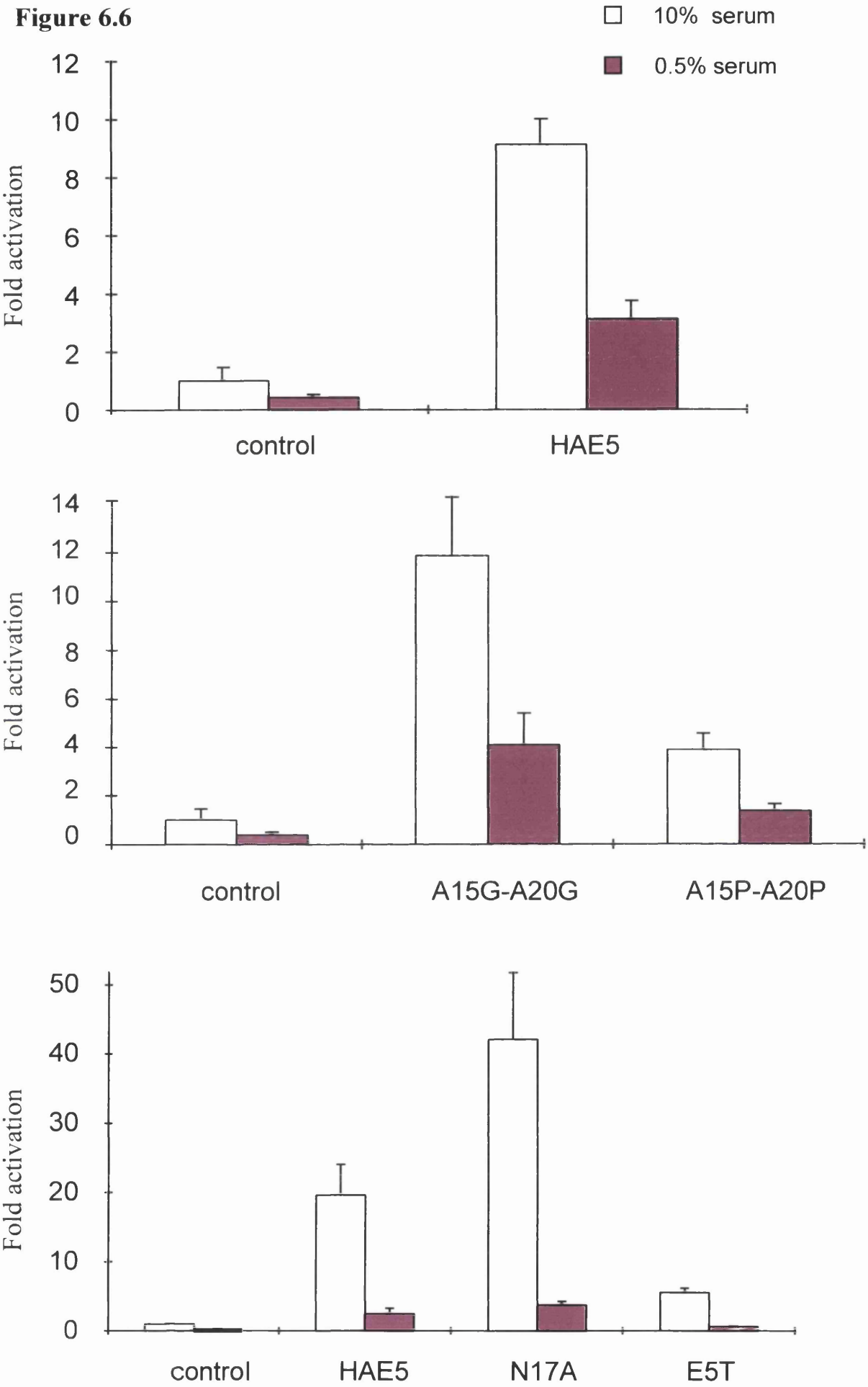
6.2.3.2 E5 mutants stable transformants

NIH3T3 cells were seeded at 0.5×10^6 in a 90 mm tissue culture dish the day before transfection, two dishes for each transfection class (Table 6.2). Cells were transfected with 10 µg of the appropriated plasmids by using the calcium phosphate method as detailed in section 2.2.2.6. After 21-28 days of drug selection, with medium containing 500 µg/ml of G418, resistant colonies were pooled together and population expanded. (Cell lines expressing pZipneo empty vector, E5 wild type, N17S, N17Y, N17A and E5T mutant have been produced by Dr. Vincent O'Brien,

Figure 6.6 Trans-activation of the human cyclin A promoter by E5 wt and its mutants

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 value from triplicate samples are given for a representative experiment. The reading obtained with control cells (NIH3T3 transiently transfected with pZipneo empty vector) was set at 1, and the luciferase reading of the samples were normalised to the control reading. Standard deviation (bar) was calculated for each transfection class. The experiments were performed at least twice.

Figure 6.6



cell lines expressing E5/KDEL and E5/GGEV mutants have been produced by myself).

Table 6.3 Stable transfection classes

pZipneo
pZipneo E5
pZipneo N17S
pZipneo N17Y
PZipneo N17A
pZipneo E5T
pZipneo E5/KDEL
pZipneo E5/GGEV

N.B. All constructs are HA tagged

6.2.3.3 Detection of mutant transcripts by RT-PCR in stable transformants

There are no good antibodies against E5 protein and the detection of the HA tagged protein by western blot using an anti-HA antibody has never been successful thus the cell lines were analysed for E5 transcripts by RT-PCR (2.2.2.9).

Total cellular RNA was isolated using RNazol extraction as detailed in section 2.2.2.9. 1 µg of RNA was used for RT-PCR using a Perkin Elmer Kit and 0.15 µM of appropriated primers (Table 2.1). As control for DNA contamination (all samples had been treated with DNase) an identical reaction mix was prepared for each sample excluding reverse transcriptase. To verify that the products obtained had the correct length a PCR reaction was performed with pZipneoHAE5 plasmid as template. Finally, to check the reaction parameters and the quality of the RNA, parallel mixes were set up utilising the RNA samples with primers specific for actin mRNA.

The detection of the actin transcripts in all cell populations (Figure 6.7 a, b) and the absence of products in reactions without reverse transcriptase confirmed the success of the RNA extraction and DNase treatment procedures. In all transfectant classes with the exclusion, as expected, of the pZipneo cells, there were detectable products, which indicate that the E5 and its mutant genes were transcribed (Figure 6.7 a, b).

Figure 6.7 a, b Detection of E5 wt, N17S, E5T, E5/KDEL, E5/GGEV, N17Y and N17A transcripts by RT-PCR

Upper panels: RT-PCR was performed using 1 µg of total RNA and 0.15 µM of specific primers for E5 wt and its mutants; pZipHAE5 (plasmid) was used as template to confirm the correct length of the transcripts; a parallel reaction was performed using a mix minus MULV reverse transcriptase (-RT) to check for genomic DNA contamination. RT-PCR products of ~ 185 bp were detected for E5 wt, the N17S, N17Y, N17S and E5T and these co-migrated with the plasmid control; because the addition of 18 bp at the 3' end of E5 ORF, E5/KDEL and E5/GGEV amplicons showed a different electrophoretic mobility.

The lower panels show the products obtained from parallel mixes set up utilising the RNA samples (0.1 µg) with primers specific for actin mRNA (245 bp) to check the reaction parameters and the quality of the RNA. Also these reactions were performed plus (+RT) and minus (-RT) reverse transcriptase step.

All mutants were sequence verified.

Figure 6.7 a

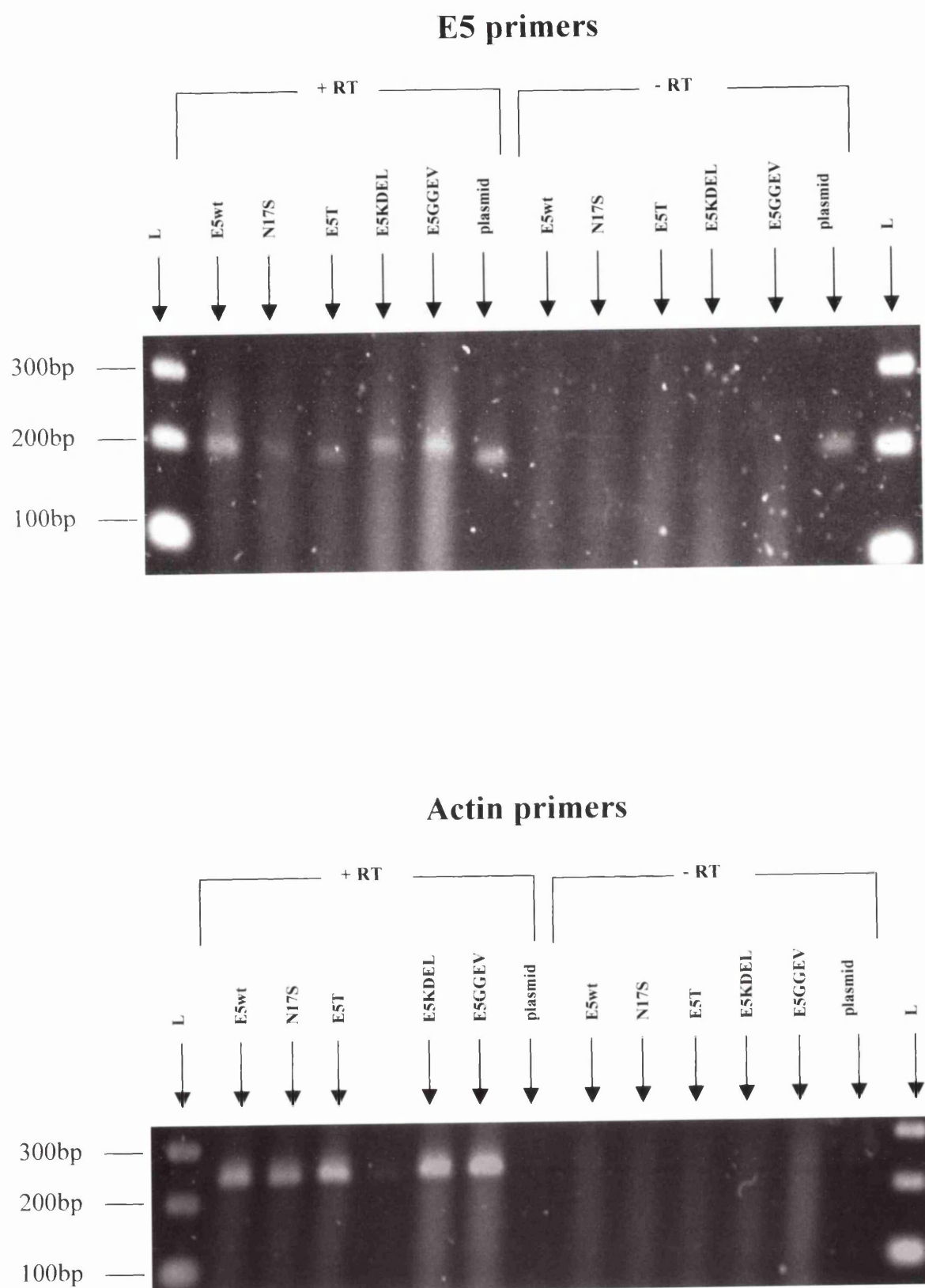
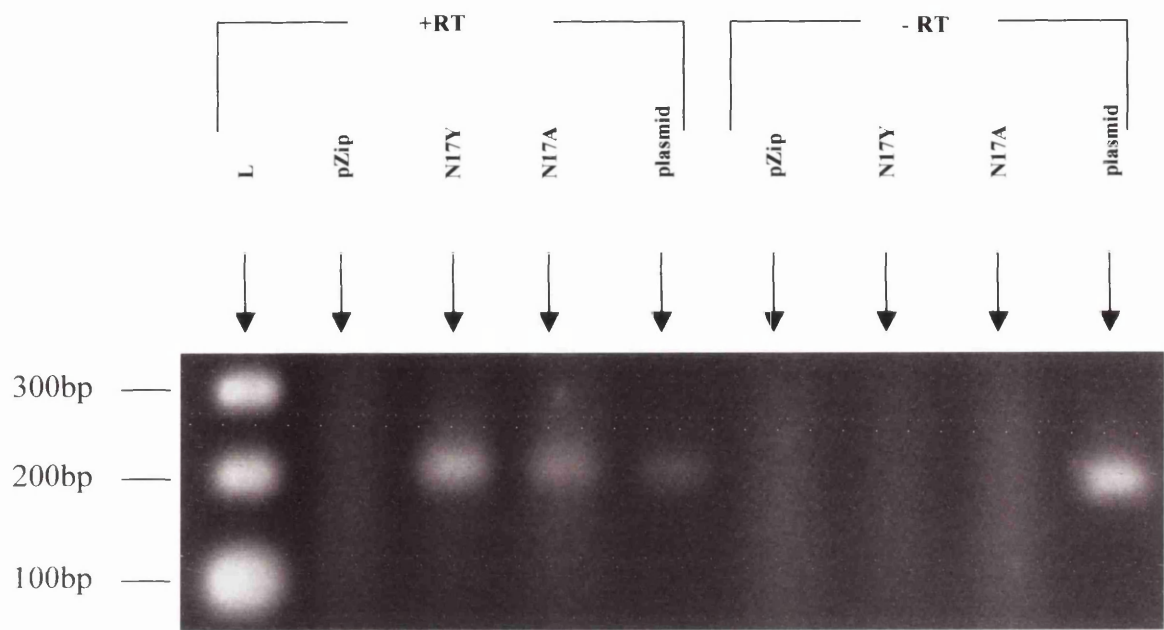
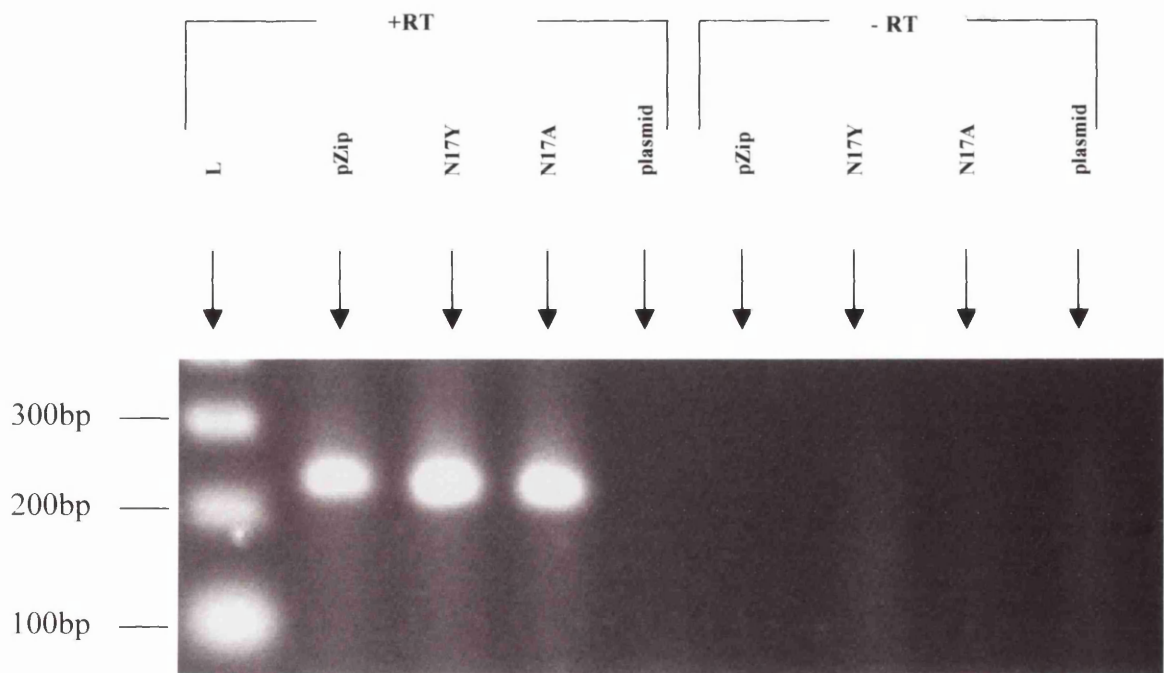


Figure 6.7 b

E5 primers



Actin primers



6.3 Conclusions

Studies using GFP tagged forms of E5 mutants demonstrate that all E5 mutants analysed so far share with the wild type E5 their cellular location: the Golgi apparatus. Therefore a different cellular location cannot be the reason for the different transforming abilities among the mutants. Moreover, the fact that E5 wt and its partially transforming, non transforming and hypertransforming forms were all in the Golgi apparatus suggests that this cellular location may be necessary but is not sufficient for E5 transforming ability (see Chapter 7). It is interesting to note that the modification of the asparagine in position 17 to serine, tyrosine or alanine in the E5 hydrophobic body, does not interfere with the Golgi location; the hydrophilic tail is not required for the Golgi location and the first 32 amino acids of E5 encode sufficient information to locate the GFP-E5T mutant to this cellular compartment. In addition, as observed in Chapter 3, the disruption of the α -helix does not interfere with the Golgi location of E5.

In NIH3T3 cells, E5 mutants show a similar profile of cyclin A promoter transactivation as seen in primary bovine fibroblasts. Moreover the promoter activation correlates with transformation ability (O'Brien and Campo, 1998). The study of the transformed phenotype of NIH3T3 stably expressing N17S, N17Y, N17A, E5/KDEL and E5/GGEV would add important information to our understanding of E5. Unfortunately this part of the project could not be completed because of unforeseen problems and lack of time.

CHAPTER 7: DISCUSSION

7.1 Introduction

The human papillomaviruses (HPVs) are the most important aetiological agents of cervical squamous carcinoma and HPV-16 and HPV-18 have recently been declared as human carcinogens by the International Agency for Research on Cancer (IARC). It is clearly a major research interest to investigate how the virus (specifically its transforming proteins) interferes with its host to induce carcinogenesis. However many technical obstacles have made the studies difficult. The infection of human beings with HPV for experimentation is obviously unethical and HPVs do not reproduce or induce cancers when not in their natural host. Moreover, these viruses are not easy to culture *in vitro*. Although these difficulties have been partially overcome with the development of new technical approaches (i.e. rafts culture), the best alternative to investigate the virus properties remains the use of animal papillomaviruses. The Bovine Papillomavirus type 4 (BPV-4), in particular, is a valuable model for HPV-associated carcinogenesis. Like HPVs, BPV-4 is involved in cancer development in its own host, providing the opportunity to study carcinogenesis in a natural system. Moreover, BPV-4 infection is limited to the mucous epithelia of the alimentary tract and this tissue specificity is shared with HPVs. Finally, for BPV-4, the progression of papillomas to cancers has been experimentally reproduced.

One of the two transforming proteins of BPV-4 is E5, a small, 42 amino acids long, hydrophobic protein. E5 is localised to the endomembrane compartments of primary bovine fibroblasts (Pennie et al., 1993) and is expressed in the basal and suprabasal layers of early papillomas *in vivo* (Anderson et al., 1997). The small dimension, the high hydrophobicity and the membrane location are features shared with the members of the E5 family proteins from animal and human papillomaviruses.

E5s appear to transform cells by enhancing activation of the intrinsic tyrosine kinase activity of growth-factor receptors, interfering with the cell cycle machinery and disrupting pH homeostasis in endomembranes.

BPV-1 E5 transforms cells by activating the platelet-derived growth factor β receptor (PDGF β -R) in a ligand dependent fashion; it induces receptor dimerisation and activation, trans-phosphorylation and recruitment of cellular signalling proteins to the receptor (Petti et al., 1991; Lai et al., 1998; Klein et al., 1998). HPV-16 E5 affects the metabolism of EGF-R. Cells expressing HPV-16 E5 display elevated levels of EGF-R on their surface (Straight et al., 1993; Straight et al., 1995; Crusius et al., 1998), and a sustained activation of the mitogen activated protein kinase (MAPK) cascade in response to EGF treatment (Gu and Matlashewski, 1995; Ghai et al., 1996; Crusius et al., 1997). BPV-4 E5 transformation is correlated with transactivation of the cyclin A promoter, up-regulation of cyclin D and cyclin A expression and associated kinase activities and, unusually, with over-expression of the cyclin dependent kinase inhibitor p27 (O'Brien and Campo, 1998; O'Brien et al., 2001).

All E5s bind to the highly conserved 16 kDa subunit c/ductin (16K) a component of the vacuolar proton ATPase (V-H⁺-ATPase) and of the gap junction (Goldstein et al., 1991; Finbow et al., 1991; Conrad et al., 1993; Faccini et al., 1996). This physical interaction may impair V-H⁺-ATPase function and be responsible for the lack of acidification of endosomes and Golgi apparatus (Straight et al., 1995; Schapiro et al., 2000). Finally BPV-1 E5, BPV-4 E5 and HPV-16 E5 down-regulate gap junction intercellular communications (Oelze et al., 1995; Faccini et al., 1996; Ashrafi et al., 2000)

Understanding the mode of action of E5 proteins will help clarify viral transformation and replication mechanisms, cellular signal transduction pathways and organelle functions. This Thesis aims to contribute to these investigations expanding the information on BPV-4 E5 transforming mechanisms with regard to:

- (i) The importance of the subcellular location of E5 for its transforming activities.
- (ii) The signal pathways utilised by E5 for cell transformation.

7.2 Cellular localisation of BPV-4 E5

The hydrophobic nature of BPV-4 E5 has rendered its purification extremely difficult because the protein is insoluble. Consequently antibody reagents directed against E5s are limited and not very efficient. In the attempt to obtain an efficient

detection system for routinely visualising E5, two forms of the viral protein fused with the green fluorescence protein (GFP) were constructed (GFP-E5 and E5-GFP).

GFP-E5 is mainly localised in the Golgi apparatus and only partially present in endoplasmic reticulum (ER) (Figures 3.2 and 3.4). These results are in agreement with BPV-4 E5 subcellular location in transiently transfected PalFs (Pennie et al., 1993) and consistent with the intracellular location of BPV-1 E5 and HPV E5s (Burkhardt et al., 1989; Conrad et al., 1993; Sparkowski et al., 1995; Schapiro et al., 2000).

BPV-4 E5 has been subjected to a genetic analysis that revealed the crucial role of specific amino acids in cell transformation. Mutation of the asparagine (N) residue in position 17 to serine (N17S), tyrosine (N17Y) or alanine (N17A) produced forms of BPV-4 E5 partially transforming, non transforming and hypertransforming respectively; a truncated form of E5 (E5T), lacking the 12 hydrophilic amino acids, was transformation defective (O'Brien and Campo, 1998; O'Brien et al., 1999).

Our investigation on the cellular location of these different mutants revealed that they all localise mainly in the Golgi apparatus and apparently their distribution does not differ from wild type (Figures 6.3 and 6.4 a, b). These results show that the difference in transformation activities of the mutants is not dependent on their different cellular localisation, suggesting the conclusion that the presence in the Golgi apparatus may be necessary but is not sufficient for cell transformation. Moreover, a single modification in the hydrophobic body does not influence the cell location of the protein. Likewise all BPV-1 E5 mutants at position 17 have been localised in the Golgi compartment, confirming that mutations in the transmembrane domains are usually tolerated for Golgi location. Moreover the first 30 amino acids of BPV-4 E5 enclose the information required to target the protein to the Golgi, as the mutant lacking the last 12 amino acids still localises in this organelle.

The BPV-4 E5 mutant A15P-A20P also localises in the Golgi, therefore changing the alanine residues at position 15 and 20 to a proline, which has the property of forcing a bend in the amino acid chain and of disrupting the α -helix, does not interfere with the protein cellular location. These results suggest that an intact secondary structure is not required to localise E5 to the lipids bilayers and the high hydrophobicity of the N-terminus is sufficient to target the viral protein to endomembranes. Alternative it is possible that the introduction of the proline residues has not affected the α -helix conformation (Figure 3.7).

The importance of the subcellular location and its relation to viral transformation ability has been thoroughly investigated in the case of BPV-1 E5. The use of a form of BPV-1 E5 bearing a KDEL retrieval signal (section 3.3.2) demonstrated that the Golgi location is required for BPV-1 E5 transforming abilities (Sparkowski et al., 1995). BPV-1 E5/KDEL was expressed in the ER and could not induce focus formation in mouse fibroblasts (Sparkowski et al., 1995). The authors hypothesised that the E5/KDEL-PDGFR complexes, although still detectable, were sequestered in the ER where the growth factor receptor was unable to transmit a mitogenic signal. On the contrary, the PDGFR in complex with the wild type form of E5 could be activated in the Golgi apparatus or move, with the viral protein, to the plasma membrane (Chapter 1). Although necessary, the presence in the Golgi apparatus is not the only requisite for cell transformation as demonstrated by mutants of BPV-1 E5 still located in the Golgi but unable to induce cell transformation.

The addition of the KDEL signal to the C-terminus of BPV-4 E5 has failed to retain the viral protein in the ER (Figure 3.6). There could be several reasons for why GFP-E5/KDEL is still localised mainly in the Golgi compartment: (i) it is known that proteins bearing the KDEL amino acids sequence although resident in the ER can yet slip out of it, they are in fact retrieved from Golgi in a sorting process that returns them to the ER. It is therefore possible that an over expression of ligands causes a saturation of the retrograde mechanism and allows protein to escape the control point and move towards the Golgi compartment; (ii) BPV-4 E5 could be a type I transmembrane protein with a cytoplasmic C-terminus. This means that the KDEL sequence is not facing the endomembranes lumen and it cannot be recognised by the KDEL-receptors, which reside in the ER-Golgi intermediate compartment. The last model is not in agreement with what previously hypothesised by O'Brien & Campo (1998) and need further experimental supports. It is unlikely that GFP contributes to the Golgi location of GFP-E5/KDEL. The use of GFP to tag transmembrane proteins (type I or type II) is widespread and GFP does not interfere with the protein assembly, or transport within the cell organelles (Girotti and Banting, 1996; Simonova et al., 1999); see below.

Further findings corroborate the importance of the Golgi location for BPV-4 E5 and BPV-1 E5: a perfect correlation has been found between the ability of BPV-1

E5 to induce Golgi alkalinisation and to transform cells (Schapiro et al., 2000). These results suggest that the disruption of the normal activity of the vacuolar ATPase in the acidification of endomembrane compartments plays a role in the oncogenic ability of BPV-1 E5. It is still unknown whether BPV-4 E5 can bind to Growth Factor Receptors (GFRs); however, it can complex, *in vitro*, with 16K protein (Faccini et al., 1996). Moreover, NIH3T3 expressing BPV-4 E5 show alkalinisation of the Golgi compartment (Schlegel, personal communications) pointing to the interaction with the V-H⁺-ATPase activity as a possible transforming mechanism also for BPV-4 E5.

Recently it has been shown that when expressed in fibroblasts (PalFs and NIH3T3) BPV-4 E5 induces down-regulation of the surface MHC I (histocompatibility complex class I) (Ashrafi et al., 2002). Although the specific mechanisms is unknown, it is possible that the transport of the MHC I to the surface is inhibited because of the Golgi location of E5 and/or the consequent Golgi alkalinisation.

Although all hypotheses of how the presence in the Golgi compartment could contribute to cell transformation by E5 are still under investigation, it is clear that this is an extremely strategic location to control or regulate the transport/processing of itinerant proteins and lipids, and to influence various cellular processes either by direct interaction with targeted proteins or indirectly by modifying the organelle environment (alkalinisation) (Shields and Arvan, 1999).

Specific amino acid motifs and/or conformation of particular proteins determine their destination to various cellular compartments. In the absence of such targeting information, a protein that is translocated into the ER follows a 'default' pathway, that leads, in animal cells, to the expression of membrane proteins on the cell surface. Surprisingly GFP-E5 could not be detected on the plasma membrane (section 3.3.4). However, the amino acid sequence of E5 does not present a known targeting motif for the ER or Golgi. It is known that a retention system for the Golgi compartment exist (Machamer, 1993; Munro, 1998), however unlike the ER localisation signals, which are usually exposed to soluble factors and are involved mainly in protein-protein interaction, Golgi localisation involves lipid-protein interaction as well as protein-protein interactions (Machamer, 1993). One proposed model for Golgi retention correlates the length of the transmembrane domains with the progressive enrichment of cholesterol in the plasma membrane over that in the

ER (Bretscher and Munro, 1993). If cholesterol was gradually concentrated in transport vesicles throughout the secretory pathway, a cholesterol gradient would exist. Plasma membrane proteins usually have longer transmembrane segments and would be more likely to associate with thicker membranes and be transported forward, whereas Golgi-resident proteins, which have shorter transmembrane domain on average, would be excluded from the transport vesicles and thus be retained in the Golgi. This mechanism, which is non-saturable and independent on the exact sequence of transmembrane domain, could explain the retention of BPV-4 E5 in the Golgi compartment.

Similarly to BPV-4 E5, other members of E5 family are located principally within subcellular compartments (Burkhardt et al., 1989; Conrad et al., 1993) and appear at the cell surface only when overexpressed, although this aspect has not been extensively investigated. It has been hypothesised that, at least in the case of BPV-1 E5, the progression to the cell surface may not be required for cell transformation. BPV-1 E5 could, for example, activate PDGF β -R from the Golgi apparatus, without necessarily moving to the plasma membrane. There are contradictory reports in literature on the ability of GFRs to signal from an intracellular compartment. In some studies the *v-sis* oncogene has been shown to transform cells through an autocrine activation of PDGF β -R; in these studies the receptor activation occurs in intracellular compartments, before receptor maturation is complete and before the receptor has reached the cell surface (Keating and Williams, 1988). Others, however, demonstrated that *v-sis*/PDGF-R interaction could not signal functionally from the proximal Golgi apparatus (Fleming et al., 1989) leaving the subject an open matter.

The influence of the GFP tag in the retention of E5 in the Golgi could not be definitely excluded, however it could be considered unlikely for different reasons. First, both GFP-E5 and E5-GFP proteins localised in the Golgi apparatus, suggesting that E5 itself is the target sequence for this organelle. Second, we used a fragment (PEP; 50 amino acids) of the rabbit neutral endopeptidase 24.11 (Simonova et al., 1999), that presents similar size to BPV-4 E5, fused with GFP. We demonstrated that GFP does not interfere with PEP trafficking through cell compartments despite the difference in size between the two proteins (Figure 3.10). We conclude that it is unlikely that GFP affects BPV-4 E5 cellular location.

7.3 Cell transformation by BPV-4 E5

The decision to divide and progress through the cell cycle requires the integration of extracellular and intracellular signals (see below). The environmental signals are the presence/absence of particular growth factors and of the extracellular matrix (ECM). Growth factors and ECM proteins stimulate signal-transduction pathways by binding to their receptors, typically tyrosine kinase receptors (RTKs) and integrins. Ultimately these pathways transduce information to the cell cycle machinery.

In normal cells the presence of growth factors and the attachment to substrate are necessary for cell division, in contrast tumour cell proliferation is generally independent of both adhesion and growth factors. Indeed, many or most oncogenes, such as v-Ras or simian virus 40 (SV40) Large T, induce cells transformation that leads to anchorage and serum-independent proliferation.

Accordingly NIH3T3 cells expressing BPV-4 E5 do not exit the cell cycle after serum withdrawal and proliferate when maintained in suspension. However, two mutants of the viral protein, N17S and the chimera 1E5N-4E5C (Chapter 6), induce anchorage-independent growth in PalFs but do not allow cell proliferation in absence of mitogens, suggesting the existence of two independent mechanisms by which E5 confers these phenotypes. Here we show for the first time that the segregation of the two phenotypes occurs also in NIH3T3. GFP-E5 cells can grow in suspension culture (Figure 4.6), demonstrating that the sole expression of GFP-E5 is sufficient to transform NIH3T3, as judged by growth in suspension. Thus GFP-E5 can override the control mechanisms that arrests anchorage-dependent cells in late G1 phase when maintained in suspension (O'Brien and Campo, 1998; O'Brien et al., 1999). However anchorage-independent, 3T3-GFP-E5 are not able to proliferate in low serum (Figures 4.4 and 4.5) demonstrating that the addition of GFP at the N-terminus of E5 disturbs the ability of the wild type protein to maintain cell cycle progression in the absence of mitogens (O'Brien and Campo, 1998; O'Brien et al., 1999). GFP-E5 is a fundamental tool to study how this segregation occurs in NIH3T3 cells, a system that allows studies of the viral protein transforming mechanisms without the influence of other viral oncoproteins. Moreover, contrary to N17S or 1E5N-4E5C mutants, it is possible to verify GFP-E5 protein expression through

direct visualisation by confocal/fluorescence microscopy and, for the first time, by western blotting detection (Figure 4.2).

7.4 BPV-4 E5 and the cell cycle

The progression through the cell cycle is dependent on phosphorylation of key regulatory proteins by cyclin dependent kinases (CDKs), which in turn are regulated in a complex fashion by association with cyclins, phosphorylation and dephosphorylation and binding to CDK inhibitors (CKIs) (Sherr and Roberts, 1995). One of the most important targets of cyclin/CDK phosphorylation activity is the retinoblastoma protein (pRb). pRb phosphorylation releases the transcription factors E2F, and this event, at the mid-G1-phase, determines the cell irreversible commitment to cell division (Chapter 1 and Chapter 5).

DNA viruses are dependent on their host replicative machinery and must induce cells to enter the S phase to create the environment necessary to viral DNA synthesis. The ability of adenovirus, papillomavirus, SV40, and other viruses to extend the proliferative capacity of their host cells is a direct result of expression of viral proteins that override growth-suppressive signals that control cell cycle progression. Adenovirus E1A, SV40 Large T and HPV E7 can all form complexes with pRb and cause the release of E2F, bypassing the requirement for cyclin D-associated kinase activity (Munger et al., 1989b; Wang et al., 1993; DeCaprio et al., 1988); moreover, adenovirus E1B, SV40 Large T and HPV E6 interact and inactivate p53 protein (Zantema et al., 1985; Werness et al., 1990; Funk and Galloway, 1998). Clearly these three different viruses have adopted very similar strategies to de-regulate cell growth.

It has emerged recently that tumour suppressors are not the only victims of viral opportunism and that both CKIs and cyclins are vulnerable to disruption by several viral gene products (reviewed in Swanton and Jones, 2001). HPV E7 affects, by direct binding, the activity of p27 and p21, leading to an increase in CDK2-associated kinase activity (Zerfass-Thome et al., 1996; Jones et al., 1997; Funk et al., 1997). In NIH3T3 cells, E7 leads to constitutive expression of cyclin E and cyclin A proteins and their associated kinase activity (Zerfass et al., 1995). In addition, E7 induces anchorage-independent growth via activation of cyclin A expression and prevention of the inhibition of cyclin E dependent kinase activity (Schulze et al., 1998).

BPV-4 E5 influences the cell cycle progression via up-regulation of cyclin A (O'Brien and Campo, 1998; O'Brien et al., 1999). E5 expression in NIH3T3 cells promotes transcription of a heterologous cyclin A promoter, increases the expression of endogenous cyclin A protein and elevates the cyclin A-associated kinase activity in growth conditions where cyclin A expression is diminished in control cells (O'Brien and Campo, 1998). In PalF cells E5 expression promotes transcriptional transactivation of a heterologous cyclin A promoter in all growth conditions tested (Ashrafi, 1998; O'Brien et al., 1999). A strong relationship exists between cyclin A promoter transactivation and cell transformation: mutants of E5 able to transactivate cyclin A promoter confer to cells the ability to grow in suspension and mutants that strongly transactivate the promoter permit growth in low serum.

This relationship is maintained in GFP-E5 cells: proliferation of GFP-E5 cells in normal serum or in suspension is always accompanied by increased cyclin A promoter activity and conversely the lack of cyclin A promoter transactivation is associated with the failure of the cells to proliferate in low serum (Figures 5.3 and 5.9). Moreover, cyclin A expression and associated kinase activity present the same pattern, being elevated in suspension and down-regulated in low serum (Chapter 5). These observations support the conclusion that E5-mediated cell transformation is due to the ability of the viral protein to de-regulate cyclin A expression and associated kinase activity.

During the cell cycle, cyclin A gene expression is strictly regulated at the level of transcription: the protein is expressed at the G1/S transition and through the S phase (Henglein et al., 1994). It has been shown that the adhesion requirement is likely to reflect a cell cycle check-point in late G1 phase of the cell cycle (Guadagno and Assoian, 1991). Cells arrested by suspension fail to produce cyclin A, however ectopic expression of cyclin A enables the cells to bypass the adhesion requirement, implicating cyclin A as the major target of cell cycle control by anchorage-signalling pathway (Guadagno et al., 1993). In agreement with these observations also in the case of E5 transformed cells it is the up-regulation of cyclin A that mediates their ability to grow in suspension.

In NIH3T3 cells the expression of cyclin D1 and cyclin E is anchorage-independent, however, their associated kinases are inactivated, mainly by the inhibiting activity of p27 (Schulze et al., 1996). As a consequence pRb is not

hyperphosphorylated/inactivated, E2F is not released and cyclin A is not expressed. In E5 and GFP-E5 transformed cells the situation is different. Although cyclin D1 and cyclin E are expressed in suspension, no hyperphosphorylated forms of pRb could be detected, suggesting that the cyclins/CDKs complexes are not active. However, a form of p107 with a different electrophoretic mobility, probably due to protein phosphorylation, is observed in E5 and GFP-E5 cells. Cyclin E/CDK2 kinase activity is only slightly higher than in control cells and probably not sufficient to induce pRb deactivation, but nevertheless cyclin A is up-regulated. Therefore cyclin A expression and associated kinase activity are independent from cyclin D1-associated kinase activity and pRb deactivation (Chapter 5). It is known that pocket proteins regulate cyclin A expression but their exact mechanisms remains to be elucidated (Schulze et al., 1995; Philips et al., 1998). Some reports indicate p107/E2F complexes as the principal effectors (Schulze et al., 1995; Zerfass-Thome et al., 1997) and exclude the requirement of an inactivated pRb (Henglein et al., 1994). However, experiments with pRb-null and p107-null mouse embryo fibroblasts emphasize that pRb is important for cyclin A gene expression (Philips et al., 1998; Philips et al., 1999). In agreement with the former model, p107/E2F complexes in E5 transformed cells could regulate cyclin A expression.

The independence of cyclin A expression from cyclin D1 is supported by the data obtained using MAPK inhibitors. The mitogens-activated protein kinase (MAPK) cascade, Ras-Raf-MEK-ERK, is a well characterised response to both receptor tyrosine kinase (activated by growth factors) and integrins activation (by ECM) (Danen and Yamada, 2001). Several studies have established a link between ERK activation and cell proliferation: expression of dominant negative ERK or antisense ERK inhibits proliferation of fibroblasts (Pages et al., 1993). Activation of MEK/ERK pathways leads to the induction of cyclin D1 mRNA and the use of a MEK inhibitor (PD98059) or a dominant negative ERK, results in decreased expression of cyclin D1 mRNA and protein (Weber et al., 1997; Balmain and Cook, 1999). In E5 cells cyclin D1 expression is independent from MAPK activation: in suspension cyclin D1 expression level is unaltered after the addition of MEK inhibitors (Figure 5.13). Moreover, cyclin A expression is independent from MAPK activity and cyclin D1 expression. In E5 cells not only the normal sequential activation of cyclins is altered but the transduction cascade that links the extracellular

signals to intracellular signals is dispensable. In our knowledge this is the first report of ERK 1/2-independent cyclin A expression.

It has been previously hypothesised that in E5 cells, cultured in adhesion, the principal cyclin-dependent kinase companion of cyclin A is CDK2 (O'Brien and Campo, 1998). In suspension, no CDK2 could be detected in complex with cyclin A in E5 and GFP-E5 cells, and only cdc2 could be precipitated with cyclin A. In normal cells cyclin A/cdc2 kinase activity is required for passage through G2 and enter in mitosis (Pagano et al., 1992). At this moment it is not known if, in E5 transformed cells, cyclin A/cdc2 complexes are active also in S phase or only in G2/M phase.

In light of these results it is important to establish which cyclin-dependent kinase is associated with cyclin A in adhesion culture.

BPV-4 E5 transformation is accompanied by a surprisingly up-regulation of p27 in all condition tested (O'Brien and Campo, 1998), although the cells continue to proliferate. In adherent E5 cells, the up-regulated and active cyclin D1/CDK complexes sequester p27 and prevent its repressing activity on CDK2 activity (O'Brien et al., 2001). In suspension p27 is associated mainly with cyclin E/CDK2 complexes and to a lesser extent with cyclin A/cdc2 (Figure 5.12). This difference could explain why the kinase activity of cyclin A is higher than the activity of cyclin E. GFP-E5 cells do not present an up-regulation of p27. Both cyclin E/CDK2 and cyclin A/cdc2 complexes are equally associated with p27 although they show a difference in kinase activity (Figure 5.12). p27 acts in G0 and early G1 to inhibit G1 cyclins/CDKs, with the primary target being cyclin E/CDK2 complexes (Slingerland and Pagano, 2000), it is possible therefore that p27 is not active as an inhibitor of cyclin A/cdc2 complexes in G2 and M phases of the cell cycle. A recent report, however, shows that p21 (a CKI structurally related to p27) contributes to the regulation of the G2/M transition (Niculescu et al., 1998). It is known that cyclin/CDK complexes must overcome an inhibitory threshold of p27 expression level to become catalytically active (Polyak et al., 1994). It is therefore possible that in GFP-E5 cells the stoichiometric balance between p27 and cyclin A/cdc2 complexes is in favour of the latter, allowing cell cycle progression.

7.5 Conclusions

Work with GFP-E5 reveals that E5 has at least two transforming mechanisms: 1) via sustained activation of cyclin D1 and cyclin D1-associated kinase activity and 2) via up-regulation of cyclin A and cyclin A-associated kinase activity. Activation of cyclin A and expression of cyclin D1 are mitogens and adhesion-independent. However, the kinase activation of cyclin D1 appears to be dependent on adhesion to substratum. Previous studies have already revealed that in E5 cells cyclin E protein level and associated kinase activity are not up-regulated (O'Brien and Campo, 1998), suggesting an alternative way to bring about transcriptional activation of cyclin A gene. Here we show that ERK 1/2 activation and cyclin D1 are dispensable for cyclin A expression at least in suspension culture. The cyclin A-associated activity detected in transformed cells appears to be associated mainly, if not exclusively, with cdc2 (Chapter 5).

We propose that the up-regulation of cyclin A and associated kinase activity (mainly cdc2) is the fundamental requisite for anchorage-independent growth of E5 transformed cells. This activation occurs independently from MAPK pathway and cyclin D1 expression and activation. The state of the cell cycle components in E5 transformed cells is summarised in Tables 7.1, 7.2, 7.3.

It has been shown recently that another viral oncoprotein, BPV-1 E5, transforms NIH3T3 cells without inducing ERK phosphorylation (Suprynowicz et al., 2000), although the interaction and activation of PDGF β -R is considered the principal transforming pathway of the viral protein (Chapter 1), (Petti et al., 1991; Petti and DiMaio, 1994; Goldstein et al., 1994). This suggests that downstream effectors of growth factors, other than the MAPK, are responsible for cell cycle progression in E5 transformed cells. Interestingly, mutants of BPV-1 E5, not able to induce PDGF β -R transphosphorylation, can sustain phosphorylation of PI3-K and induce anchorage-independent growth, but not proliferation in low serum (Suprynowicz et al., 2000). These results suggest a link between PI3-K activation and promotion of anchorage-independent growth. Moreover, inducible expression of a constitutively active PI3-K in rat embryo fibroblasts promotes anchorage-independent growth but does not eliminate the growth factor requirements (Klippel et al., 1998). In light of this observation, PI3-K pathway becomes a potential candidate to explain the ability BPV-4 E5 cells to grow in suspension independently from the

MAPK pathway. PI3-K phosphorylates the 3'-OH position of the inositol ring of inositol phospholipids, activating a cascade of events that leads to the phosphorylation of various targets such as Protein Kinase B (PKB/AKT) and to the activation of the Rho family of small G-proteins (including CDC42, Rac, and Rho). PKB/AKT, a serine/threonine kinase, is involved in cell survival, through a putative downstream target p70S6K required for G1 progression in a variety of cells (Chung et al., 1994). The Rho family has pleiotropic functions: control actin cytoskeleton, regulate stress-activated Map kinase p38 and JNKs and activation of transcription factors (reviewed in Cantrell, 2001). Moreover, constitutive activation of FAK, CDC42 and Rho leads to cell proliferation that is anchorage-independent but growth factor-dependent (Schwartz, 1997).

It has been shown that mitogens and the cytoskeleton play different roles in cyclin A expression. Mitogens are required to phosphorylate and activate CREB, an transcription activator of cyclin A promoter (Figure 7.1), but disruption of cytoskeleton does not influence this step. However, phosphorylation occurs well before promoter activity is detected, thus a second “stimulating” step, sensitive also to cytoskeleton disruption, is required to time cyclin A expression (Bottazzi et al., 2001). It is possible that the requirement of mitogens in GFP-E5 cells reflects the need to free cyclin A promoter from the repressor, however the second event may occur independently from the state of the cytoskeleton.

Considering all the above observation we propose the following model for E5 transformation. The Golgi is a critical location for controlling membrane trafficking and cell signalling. E5 targets key regulators of signalling complexes that assemble and/or are *en route* through the organelle. The Golgi could become the new decisional centre, freeing the cells from extracellular constraints (nutrients and adhesion) and leading to cell transformation.

7.6 Future prospective

More data are required to validate the model proposed. It is important to define what pathways, other than MAPK, are de-regulated by E5 and determine the ones involved in cell transformation. As mentioned above PI3-K is considered to be the first candidate. Using a PI3-K specific inhibitor (LY 294002) it will be possible to verify the role of PI3-K in the anchorage- and mitogens-independent cell progression of E5

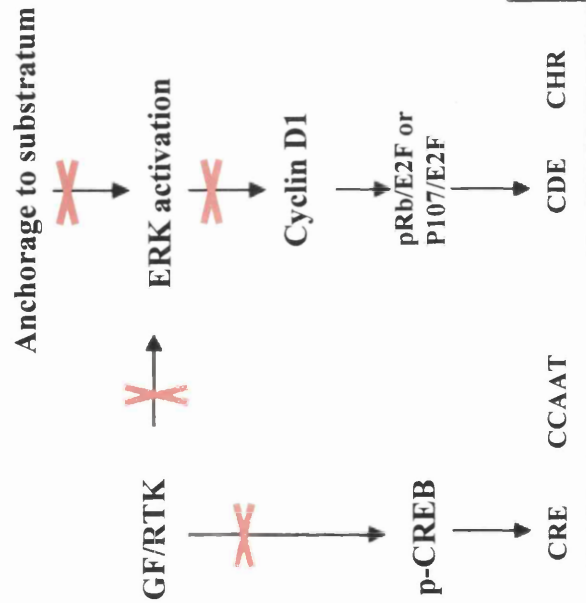
expressing cells. This will elucidate the pathway/s responsible for Golgi-nucleus communication. Moreover, it is necessary to clarify how the sequential events of the cell cycle have been deranged by E5. Further investigations are required to prove the independence of cyclin A expression from pocket protein deactivation. The use of a dominant negative form of pRb could be extremely informative. This form of pRb presents mutated CDKs phosphorylation sites and it cannot be inactivated by hyperphosphorylation (Zhang et al., 2000). It would be interesting to express this form of pRb in E5 cells and to analyse their cell cycle profile.

The surprising observation that only cyclin A/cdc2 complexes could be detected in E5 cells needs to be confirmed. Moreover the principal CDK companion of cyclin A in adherent E5 cells needs to be determined.

Here we have established that residence of E5 in the Golgi apparatus is not the only requirement for cell transformation. Moreover, this subcellular location has not yet been proved necessary for BPV-4 E5 transforming activities. It will be important to create a form of E5 that does not localise in the Golgi and to study its effect on cell transformation. A fusion form of GFP-E5 with the fragment of the rabbit neutral endopeptidase (PEP) could be a good candidate. In addition, the effect of transforming and non-transforming E5 mutants on Golgi alkalinisation will help to clarify the association between this event and cell transformation by E5.

Finally, GFP-E5 can be used to detect proteins that interact with BPV-4 E5. This has been difficult to pursue until now, due to the absence of good antibodies against E5.

E5 wt cells



GFP-E5 cells

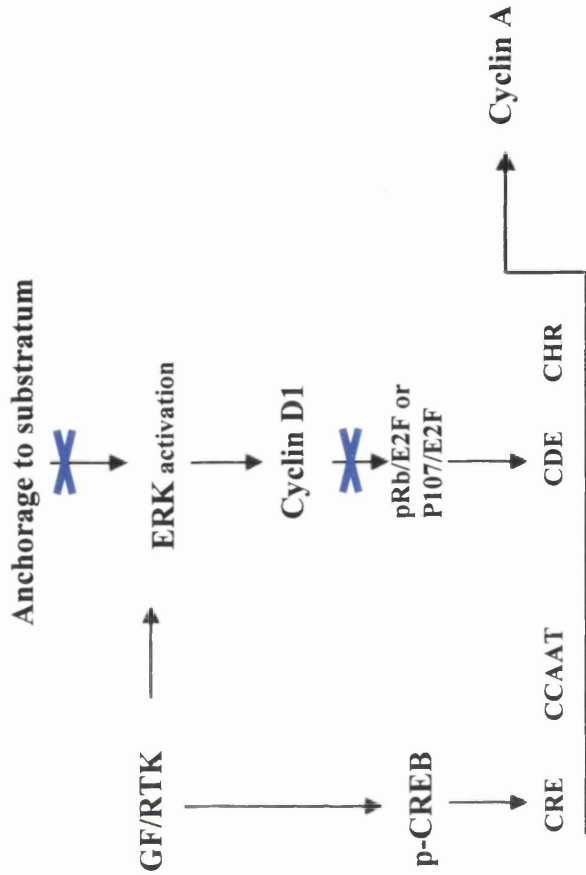


Figure 7.1 Possible mechanisms for the induction of Cyclin A expression by E5 wt and GFP-E5.

Activation of a receptor tyrosine kinase (RTK) by growth factor (GF) leads to the phosphorylation of cyclin-AMP (c-AMP) responsive element binding protein (CREB) in early G1 phase. Receptor tyrosine kinase also cooperate with the organised actin cytoskeleton to sustain ERK activity and to regulate cyclin D1 expression, pocket proteins phosphorylation and occupancy of the cell cycle-dependent element (CDE) and the cell cycle homology region (CHR). In E5 cells (left panel) cyclin D1 expression and possibly phosphorylation of CREB are independent from GF. In suspension cyclin D1 is still sustain independently from ERK activation and potentially still active. In GFP-E5 cells GF are required to induce CREB phosphorylation while occupancy of CDE/CHR might be independent from adhesion to substratum (modified from Bottazzi et al., 2001).

Table 7.1 Expression level and associated kinase activity of cyclins in cells cultured in 0.5% serum

	Cyclin D1		Cyclin E		Cyclin A	
	Express	Kinase	Express	Kinase	Express	Kinase
Z3T3	↓	↓	↓*	↓*	↓	↓
E5	↑	↑	↓*	↓*	↑	↑
GFP	↓	↓	N/D	N/D	↓	↓
GFP-E5	↓	↓	N/D	N/D	↓	↓

Table 7.2 Expression level and associated kinase activity of cyclins in cells cultured in suspension

	Cyclin D1		Cyclin E		Cyclin A	
	Express	Kinase	Express	Kinase	Express	Kinase
Z3T3	↓	N/D	=	=	↓	↓
E5	↑	N/D	=	=	↑	↑
GFP	↓	N/D	=	=	↓	↓
GFP-E5	↑	N/D	=	=	↑	↑

Table 7.3 Expression level and associated kinase activity of cyclins in cells cultured in suspension after treatment with MAPK inhibitors

	Cyclin D1		Cyclin E		Cyclin A	
	Express	Kinase	Express	Kinase	Express	Kinase
Z3T3	↓	N/D	N/D	N/D	↓	N/D
E5	↑	N/D	N/D	N/D	↑	N/D
GFP	↓	N/D	N/D	N/D	↓	N/D
GFP-E5	↓	N/D	N/D	N/D	↑	N/D

↓ down-regulated

↑ up-regulated

= no or little change

N/D =not determined

* (O'Brien and Campo, 1998)

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