The role of p53 in cell transformation by BPV-4.

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ABSTRACT

Bovine papillomavirus-type 4 is associated with benign papillomas which can progress to carcinoma in cattle grazing on bracken fern. Bracken is known to contain immunosuppressants and cocarcinogens which can act as cofactors and these are known to be required for progression *in vivo*. One of the best characterised of these cofactors is the mutagen quercetin. BPV-4 has been shown to be capable of partially transforming primary bovine fibroblasts (PalF) cells *in vitro* in the presence of an activated *ras* and when these cells are additionally treated with quercetin they become tumourigenic in nude mice.

The transforming functions of BPV-4 are encoded by the E7 and the E8 ORF's. BPV-4 is unusual in that it does not possess an E6 ORF. E6 has been shown to be one of the major transforming oncoproteins of the human papillomaviruses and is known to bind and degrade the tumour suppressor protein p53. This abrogation of the p53 protein is known to contribute to tumour progression by the high risk papillomaviruses such as HPV-16 and 18. As BPV-4 does not encode E6 functions, this would suggest that if p53 dysfunction is important in BPV-4 cell transformation, it may occur by alteration of the cellular gene.

In order to look at possible p53 mutations in alimentary cancer of cattle, a 14kb genomic clone containing wild type p53 sequence was isolated from a bovine liver genomic DNA library and sequenced. This clone was used to localise bovine p53 to chromosome 19q15 which is known to be syntenic with human chromosome 17 and mouse chromosome 11 both of which harbour the p53 gene.

Immunocytochemical analysis of BPV-4 derived lesions demonstrated that elevated levels of p53 protein were detectable in papillomas but not in carcinomas. BPV-4 E7 was found to be present at all stages of papillomatosis as was BPV-4 viral DNA as detected by *in-situ* hybridisation. Neither viral DNA nor BPV-4 E7 were detected in normal palatine tissue or in carcinomas which is coincident with the loss of viral DNA previously reported for tumours *in vivo*. The elevated levels of p53 in some of the papillomas were shown to be a result of the presence of mutations in p53 exon 7 as determined by SSCP-PCR and sequencing. Mutant p53 was also detected in two metastases of upper alimentary canal tumours.

The addition of mutant p53 to PalF cells *in vitro* in the presence of BPV-4, *ras* and HPV-16E6 caused these cells to become tumourigenic in nude mice. In these tumorigenic PalF cell lines, mRNA expression of the BPV-4 viral oncoprotein E8 was shown to be elevated. Cotransfected mutant p53 mRNA expression was also detectable. This would suggest that p53 mutation plays a role in tumourigenic progression by BPV-4 both *in vivo* and *in vitro*.

In this study we demonstrate that the lack of an E6 in BPV-4 may be compensated by the presence of a mutated p53 gene in BPV-4 induced carcinomas. These p53 mutations may arise due to cofactors present in bracken fern, and p53 mutations were detected in BPV-4 and *ras* transfected PalF cells that had been treated with quercetin. However, p53 mutations were detected in transfected PalF cells which had not been exposed to quercetin treatment. We also demonstrate that the additional functions provided by HPV-16 E6 (16E6) *in vitro* are at least in part, independent of the p53 binding and degradation function. The data suggests that an exogenous 16E6 can supplement *in vitro* other functions of BPV-4 which are responsible for progression *in vivo*. HPV-16 E6 was also demonstrated to possess a function confering anchorage independence, which mapped to the cysteines present in the zinc finger motifs of the protein.

This study has demonstrated BPV-4 lacks more than one function that can be provided by an exogeneous E6 oncoprotein and that these functions may be independent of p53. In addition, p53 mutations were shown to be present in BPV-4 induced papillomas and carcinomas.

'Everything in this book may be wrong'.

Richard Bach
Illusions: The story of the reluctant messiah.

This thesis is dedicated to Mum, Dad and Brian for their encouragement and support throughout, for putting up with me and to the animals for helping me keep my sanity. I would also like to thank Claire, Alison, Lesley, Marcia, Jane and Debbie who were always there for me and especially Susie for all the mucking out and wine.

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Abbreviations

AI	anchorage independence
APS	ammonium persulphate
BPV-4	Bovine papillomavirus type-4
BSA	Bovine serum albumin
CsCl	Caesium chloride
Ci	Curie
dATP	2' deoxyadenosine 5'-triphosphate
dCTP	2' deoxycytidine 5'-triphosphate
DIG	digoxigenin
DEPC	diethyl pyrocarbonate
DMBA	7,12-dimethylbenz(a)anthracene
DMEM	Dulbecco's modified Eagles medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleosidetriphosphates
E.coli	Eschericia coli
EDTA	EthyleneDiamineTetraAceticacid (sodium salt)
EtBr	Ethidium bromide
EtOH	Ethanol
FCS	Foetal calf serum
HBS	HEPES buffered saline
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HPV	Human papillomavirus
KAc	Potassium acetate
λ	bacteriophage lambda
LB	Luria-Bertani broth
LMP	Low melting point
M	Molar
MBq	Megabecquerels
MDE	Mutation Detection Enhancement
MEM	Minimal essential medium
mg	milligrams
µg	micrograms
$MgCl_2$	magnesium chloride
ml	millilitre

µl	microlitre
min	minute
mM	millimolar
µM	micromolar
MOPS	3-(N-Morpholino)Propanesulfonic Acid
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide;thiazol blue
NaAc	sodium acetate
NaCl	sodium chloride
ng	nanograms
NaOH	sodium hydroxide
^{o}C	degrees Centigrade
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PE	Phosphate buffered EDTA
Rb	Retinoblastoma protein
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
mRNA	messenger ribonucleic acid
rpm	revolutions per minute
sec	seconds
SSCP	Single Strand Conformational Polymorphism
Taq pol	*Thermus aquaticus* DNA polymerase
TBS	Tris-buffered saline
Temed	N,N,N',N'-tetramethylethylenediamine
TPA	12-*o*-tetradecanoyl-phorbol-13-acetate
U	units
v/v	volume per volume
V	volts
W	watts
w/v	weight per volume
%	percentage

Solutions

1xTE	10mM Tris.Cl, 1mM EDTA pH7.5
1xTBE	0.09M Tris-borate, 2mM EDTA
Stop buffer	0.05% bromophenol blue, 0.05% xylene cyanolFF, 20mM EDTA, 95% formamide
KAc	60ml 5M KAc, 11.5ml glacial acetic acid, 28.5ml distilled water
2xHBS	280mM NaCl, 50mM HEPES, 1.5mM Na_2HPO_4, pH to 7.1 with 0.5M NaOH. Filter sterilise with 0.4micron filter.
L-Broth	10g bactotryptone, 5g yeast extract, 10g NaCl, pH to 7.0 with NaOH make up to 1 litre.
Superbroth Solution A:	24g bactotryptone, 48g yeast extract, 10ml glycerol made up to 1800ml and autoclaved as 4x450ml aliquots.
Solution B:	62.5g K_2HPO_4(anhydrous), 3.8g KH_2PO_4(anhydrous) made up to 500ml and autoclaved. Mix 50ml of solution B with 450ml solution A before use.
10xMOPS	0.2M [3-(N-morpholino)propanesulfonic acid], 50mM NaAc, 10mM EDTA, pH to 7.0 with NaOH and make up to 1 litre with milliQ water.
20xSSC	3M NACl, 0.3M Tri-Na citrate
100xDenhardts	2% Ficoll 400, 2% polyvinylpyrrolidine, 2% BSA, filter sterilised and stored at -20°C
10xSM buffer	1M NaCl, 0.1M $MgSO_4$, 0.35M Tris.Cl

1xDMEM	100mls 10xDMEM, 10mls glutamine (200mM), 50mls 7.5%NaCO3, 10mls Na pyruvate, 1% penicillin and streptomycin, 10% FCS.
TBS	0.05M Tris.Cl, 0.15M NaCl
in-situ hybridisation mix	50% formamide, 5xDenhardts, 0.1%SDS, 50mM Tris.Cl pH7.4, 10mM EDTA, 3xSSC, 10% dextran sulphate and 250µg/ml denatured salmon sperm DNA.
Electrophoresis sample buffer (Northern blots)	750µl formamide 240µl formaldehyde 200µl 50% glycerol 150µl 10x MOPS 7µl Bromophenol blue

Single Letter Amino Acid Codes

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

CHAPTER ONE

INTRODUCTION

1.1 Papillomaviruses and Cancer

Cancer is a multistage process and requires many changes to take place in order for progression to occur. Most of these changes are a result of a combination of activation of cellular protooncogenes, inactivation of tumour suppressor genes, and environmental cofactors. This would suggest that cancer is a result of an acccumulation of a number of events gradually leading to malignancy (reviewed in Vogelstein and Kinzler, 1993). Several viruses have been identified which can also contribute to these processes in humans and other mammals and in this introduction I intend to first discuss papillomavirus infection involved in cancer processes, the role of the tumour suppressor gene p53 and then deal with papillomaviruses and their oncoproteins. Finally I will discuss the link between these and the aims of my project.

One of the best characterised of the oncogenic viruses are the papillomaviruses which are a member of the papovaviridae family. Papillomaviruses were first isolated from cottontail rabbits by Shope and Hurst in 1933 and they have been identified as the etiological agents of a variety of benign epithelial lesions commonly known as warts (Pfister, 1987). These lesions normally regress (Lutzner, 1985) but some are capable of undergoing malignant progression to squamous cell carcinoma, however, virus alone is not sufficient to induce progression. Epidemiological evidence has implicated many environmental agents as sources of cocarcinogens in papillomavirus associated carcinogenesis (Smith *et al*, 1987). The synergism between papillomaviruses and cofactors has been studied in rabbits (Rous and Freidewald, 1944), cattle (Campo and Jarrett, 1986) and EV (epidermodysplasia verruciformis) patients (Orth, 1987). These studies have demonstrated the importance of environmental carcinogens and immunosuppresants in aiding malignant transformation by the virus *in vivo*

(reviewed in Khare *et al*, 1995) and that papillomavirus infection is a key event in the development of neoplasia.

Papillomaviruses infect cells and replicate within differentiating keratinocytes. HPV infection usually results in a localised epithelial cell proliferation (Croissant *et al*, 1985) which will regress or persist. For certain HPV types further progression leads to premalignant events and can result in the development of cancer (Vousden, 1990). The link between papillomaviruses and differentiation makes them difficult to grow in conventional cell culture systems and the main problem with papillomavirus research is the inability to propagate the virus *in vitro*. Recent advances in *in vitro* HPV expression systems have provided powerful new methods for the study of viral mechanisms; these methods include the organotypic or raft culture system which mimics the differentiating epithelium that is the natural target tissue of the virus (Meyers and Laimins, 1992). The production of virus like particles (VLP) is also possible by exploiting the ability of the papillomavirus major capsid protein L1 to self assemble. Thus the study of viral attachment and entry into the cell is now feasible (Schiller and Roden, 1995).

Papillomavirus DNA is generally present as extrachromasomal episomes in benign lesions, however, in high grade lesions, viral DNA is frequently integrated with only the E6 and E7 open reading frames (ORF) remaining intact (Smotkin and Wettstein, 1986). Integration appears to be a non-specific event although it has occasionally been observed to occur in the vicinity of cellular oncogenes (Coutourier *et al*, 1991). This disruption of the regulatory papillomavirus genes is suspected to result in uncontrolled expression of E6 and E7 (Schwarz *et al*, 1985). In the case of some papillomavirus infections such as bovine papillomavirus type-4 (BPV-4), viral DNA is lost during the carcinogenic process and does not appear to be

required for maintenance of the transformed phenotype (Campo *et al*, 1985). This will be dealt with in greater detail later in this thesis.

There are 70 cloned and sequenced human papillomaviruses and the total number of human papillomavirus types is nearer 100 when the unclassified and incomplete viral data are included (Van Ranst *et al*, 1994). Generally they are classified as either cutaneous or mucosal viruses according to their tissue tropism and clinical manifestation although there are papillomaviruses which are capable of infecting both tissue types such as human papillomaviruses HPV-57, HPV-7 and HPV-2 (de Villiers, 1989; Van Ranst, 1993). Of all the papillomavirus types at least 27 cause anogenital infection with HPV-6, 11, 16, 18, 31, 33 and 35 being the most prevalent. These are then subdivided as high or low risk papillomavirus infections depending on the clinical outcome, i.e, HPV 6 and 11 are classed as low risk resulting in benign lesions such as condylomata acuminata, however, HPV-16 and 18 are high risk resulting in lesions which can progress to carcinoma (Vousden, 1989). Other differences between high and low risk will be discussed later with respect to viral oncoproteins and their interactions with other cellular proteins. The epidemiology of the papillomaviruses has been well studied but the molecular mechanisms of infection and progression are still being characterised. As many papillomavirus types have been implicated as the etiological agents of many cancers, it is important to determine the nature of papillomavirus infection and their transformation mechanisms in order to prevent or treat infection.

This thesis examines the role of p53 in BPV-4 associated carcinogenesis and before considering the viral contribution to the carcinogenic process, I will first discuss the role of p53 as a tumour suppressor.

1.2 The tumour suppressor p53

The p53 tumour suppressor gene has been shown to be the most commonly mutated locus in a wide range of human cancers (Hollstein *et al*, 1991). It was first identified as a cellular protein associated with SV40 large T antigen (Lane and Crawford, 1979) and originally thought to be a oncogene. However, p53 activity as a tumour suppressor became increasingly apparent, for example, wild type p53 can inhibit the *in vitro* transformation of primary rodent cells by a variety of oncogenes, wheras tumour derived mutants have lost this capacity (Eliyahu *et al*, 1989). The wild type protein is a potent transcriptional transactivator (Fields and Jang, 1990; Weintraub, 1991) which stimulates genes that negatively regulate cell growth and division, suppresses genes involved in cell proliferation (Ginsberg *et al*, 1991a) and is integral to a cellular checkpoint in response to DNA damage (Kuerbitz *et al*, 1992). A variety of genotoxic agents can induce an accumulation of the wild type p53 protein in the nucleus of mammalian cells which can lead to either growth arrest or to apoptosis (Yonish-Rouach *et al*, 1991) in a p53 dependent manner. p53 has also been implicated in cell differentiation (Shaulsky *et al*, 1991).

The human p53 gene is located on chromosome 17p13.1 and is 20 kilobases (kb) in length (Benchimol *et al*, 1985). The gene (see figure 1.1) itself contains 11 exons, the first of which is non-coding and separated from the rest by 10kb of intron (Lamb and Crawford, 1986). The human p53 promoter appears to lack either a TATA or a non-TATA initiated transcription site. However, the murine p53 promoter contains sequences commonly found in non-TATA box promoters such as a GC-rich region containing a Sp1 binding site. The processed human transcript is 2.5kb long and yields a 393 amino acid nuclear phosphoprotein with a molecular weight of 53 kilodaltons (kDa).

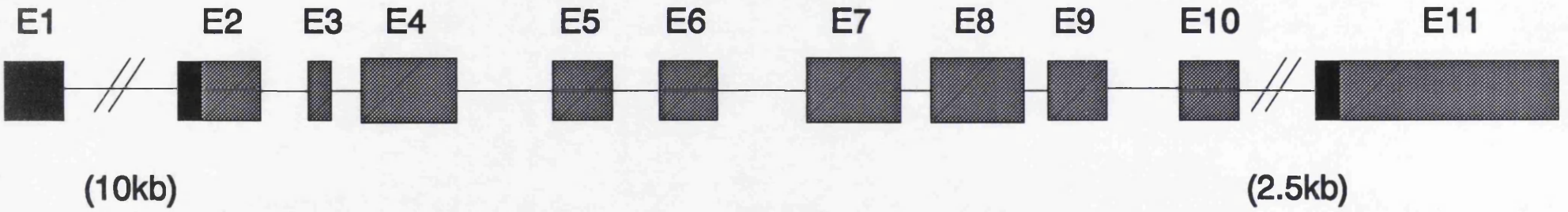


Figure 1.1 The human p53 gene. The coding exons are indicated by the hatched boxes, and the solid black boxes indicate those exons which are not translated.

p53 is present in almost all cell types although expression of the wild type protein is virtually undetectable. The half life of the wild type p53 protein in non-transformed cells is between 6 and 20 minutes but in transformed or tumour cells this is increased by 5-100 fold as the mutant proteins have increased stability (Ginsberg *et al*, 1991b). Many cellular proteins which form complexes with p53 and DNA damaging agents also increase the half-life of p53 and raise steady state levels to those which can be detected immunohistochemically (Matlashewski *et al*, 1986).

Generally in tumours, one p53 allelle is found in the mutant form and the second wild type allelle is rearranged or lost by deletion. Expression of mutant p53 protein is common and the mutant protein has been demonstrated to bind the wild type protein *in vitro* to form inactive aggregates. These p53 protein aggregates are usually dysfunctional although gain of function mutations have also been observed and some are able to enhance cell transformation although the mechanism is unclear. Dittmer *et al*, (1993) have shown that expression of murine or human mutant p53 in the absence of endogeneous p53 can confer enhanced tumourigenicity upon cells. These p53 mutants Ala143, His175, Trp248 and His273 were capable of activating transcription from the multi drug resistance gene (MDR-1) enhancer-promoter element in Saos-2 cells. These cells are derived from human osteosarcomas which lack endogeneous expression of wild type p53 (Dittmer *et al*, 1993). This transactivating ability is absent in the wild type p53 protein which represses the MDR-1 gene promoter thus the mutant proteins appear to predispose the cell to tumourigenicity by acquiring a new function in the absence of a wild type allele. This data would suggest that a total loss of p53 expression is not as detrimental as the presence of certain mutant p53 proteins which may provide the cell with some new function conferring growth advantage rather than just inactivation. It has been shown that in the majority of breast tumours, wild type p53 has been relegated to the cytoplasm and is

inactive (Moll *et al*, 1992). This may indicate the influence particular environmental carcinogens could have for specific tissues on the p53 gene or other cellular factors not yet found.

When a comparison is made between species, there exist five regions of 90-100% homology within the p53 protein. These regions are extensively conserved from Xenopus through to primates suggesting a role of biological importance (Soussi *et al*, 1990). Most mutations that occur in the p53 protein such as deletions, insertions, point and misense mutations have been mapped to these conserved domains and throughout the central DNA binding region of the protein. These highly mutated sites have been termed hotspots and correspond to residues which have been found to be mutated in a variety of human tumours. Mutations at residues 175 (CRIII), 248 (CRIV), and 273 (CRV) are more common, but do not appear to be associated with particular forms of cancer (Levine *et al*, 1991). It should be noted however that localisation of a mutation within the gene does not predetermine its effect.

As well as these somatic mutations, p53 germline mutations which are inherited have been identified which have been shown to increase the susceptibility of certain families to cancer (Srivastava *et al*, 1990). This condition is known as Li Fraumeni Syndrome (LFS) and these families develop a wide spectrum of multiple primary tumours and soft tissue sarcomas at an early age. Non affected individuals have two wild type p53 alleles while affected individuals acquire the wild type p53 and a mutant allele. In tumours that develop the wild type p53 allele is lost (Vogelstein, 1990; Srivastava *et al*, 1993).

The importance of p53 as a tumour supressor cannot be ignored as >60% of human tumours contain elevated levels of mutant p53 (Hollstein *et al*, 1991). Although p53 mutations occur in many cancers, other mechanisms

of p53 abrogation have been identified in tumours where the p53 protein is wild type, and these mechanisms will be discussed later.

The human p53 protein can be divided into three domains as illustrated in figure 1.2, (adapted from Deppert, 1994);

Firstly, the N-terminal domain is highly acidic and largely alpha-helical (Sturzbecher *et al*, 1992). AA 1-42 are required for transcriptional activation (Unger *et al*, 1992). TATA-box binding protein (TBP) binding and Adenovirus oncoprotein E1b p55 also bind in this region (Levine, 1990).

The central portion of the molecule is proline rich and highly hydrophobic. The highly conserved regions II to V are located in this region (Soussi *et al*, 1990) as is the SV40 large T antigen binding site and the p53 sequence specific DNA binding domain. The core portion of p53 (AA102-292) fold to a structural domain containing this sequence specific DNA binding function and Cho *et al* (1994) have crystallised this structure in the presence of a consensus DNA site (Cho *et al*, 1994). The prevalence of mutations are found in the central 200AA as shown in figure 1.2 suggesting that this region is a target site for p53 inactivation in tumours. The p53 binding site consists of four copies of the consensus 5'-PuPuPuC(A/T)-3' (El Diery *et al*, 1992).

Figure 1.3 illustrates part of the p53 protein sequence and the secondary structures of this region. The majority of conserved region IV is located in the L3 loop of the protein. The amino acids in loop 3 are involved in interactions with the minor groove of the DNA consensus site. Arg^{248} and Arg^{249} are among the most commonly mutated amino acids in the whole p53 protein (Hollstein *et al*, 1991) and 30% of p53 mutations are found in the L3 loop (Levine *et al*, 1994). This suggests the importance of this loop in protein-DNA interactions and that mutation in this region would lead to an inactivated protein. In general, mutations were found to be most frequent in

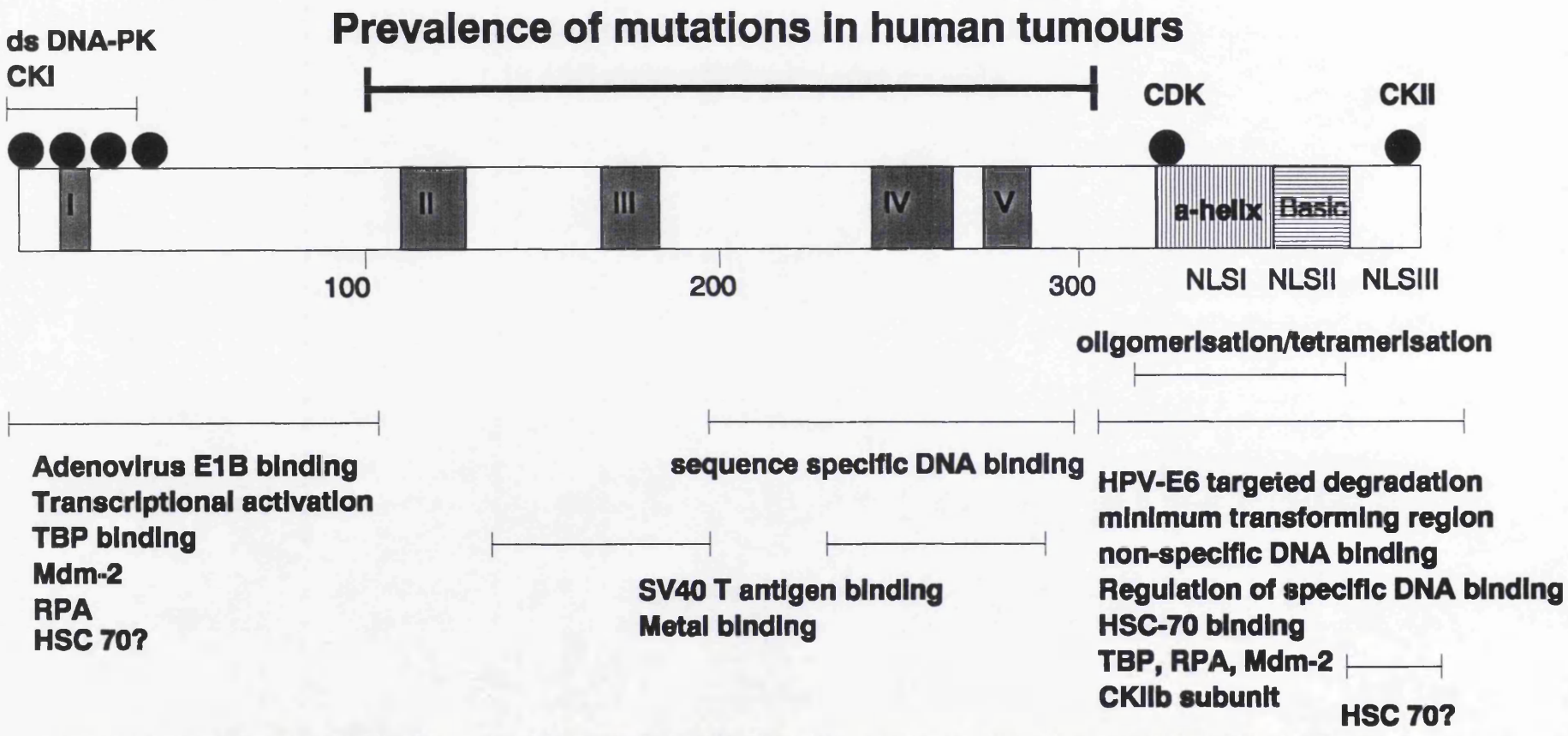


Figure 1.2 Diagram of the human p53 protein indicating the conserved regions and functional domains.

● **Phosphorylation site** ⊢⊣ **Bar indicates 'hotspot'region where the majority of mutations are found.**

NLS - nuclear localisation signals I - III, CK - casein kinase, CDK - cyclin dependant kinase, dsDNA-PK - double strande d protein kinase, TBP - TATA binding protein, RPA - replication protein A, HSC 70 - heat shock cognate protein, I - V conserved regions.

those regions of the p53 protein core domain that closely contacted the DNA (Cho *et al*, 1994). There are zinc binding sites in the central region and zinc is necesssary for DNA binding activity (Pavletich *et al*, 1993), maintenance of the wild type conformation appears to be dependent on metal binding (Hainaut and Milner, 1993).

The p53 protein forms a tetramer via an oligomerisation domain in the C-terminus at residues 311-364 (Pavletich *et al*, 1993) and p53 tetramerises as a dimer of dimers. The crystal structure of the tetramerisation domain (residues 325-356) has been determined (Jeffrey *et al*, 1995) and Clore *et al* (1994) have shown the 3-D structure of the oligomerisation domain (residues 319-360) by multidimensional NMR (Clore *et al*, 1994). Oligomerisation of the p53 protein is essential for its tumour suppressor function and mutations in this domain have been shown to inactivate this p53 function (Ishioka *et al*, 1995). *In vivo*, oligomerisation deficient p53 cannot transactivate via p53 binding sites (Peietenpol *et al*, 1994) and deletion mutants in the oligomerisation domain are also defective with regard to DNA binding (Hupp *et al*, 1993) despite retaining the central DNA binding domain.

Point mutations at residues 330, 334, 335 and 349, are located at crucial sites in the β-sheet structures of the dimers but it has not been established whether they have lost their tumour suppressor activity. It has been suggested that disruption of these dimers potentially destabilises the tetramer, *in vitro* tetramerisation is not however, essential for DNA binding as the core domain is capable of binding on its own. This would suggest that these C-terminal domains are important structurally in facilitating correct DNA binding for p53 function.

The C-terminal basic region (residues 330-393) appears to be involved in non-specific DNA binding (Pavletich *et al*, 1993) and is capable of forming an amphipathic helical structure (Kern *et al*, 1991a).

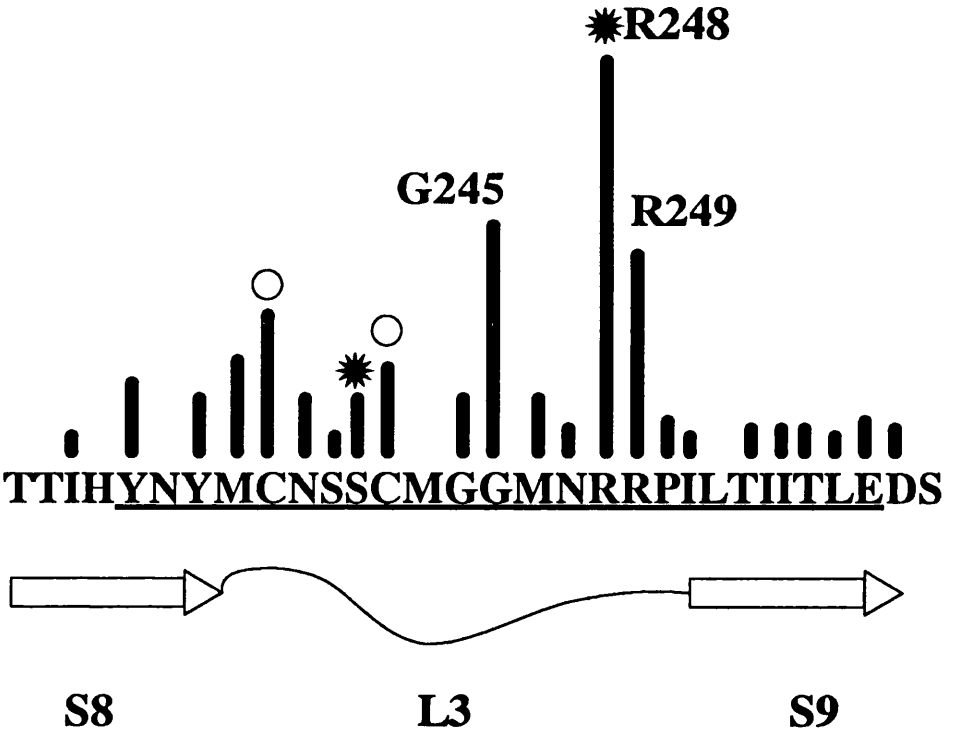


Figure 1.3 Partial sequence of p53 core domain residues 230-260. Conserved region IV is underlined and the ribbon drawing represents part of the secondary structure elements of the core domain. The circles represent those residues involved in zinc binding and the asterisks indicate the residues involved in DNA binding. S8 and S9 are β-sheet structures and L3 is the loop structure. The bars indicate the frequency of mutation for the residue.

The C-terminal domain has considerably more characterised functions than the other two regions yet few tumour derived mutations map to this region (Ishioka *et al*, 1995). However, it is now apparent that different mutations produce functionally different proteins and may exert conformational changes in the protein possibly altering p53 in this manner. Milner (1994) proposed a conformational model for p53 switching between its suppressor and activator forms which may be controlled in the C-terminus. Conformational regulation is apparent as the wild type p53 protein undergoes conformational change during G1 and adopts a mutant form which may prevent binding to DNA or proteins (Milner and Watson, 1990). However, the changes that occur as a result of mutation may not be as subtle as a conformational shift, but may result in a denatured protein. Thus the role of p53 as a tumour suppressor and as a transcriptional activator can be disrupted since mutant conformations may prevent activation of the necessary genes required for growth suppression.

The p53 protein is multiply phosphorylated by several different protein kinases (reviewed in Meek, 1994). Phosphorylation of murine p53 at serine 386 by casein kinase II has been demonstrated to be required for its tumour suppressor functions *in vitro* and possibly *in vivo* (Meek *et al*, 1990). Mutation of serine 386 abolishes the tumour suppressor activity of p53 (Milne *et al*, 1992) and serine 386 is also the proposed attachment site for an unusual RNA moiety. It is at present unknown whether serine 386 is a site for regulatory phosphorylation, covalent modification or both, however, recent data demonstrating p53 as a RNA binding protein may help elucidate further biological functions of p53 (Oberosler *et al*, 1993). The N-terminus is phosphorylated by casein kinase I (CKI), double stranded DNA protein kinase (ds DNA-PK) and it has been reported that dephosphorylation occurs via protein phosphatase 2A (PP2A) (Wang and Eckhart, 1992). Serine 309 in mouse p53 protein (ser-315 in human) is phosphorylated by CDKI (p34cdc2) suggesting that cell cycle dependent phosphorylation might control p53

(Bischoff *et al*, 1990). The ability of p53 to act as a tumour suppressor appears to correlate with an increase in phosphorylation of the protein and it has been shown that p53 becomes increasingly phosphorylated on entering S phase of the cell cycle (Meek and Street, 1992). This results in a p53 protein which will activate other cell cycle regulators such as waf1/cip1 (El-Diery *et al*, 1993) which is induced by expression of wild type p53 and will be discussed later.

Marston *et al* (1994) also demonstrated that human p53 mutated at the CKII and p34cdc2 sites also had no effect on transcriptional activity suggesting that p53 phosphorylation is not simply an on-off mechanism but may regulate other functions or act as a more subtle method of regulation.

1.3 p53 and its biological functions.

No single major function has yet been determined for p53 although involvement in many biological processes is clear from both its protein binding activities, which I will deal with subsequently, and its inactivation in most human tumours. The most likely function for p53 is as a transcription factor and when fused to the Gal4 DNA-binding domain of yeast, p53 has been shown to activate transcription from promoters containing Gal4 binding sites. Gene fusion of the N-terminus of p53 (AA 1-73) or of the whole p53 protein to Gal4 will promote the expression of a reporter gene in yeast or in mammalian cells in culture (Raycroft *et al*, 1990). This positive regulation by the p53 protein is in a sequence specific manner. p53 only activates transcription when the target contains the p53 consensus binding site (El Diery *et al*, 1992). Several different p53 binding motifs have been identified (Kern *et al*, 1991a; Funk *et al*, 1992) confirming the complexity of p53-DNA interactions. A specific p53 element has also been identified which is only bound by wild type p53; this target sequence is found in the waf1/cip1 gene

(El-Diery *et al*, 1993), the *mdm-2* gene (Juven *et al*, 1993) and the muscle creatine kinase (MCK) promoter (Weintraub *et al*, 1991). The regulatory region of the MCK gene contains a p53 responsive element that will activate a MCK-CAT reporter gene. Mutant p53 cannot activate transcription in this manner and displays a dominant negative effect when cotransfected with wild type constructs. However, some mutant p53 proteins can still bind to DNA and do not lose their ability to activate transcription (Zhang *et al*, 1993; Dittmer *et al*, 1993). This is in contrast to previous reports that mutant p53 proteins fail to bind DNA via the consensus element (Bargonetti *et al*, 1991). This may be explained by the location of the mutants studied with respect to the core domain and the oligomerisation/tetramerisation domains. One of the mutants used by Dittmer *et al*, (1993), Ala^{143}, is in the hydrophobic β-sandwich of the core domain. This domain is not directly linked to the DNA binding surface and therefore may not exert a marked effect on p53 transcriptional activation.

p53 can also regulate its own transcription, however, a direct interaction with its own promoter has not been demonstrated. Mutant p53 defective for transformation and transactivation cannot activate the p53 promoter. The p53 responsive element within the p53 gene has been suggested to be the NF-κB motif (Deffie *et al*, 1993). The cellular encoded transcription factor USF which is involved in the transcription of a number of cellular and viral genes can also bind to the p53 promoter possibly regulating p53 expression (Reisman and Rotter, 1993). Thus p53 transactivation of transcription factors or p53 interactions with other cellular proteins may act to regulate its own transcription.

As previously mentioned, wild type p53 levels can be increased by agents that result in DNA damage. Kastan *et al*, (1991) showed that this increase in p53 levels mediated a growth arrest at G1 of the cell cycle. Cell cycle arrest is thought to prevent the genomic instability associated with many

cancers by allowing time for DNA repair or when this is not possible by inducing programmed cell death or apoptosis (Perry and Levine, 1993, Lane, 1993). This illustrates a physiological state where cells express high levels of wild type p53 and consequently either arrest growth or apoptose. Mutant p53 or p53 bound by other proteins cannot perform these functions and so increase the possibility of genetic mutation.

p53 can function as a cell cycle checkpoint determinant by arresting the cell at the transition between G1 and S phase (Livingstone *et al*, 1992). This is supported by studies on cells from patients with the cancer prone disease Ataxia Telangiectasia (AT) in which the Growth Arrest and DNA Damage gene (*GADD45*) is absent (Kastan *et al* 1992). Cells from an AT lymphoblastoid cell line are radiosensitive and the p53 protein does not increase in response to ionising radiation. In normal cells, ionising radiation induces an increase in GADD45 and p53 levels and it was demonstrated that the AT gene which is defective in this syndrome is responsible for increased p53 levels in response to radiation. Furthermore, *GADD45* (which contains a p53 responsive element) is dependent on a functional p53 for its induction (Kastan *et al*, 1992). Thus the lack of GADD45 or p53 response following irradiation of AT cells allows inappropriate cell survival leading to further mutation. As well as responding to radiation induced damage, accumulation of wild type p53 in the nucleus has been demonstrated to occur in response to cytotoxic anti-cancer drugs and agents such as mitomycin C, cisplatin, etoposide and actinomycin D (Fritsche *et al*, 1993).

DNA damage also results in the induction of the waf1/cip1 gene, expression of which is regulated by p53 (Liu and Pelling, 1995). Waf1/cip1 is a major candidate as a downstream mediator of p53 function (El Diery *et al*, 1993), and is a potent cyclin dependent kinase (CDK) inhibitor which interacts with many cyclins and CDK's (Harper *et al*, 1993; Xiong *et al*, 1993). Waf1/cip1 can also act as a suppressor of cell growth (El Diery *et al*,

1993), however, a Val-Ala 138 mutant p53 was unable to induce waf1/cip1 expression while still retaining the ability to arrest cell growth of rat embryo fibroblasts (Hirano *et al*, 1995), suggesting that p53 can arrest cell growth in the absence of waf1/cip1 induction. Thus p53 induced growth arrest may occur via alternative pathways to prevent the accumulation of genetic damage.

It is important to mention that additional checkpoints exist in G2 and S that do not involve p53 (reviewed in Weinert and Lydall, 1993) as identified by Rad9 mutants in yeast (Weinert and Hartwell, 1990) and the loss of RCC1 in mammalian cells (Nishitani *et al*, 1991). These may also contribute to cellular control in the absence of p53.

Apoptosis or programmed cell death is widespread in multicellular organisms and plays an important role in developmental processes. Apoptosis has been shown in thymocytes to be induced by DNA damaging agents via a p53 dependent pathway (Clarke *et al*, 1993). This pathway appears to respond to single strand breaks in the DNA. High levels of p53 can induce apoptosis, yet can also induce growth arrest without apoptosis as already discussed. p53 has been shown to be essential to the process as p53 null mice (Donehower *et al*, 1992) show no apoptotic response even to high levels of radiation (Lowe *et al*, 1993). A p53 independent apoptotic pathway exists as evident by the normal development of homozygous p53 null mice with functional immune and reproductive systems, although these animals are predisposed to tumour formation by the age of six months (Clarke *et al*, 1993). The mechanism by which p53 induces apoptosis is unclear, however other cellular proteins have been identified which are involved in the p53 dependent apoptotic response. p53 is known to induce a reduction in the expression of *bcl-2* and an increase in *bax* expression (Miyashita *et al*, 1994). The bcl-2 protein can block p53 induced apoptosis (Wang *et al*, 1993) and bax can form hetrodimers with bcl-2 abrogating its ability to suppress apoptosis (Oltvai *et al*, 1993).

Increased levels of bcl-2 have been observed in some tumours and p53 is either lost or mutated. Thus, these proteins regulate the balance between life and death of the cell and loss of p53 mediated repression of bcl-2 may lead to bcl-2 overexpression resulting in inappropriate cell survival.

1.4 p53 and other proteins

p53 has been shown so far to be inactivated by mutation or loss however, abrogation of p53 function also occurs as a result of binding to viral oncoproteins. p53 also binds cellular proteins and I will deal with these first.

The murine double minute gene product (*mdm-2*) is a cellular protein which has been shown to form complexes with p53 (Momand *et al*, 1992). *Mdm-2*, a cellular oncogene, was initially characterised as an amplicon on the double minute chromosomes of a spontaneously transformed, tumourigenic murine cell line (Fakarazedah *et al*, 1991). The human *mdm-2* gene on chromosome 12q13-14 has also been observed to be amplified in many human sarcomas leading to the inactivation of wild type p53 by complex formation with the *mdm-2* gene product mdm-2P^{90} (p95) (Oliner *et al*, 1992). In cases where *mdm-2* amplification occurs p53 mutations are rarely present (Oliner *et al*, 1992) although human and mouse *mdm-2* gene products have been shown to bind to mutant as well as wild type p53. *mdm-2* expression is induced by wild type p53 suggesting that *mdm-2* is a target for activation by p53 (Barak *et al*, 1993). As mdm-2P^{90} can act as an antagonist of p53 activity, this induction of *mdm-2* may autoregulate p53 activity in cells. Overexpression of mdm-2P^{90} inhibits the ability of p53 to transactivate genes whose promoters contain p53 binding sites, and leads to enhanced tumourigenicity (Finlay, 1993). Recently mdm-2P^{90} was shown to stimulate the S-phase inducing transcription factors E2F-1/DP1 (Martin *et al*, 1995).

This response suggests that mdm-2^{p90} can both abrogate p53 tumour suppressor functions and enhance cell growth.

Hypophosphorylated Rb is also associated with the transcription factors E2F-1/DP1 as heterodimers preventing the transcriptional activity of E2F-1 (Krek *et al*, 1993). Rb-1 was first identified as deleted or mutated in the familial malignancy retinoblastoma (Rb) (Goodrich and Lee, 1993) and is known to play an important part in control of the cell cycle (Goodrich *et al*, 1991). Rb is phosphorylated in a cell cycle dependent manner and is hypophosphorylated in G0/G1 and at the G1/S boundary Rb becomes phosphorylated at serine residues by a cdc2-like protein kinase (Goodrich and Lee, 1993; Munger and Phelps, 1993). Xiao *et al* (1995) have shown that mdm-2 interacts with Rb both physically and functionally, and, as with p53, inhibits the growth regulatory functions provided by Rb. Thus, both p53 and Rb may be negatively regulated by the product of the single cellular protooncogene mdm-2^{p90}.

It is apparent that loss of both Rb and p53 tumour suppressor functions leads to inappropriate cell survival creating an enhanced environment for cell proliferation. This situation is mimiced by DNA tumour viruses which target both p53 and Rb as will be discussed in section 1.5.

Wild type p53 can repress transcriptional activity from various promoters, for example, the *fos* basal promoter (Seto *et al*, 1992; Liu *et al*, 1993). This occurs through the binding of the basal transcription factors by p53 (Ragimov *et al* 1993) thus preventing these factors binding to the TATA motif and activating transcription. Both wild type and mutant p53 can form a specific complex *in vitro* with the human but not the yeast TATA binding protein (TBP) (He *et al*, 1993), however only wild type p53 can prevent the stable binding of the TBP to the TATA motif which is the initial step in the formation of a preinitiation complex (Ragimov *et al*, 1993). This could be the molecular basis of the transcriptional repressor activity of p53 which is also

implicated in growth arrest, i.e, activation of p53 leads to an increase in p53 protein and the binding of other cellular proteins which halt cell cycle progression. Unlike p53 transcriptional activation, there appears to be no specific sequence requirement for repression of transcription (Ginsberg *et al*, 1991a). p53 binds origins of replication and the human and yeast Replication protein A (RPA) is bound by the N-terminus of p53 resulting in the inhibition of initiation of DNA replication (Dutta *et al*, 1993). It does so by preventing the binding of RPA to single stranded DNA *in vitro*, however no homologue of p53 has yet been identified in yeast, although RAD9 is a possible candidate (Prives, 1993). SV40 large T antigen binds to RPA to facilitate SV40 replication. If p53 binds RPA, then this may prevent SV40 replication illustrating the requirement for DNA tumour viruses to inactivate p53 in order to replicate in the cell.

In contrast p53 has been shown to downregulate HPV LCR expression independent of the TBP interaction (Desaintes *et al*, 1995) suggesting there are as yet unidentified proteins controlling this process.

Interestingly, p53 which has been associated with the response to DNA damage, regulates the expression of the Hsp70 stress/heat shock protein. Hsp70 is expressed in late G1/early S phase of the cell cycle and can be induced by environmental factors. The hsp70 promoter contains the CCAAT element through which transcription is stimulated by the CCAAT binding factor (CBF) stimulating transcription from the hsp70 promoter (Agoff *et al*, 1993). p53 binds to CBF preventing activation of the hsp70 gene again supporting p53 action as a repressor rather than activator of transcription. Hsp70 protein is associated with Arg^{175} p53 mutants (Hinds *et al*, 1990). This amino acid is suspected to have a critical role in stabilising the L2-L3 loops of the core domain. Hsp70 is know to associate with denatured proteins prior to their degradation in the cell. The association of mutant p53 with

hsp70 may occur as a result of the unfolded p53 protein being targeted for removal from the cell.

p53 binds to numerous different proteins and has been implicated in so many biochemical and biological functions that it is difficult to determine which are physiologically relevant, although it is possible they all are. It is as yet unclear how p53 functional activities are controlled, for example, by DNA damage. Another puzzle is how p53 directs the choice between apoptosis and growth arrest. This may depend on the interactions of other cellular proteins such as Rb, which along with p53 expression, can direct the cell to undergo either growth arrest or apoptosis. Overexpression of Rb rescued HeLa cells from p53 induced apoptosis (Haupt *et al*, 1995). Thus, if Rb is reduced and wild type p53 increased, then apoptosis will occur and if both p53 and Rb levels are raised then growth arrest occurs. Therefore, viruses require to inactivate both proteins to survive and replicate in the cell.

1.5 p53 and viral oncoproteins

From the above data it is clear that p53 is a crucial protein in regulating cell proliferation. Cellular inactivation of p53 leads to a potential tumourigenic situation where cells are allowed to progress and gain genetic abnormalities. Viruses have exploited this potential and evolved mechanisms which disrupt p53 functions thus creating a favourable environment for viral production.

The viral oncoproteins encoded by the DNA tumour viruses have been shown to target the p53 protein and prevent its functioning as a tumour supressor. The SV40 large T antigen was the first to be found to bind wild type p53 (Lane and Crawford, 1979). It blocks p53 binding to DNA preventing p53 transcriptional activation (Bargonetti *et al*, 1992).

The Adenoviruses express two oncogenes, E1a and E1b which co-operate to transform cells (Yew and Berk, 1992). E1a binds Rb-1 (p105) and acts as the major transforming protein of the virus. Non-oncogenic human Adenovirus Type 5 E1b p55 protein binds p53 and sequesters it to the cytoplasm (Sander *et al*, 1993), however, the oncogenic Adenovirus Type 12 (Ad12) E1b does not form a detectable association with p53 (Zantema *et al*, 1985). In Ad12 transformed cells p53 is wild type and is present in the nucleus at high levels. This p53 also contributes to the transformation of the cells and demonstrates properties normally associated with mutant p53. However, E1b from both Ad5 and Ad12 is involved in the transformation of cells in culture, contributes equally well to transformation and can inhibit transcriptional transactivation mediated by p53 (Sander *et al*, 1993). How Ad12 E1b functions without complexing to p53 is unclear. Nevertheless, p19 and p55 encoded by E1b can protect cells from p53 induced apoptosis which occurs in cells expressing E1a alone (Debbas and White, 1993). Mutant Ad5 E1b proteins which are defective in p53 binding are unable to transform and cannot block p53 induced transcription. Thus, both E1a and E1b are required as both Rb and p53 need to be inactivated for immortalisation to occur.

The oncoproteins of the papillomaviruses are also known to interact with p53 and Rb-1 (p105) in order to abrogate their functions and facilitate transformation. The E6 oncoprotein of HPV-16 and 18 binds the tumour suppressor protein p53 (Werness *et al*, 1990) and the E7 oncoprotein binds to the retinoblastoma gene product p105 (Rb-1) (Munger *et al*, 1989), however, these aspects will be dealt with separately.

1.6 Papillomavirus Genome Organisation

The focus of this thesis is to consider the role of p53 in BPV-4 associated carcinogenesis. The following sections will deal with

papillomaviruses in general and then with Bovine papillomavirus type-4 (BPV-4) in particular.

Papillomaviruses have a circular double stranded genome of approximately 7-8 kb in length (reviewed in Jackson and Campo, 1992). The papillomaviruses all essentially possess the same genomic organisation as illustrated in figure 1.4; however functional differences do exist between the proteins encoded by different papillomaviruses as will be discussed later.

The viral genes are divided into those which are expressed early (E) or late (L) in the viral life cycle. The E1 to E8 ORFs are expressed at the early stages and are involved in viral replication, transcription and transformation with the exception of the E4 ORF which is expressed at later stages of viral production along with the structural proteins L1 and L2.

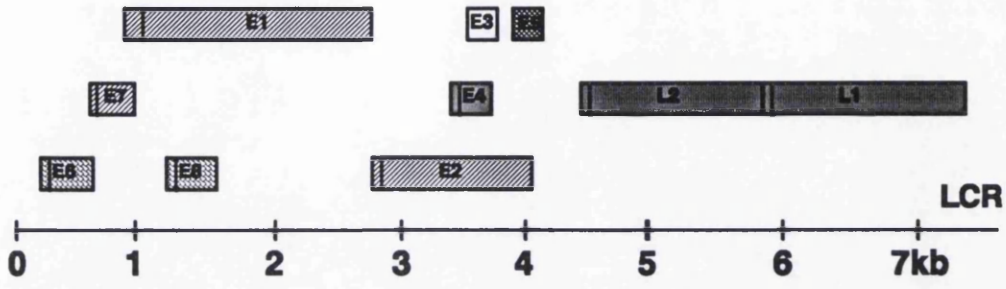
Immediately upstream of the early region is the non-coding Long Control Region (LCR) which contains the viral origin of replication, promoters, enhancer elements and cis acting sequences (McBride *et al*, 1989). The E1 and E2 ORF's bind to specific sites within the LCR and are essential for viral DNA replication *in vivo* and must be expressed together for viral replication to take place (reviewed in Ham *et al*, 1991). E2 products modulate papillomavirus gene expression and viral replication, thus both transcription and viral DNA replication are regulated through elements in the LCR.

Papillomavirus Proteins

Papillomavirus genomes encode transforming proteins which create an expanded cell population as a favourable environment for viral replication.

The proteins encoded by the papillomaviruses differ in their functions, for example, the E6 oncoproteins of the high risk papillomaviruses HPV-16 and 18 bind and degrade the tumour suppressor p53. In contrast to the high risk HPV E6 proteins, the low risk E6's of HPV-6 and 11 are not capable of high affinity binding to p53 and consequently do not facilitate degradation of

BPV-1



BPV-4

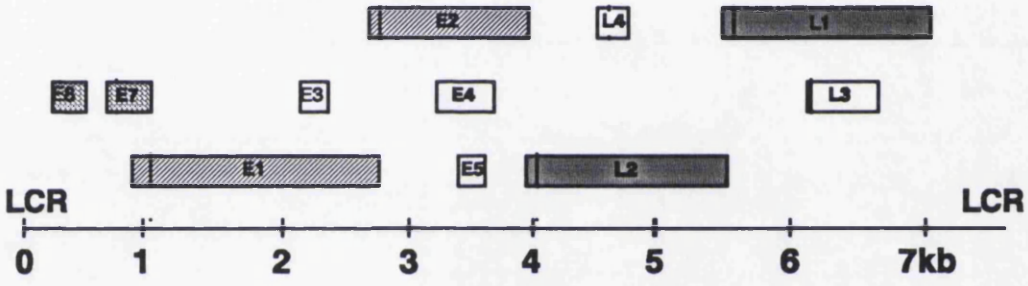


Figure 1.4 Genomic organisation of BPV-1 and BPV-4.

Early ORF's involved in replication and transcription

Early ORF's involved in transformation

Later structural ORF's

ORF's which have an as yet undefined function.

n.b E4 proteins are known to form a filamentous network which interacts with cytokeratins (Roberts *et al*, 1993), see text, page 34.

p53 (Werness et al, 1990; Scheffner et al, 1990). In this section I will deal with the papillomavirus proteins and their functions in general and discuss BPV-4 in section 1.7. Although an E6 ORF is not present in the BPV-4 genome, HPV-16E6 is important in the experimental aspects of this thesis and therefore will be discussed in greater detail.

The E6 oncoprotein

The E6 oncoprotein of the papillomaviruses is a small acidic protein of approximately 150 amino acids with four C-X-X-C motifs which comprise two characteristic zinc finger domains (see figure 1.5). These motifs are conserved throughout the E6 encoding papillomaviruses and there is evidence that E6 binds zinc *in vitro* but not *in vivo* (Barbosa *et al*, 1989). These zinc finger domains are known to be important but only major structural alterations appear to affect transformation. The functional domains of HPV-16 (16E6) and HPV-18 E6 (18E6) and BPV-1 E6 protein have been dissected (Crook *et al*, 1991a; Pim *et al*, 1994; Vousden *et al*, 1989) and in BPV-1 E6, the conserved cysteine motifs are important for the transformation of C127 cells (Vousden *et al*, 1989). E6 is capable of partially transforming cells on its own although it requires the cooperation of the other major papillomavirus transforming oncoprotein E7 in order to fully transform cells (Munger *et al*, 1992). Both E6 and E7 have *in vitro* transforming activities although E6 is usually less active. E6 cooperates with E7 to immortalise primary human foreskin keratinocytes (Hawley-Nelson *et al*, 1989) and E6, like E7, can also cooperate with *ras* to transform primary rodent cells (Storey and Banks, 1993). HPV-6 and 11 E6 failed to cooperate with *ras* in the BMK transformation assay (Storey and Banks, 1993) demonstrating the different properties of E6 proteins from oncogenic and non-oncogenic papillomaviruses. Pim *et al* (1994) mapped the domains of 18E6 responsible for cooperation with *ras* in the immortalisation of primary rodent cells, *in*

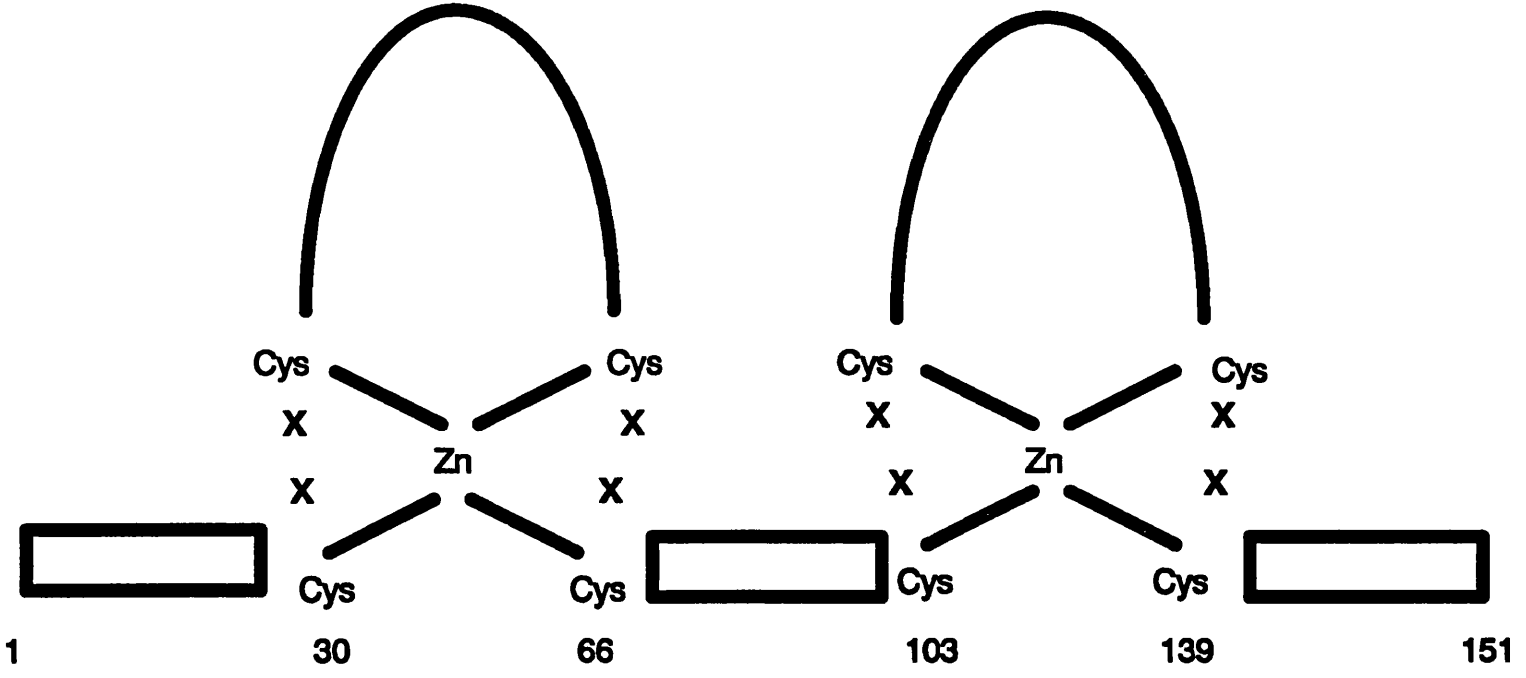


Figure 1.5 Schematic representation of the HPV-16 E6 protein and its zinc finger structures. The numbers indicate the amino acid position of the zinc finger motifs. Cys - cysteine residue, Zn - zinc atom, X - any amino acid.

vitro degradation of p53 and the inactivation of p53 function *in vivo.* Immortalisation appears to be independent of p53 and requires the C-terminal portion of the protein. The ability of 18E6 to abolish p53 transactivation is independent of p53 degradation. This would suggest that this function is mediated via the inhibition of p53 binding to its recognition sequence (Pim *et al*, 1994; Thomas *et al*, 1996).

E6 also has functions as a transcriptional transactivator (Sedman *et al*, 1991; Desaintes *et al*, 1992) and Sedman *et al* (1991) have shown that both high and low risk papillomaviruses activate transcription at a similar level, thus this function may not be related to transformation. This is supported by the transactivating and transformation functions of E6 being separable as determined by the mutational analysis described previously (Pim *et al*, 1994).

E6 and p53 interaction

HPV-16E6, HPV-6E6 and BPV-1E6 can all immortalise cells albeit the latter less efficiently, however, it appears that only E6 of the high risk human papillomaviruses associates with p53 in detectable complexes (Werness *et al*, 1990). The E6 genes from HPV-5, HPV-8, BPV-1 and CRPV have oncogenic properties yet many studies confirm that these E6 proteins do not interact with p53 (Keen *et al*, 1994; Lechner *et al*, 1992; Werness *et al*, 1990). In contrast, a study by Band *et al* (1993) showed that E6 of BPV-1 does bind p53 with low affinity and Crook *et al* (1991a) have shown that HPV-6E6 can bind p53 with reduced affinity but cannot degrade it. What function E6 serves in the low risk HPV's and whether it binds to wild type p53 with lower affinity is still unclear although new E6 functions now being elucidated may be involved.

The E6 oncoprotein binds p53 via an E6 associated protein [E6-AP], a cellular protein with an approximate molecular weight of 100 kDa (Huibregtse *et al*, 1991; Huibregtse *et al*, 1993a). The N-terminal 212AA of E6-AP are

not required for complex formation or for HPV-16E6 mediated degradation of p53 (Huibregtse *et al*, 1993b). E6 from the high risk papillomaviruses promotes the degradation of p53 *in vitro* by ATP-dependent hydrolysis which is suspected to be via the ubiquitin dependent protease pathway (Scheffner *et al*, 1990; Scheffner *et al*, 1993). However, this E6 induced degradation may be targeted to other proteins. The E7 protein of the HPV's are involved in the binding of the cellular retinoblastoma product pRB. When the N-terminus of HPV-16 E7 is fused with full length HPV-16 E6 then the pRb protein is directed for degradation. Although the E6 proteins of HPV-11 and HPV-6 E6 proteins do not bind to p53 and therefore do not target it for degradation, their E7 fusion proteins were still capable of degrading pRb. This suggests that the major functional differences between the E6 proteins of the high and low risk HPV's may lie in the specific cellular proteins they target for degradation.

To determine if this function was confined to the anogenital HPV's, additional fusions of HPV-5, BPV-1 and CRPV E6 were constructed. When fused to E7, all bound to pRb but failed to promote its degradation (Scheffner *et al*, 1992a). The E6-AP was not required in the E6/E7 fusion protein complexes to facilitate degradation (Scheffner *et al*, 1992a) as binding was mediated via E7 (Huibregtse *et al*, 1993b). Thus, the anogenital E6 oncoproteins may possess the common ability to facilitate the degradation of any protein they may associate with, which may lead to the identification of additional cellular targets.

Additional functions of E6

E6 is known to bind at least seven cellular proteins including p53, p212, p182, p100, p81, p75 and p33 (Keen *et al*, 1994). p100 is suspected to be the E6-AP protein and along with p33 are the only two directly bound to E6, the binding of the other proteins appear to be mediated via cellular factors. Histone H1 kinase activity was associated with HPV-6, 11, 16 and 18E6, but

not HPV-5 or 8E6. This E6 associated kinase activity phosphorylated the E6 associated protein pp182. This kinase activity appeared to be associated with the mucosal HPV types 6, 11, 16 and 18 and not with the cutaneous HPV-5 and 8E6. Thus E6 may alter the status of cellular proteins and redirect their functions.

Recently, a protein described as E6-BP was identified and characterised (Chen *et al*, 1995). E6-BP was found to be identical to the endoplasmic reticulum calcium binding protein, ERC55 (Weiss *et al*, 1994) a protein of 317 AA with six calcium binding motifs. E6-BP formed complexes with the E6 proteins of other high risk HPV's 16, 18 and 31 and BPV-1, but was not found to bind to the E6 proteins of the low risk HPV-6 and 11. The transformation activity of BPV-1 E6 mutants (Vousden *et al*, 1989) was examined and found to correlate with the ability of the mutant E6 proteins to bind to E6-BP, ie, those mutants defective for transformation could no longer bind E6-BP. E6-BP was also found to associate with E6-AP the cellular protein that links E6 and p53 in their degradation complex, and immunofluorescent studies colocalised E6-BP to cells expressing E6 (Chen *et al*, 1995). Thus, there may be a correlation between the risk of cervical cancer as a result of HPV infection, and the ability of E6 to bind E6-BP. The binding of E6 to E6-BP may be independent of p53 as no p53 was found in any of the complexes studied (Chen *et al*, 1995).

Calcium is essential for cell differentiation and keratinocytes infected with E6 and E7 are resistant to differentiation (McCance *et al*, 1988; Schlegel *et al*, 1988). Thus, E6 association with the calcium binding E6-BP may be significant in the ability of HPV to control the differentiation of infected cells.

E6 and cervical cancer

The binding of E6 to p53 results in an abrogation of G1 arrest in response to DNA damage (Kessis *et al*, 1993) as can expression of HPV-16

E7 and E1a (Vousden *et al*, 1993). However, recently Butz *et al* (1995) described residual wild type p53 transactivating activity in HPV positive cancer cell lines. They demonstrated that genotoxic treatment of these cell lines resulted in an increase in nuclear p53 protein levels and an increase in *waf1/cip1* mRNA confirming the presence of functional p53 in cells expressing an E6 protein (Butz *et al*, 1995). The expression of E6 leading to the binding and degradation of p53 in HPV positive cell lines, is not functionally equivalent to the mutation of p53 in HPV negative cancers. Although the E6/p53 interaction is still important for progression of HPV induced cervical carcinogenesis, in the metastatic pathway other processes are possibly required, i.e, other E6 functions that do not depend on p53. Cervical cancer cell lines lacking HPV DNA sequence appear to possess mutant p53 and the tumours from which these cell lines were derived were much more aggressive and invasive than those of HPV positive tumours (Crook *et al*, 1991b). So far no mutations in the p53 gene have been observed in HPV positive cancer cell lines and expression of wild type p53 is low (Srivastava *et al*, 1992), however, metastases from HPV positive carcinomas, were observed to contain mutant p53 (Crook and Vousden, 1992; Crook *et al*, 1992). Thus, this difference in p53 status may explain the poorer prognosis in HPV negative cancers where no wild type p53 is present.

The E7 oncoprotein

The E7 proteins of the HPV's associated with anogenital lesions are acidic nuclear phosphoproteins of approximately 100 amino acids in length (Greenfield *et al*, 1991). The N-terminal 38 amino acids of the E7 proteins have been shown to be structurally and functionally related to the adenovirus E1a protein and to the homologous parts of SV40 large T antigen (Phelps *et*

al, 1988). These regions are involved in the binding of the p105 product of the retinoblastoma gene (Rb-1) (Munger *et al*, 1989). E7 possesses two cys-x-x-cys zinc binding domains and a CKII phosphorylation site as illustrated in Figure 1.6.

E7 and cell transformation

HPV16 DNA is tumourigenic in NIH3T3 cells and HPV-16E7 can cooperate with *ras* to to fully transform primary rodent cells (Storey *et al*, 1988). The E7 proteins require intact zinc finger motifs for binding, dimerisation and transformation but not for Rb binding (McIntyre *et al*, 1993). The E7 proteins of the high risk HPV's appear to have a greater transformation potential than the E7 proteins of the low risk HPV's. This appears to correlate with an increased binding affinity of the high risk E7 proteins for pRb (Munger *et al*, 1991; Barbosa *et al*, 1991). Barbosa *et al* (1991) investigated whether *in vitro* biological activities of E6 and E7 varied among the HPV's of different oncogenic potentials by studying the complementation of E6/E7 to induce anchorage independent growth in NIH3T3 cells and human keratinocyte immortalisation assays. HPV-6 E7 demonstrated a considerably reduced ability to induce anchorage independent growth in comparison to HPV-16 and 18E7. E6 and E7 from the high risk papillomaviruses were interchangeable, however, E6 from HPV-6 or 11 was unable to complement E7 from HPV-16 or 18 in keratinocyte immortalisation. E7 of low risk HPV types was found to lack the potential to undergo splicing events which result in efficient translation of high risk E7 suggesting the efficiency of transcription may affect E7 activity *in vitro* (Barbosa *et al*, 1991). Phosphorylation of E7 by CKII also varies according to oncogenic potential (Barbosa *et al*, 1990). Thus, the biological activities of E6 and E7 are intrinsically different between the high and low risk papillomaviruses.

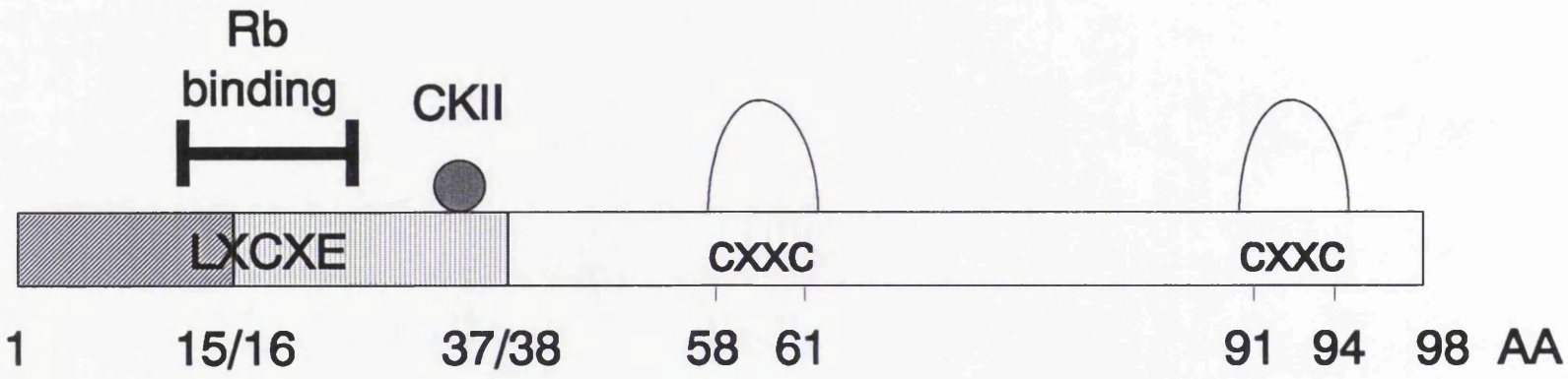


Figure 1.6 HPV-16 E7 oncoprotein.

Region of E7 which is homologous to CRI of Adenovirus type 5 E1a(Ad5 E1a).

Region of E7 homologous to CRII of Ad5E1a.

The CKII phosphorylation site is located at serines 31 and 32, the two zinc finger motifs located at amino acids 58-61 and 91-94 are involved in the dimerisation of the protein.

E7 and pRb interactions

In HPV-16 E7 mutation of the pRb binding sites will abolish transformation (Storey *et al*, 1990; Watanabe *et al*, 1990; Phelps *et al*, 1992). Studies with chimaeric high and low risk E7 proteins demonstrated that the biological properties are dependent on the N-terminal portion and that the C-terminal portion can be altered without effect (Munger *et al*, 1991). 16E7 competitively binds the hypophosphorylated form of Rb in G1 releasing E2F which activates transcription. The Rb binding domain of E7 contains the motif LXCXE which has also been found in cyclins (Ewen *et al*, 1993) and is conserved between E7 proteins from different papillomavirus types. Mutations in the Rb binding domain of both HPV-16E7 and BPV-4E7 abolish transformation of primary fibroblast cultures *in vitro* (Phelps *et al*, 1992; GJ Grindlay; personal communication). In contrast, other studies have shown that mutation in the Rb binding domain does not alter the transformation capabilities of E7. Jewers *et al* (1992) showed that 16E7 and *ras* immortalised human primary kertatinocytes and that alteration of both Cys24 and Glu26 to Glycine had no effect on transformation (Jewers *et al*, 1992). Mutant CRPV E7 which was not capable of binding Rb, could still induce warts in rabbits (Defeo-Jones *et al*, 1993). This is supported by the observations of Ciccolini *et al* (1994) that the efficiency of binding Rb does not correlate with the transforming capacity of the HPV E7s. Thus E7/Rb interaction alone is not sufficient for transformation and more than one E7 target is required to be inactivated.

E7 also associates with cyclin A and the p33CDK2 kinase (Tommasino *et al*, 1993) possibly via the Rb related protein p107 (Ewen *et al*, 1992). It may be that E7 functions to divert this kinase activity to other proteins, i.e, phosphorylation of viral proteins in order to facilitate viral replication. This would be analogous to the control of SV40 replication by phosphorylation of the SV40 large T antigen (McVey *et al*, 1989).

The E4 protein

The E4 ORF encodes a protein of 124 amino acids which is expressed in large amounts in the cytoplasm of the upper differentiating layers of the papilloma (Doorbar *et al*, 1986; Anderson *et al*, 1997). The E4 proteins are expressed late in papilloma development and E4 levels vary with papilloma type. In cutaneous lesions, E4 is expressed upwards from the basal cell layers. This differs from mucosal infections where E4 is expressed in the spinous layer and above. E4 proteins form filamentous networks which colocalise with cytokeratin intermediate-filament networks when expressed in SV40 transformed keratinocytes. HPV-16 E4, but not HPV-1 E4, can disrupt this keratin network (Doorbar *et al*, 1991; Roberts *et al*, 1993) again demonstrating the differences existing between papillomavirus types and the potential for oncogenesis.

The roles of the E4 proteins are uncertain although it is thought that they may allow disruption of the normal processes of keratinocyte differentiation to favour virion maturation in productive infection and possibly release of virus particles in the cells of the granular layer as has been observed for the late proteins of other viruses (Brietburd *et al*, 1987) and/or viral DNA replication (Doorbar *et al*, 1986) so as to favour high level virus production.

The late proteins

L1 and L2 are the capsid proteins of which L1 is the major component and highly conserved between papillomavirus types. They form the icosahedral hexagonal and pentagonal segments of the viral capsomere (reviewed in Zhou and Frazer, 1995). L1 and L2 are expressed at the later stages of viral infection and they are present higher up in the spinous and granular layers of the papilloma. The function of the L2 minor capsid protein is not clear although several have been implicated, such as interaction with the viral DNA to facilitate virion assembly and targeting of L1 to the nucleus

(Zhou and Frazer, 1995). L1 is capable of self assembly into virus like particles (VLP's) within intact cells in vitro (Zhou *et al* 1993). These VLP's are useful in the analysis of how the virus may enter the cell as the nature and distribution of cellular receptors is unknown and production of VLP's and advances in technology hopefully will allow us to overcome the problem of propagation of papillomavirus *in vitro* and allow the receptors and mechanism of entry to be studied and thus how papillomavirus infection is achieved.

1.7 Bovine Papillomaviruses

There are six known types of bovine papillomavirus (BPV) which are divided into two subgroups A and B according to their molecular and immunological homology (Jarrett *et al*, 1984). The subgroup A bovine papillomaviruses are BPV-1, BPV-2 and BPV-5; subgroup B contains BPV-3, BPV-4 and BPV-6. Viruses in subgroup A cause fibropapillomas which produce extensive skin lesions and can also infect the epithelial lining of the bladder although such lesions are not productive. Subgroup B viruses are epitheliotropic and infection does not involve fibroblastic cells. Both BPV-2 and BPV-4 have been implicated in cancer in cattle (Campo *et al*, 1992; Campo *et al*, 1980; Campo and Jarrett, 1986) and this association has been experimentally reproduced (Campo *et al*, 1994a; Gaukroger *et al*, 1993; Gaukroger *et al*, 1991; Campo and Jarrett, 1986). Bovine papillomaviruses from subgroup A have also been identified in other species including the sarcoids of donkeys and horses (Reid *et al*, 1994) and papillomas from llama (Peauroi *et al*, 1995).

BPV-1 DNA was first sequenced by Chen *et al* (1982), and as the most extensively studied papillomavirus, BPV-1 has been used as a model to determine the molecular biology of the other papillomaviruses. All of the significant ORF's are known to be located on one DNA strand of 7.495kb (Chen *et al*, 1982). This transforming region contains the 'early' ORF's E1,

E2, E6, E7, E8 and E5. The oncoproteins of BPV-1 are E5, E6 and E7. BPV-1 E5 can independently transform NIH3T3 (Schiller *et al*, 1986) and C127 cells (Yang *et al*, 1985) and mutations in E5 that leave E2 and E4 intact result in a loss of transformation potential. BPV-1 E6 is also capable of transforming C127 cells (Schiller *et al*, 1984). E1 has a role in viral replication and E2 is involved in the control of transcription. E3 and E8 have to date no homologies to suggest a function (Giri and Danos, 1986).

BPV-4

BPV-4 infects the mucosal epithelium of the alimentary tract in cattle (Campo *et al*, 1980). Infection results in benign papillomas which can progress with high frequency (30%) to carcinomas in cattle grazing on bracken fern (Jarret *et al*, 1978) as illustrated in figure 1.7. Bracken fern is known to contain immunosuppressants, cocarcinogens and mutagens (Evans I.C *et al*, 1982; Evans W.C *et al*, 1982) and immunosuppression results in widespread persistent papillomatosis which provides the opportunity for malignant progression in the presence of other factors (Campo and Jarret, 1986).

Like the other papillomaviruses, BPV-4 possesses both early and late ORFs. The 'early' region comprises approximately two-thirds of the genome and encodes proteins that control the replication and transcription of the viral DNA. These ORFs are involved in cell transformation by BPV-4. The 'late' region encodes the viral capsid proteins and a region of about 500bp defines the LCR which contains the control elements which determine viral functions. Comparison of the BPV-4 genome with BPV-1 reveals several differences (see Figure 1.4). BPV-4 has neither an E5 nor an E6 ORF, and no known region of E6 homology has been identified in the BPV-4 genome (Jackson *et al*, 1991), however, the product of the BPV-4 E8 ORF has been shown to possess a similar hydrophobicity profile and putative transmembrane structure

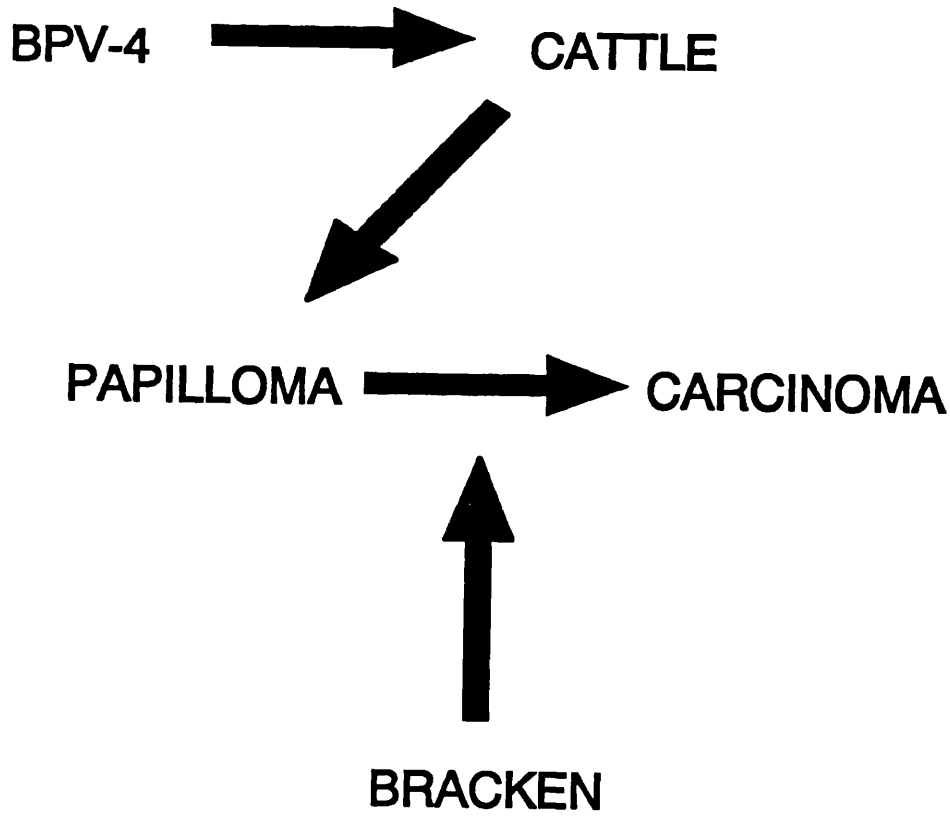


Figure 1.7 Schematic representation of events which are known to occur *in vivo* as a result of infection with BPV-4 in the presence of cofactors.

as BPV-1 E5. Like BPV-4, the other B subgroup papillomaviruses lack an E6 ORF.

The Long Control Region (LCR)

Like the HPVs, BPV-4 gene expression is regulated by both virally encoded and cellular transcription factors. Several positive and negative elements have been identified within the BPV-4 LCR which bind transcriptional factors, including three negative regulatory elements known as NR1, NR2 and NR3.(Jackson and Campo, 1991; Jackson and Campo, 1995a). Of these negative elements NR2 has been studied in detail. Deletion or mutation of the NR2 element in the LCR leads to an increase in enhancer activity in fibroblastic cell lines. The C/EBPβ subunit has been shown to complex with the NR2 element by EMSA and UV crosslinking experiments (McCaffrey and Jackson, 1994). C/EBPβ transcription factor functions are derepressed by the activation of *ras* (Kowenz and Leutz, 1994). If *ras* activation can exert an effect on regulation of BPV-4 transcription via C/EBPβ, this may in part explain the requirement for activated *ras* for BPV-4 transformation *in vitro* which will be discussed later.

The functions of the BPV-4 early proteins are similar to those of BPV-1. The late structural proteins BPV-4 L1 and L2 have been extensively investigated as anti-BPV vaccines. As this aspect of the BPV-4 biology is not relevant to this thesis it will not be discussed further.

The BPV-4 E2 protein

The E2 ORF encodes a protein with a predicted molecular weight of 45-48kDa which has been shown to bind DNA as a dimer (Ham *et al*, 1991). The BPV-4 LCR contains three consensus binding sites for the viral E2 transcription factor which mediate the action of the E2 protein and a fourth

degenerate site. The E2 protein can influence LCR promoter activity in primary foetal bovine keratinocytes (PalK) cells. E2 acts as a transcription regulator and at low concentrations, full length BPV-4 E2 can mediate transactivation of the BPV-4 LCR. At higher concentrations, E2 was found to completely repress transcription from the LCR (Jackson and Campo, 1995a). Mutational analysis of the LCR demonstrated that the four E2 sites each perform different functions in the control of transcription and that competition between cellular transcription factors and viral E2 proteins is essential in regulating the level of viral gene expression during papilloma development (Jackson and Campo, 1995a).

The E8 protein

E8, a 42 amino acid hydrophobic protein, is one of the oncoproteins of BPV-4 (Pennie *et al*, 1993) which, when coexpressed with BPV-4 E7 in PalF cells, confers anchorage independent growth (Cairney and Campo, 1995). E8 interacts with the 16k ductin protein (Faccini *et al*, 1996) a component of gap junctions and of the vacuolar ATPase proton pump (Finbow *et al*, 1991). BPV-1 E5 will also bind 16K ductin as will HPV-16 E5 and HPV-6 E5 (Goldstein *et al*, 1991). In PalF cells transformed by BPV-4 and an activated *ras*, expression of BPV-4 E8 has been shown to be associated with the loss of gap junctional intercellular communication (GJIC) (Faccini *et al*, 1996). This agrees with the study of Oelze *et al* (1995) who found that HPV-16 E5 expressed in a human keratinocyte cell line, inhibited GJIC to a similar extent. As previously mentioned, BPV-4 E8 is similar to BPV-1 E5 (Jackson *et al*, 1991). E5 alone will transform cells (Schiller *et al*, 1986; Yang *et al*, 1985), however, overexpression of E8 in PalF cells on its own results in accelerated senescence and cell death (Pennie *et al*, 1993; Cairney and Campo, 1995).

The E7 protein

The major transforming gene of BPV-4 is E7, which possesses two Cys-X-X-Cys motifs and a potential p105 (Rb) binding domain, although it lacks the casein kinase II site which is a feature of the E7 proteins of the high risk HPVs (Jaggar *et al*, 1990). Although the functions of the BPV-4 E7 protein have not yet been studied in detail, this protein may have similar or even identical functions to HPV-16 E7.

Although it is not known if BPV-4 E07 (4E7) binds Rb, mutation of the pRb binding domain or deletion of the second Cys-X-X-Cys motif abolish transformation, demonstrating the importance of these sites and the role of E7 in cell transformation (Campo *et al*, 1994b).

PalF cells are only partially transformed by 4E7 in the presence of an activated *ras* and require the addition of 4E8 for anchorage independent growth (Pennie *et al*, 1993). HPV-16E7 (16E7) transformed PalF cells are anchorage independent even in the absence of BPV-4 E8 suggesting that 4E7 may lack functions present in 16E7 (GJ Grindlay and E Wagner; personal communication).

For the oncogenic HPV's, E6 is one of the transforming oncoproteins. It is unclear why BPV-4 does not require E6 and one of the aims of this thesis is to answer this question by examining the E6 functions that BPV-4 lacks.

BPV-4 and Cell Transformation

BPV-4 can transform established cell lines, induce cellular DNA amplification (Smith *et al*, 1989) and rearrangements have been found in the bovine *ras* gene in cancers (McCaffrey *et al*, 1989; Campo *et al*, 1990). Primary epithelial cells are the natural host of the virus and provide a more accurate system in which to study the transformation functions of BPV-4 *in*

vitro, thus more recently primary cells have been derived from the palate of a bovine foetus and cultured as fibroblasts (PalF) or keratinocytes (PalK).

BPV-4 has an absolute requirement for activated *c-Ha-ras* to induce transformation of PalF cells *in vitro* (Jaggar *et al,* 1990) and even then is still only capable of partial transformation, the addition of HPV-16E6 being required to immortalise the cells (Pennie *et al,* 1993). HPV-16 is capable of transforming immortalised rodent cell lines to tumourigenicity (Yasumoto *et al,* 1986) and in the presence of an activated *c-Ha-ras* can transform primary rodent kidney epithelial cells (Crook *et al,* 1988) demonstrating a requirement for activated cellular oncogenes for *in vitro* transformation of primary cells by papillomaviruses.

One of the best characterised mutagens contained in bracken is the flavonoid quercetin (5,7,3',4'-tetrahydroxyflavone). Quercetin is known to adduct DNA (Rahman *et al,* 1990), induce mutation in both bacteria and mammalian cells (Bjieldanes and Chang, 1977; Ishikawa, 1987) and cause clastogenic damage (Ishidate, 1988). In relation to the latter point, it has been observed that cattle fed on bracken fern have a high incidence of chromosomal abnormalities (Moura *et al,* 1988). Quercetin is also capable of inhibiting ATP binding by activating phosphatases and downregulating kinases in chick embryo fibroblasts (Van Wart Hood *et al,* 1989).

When PalF cells transfected with BPV-4 and activated *ras* are treated with quercetin for 48 hours they become tumourigenic, thus confirming the association of cofactors present in bracken with neoplastic progression of BPV-4 induced papillomas *in vivo* (Campo and Jarrett, 1986; Cairney and Campo, 1995; Pennie and Campo, 1992). The minimum requirement for tumourigenicity in conjunction with quercetin treatment is the presence of the BPV-4 E7 oncoprotein and activated *ras* (Campo *et al,* 1994b; Cairney and Campo, 1995). These BPV-4 and quercetin treated PalF cells were found not

to contain any chromosomal abberations or cellular rearrangements/amplifications (Cairney and Campo, 1995), however, it should be noted that previous studies demonstrating the mutagenic nature of quercetin utilised established cell lines and not primary cells. PalF and PalK cells which have been treated with quercetin demonstrate an upregulation in LCR activity which may result in increased transcription of the viral genes. This may lead to an increase in production of viral proteins and a more transformed phenotype (JA Connolly; personal communication). This is supported by an increase in transformation efficiency observed when 4E2 is cotransfected with BPV-4 E7/E8 under control of the homologous LCR in the presence of an activated *ras* (Jaggar *et al*, 1990).

Aims of this PhD thesis

As discussed, studies have demonstrated the link between papillomavirus oncoproteins and the abrogation of cellular functions which control cell proliferation. HPV proteins encoded by the early genes subvert normal regulatory pathways of infected cells to accomodate viral replication. This is accomplished by interactions with cellular proteins including tumour suppressor products as illustrated in figure 1.8. As a result of this viral targeting, the potential for uncontrolled cell growth in the presence of other factors is apparent. It is necessary to dissect these viral mechanisms in order to understand the role of papillomaviruses in the carcinogenic process. The aims of this thesis, in elucidating the role p53 plays in the transformation of cells by BPV-4 in the absence of an E6 protein, are discussed below.

BPV-4 is unusual in that it does not possess an E6 open reading frame like the other known papillomaviruses. Sequencing of the closely related BPV-3 and BPV-6 demonstrated an identical genome arrangement for all three B subgroup bovine papillomaviruses. For this reason we wished to

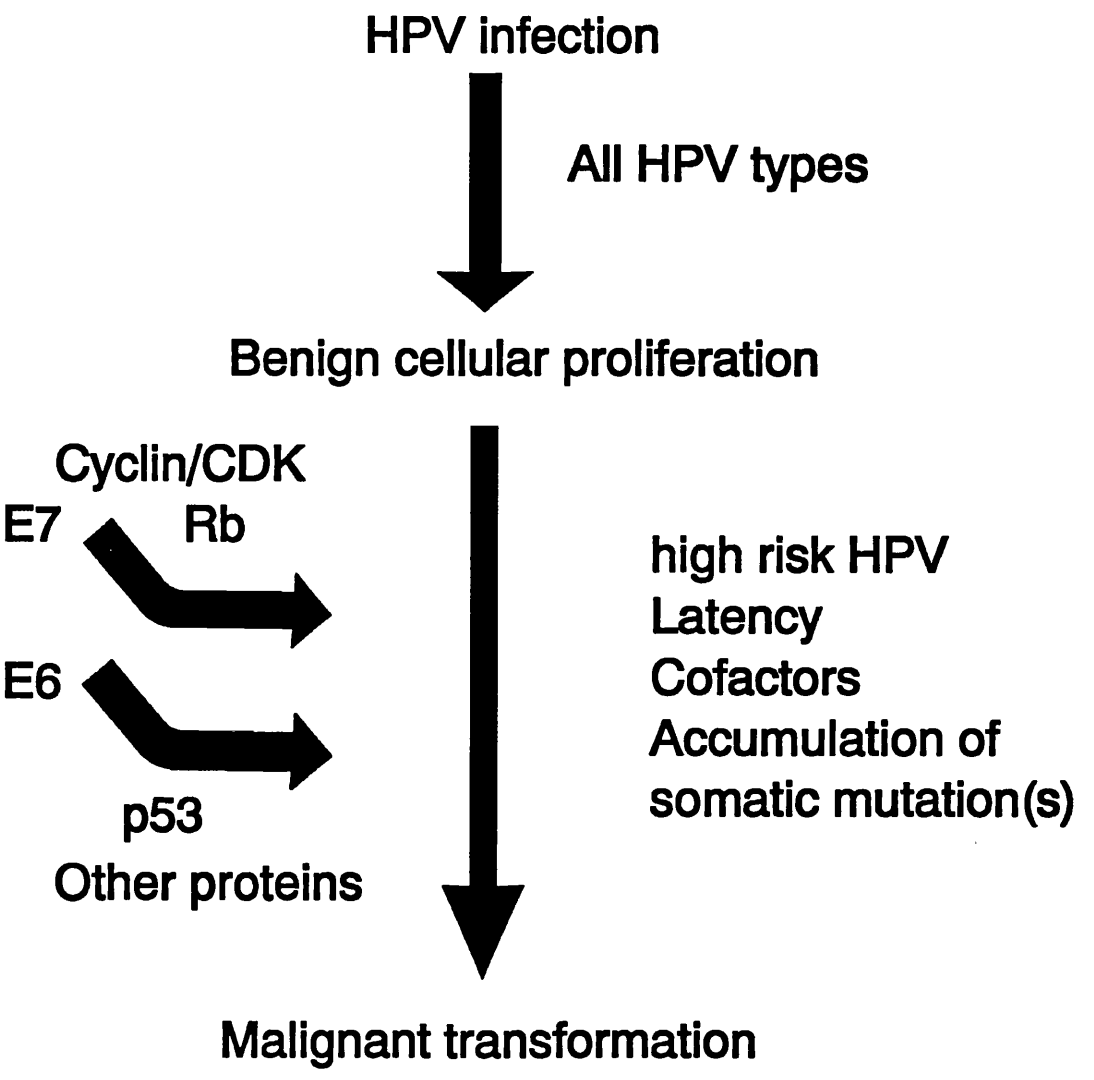


Figure 1.8 Summary table of the E6 and E7 proteins and their contribution to the malignant progression of HPV infected cells

investigate whether p53 inactivation is important in BPV-4 associated carcinogenesis and to determine if viral proteins play a role or if an alternative mechanism of p53 abrogation exists for BPV-4. The E6 proteins of HPV's 16 and 18 have been shown to cooperate with E7 in the transformation of keratinocytes in culture, to promote the degradation of p53 and to be capable of transcriptional transactivation. Since BPV-4 does not possess an E6 ORF, yet is capable of successful infection and propagation *in vivo* and malignant transformation both *in vitro* and *in vivo* in the presence of chemical cofactors, this raises the question of whether E6 functions are unneccessary or whether these functions are provided by another viral or host protein. In the case of BPV-4, it might be predicted that *p53* gene expression and function should be unaltered in papillomas in the absence of an E6 product, and that *p53* mutations, perhaps induced by environmental factors, will have occurred during progression to carcinoma. On the other hand BPV-4 may have evolved another mechanism of evading p53 growth suppression; such an alternative pathway might provide useful insights into the mechanism of BPV-4 transformation and p53 function as a tumour suppressor gene.

It was thus decided to determine the status of *p53* in BPV-4 derived lesions and to establish the effect mutant p53 could exert on cells transformed with BPV-4. This required the cloning and sequencing of the wild type bovine p53 gene and analysis of p53 status of BPV-4 induced lesions of cattle.

Amplification of the *mdm-2* locus and consequent overproduction of the p53 binding mdm-2 protein appears to be a mechanism by which cells may escape p53-regulated growth control. The transforming region of BPV-4 DNA has been shown to induce cellular DNA amplification, therefore, the copy number of the bovine *mdm-2* gene in papillomas and carcinomas was evaluated.

In order to look for possible mutations in *p53* in papillomas and carcinomas we set out to clone and sequence the bovine *p53* gene. It is known

that one of the effects of *p53* mutations can be to elevate steady state levels of the protein to an amount that can be detected by immunostaining. A range of antibodies to human and mouse p53 were available and the highly conserved nature of p53 suggested that some of these antibodies would react with bovine p53. This would allow us to determine levels of p53 protein expression in papillomas and carcinomas.

BPV-4 DNA is able to partially transform primary bovine fibroblasts (PalF) in the presence of activated *ras*; the additional presence of HPV-16E6 renders these cells immortal. Thus BPV-4 does appear to lack at least some of the functions which can be provided by HPV-16 E6. As mentioned above, one of the known targets for the E6 protein of the oncogenic HPV's is the p53 tumour suppressor gene product. We therefore investigated whether HPV-16 E6 was able to cooperate with BPV-4 in the transformation of primary cells derived from *p53* knockout mice, to test whether HPV-16 E6 provides transformation functions in addition to its role in p53 degradation.

MATERIALS

CHAPTER TWO

2.1 Molecular Biology

Chemicals

Agarose	Bethesda Research Laboratories,
Low melting point (LMP) Agarose	U.S.A
Ammonium Chloride	Aldrich Chemical Company, U.K
2X MDE gel mix	AT Biochem Ltd (FMC
(Mutation Detection Enhancment)	Bioproducts, Denmark)
Bactotryptone	Difco Laboratories, U.S.A
Bactoagar	
Calcium chloride(Analar)	BDH , U.K
Citric acid	
D-Glucose	
Dimethylformamide	
Formaldehyde	
Glycerol	
Magnesium chloride	
Na_2HPO_4 (di-sodium hydrogen orthophosphate)	
PEG (Polyethylene Glycol)	
Potassium chloride(Analar)	
Potassium hydroxide(Analar)	
Repelcote silicone treatment	
Sodium chloride(Analar)	
Caesium chloride	Boehringer Mannheim, Germany
IPTG (isopropyl-β-D-thiogalactopyranoside)	
X-gal (5-bomo-4-chloro-3-indolyl-β-D-galactopyranoside)	
RNAzol B	Cinna-Biotecx Laboratories Inc, U.S.A
Formamide	Fluka Chemika-Biochemika AG, Switzerland

Ammonium persulphate (APS)	Fisons Scientific Equipment, U.K
Boric acid	
Chloroform	
EDTA sodium salt (EthyleneDiamineTetraAceticcid)	
Glycine	
Isopropanol	
K_2HPO_4 (Potassium hydrogen orthophosphate)	
Magnesium sulphate	
Maltose	
Methanol	
Potassium acetate	
Sodium Dodecyl sulphate (SDS)	
Sodium hydroxide pellets	
Sucrose	
Tri-sodium citrate	
Urea	
Tris base	Gibco BRL Life Technologies
Ethanol	James Burrough Ltd, U.K.
Phenol	Rathburn Chemicals Ltd, U.K
30% (w/v) acrylamide:0.8% bisacrylamide	Severn Biotech Ltd, U.K
40%(w/v) acrylamide: bisacrylamide	
Ethidium bromide	Sigma Chemical Company, U.K
Ficoll 400,000	
MOPS (3-(N-Morpholino)Propanesulfonic Acid)	
Polyvinylpyrrolidone	
TEMED	
Tween 20 (Polyoxyethylene sorbitan monolaurate)	
Yeast extract	Beta Laboratories, U.K

Radiochemicals

α-[^{32}P]-dCTP (400Ci/mmol)	Amersham International
γ-[^{32}P]-ATP (3000Ci/mmol)	plc, U.K
α-[^{35}S]-dATP	

Nucleic Acids

ΦX 174 HaeII digested DNA	Bethesda Research Laboratories, U.S.A
λ HindIII digested DNA	
RNA ladder	Gibco BRL Life Technologies
pC53-SN3, pC53-SCX3	Dr.B.Vogelstein (Baker *et al*, 1990).
pJ4Ω vector, pJ4Ω 16E6 and the E6 mutants	Dr.K.Vousden (Storey *et al*, 1988; Crook *et al*, 1991a).
pIC20H	Dr Maria Jackson (Marsh *et al*, 1984).
MDM-2 cDNA pMDMFL4	Dr.B. Vogelstein (Oliner *et al*, 1992).
pT24 (6.6kb human c-Ha-ras in pUC13)	Ros McCaffrey (Santos *et al*, 1982)
pBPV-4 (whole genome BPV-4 in pAT153)	Margaret Cairney (Campo and Spandidos, 1983).
pBabehygro	Dr. S.Barnett
pHSV-βGAL	Ros McCaffrey
pzipneo	Ros McCaffrey

Enzymes and kits

Alkaline phosphatase T4 polynucleotide kinase	Northumbria Biologicals Ltd, UK
Bovine liver genomic DNA library	Clontech, Cambridge Bioscience, U.K
Geneamp DNA PCR kit Geneamp thinwalled reaction tubes	Perkin-Elmer Cetus, U.S.A

DNA sequencing kit (dye terminator cycle sequence ready reaction)

Microspin S-400 HC columns Sephadex G-25 columns	Pharmacia Biotech, Sweden
Proteinase K	Bethesda Research Laboratories, U.S.A
Random priming kit Calf Intestinal Alkaline Phosphatase (CIP)	Boehringer Mannheim, Germany
Restriction enzymes T4 DNA ligase BioNick translation system	Gibco BRL Life Technologies
Taq polymerase	Promega
PCR-script SK+ cloning vector kit	Stratagene
Sequenase version 2.0 kit	United States Biochemical Corporation, U.S.A
Wizard magic PCR prep kit Wizard magic miniprep kit	Promega, U.K

Miscellaneous

Sterile bacteriological plates	Bibby-Sterilin Ltd, U.K
X-OMAT AR X-ray film	Eastman Kodak Company, U.S.A
0.2μM filter membrane	Pall Biodyne Membranes Ltd
E.Coli DH5-α competent cells	Gibco BRL Life Technologies
Whatman 3MM filter paper	Whatman International Ltd, U.K
Hybond -N+ nylon membrane	Amersham International plc, U.K

2.2 Tissue Culture

Cell Lines

PalF	Primary foetal bovine palate fibroblasts, pass #3, stocks made by Margaret Cairney (PhD thesis). Palate was removed from a foetus of less than 5 months gestation, obtained from Glasgow University Veterinary School.
p53 null	Primary fibroblasts derived from the skin removed from newborn p53 null mice (B95). These mice were homozygous null for p53 and were provided by Debbie Stuart. Transfection criteria for these cells were determined by transfecting pHSV-βGAL by various methods and determining the levels of β-galactosidase produced from the lysed cells. This demonstrated that $CaPO_4$ transfection was the best method to ensure maximum plasmid entry into cell. Kill curves using different concentrations of hygromycin were carried out to determine amount required to select resistant colonies from non-resistant background.

Chemicals

Silicon grease	BDH Analar, U.K.
Giemsa stain	
Hygromycin B	Boehringer Mannheim, Germany
DMSO	Fisons Scientific Equipment, U.K
Methanol	
MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide;thiazol blue)	Sigma Chemical Company, U.K
HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])	
Trypan blue stain	Gibco BRL Life Technologies Ltd, U.K
G418 sulphate (Geneticin)	

Media

Dulbeccos modified Eagles medium (10X DMEM) F-10 HAM nutrient mix (10x) Foetal Calf Serum (FCS) L-Glutamine (200mM) MEM amino acids (50x) Penicillin (100x) SLM Sodium bicarbonate (7.5%) Sodium pyruvate (100mM) Streptomycin (100x)	Gibco BRL Life Technologies Ltd, U.K
Trypsin Solution(10x)	Worthington Biochemical Company, U.K

Miscellaneous

Sterile plastic flasks and plates	Becton DickinsonU.K Ltd.
96 and 24-transwells	Costar Corporation, U.S.A.
MF-1 NuNu mice	Harlan Olac Ltd, U.K.
Cryotubes	Nunc, Denmark.

2.3 Immunocytochemistry and *in-situ* hybridisation

Antibodies

CM-1 was provided by Biogenex Ltd. Anti-BPV-4 E7 was produced using multiple peptides in rabbit by G.Joan Grindlay (McGarvie *et al*, 1995), BPV-4 E8 was also a polyclonal antiserum generated by inoculation of a peptide from the carboxyl terminus of E8 conjugated to KLH (peptide 67) as described in Pennie *et al* (1993).

Anti digoxigenin-11-dUTP	Boehringer Mannheim, Germany

Chemicals

Aquamount	BDH Analar, U.K.
Bovine Serum Albumin fraction V	Sigma Chemical Company, U.K
Powdered trypsin	Difco Laboratories, U.S.A
Hydrogen peroxide	Fisons Scientific Equipment, U.K.
Xylene	
DPX mounting medium	Surgipath Medical Industries Inc.
Scotts tap water concentrate	U.S.A
Harris's Hematoxylin	
Eosin	
Lugol's Iodine	
Sodium thiosulphate	
Oxoid PBS tablets	Unipath Ltd, U.K

Kits

DIG nucleic acid detection kit	Boehringer Mannheim, Germany
Vectastain ABC peroxidase kit	Vector Laboratories, U.K
anti-rabbit IgG	

Miscellaneous

Digoxigenin-11-dUTP	Boehringer Mannheim, Germany
Coverslips	Chance Propper Ltd, U.K

METHODS

CHAPTER THREE

3.1 Molecular Biology Techniques

3.1.1 Large scale preparation of plasmid DNA

A 10ml overnight culture of *E.coli* containing plasmid DNA, wasgrown in the presence of 100μg/ml ampicillin, was transferred to a 1 litre flask and incubated overnight at 37°C with shaking. All cells were grown in LB except for pZipneo and pbabehygro which were grown in high nutrient superbroth to ensure maximum yield. Bacteria were spun down at 5000rpm in a Sorvall RC5B (GS-3 rotor) for 10min and the supernatant removed. The bacterial pellet was resuspended in 40ml of TEG (25mM Tris.Cl pH7.5, 1mM EDTA, 50mM Glucose), 2 volumes of freshly made 0.2M NaOH/1%SDS were added, mixed by inversion and left on ice for 10min. 1.5 volumes of ice cold 3M KAc was then added, mixed by inversion and incubated on ice for 30min. The chromosomal clot was pelleted at 8000rpm for 10min Sorvall RC5B (GS-3 rotor) and the supernatant filtered through gauze. 0.6 volume of isopropanol was added and left at room temperature for at least 15min to precipitate DNA, followed by centrifugation at 8000rpm for 15min in Sorvall RC5B (GS-3 rotor). The DNA pellet was resuspended in 6.5ml of 1xTE (10mM Tris.Cl, 1mM EDTA pH7.5), and the volume made up to 8ml with 1xTE. 10g of CsCl was added (resulting in a density of 1.395) and dissolved at 37°C for 10min, 880μl of Ethidium bromide (EtBr) (10mg/ml) was added to visualise the DNA band after centrifugation. Samples were spun in a Sorvall ultracentrifuge using 11.5ml Sorvall polyallomer tubes in a T1270 rotor for 24 hours at 50,000rpm, 20°C. The plasmid band was then removed using a 24 gauge needle and transferred to a 15ml falcon tube. The EtBr was removed by several extractions with 5M NaCl saturated isopropanol and the CsCl removed by dialysing the sample for 3x30 min in 1x TE. DNA was then ethanol precipitated with 1/10 volume 3M NaAc pH5.2 and 3 volumes of ethanol at -

20°C overnight.. The concentration was determined by reading the A_{260} and the purity determined by the ratio $A_{260/280}$ which was typically 1.8-1.9. Any plasmids which were used for cell transfection purposes were purified using two rounds of CsCl gradient and sterilised by ethanol precipitation; for plasmids used in standard laboratory experiments one round of purification was sufficient.

3.1.2 Gel Electrophoresis

All analysis was carried out on either agarose or polyacrylamide gels. Agarose gels were run using Flowgen apparatus and varied from 0.8% to 2% according to the size of the fragment to be visualised. Gels were cast in 1x TBE and 0.5μg/ml EtBr was added to both the gel and the running buffer, with the exception of gels to be Southern or Northern blotted. The separated DNA was visualised using short wave UV light and photographed using polaroid type 57 high speed film or an Appligene imager.

Denaturing polyacrylamide gels were run in 1xTBE buffer and either 6 or 15% acrylamide (40%w/v) used for sequencing and oligo purification respectively. Polyacrylamide gels were run using Cambridge Electrophoresis DNA sequencing apparatus.

3.1.3 Restriction digests

Plasmid DNA was incubated with 10u of enzyme per 1μg DNA in the buffered solution suggested by the manufacturer. Incubations were at the specified temperature for 1-2 hours unless otherwise stated. Digested fragments were analysed by gel electrophoresis as described above. Restricted fragments were recovered from the gel as described in 3.1.5.

3.1.4 Oligonucleotide Synthesis

Oligonucleotides were synthesised using a 1) Applied Biosystems 381A DNA synthesiser or 2) Applied Biosystems 392 DNA/RNA synthesiser as instructed by the manufacturers. Oligonucleotides were provided either as: 1) a precipitate in a column which was broken open and the oligonucleotides beads tipped into a vial of ammonia, or 2) already eluted into ammonia. The oligonucleotides were deprotected overnight at 55°C and separated form the beads by centrifugation, if provided as described in (1), then ethanol precipitated and resuspended in distilled water. The concentration was then determined by A_{260} and the oligonucleotides were either used in this form crude or after gel purification as described below.

3.1.5 Purification of DNA fragments

a) Isolation of oligonucleotides or PCR fragments from polyacrylamide gels.

Oligonucleotides were purified on 2mm thick 15% denaturing polyacrylamide gels as described above. 50μg of crude oligonucleotides was mixed with an equal volume of formamide and heated to 85°C for 10min before loading onto the gel. The gel was prerun for 30min prior to loading and then run at 35W for 3-4 hours. A dye lane (0.05% bromophenol blue, 0.05% xylene cyanol FF, 20mM EDTA, 95% formamide) was run alongside the sample to indicate how far oligonucleotides had electrophoresed. The gel was then removed from the plates and covered with saranwrap. A precoated thin layer chromatography plate (SILG25, UV254) (Macherey-Nagel, Germany) was placed underneath the gel and a handheld shortwave UV light shone onto the gel to visualise the bands by shadowing. The oligonucleotides band was excised and placed in an eppendorf. 1ml of oligonucleotides elution buffer (0.5M ammonium acetate, 10mM magnesium acetate, 1mM EDTA pH8.0, 0.1% SDS) was added and the tube incubated overnight at 37°C with shaking. The acrylamide was pelleted by centrifugation for 5min at full speed in a

microfuge (Anderman 5415C) and the supernatant removed to a 15ml Falcon tube. A further 500µl of elution buffer was added to the remaining acrylamide, vortexed briefly, centrifuged and the supernatant added to the previous supernatant. The oligonucleotides DNA was ethanol precipitated as described in 3.1.1.

b) Isolation using low melting point (LMP) agarose gel

The band of interest in approximately 300-500µl of agarose was excised from the gel and placed in an eppendorf. 500µl of TE was added to the gel piece and the agarose melted at 65°C for 10min. The solution was then extracted twice with tris-buffered phenol, twice with phenol/chloroform followed by two chloroform (24:1 chloroform/isopropanol) extractions to remove contaminating agarose and the remaining aqueous phase was ethanol precipitated.

c) Isolation using DE81 filter paper.

The DNA band was excised from the gel, wrapped on three sides by a piece of whatman DE81 paper (soaked in 2.5M sodium chloride for 3 hours then washed 5x in dH_2O - stored at 4°C in 1mM EDTA pH8.0) and the 'parcel' placed back into the gel space with the open end facing upwards. This was then electrophoresed at 100V for a further 30min and the gel piece and filter paper removed. The filter paper was checked for the presence of DNA by short wave UV light, the excess paper trimmed and the remainder placed in an eppendorf and macerated in 450µl of elution buffer (1M sodium chloride, 50mM Tris.Cl, 1mM EDTA pH7.5) and the tube vortexed briefly. The tube was incubated at 37°C for 15min or 65°C for 3min if the fragment was larger than 2kb. The supernatant was removed and the DNA precipitated by the addition of 1.1ml of ethanol.

d) Isolation of DNA using polyallomer wool.

If the fragment to be isolated was less than 1kb then the agarose plug removed from the gel was spun through sterile polyallomer wool as described in Sambrook *et al* (1989) and the eluted DNA ethanol precipitated.

3.1.6 Cloning Techniques

DNA was subcloned into the vector pIC20H for sequencing purposes. 5μg of pIC20H was digested with 20U of the appropriate enzymes, ethanol precipitated and resuspended in 89μl of 1xTE. 10μl of 10xCIP buffer and 6U of calf intestinal phosphatase (CIP) was added and incubated at 37°C for 30min. The reaction was stopped using 5mM EDTA, 0.5% SDS and proteinase K (50μg/ml) and heated to 75°C for 10 min. The sample was then cooled to room temperature and phenol/chloroform extracted. DNA was precipitated by adding 1/10 volume of 3M NaAc pH7.0, and 3 volumes of EtOH. Following centrifugation at full speed in microfuge (Anderman 5414C) for 30min, DNA pellet was resuspended in 15μl of distilled water and 3μl used per reaction.

The DNA to be cloned was restricted and mixed with vector, and the volume of the mixture was increased to 100μl with dH_2O. After one phenol/chloroform extraction and then ethanol precipitation. DNA pellet was resuspended in 10μl of ligation buffer and 4U of T4 DNA ligase. Reaction was incubated at RT for 2 hours or at 16°C overnight. 3μl of the ligation mixture was then used to transform library efficiency DH5-α competent cells (F^-, *deo*^{R}, *rec*A1, *end*A1 D6 *hsd*R17 (r_k^-, m_k^+), *sup*E44, λ^-*thi*-1, *gyr*A, *rel*A1) according to the manufacturers instructions. Blue/white colour selection was used to detect recombinant plasmids on L-agar plates containing 100mM IPTG / 5% X-GAL.

PCR products were either directly sequenced as described in 3.1.12 or cloned into PCRscript vector (Stratagene) as described in the manufacturers protocol.

3.1.7 Probe preparation

100μg of pC53-SN3 was digested with BamHI to excise 1.8kb insert from vector, the fragment was then gel purified and digested with SmaI. The 1.58kb fragment was cut from the gel and digested with StyI to give a fragment of 0.56kb. This was again gel purified and recovered from the gel by spinning through polyallomer wool as described above. The fragment was further purified by passing through a Wizard magic PCR prep column and resuspended in 1xTE. This was then used as the p53 probe CR345 and contained conserved regions III, IV and V.

100μg pMDM-FL4 was restricted with ApaI to excise the 1.3kb insert. The insert was purified on a 0.8% LMP agarose gel as described in 3.1.5, and further purified using a Wizard PCR prep column.

Probes were either labelled using random priming and α-[^{32}P]-dCTP or end labelled with γ-[^{32}P]-ATP and unincorporated nucleotides removed by purification on a Sephadex G-25 column as instructed by the manufacturers.

3.1.8 Southern and Northern Hybridisation

Southern blotting: 30μg DNA was restricted overnight with 100u of appropriate enzyme and ethanol precipitated and resuspended in 20μl of dH_2O. Samples were run on a 0.8% agarose gel and transfered to Hybond N+ nylon membrane using the alkali blotting procedure (Sambrook *et al*, 1989). Hybridisation was carried out according to the manufacturers instructions.

Northern blotting was carried out using 20μg of total RNA in a volume of 5-10μl of DEPC-H_2O diluted in 5 volumes of freshly prepared Electrophoresis sample buffer. The sample was denatured at 65^{o}C for 15min

and 3µl of EtBr (1mg/ml) added. The RNA was electrophoresed on a 1.25% agarose gel (containing 5% formaldehyde) in 1xMOPS buffer. The gel was washed twice for 20min in dH_2O and then blotted as described in Sambrook *et al* (1989), using Hybond-N+ in 20xSSC overnight. RNA was fixed to the filter by UV autocrosslinking using a Stratalinker (Stratagene). The filter was then washed briefly in 2xSSC and either stored at 4°C or hybridised immediately.

The RNA prehybridisation mix was made as follows:

5xSSC pH7.4, 5x Denhardts, 50% (v/v) deionised formamide. This was mixed and then SDS added to a final concentration of 0.5%. Prehybridisation mix was always made fresh and only used if the final solution was clear.

40ml of prehybridisation mix plus 800µl of 10mg/ml salmon sperm DNA which had been autoclaved and sonicated with 1min bursts at maximum amplitude for 10min, was added to a single bagged filter and incubated at 42°C for 4 hours. The probe (prepared as described above) was then added and hybridised overnight at 42°C.

Filters were washed twice in 2xSSC, 0.1%SDS for 20min at room temperature or as described and then autoradiographed using XR-OMAT fast film.

3.1.9 Library Screening and λ DNA preparation

A bovine liver genomic library constructed in EMBL-3 λ phage (Clontech) was used to infect Y1090r- (*ara*D139, *hsd*R (r_k-, m_k+) *mcr*B+, *rps*^{L}, *sup*F, *trp*C22::Tn10, Δ*lac* U169, Δ*lon*, F$^-$, λ$^-$ (pMC9) host cells provided with the library. The library was titred according to the manufacturers instructions on arrival.

20ml cultures of Y1090r- cells were grown overnight at 37°C in the presence of 0.2% maltose. The cells were then transferred to 50ml Falcon tubes and spun in a Beckman J-6B centrifuge at 3000rpm for 10min. The

Y1090r- cells were then resuspended in 10ml of 10mM $MgSO_4$ and the $O.D_{550}$ read. An O.D of 1.0 was calculated to be $8x10^8$ cells. 200µl of a 1/2000 dilution of the library in SM buffer (10x; 1M NaCl, 0.1M $MgSO_4$, 0,35M Tris.Cl pH7.5 autoclaved and stored at 4°C) was added to $9x10^8$ Y1090r- cells in a Falcon 2059 polypropylene tube and incubated at 37°C for 15min. This was added to a bottle containing 50ml of top agarose (50ml LB, 0.36g agarose autoclaved and equilibrated to 42°C). This was poured onto a $25x25cm^2$ square LB (1mM MgSO4) agar plate to give approximately 30,000 plaques per plate. Plates were then incubated overnight at 37°C and then chilled in the cold room for about 1 hour. Duplicate plaque filter lifts were taken using Pall Biodyne membrane. These filters were hybridised to the CR345 fragment of the human p53 cDNA using the hybridisation technique for southern blotting as described above. Positive plaques were picked using a glass pipette and the plugs stored in 1ml of SM buffer with a drop of chloroform.

Lambda DNA was purified by the liquid lysate method as follows: 250µl of phage was incubated with 100µl of Y1090r- cells at 37°C for 15min. 4ml of LB and 5mM $CaCl_2$ was added and incubated overnight at 37°C. 75µl of chloroform was added and the tube incubated at 37°C with shaking for 15min. The cell debris was then pelleted by spinning in a Beckman J6-B centrifuge at 3000rpm for 10min at 4°C. 4µl of DNAse1 (1mg/ml) and 0.4µl of RNAseA (10mg/ml) was added to the supernatant which was then incubated at 37°C for 30min. An equal volume of 20%PEG/2M NaCl in SM buffer was added and the DNA precipitated on ice for 1 hour. The DNA was pelleted by centrifugation in a Beckman J6-B at 3000rpm for 20min at 4°C. The pellet was resuspended in 0.5ml of SM buffer and extracted with chloroform x1. Following centrifugation at full speed in Anderman (5415C) microfuge for 5min, the aqueous phase was transferred to a fresh eppendorf. 5µl of 10% SDS, 5µl of 0.5M EDTA and 0.5µl ProteinaseK (10mg/ml) was added to the

DNA followed by incubation at 68°C for 1 hour. Any contaminating proteins were removed by 1x phenol/chloroform and 1x chloroform extraction and the DNA then ethanol precipitated and resuspended in 1x TE (made with SM buffer).

3.1.10 DNA Extraction from Paraffin Sections

Sections were cut 4-5 microns thick using a microtome and put into eppendorfs. Three sections of normal bovine tissue were cut in between each tumour sample to prevent cross-contamination. 400µl of xylene was added to each sample, vortexed and left for 1min. The section was pelleted by centrifugation for 2min at full speed in a microfuge Anderman (5415C) and the waste xylene removed. The samples were dried on a heat block at 50°C for approximately 3 hours and 100µl of sample buffer (SPB) was added to the dried section. 10µl was removed and made up to 100µl with SPB and proteinase K at 0.5mg/ml (made fresh) and sonicated in a 50°C water bath for 10min. The samples were then boiled for 10min and the denatured proteins pelleted by centrifugation for 5min at full speed in a EC5415 microfuge. The supernatant was removed and 5µl was used in each PCR reaction.

3.1.11 Polymerase Chain Reaction (PCR)

All PCR reactions were carried out an 9600 Perkin Elmer Cetus PCR machine.

DNA-PCR was carried out using 1μg of genomic DNA, 1xPCR buffer as provided by the manufacturer, 1.5mM $MgCl_2$, 200μM dNTP's, 0.5μM primers and 2.5u of Taq polymerase in a 100μl PCR reaction. The primers used in the PCR are described in table 3.1.11A. The standard programme used throughout these experiments was;

94°C for 5min / (94°C 15sec, 54°C 15sec, 72°C 30sec) x35 cycles / 72°C for 5min / (extension cycle) and then 4°C hold. Samples were maintained at 4°C or -20°C until required.

3.1.12 Sequencing

Sequencing of DNA was carried out using either the dideoxy termination method of manual sequencing with a sequenase version 2.0 kit, or by using the ABI 373A automated sequencer. Dideoxy reactions were carried out according to the manufacturers instructions. Manual sequencing reactions were run on a prewarmed 6% denaturing polyacrylamide gel for 1.75 hours at 55W. The gel was then dried at 80°C for 2 hours and autoradiographed.

Automated cycle sequencing was carried out as follows. The region to be sequenced was amplified by a 20μl PCR reaction and the PCR products purified on a miniprep column (Pharmacia Biotech). 7μl of the 50μl purified product was mixed with 32ng of primer in a total volume of 12μl and 8μl of premix (dye terminator cycle sequence ready reaction) added. The method of Taq terminator sequencing was as follows;

96°C 2min, (96°C 15sec, 50°C 1sec, 60°C 4min) x25 cycles. The samples were then cooled to 4°C. The products were precipitated with 2μl 3M NaAc pH4.5 and 50μl EtOH at -70°C for 15min. The samples were then spun and the DNA pellet washed in 250μl 70% EtOH. The pellet was dried in

a speedivac for 5min then processed for loading in ABI373A according to the manufacturers protocols.

3.1.13 SSCP-PCR Analysis

A 50μl PCR reaction was prepared as follows: 1x PCR buffer, 2.5mM $MgCl_2$, 100ng genomic DNA, 40μM dNTP's, 0.1μl α-[^{32}P]-dCTP (1.11MBq), 100ng of each primer and 2.5u of Taq polymerase. PCR with LS7A and LS7B (see table 3.1.11B) was used for DNA purified from tumour tissue using the programme:

94°C 2min, /(94°C 30secs, 65°C 30secs, 72°C 30secs) x30 cycles/ 4°C until required. A nested PCR reaction was used for DNA purified from cells and paraffin embedded sections. For the nested PCR, the first round was carried out using the above programme with an annealing temperature of 55°C and primers LS7A' and LS7B' as described in table 3.1.11B. 10μl of this reaction was used in the second round at an annealing temperature of 65°C, using primers LS7A and LS7B.

SSCP gels were run using Gibco BRL S2 sequencing appararus. 3μl of 50μl PCR products were mixed with 7μl of stop buffer (95% formamide, 20mM EDTA, 0.05% bromophenol blue, 0/05% xylene cyanol FF). The samples were denatured at 80°C for 4min and snap cooled immediately in an ice water bath to prevent reannealing of DNA strands. 4μl was loaded onto a 6 and 10% polyacrylamide or 0.5x and 1x MDE SSCP gels and run overnight at 4°C or room temperature. Gels were run with 1x TBE buffer and the room temperature gels contained 5% glycerol.

Gel mixes were as follows:

6% /10%

15mls/25mls acrylamide (30%, 0.8%w/v)

7.5mls 10xTBE

52.5mls/42.1mls dH_2O to 75mls then add 150µl freshly made 25% APS and 75µl TEMED.

1xMDE

37.5mls MDE mix

4.5mls 10xTBE

29.78mls dH_2O to 75mls then as APS and TEMED added as above.

The gels were then dried and autoradiographed as described in 3.1.12.

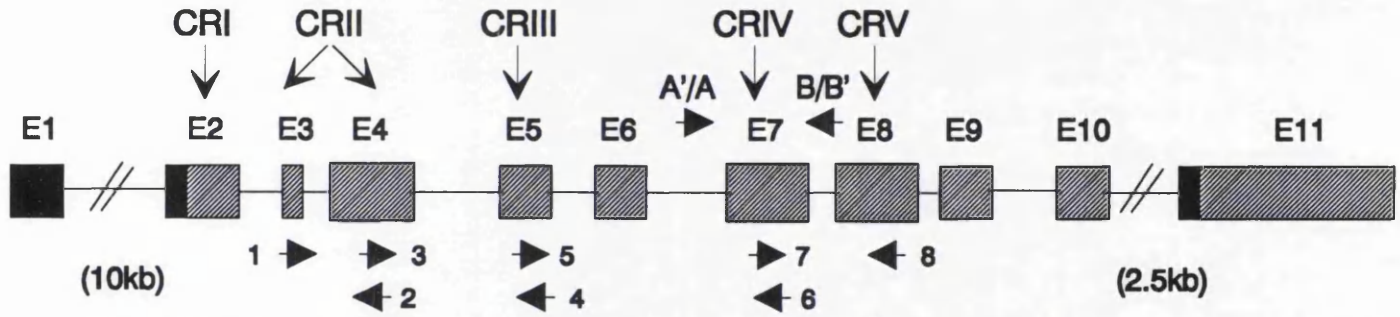


Figure 3.1.11 The human p53 gene. Coding exons are indicated by the hatched boxes, non-coding exons in black. The conserved regions are indicated by the roman numerals and the oligonucleotides designed by the numbered arrows. The tables on the facing page describe the oligonucleotides used in further detail.

Table 3.1.11A

No.	Oligonucleotide	Sequence (5'>3")
1	CRI5'	ccc tct gag tca gga
2	CRII3'R	aca gac ttg gct gt
3	CRII5'	aag acc tgc cct gtg cag
4	CRIII3'R	gcg ctc atg gtg ggg gca
5	CRIII5'	gct gcc ccc acc atg agc gct g
6	CRIV3'R	ctg gag tct tcc agt gtg at
7	CRIV5'	tac cac cat cca cta caa
8	CRV3'R	acc tca aag ctg ttc cgt cc

Table 3.1.11B

No.	Oligonucleotide	Sequence (5'>3")
A	LS7A	cct gtg ctc tct cca gat cg
A'	LS7A1	cca caa ttg aga aac agg ct
B	LS7B	tag gga gca agc tag agt gg
B'	LS7B1	ctc tta cac cat ccg cc

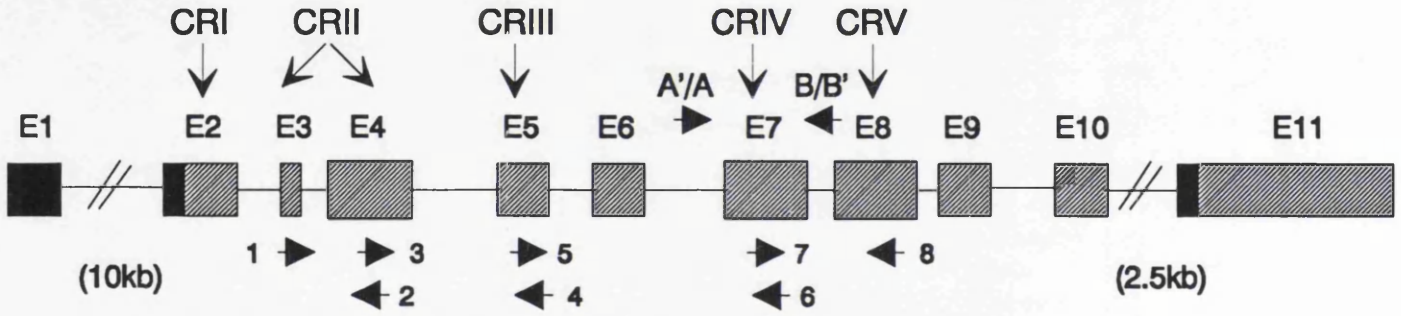


Figure 3.1.11 The human p53 gene. Coding exons are indicated by the hatched boxes, non-coding exons in black. The conserved regions are indicated by the roman numerals and the oligonucleotides designed by the numbered arrows. The tables on the facing page describe the oligonucleotides used in further detail.

Table 3.1.11A

No.	Oligonucleotide	Sequence (5'>3")
1	CRI5'	ccc tct gag tca gga
2	CRII3'R	aca gac ttg gct gt
3	CRII5'	aag acc tgc cct gtg cag
4	CRIII3'R	gcg ctc atg gtg ggg gca
5	CRIII5'	gct gcc ccc acc atg agc gct g
6	CRIV3'R	ctg gag tct tcc agt gtg at
7	CRIV5'	tac cac cat cca cta caa
8	CRV3'R	acc tca aag ctg ttc cgt cc

Table 3.1.11B

No.	Oligonucleotide	Sequence (5'>3")
A	LS7A	cct gtg ctc tct cca gat cg
A'	LS7A1	cca caa ttg aga aac agg ct
B	LS7B	tag gga gca agc tag agt gg
B'	LS7B1	ctc tta cac cat ccg cc

Figure 3.1.11 The human p53 gene and the conserved regions to which the oligonucleotides for DNA-PCR were designed. Table 3.1.11A lists the sequences of the oligonucleotides used in the PCR of the bovine p53 gene and the size of the resulting products. Table 3.1.11B lists the outer and inner primers used for the SSCP-PCR. These were designed to the flanking intronic sequence of bovine p53 exon 7 as determined by sequencing of the PCR products derived from genomic bovine liver DNA and the lambda clone isolated from the genomic library.

3.2 Cell Culture and Analysis

3.2.1 Growth of PalF cells

Cells derived from the palate of a bovine foetus were cultured in 1XDMEM. They were seeded at approximately 10^5 cells per $80cm^2$ (T80) flask and grown at 37°C with air containing 5% (v/v) CO_2 until almost confluent. At this stage the cells were washed with PBS and trypsinised with 10% (v/v) trypsin diluted in PE. 10ml of SLM containing 10% FCS was added to the flask to inactivate the trypsin and cells then centrifuged at 1000rpm for 5min in a MSE (Centaur 2), resuspended in fresh medium and reseeded.

Stocks of cells were kept frozen in liquid nitrogen. The cells were trypsinised as described above and resuspended in ice cold 1xDMEM containing 25% FCS and 10%DMSO, at a concentration of 10^6 cells per ml. Cells were aliquoted into cryotubes and placed in a polystyrene box. Cells were frozen at -70°C overnight before transfer to liquid nitrogen. Gradual freezing of the cells ensures viability on thawing. Cells were quickly thawed at 37°C and then transferred to a universal containing prewarmed 1xDMEM, centrifuged at 1000rpm for 5min in a MSE (Centaur 2) then resuspended in 1xDMEM and supplements as described above and replated as required.

3.2.2 Growth of p53 null mouse fibroblasts

Fibroblast cells were derived from 2 week old p53 knockout mice by dissecting skin tissue which was macerated and grown in 1x DMEM containing 5% penicillin, 5% streptomycin and 10% FCS. These skins were cultured until sufficient cells had grown out from the tissue to be trypsinised and expanded. Cells were grown and subcultured as described above for the PalF cells.

3.2.3 Transfection of cultured cells

All cells were transfected by the $CaPO_4$ method as described by Gorman (1985) using a total of 20μg of DNA including 2μg of pZIPneo or pBabehygro encoding geneticin and hygromycin resistance respectively, for selection.

$5x10^5$ PalF cells were plated into a T80 flask on day 1. On day 2, the DNA to be transfected was made up to 20μl and added to a tube containing 100μl 2M $CaCl_2$, 100μl 0.1xTE and 280μl of distilled water. This was mixed by adding slowly, dropwise to a separate tube containing 500μl 2xHBS. The DNA mixture was incubated at room temperature for 30min to allow a precipitate to form, and then 1ml added to a T80 flask containing 15ml medium (or 500μl total to a T25 containing 5ml medium for p53 null cells). On day 3, the cells were washed 1x in PBS and refed with fresh 1xDMEM and supplements. On day 4, the cells were trypsinised as described above and reseeded in a T175 flask. PalF cells were then selected for resistant colonies for a period of 3-5 weeks with 500μg/ml of Geneticin (G418), p53 null fibroblasts were selected for resistance for 2-3 weeks with 125μg/ml of Hygromycin B. After this period the colonies were then counted and either ring cloned or fixed with methanol for 10min and then stained with 10% Giemsa for 10min and photographed. Ring cloning was carried out as follows. The flask was cut open, the medium aspirated out and the cells washed in PBS. A 10mm cloning ring coated with silicon grease at the base was then placed over the colony and 200μl of trypsin prewarmed to 37°C was added for approximately 5min. The trypsinised cells were then transferred to a 24-well plate along with 2ml of medium and returned to the 37°C incubator. Cells were expanded and stocks frozen down in 10% DMSO, 25% FCS in 1x DMEM as described above. The PalF cells were continually passaged at identical cell densities for about 4-6 months to determine if the cell lines

established were immortal, cells were also grown in Methocel to determine anchorage independent growth and assayed for their growth rates (see MTT assay as described below). All solutions were filter sterilised and the plasmids sterilised by ethanol precipitation.

3.2.4 RNA extraction from Cell lines

Cells were grown in a 175 cm^2 (T175) flask until confluent and washed twice with ice cold PBS. 10ml of RNAzol was added to the flask which was then left on ice for 15min. The lysate was pipetted into a Falcon 2059 tube and 1ml of chloroform added, vortexed and then left on ice for 15min. The tubes were centrifuged in a Sorvall RC5B (HB6 rotor) at 10,000rpm for 15min at 4°C. The upper, aqueous phase was transferred to a fresh tube and an equivalent volume of isopropanol added. The samples were mixed and left at -20°C overnight and the RNA pelleted by centrifugation in a Sorvall RC5B (HB6 rotor) at 10,000rpm for 15min at 4°C. The pellet was partially resuspended in 75% ethanol (made with DEPC H_2O), transferred to an eppendorf and kept at -70°C until required. Before use the RNA was pelleted in a microfuge at full speed at 4°C for 30min, dried on a speedivac and then resuspended in 50-100µl of DEPC H_2O.

3.2.5 Transformation assays

3.2.5.1 Anchorage independent growth

Cells were tested for viability by the addition of 20µl of trypan blue stain (0.4% in 0.85% saline) to 80µl of cells suspended in 1xDMEM. Only the green (viable) cells were counted and $2x10^5$ cells added to 15ml Methocel medium. The cells and Methocel were poured to bacterial petri dishes and left for 10-14 days before colonies were counted and photographed with PanF 50 technical film.

Methocel medium was made up as follows:

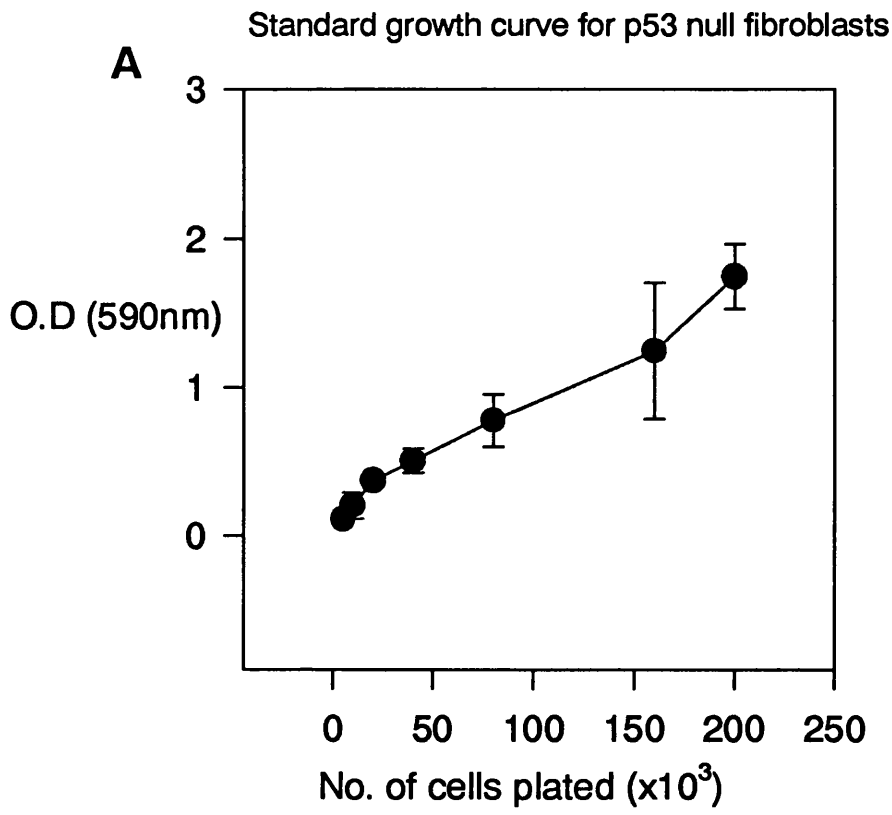
3g of Methocel agar and 200ml of distilled water was autoclaved and stirred at 4°C overnight to dissolve agar. 22ml 10x F10-HAM, 100ml FCS, 2.5ml 200mM glutamine, 5ml 7.5% sodium bicarbonate, 4ml sodium pyruvate, 4ml MEM-amino acids and 2% penicillin and streptomycin was added.

3.2.5.2 Analysis of cell growth rates (MTT assay)

$5x10^3$ viable cells were plated out in quadruplicate, in ten 96-well plates for each cell line established. Samples for each cell line were assayed at 24 hour intervals as follows: 20µl of MTT was added to the well and incubated at 37°C for 3 hours. The medium was then aspirated off and 100µl of DMSO was added to each well. The plate was shaken at room temperature for 10min. The absorbance was read at 590nm, against a medium only blank. This was carried out over a period of ten days. A calibration curve for untransfected cells was determined to confirm that O.D increased proportionally with cell number. Figures 3.2.4.2 A and B illustrate these standard curves.

3.2.5.3 Tumourigenicity in nude mice

Four $175cm^2$ flasks of cells were trypsinised to yield $3x10^7$ viable cells which were resuspended in 300µl of PBS. 100µl of the cell suspension was inoculated into an athymic nude mice at a single injection site. A minimum of three mice were used to analyse individual clones for each cell line. The mice were then checked weekly until tumours arose. Tumours were scored as positive if remaining after 12 weeks. Mice were kept for 20-30 weeks or until they had to be sacrificed as a result of tumour load. The tumours were then excised and either placed in formalin for paraffin embedding or frozen immediately in liquid nitrogen.



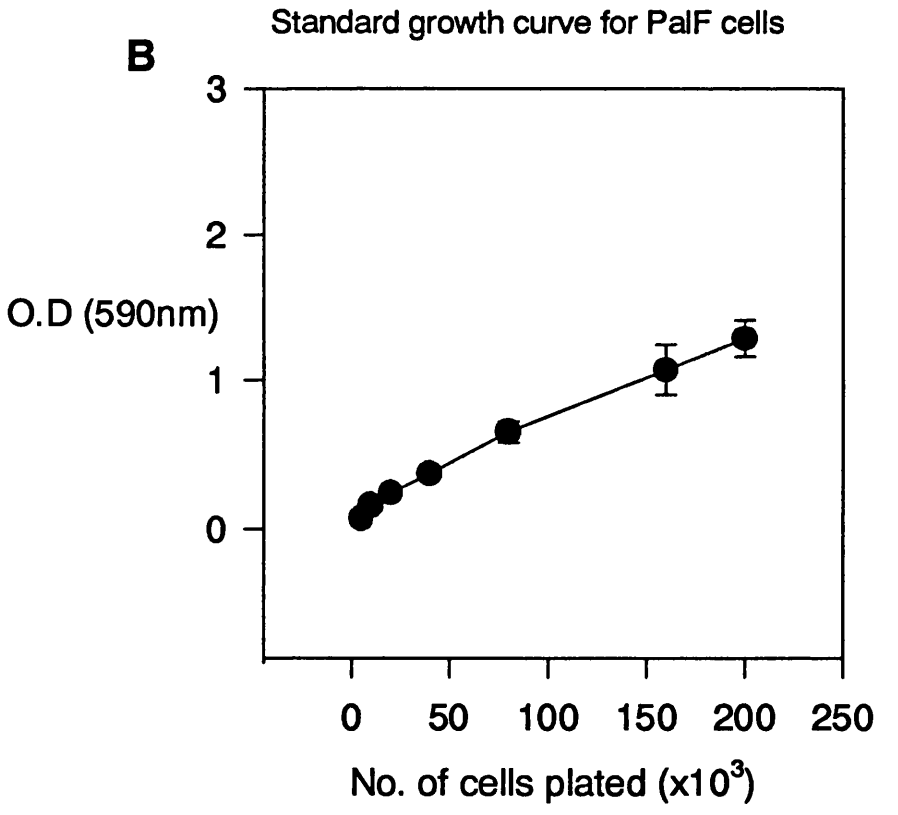


Figure 3.2.5.2 MTT assay standard curves

3.3 Immunocytochemistry

Tissue from cattle that were infected with BPV-4 was excised, placed in formalin and then embedded in paraffin. Sections were then cut 4-5 microns thick using a microtome and floated onto 3-aminopropyltriethoxysilane (APES) (Sigma-Aldrich Co.,U.K) coated slides (Diagnostic Pathology Lab 5, Veterinary Pathology, University of Glasgow). Sections were stained using the Vectastain ABC peroxidase kit according to the manufacturers instructions with the following modifications. After sections had been taken to water they were immersed in Lugols iodine for 2.5min then cleared in 5% sodium thiosulphate. Antigen retrieval by trypsinising in 0.1% (w/v) trypsin, 0.1% (w/v) $CaCl_2$ in TBS or by microwaving the sections in citrate buffer (0.01M Citric acid pH6.0) was carried out before blocking the sections with normal goat serum. Sections were incubated overnight at $4^{o}C$ with the primary antibody. Diaminobenzidine (DAB) was used to visualise the reaction and resulted in a brown stain. Sections were then counterstained with the blue nuclear stain haemotoxylin. The cells were then photographed using E6 tungsten slide film.

CM-1 antibody was used at the concentration supplied by the manufacturer. Anti-BPV-4 E7 and Anti-BPV-4 E8 were used at 1/200 and 1/2000 respectively. Antibodies were diluted in 0.1% BSA in PBS, pH7.6.

3.4 *In-situ* hybridisation

The whole BPV-4 genomic insert was excised from the vector pAT153 using BamHI and 500ng labelled with digoxigenin-11-dUTP according to the manufacturers instructions for *in-situ* hybridisation. 2µl of doubling dilutions of the labelling reaction were spotted onto Hybond-N membrane and immunological detection used to check the success of the labelling reaction using a DIG nucleic acid detection kit (Boeringher Mannheim) as instructed by the manufacturer. The paraffin sections were dewaxed in xylene and hydrated through graded alcohols to water as follows: 10min xylene, 5min 100% EtOH, 5min 70% EtOH, 5min 50% EtOH, 5min tap water. The sections were then rehydrated in PBS, pH7.2 for 5min, immersed in 0.1M glycine/PBS for 5min and immersed in 0.3% Triton X-100 for 15min and finally washed twice with PBS for 3min. The sections were then incubated with proteinase K (10µg/ml) in 0.1M Tris.Cl pH8.0, 50mM EDTA at 37°C for 30min. The sections were then fixed with 4% paraformaldehyde/PBS for 5min and washed twice with PBS for 3min. The sections were immersed in 0.25% acetic anhydride/0.1M triethanolamine pH8.0 for 10min and prehybridised with 50% formamide, 2xSSC at 37°C for 2 hours. The slides were then drained and 20µl *in-situ* hybridisation mix added containing a 1/10 dilution of the BPV-4 DNA probe. The sections were covered with glass coverslips, heated at 80°C for 10min, and then incubated in a moist chamber overnight at 42°C. The coverslips were removed by immersion in 4xSSC and the sections washed for 10min in 4xSSC, 2xSSC for 10min and 0.1xSSC at 37°C for 30min. The DIG detection reaction was carried out according to the manufacturers instructions with a 1/200 dilution of the anti-DIG antibody. The sections were then counterstained with 5% aqueous eosin and mounted in a permanent water mountant. The above method was adapted from Hamid *et al* (1987).

3.5 Fluorescence In-Situ hybridisation (FISH)

Metaphase chromosomes were prepared with standard methods from peripheral blood lymphocytes obtained from cattle. The bovine p53 lambda clone and the cosmids ETH3, ETH1 and MAP1B, were labelled by the incorporation of biotin-14-dATP using the BioNick nick translation kit. In-situ hybridisation, stringency washes and detection of the biotinylated probes with fluorescein-avidin DCS and one round of amplification with biotinylated anti-avidin antibody (Vector Laboratories), were carried out as described by Toldo *et al* (1993). Preparations were mounted in Vectorsheild antifade medium (Vector Laboratories) and were viewed with a BioRad MRC600 confocal laser scanning microscope.

3.6 Production of antibody against bovine p53

The bovine p53 peptide sequence CSDYSDGERRGLWMG was chosen based on the human and mouse epitopes mapped by Yewdell *et al* (1986). This peptide was synthesised and conjugated onto the large antigenic peptide KLH (Keyhole Limpet Haemocyanin) by Thistle Peptide Services (Dept. of Biochemistry, University of Glasgow). Two rabbits were inoculated with 100μg of KLH-bp53 immunogen at weekly intervals. The peptide was mixed with 600μl of adjuvant and syringed with a 24 gauge needle to produce an emulsion. The first injection was mixed with Freunds complete adjuvant and the second and third injections with Freunds incomplete adjuvant. After the third injection (preboost) a test bleed was taken to ensure antibodies were being produced. After 8-10 weeks a fourth injection (boost) of immunogen was given and 10 days after the fourth injection the rabbits were euthanased and bled out. The blood was incubated at 37^{o}C for 30min and the clots 'ringed' with a glass pasteur pipette. The blood was then stored overnight at 4^{o}C and centrifuge at 2000rpm for 5min in a Beckman J6-B. The serum supernatant was then aliquoted and stored at -70^{o}C until use. Figure 3.6 illustrates the

antibody titre achieved after the 3rd injection (preboost) and after the 4th injection (boost). This antibody was also tested on paraffin sections and staining compared with that of CM-1.

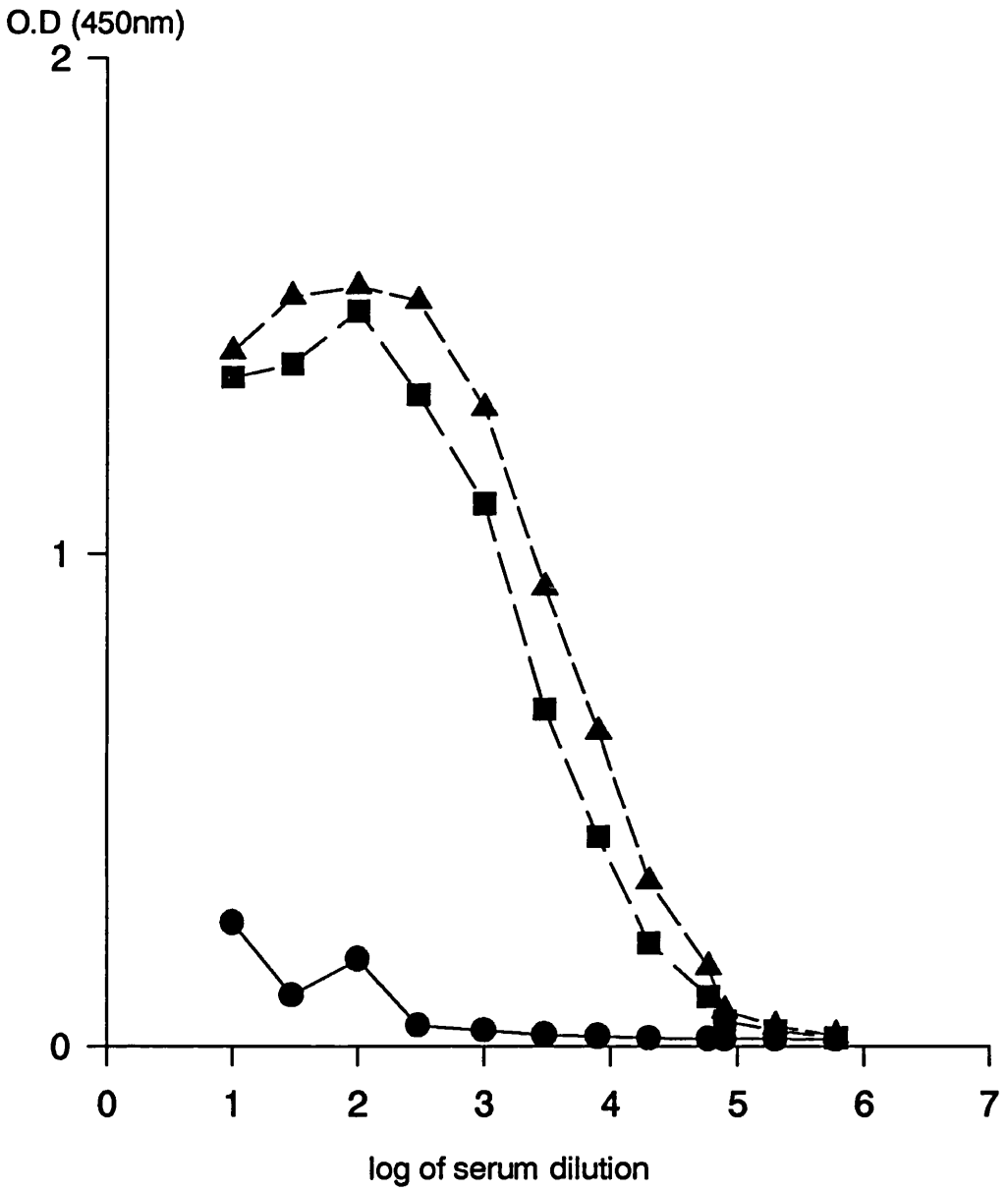


Figure 3.6 Antibody titre for rabbit 1 inoculated with KLH-bp53.

RESULTS

CHAPTERS FOUR - SIX

Introduction

BPV-4 lacks an E6 ORF, as do other members of the subgroup B papillomaviruses. All other known papillomaviruses encode an E6 oncoprotein and E6 has been demonstrated to contibute to cell transformation in cooperation with the E7 oncoprotein (Storey *et al*, 1994; Munger *et al*, 1992; Sedman *et al*, 1991, Hawley-Nelson *et al*, 1989). One function of the E6 oncoprotein from the high risk papillomaviruses is to bind and degrade the tumour suppressor p53 (Scheffner *et al*, 1993; Scheffner *et al*, 1992b; Werness *et al*, 1990; Scheffner *et al*, 1990). As BPV-4 lacks an E6, it was unclear if and how p53 functions were abrogated during papilloma formation by BPV-4 and progression of these lesions to carcinoma. It was suggested that p53 was altered or that other factors were involved in these processes (Jackson *et al*, 1991). These observations raised several questions which have been addressed in this thesis.

1) What is the status of the bovine *p53* gene in BPV-4 associated papillomas and carcinomas?

2) Does BPV-4 lack E6 associated functions?

3) Can the addition of an exogeneous HPV-16E6 confer additional functions *in vitro*?

Chapters Four and Five will deal with the isolation and characterisation of the wild type bovine *p53* gene and the p53 status in BPV-4 associated papillomas and carcinomas. Chapter Six describes the *in vitro* transformation of primary foetal bovine fibroblasts (PalF) and p53 deficient mouse fibroblasts by BPV-4 and HPV-16E6. The effect of the addition of an exogeneous human mutant p53 to PalF cells in the presence of BPV-4 will also be described. Transfection of p53 deficient fibroblasts with BPV-4 and HPV-16E6 will determine if any additional functions provided by HPV-16E6 are p53 independent.

CHAPTER 4

Characterisation of bovine p53

4.1 p53 isolation

To determine the bovine p53 status, the wild type *p53* gene was isolated for comparitive studies.

A human wild type *p53* cDNA probe was prepared as described (materials and methods section 3.1.7) and used to screen a bovine liver genomic DNA library. Positive plaques were visualised by southern hybridisation and subjected to four rounds of plaque purification until all of the plaques were positive as illustrated in figure 4.1.1. Four of the positive plaques were picked for further characterisation and the phage DNA isolated as described (materials and methods section 3.1.9), one isolate was selected for further analysis. The phage DNA was digested with several restriction enzymes and the size of the resulting fragments estimated according to the molecular weight markers. Figure 4.1.2A illustrates the ethidium bromide stained restriction fragments and the subsequent southern hybridisation (Figure 4.1.2B) to confirm the presence of *p53* DNA sequences.

Digestion of the isolated phage DNA with the restriction enzyme BamHI released an insert of 14kb from the lambda vector DNA (Figure 4.1.2A, lane 2). EcoRI cut lambda DNA yielded a fragment of 8kb and two bands of 3kb and 2.5kb respectively (Figure 4.1.2A, lane 3), SstI digests resulted in only two fragments suggesting that this restriction site may lie within the insert as there are no SstI sites within the lambda vector DNA (Figure 4.1.2A, lane 4). The XbaI digests yielded fragments at 4.6kb, 4.0kb and a band at about 15kb which incorporated the 9kb lambda fragment of the lambda vector DNA (Figure 4.1.2A, lane 5). XhoI did not cut the the phage DNA suggesting that

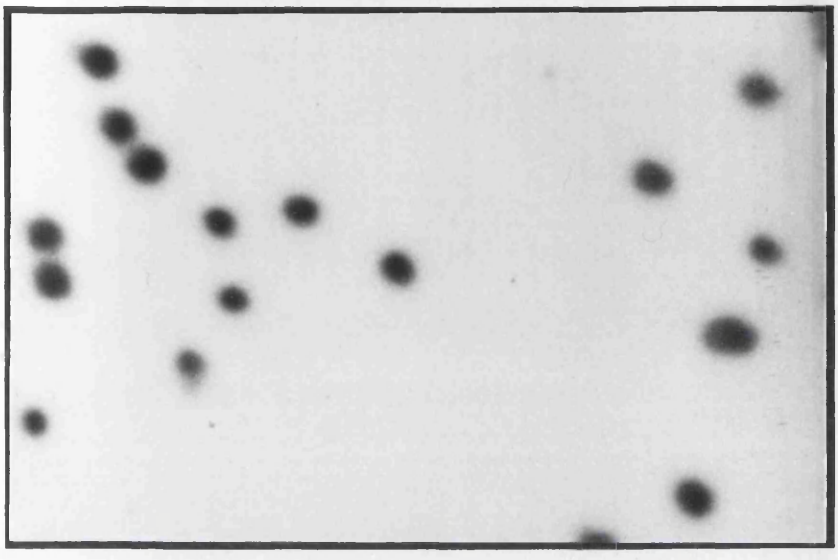


Figure 4.1.1 Fourth round screening of selected positive plaques and Southern hybridisation with the human *p53* cDNA fragment probe (CR345) to plaque lift filter. The dark spots on the autorad indicate positive plaques which corresponded exactly to the plaques on the agar plate (data not shown). Four of these plaques were picked and phage DNA isolated as described (materials and methods section 3.1.9).

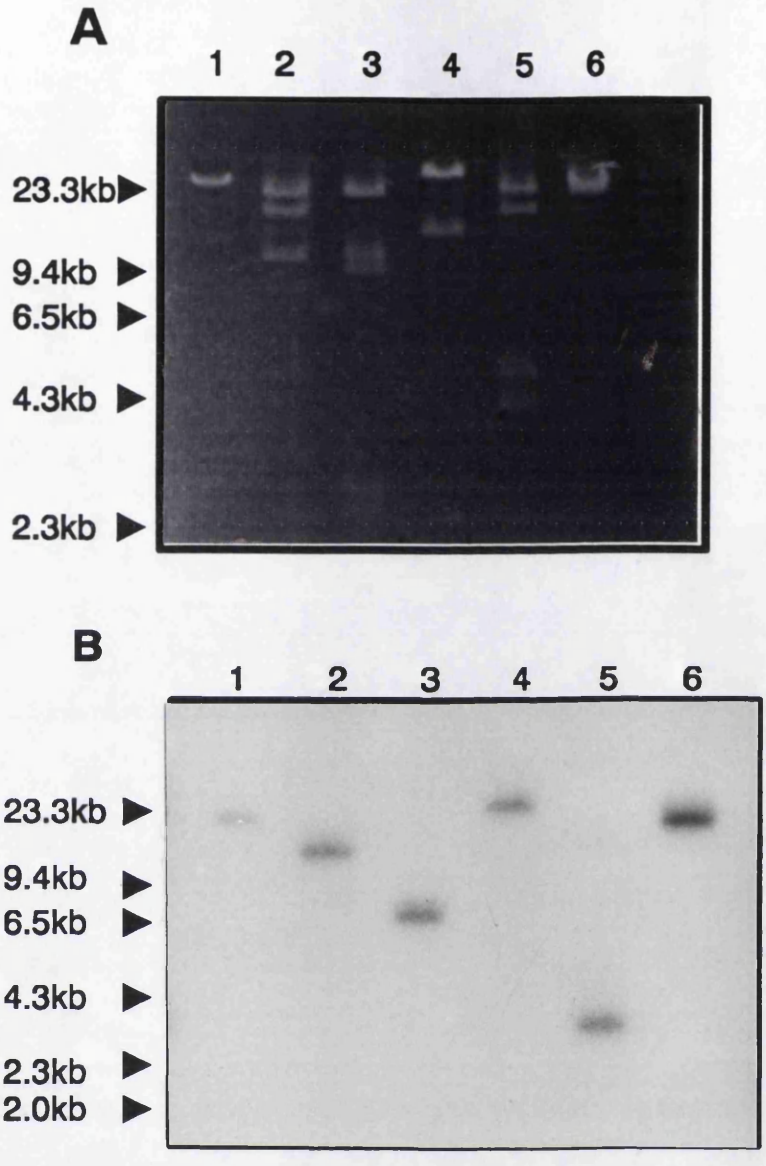


Figure 4.1.2 Restriction digests of isolated lambda phage A) stained with ethidium bromide and B) southern hybridisation using CR345 of human p53 as probe. The phage DNA was digested with the following enzymes: Lane 1: undigested λ phage DNA, 2: BamHI cut, 3: EcoRI cut, 4: SstI cut, 5: XbaI cut, 6: XhoI cut. Positions of the λ HindIII cut markers are indicated on the left.

there are no XhoI sites within the insert (Figure 4.1.2A, lane 6).

Southern hybridisation with the human *p53* cDNA fragment probe (materials and methods section 3.1.7) revealed that the region of the bovine *p53* gene containing the sequences corresponding to conserved regions 3, 4 and 5 (CR345) of the human cDNA probe were present in the 14kb, 8kb, 29kb and 4.0kb for digests BamHI, EcoRI, SstI and XbaI respectively (figure 4.1.2B).

Oligonucleotide primers designed to match the conserved regions of human p53 (materials and methods section 3.1.11), were used to generate PCR products from both bovine liver genomic DNA and DNA from the isolated lambda phage clone. Figure 4.1.3 illustrates the PCR products generated from both the bovine and the human control DNA. The predicted sizes for amplification of human *p53* conserved regions (CR) 1 to 2, 3 to 4 and 4 to 5, are 520bp, 767bp and 470bp respectively (Lamb and Crawford 1986; human p53 cDNA Genebank accession number KO1399) . The observed bands amplified from the human genomic DNA of approximately 500bp (CR1/2), 750bp (CR3/4) and 500bp (CR4/5) are indicated in figure 4.1.3A and B. The amplified product for bovine *p53* conserved region 1 to 2 was 800bp, for conserved region 3 to 4, 280bp (Figure 4.1.3A) and conserved region 4 to 5 around 480bp (Figure 4.1.3B). The PCR products generated from bovine *p53* conserved regions 1/2 and 3/4 appear to be larger and smaller than the human *p53* products respectively (Figure 4.1.3A). This would suggest that the intronic sequence between bovine *p53* conserved region 1 and 2 (exons 2 to 5) and conserved regions 3 and 4 (exons 5 to 7), varies in the bovine *p53* gene in comparison to the human p53 sequence. The predicted size for amplification of the ovine *p53* gene conserved regions 3 to 4 is less than 500bp (Dequiedt *et al*, 1995a), which is smaller than that of the human *p53* gene suggesting that this

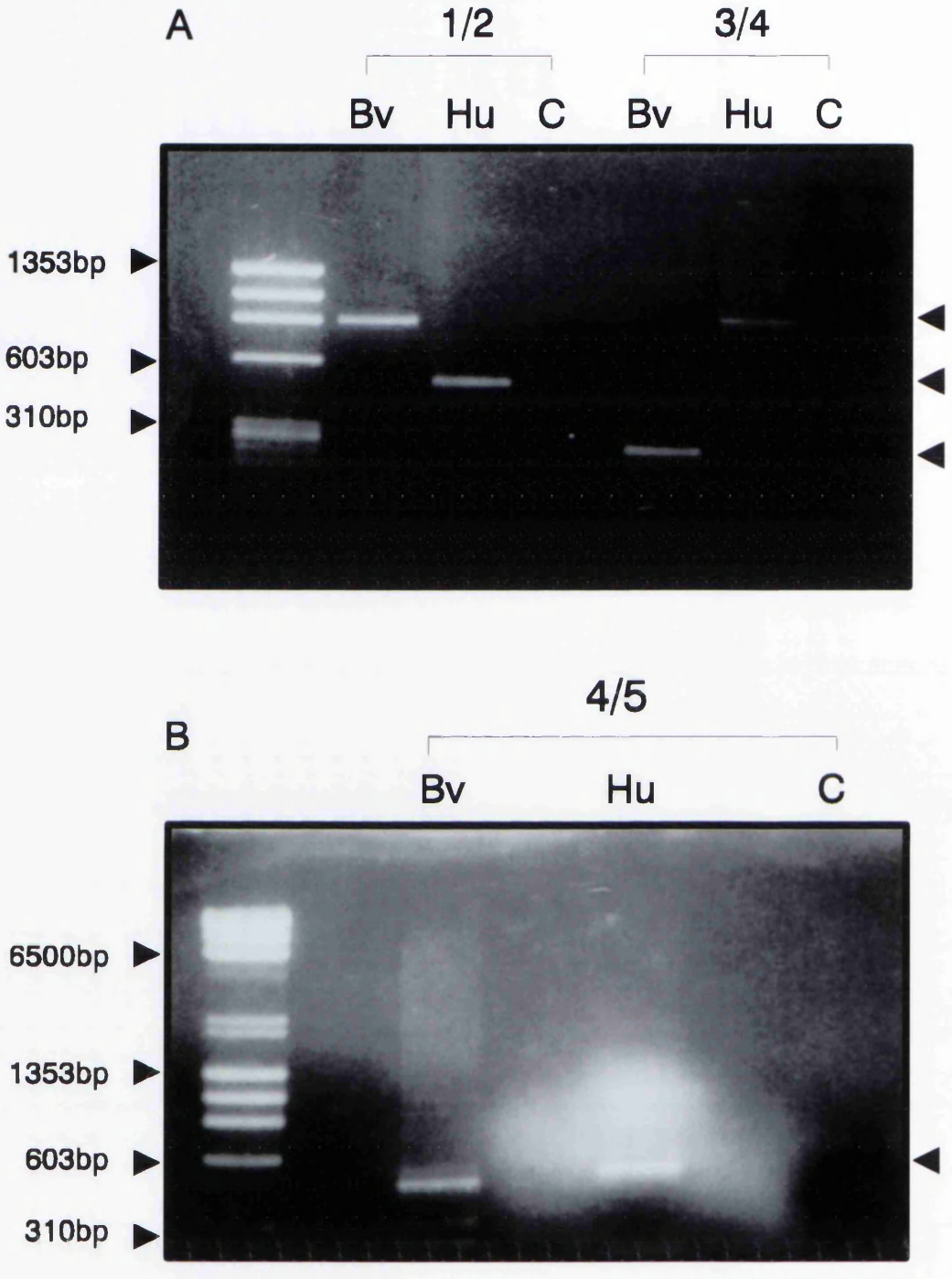


Figure 4.1.3 PCR products generated from both human and bovine genomic DNA. Figure A illustrates the amplification of p53 conserved regions 1 to 2 and 3 to 4.
Figure B illustrates amplification of conserved regions 4 to 5.
Bv - bovine, Hu - human, C - negative control primers only.

difference may be due to species variation as ungulates are several million years evolutionarily diverged from man. Amplification of a bovine pseudogene appears unlikely as only a single product was amplified.

The PCR products from both bovine genomic DNA and the lambda isolate were cloned into PCRscript (Stratagene) (materials and methods 3.1.6) and both manual and automated sequencing was used (materials and methods 3.1.12) to confirm the presence of *p53* sequences. Sequencing of the fragments representing conserved regions 3/4 and 4/5 generated the sequence shown in figure 4.1.4A and B. The data shown here confirms that the amplified PCR products did indeed contain sequences of the corresponding bovine *p53* gene as previously suggested by Southern hybridisation. The complete cDNA sequence of the bovine *p53* cDNA (387 AA) has recently been published by Dequiedt *et al*, (1995b). The sequence obtained from the PCR products was in complete agreement with that of Dequiedt *et al* (1995b), however, additional sequences flanking exon7 of bovine *p53* were characterised as shown in figure 4.1.4A.

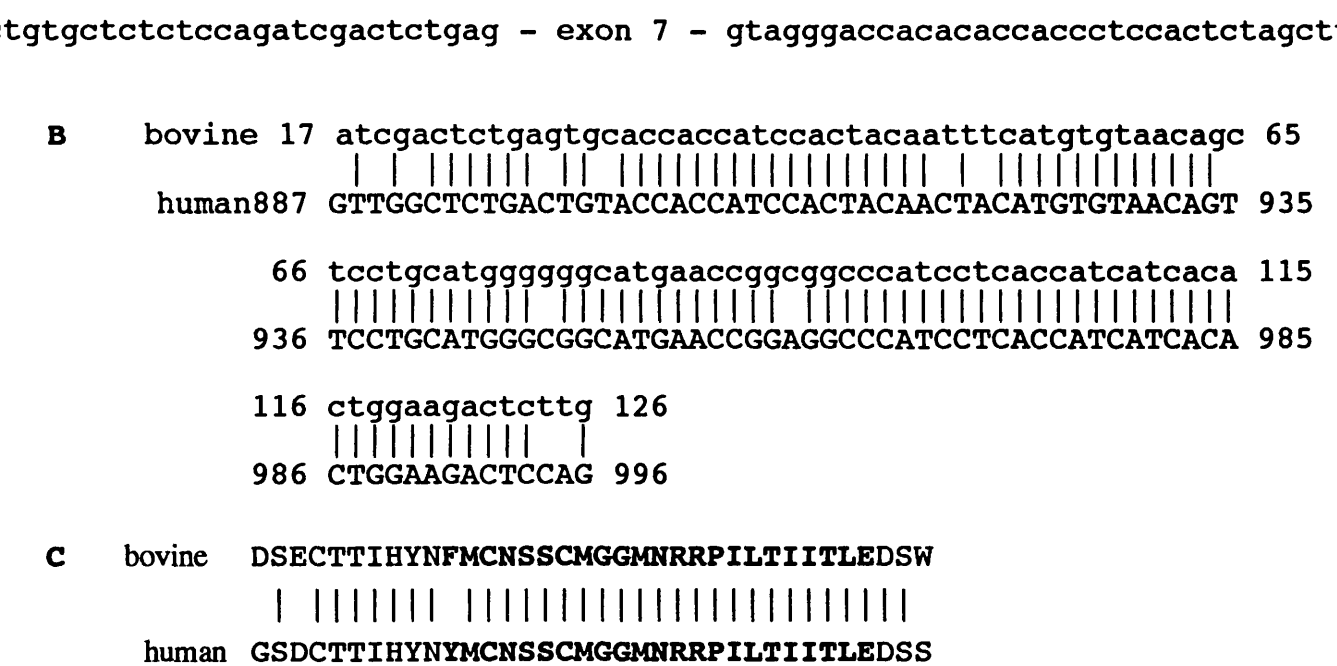


Figure 4.1.4 A, Flanking intronic sequence for bovine p53 exon 7. B, Bovine p53 sequence aligned with human p53 exon 7 sequence from 5' to 3' splice junction (accession number KO3199). Data shows 89% homology between the two species at the nucleotide level. C, Translation of the bovine exon 7 aligned with human p53 exon 7 demonstrating only one amino acid change (96% homology) over conserved region IV (indicated in bold). The vertical lines indicate the homology of the nucleotides or amino acids.

4.2 Chromosomal localisation of bovine *p53*

The phage DNA isolated from the bovine liver genomic DNA library was used to localise the bovine *p53* gene to its specific bovine chromosome. The bovine *p53* lambda clone was biotinylated and hybridised to metaphase chromosomes isolated from bovine peripheral blood lymphocytes by fluorescence *in-situ* hybridisation (FISH) as described (materials and methods section 3.5). Arranging the chromosomes by size indicated that bovine p53 was present on one of chromosomes 19-22, however the acrocentric nature of bovine chromosomes makes them difficult to assign by size alone. Chromosomal markers specific for loci on bovine chromosomes 19, 20 and 21 were available and are indicated in Table 4.2.1 below. To date, there are no specific markers available for bovine chromosome 22.

Marker	Chromosome
ETH 3 (Barendse *et al*, 1994)	19
MAP1B (Barendse *et al*, 1994)	20
ETH 1 (Toldo *et al*, 1993)	21

Table 4.2.1 Chromosomal markers used to colocalise bovine *p53*.

The above cosmids were biotinylated and cohybridised with the bovine *p53* lambda clone. *In-situ* hybridisation of the cloned bovine *p53* gave a signal near the centromere on one pair of bovine metaphase chromosomes. Neither ETH1 (figure 4.2.1A), nor MAP1B (data not shown) colocalised with the bovine p53 clone as a signal was detected on two separate pairs of chromosomes. FISH of

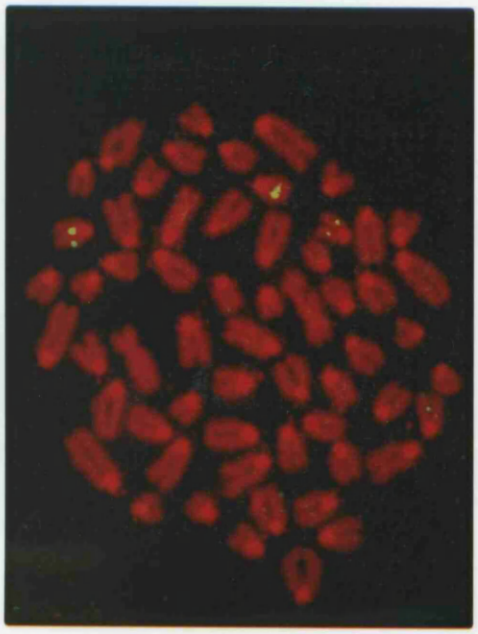
Figure 4.2.1 Chromosomal localisation of the bovine *p53* gene by FISH. Chromosome spreads of bovine peripheral blood lymphocytes were stained with propidium iodide and hybridised with the fluorescent probes.

Panel A demonstrates that bovine *p53* lambda clone does not colocalise with the ETH1 (21q17) probe.

Panel B illustrates that the bovine *p53* clone and the chromosome 19qter probe ETH3 cohybridise *in situ* to the same chromosome.

Panel C depicts the bovine *p53* gene assignment to 19q15 according to the ISCNDA 1989 (1990).

A



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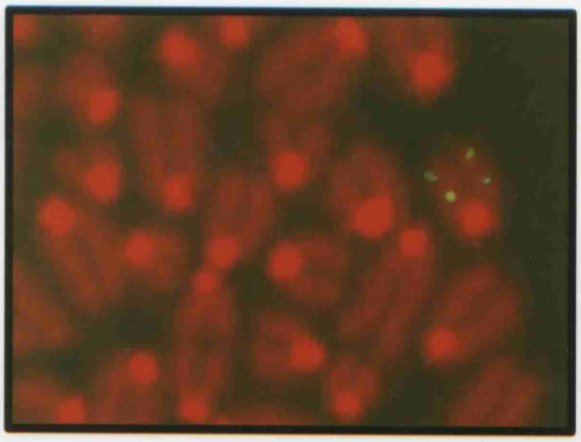

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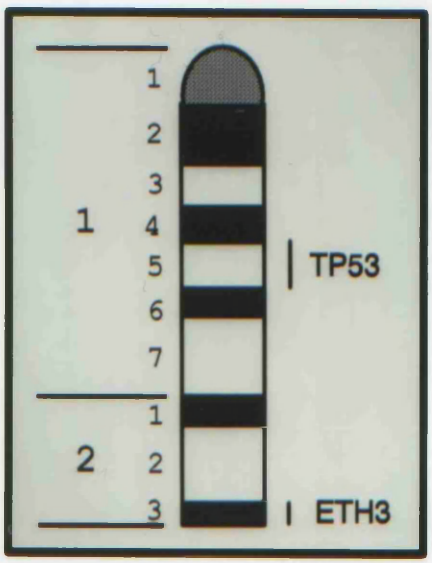


the ETH 3 probe alone confirmed that it hybridised near the telomere of chromosome 19 (Toldo *et al*, 1993). Cohybridisation of both ETH3 and the bovine *p53* lambda clone gave four fluorescent signals on chromosome 19 as illustrated in figure 4.2.1B. As previously observed, the bovine *p53* probe hybridised near the centromere and the ETH3 signal was telomeric. Figure 4.2.1C, illustrates the position of the bovine *p53* with respect to ETH3, with p53 located proximal to the centromere on band 19q15.

Mammalian species genome mapping has proceeded to such a point that selected genetic loci can serve as anchored reference markers for the comparitive genetic analysis of mammalian and vertebrate species (O'Brien *et al*, 1993). For example, Homeobox region 2 (HOXB), the protein kinase C α polypeptide (PRKCA) and the growth hormone (GH) gene are all located on bovine chromosome 19 (Fries *et al*, 1993; Gunawardana and Fries, 1992; Hediger *et al*, 1990). The above genes have been localised to human chromosome 17qter (O'Brien *et al*, 1993; Leach *et al*, 1989; Benchimol *et al*, 1985). Similar genes have also been identified in mouse and are listed in table 4.2.2. This would suggest that the chromosomal localisation of these genes are conserved among species.

Gene	Bovine	Mouse	Human
p53	19q15 (Coggins *et al*, 1995)	11 (Rotter *et al*, 1984)	17p13 (Benchimol *et al*, 1985)
HOXB (HOX-2)	19qter (Gunawardana and Fries, 1992)	11 (O'Brien *et al*, 1993)	17q21-22 (Leach *et al*, 1989)
GH	19qter (Hediger *et al*, 1990)	11 (Buckwalter *et al*, 1991)	17q23-25 (Leach *et al*, 1989)

Table 4.2.2 Syntenic relationships between species.

Conclusions and Discussion

To determine the status of the bovine *p53* gene in BPV-4 associated carcinogenesis, it was necessary to clone and sequence the wild type bovine p53 gene. A lambda clone isolated from a bovine liver genomic DNA library was shown to contain *p53* related sequences by southern hybridisation with a human p53 fragment cDNA probe. PCR products from the lambda clone were sequenced and found to be homologous to the conserved regions of p53 identified from other species. The recently isolated bovine *p53* cDNA sequence (Dequiedt *et al*, 1995b) was also in agreement with our data.

Chromosomal localisation of the bovine *p53* gene to 19q15 (Coggins *et al*, 1995) confirmed that bovine chromosome 19 was syntenic with both the short arm of human chromosome 17 and mouse chromosome 11 suggesting that this part of the chromosome is conserved among species.

Thus, bovine p53 gene was found to be homologous to human p53 as identified by sequencing of its conserved regions and to be syntenic with both human and mouse in its chromosomal localisation.

CHAPTER 5

Status of bovine p53 in BPV-4 associated papillomas and carcinomas

Introduction

BPV-4 infects the mucosa of the alimentary tract and results in the formation of benign papillomas. Additional cofactors such as immunosuppressants and mutagens found in bracken fern, provide the opportunity for a widespread, persistent infection to occur. The result of this can be progression to carcinoma of the alimentary canal. The morphogenesis of papillomas induced by BPV-4 proceeds through a series of distinct stages based on size, appearance and histology (Jarrett, 1985). Raised plaque-like lesions or stage I papillomas appear a few weeks post infection with BPV-4 and productive viral infection is apparent at these stages. Stage II lesions possess an archetypal papilloma structure with keratinised fronds and large amounts of viral DNA are present in the upper differentiating layers of these papillomas. After several months the papillomas progress to the mature stage III form which is similar to stage II except that levels of viral replication and virion production are reduced. Most papillomas regress spontaneously through a cell mediated immune response (stage IV) and recent evidence indicates that infiltrating lymphocytes have a role in this process (Knowles *et al*, 1996). The normal bovine alimentary mucosal epithelium consists of i) a cubiodal, mitotically active basal cell layer, ii) suprabasal cell layer, iii) spinous layer and iv) a squamous layer (illustrated in figure 5.1). In the papilloma,

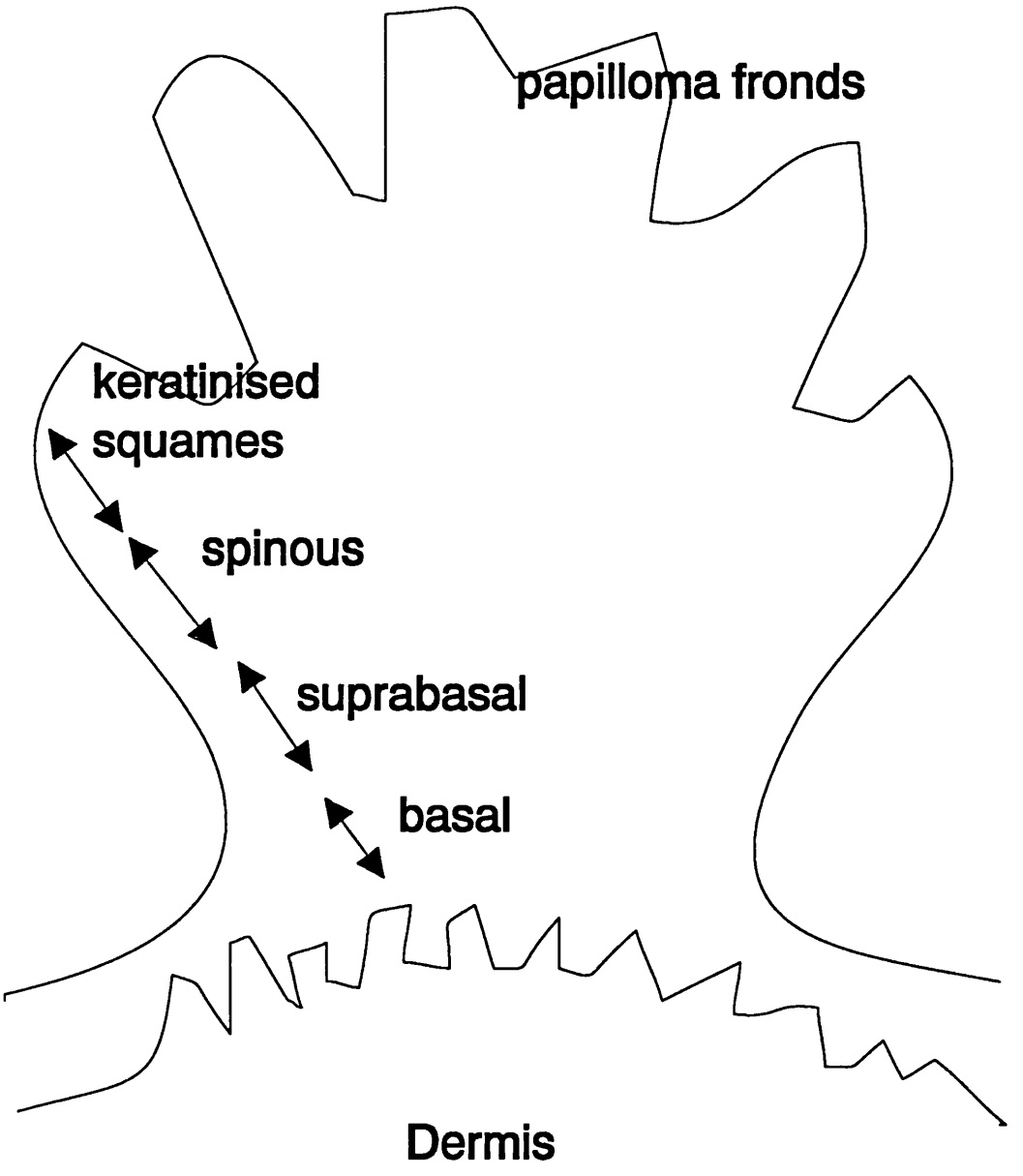


Figure 5.1 Schematic representation of the differentiated layers in a mucosal papilloma.

these layers differentiate and the squamous layer becomes cornified on the external surface of the papilloma.

5.1 Are p53 levels elevated in BPV-4 induced papillomas?

Introduction

To determine the p53 status in BPV-4 associated papillomas, a panel of samples representing the progression described above were removed from animals experimentally infected with BPV-4. An oesophageal carcinoma which had arisen from a BPV-4 induced papilloma was included to represent the advanced state when environmental cofactors are present.

Wild type p53 is undetectable by immunocytochemistry due to the short half life of the protein (Oren *et al*, 1981; Matlashewski *et al*, 1986). Stabilisation of the p53 protein by mutation or complex with other cellular or viral factors allows p53 detection with antisera as does upregulation of the wild type p53 protein by DNA damage (Bartek *et al*, 1990a, 1990b; Bartek *et al*, 1991; Kastan *et al*, 1991).

In vitro studies have shown that BPV-4 E7 (4E7) is necessary for morphological transformation of primary bovine foetal palate cells (Pennie *et al*, 1993). 4E7 possesses conserved motifs which contribute to the transforming and immortalising activity of other E7 homologues (Barbosa *et al*, 1990; Storey *et al*, 1990; Watanabe *et al*, 1990; Phelps *et al*, 1992; McIntyre *et al*, 1993). Cattle vaccinated with a 4E7 subunit vaccine demonstrated early regression of BPV-4 induced papillomas (Campo *et al*, 1993) confirming that 4E7 has a role in the progression of BPV-4 infected lesions.

To determine if the bovine p53 protein was stabilised when viral infection was present, we analysed serial sections of experimentally induced BPV-4 lesions. The presence of viral DNA and expression of the 4E7 oncoprotein was also determined.

Results

p53 and 4E7 proteins were detected by immunocytochemistry and viral DNA detected by *in-situ* hybridisation (materials and methods sections 3.3 and 3.4).

Both normal palatine tissue and the oesophageal carcinoma were examined for the presence of viral DNA, 4E7 and p53. Neither E7, nor p53 could be detected by immunocytochemistry in normal palate tissue (Figure 5.1.1A; E7 antiserum) or in the carcinoma (Figure 5.1.1B; p53 antibody). Viral DNA was absent in normal palate tissue and in the oesophageal carcinoma (Figures 5.1.1C and 5.1.1D). This correlated with previous findings that BPV-4 DNA is lost during carcinogenesis and is not required for maintenance of the transformed phenotype (Campo *et al*, 1985).

The absence of 4E7 in normal mucosal palatine tissue was expected as no viral infection was present. Lack of 4E7 expression in the carcinoma was attributed to the loss of viral DNA at this stage. As mentioned above, wild type p53 is virtually undetectable by immunocytochemistry and it is assumed that this is the case in the normal palatine tissue sample as no p53 was detected (Figure 5.1.1B). The absence of p53 staining in the carcinoma may indicate that p53 expression is unaltered. Alternatively, the p53 antibody, CM-1, may

Figure 5.1.1 (on facing page).

Immunocytochemistry of normal palatine tissue and oesophageal carcinoma.

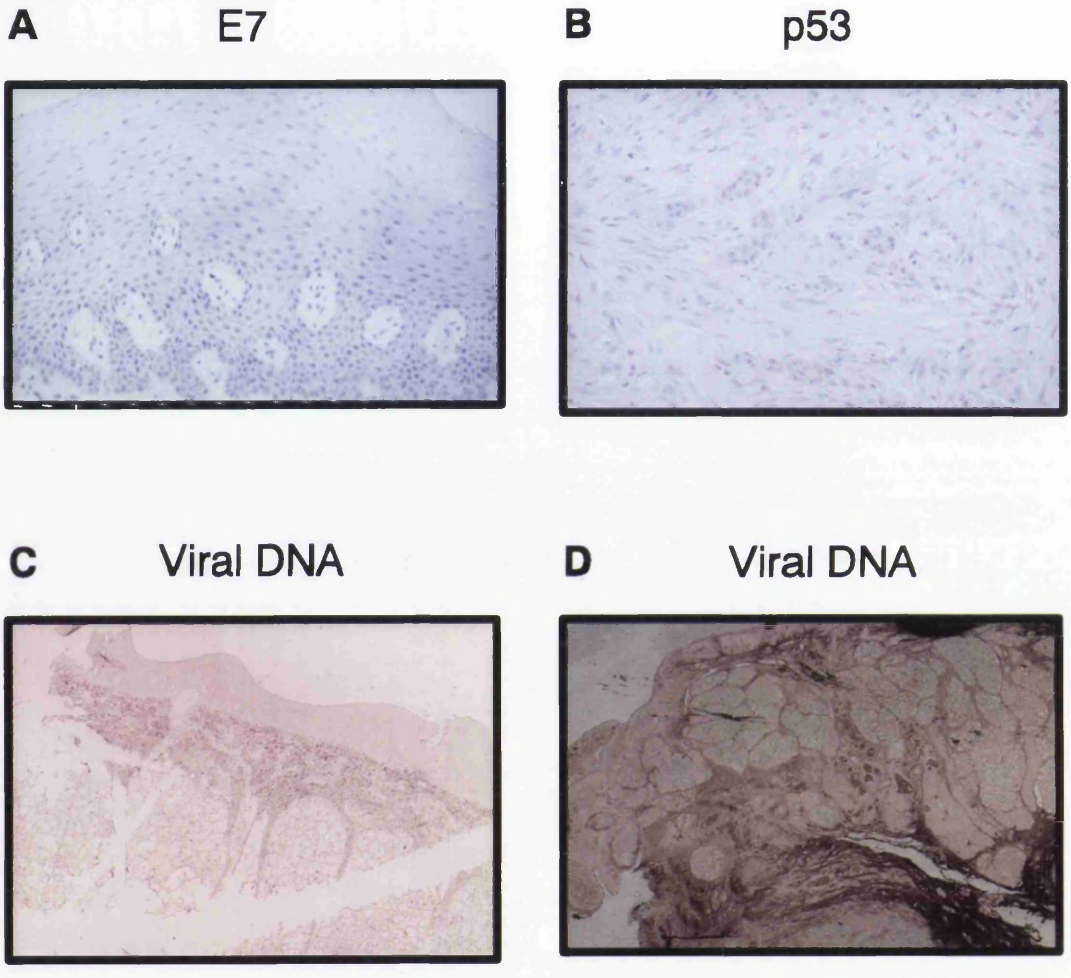
The absence of immunoperoxidase detection of;

A: the BPV-4 transforming protein E7 in normal palatine tissue; compare with the positive staining in Figure 5.1.3 A-C;

B: the tumour suppressor protein p53 in an oesophageal carcinoma; compare with positive staining shown in Figure 5.1.4A and B. Magnification is X50 in A and X100 in B.

Absence of detection of viral DNA by *in-situ* hybridisation.

Sections of normal (C) and oesophageal carcinoma tissue (D) were incubated with a BPV-4 DNA probe in the hybridisation mixture. Compare with positive *in-situ* hybridisation in Figure 5.1.2A, D and E. Magnification is X 25 in both C and D.


A
E7
B
p53
C
Viral DNA
D
Viral DNA

not recognise mutated bovine p53, however, this is unlikely, as the antibody was raised against the N-terminus of the protein and mutations generally occur in the central domain of p53.

Another possibility is that the p53 gene may be deleted resulting in the absence of a p53 protein.

Analysis of the papillomas by *in-situ* hybridisation detected high copy numbers of viral DNA in the differentiating spinous and squamous layers of papillomas (Figures 5.1.2-5.1.5). Few copies of viral DNA were present in the plaque-like lesion (Figure 5.1.2C) although this does not exclude viral DNA from other cells. Detection of viral DNA by *in-situ* hybridisation is limited to the presence of greater than 30 viral copies per cell. Viral copy numbers were high in stage I papillomas (Figure 5.1.2A) and positive staining was absent in the control sections (Figure 5.1.2B). At stage II, higher levels of viral DNA were detected (Figure 5.1.2D) and at stage III, viral DNA was present in all fronds of the papilloma (Figure 5.1.2E).

Expression of the 4E7 oncoprotein varied depending on the stage of the papilloma and was found to be both cytoplasmic and nuclear. A stage I hyperplastic papilloma demonstrated that 4E7 was expressed in the cytoplasm (Figure 5.1.3A). 4E7 expression was detected from the suprabasal to the superficial spinous cell layers. At stage II and III, 4E7 expression was found to be both nuclear and cytoplasmic. Cytoplasmic 4E7 was expressed in the spinous layers (Figure 5.1.3B) and nuclear 4E7 expressed in the basal and suprabasal cell layers (Figure 5.1.3C). As a negative control, a 1 in 10

Figure 5.1.2 (on facing page)

Detection of viral DNA by *in-situ* hybridisation.

Sections taken from a stage I papilloma were incubated with a BPV-4 genomic probe (A) or without probe (B) in the hybridisation mixture. Sections of a plaque-like (C), stage II (D) and stage III (E) papillomas incubated with BPV-4 genomic probe.

Arrows indicate the positively stained cells.

Magnification X 25 (A,B and D); magnification X 50 (C and E).

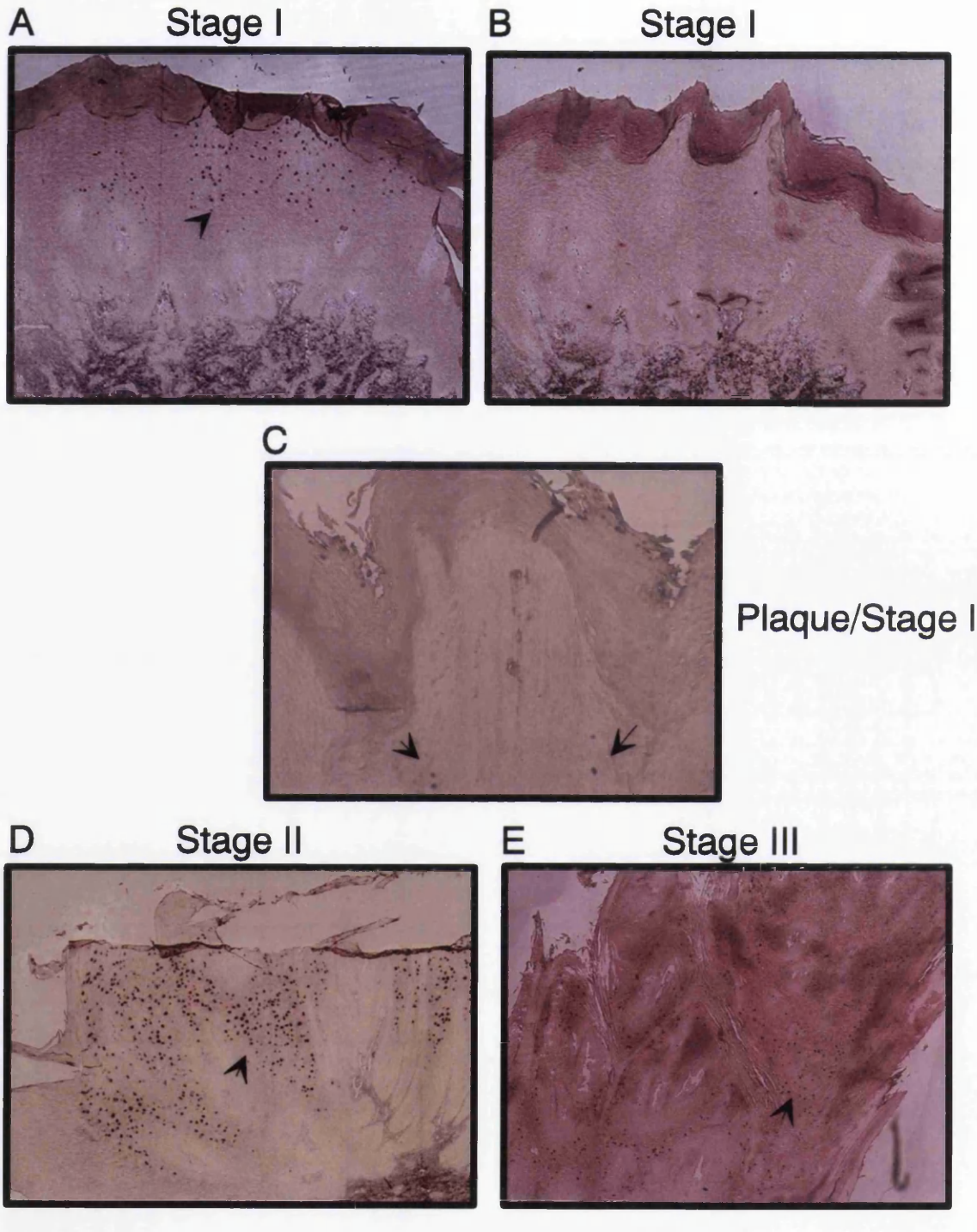

A
Stage I
B
Stage I
C
Plaque/Stage I
D
Stage II
E
Stage III

Figure 5.1.3 (on facing page)

Immunoperoxidase detection of the BPV-4 transforming protein E7.

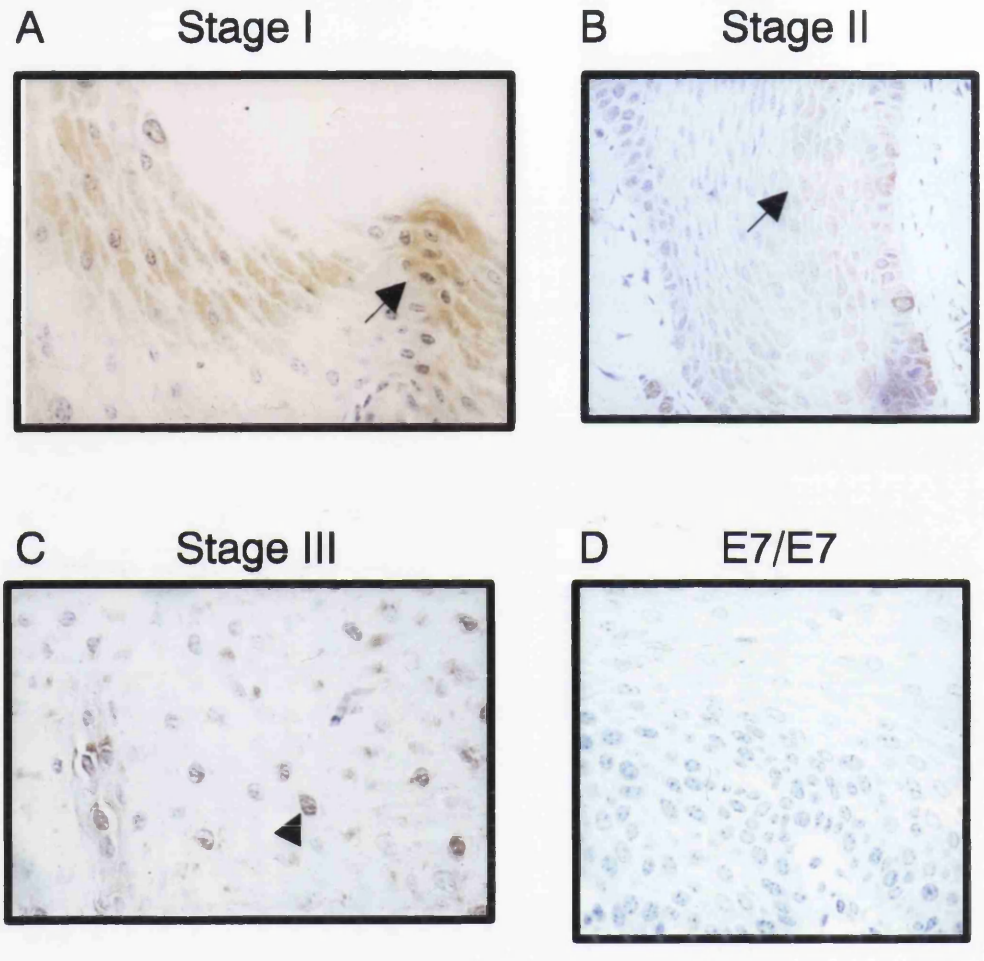
Stage I, II and III papillomas incubated with 4E7 primary antiserum

(A, B and C respectively), and competed antiserum (D).

Arrow indicates cells staining positively in the cytoplasm and

the arrowheads indicate nuclear staining.

Magnification X 20 (A and B), magnification X 100 (C and D).


A
Stage I
B
Stage II
C
Stage III
D
E7/E7

dilution of the 4E7 antiserum was competed with 200μg of 4E7 synthetic oligopeptides (Chandrachud *et al*, 1994; McGarvie *et al*, 1995). No positive cells were detected in the control section (Figure 5.1.3D).

Elevated levels of p53 protein were detectable in papillomas at all stages (Figure 5.1.4 and 5.1.5). p53 protein expression was consistently nuclear and found in the basal and suprabasal cell layers at both early and late stages of progression (Figure 5.1.4A and B).

The stage IV lesion was assigned this status on the histological observation of the disappearance of virus producing cells and due to the presence of infiltrating mononuclear cells in the cores of the two fronds. Sub-epithelial dermal tissue was absent from this sample and so it was not possible to confirm the heavy lymphocytic infiltration into the derma which typifies regressing papillomas (Knowles *et al*, 1996). Only a few cells were observed to contain viral DNA (Figure 5.1.5A). However, no 4E7 expression was detected in this lesion (Figure 5.1.5B). On the contrary, the p53 antibody did react in this papilloma and incubation of the section with preimmune serum eliminated the positive staining (Figures 5.1.5D and 5.1.5C respectively). In agreement with previous observations (Bartek *et al*, 1990a, 1990b; Bartek *et al*, 1991), this suggested that the p53 protein was stabilised in this regressing lesion.

Figure 5.1.4 (on facing page)

Immunoperoxidase detection of the tumour suppressor p53.

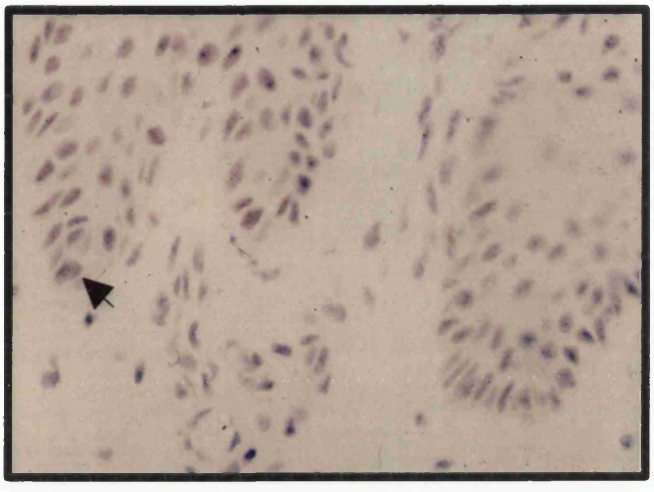
Stage I and II papillomas incubated with p53 antibody

CM-1, (A and B respectively) and preimmune antiserum (C).

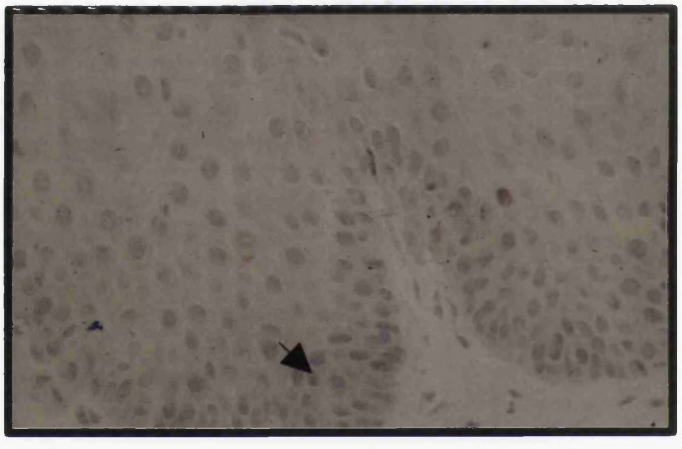
Arrows indicate positive cells.

Magnification X 100.

A Stage I



B Stage II



C preimmune Stage II

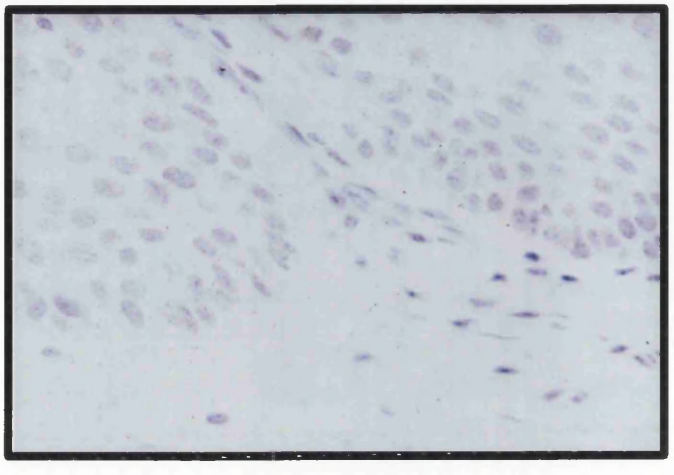


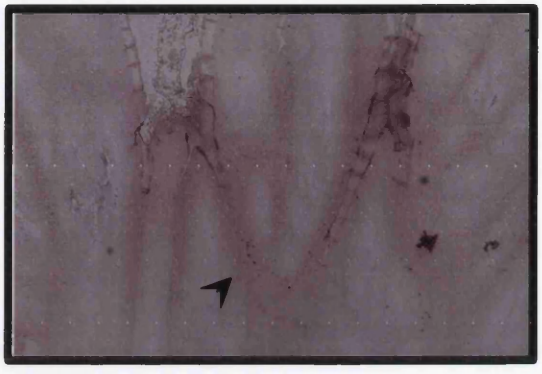
Figure 5.1.5 (on facing page)

Histochemistry of stage IV papilloma.

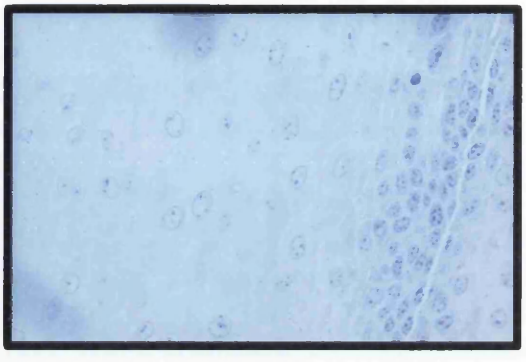
Virus detection by *in-situ* hybridisation with BPV-4 genomic probe (A), incubated with E7 antiserum (B), preimmune antiserum (C), and p53 antibody CM-1 (D). Arrows indicate positive cells.

Magnification X 25 (A), X 50 (C), X 100 (B and D).

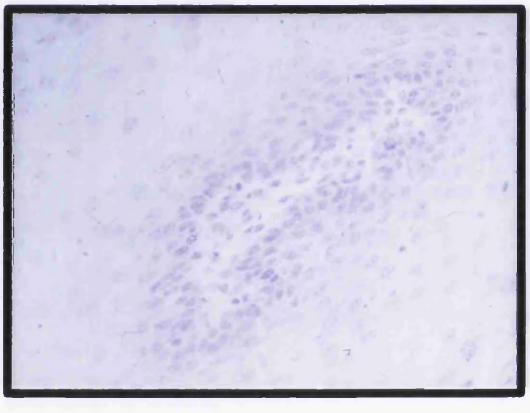
A Viral DNA



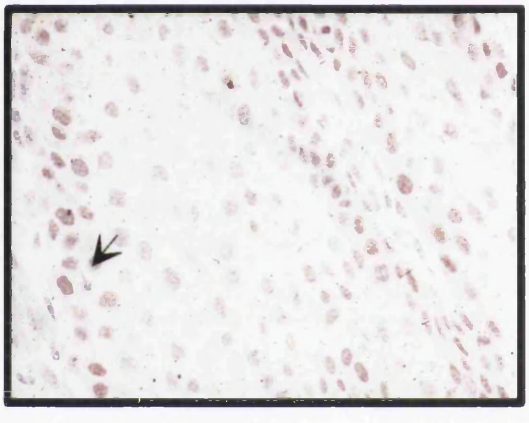
B E7



C preimmune



D p53



Conclusions and Discussion

The above data are summarised in table 5.1.1 on the following page.

No viral DNA, 4E7 or p53 antigens were detected in the normal palatine tissue or in the carcinoma. Viral DNA was detected at all of the papilloma stages as was the p53 protein. 4E7 was detected in papillomas from stage I to III but not in the plaque or stage IV lesions.

Demers *et al* (1994b) showed that after DNA damage, cells expressing HPV-16 E7 demonstrated an increase in p53 levels. It was initially thought that this may be the case for the BPV-4 induced papillomas, however, in the stage IV lesion, p53, but not 4E7, was detected suggesting that there is no relationship between these two proteins and that p53 stabilisation has occurred via another pathway. p53 is induced by DNA damage (Kastan *et al*, 1991; Bartek *et al*, 1991; Yonish-Rouach *et al*, 1991; Kuerbitz *et al*, 1992) and it may be that the experimental injection of a large dose of virus into the palate is sufficient to cause p53 induction. However, it is unknown if this state would persist through the maturation stages of the papillomas.

It is unclear why 4E7 is differentially detected in the cytoplasm and the nucleus. No correlation between 4E7 and p53 could be made and so the presence of 4E7 in the cytoplasm and in the nucleus may be dependent on other cellular factors. Analogous to HPV-16, BPV-4 E7 transport to the nucleus may be mediated by conserved residues centred around the putative pRb binding domain (Fujikawa *et al*, 1994). It is possible that expression of E7 in the nucleus may be masked due to an association with cellular anti-oncogenes or conformational changes as a result of these interactions. This is unlikely as trypsinisation of sections was employed to maximise epitope

Animal no.	Section no.	Description	Viral DNA	E7 protein	p53 protein
94/2309J	94/2309-1	normal palate	-	-	-
203	93/6975-1	plaque	+	-	+(n)
116088/67E	94/1379-1	stage I papilloma	+	+(c/n)	+(n)
116088/241	94/752-1	stage I/II papilloma	+	+(c)	+(n)
116088/227	94/715-1	stage II papilloma	+	+(c/n)	+(n)
116088/227	94/715-2	stage II/III papilloma	+	+(c/n)	+(n)
116088/207	94/713-1	stage III papilloma	+	+(c/n)	+(n)
116088/78	94/3566-1	regressing lesion	+/-	-	+(n)
119513	92/1785-2	carcinoma	-	-	-

Table 5.1.1 Immunohistochemistry and *in-situ* analysis of paraffin embedded sections of BPV-4 experimentally infected lesions.

+ indicates section positive for protein expression, or viral DNA, - negative. n: nuclear staining; c: cytoplasmic.

Sections treated with pre-immune sera, competed antisera and hybridisation buffer only (*in-situ*) were all negative.

detection in combination with polyclonal antiserum which may recognise additional immunoreactive regions of 4E7 (Chandrachud *et al*, 1994). Potential interactions between 4E7 and nuclear factors appear to be possible only in a particular population of differentiating cells, i.e., the basal and suprabasal layers of the papilloma. This specific localisation of 4E7 may be sufficient to provide an abnormal proliferative signal in the basal cells to induce differentiation into a mature papilloma. The cytoplasmic expression of 4E7 was closely associated with cells where viral DNA was present in the upper epithelial layers of the papilloma. It may be that these cells are differentiated enough to allow viral production and E7 functions are no longer required, thus E7 may be present in the cytoplasm prior to removal from the cell.

Thus, bovine p53 was elevated in experimentally BPV-4 induced papillomas, this was accompanied by the presence of viral DNA and the expression of the 4E7 oncoprotein.

5.2 Detection of bovine *p53* mutations in exon 7 *in vivo*

Introduction

Although we had established that p53 protein levels were elevated in the papillomas experimentally induced by BPV-4 infection, we did not know if this stabilisation was a direct result of a mutation in the *p53* gene. As the bovine *p53* exon 7 had been fully characterised (Chapter 4.1) we used a technique known as Single Stranded Conformational Polymorphism, Polymerase Chain Reaction (SSCP-PCR) to determine whether mutation had occurred in this exon in the experimentally induced papillomas.

SSCP-PCR is used to detect the presence of mutations in DNA and allows the analysis of large sample numbers with only the positive samples sequenced for mutations. Denatured SSCP-PCR samples are run on a non-denaturing polyacrylamide gel and the DNA adopts a single stranded conformation dependent on intramolecular interactions. The conformation of the DNA and therefore the mobility of the DNA on the gel is dependent on the nucleotide sequence. This method is capable of detecting a single point mutation in 99-100% of all samples screened (Hayashi, 1991).

SSCP-PCR analysis was also used to determine the status of the bovine *p53* gene from naturally occurring BPV-4 associated carcinomas and other lesions taken from experimentally infected cattle.

A normal control bovine DNA was used at all times and run on the gels as a denatured and non-denatured standard. Samples were run on 6 or 10% gels at room temperature or at at 4^{o}C. Glycerol was added to the room temperature gels at a concentration of 5-10% to ensure maximum detection of mutations.

Primary bovine foetal palate fibroblasts (PalF) transfected *in vitro* with BPV-4 were also analysed. DNA from the experimental papillomas described in Chapter 5.1 was prepared from paraffin sections (materials and methods section 3.1.10). Oligonucleotide primers were designed and SSCP-PCR carried out as described (materials and methods section 3.1.11) to amplify bovine *p53* exon 7. Sequencing of positive samples was used to confirm the presence of *p53* exon 7 mutations.

Results

Urinary bladder and alimentary canal cancers were available from a previous experiment designed to reproduce the synergism between BPV-4 and bracken *in vivo* (Campo *et al*, 1992; Campo *et al*, 1994a). From the above experiment DNA samples from animals 30 and 32 were examined. Both of these animals had received multiple inoculations of virus in the soft palate and were fed bracken fern. None of the cancers removed from animal 30 were found to contain BPV-4 DNA. The bladder cancer from animal 32 contained multiple copies of episomal BPV-2 DNA (Campo *et al*, 1992). Table 5.2.1A lists the experimental samples described above. Table 5.2.1B lists the experimental papillomas as described in Chapter 5.1 and an experimentally induced pharyngeal papilloma from the same BPV-4 and bracken experiment as the cancers (Campo *et al*, 1992).

No mutations in bovine *p53* exon 7 could be detected in experimental carcinomas. None of the cancer DNA tested in table 5.2.1A demonstrated any change in their mobility on the non-denaturing gel with respect to the normal bovine control (Figure 5.2.1A and B). Heteroduplex analysis was also carried

Table 5.2.1 (on facing page)

Experimental papillomas and carcinomas examined by SSCP-PCR for the presence of *p53* mutations in exon 7.

Table A, experimental carcinomas and B, experimental papillomas. The lesions described in table A, were previously examined for alterations in the bovine *ras* gene, and none could be detected (Campo *et al*, 1990).

DNA was isolated from either paraffin embedded sections of BPV-4 derived lesions (PE) or tumour tissue (D). The 116088 animals were infected with multiple inoculations of BPV-4 in the soft palate and received no other treatment. Animals 78529/30 and 32 were fed bracken fern and infected with BPV-4, animal 78529/9 was treated with the immunosuppressant azothioprine and infected with BPV-4 (Campo *et al*, 1992). All papillomas were removed from the palate with exception of the pharyngeal papilloma from animal 78529/9. (X) indicates an as yet undetermined *p53* mutation.

A

Lane	Animal no.	Type of lesion	*p53* exon 7
1	78529/32 (134185)	bladder carcinoma (D)	wild type
2	78529/30 (30/85)	bladder carcinoma (D)	wild type
3	78529/30 (30/85)	colon adenoma (D)	wild type
4	78529/30 (30/85)	colon adenoma (D)	wild type
5	78529/30 (30/85)	lymph metastasis (D)	wild type
6	78529/30 (30/85)	small intestine adenoma (D)	wild type

B

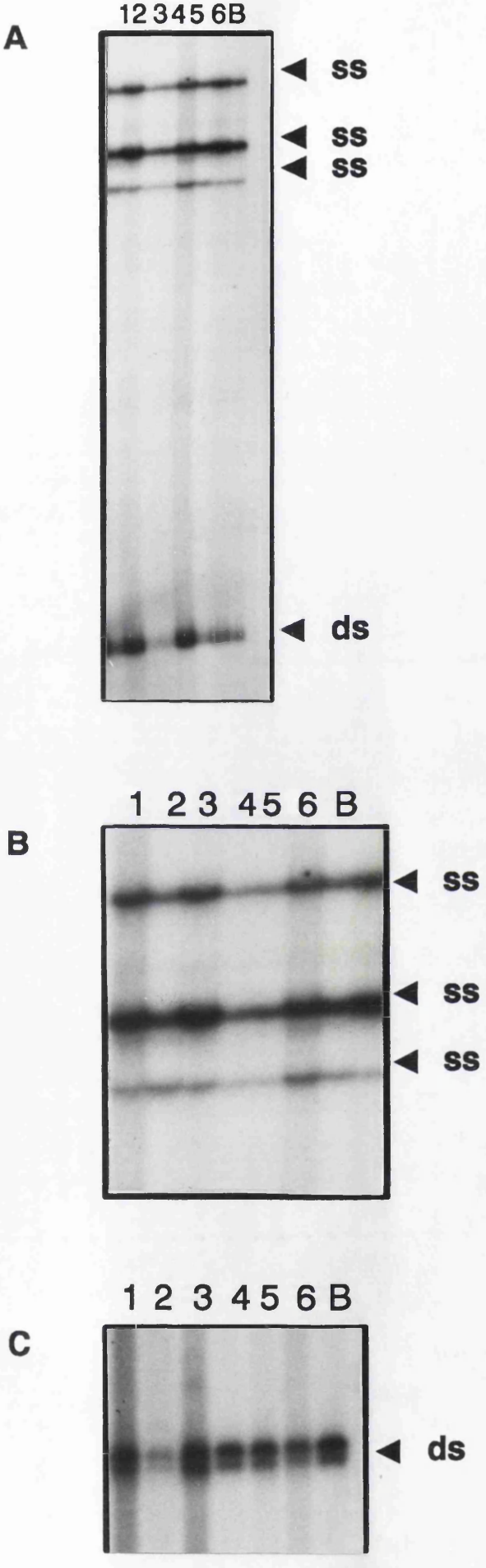
Lane	Animal no.	Type of lesion (source)	*p53* exon 7
1	78529/9	pharyngeal papilloma (D)	wild type
2	203	palate plaque (PE)	wild type
5	116088/67E	Stage I papilloma (PE)	mutant (X)
4	116088/241	Stage I/II papilloma (PE)	wild type
6	116088/227	Stage II papilloma (PE)	wild type
7	116088/227	Stage II/III papilloma (PE)	wild type
8	116088/207	Stage III papilloma (PE)	mutant (X)
3	116088/78	Regressing lesion (PE)	mutant (X)

Figure 5.2.1 (on facing page)

SSCP-PCR analysis of experimental carcinomas.

Figures A and B: Lanes 1 to 6 correspond to the samples described in table 5.2.1A. Lane B indicates the normal control bovine genomic DNA. Mobility of single stranded (ss) DNA and double stranded (ds) DNA are indicated by the arrows. Figure B is a close-up of the single stranded bands represented in figure A. PCR products labelled with [α-^{32}P]dCTP were electrophoretically separated on a 10% polyacrylamide run at room temperature in the presence of 5% glycerol with a cooling fan for 16hours, 800V, constant current.

Figure C: Heteroduplex mapping of the above negative samples to confirm that no mutations in *p53* exon 7 were present. Samples were run on a 0.5 X MDE gel with 10% glycerol at 6W constant power for 14hours.


A
1 2 3 4 5 6 B
ss
ss
ss
ds
B
1 2 3 4 5 6 B
ss
ss
ss
C
1 2 3 4 5 6 B
ds

out on these samples to ensure optimum mutation detection within exon 7. In heteroduplex analysis, resolution is based on conformational differences in double stranded molecules (Keen *et al*, 1991) as opposed to single stranded molecules as utilised in the SSCP analysis. Analysis of the cancers using the heteroduplex technique also suggested that no alterations in the bovine *p53* exon 7 were present (Figure 5.2.1C).

Only the bladder cancer was found to contain BPV-2 viral sequences; the colon and small intestine adenomas are not associated with the presence BPV sequences (Campo *et al*, 1992; Campo *et al*, 1994a) and there appeared to be no detectable mutation in *p53* exon 7. This does not rule out the possibility that mutations in other exons of bovine *p53* are present or that inactivation of p53 by alternative mechanisms have occurred in these cancers. BPV-2 is a member of the subgroup A BPVs and does retain an E6 ORF. There is no evidence that BPV-2 E6 is capable of binding and degrading the p53 tumour suppressor, however, if this is possible then it would be expected that low levels of wild type p53 would be present as described for HPV positive carcinomas (Butz *et al*, 1995). The precursors of the colon and small intestine adenomas taken from the experimental animals are as yet unknown. These tumours have not been shown to arise as a result of BPV-2 or BPV-4 associated infection but are often present in animals grazing on bracken fern with upper alimentary tract tumours (MS Campo; personal communication). They may have arisen as a direct aetiological effect of the cattle feeding on bracken fern. Bracken possesses immunosuppressants and cocarcinogens and the importance of cofactors in the progression of papillomavirus lesions has been reviewed (Jackson and Campo; 1995b) however, the effects from constant bracken feeding and absorption in the gut of the animal is unclear. Animals fed on bracken alone, are severely immunosuppressed and can suffer from acute

bracken poisoning (Campo *et al*, 1992) and as previously demonstrated, these animals suffer from cytogenetic abnormalities (Moura *et al*, 1988). These tumours may arise in the gut mucosa as a result of exposure to many, as yet unknown, breakdown products of bracken fern.

Three of the experimental papillomas (Table 5.2.1B; figure 5.2.2), all of which had arisen as a result of infection with BPV-4, did demonstrate an altered mobility on the non-denaturing gels. The papilloma DNA samples in lane 1 (Figure 5.2.2A) and lanes 3 and 5 (Figure 5.2.2B) correspond to stage III, IV and stage I lesions respectively. Although these samples were sequenced, technical difficulties did not allow the mutation in each sample to be fully characterised (See figure 5.2.4C and D). Sequencing did however indicate that these samples may be heterozygous, suggesting that both a wild type and mutant gene were present (Figure 5.2.4D).

In Chapter 5.1, immunocytochemistry showed that the bovine p53 protein was stabilised. This stabilisation in the experimental stage I, III and IV papillomas, appears to be as a result of a mutation in exon 7 of the bovine *p53* gene. Mutant conformations of p53 proteins are known to be more stable and are detectable by immunocytochemistry (Iggo *et al*, 1990; Bartek *et al*, 1990a; Bartek *et al*, 1991). Although the other experimental papillomas did not appear to possess a mutation in exon 7, they may have as yet unidentified mutations in other exons.

Figure 5.2.2 (on facing page)

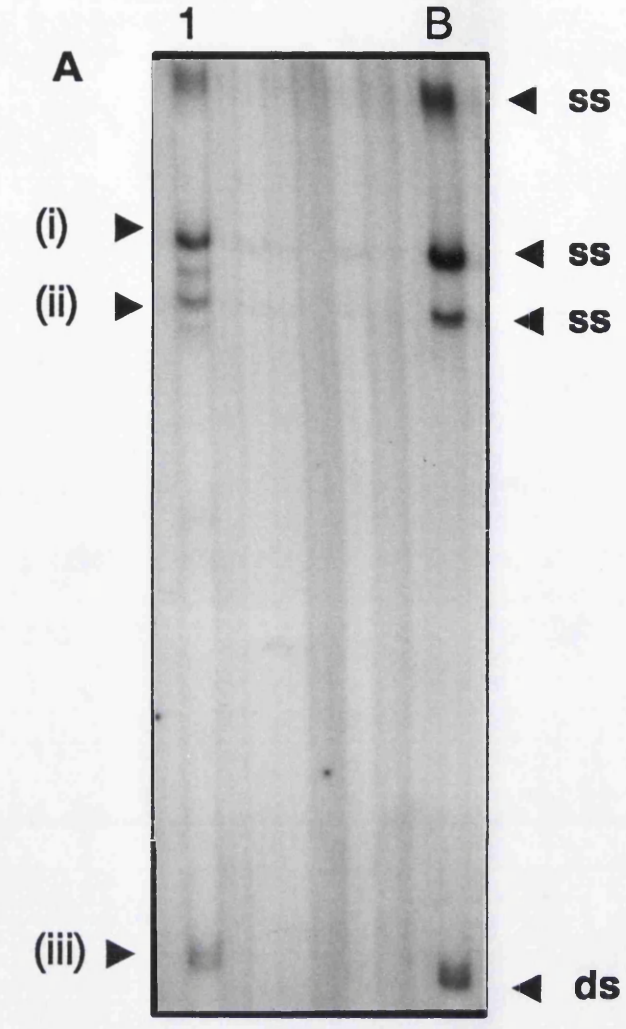
SSCP-PCR analysis of experimental papillomas.

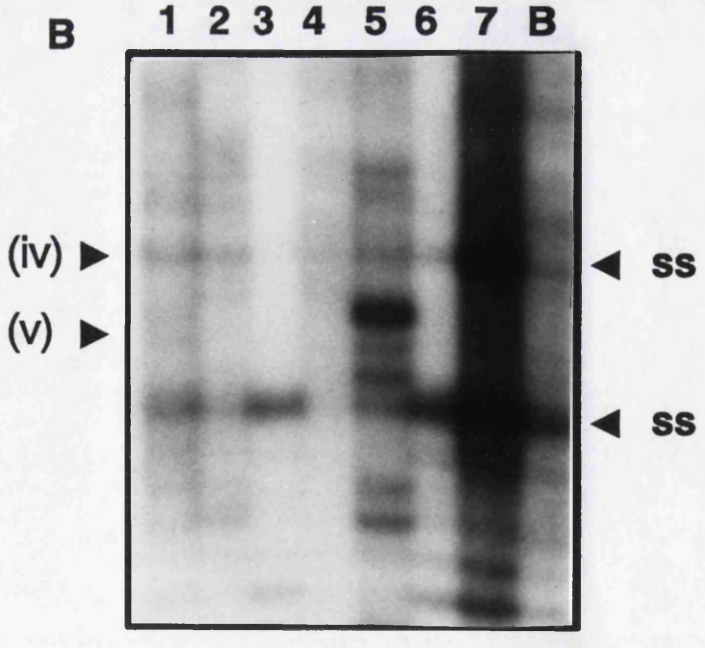
In lane 1 of figure 5.2.2A, the arrows (i and ii) indicate the presence of a second single stranded (ss) species and a decreased mobility of the double stranded (ds) DNA (iii). The sample in lane 1 corresponds to sample number 8, in table 5.2.1B. All samples in lanes 2 to 8 demonstrated the same pattern as the normal control bovine DNA (lane B) when overexposed.

The lanes in panel A and panel B correspond to papillomas described in table 5.2.1B.

In lanes 1, 2, 4, 6, and 7, showed the same pattern as the normal control bovine DNA (lane B). Lane 7 is overexposed in this autoradiograph, however, a decreased exposure time confirmed that the mobility of the ss DNA was consistent with the normal control bovine DNA. The DNA sample in lane 3 demonstrated the loss of the upper ss DNA band (arrow iv), and in lane 5, the ss band had an increased mobility (arrow v).

As described in figure 5.2.1, PCR products were labelled with [α-^{32}P]dCTP and were electrophoretically separated on was a 10% polyacrylamide gel, run at 4^{o}C with no cooling fan at 800V, constant current for 17hours (autoradiograph shown in panel A); in panel B the samples were electrophoresed in 1xMDE at at 4^{o}C with no cooling fan at 800V, constant current for 15hours (autoradiograph shown in panel B).


A
1
B
ss
(i)
ss
(ii)
ss
(iii)
ds


B
1 2 3 4 5 6 7 B
(iv)
ss
(v)
ss

To compare the p53 status determined in the experimentally induced lesions, naturally occurring BPV-4 associated cancers and papillomas were obtained from diseased animals referred to the University of Glasgow Veterinary School from bracken infested areas. Table 5.2.2A and B, describe the cancers and papillomas examined. Figure 5.2.3A and B, illustrate the altered mobility of these samples on the non-denaturing SSCP-PCR gels. The majority of the cancers and the two papillomas examined did not possess alterations in the bovine *p53* exon 7. Two metastases, both isolated from the same animal, were found to possess a mutation at bovine p53 codon 243 altering a proline residue to a threonine. The nucleotide sequence which encodes codon 243 in bovine p53 is altered from CCC to ACC (Figure 5.2.4A, B and C). Figure 5.2.4A depicts the wild type sequence 5'>3' for *p53* exon 7 at codon 243. Figure 5.2.4B and C illustrate the forward (5'>3') and reverse (5'<3') priming of *p53* exon 7 from the thyroid metastases DNA. An increase in the number of secondary species present in figure 5.2.4C is due to inadequate priming with the reverse primer LS7B (Methods Figure 3.1.11; Table 3.1.11B). The apparent heterozygosity may be as a result of the use of genomic DNA possibly contaminated with wild type *p53* sequences. It is more likely this is due to self annealing by the LS7B primer which leads to repriming further along the DNA strand causing an increase in the secondary species present. The primary cancer of these metastases was not available for analysis.

30% of mutations in the p53 gene are located in CRIV which encompasses exon 7 in human cancers (Hollstein *et al*, 1991; Levine *et al*, 1994; Cho *et al*, 1994). As previously mentioned in Chapter One, the importance of conserved region IV is apparent in that the L3 loop is required for DNA binding by p53.

Table 5.2.2 (on facing page).

Naturally occurring papillomas and carcinomas examined for *p53* mutations in exon 7.

DNA was isolated from either paraffin embedded sections of BPV-4 derived lesions (PE) or from tumour tissue (D). The activation of *ras* sequences in the cancers was previously determined by restriction enzyme and hybridisation analysis, and by DNA-mediated transformation of NIH3T3 cells (Campo *et al*, 1990). The *p53* status is indicated as either wild type or mutated at bovine p53 residue 343 (P243T). nd - not determined.

A

Lane	Animal no.	Type of lesion (source)	*ras* activation	*p53* status
1	80931	rumen carcinoma (D)	yes	wild type
2	109594/J	oesophageal carcinoma (D)	n.d	wild type
3	72856	rumen carcinoma (D)	no	wild type
4	74659	thyroid metastasis (D)	no	P243T
5	75668	cardia carcinoma (D)	yes	wild type
6	74659	lymph metastasis (D)	no	P243T
7	86168	rumen carcinoma (D)	yes	wild type
8	71078	oesophageal carcinoma (D)	yes	wild type
9	86168	kidney metastasis (D)	yes	wild type
10	17585	oesophageal carcinoma (PE)	n.d	wild type

B

Animal no.	Type of lesion	*p53* status
229	alimentary papilloma (D)	wild type
unknown	alimentary papilloma (D)	wild type

Figure 5.2.3 (on facing page)

SSCP-PCR analysis of naturally occurring cancers and metastases.

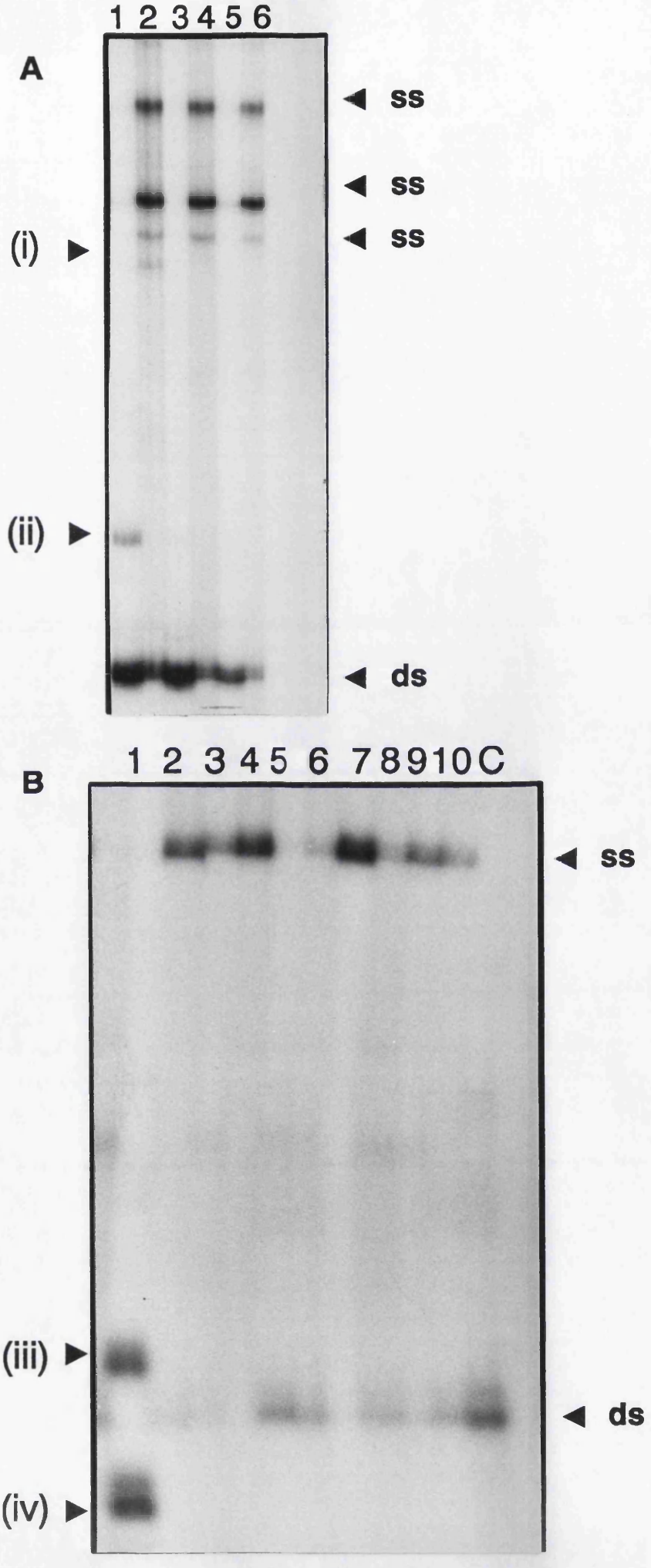
Lanes correspond to samples described in table 5.2.2A.

In panel A, lanes 1,2 and 3,4 represent cancers number 4 and 5 in table 5.2.2A respectively. Lanes are alternate non-denatured and denatured samples. Lanes 5 and 6 are the non-denatured and denatured normal control bovine DNA respectively.

In panel A, lane 1, arrow (i) indicates an extra single stranded species with an increased mobility. The decreased mobility of a second double stranded (ds) speciesis indicated by arrow (ii).

In panel B, lane 1 (sample 6 on table 5.2.2A), the single stranded species is absent and arrows (iii) and (iv) indicate two separate ds species in comparisonto one seen for the normal bovine genomic DNA control (C).

PCR products were electrophoresed on a 10% polyacrylamide gel at $4^{o}C$ without cooling fan at 800V, constant current for 19hours.


A
1 2 3 4 5 6
ss
ss
ss
(i)
(ii)
ds
B
1 2 3 4 5 6 7 8 9 10 C
ss
(iii)
(iv)
ds

The adjacent AAs arginine R248 and R249 are critical in the contact with the minor groove of DNA bound by the core domain of the p53 protein (Cho *et al*, 1994). Mutation of R248 results in the loss of critical DNA contacts which may destabilise the p53 DNA interaction (Cho *et al*, 1994). The *p53* exon 7 mutation found in the bovine metastases changed AA 243 from a proline (P) to a threonine (T). This corresponds to AA 250 in human p53 which is known to be altered in cancers (Hollstein *et al*, 1991) and is located in the L3 loop as determined by the crystal structure of human p53 (figure 1.4; Cho *et al*, 1994).

Proline is an aliphatic imino acid and has a secondary amine group, in contrast to the aliphatic hydroxyl group of the amino acid threonine (T). Proline leads to bending of the polypeptide chain and the substitution of P with T could result in an altered protein conformation disrupting the DNA binding domain. Although this mutation has not been tested *in vitro*, it could be suggested that the DNA binding function of bovine p53 could be abrogated by this alteration.

These data suggest that p53 mutations occur in exon 7 under certain experimental conditions and are more prevalent in papillomas than in carcinomas. Of the field cases analysed, only two mutations could be detected and these were isolated from a thyroid and a lymph node metastases taken from the same animal. Unfortunately, no normal tissue or material from the primary carcinoma of this animal was available for analysis, therefore it cannot be determined if this alteration is a polymorphism or a true mutation although alteration at this amino acid has been shown to occur in human cancer (Hollstein *et al*, 1991).

All samples that did not demonstrate any band shifts by SSCP-PCR analysis (Figure 5.2.1A, B and C) possessed wild type *p53* exon 7 sequence as determined by random sequencing of negative samples.

Figure 5.2.4 (on facing page).

Electropherograms from ABI automated sequencer.

Panel A illustrates the wild type sequence (5'>3') for bovine *p53* exon 7 from nucleotides 725-733 (Dequiedt *et al*, 1995b). The arrow indicates the nucleotide which is altered in panel B.

Panel B illustrates the altered nucleotide sequence (5'>3') of bovine *p53* exon 7 found in the thyroid metastases as described in Table 5.2.2. The nucleotide which is altered from C to A causes a change from a proline to a threonine at bovine p53 residue 243 (Dequiedt *et al*, 1995b).

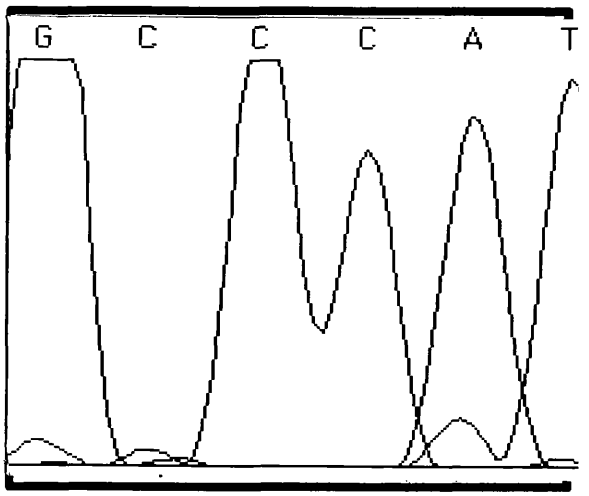
Panel C depicts the opposite strand (5'<3') of that indicated in panel B (see text page 117).

Panel D indicates sequence 'noise' and weak signals generated from DNA isolated from the cell line QOF24 (Table 5.2.3). This pattern was observed for the majority of sequences analysed when amplified from DNA purified from cell lines.

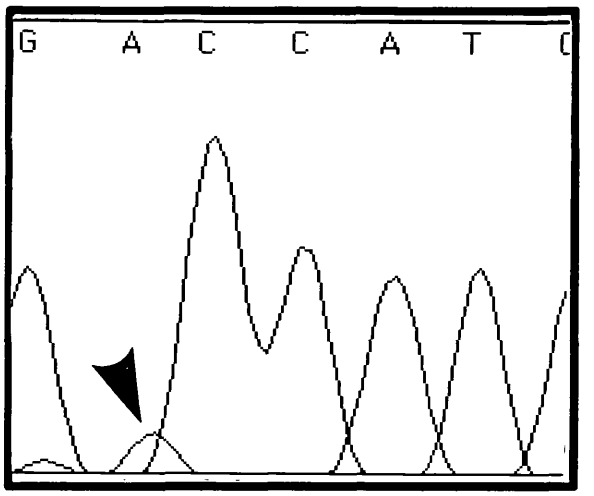
Panel E indicates the sequence generated from DNA isolated from the Stage III experimental papilloma (Table 5.2.1) which was positive for *p53* exon 7 mutation by SSCP-PCR. This pattern of sequence is indicative of heterozygous DNA, i.e., two *p53* exon 7 DNA sequences are present. The arrowhead indicates the presence of a possible C to T transversion in the papilloma DNA sequence. This would suggest that a mutation is indeed present but the number of undetermined nucleotides (N) are too numerous to confirm the exact location of the mutation.

Maximum scale deflection is 1200 in all cases.

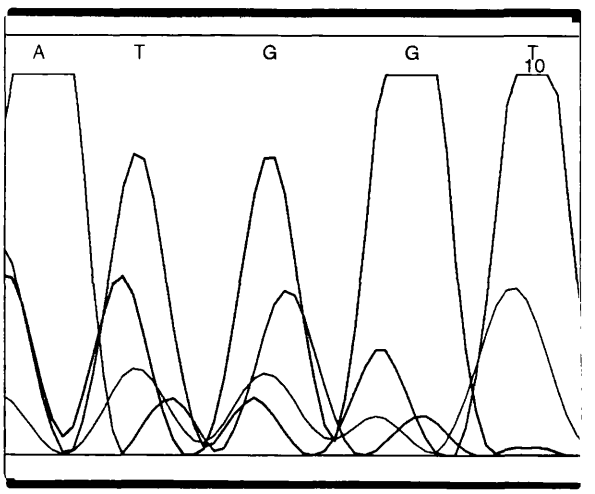
A



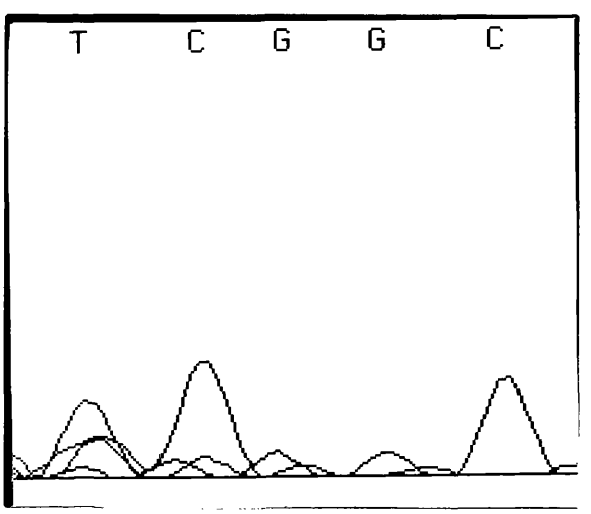
B



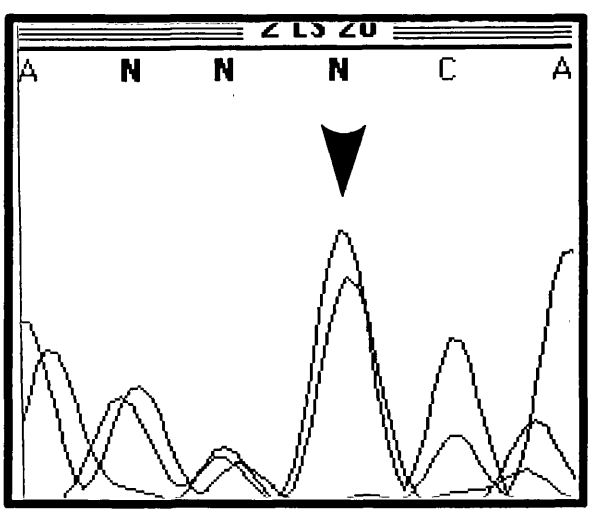
C



D



E



Detection of bovine *p53* mutations in exon 7 *in vitro*

In vitro transfected PalF cell lines which had been previously characterised, (M Cairney, PhD thesis) were also analysed for the presence of a mutation in *p53* exon 7. These cell lines are described in table 5.2.3. The role of bracken fern in alimentary canal cancer in cattle has been previously described (Jackson and Campo, 1995b; Campo and Jarrett, 1986; Jarrett *et al*, 1978) and the mutagenic component quercetin is known to confer tumourigenicicty to primary bovine fibroblasts *in vitro* (Pennie and Campo, 1992; Cairney and Campo, 1995). PalF cells transfected *in vitro* with BPV-4 whole genome or sub-genomic fragments and treated with the mutagen quercetin, known to be a component of bracken fern, a cofactor in BPV-4 associated carcinogenesis (Pennie and Campo, 1993; Cairney and Campo, 1995) were examined for mutations in *p53* exon 7. Due to the absence of an E6 protein, it was possible that environmental cofactors may influence the status of the bovine *p53* gene, and there may be a correlation, with respect to the p53 status, between lesions *in vivo* and transformed bovine cells *in vitro*.

In lanes 1, 8 and 10, the upper single stranded band (indicated by arrow (i) on figure 5.2.5) appeared to have a reduced mobility in comparison to the wild type pattern. The sample in lane 1 also appeared to have a double stranded doublet (indicated by arrow (ii) in figure 5.2.5) as opposed to a single double stranded band as observed for the wild type *p53* exon 7 pattern. The samples in lane 5 and lane 12 (Figure 5.2.5) were considered positive as additional bands were present in lane 5 and on increased exposure only the lower single stranded band was evident in lane 12. Five out of the thirteen cell lines examined were found to contain a putative *p53* exon 7 mutation (Figure 5.2.5), these data were not confirmed by sequencing analysis again due to technical

difficulties (see Figure 5.2.4). However, the quercetin treated PalF cell lines examined for *p53* mutation in exon 7, were found to possess a pattern similar to that illustrated in figure 5.2.4D suggesting that the samples may be heterozygous. No correlation could be drawn between the suspected exon 7 mutations and quercetin treatment. An interesting observation is that *p53* exon 7 mutations did not appear in the cell line that was tumourigenic in nude mice. *p53* exon 7 mutations in the *in vitro* transformed cell lines may have arisen spontaneously through continuous passage in culture. The cumulative effects due to the presence of cofactors in the progression of papillomas to cancers *in vivo* occurs over long periods of time. The ability of a single mutagenic component (quercetin) in bracken fern to mimic this function *in vitro*, is unlikely as quercetin exposure in the PalF cells is limited to 48 hours and would be insufficient to induce genetic changes over this short time period.

Cytogenetic analysis of quercetin treated PalF cells and screening with minisatellite probes did not detect any gross chromosomal changes (M Cairney; PhD thesis). The generation of *p53* gene mutations in cell culture has been observed previously (Harvey and Levine, 1991; Rittling and Denhardt, 1992). Rat embryo fibroblasts transfected with HPV16E7 and *ras* occasionally sustained a recessive p53 mutation and acquired a selective growth advantage in culture (Peacock *et al*, 1990; Peacock and Benchimol, 1994).

No correlation could be found between treatment with quercetin, immortality or tumourigenicity of the PalF cells and the presence of a putative mutation in *p53* exon 7. However, *p53* mutations did seem to occur when subgenomic fragments of BPV-4 were expressed in the presence of quercetin. Since several of the cell lines did not possess *p53* exon 7 mutations, it is possible that mutations are present in additional exons or that some other aspect of the pathway has been interrupted.

Table 5.2.3 (on facing page) Analysis of mutations in *p53* exon 7 in cell lines generated as follows.

PalF cells were treated with 0, 20 or 40 mM quercetin for 48 hours as indicated then allowed to recover for 24 hours followed by transfection with the indicated plasmid DNA and selected for 3-5 weeks with G418 (Geneticin) at 500μg/ml. The addition of HPV-16E6 is required to immortalise PalF cells *in vitro*, however, this can be overcome by the additon of quercetin (Pennie et al, 1993; Cairney and Campo, 1995)

Y - yes, N - no, N.D - not determined.

B - BPV-4, R - human c-H-*ras*, E6 - HPV-16 E6 oncoprotein, E7 -BPV-4 E7 oncoprotein, E7/E8 - plasmid encoding the ORF of both the BPV-4 E7 and E8 oncoproteins.

Sample numbers correspond with the lanes depicted in figure 5.2.5.

Sample No.	Cell line	Contents	[Quercetin] mM	*p53* exon 7 mutation	Immortality	Tumourigenicity
1	QOD poly	B+R	0	Y	N	N
2	Q2XD2	B+R	20	N	Y	N
3	QOE10	B+E6+R	0	N	Y	N
4	Q2XE4	B+E6+R	20	N	Y	N
5	QOF24	E7+R	0	Y	N	N
6	Q2XF1	E7+R	20	N	Y	N
7	QOG19	E7+E6+R	0	N	Y	N
8	Q2XG14#6	E7+E6+R	20	Y	Y	N
9	QOH3	E7/E8+R	0	N	N	N
10	Q2XH5	E7/E8+R	20	Y	Y	N
11	QOI19	E7/E8+E6+R	0	N	Y	N
12	Q2XI20	E7/E8+E6+R	20	Y	Y	N.D
13	Q3D	B+R	45	N	N	Y

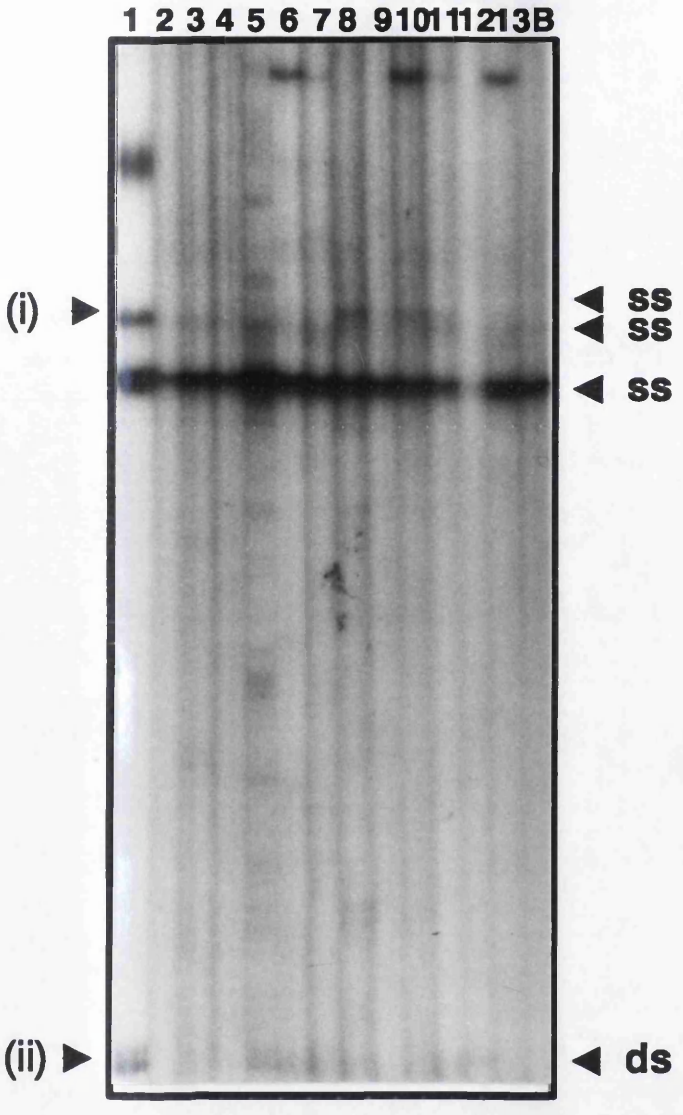


Figure 5.2.5 SSCP-PCR of bovine *p53* exon 7 from DNA extracted from BPV-4 transfected PalF cell lines described in table 5.2.3.
Lanes 1-13 correspond to sample numbers indicated in table 5.2.3, B indicates the normal bovine genomic DNA control.
Samples were electrophoresed on a 1xMDE gel containing 5% glycerol, with cooling fan at 800V for 16hours at room temperature.

Conclusions and Discussion

Mutations were found in exon 7 of bovine *p53* both *in vitro* and *in vivo*. *p53* mutations which result in the production of a disrupted protein are usually accompanied by a loss of heterozygosity (LOH), i.e, loss of the remaining wild type *p53* allele (Levine *et al*, 1991). The technical difficulties encountered in sequencing *p53* exon 7 for some of the samples may be attributed to the presence of both wild type and mutant *p53* sequences. Mutation P243T in the bovine metastases was easily detected and may indicate that only a mutant *p53* sequence was present. It could be suggested that perhaps the remaining wild type *p53* allele has been lost from these metastases. This *p53* mutation may encode a disrupted p53 protein and as mentioned, this mutation may abrogate the ability of p53 to bind DNA, however, this mutation has not yet been tested *in vitro*.

All of the non-BPV-4 associated lesions possessed a wild type *p53* exon 7 as did normal palate, control PalF cells and liver DNA. Two of the metastatic tumours from the group of field cases of alimentary tract cancers were found to have an alteration in *p53* exon 7. Of the experimental carcinomas which were not associated with BPV-4, none were mutated in exon 7.

A summary of the data on exon 7 *p53* mutations from the previous tables is given in table 5.2.4.

None of the experimental carcinomas indicated the presence of a mutation in *p53* exon 7, however, 38% of the experimental papillomas, which had been subjected to high titres of virus only, demonstrated mutations in exon 7 as determined by SSCP-PCR. Overexpression of the bovine p53 protein had been previously detected at all papilloma stages as described in Chapter 5.1. This

overexpression correlated with a mutation in *p53* exon 7 for the stage I, III and IV papillomas. Although no mutations were detected in the remaining experimental papillomas, a strong correlation has been observed between high level expression of the p53 protein and point missense mutations in the *p53* gene in other systems (Iggo *et al*, 1990; Bartek *et al*, 1990a, 1990b; Bartek *et al*, 1991). This would suggest that mutations may be present in other exons of the bovine *p53* gene not identified in this study.

p53 mutations could not be detected in naturally occurring papillomas, however, 20% of the naturally occurring carcinomas examined were positive for mutation in *p53* exon 7. The absence of mutations in the experimental carcinomas may be a result of contamination with residual wild type *p53* arising from normal tissue which was excised with the tumour, or that mutations may exist in other exons. This could also explain the inability to detect p53 mutations in the majority of field cases. The fact that p53 mutations are prevalent in exon 7 in experimental cases of papillomas may reflect the difference between animals which receive high doses of virus experimentally and persistent infection over many years in naturally occurring cancers. Alterations in the *ras* gene appear to be more prevalent than *p53* mutation in the field carcinomas although the exact exon in which bovine *ras* is mutated has not been determined.

The data accumulated suggests that mutations in *p53* exon 7 are not a result of the cocarcinogens and mutagens present in bracken fern. The animals from which the experimental papillomas came, had not been fed bracken fern, yet high levels of p53 protein were detected. In the case of the transfected cell lines, there appeared to be no correlation between exon 7 mutation and treatment with the mutagen quercetin. Although the presence of cofactors is a requirement for progression, it could be suggested that these p53 mutations

have not arisen as a result of a single specific mechanism, i.e., the presence of environmental cofactors, but as a spontaneous event.

	No.tested	No.positive	*p53* exon 7 mutation
Experimental papillomas	8	3	Unidentified
Experimental carcinomas	6	0	None
Field cases of papillomas	2	0	None
Field cases of carcinoma	10	2	P243T
Cell lines	13	5	Unidentified

Table 5.2.4 Summary of the *p53* exon 7 mutations present in various BPV derived lesions as determined by SSCP-PCR analysis.

5.3 Cellular amplification

Introduction

As described in Chapter One, the B subgroup of the BPVs lack an E6 ORF, the product of which is responsible for p53 binding and degradation in the HPVs. In the previous chapter, we demonstrated that some BPV-4 induced papillomas and cancers had a *p53* mutation in exon 7, although we did not know if *p53* gene was altered in other exons. Most of the tumours possessed a wild type *p53* exon 7 sequence. It is possible that the *p53* gene may be mutated in other exons or that alternative mechanisms of p53 inactivation may have occurred in tumours as a result of the lack of an E6 protein. BPV-4 has been found to induce cellular amplification of ARS sequences (Smith *et al*, 1993; Smith and Campo, 1989), and abrogation of wild type p53 is known to be accompanied by cellular amplification of *mdm-2* in many human sarcomas (Oliner *et al*, 1992; Ladanyi *et al*, 1993). The human *mdm-2* is a single copy gene which is amplified in human tumours, therefore, the genomic copy number of the bovine *mdm-2* gene in several BPV induced lesions was evaluated.

Several papillomas and cancers which have been characterised in Chapter 5.2, were chosen for analysis of the bovine *mdm-2* gene. The samples were as follows; the thyroid and lymph node metastases (Table 5.2.2A), two experimentally induced lesions (a cancer of the bladder and a colon adenoma; Table 5.2.1A), and two naturally occurring oesophageal papillomas (Table 5.2.2B). The *mdm-2* status in an adenomatous plaque and normal control bovine liver DNA was also determined.

Results

DNA samples from BPV associated papillomas and carcinomas were examined and the DNA was digested overnight with either of the restriction enzymes HindIII or EcoRI. Southern hybridisation was carried out using a human *mdm-2* fragment cDNA probe (nt 161-1283) (Figure 5.3.1a). Fakarazedeh *et al* (1991) showed that the *mdm-2* gene is conserved across species. The Southern blot was stripped by washing in boiling 0.1% SDS and reprobed with a 500bp fragment of the c-Ha-*ras*-3 pseudogene isolated from a 3.1kb BamHI fragment of bovine genomic DNA (McCaffrey *et al*, 1989) (Figure 5.3.1B). The bovine Ha-*ras*-3 sequence contains no introns and is believed to be a non-functional processed pseudogene (McCaffrey *et al*, 1989). Bovine Ha-*ras*-1 is altered in bovine cancers of the alimentary canal as previously mentioned (Campo *et al*, 1990). c-Ha-*ras*-3 hybridised to two fragments in the EcoRI digested bovine genomic DNA of 4.3kb and 2.3kb (Figure 5.3.1B) as described by McCaffrey *et al*, (1989). The human *mdm-2* cDNA probe hybridised to three fragments at approximately 8kb, 4kb and 2.1kb respectively (Figure 5.3.1A). The fragment indicated at 1.3kb (Figure 5.3.1B) coincided with a satellite DNA band in the ethidium bromide stained gel suggesting that satellite-like repetitive sequences were present in the probe (Figure 5.3.1A). The *mdm-2* gene is known to possess highly repetitive Alu sequences at the 3' end of the gene (B. Vogelstein; personal communication) and although the cDNA probe was digested to avoid this region, incomplete digestion of the DNA may have resulted in carryover of these sequences during purification of the insert.

The above experiment was repeated using the HindIII restricted DNA which was hybridised with the human *mdm-2* fragment cDNA probe (Figure 5.3.2A),

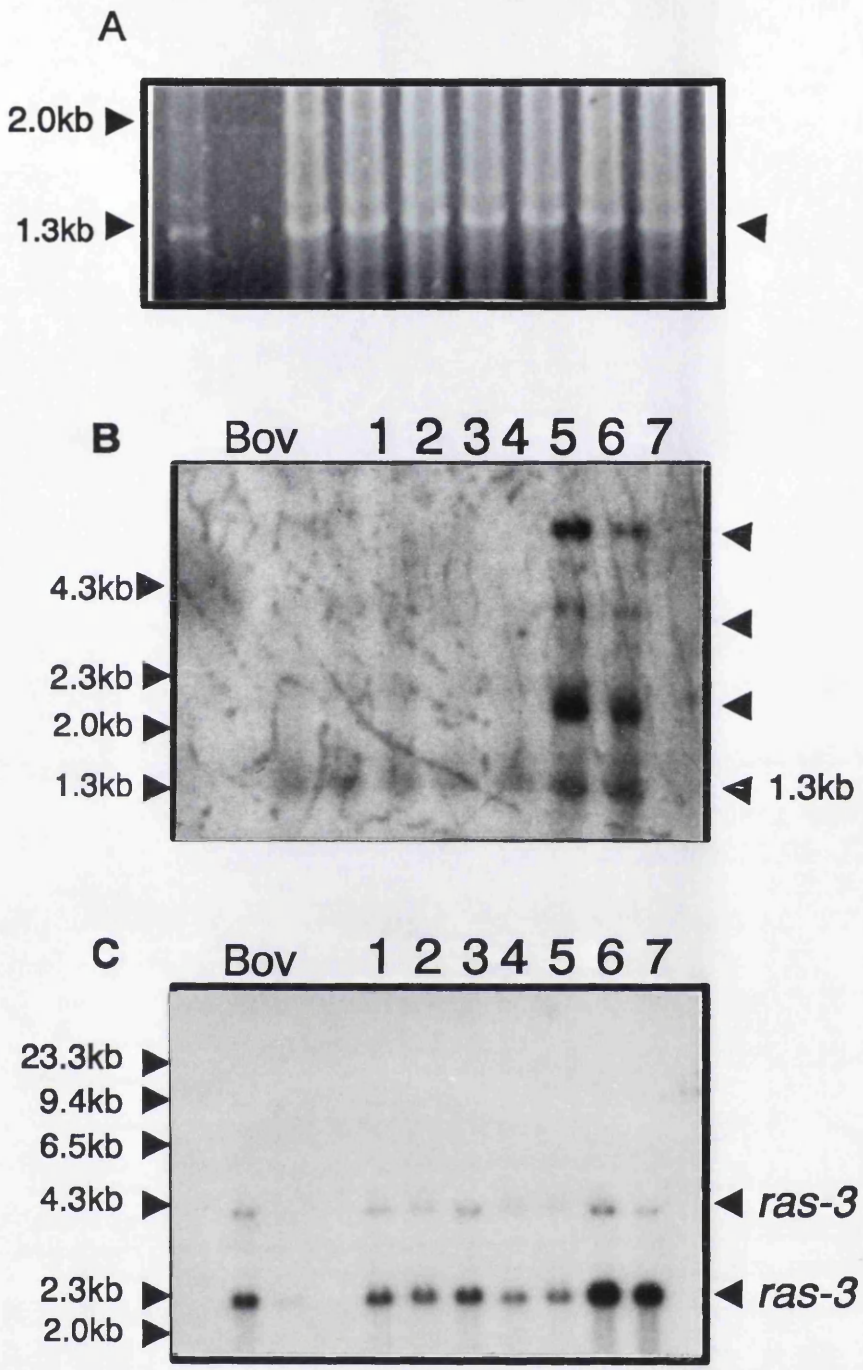


Figure 5.3.1 Southern hybridisation of EcoRI digested bovine tumour DNA with the human *mdm-2* gene and the bovine pseudo-*ras*-3 gene.
In lane1: EcoRI digested DNA from thymus tumour, lane 2: adenomatous plaque, 3: irregular lymph node, 4: adenoma, 5: bladder carcinoma, 6: alimentary papilloma, 7: alimentary oesophageal papilloma.
Bov: normal bovine liver genomic DNA.
In panel A, the arrow indicates the minisatellite band present at 1.3kb in the ethidium bromide stained gel. Panel B illustrates the three bands for EcoRI digested bovine DNA southern hybridised with the *mdm-2* gene, panel C shows the 4.3kb and the 2.3kb fragments of the bovine pseudo-*ras*-3 gene.

no satellite sequences were present on the ethidium stained gel. The Southern blot was stripped and reprobed using a cDNA encoding exon 2 of the mouse glyceraldehyde-3-phosphate dehydrogenase gene (*gapdh*) (Figure 5.3.2B).

The bovine *mdm-2* gene appears to be about 13-14kb in size as determined by the size of the fragments resulting from the restriction digests (Figure 5.3.1A and figure 5.3.2A). The human *mdm-2* gene is approximately 16kb in size (Oliner *et al*, 1992).

No amplification of the bovine *mdm-2* gene was apparent in either the EcoRI cut (Figure 5.3.1) or the HindIII cut (Figure 5.3.2) bovine DNA with respect to the loading control. To confirm this, densitometry was carried out on the original autoradiographs as shown in figure 5.3.2. The autoradiographs were scanned using an ARCUS II (420oe) scanner and analysed by the Quantity One gel analysis program (PDI, Inc.). The O.D of three equivalent regions with a scanning width of 5mm on the autoradiograph, were analysed for each lane and the peak O.D calculated. The tracing of the bands analysed in lane 6 on both figures 5.3.2 A and B, were twelve-fold greater than those bands in lanes 1, 5 and 7 (Figures 5.3.2A and B).

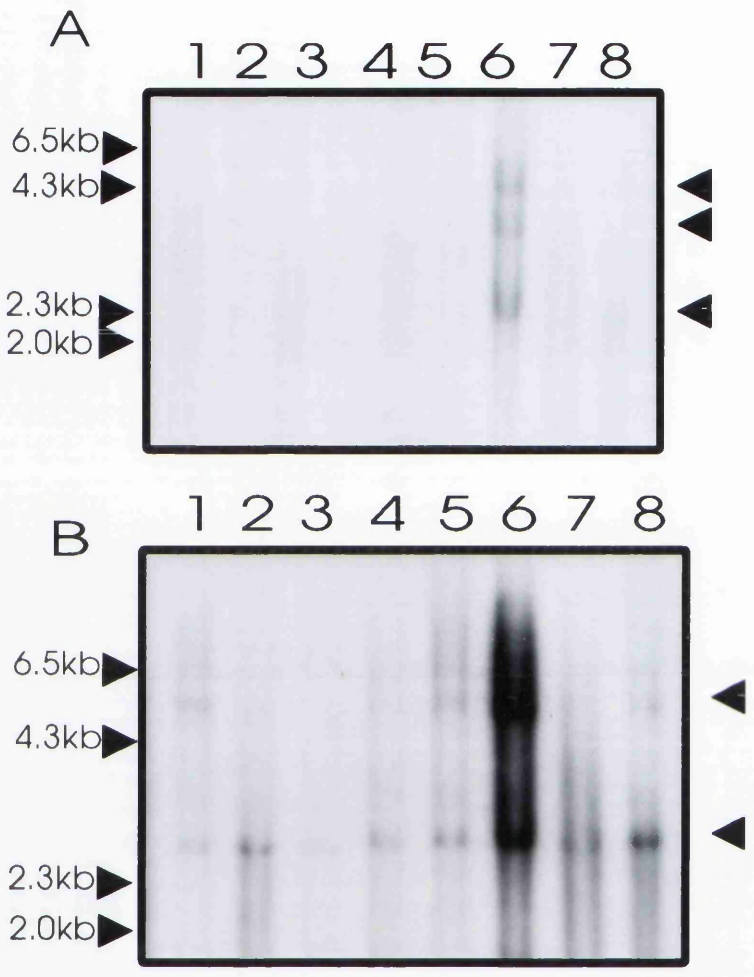


Figure 5.3.2 Southern hybridisation of HindIII digested bovine tumour DNA with the human *mdm-2* and the mouse *gapdh* gene.
Panel A and B illustrate the bands (indicated by the arrows) after southern hybridisation with mdm-2 cDNA probe (A), and the mouse gapdh cDNA probe (B).
Lane 1, bovine liver genomic DNA; lane 2, DNA from thymus tumour; lane 3, adenomatous plaque; lane 4, irregular lymph node; lane 5, adenoma; lane 6, bladder carcinoma; lane 7, alimentary papilloma; lane8, alimentary oesophageal papilloma.

Conclusions and Discussion

The *mdm-2* gene is known to be amplified and act as an oncogene in human sarcomas (Oliner *et al*, 1992; Ladanyi *et al*, 1993). This amplification lead to the mdm-2 protein binding the tumour suppressor p53 (Oliner *et al*, 1992), preventing the p53 DNA binding activity (Zauberman *et al*, 1993) and p53 mediated transactivation (Momand *et al*, 1992). It was not clear what the status of the bovine *mdm-2* gene would be, and the copy number of the *mdm-2* gene was analysed in BPV associated papillomas and carcinomas by southern hybridisation using the human *mdm-2* fragment cDNA probe. Of the lesions tested, in both BPV-4 related and non-BPV associated, the *mdm-2* gene was not amplified. Amplification at the transcriptional level cannot be ruled out, as in some leukaemias *mdm-2* mRNA levels were increased (Buesos-Ramos *et al*, 1993) although expression of the bovine *mdm-2* mRNA was not assessed. In tumours expressing high levels of *mdm-2*, *p53* is generally found to be wild type. Our data suggest that in the lesions examined, only the thyroid metastases was previously shown to possess a mutation in exon 7, the remaining lesions had a wild type *p53* gene, at least in exon 7.

It was concluded that *in vivo*, both the BPV-4 and non-BPV related tumours did not possess any alteration in the copy number of the bovine *mdm-2* gene. Further investigation of the *mdm-2* status in other BPV-4 associated lesions is required in order to confirm these observations. If *p53* mutations are present in other exons, it would be expected that no amplification of the bovine *mdm-2* gene would be detected.

CHAPTER 6

In vitro analysis of the effect of an exogeneous mutant p53 and HPV-16E6

Introduction

BPV-4 does not possess an E6 oncoprotein (Jackson *et al*, 1991), and one of the original questions asked was, does BPV-4 lack E6 associated functions and can an exogeneous HPV-16E6 (16E6) confer additional functions *in vitro*?

Previous experiments have demonstrated that the addition of 16E6 to BPV-4 transfected PalF cells *in vitro* confers immortality (M Cairney, PhD thesis, Pennie *et al*, 1993). This also raises the question as to whether these functions are a result of p53 inactivation or are there other functions of 16E6 involved. Two approaches were taken to investigate whether mutant p53 could enhance transformation by BPV-4; to cotransfect an exogeneous mutant p53 with BPV-4 into PalF cells (Chapter 6.1), and to cotransfect BPV-4 and 16E6 into cells from p53 deficient mice to determine if additional 16E6 functions were p53 independent (Chapter 6.2).

The following experiments were carried out in parallel with the analysis of the p53 status and so a human mutant *p53* cDNA was used due to the lack of a bovine mutant cDNA.

6.1 *In vitro* transfection of primary bovine foetal palate fibroblasts (PalF)

The *p53* gene is commonly mutated in human carcinogenesis and p53 protein inactivation appears to be involved in tumourigenesis associated with the high risk HPVs 16 and 18 (reviewed in Vousden, 1993), but does it play a part in BPV-4 associated carcinogenesis?

BPV-4 DNA transfected in the presence of an activated human Ha-*ras,* is capable of partially transforming PalF cells *in vitro.* On addition of an exogeneous 16E6 these cells become immortal.

The p53-SCX3 mutant human *p53* cDNA from colorectal tumour CX-3, (Baker *et al,* 1990) is altered in exon 5 at codon 143 and valine is substituted by alanine (V143A). Table 6.1.1 lists the known functions of this mutant p53(V143A).

This construct was cotransfected into PalF cells in combination with BPV-4, *ras* and HPV-16E6(16E6) to determine the effect of adding an exogeneous mutant p53. HPV-16E6 was included in the transfection as previous experiments demonstrated a requirement for 16E6 to confer immortality to PalF cells.

It may be that mutant p53 can substitute for 16E6 functions in PalF cells *in vitro* and perhaps provide them *in vivo.*

p53 (V143A)	Reference
Found in human colon cancer	Baker *et al*, 1990
Decreased ability to bind DNA	Kern *et al*, 1991b
Can substitute for 16E6 in the immortalisation of human keratinocytes	Sedman *et al*, 1992
Stimulates expression of the MDR1 gene	Dittmer *et al*, 1993
Tumourigenic in murine fibroblast cell line (10)3	Dittmer *et al*, 1993
Did not affect the growth of the non-tumourigenic adenoma derived cell line AA/C1	Williams *et al*, 1994

Table 6.1.1 Characterised features of the human mutant cDNA p53 SCX-3 (V143A).

Results

PalF cells were cotransfected with BPV-4, ras, 16E6 and mutant p53 (V143A) as described (materials and methods section 3.2.3), the cells were selected with 500μg/ml G418 and the resulting colonies picked and analysed for transformation. The data for the PalF transfections are indicated in table 6.1.2.

No colonies were established from cells transfected with pZipneo alone, nor in the cells transfected with wild type human p53 regardless of the other oncogenes present (PFG; Table 6.1.2). Wild type p53 has been previously shown to suppress transformation by oncogenes in primary rat embro fibroblasts and human foreskin keratinocytes (Eliyahu *et al*, 1989; Finlay *et al*, 1989; Sedman *et al*, 1992). It is unclear whether the lack of colony formation in the presence of wild type p53 is a result of apoptosis or a toxic effect due to overexpression of wild type p53. Expression of an exogeneous wild type p53 can induce apoptosis in p53 negative myeloid leukemia cells (Yonish-Rouach *et al*, 1991), human colon carcinoma cells (Shaw *et al*, 1992) and murine erythroleukemia cells (Ryan *et al*, 1993). It is possible that this apoptotic response results in the inhibition of colony formation in PalF cells.

When mutant p53 was included in the transfection (Table 6.1.2; PFD and PFF), two-three times as many colonies were produced in comparison to cells transfected without mutant p53 (Table 6.1.2; PFA and PFB) suggesting that mutant p53 enhances cell transformation. Sedman *et al* (1992) demonstrated that the cotransfection of p53(V143A) with 16E7 stimulated proliferation in human foreskin keratinocytes, whereas cotransfection of wild type p53 with 16E6 or 16E7 did not induce proliferation.

Table 6.1.2 (on facing page) Analysis of transformation of PalF cells. C4Ta2a is a tumorigenic cell line derived from NIH3T3 cells containing BPV-1 and BPV-4 (Smith and Campo, 1988).

* Average of three experiments. (S.E) indicates the standard error for the experiment.

The figures in brackets indicate the number of clones positive over the number of clones tested

❄ The figures in brackets indicate the average size of the tumour attained within 6-8 weeks in mm. pZipneo is the G418 resistant plasmid which confers the selectable marker. n.d - not determined.

Cell line		Average no. of colonies /T175 flask* (S.E)	Morphological Transformation	Anchorage Independent Growth #	Immortality	Tumourigenicity (no. mice positive/ no. mice tested) ❄
	PalF cells	0	no	no (3/3)	no	0/5
	(+*ras*+pZipneo)					
	ras	2 (2)	yes	no (4/4)	no	n.d
PFA	BPV-4	7 (3)	yes	yes (6/6)	no	0/6
PFB	BPV-4+16E6	17 (5)	yes	yes (6/6)	yes	0/13
PFC	mt p53	11 (2)	yes	yes (3/3)	no	0/12
PFD	mt p53+BPV-4	23 (3)	yes	yes (7/7)	yes	0/8
PFE	mtp53+16E6	14 (1)	yes	n.d	n.d	n.d
PFF	mtp53+BPV-4+16E6	37 (5)	yes	yes (8/8)	yes	13/15 (6x7mm)
PFG	wtp53+BPV-4+16E6	0	no	n.d	n.d	n.d
	C4Ta2a	n.d	yes	yes (3/3)	yes	8/8 (10x10mm)

The extent of transformation was measured as four separate parameters; morphological transformation, anchorage independent growth, immortality and tumourigenicity in nude mice. Figure 6.1.1 illustrates the morphological transformation of the transfected PalF cells. Cells which were morphologically transformed grew as raised clusters (Figure 6.1.1B-D), whereas untransformed cells continued to grow as a monolayer on the surface of the flask (Figure 6.1.1A). Six clones were picked from each flask containing transformed colonies for further analysis. Only a few colonies grew in the flasks containing PalF cells transfected with neomycin resistant plasmid pZipneo. The pZipneo colonies remained flat and it was not possible to expand these colonies for more than several passages. No colonies formed in any of the transfections containing an exogeneous wild type p53.

Clones were tested for the ability to grow in 1% methocel. Figure 6.1.2 illustrates those clones which were capable of anchorage independent growth.

Untransfected PalF cells and those transfected with *ras* alone, were not capable of growth in soft agar. The PFA, PFB, PFC, PFD and PFF cell lines were all capable of anchorage independent growth as was the control cell line C4Ta2a. When 16E6 was present in addition to BPV-4 and *ras* (PFB), the anchorage independent colonies appeared to be larger in size in comparison to PFA. To determine if this difference in colony size was a function of an increased rate growth, these transformed cell lines were analysed using the MTT assay (materials and methods section 3.2.4.2), in which the O.D measurement is proportional to the number of cells present. Figure 6.1.3 shows the MTT assay data and the rates of growth of the transformed cell lines.

When 16E6 was present in the transfection (Table 6.1.2; PFB and PFF), the growth rates were significantly higher than in the other transfected cell lines

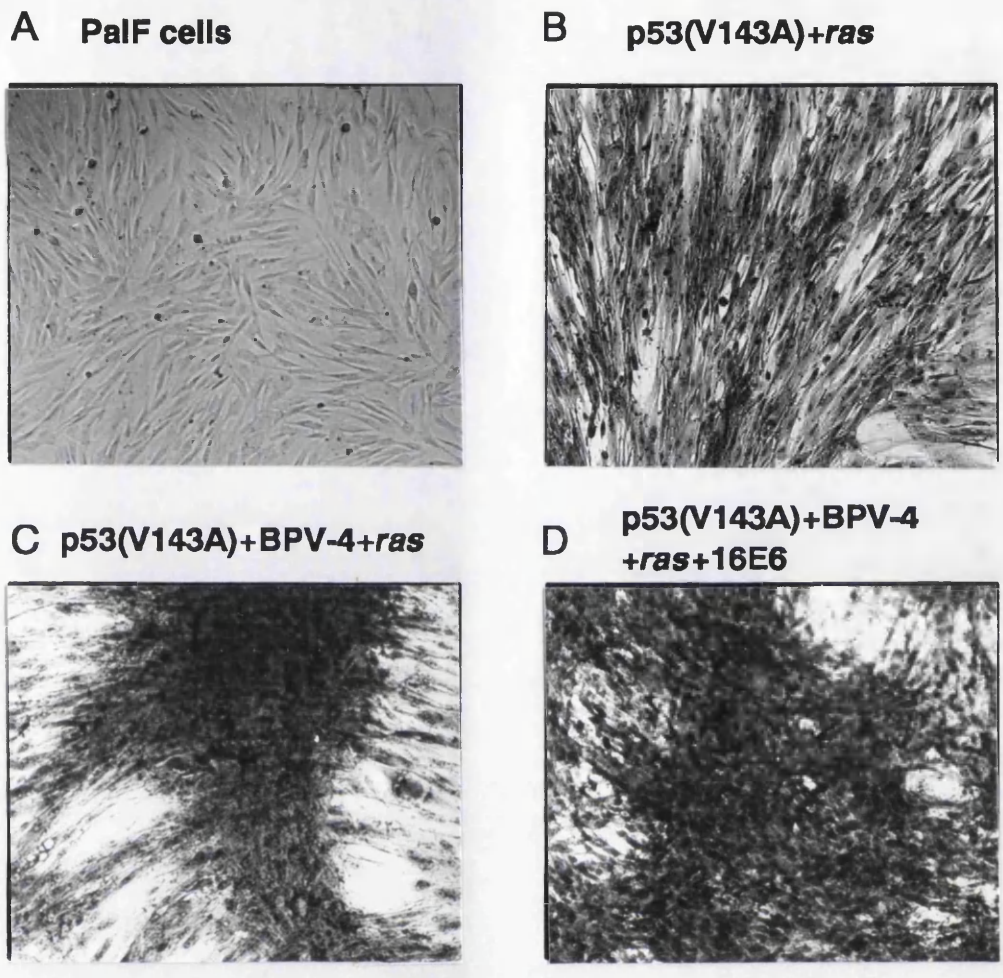


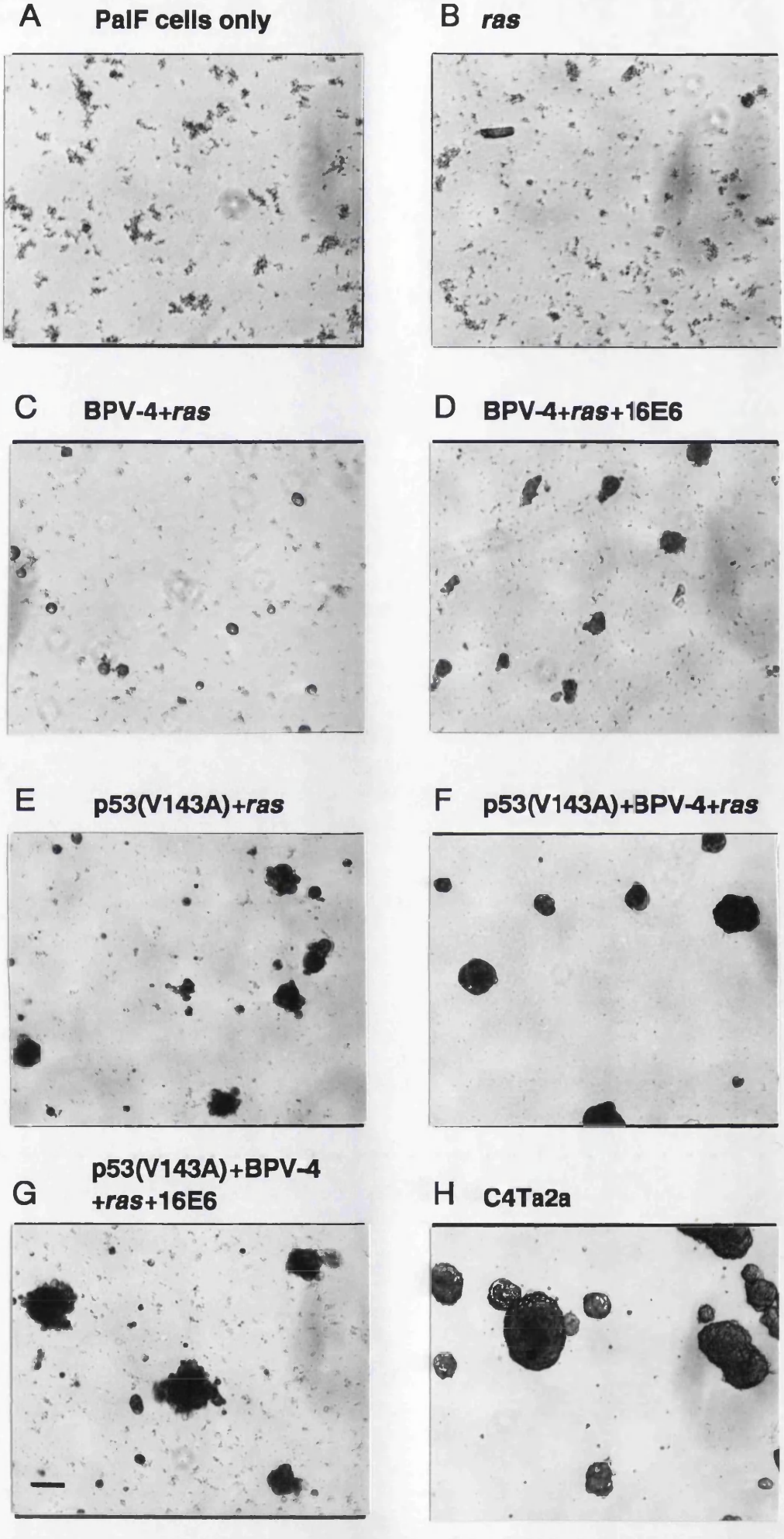
Figure 6.1.1 Examples of morphological transformation of transfected PalF cells.
Cells were stained with 10% Giemsa and photographed using PanF50 film. Panel A; untransfected PalF cells, panel B; PalF cells transfected with mutant p53(V143A) and *ras*, panel C; PalF cells transfected with mutant p53(V143A), BPV-4 and *ras* , and panel D; PalF cells transfected with mutant p53(V143A), BPV-4, *ras* and HPV-16E6.
Magnification X 125.

Figure 6.1.2 (on facing page)

Anchorage independent growth of transfected PalF cells.

$1x10^5$ cells from each cell line was added to 1% methocel and grown for 14 days at 37°C. Untransfected PalF cells or cells transfected with *ras* alone were not capable of growth in soft agar (A and B). PalF cells transfected with BPV-4 and additional oncogenes were capable of growth in soft agar (C-G). C4Ta2a is the tumourigenic cell line used a a positive control (H).

The bar in panel G indicates 100μm. Magnification X 60.


A
PalF cells only
B
ras
C
BPV-4+ras
D
BPV-4+ras+16E6
E
p53(V143A)+ras
F
p53(V143A)+BPV-4+ras
G
p53(V143A)+BPV-4
+ras+16E6
H
C4Ta2a

PFA and PFD (Figure 61.3). Mutant p53 did not appear to substitute for this 16E6 function (PFD) or to enhance the growth rate as no significant difference was observed between the PFB and the PFF cell lines. It may be possible that this increase in growth provided by 16E6 does not function via a p53 dependent pathway.

All of the clones tested were immortal with the exception of PFA which confirmed previous obsevations that an additional function can be provided by 16E6 and is a requirement for immortalising PalF cells (M Cairney, PhD thesis; Pennie *et al*, 1993). However, in the absence of 16E6, mutant p53 could also immortalise PalF cells when cotransfected with BPV-4 and *ras* (PFD).

The transfected PalF cell lines were found to be capable of anchorage independent growth as shown in figure 6.1.2, however, only the PFF cell lines were tumourigenic in nude mice (Table 6.1.2; Figure 6.1.4).

Subcutaneous injection of nude mice with cell lines PFC and PFD resulted in the appearance of small nodules which disappeared after three to four weeks. This is indicative of a reaction at the site of injection and not as a result of tumour formation. The PFF clones tested however, were tumourigenic and the tumours persisted for longer than 12 weeks. Tumours arose in the nude mice within 3 weeks and reached a maximum size of 7-8mm in diameter at 8 weeks then regressed slightly remaining at approximately 5mm in diameter. Animals displaying no tumours after 12 weeks were considered tumour free. Thus, in the absence of 16E6 PalF cells were not tumourigenic in nude mice and 16E6 was still providing additional functions that BPV-4 lacked *in vitro* even in the presence of mutant human p53. The tumours which had arisen as a result of inoculation of the mice with the PFF cell line, were excised from the mice and embedded in parafin wax for immunohistochemical analysis.

Figure 6.1.3 (on facing page)

Growth rates of transfected PalF cells as determined by MTT assay.

Graph A PalF cells transfected with BPV-4, *ras* and HPV-16E6.

Graph B as for graph A with the addition of mutant p53 (V143A).

Control PalF cells were analysed at passage 3.

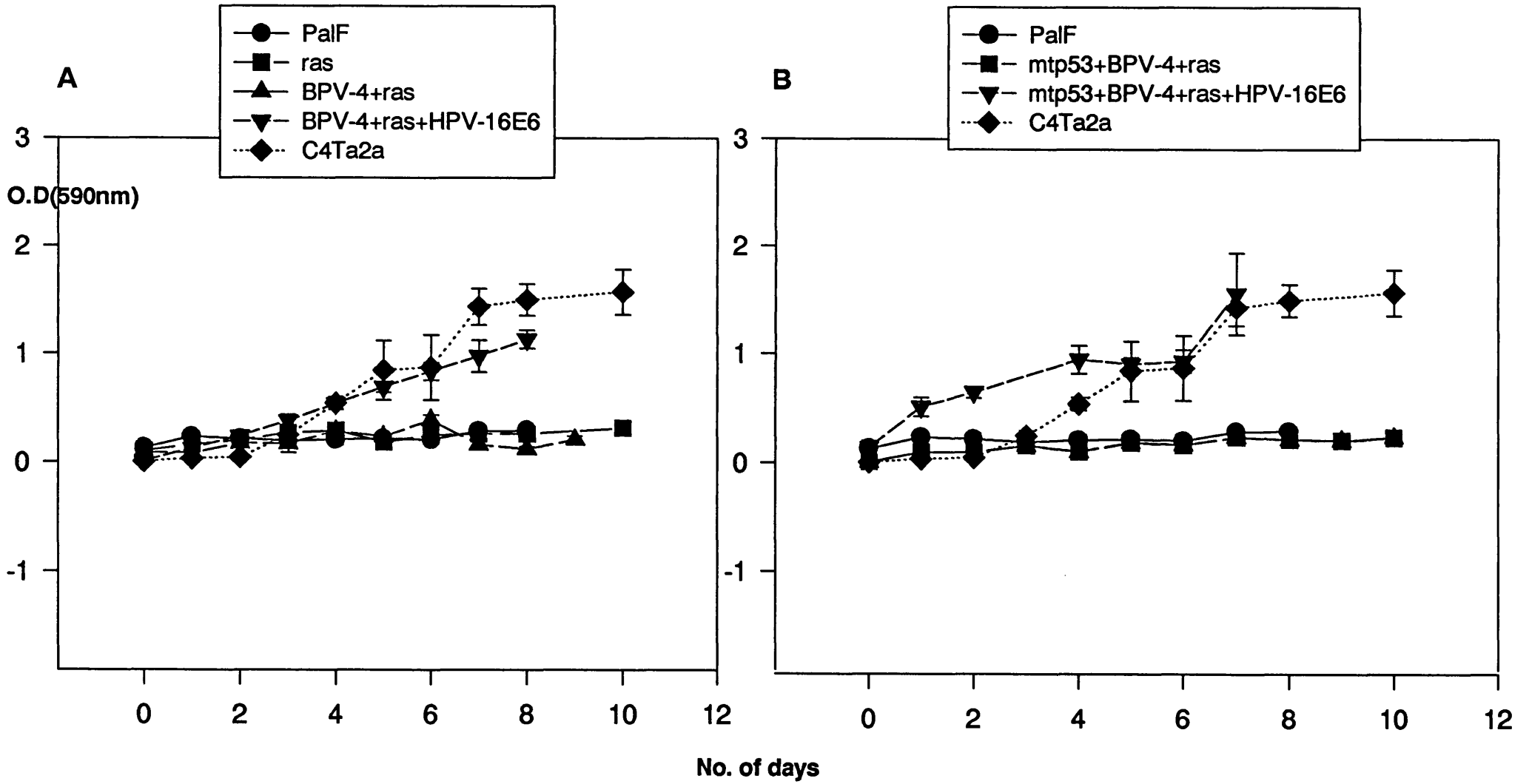

A
PalF
ras
BPV-4+ras
BPV-4+ras+HPV-16E6
C4Ta2a
O.D(590nm)
3
2
1
0
-1
0
2
4
6
8
10
12
B
PalF
mtp53+BPV-4+ras
mtp53+BPV-4+ras+HPV-16E6
C4Ta2a
No. of days

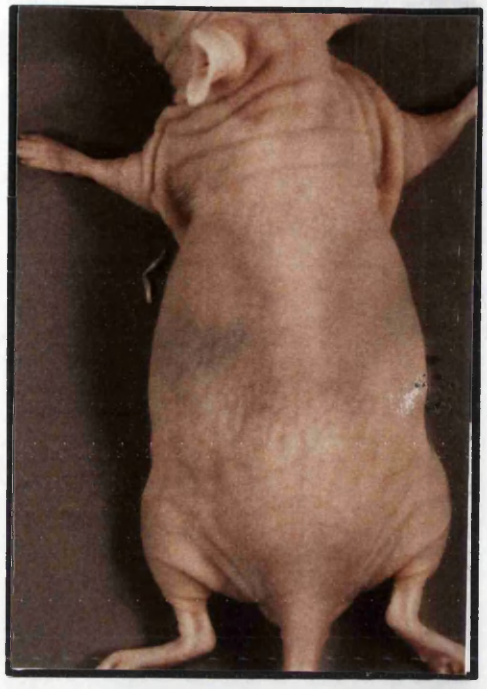
Figure 6.1.4 (on facing page)

Tumourigenicity of transformed PalF cell lines in nude mice.

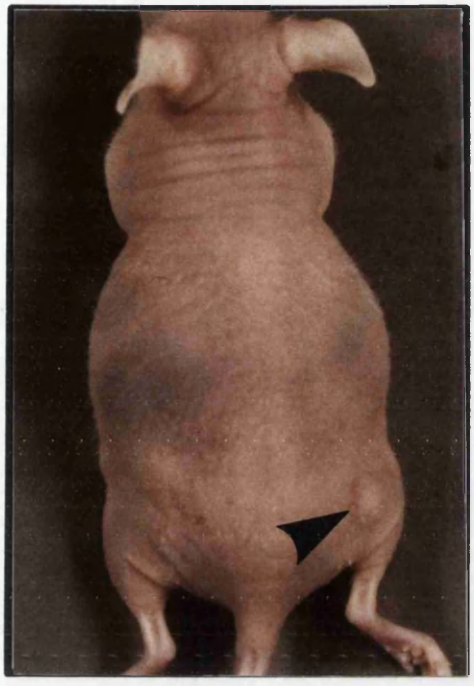
No tumours were present after 12 weeks in mice inoculated with untransfected PalF cells or the non-tumourigenic cell lines (A). The PFF cell line was transfected with BPV-4, *ras*, 16E6 and mutant p53 was positive after 12 weeks (B). The tumourigenic cell line C4Ta2a was used as a positive control (C).

Arrows indicate tumours, bar indicates 1cm.

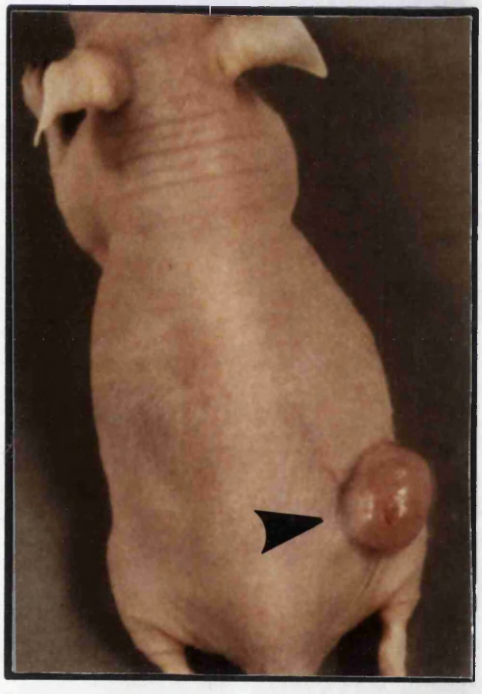
A



B



C



=1cm

The transfected cell lines were analysed by Northern hybridisation for the expression of the viral E7 and E8 mRNAs. Expression of *p53* and the bovine cellular ductin transcripts were also examined. Ductin is a 16kd proteolipid of the vacuolar H^+-ATPase and is also a constituent of the connexon channel of gap junctions. PalF cells transformed with BPV-4 E8 show a reduction in cell-cell communication and ductin is known to bind to BPV-4 E8 *in vitro* (Faccini *et al*, 1996). Ductin interactions are thought to play a role in gap junctional intercellular communication (GJIC) and it is possible that in tumourigenic cell lines, the cells have lost the ability to communicate and ductin expression may be altered.

E8 mRNA was expressed only in the PFF cell lines which were tumourigenic (Figure 6.1.5A). No E8 mRNA could be detected in any other cell lines. E7 mRNA could be detected in all of the cell lines but not in untransfected PalF cells (Figure 6.1.5B). All of the cell lines expressing E7 and E8 were transfected with whole genome BPV-4 DNA.

p53 transcripts were only detected in those cell lines which had been transfected with p53 (V143A) suggesting that only mutant *p53* was expressed at detectable levels and not the endogeneous wild type *p53* (Figure 6.1.6A). Figure 6.1.6A illustrates three cell lines transfected with p53(V143A) which do not appear to express mutant p53, however, a longer exposure of the autoradiograph revealed that p53 transcripts were indeed present and these cell lines were also positive.

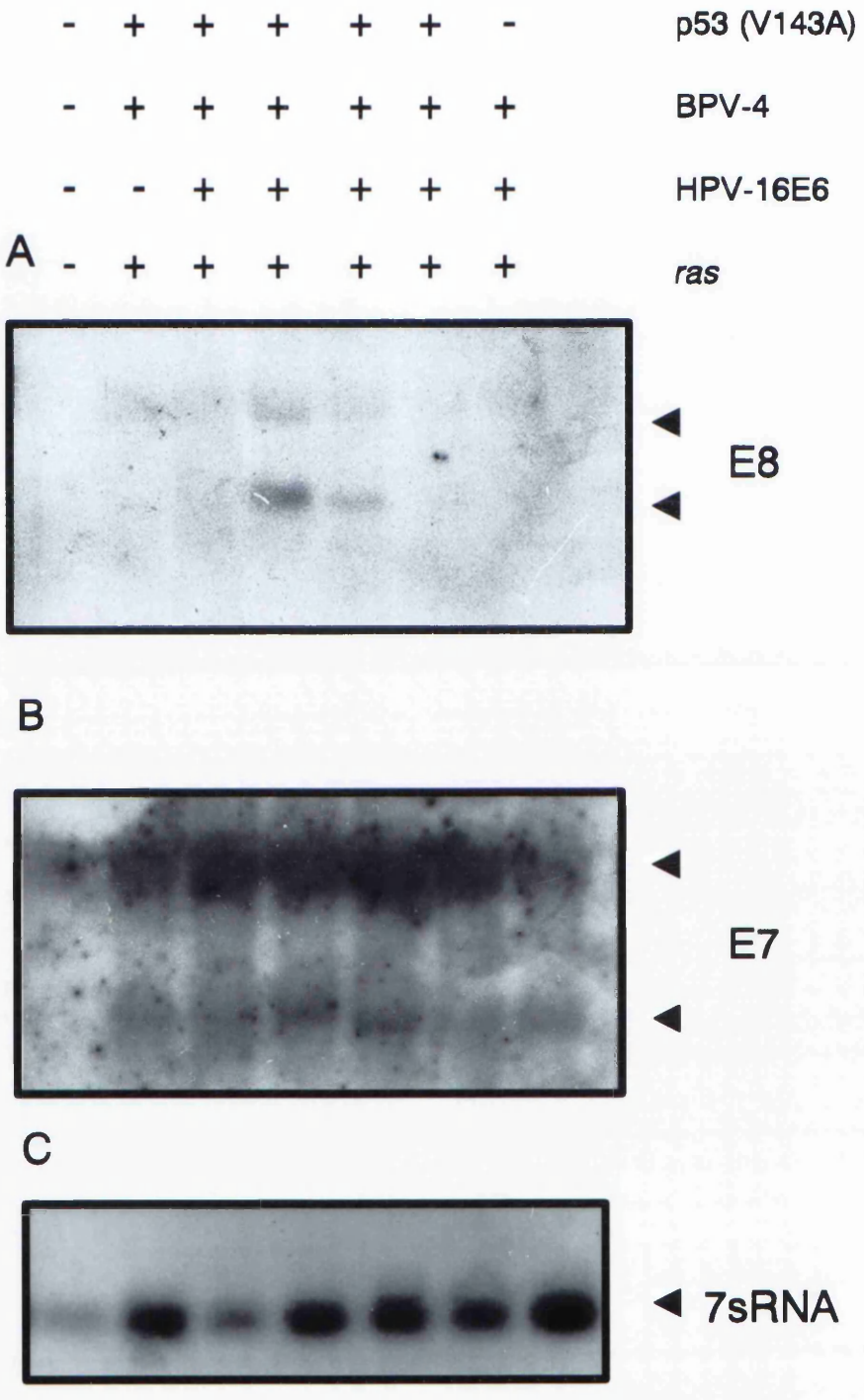


Figure 6.1.5 Northern blot analysis of transfected PalF cell lines. Panel A E8 mRNA, panel B E7, and panel C 7s RNA loading control.

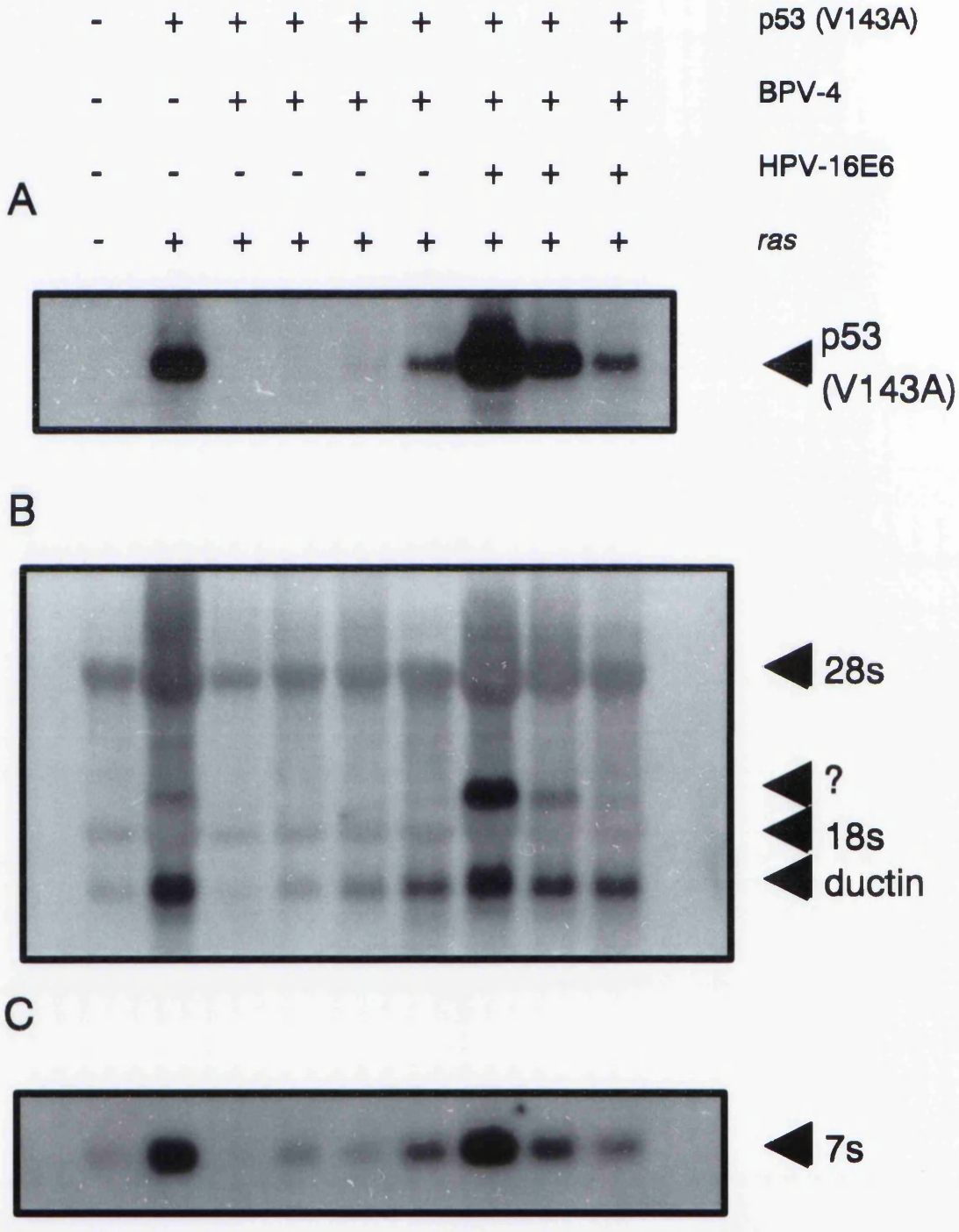


Figure 6.1.6 Northern blot analysis of transfected PalF cell lines. Panel A depicts RNA hybridised with the mutant p53 (V143A) probe, panel B illustrates RNA hybridised to the bovine ductin cDNA probe 1.3kb fragment, 18s and 28s indicate the rRNA fragments, and panel C 7s RNA loading control.

The 1.3kb ductin mRNA was unaltered (figure 6.1.6B) with respect to the 7s RNA loading control, however, another mRNA of 2.1kb in size was observed. This mRNA was apparent in cell lines which had been previously found to be expressing the E8 mRNA (tumourigenic cell line PFF). The 2.1kb mRNA was also present in the cell line PFC transfected with mutant p53 and Ha-*ras* (Figure 6.1.6B). It is possible that this may be a connexin/ductin homologue or a splice variant of the ductin message. As the 2.1kb mRNA was detected in certain cell lines, including those expressing E8, it is possible that this unidentified mRNA may be involved in the loss of GJIC in cells and possibly be induced as a result of E8 expression. Another possibility is that mutant p53 and not E8 may induce the 2.1kb mRNA as it was observed in cell line PFC albeit at a lower level than PFF. The 2.1kb mRNA was not apparent in any of the other cell lines transfected with mutant p53, i.e, PFD, however, it is possible that the 2.1kb mRNA is present in all cell lines but expressed at different levels. Budunova *et al* (1995) showed that three gap junction proteins, connexins 26, 43 and 31.1 are differentially expressed during mouse skin carcinogenesis. Connexin 26 mRNA was not expressed in untreated mouse epidermis but was found to be increased 45-50 fold in the hyperplastic epidermis of TPA treated mice and in papillomas of DMBA/TPA treated mice. Budunova *et al* (1995) suggested that gap junctions composed of different connexins may work as selective filters for growth factors and secondary messengers of different signal transduction pathways.

This 2.1kb mRNA may provide evidence of novel expression during loss of cell-cell communication in PalF cells and may be important in BPV-4 associated carcinogenesis. Other components of gap junctions may be altered

Figure 6.1.7 (on facing page)

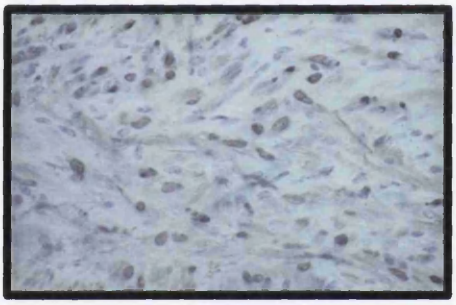
Detection of the BPV-4 and cellular transforming proteins in PFF induced tumours.

Nude mouse tumour section incubated with BPV-4 genomic probe (A) by *in-situ* hybridisation. Immunoperoxidase detection of p53 (B), E8 (C) and E7 (D) with primary antiserum and competed antiserum (E).

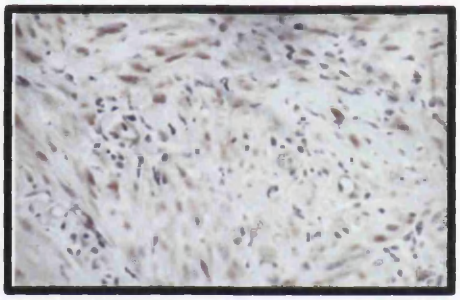
Magnification X 100.

A Viral DNA

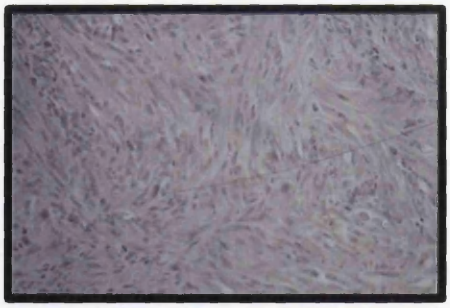
B p53



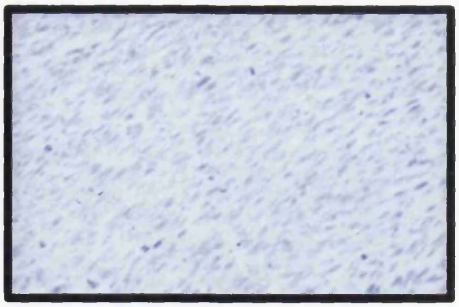
C E8



D E7



E E7/E7



in these cell lines which have not yet been characterised and this unidentified mRNA is currently being investigated by a research group in the Beatson Institute for Cancer Research.

Therefore, although E8 mRNA levels are increased, ductin RNA is not altered, thus ductin transcript levels do not vary in tumourigenic cell lines and ductin may be altered post-translationally.

As viral transcripts were detected in the tumourigenic cell lines, PFF, the excised tumours from the mice were analysed for the presence of viral E7 and E8 proteins, viral DNA and p53 protein (Figure 6.1.7). E7, E8 and p53 were detected by immunocytochemistry (Figure 6.1.7D, C and B respectively), but no viral DNA was detected by *in-situ* hybridisation (Figure 6.1.7A). As mentioned in Chapter Four, *in-situ* hybridisation is limited to the detection of greater than 30 copies of viral DNA per cell, therefore, viral copy number may be below the limits of detection in these tumours. Immunoperoxidase detection (Figure 6.1.7B-E) confirmed that the viral proteins are continuously expressed after inoculation into the nude mouse.

Conclusions and Discussion

In parallel with the analysis of naturally occurring and experimental tumours in cattle, experiments were carried out to test the role of mutant p53 and HPV-16E6 in *in vitro* transformation by BPV-4. At this time, the bovine cDNA had not yet been cloned and the human mutant p53 cDNA (V143A) was available. In the presence of *ras* and HPV-16E6 (16E6), cells transfected with BPV-4 and mutant p53 became tumourigenic in nude mice. Without 16E6 these cells were not tumourigenic, however, in the absence of mutant p53 the cells were not tumourigenic either. The BPV-4 and *ras* transfected PalF cells were immortal in the presence of either mutant p53 or 16E6. Previous data demonstrated that 16E6 is required to confer immortality to PalF cells transformed with BPV-4 and *ras* (Pennie *et al*, 1993; M Cairney, PhD thesis). 16E6 cooperates with E7 to immortalise primary human foreskin keratinocytes *in vitro* (Sedman *et al*, 1991). Sedman *et al* (1992) have also shown that transfection of human keratinocytes with 16E7 and the mutant p53 (V143A), can substitute for 16E6 in an immortalisation assay. Mutant p53 substituted for this E6 function lacking in BPV-4 *in vitro* and could replace the lack of an E6 ORF *in vivo*. Storey *et al* (1995) have demonstrated that the expression of an inducible 18E6 and EJ-*ras* results in the immortalisation of baby mouse kidney cells (BMK). In the absence of dexamethasone-induced 18E6, the cells arrested at the G0/G1 point of the cell cycle. This may explain the requirement for 16E6 to immortalise PalF cells. Storey *et al* (1995) also showed that those 18E6 dependent clones which reverted to dexamethasone independent growth, possessed a mutation in one of the p53 allelles supporting previous reports that mutations in the p53 gene can substitute for E6 functions in BMK cells (Storey and Banks, 1993). Mutant p53 has been implicated in the immortalisation

process in cell culture (Harvey and Levine, 1991; Rittling and Denhardt, 1992). Ulrich *et al* (1992) showed that the loss or mutation of p53 correlated with the immortalisation of *v-src* transformed chick embryo fibroblasts and the expression of a foreign mutant p53 promoted the immortalisation of rodent fibroblasts (Jenkins *et al*, 1984; Rovinski and Benchimol, 1988). Thus 16E6 and mutant p53 appear to have distinct pathways by which they can both immortalise cells. Both of these pathways are functional in BPV-4 transformed cells *in vitro*, therefore although BPV-4 lacks an E6 protein *in vivo*, the presence of a mutant p53 could replace this missing function. This is supported by the findings that BPV-4 induced papillomas and carcinomas possess *p53* mutations in exon 7 although these mutants have not been tested *in vitro*.

The requirement of both 16E6 and mutant p53 for tumourigenicity *in vitro* suggested that BPV-4 lacks more than one function required for cellular transformation. Certain p53 mutants are thought to inhibit the growth suppressive properties of wild type p53 by forming inactive hetero-oligomers (Eliyahu *et al*, 1988; Finlay *et al*, 1989; Rovinski and Benchimol, 1988) and it may be that the formation of heterocomplexes of mutant and wild type p53 are necessary for tumorigenicity of BPV-4 transfected PalF cells *in vitro*. p53(V143A) is no longer capable of binding DNA (Kern *et al*, 1991b) however, the mutant protein may still retain the ability to bind other cellular factors to form active complexes which can affect the transcription of target genes. Desaintes *et al* (1995) showed that wild type p53 could repress transcription of HPV, the mutant p53(V143A) exhibited no repression of HPV transcription. This would suggest that p53(V143A) may not affect the transcription of BPV-4 genes *in vitro* and contributes to transformation via other mechanisms. Thus, although p53 and 16E6 appear to have independent pathways which lead to immortality, the requirement for both p53 and 16E6 to

confer tumourigenicity to PalF cells in the presence of BPV-4 suggests that these pathways are complementary. Possible mechanisms by which mutant p53 and HPV-16E6 contribute to both immortality and tumourigenicity will be discussed in Chapter Seven.

Analysis of the growth rates of the BPV-4 transformed cell lines showed that 16E6 contributed a growth advantage regardless of the presence of mutant p53. This function may be p53 independent, as Ishiwatari *et al* (1994) showed that degradation of p53 is not sufficient for a growth stimulatory effect in human embryonic fibroblasts.

Both E8 and E7 mRNAs were found to be expressed in the tumourigenic cell lines, and E8 and E7 proteins were detected in the tumours.

Liu *et al* (1995) have shown that levels of E7 expression in baby rat kidney cells correlate with its transforming potential. The presence of mutant p53 or 16E6 may induce an increase in BPV-4 E7 levels leading to enhanced transformation. Crook *et al* (1991c) showed that mutant p53 potentiated the transforming functions of E7 in baby rat kidney cells. Their data suggested that E7 and p53 function in separate yet complementary pathways. Coexpression of E6 is known to increase levels of E7 (Halbert *et al*, 1991) and transformation appears to depend on enhanced expression of viral genomes. This has been demonstrated with the use of glucocorticoids (Pater *et al*, 1988) and strong heterologous promoters (Kanda *et al*, 1988). Thus increased levels of E7 mRNA and hence protein, can lead to accelerated transformation.

Expression of E8 and the loss of gap junctional communication may contribute to tumourigenicity. Initiated cells which have lost their ability to communicate with the surrounding normal cells are important for the clonal expansion of a population of cells capable of progressing. Although no direct

interaction between ductin and E8 has been observed in PalF cells, expression of E8 protein in the tumours extracted from the nude mice may indicate that these cells have lost their ability to communicate.

The presence of viral DNA is not required for maintenance of the transformed phenotype and is not detected in BPV-4 associated cancers (Campo *et al*, 1985). Viral proteins were detected in the nude mouse tumours confirming the presence of viral DNA, although no DNA was detected by the *in-situ* technique used. The short term culture of PalF cells *in vitro* may not mimic latent BPV-4 infection *in vivo*, and the presence of viral DNA may still be required to maintain the cell phenotype. These tumours may represent an intermediate stage of transformation which supports viral replication and viral protein production. Loss of viral DNA may be a consequence of long term transformation in combination with an accumulation of genetic alterations *in vivo*.

Thus, BPV-4 appears to lack several functions *in vitro* which can be provided by HPV-16E6 and mutant p53. These functions appear to be separate but can interact to enhance transformation.

6.2 Additional functions provided by 16E6 in transformation by BPV-4 *in vitro*.

The addition of an exogeneous 16E6 is required to confer immortality to BPV-4 transfected PalF cells *in vitro* and this function can be substituted by the addition of mutant p53. It is apparent that p53 mutations may be present *in vivo*, and BPV-4 is still lacking functions *in vitro* which can be provided by 16E6. PalF cells only become tumourigenic when both 16E6 and mutant p53 are present suggesting that the additional 16E6 functions are independent of the p53 growth suppressive pathway. To establish if 16E6 was capable of cooperating with BPV-4 in the absence of a functional p53 gene, p53 deficient mouse fibroblasts were transfected with BPV-4, *ras* and 16E6 as described (materials and methods section 3.2.3). p53-deficient mice were derived by the introduction of a null mutation into the p53 gene by homologous recombination (Donehower *et al*, 1992). Fibroblast cultures were derived from the skins of these homozygous p53 null mice as described (materials and methods section 3.2.2).

Results

In all transfections, the cells were morphologically transformed (Figure 6.2.1 B-H) in comparison to the untransfected p53 deficient fibroblasts (Figure 6.2.1A) and as described in table 6.2.1A. However, in cells transfected with 16E6 alone, the colonies had a much flatter appearance than the other transformed cells (Figure 6.2.1B). BPV-4 and 16E6 cooperated to produce

Figure 6.2.1 (on facing page)

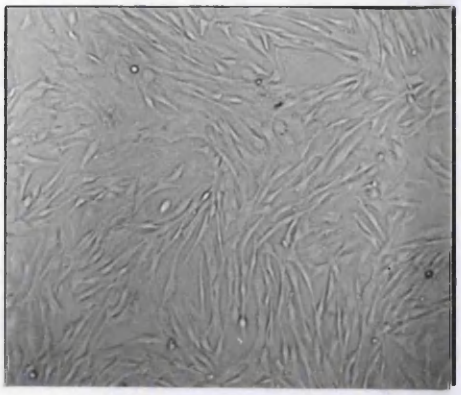
Morphological transformation of transfected p53 deficient fibroblasts.

The cells were transformed when transfected with plasmids (B-H)

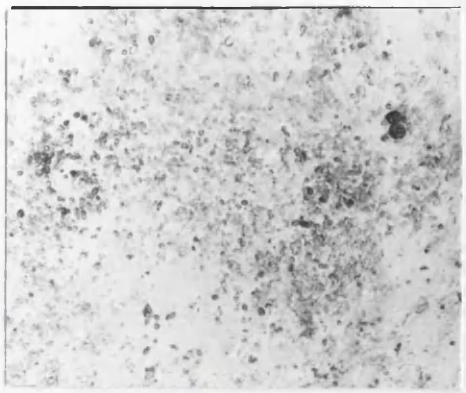
in comparison to the untransfected p53 deficient fibroblasts (A).

Magnification X 60.

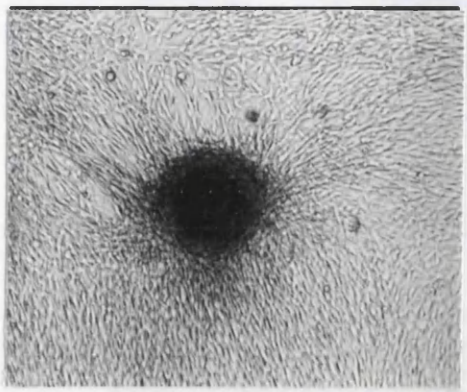
A p53 null fibroblasts



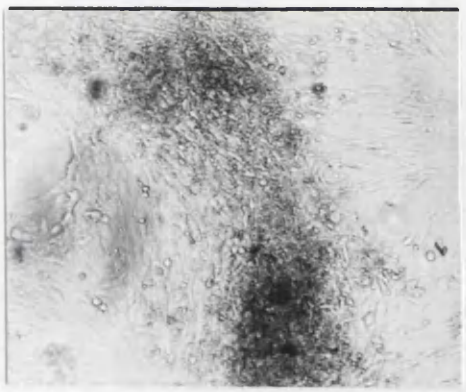
B *16E6*



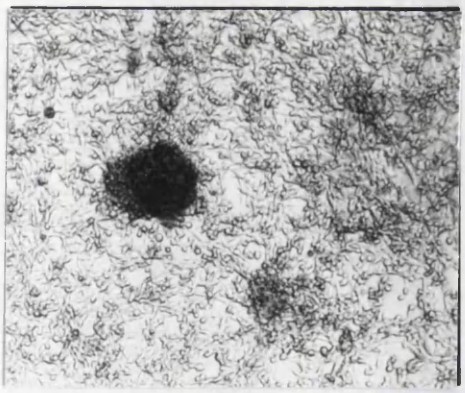
C *ras*



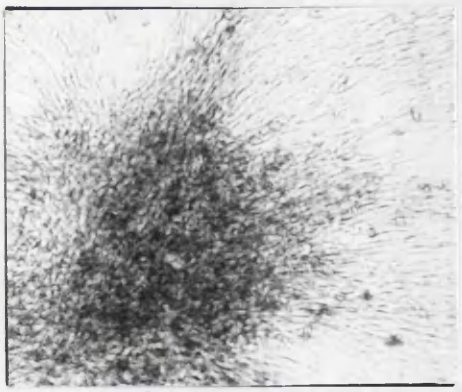
D BPV-4



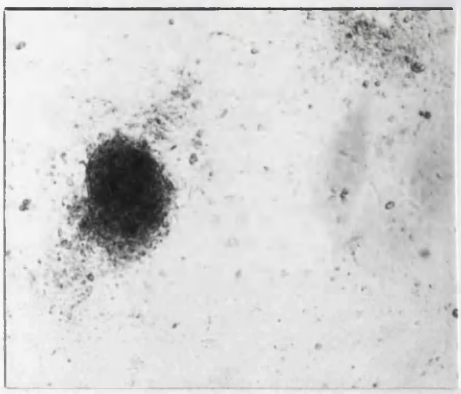
E 16E6+*ras*



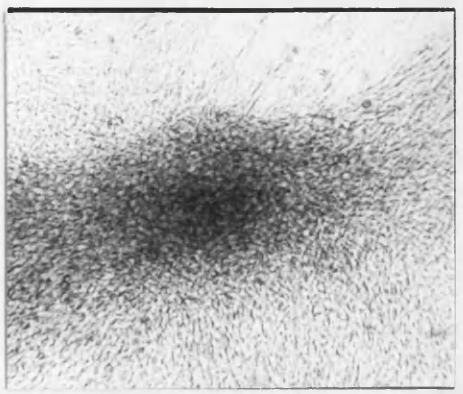
F *BPV-4+ras*



G BPV-4+16E6



H BPV-4+*ras*+16E6



A

	Morphological Transformation
(+hygro)	
pBabeHygro	-
BPV-4	+
ras	+
16E6	+
BPV-4+*ras*	+
ras+16E6	+
BPV-4+16E6	+
BPV-4+*ras*+16E6	+

B

	No. of colonies / Experiment			
	1	**2**	**3**	**Average**
pbabehygro	0	0	0	0
BPV-4	3	4	3	3
ras	1	7	1	3
16E6	3	2	1	2
BPV-4+ras	4	12	9	8
ras+16E6	10	9	9	9
BPV-4+16E6	10	12	14	12
BPV-4+*ras*+16E6	6	16	11	11

Table 6.2.1 Transformation efficiency of BPV-4, *ras* and HPV-16E6 constructs in primary p53 deficient mouse fibroblasts. Table A, shows if cells were transformed and table B, indicates the number of colonies formed for three separate transfections.

four to six fold as many colonies as when BPV-4 and 16E6 were transfected on their own (Table 6.2.1B). This effect appears to be synergistic as BPV-4 and 16E6 together produced two fold more colonies than the added number of colonies (five) of BPV-4 and 16E6. The significance of this observation cannot be determined as the colony numbers counted for the transfections were very small.

The additional presence of *ras* in the transfection did not appear to create a significant difference to the resulting number of colonies.

Six separate colonies were picked from each transfection and analysed for the ability to grow in 1% methocel. Untransfected p53 deficient fibroblasts and those transfectants containing BPV-4, *ras* or 16E6 on their own were not capable of anchorage independent growth as illustrated in figure 6.2.2A-D. However, in combination, as indicated in table 6.2.2, the transfected p53 deficient fibroblasts grew in 1% methocel (Figure 6.2.2E-H). The numbers of colonies per dish was constant regardless of the transfected plasmids as described in table 6.2.2.

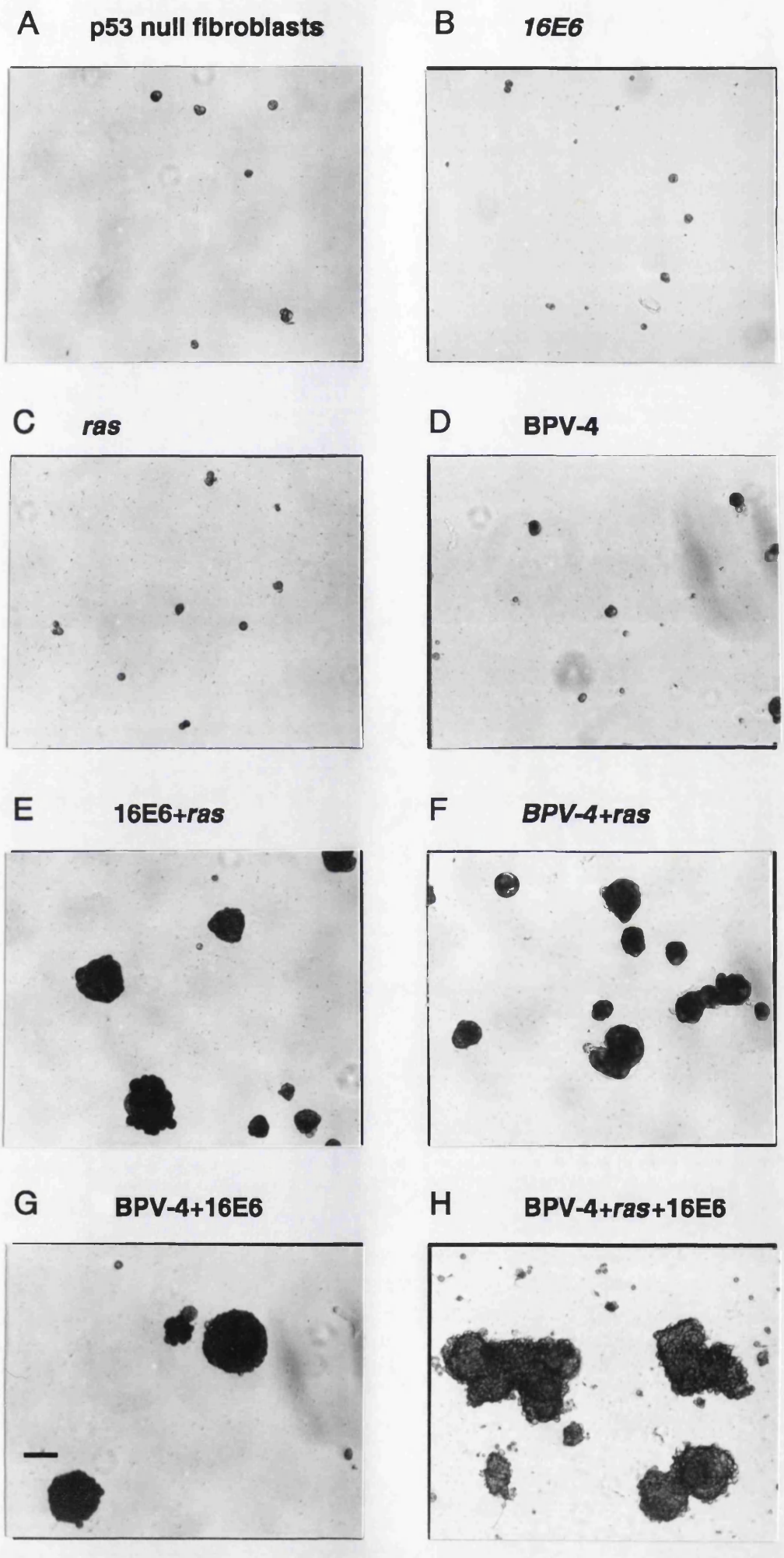
The growth rates of these transfectants was analysed by the MTT assay (Figure 6.2.3). The rates of growth for untransfected p53 deficient fibroblasts and a cell line derived from a facial tumour excised from a p53 homozygous null mouse were also determined as controls. Those cells transfected with BPV-4 alone grew at a rate equivalent to that of the untransfected p53 deficient fibroblasts (Figure 6.2.3A). p53 deficient fibroblasts transfected with *ras* or 16E6, appeared to have a reduced rate of growth in comparison to the other cell lines (Figure 6.2.3A).

Overexpression of an activated *ras* gene in the rat embryo fibroblast line REF52 results in a growth arrest at the G1/S or G2/M boundary of the cell cycle (Hicks *et al*, 1991). The reduction in the growth rate of p53 deficient

Figure 6.2.2 (on facing page)

Anchorage Independent growth of transfected p53 deficient fibroblasts. Untransfected cells and those transfected with BPV-4, *ras* or 16E6 alone were not capable of growth in soft agar (A-D). Cotransfection of the above did allow the cells to grow in soft agar (E-H).

Magnification X 60. Bar on panel G indicates 100μm.


A
p53 null fibroblasts
B
16E6
C
ras
D
BPV-4
E
16E6+ras
F
BPV-4+ras
G
BPV-4+16E6
H
BPV-4+ras+16E6

	Anchorage Independent Growth	Average no.* Anchorage Independent Colonies /cm^2
(+hygro)		
pBabeHygro	-	0
BPV-4	-	0
ras	-	0
16E6	-	0
BPV-4+*ras*	+	96
ras+16E6	+	96
BPV-4+16E6	+	94
BPV-4+*ras*+16E6	+	97

Table 6.2.2 Anchorage independent growth of transfected p53 deficient fibroblasts. The number of colonies determined for the clones analysed was not significantly different. + - indicates cell line positive for anchorage independent growth.

* Six separate clones from each transfection were assayed for anchorage independent growth and the average number of colonies counted on six separate sites on each dish.

fibroblasts transfected with activated *ras,* and the low number of colonies generated as a result of transfection (Table 6.2.1B) may be due to a negative growth regulation induced by *ras*; however, it is unclear what negative growth regulatory mechanisms, if any, 16E6 is exerting in these cells. p53 deficient fibroblasts are known to grow at a high rate during continuous passage (Harvey *et al,* 1993).

The additional presence of 16E6 and *ras* with BPV-4 did not appear to significantly increase or decrease the growth rate of the p53 deficient mouse fibroblasts (Figure 6.2.3B) in comparison to BPV-4 alone (Figure 6.2.3A). BPV-4 did however, overcome the negative growth regulation which was previously demonstrated in the presence of *ras* alone (Figure 6.2.3A) when both were transfected into the p53 deficient fibroblasts (Figure 6.2.3B). The negative effect of 16E6 however, was not released by the addition of BPV-4 (Figure 6.2.3B).

Thus, 16E6 appeared to slow the growth of the cells except when both BPV-4 and *ras* were present.

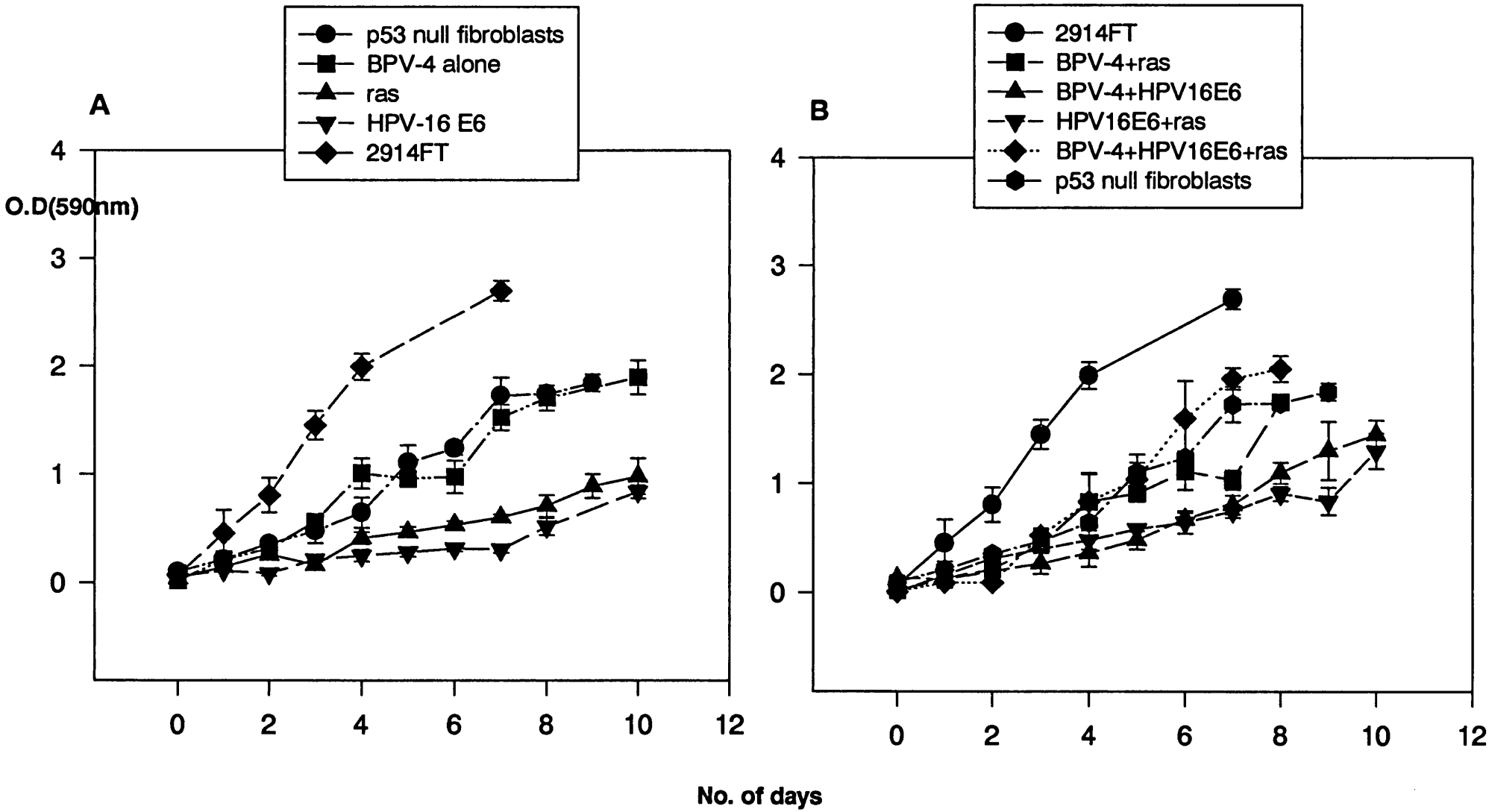
Figure 6.2.3 (on facing page)

Growth rates of transfected p53 deficient fibroblasts as determined by MTT assay.

Graph A illustrates the growth rates of p53 deficient fibroblasts transfected with BPV-4, *ras* or 16E6 alone (in the presence of pbabehygro).

2914FT is a cell line derived from a facial tumour explant taken from a p53 deficient mouse.

Graph B illustrates the growth rates of p53 deficient fibroblasts transfected with a combination of the above plasmids. The p53 untransfected deficient fibroblasts were assayed at passage #10.


A
p53 null fibroblasts
BPV-4 alone
ras
HPV-16 E6
2914FT
O.D(590nm)
4
3
2
1
0
0
2
4
6
8
10
12
B
2914FT
BPV-4+ras
BPV-4+HPV16E6
HPV16E6+ras
BPV-4+HPV16E6+ras
p53 null fibroblasts
4
3
2
1
0
0
2
4
6
8
10
12
No. of days

Conclusions and Discussion

As BPV-4 lacked transformation functions provided by 16E6 *in vitro,* further analysis of the BPV-4/16E6 interaction was carried out. Cells lacking an endogeneous p53 protein were transfected with BPV-4 and 16E6 to establish if the additional functions were indeed p53 dependent. 16E6 was found to provide transforming functions, i.e., anchorage independent growth, for p53 deficient cells transformed by BPV-4 *in vitro* suggesting that the additional functions of 16E6 were indeed p53 independent. Although it is apparent that BPV-4 and 16E6 can cooperate to transform p53 deficient mouse fibroblasts in the absence of *ras*, there is not a significant effect on the growth of the cells except in the presence of BPV-4, 16E6 and *ras*. The presence of 16E6 conferred a growth advantage to transfected PalF cells (Figure 6.1.3). It may be that the growth advantage provided by 16E6 in PalF cells is a p53 dependent function as it is absent in the p53 deficient fibroblasts. The growth advantage seen in the PalF cells may also be a function of *ras* as BPV-4 and *ras* cooperate to increase the growth rate of the p53 deficient fibroblasts. This cooperation may occur in the PalF cells as an activated *ras* is an absolute requirement for transformation, no colony formation occurs in the absence of an activated H-*ras*. This seems a more likely explanation as E6 is known to cooperate with EJ-*ras* in murine cells independent of its ability to associate with p53 (Pim *et al*, 1994). This would suggest that 16E6 cooperates with *ras* in both PalF cells and p53 deficient mouse fibroblasts to enhance the growth rates of these cells. *ras* activation may be an early event in BPV-4 transformation although activation of *ras* has only been observed in cancers (Campo *et al*, 1990).

BPV-4 and 16E6 synergised to transform p53 deficient cells *in vitro*. These cells were capable of anchorage independent growth, but it is not known if they are tumourigenic in nude mice. The initial observation was that *ras* was no longer required for transformation in the absence of p53, suggesting that loss of p53 can replace activation of *ras*. Alternatively, it may be that the *ras* gene has already been inactivated in these cells, as in p53 deficient cells only about a third of the cells contain the normal number of chromosomes and the rest of the cells are aneuploid after only three passages (Harvey *et al*, 1993).

Other functions of 16E6 may contribute to transformation in p53 deficient cells as 16E6 has been shown to repress AdE2 promoter activity independent of p53 (Etscheid *et al*, 1994). Keen *et al* (1994) have demonstrated that 16E6 binds other cellular proteins in addition to p53. The functions of these proteins have not yet been characterised and may be important in p53 independent pathways of transformation.

6.3 Further analysis of the HPV16-E6 transforming functions independent of p53.

To further characterise these apparent p53 independent functions and to further define the region of the E6 oncoprotein which could cooperate with BPV-4, a panel of 16E6 mutants (Crook *et al*, 1991a) as described in table 6.3.1, were cotransfected into p53 deficient fibroblasts together with BPV-4 DNA.

E6 is an oncoprotein of 151 amino acids (Figure 1.6). As discussed previously, the E6 protein has functions which vary according to the relative risk of the papillomavirus infection. Certain amino acids have been found to be conserved between those E6 oncoproteins of the oncogenic or high risk HPVs. Point mutations were targeted to the conserved Cys-x-x-Cys motifs (Gly 103 and Gly 136) and the amino acids conserved among E6 oncoproteins encoded by the oncogenic HPV types (YYH, Δ111-115 and Δ123-127) (Figure 6.3.1A). The latter mutations were designed to result in the substitution with those residues found in the benign (low risk) HPV types. A premature termination mutation which resulted in the expression of a truncated E6 protein (Δ110) (Figure 6.3.1A), and chimaeric proteins consisting of the N and C-terminal regions of HPV-16 and HPV-6 E6 as well as the wild type HPV16 and HPV6 E6 proteins were also examined for their ability to cooperate with BPV-4 (Figure 6.3.1B and C). The mutants described above are illustrated in figure 6.3.1 and table 6.3.1.

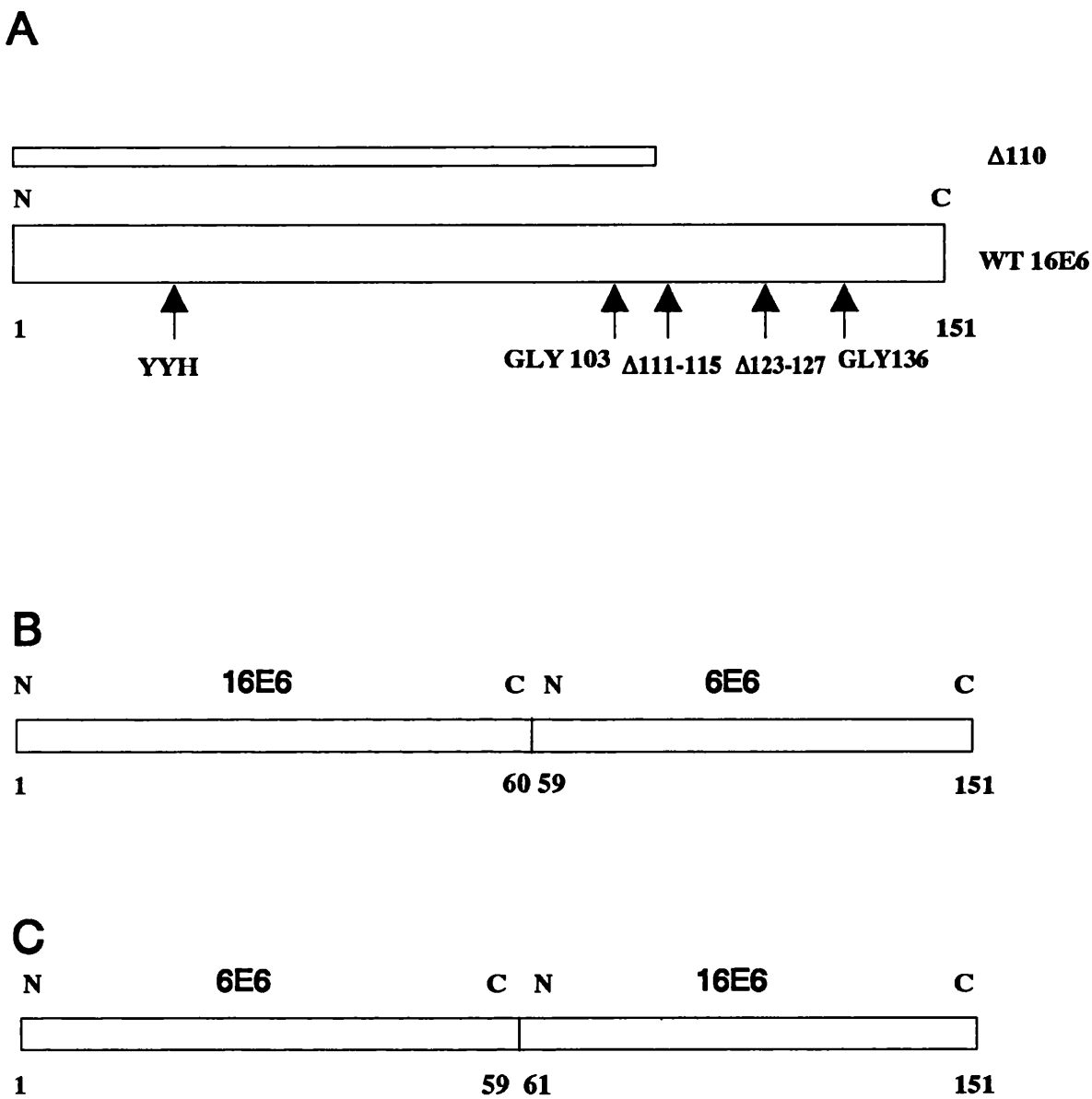


Figure 6.3.1 Location of the mutations in HPV-16E6 and the structure of the chimaeric proteins.

Construct	Alteration	p53 binding	p53 degradation	Transactivation
wt 16E6	none	+	+	+
wt 6E6	none	+	-	+
YYH	AA45Y,46Y,47H	+	-	+
Δ110	Deletion AA111-151	-	nd	-
Δ111-115	Deletion AA111-115	-	-	+
Δ123-127	Deletion AA123-127	+	+	-
GLY103	Cys103Gly	+	nd*	nd*
GLY136	Cys136Gly	+	nd	nd
6/16	N6E6, C16E6	+	-	+
16/6	N16E6, C6E6	+	+	+

Table 6.3.1 Summary of the wild type and mutant HPV-16E6 plasmids and their functions (Crook *et al*, 1991a). nd - not determined.

* - Although not determined for HPV16E6, a similar mutation in HPV-18E6 demonstrated reduced ability (less than 50%) to degrade p53 but was still capable of transactivating the adenovirus E2 promoter (Pim *et al*, 1994).

Results

All of the mutants described above were transfected singly and in the presence of BPV-4 into p53 deficient mouse fibroblasts. Figures 6.3.2 to 6.3.5 are a representation of the extent of colony formation observed when the transfectants were stained with 10% Geimsa after two to three weeks.

All of the mutants retained the ability to morphologically transform the p53 deficient fibroblasts and to form colonies both when transfected on their own or with BPV-4. Table 6.3.2 summarises the average number of colonies obtained as a result of five separate transfection experiments.

In comparison to the synergy of BPV-4 and 16E6, none of the mutants or 6E6 cooperated with BPV-4 to transform the p53 deficient fibroblasts as efficiently as wild type 16E6, although all of the mutants were capable of morphologically transforming the cells (Figure 6.3.6). The numbers of colonies obtained for each experiment varied, however the appearance of the stained flasks was similar throughout the separate experiments. It is possible that due to the small flasks used colonies were not counted consistently, as it is obvious in figures 6.3.2 to 6.3.5 that colonies merged into one another and it was difficult to determine the boundary of one colony and that of the next.

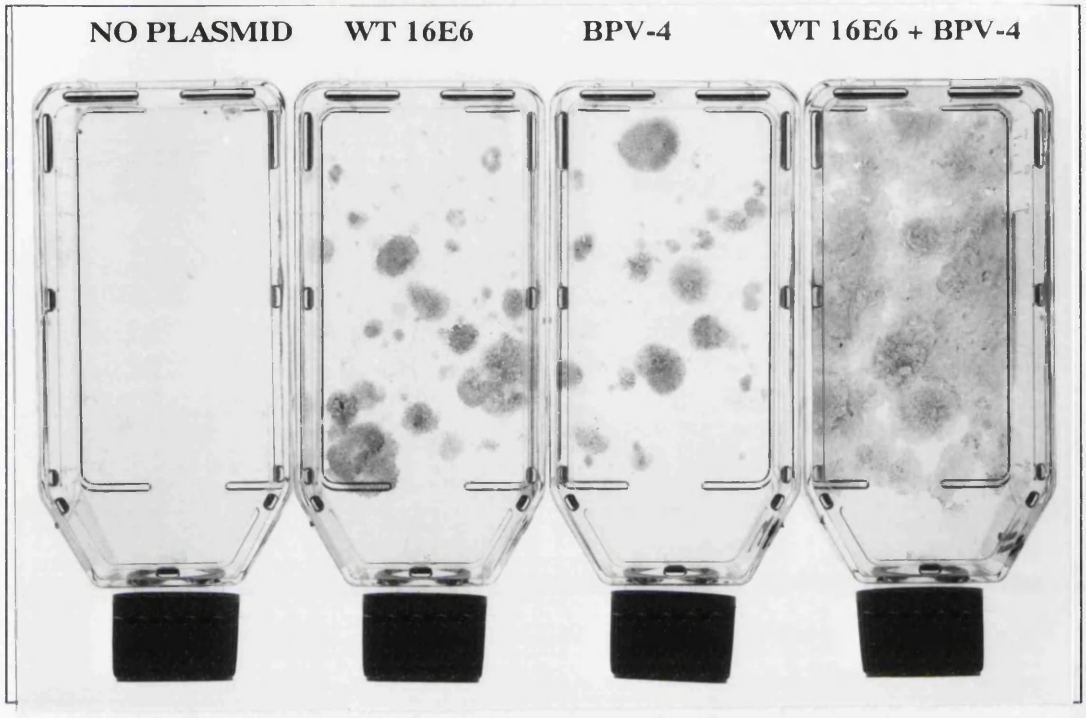


Figure 6.3.2 Wild type 16E6 plasmid transfected into p53 deficient fibroblasts with and without BPV-4 DNA followed by selection with hygromycin (125μg/ml) for 2-3 weeks. Cells in T25 flasks were then stained with 10% Giemsa and photographed. All transfection mixes contained pbabehygro plasmid for selection and 'no plasmid' indicates the control transfection with pbabehygro alone.

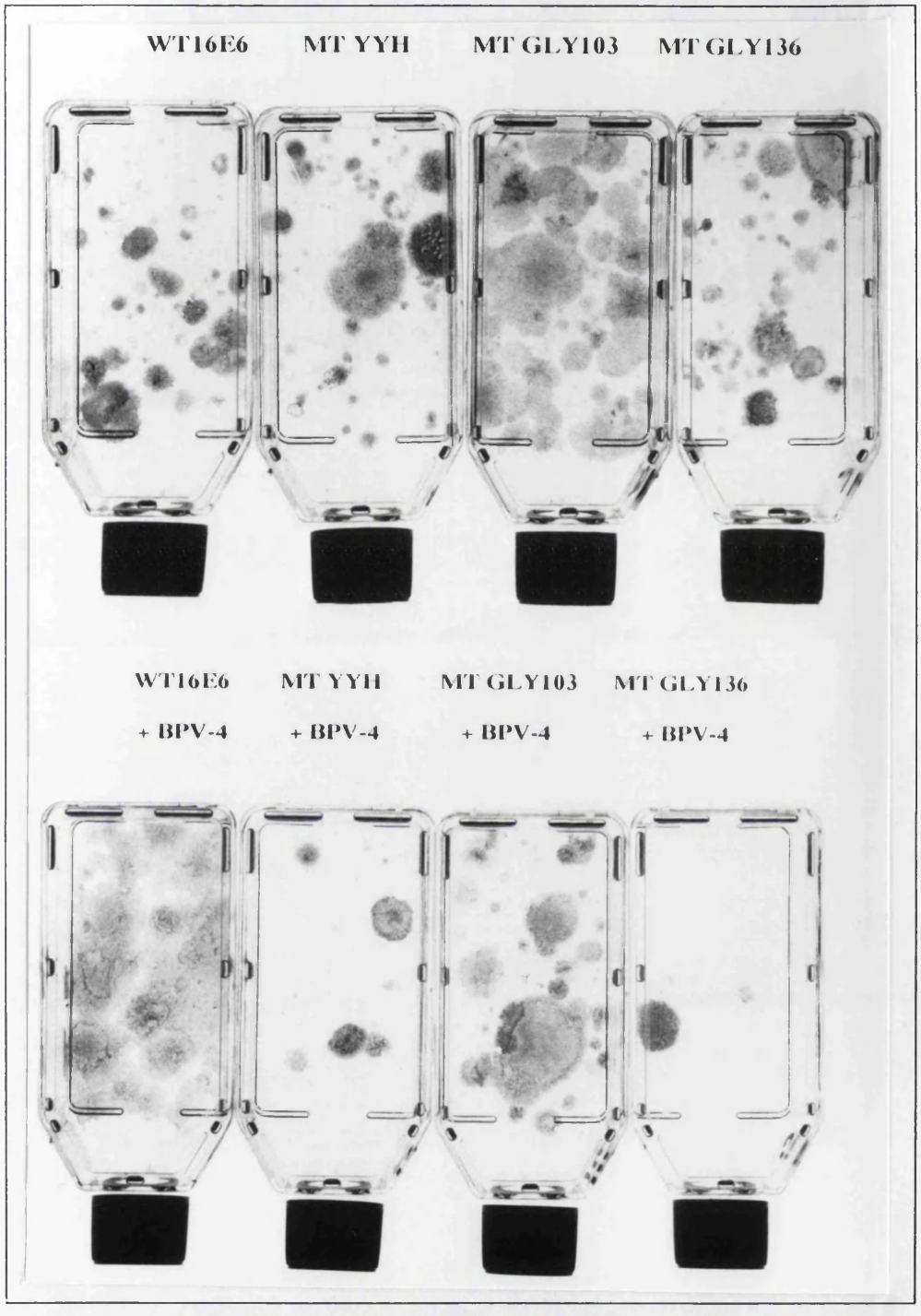


Figure 6.3.3 Mutant E6 plasmids transfected into p53 deficient fibroblasts with and without BPV-4 DNA. Other details as for figure 6.3.2.

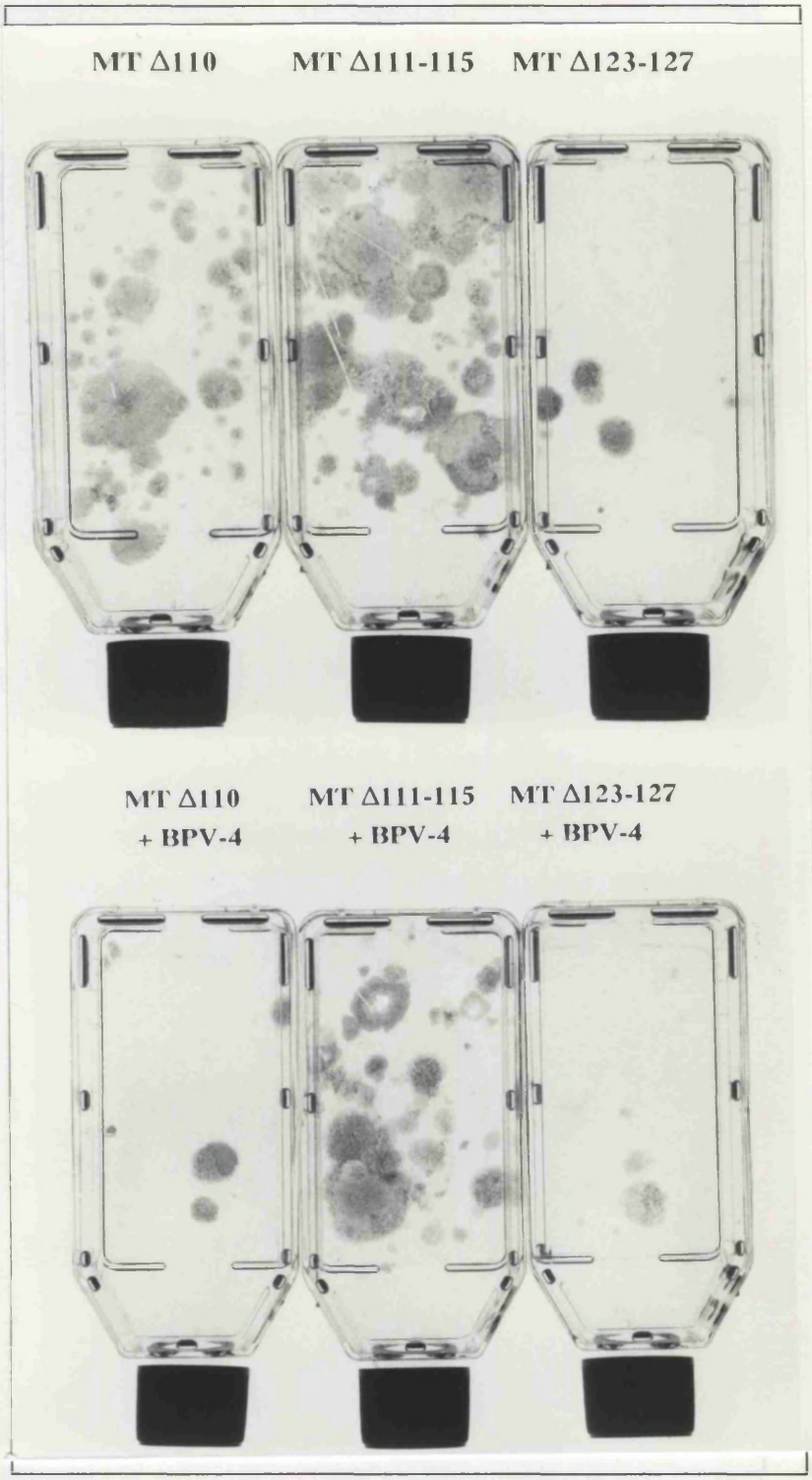


Figure 6.3.4 Mutant E6 plasmids transfected into p53 deficent fibroblasts with and without BPV-4 DNA. Other details as for figure 6.3.2.

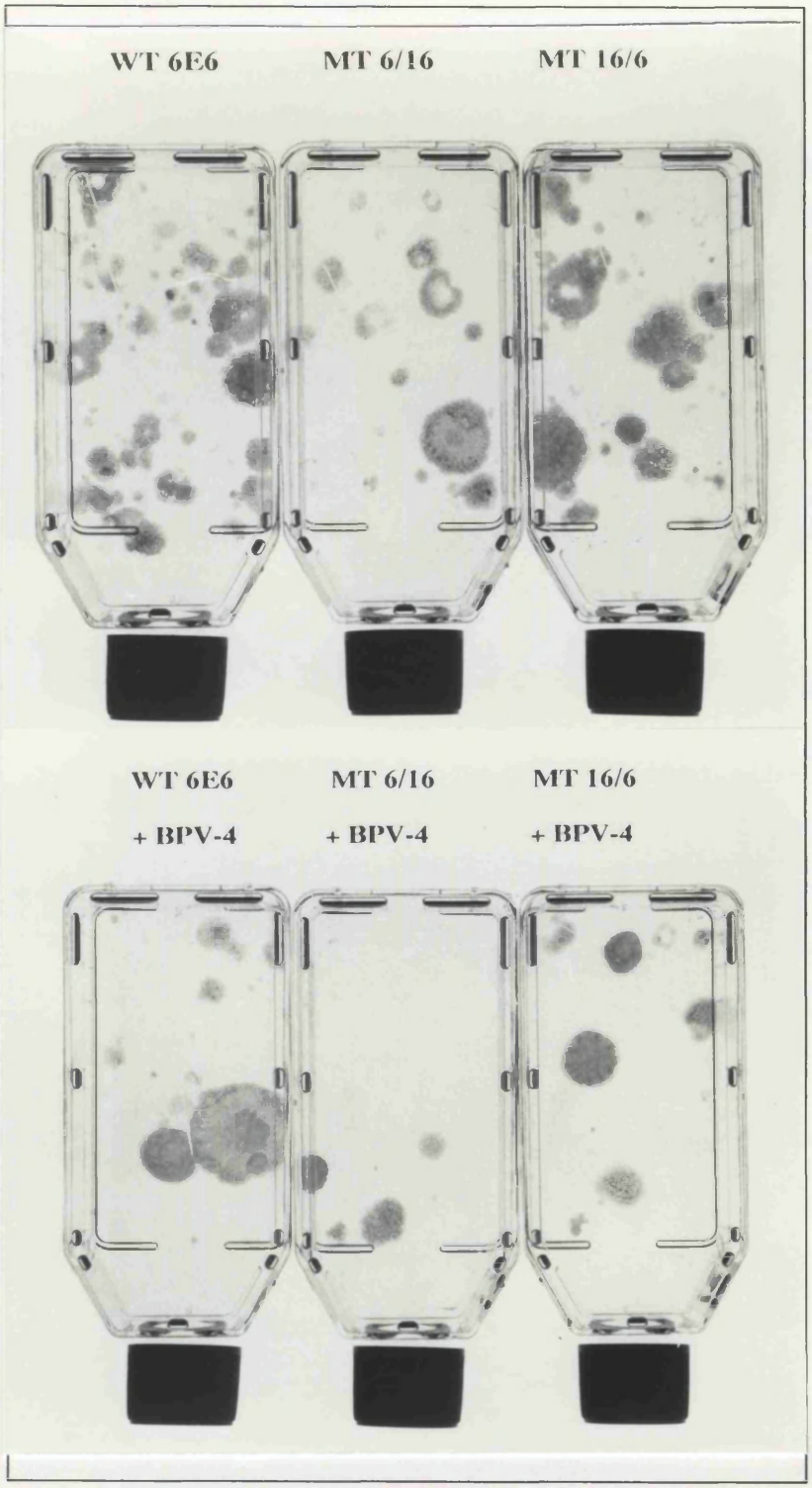


Figure 6.3.5 HPV6E6 and 6E6/16E6 chimaeric plasmids transfected into p53 deficient fibroblasts with and without BPV-4 DNA. Other details as for figure 6.3.2.

Table 6.3.2 Summary of the number of transformed colonies (*average of five separate experiments) and the % of the transformed colonies picked per flask that were positive for growth in 1% methocel. pbabehygro was present in all transfections. Figures in brackets indicate the standard error.

Transfection	Average no. of colonies / T25 flask* (S.E)	% clones anchorage independent (colonies positive/colonies tested)
BPV-4	4 (0.9)	0
16E6	5 (0.9)	0
BPV-4+16E6	20 (1.4)	100
6E6	8 (1.0)	0
BPV-4+6E6	6 (1.6)	50
Δ110	8 (1.8)	0
BPV-4+Δ110	9 (2.0)	0
YYH	8 (1.6)	0
BPV-4+YYH	8 (1.9)	50
GLY103	8 (1.0)	0
BPV-4+GLY103	4 (1.0)	25
GLY136	5 (0.8)	0
BPV-4+GLY136	5 (0.9)	50
6/16	8 (1.0)	0
BPV-4+6/16	8 (1.4)	50
16/6	5 (1.3)	0
BPV-4+16/6	5 (1.6)	75
Δ111-115	6 (1.3)	0
BPV-4+Δ111-115	4 (0.9)	75
Δ123-127	4 (0.9)	0
BPV-4+Δ123-127	4 (0.8)	75

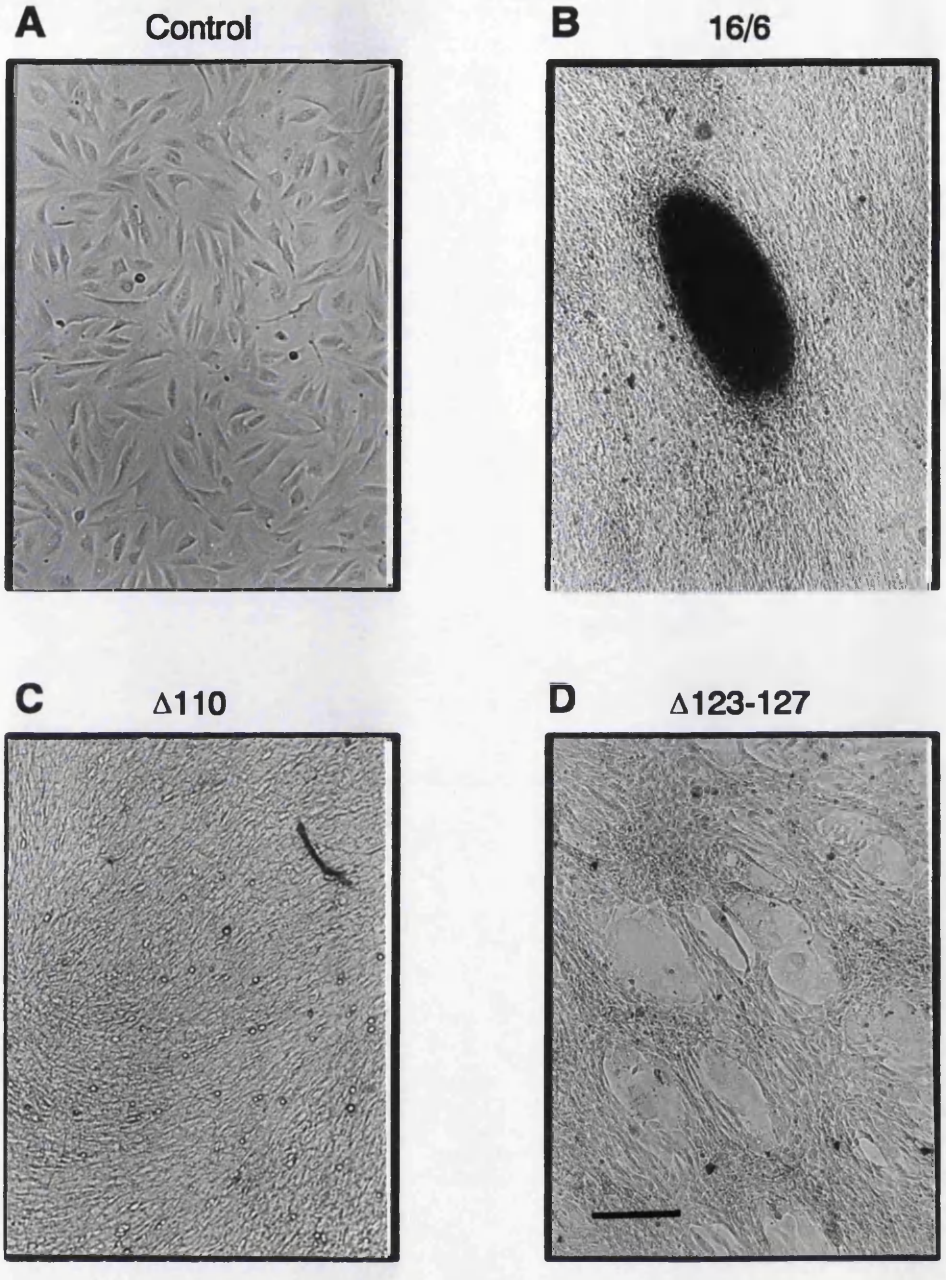


Figure 6.3.6 Examples of morphological transformation of p53 deficient fibroblasts transfected with mutant 16E6. Panel A, untransfected p53 null fibroblasts, panel B, cells transfected with chimaeric mutant 16/6, panel C, cells transfected with deletion mutant Δ110, and panel D, cells transfected with Δ123-127. Magnification X125 (A), X 60 (B-D). Bar in panel D indicates 100μm.

The transfectants were analysed for the ability to grow in 1% methocel (Table 6.3.2; figure 6.3.7) and their growth rates determined (Figure 6.3.8). None of the transfectants containing mutant 16E6 plasmids alone were capable of anchorage independent growth (Table 6.3.2). However, in cooperation with BPV-4, only mutant Δ110 lost the ability to confer anchorage independent growth. Mutants Gly103 and Gly136 were diminished in their ability to cooperate with BPV-4 and alteration of Cys103 to Gly103 reduced the percentage of colonies positive for anchorage independent growth to only 25%. The presence of intact cysteine residues in both zinc finger motifs of 16E6 appears to be required for cooperation with BPV-4 and anchorage independent growth of the p53 deficient cells. The alteration and deletion of residues conserved among the oncogenic HPVs also led to a reduction in the number of colonies capable of anchorage independent growth. This may suggest that the ability of 16E6 to induce anchorage independent growth in the presence of BPV-4 in p53 deficient cells is considerably reduced in the low risk HPVs and a function of the oncogenic HPVs. From the data shown, anchorage independent growth did not appear to correlate with the mapped p53 binding and degradation functions or transactivation which would suggest that the ability to confer anchorage independent growth in the absence of p53 may be an as yet unidentified function of 16E6.

The MTT assay as shown in figure 6.3.8, demonstrated that although there was no significant difference in the growth rates of either 16E6 or 6E6 in conjunction with BPV-4 or the chimaeric constructs (figure 6.3.8A and B), mutant YYH (Figure 6.3.8C), Gly 136 (Figure 6.3.8D), and Δ111-115 (Figure 6.3.8E) demonstrated increased rates of growth in cooperation with BPV-4. It is unclear how this cooperation is occurring as the wild type E6 constructs do not increase the rate of growth confirming the previous observations that the growth advantage in the presence of BPV-4 is possibly *ras* dependent.

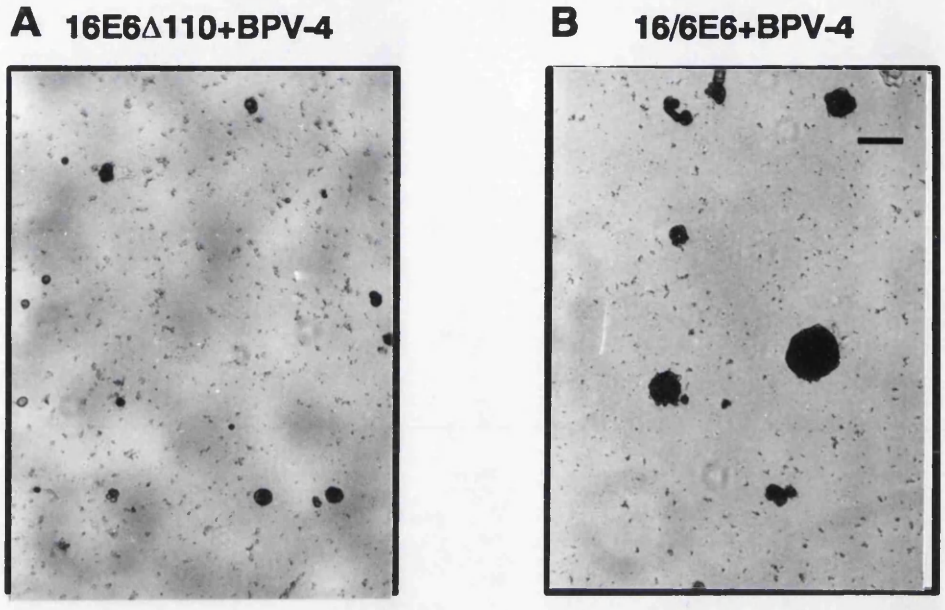
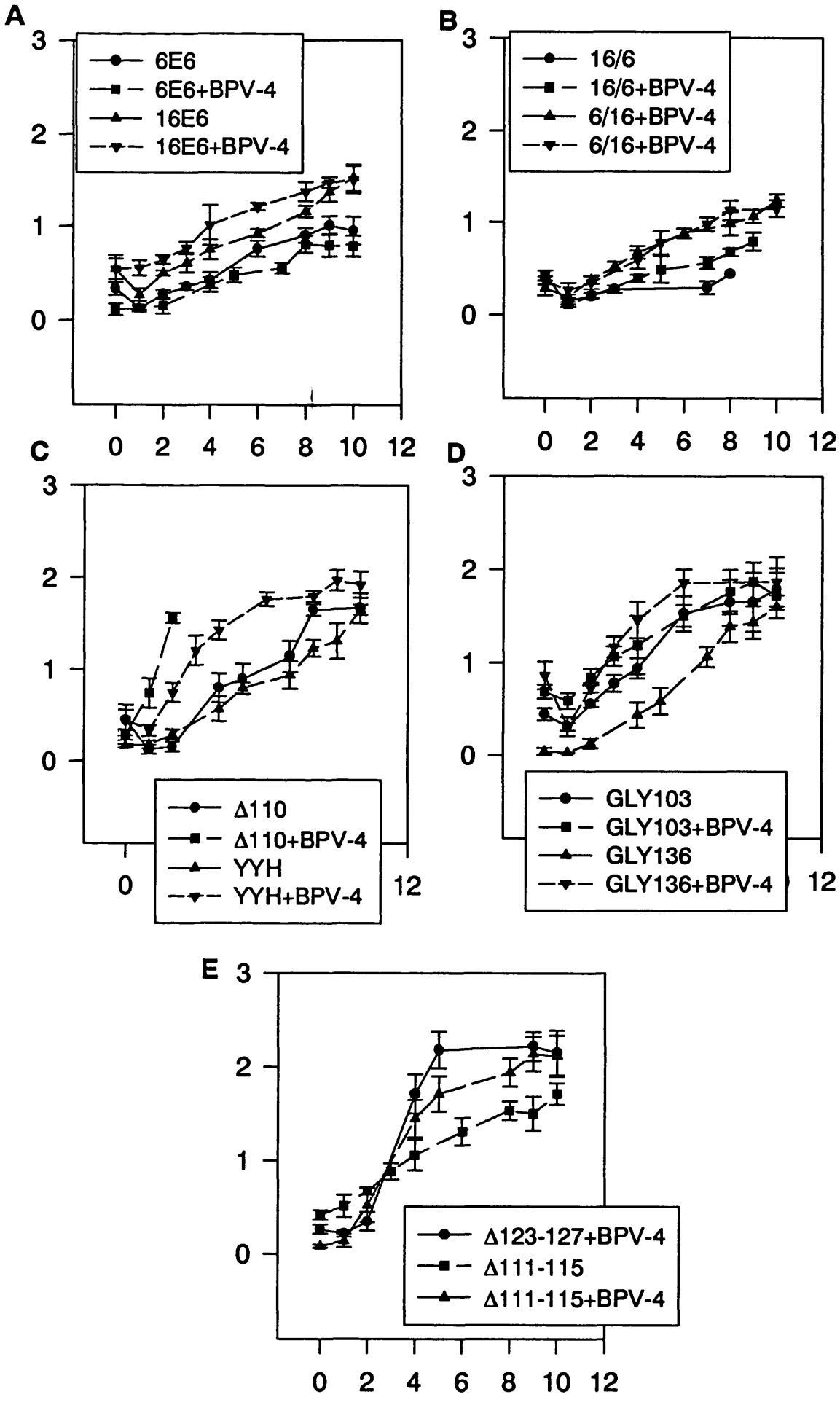


Figure 6.3.7 Anchorage independent growth of p53 deficient fibroblasts transfected with mutant 16E6 and BPV-4. Panel A; cells transfected with Δ110 and BPV-4 are negative for A.I growth and panel B; cells transfected with chimaeric 16/6 and BPV-4 which were positive for A.I growth.
Magnification X 60. Bar in panel B indicates 100μm.

Figure 6.3.8 MTT assay of growth rates for transfected p53 deficient fibroblasts. Graphs A-D illustrate the average growth rates from two separate experiments.


A
6E6
6E6+BPV-4
16E6
16E6+BPV-4
B
16/6
16/6+BPV-4
6/16+BPV-4
6/16+BPV-4
C
Δ110
Δ110+BPV-4
YYH
YYH+BPV-4
D
GLY103
GLY103+BPV-4
GLY136
GLY136+BPV-4
E
Δ123-127+BPV-4
Δ111-115
Δ111-115+BPV-4

These mutants may demonstrate the interactions of 16E6 with other proteins which allow cooperation with BPV-4 to increase the growth rate of the p53 deficient fibroblasts. It may be necessary to further dissect BPV-4 and look at the sub-genomic components in cooperation with the mutant E6 constructs to accurately define the functions BPV-4 lacks.

Conclusions and Discussion

The use of 16E6 mutants to further characterise the BPV-4/16E6 interaction demonstrated that the cysteine residues in the zinc finger motifs of 16E6 are required for the interaction with BPV-4 to confer anchorage independent growth. Crook *et al* (1991a) showed that all of the HPV-16E6 mutants except for Δ110 could efficiently transactivate the adenovirus E2 promoter. This suggests that they are expressed as functional proteins and differences in their abilities observed here and their previously characterised functions are not attributable to a lack of expression or instability of the mutant proteins. Analysis of both HPV-16 and HPV-18 E6 mutants has led to the observation that p53 degradation and the transactivation activities of E6 are separable (Crook *et al*, 1991a; Pim *et al*, 1994) and p53 independent functions of E6 are apparent in these cells. In the absence of p53 other proteins may be targeted for degradation as has already been demonstrated (Scheffner *et al*, 1992a).

The anchorage independent functions provided by 16E6 in the presence of BPV-4 appear to be p53 independent and so may the cooperation between BPV-4 E7 and E8 oncoproteins to induce anchorage independent growth of PalF cells (Pennie *et al*, 1993; M Cairney, PhD thesis). Thus, although p53 inactivation is important in BPV-4 transformation it may not be involved in anchorage independent growth of cells. It is necessary to confirm which

BPV-4 oncoprotein interacts or cooperates with 16E6 to enable the cells to grow in soft agar.

Summary

p53 protein levels were found to be elevated in experimentally induced papillomas from animals inoculated in the palate with purified BPV-4. 38% of these experimental papillomas were found to possess putative mutations in bovine *p53* exon 7. The naturally occurring papillomas and carcinomas had a lower incidence of mutation in *p53* exon 7, but this does not rule out the presence of other mutations. The cell lines were also shown to possess putative mutations. Previous data suggest that spontaneous mutations arise in cell culture on transfection with the HPV viral oncogenes and the nature of the mutations suggest that they are not attributable to a single mechanism.

Addition of an exogeneous mutant p53 to PalF cells in the presence of BPV-4, *ras* and HPV-16E6 (16E6) led to tumourigenicity. BPV-4 was indeed found to be lacking in functions which could be provided by 16E6, some of which appeared to be independent of the p53 binding and degradation pathway. The presence of mutant p53 *in vitro* was not required for morphological transformation or anchorage independent growth in PalF cells transformed BPV-4, however, mutant p53 was capable of substituting for 16E6 to confer immortality to PalF cells and was required for tumourigenicity in nude mice. Partial transformation by BPV-4 *in vitro* does not appear to require a mutant p53 but the progression to carcinoma may involve *p53* mutations which give the cells a selective advantage and make them targets for further alteration.

In cervical cancer, p53 status is varied depending on the presence of HPV DNA. Generally, if HPV DNA is present then p53 is wild type and appears to possess its normal cell regulatory functions (Butz *et al*, 1995), albeit at very low levels due to the presence of E6. Absence of HPV DNA usually results in the mutation of the p53 gene. As viral DNA is lost in BPV-4

associated cancers *in vivo*, the presence of mutant p53 may be an important step in progression to carcinoma.

Complementation of BPV-4 with 16E6 in both PalF and p53 deficient cells demonstrated that BPV-4 did not encode E6-like functions. Although BPV-4 lacks the p53 binding and degradation functions of the E6 oncoprotein, this does not appear to affect the ability of BPV-4 to transform cells both *in vivo* and *in vitro*. The E6 functions lacking in BPV-4 can be partially replaced by the addition of a mutant p53 and *p53* mutations found in BPV-4 induced lesions would suggest that p53 dysfunction does indeed appear to have a role in BPV-4 transformation. The absence of an E6 ORF is not detrimental to cell transformation by BPV-4, however progression to tumourigenicity does require additional functions.

GENERAL DISCUSSION

CHAPTER 7

General Discussion

Introduction

The natural progression to carcinoma *in vivo* as a result of BPV-4 infection and the presence of cofactors is summarised in figure 7.1

Infection by BPV-4 results in benign papillomas of the alimentary canal which progress to squamous cell carcinoma in cattle feeding on bracken fern. Bracken fern has been shown to contain immunosuppressants and cocarcinogens which contribute to this process (reviewed in Jackson and Campo, 1995b).

The action of immunosuppressants present in bracken creates a widespread papillomatosis which is susceptible to further transformation. Activation of *ras* (Campo *et al*, 1990) and an increase in the numbers of EGF receptors (Smith *et al*, 1987) are also known to occur during tumour progression. The loss of viral DNA at this stage may reflect the advanced stages of progression and that the presence of virus is no longer required or that the cells are no longer suitable for viral replication.

BPV-4 lacks an E6 ORF as do the other subgroup B bovine papillomaviruses (Jackson *et al*, 1991). The E6 proteins of the oncogenic HPVs 16 and 18 bind to and degrade the p53 protein and this event is thought to contribute to transformation by these viruses (Scheffner *et al*, 1990; Werness *et al*, 1990). It is obvious that in the presence of cofactors, BPV-4 is capable of full malignant transformation *in vivo* and *in vitro* (Campo and Jarrett, 1986; Pennie and Campo, 1992; Campo *et al*, 1994b; Cairney and Campo, 1995). This does not appear to be hindered in any way by the lack of an E6 oncoprotein and raises the question whether E6 functions are necessary for BPV-4 transformation.

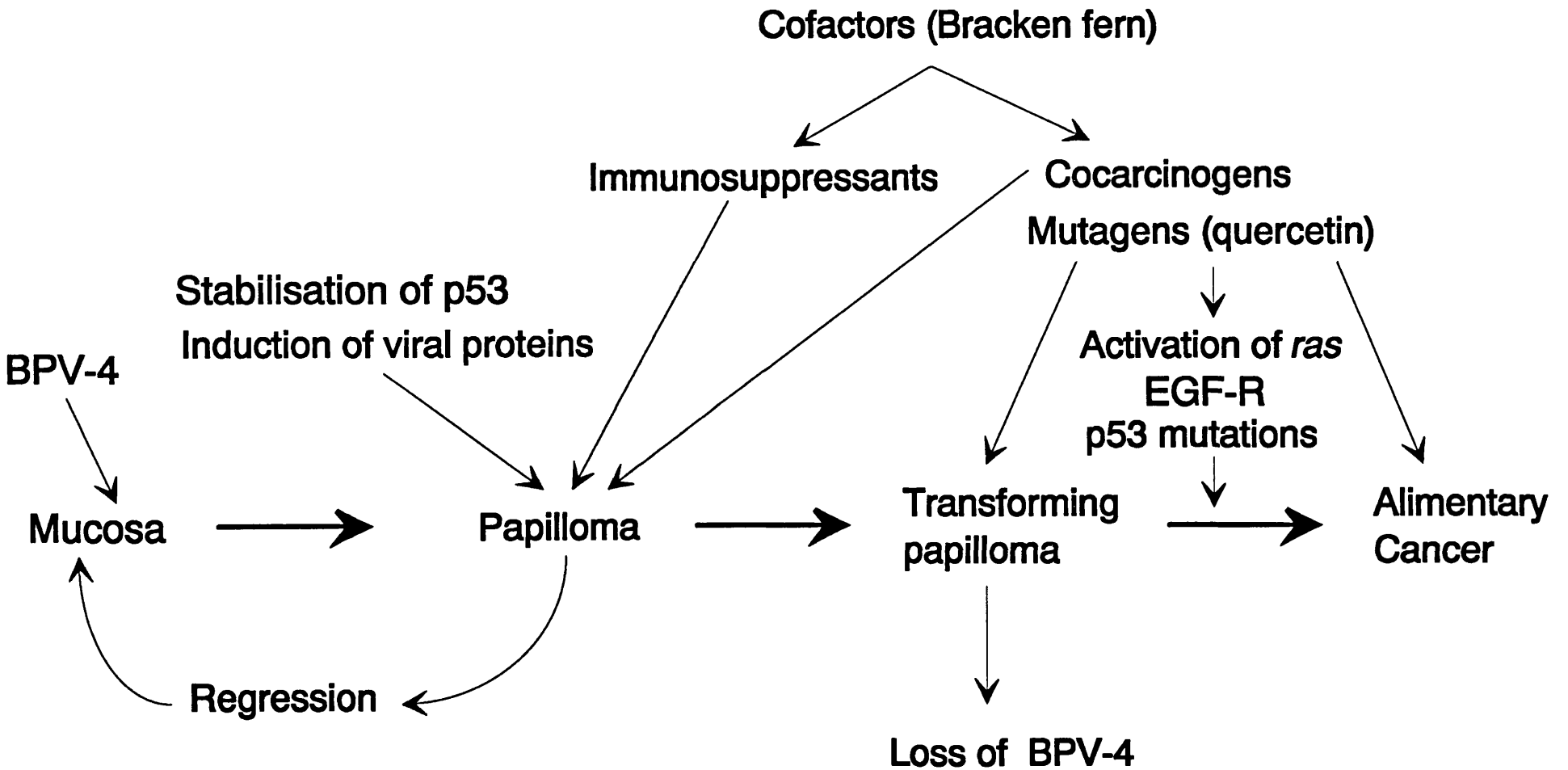


Figure 7.1 Natural history of BPV-4 infection in vivo.

The absence of an E6 ORF suggests the hypothesis that the p53 protein would be unaltered in papillomas but that *p53* mutations may occur during the progression of benign papillomas to carcinomas. Alternatively BPV-4 may have evolved another mechanism of evading p53 growth suppression.

The work in this thesis examines the status of the bovine p53 in both naturally occuring and experimentally BPV-4 induced papillomas and carcinomas. We have also studied the significance of the absence of the E6 ORF by investigating the roles of an exogenous 16E6 and p53 in BPV-4 cell transformation.

***p53* mutations *in vivo* and *in vitro*.**

p53 protein levels were found to be elevated in experimentally BPV-4 induced papillomas, however, no p53 protein could be detected in an oesophageal carcinoma. Analysis of these papillomas by SSCP-PCR suggested that mutations were present in bovine *p53* exon 7 in several of these papillomas. Technical difficulties arose in the sequencing of the DNA from these papillomas and so we were unable to confirm the exact nature of the exon 7 mutation present. The samples did appear heterozygous and evidence of a C to T transversion was identified in one of the papillomas. This could not be confirmed due to the number of unidentified nucleotides in the sequence analysis (Figure 5.2.4). It could be suggested that the mutation present in these experimentally induced BPV-4 papillomas is different from that identified in the naturally occurring metastases.

No mutations in *p53* exon 7 were found in experimentally induced urinary bladder carcinomas although these were identified as non-BPV related. Naturally occuring papillomas were examined for the presence of mutations in *p53* exon 7 and none were detected although it is not known if the p53 protein is stabilised in these papillomas. Two naturally occuring metastases harboured *p53* mutations in exon 7 altering a proline residue to a threonine at codon 243. No other *p53* exons were analysed and so it cannot be ruled out that mutations may be present in other exons.

Only two naturally occurring papillomas were analysed and found to possess a wild type *p53*. It is possible that mutations are present elsewhere in the *p53* gene and that analysis of a larger number of papillomas may also reveal mutations in exon 7. One aspect of the naturally occurring papillomas which was not investigated was that of the p53 status by immunocytochemistry. We do not know if p53 is also stabilised in these papillomas, as was the case for the experimental papillomas which demonstrated a stabilisation of the p53 gene and

a mutation in exon 7. Further characterisation of the other *p53* exons as discussed above would confirm if mutations were indeed present. Mutations have also been identified outside the highly conserved exons 5-8 (Hartmann *et al*, 1995) and these regions may harbour potential BPV-4 induced mutations.

PalF cells which had been transformed by whole genome and sub-genomic fragments of BPV-4 and treated with quercetin (M. Cairney; PhD thesis) were also analysed by SSCP-PCR. Quercetin is one of the mutagens present in bracken fern (Evans, I.C *et al*, 1982; Evans, W.C *et al*, 1982). The action of quercetin both *in vitro* and *in vivo* has been widely studied (reviewed in Jackson *et al*, 1993; Jackson and Campo, 1995b). Quercetin also synergises with BPV-4 *in vitro* to fully transform PalF cells (Pennie and Campo, 1992; Cairney and Campo, 1995). Although treatment of BPV-4 transformed PalF cells lead to a more aggressively transformed phenotype and tumourigenicity in nude mice, no quercetin-induced mutagenic effects were detected (M. Cairney, PhD thesis). Several putative *p53* exon 7 mutations were detected in both the quercetin and non-quercetin treated cell lines, and there appeared to be no correlation between the presence of *p53* mutation and quercetin treatment. The initial studies provided in this thesis suggest that *p53* exon 7 mutations may not be directly related to environmental cofactors. The animals from which the experimental papillomas were obtained did not receive any bracken fern and were only exposed to BPV-4.

We know from this thesis, that at the papilloma stage the production of viral proteins takes place and possible mutation of the *p53* gene occurs. *p53* mutation may be one of the deciding factors in conjuction with cofactors which determine whether the papilloma remains benign and regresses via an immune response (Knowles *et al*, 1996) or if it transforms and progresses to a carcinoma.

Demers *et al* (1994a) showed that wild type p53 levels were elevated in primary foreskin epithelial cells immortalised by the HPV-16 E7 (16E7) gene.

p53 is known to function as a cellular checkpoint and high levels of p53 protein associated with DNA damage, are accompanied by a growth arrest (Kastan *et al*, 1991). Demers *et al* (1994b) demonstrated that this growth arrest can be bypassed by expression of 16E7 in DNA damaged keratinocytes. Expression of the BPV-4 E7 (4E7) oncoprotein was detected at all papilloma stages but not in the regressing lesion. The p53 protein was detected in all of the papillomas examined. The stabilisation of p53 was initially thought to be a consequence of 4E7 expression, however, the absence of E7 protein in the regressing lesion suggested that this may not be the case. The lack of 4E7 expression in this lesion may represent the difference between a papilloma which is capable of further progression and one which follows the typical regression pathway. 4E7 has sequence similarity to and functional homology with 16E7 (Campo *et al*, 1994b). If 4E7, like 16E7, can bypass a p53 induced growth arrest this may result in the accumulation of genetic alterations which could contribute to the BPV-4 transformation pathway.

Transformation by papillomaviruses appears to depend on the targeting and inactivation of both the Retinoblastoma (Rb) and p53 tumour suppressor genes (Vousden *et al*, 1991; Munger *et al*, 1992). The HPV E7 proteins complex with the Rb tumour suppressor gene product (Munger *et al*, 1989). SV40 large T antigen and adenovirus E1a also complex with Rb and functional similarities have been demonstrated between these viral proteins and HPV-16E7 (Vousden and Jat, 1989). Although binding studies have not yet been carried out, BPV-4 E7 possesses a putative p105Rb binding domain and deletion or mutation of this region abolish transformation (Jaggar *et al*, 1990; GJ Grindlay, personal communication). The expression of both 4E7 and mutant p53 would be required to inactivate these tumour suppressor pathways and result in the deregulation of cellular control.

The bovine *p53* gene was localised to chromosome 19q15 (Coggins *et al*, 1995). The availabilty of a bovine *p53* genomic clone and the recently published bovine *p53* sequence (Dequiedt *et al*, 1995b) will allow further characterisation of *p53* mutations in exons 5, 6 and 8 where the major mutational hotspots have been located in human cancer (Hollstein *et al*, 1991). This would confirm the proportion of BPV-4 associated papillomas and carcinomas which do indeed possess mutations in the *p53* gene and further characterisation of the effects of these mutations by their analysis *in vitro*.

BPV-4 lacks a 16E6 associated function *in vitro* which can be substituted by mutant p53.

It has been demonstrated that the addition of an exogeneous HPV-16E6 to BPV-4 genes can confer immortality to PalF cells *in vitro*, however, these cells are not tumourigenic (Pennie *et al*, 1993; M. Cairney, PhD thesis). Morphological transformation of PalF cells by BPV-4 is dependent on the presence of an activated *ras* gene. The presence of *ras* is a requirement for the transformation of primary cells by other papillomaviruses including HPV-16 and 18 (Matlashewski *et al*, 1988; Storey *et al*, 1988; Storey and Banks, 1993).

This thesis confirms that the addition of an exogeneous 16E6 conferred immortality to BPV-4 transformed cells and showed that this function can be substituted by the presence of an exogeneous mutant p53. Other studies have demonstrated the substitution of E6 activities by *p53* mutations *in vitro* (Sedman *et al*, 1992; Storey and Banks, 1993; Storey *et al*, 1995). This would suggest that inactivation of wild type p53 is required for BPV-4 induced immortalisation *in vitro* and that in this system 16E6 and mutant p53 disrupt a common pathway.

16E6 does possess functions in addition to immortalisation as both mutant p53 and 16E6 were required for PalF cells to become tumourigenic in the presence of BPV-4. It appears that p53 inactivation is not the only E6 activity required for tumourigenicity *in vitro* and that other p53 independent E6 functions may contribute.

The E6 oncoprotein has been demonstrated to possess other functions and more recently has been shown to bind many other cellular proteins other than p53 (Keen *et al*, 1994).

The E6 oncoproteins of the oncogenic HPVs type 16 an 18 are known to interact with two cellular proteins, the tumour suppresssor p53 (Werness *et al*, 1990) and the 100kDa protein E6-AP (p100) (Huibregtse *et al*, 1991; Huibregste *et al*, 1993a). The interaction of 16E6 with E6-AP is necessary for the interaction with p53. Keen *et al* (1994) described an additional five cellular proteins pp212, pp182, p87, p75, p33 which were bound by a biologically active 16E6 protein. The proteins pp212 and pp182 were found to be highly phosphorylated and Keen *et al* (1994) showed that both p100 and p33 interact directly with 16E6. E6 from HPVs 6, 11, 16 and 18 also demonstrated the ability to bind a cellular protein kinase which phosphorylated Histone H1. This H1 kinase activity was not associated with the E6 of the cutaneous HPVs 5 and 8 (Keen *et al*, 1994). The functions of these proteins have yet to be identified and so it is difficult to postulate what mechanisms may be involved, although this confirms that E6 has a role in addition to p53 binding and degradation by its ability to complex other cellular proteins.

The recently identified E6 binding protein, E6-BP (Chen *et al*, 1995), was found to contain four potential calcium binding motifs and a putative endoplasmic reticulum retention signal. E6-BP was identical in sequence to ERC-55 an EF-hand calcium binding protein which is localised to the endoplasmic reticulum. Only E6 from the high risk anogenital HPVs 16, 31

and 18 bound E6-BP, as did BPV-1 E6. Interestingly, although BPV-1 E6 does not bind and degrade p53 *in vitro* (Werness *et al*, 1990), BPV-1 E6 was still capable of binding E6-AP suggesting that this interaction is insufficient for the interaction with p53 or that E6-AP has other functions which play a role in BPV-1 induced transformation. Chen *et al* (1995) also demonstrated that 16E6, E6-AP and E6-BP could associate in a complex with no detectable p53, which suggests that this complex may possess p53 independent functions.

Other calcium binding proteins, such as Trichohyalin which is found in the granular cells of the epidermis, target keratins and are involved in the regulation of cell structure (Lee *et al*, 1993). Calcium ions are important second messengers in the control of many biological processes such as cell growth and differentiation (reviewed in Schafer and Heizmann, 1996). The interaction of 16E6 with E6-BP may prevent the terminal differentiation of epithelial cells providing the necessary environment for viral DNA replication. McCance *et al* (1988) showed that E6 alters the differentiation of keratinocytes, and keratinocytes immortalised by HPV-16 E6 and E7 are resistant to calcium induced differentiation (Schlegel *et al*, 1988). The inhibition of cell differentiation is an important feature of transformation, although it is possible that E6 is not the only viral oncoprotein which can influence cellular calcium levels. Members of the calcium binding S100 protein family CAPL and S100b have been demonstrated to target p53. These interactions have been suggested to be involved in the depolymerisation of the cytoskeleton and the inhibition of protein kinase C (PKC) mediated phosphorylation (Parker *et al*, 1994; Baudier *et al*, 1992). In the case of BPV-4 transformed cells, the interactions of 16E6 with other cellular proteins may depend on as yet unidentified factors which *in vivo* may be substituted by the presence of cofactors or even mutant p53 leading to the disruption of cellular calcium flux.

In this thesis, we found that PalF cells transfected with BPV-4 and mutant p53 were immortal but only became tumourigenic when 16E6 was present. Evidence of the ability of 16E6 to immortalise cells was discussed in Chapter 6.1, however, no mechanisms by which this may occur were proposed. It has been shown that one function of wild type p53 may be to signal growth arrest in response to telomere shortening in senescent human cells and that mutant p53 can overcome this senescence in human fibroblasts (Bond *et al*, 1994). Wynford-Thomas *et al* (1995) have proposed a hypothesis that when a cell reaches a critical number of divisions, the telomere is sufficiently shortened to trigger activation of wild type p53. If p53 function is eliminated, i.e, by mutation, no growth arrest will occur which would account for the resulting immortal phenotype.

Klingelhutz *et al* (1996) showed that 16E6 is capable of telomerase activation in human keratinocytes and mammary epithelial cells. This function was also shown to have no correlation with p53 binding and degradation and may be p53 independent. However, the keratinocytes expressing E6 alone possessed an extended lifespan but were not immortal (Klingelhutz *et al*, 1994). This would suggest that the p53 binding and degradation function and telomerase activation by E6 are insufficient for immortalisation. It would be expected that this telomerase activation would be sufficient for immortality, however, as discussed above, p53 elimination by mutation appears to induce the immortal phenotype (Bond *et al*, 1994). Butz *et al* (1995) demonstrated that residual wild type p53 activity exists in cells expressing 16E6. This would possibly account for the inability of the keratinocytes to become immortal in this assay if the model (Wynford-Thomas *et al*, 1995) discussed above is correct. Klingelhutz *et al* (1996) could not detect telomerase activity in E6 expressing early passage human fibroblasts. It is possible that this activity is due to differences in the proliferative capacity of keratinocytes and fibroblasts, i.e, epithelial cells have a lower proliferative capacity than fibroblasts, and that

the E6 expressing fibroblasts have not reached their threshold for telomerase activation.

The addition of an exogeneous 16E6 to BPV-4 transfected PalF cells was shown to be required for their immortalisation *in vitro* suggesting that BPV-4 lacked 16E6 functions, however, this function was substituted for by the addition of an exogeneous mutant p53. This may account for the ability of BPV-4 transfected PalF cells to become immortal with the expression of either mutant p53 or 16E6, it does not however, explain why in the presence of both mutant p53 and 16E6, BPV-4 transfected PalF cells become tumourigenic. It could be suggested that telomerase has been activated in BPV-4 transfected PalF cells which express an exogenous 16E6. The additional presence of mutant p53 may provide a selective growth advantage allowing the cells to become tumourigenic.

Infection by BPV-4 may be enough to generate the clonal expansion of cells which results in a benign tumour growth (papilloma) before growth is arrested by p53 activation. Elimination of p53 by mutation would allow further proliferation and in the presence of cofactors, such as those contained in bracken fern, progression to a squamous cell carcinoma could occur.

BPV-4 does indeed lack more than one function which can be provided by the addition of an exogeneous HPV-16E6 as shown in this thesis, however it is also apparent that some of these functions can be substituted by other factors. The ability of 16E6 to associate with many cellular proteins illustrates the requirement for many different steps in the transformation process. The lack of an E6 protein in the BPV-4 genome does not have any detrimental effects on its ability to transform bovine cells and induce papillomas. The use of 16E6 as a tool to identify mechanisms of transformation by BPV-4 may reveal important aspects of viral transformation which have not yet been identified.

Expression of viral proteins in tumourigenic cell lines.

The transforming functions of BPV-4 have been mapped to the E7 and E8 ORFs. The E7 ORF is involved in morphological transformation (Jaggar *et al*, 1990) and the E8 ORF is responsible for anchorage independent growth (Pennie *et al*, 1993).

BPV-4 E7 (4E7) mRNA was expressed in all of the cell lines transfected with BPV-4, however only those cell lines which were tumourigenic in nude mice expressed BPV-4 E8 (4E8) mRNA. Immunohistochemical analysis of the tumours excised from the nude mice demonstrated expression of both 4E7 and 4E8 transforming proteins. High levels of p53 protein were also detected and accompanied expression of 4E7 as was observed in the experimentally BPV-4 induced papillomas. 4E8 protein was also detected in the experimentally BPV-4 induced papillomas (Anderson *et al*, 1996).

The similarity of 4E7 to the E7 proteins of other papillomaviruses suggest that it acts in the same way in transformation. The ability of E7 to bind Rb is essential for the transforming and immortalising properties in rodent cells (Barbosa *et al*, 1990) and as mentioned deletion of the potential p105Rb binding site abolishes 4E7 transformation functions. Crook *et al* (1989) demonstrated that continued expression of E7 was required for the maintenance of the transformed phenotype in baby rat kidney cells transformed with HPV-16 and EJ-*ras*. Expression of 4E7 in BPV-4 transfected cells *in vitro* may contribute to transformation by disruption of the Rb dependent pathway and the biological significance of 4E7 *in vivo* has been demonstrated by its expression in the nude mouse tumours and in the experimentally BPV-4 induced papillomas.

The 4E8 protein is similar to the BPV-1 E5 protein (Jackson *et al*, 1991) and both BPV-1 E5 and 4E8 have been demonstrated to bind ductin, a 16kD cellular component of both the vacuolar H^+-ATPase and gap junctions

(Goldstein *et al*, 1991; Finbow *et al*, 1991; Faccini *et al*, 1996) although no direct interaction between ductin and 4E8 has been observed in transfected PalF cells. Other viral oncoproteins such as HPV-16 E5 and HPV-6 E5 also bind to ductin (Goldstein *et al*, 1991).

E5 alone will transform cells (Schiller *et al*, 1986; Yang *et al*, 1985), however, overexpression of E8 in PalF cells in the presence of *ras* results in accelerated senescence and cell death (Pennie *et al*, 1993; Cairney and Campo, 1995). In PalF cells transformed by BPV-4 and an activated *ras*, expression of BPV-4 E8 is associated with the loss of gap junctional intercellular communication (GJIC) (M Cairney, PhD thesis; Faccini *et al*, 1996). This is supported by the study of Oelze *et al* (1995) who found that HPV-16 E5 expressed in a human keratinocyte cell line inhibited GJIC. It has also been shown that BPV-1 transformed primary bovine conjunctival fibroblasts have disrupted GJIC (W.D Pennie, PhD thesis).

This disruption of cell-cell interactions appears to be a consequence of E8 expression and lead to a population of cells isolated from the surrounding normal cells and factors which may negatively regulate cell growth. Thus both BPV-4 E7 and E8 contribute to BPV-4 induced cellular progression.

16E6 cooperates with BPV-4 to confer transformation functions independent of p53.

BPV-4 and 16E6 were found to cooperate in the transformation of p53 deficient mouse fibroblasts suggesting that not all 16E6 functions are mediated via a p53 dependent pathway. Transformation of the p53 deficient mouse fibroblasts with BPV-4 and 16E6, unlike PalF cells, did not require the presence of an activated *ras*.

BPV-4 and 16E6 transformed p53 deficient fibroblasts were capable of anchorage independent growth, but it is not known if these cells are

tumourigenic in nude mice. Activated *ras* is not a requirement for morphological transformation of the p53 deficient fibroblasts by BPV-4 (as it is in the PalF cells) but it is still required for anchorage independent growth in the absence of 16E6. These cells were not tested for tumourigenicity. Activation of *ras* is seen at the later stages of BPV-4 progression in cancers (Campo *et al*, 1990) and at the earlier stages of transformation all papillomas examined to date appear to possess a normal *ras* gene. The requirement for *ras* for *in vitro* transformation of PalF cells may suggest that it is an early event and from data in this thesis, *ras* may provide a selective advantage for the enhanced growth of PalF cells in culture. This would suggest that although *ras* is not necessary for transformation by BPV-4 and 16E6 in p53 deficient fibroblasts, it may be a requirement for tumourigenicity.

The ability of 16E6 and BPV-4 to confer anchorage independence to the p53 deficient mouse fibroblasts required intact Cys-X-X-Cys motifs and the presence of the C-terminal domain. It has been demonstrated that for the HPV-18 E7 protein intact Cys-X-X-Cys motifs are necessary for zinc binding, dimerisation and transformation (McIntyre *et al*, 1993). Crook *et al* (1991a) suggested that disruption of one residue in the Cys-X-X-Cys motifs was insufficient to destabilise the 16E6 protein as these mutants retained wild type functions. Mutants of 18E6 which fail to bind and degrade p53 were found to still cooperate with EJ-*ras* in the immortalisation of primary baby mouse kidney cells (Pim *et al*, 1994) yet transforming activity localised to the C-terminal region including the second Cys-X-X-Cys motif. Band *et al* (1993) also suggested a transforming function of E6 which is independent of a direct association with p53 in human mammary epithelial cells. The HPV E6 proteins have been shown to bind zinc and the highly conserved Cys-X-X-Cys motifs are predicted to play a role in forming this complex comparable to that predicted for zinc finger proteins (Barbosa *et al*, 1989). This 16E6 region is

important in the cooperation with BPV-4 and may suggest that zinc binding by 16E6 has a role in transformation. Our data also confirms that a p53 independent transforming activity of 16E6 localises to the C-terminal half of the protein.

This is supported by the previous observations that both mutant p53 and 16E6 were required for tumourigenicity of PalFs suggesting that p53 inactivation is not the only mechanism by which E6 transforms cells. These *in vitro* experiments demonstrating that the additional functions conferred by 16E6 may be p53 independent, could be further confirmed by the cotransfection of BPV-4 with one of the 16E6 mutants defective for p53 binding and degradation. It would be expected that if this is indeed a p53 independent function, then the immortalisation and tumourigenicity functions provided by 16E6 would not be altered in PalF cells.

The data in this thesis suggests that p53 inactivation does indeed play a role in BPV-4 induced cellular transformation. It is known that quercetin, a mutagenic component of bracken fern, can bypass both mutant p53 and 16E6 requirements *in vitro* and cause BPV-4 transformed PalF cells to become tumourigenic in nude mice (Cairney and Campo, 1995; Pennie and Campo, 1992) confirming the role of cofactors in BPV-4 induced transformation.

p53 mutation may be an early event in the transformation of BPV-4 induced papillomas as high levels of p53 protein and mutations were detected in papillomas. In the absence of 4E7 expression these papillomas may regress, however, the expression of both mutant p53 and 4E7 could provide a proliferative stimulus. The presence of immunosuppresants in bracken fern would suppress any immune response which may induce regression. Expression of 4E8 resulting in a loss of cell-cell communication would lead to the isolation of virally transformed cells from the growth inhibitory effects of surrounding untransformed cells. The contributions of these viral proteins and

the disruption of p53 regulatory functions would create a situation which, in the presence of environmental cofactors, would allow further progression to carcinoma *in vivo*.

Future Work

Further experiments have already been suggested in the general discussion however, there are some aspects which have not yet been addressed.

Bovine papillomavirus type-4 (BPV-4) does not require an E6 ORF for a successful infection cycle and papillomas induced by BPV-4 progress to carcinoma in the absence of E6. It is still unclear if other viral or host proteins provide E6 functions. 16E6 immortalises PalF cells transformed by BPV-4 E7 and ras +/- E8 suggesting that E6 does indeed provide functions not supplied by E7 and E8 (Pennie *et al*, 1993). In the presence of the mutagen quercetin, E7 is the only BPV-4 oncogene required for full transformation substituting E8 in conferring anchorage independence and 16E6 in inducing immortalisation.

16E6 is known to inhibit apoptosis by binding oligomeric and monomeric forms of p53 (Marston *et al*, 1994). It would be interesting to determine if 16E6 confers immortality by the inhibition of apoptosis and if other E6 proteins from the low risk papillomaviruses HPV-6 and 11 or BPV-1 E6 can provide the same functions. Expression of cell death/survival genes such as *bcl-2* and *bax* and the p53 induced *waf1/cip1* could be determined at various stages of cell transformation. This would raise the question as to whether the expression of these genes is altered when the cells are treated with quercetin in the presence of viral oncogenes. The status of p53 protein expression in transformed cell lines is also unclear and differences in p53 expression between quercetin and non-quercetin treated cells could be determined by immunohistochemistry.

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