Cell type specific regulation of papillomavirus transcription

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For my mum and dad

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Abbreviations

AMF-1	Activation domain modulating factor-1
AP-1	Activator protein-1
ATP	Adenosine triphosphate
BLAST	Basic logic search alignment
bp	Base pair
BPV	Bovine papillomavirus
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
C/EBP	CCAAT/enhancer binding protein
CIN	Cervical intraepithelial neoplasia
cm	Centimetres
CRPV	Cottontail rabbit papillomavirus
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
E. Coli	Escherichia Coli
ECL	Enhanced chemiluminescence
EIF3	E2 interacting factor 3
EMSA	Electromobility shift assay
FCS	Foetal calf serum
g	Gram
GST	Glutathione-S-transferase
HBS	HEPES buffered saline
HPV	Human papillomavirus

hr	Hours
HSV	Herpes Simplex Virus
kb	Kilobase pairs
kDa	KiloDalton
kg	Kilogram
l	Litre
LCR	Long control region
μg	Microgram
μl	Microlitre
MAR	Matrix attachment region
M	Molar
mg	Milligram
min	Minute
ml	Millilitre
mM	Millimolar
mRNA	Messenger ribonucleic acid
NF1	Nuclear factor-1
^{0}C	Degree centigrade
OD	Optical density (light absorbance)
ORF	Open reading frame
PalF	Primary bovine foetal palate fibroblasts
PalK	Primary bovine foetal palate keratinocytes
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PIC	Preinitiation complex
pmols	Picomole

PODs	Promonocytic leukaemia protein (PML) oncogenic domains
PRE	Promoter repressor element
RNA	Ribonucleic acid
rpm	Revolutions per minute
sec	Second
SLM	Special liquid medium
SV40	Simian virus 40
TAFs	TBP associated factors
TBP	TATA box binding protein
TEF	Transcriptional enhancer factor
TSA	Trichostatin A
tk	thymidine kinase
UV	Ultraviolet
v/v	Volume per unit volume
w/v	Weight per unit volume
YY1	Ying-yang 1

Single letter amino acid code

Alanine	Ala (A)
Arginine	Arg (R)
Asparagine	Asn (N)
Aspartic acid	Asp (D)
Cysteine	Cys (C)
Glutamic acid	Glu (E)
Glutamine	Gln (Q)
Glycine	Gly (G)

Histidine	His (H)
Isoleucine	Ile (I)
Leucine	Leu (L)
Lysine	Lys (K)
Methionine	Met (M)
Phenylalanine	Phe (F)
Proline	Pro (P)
Serine	Ser (S)
Threonine	Thr (T)
Tryptophan	Trp (W)
Tyrosine	Tyr (Y)
Valine	Val (V)

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

Abstract

All papillomaviruses have a non-coding region of approximately 500-1000 bp called the upstream regulatory region (URR) or long control region (LCR). The LCR is the transcriptional control unit of the virus. Mucosal epitheliotropic papillomaviruses, for example, BPV-4 and HPV-16 and –18, have a similar LCR organisation: a promoter region, an epithelial specific enhancer, and a highly conserved distribution of DNA binding sites for the viral E2 protein. When compared with the HPV-16 LCR, the BPV-4 LCR has a higher transcriptional response to activation by E2 in its target cell type. E2 up-regulates transcription from the BPV-4 LCR preferentially in primary bovine palate keratinocytes (PalK) when compared with fibroblasts (PalF) (Morgan et al., 1998). The BPV-4 LCR and primary bovine cell system presents a model to study the mechanisms of E2 mediated transcriptional regulation of mucosal epitheliotropic papillomaviruses and the cell type specificity of this regulation.

Insertion of multiple E2 sites upstream from the LCR promoter demonstrates that the enhanced epithelial response to E2 is not due to the epithelial specific enhancer in the BPV-4 LCR, but is a property of the BPV-4 promoter region which responds better to several transcriptional activators in PalK cells. This is a promoter specific effect as the tk promoter shows no such epithelial preference to upstream activators. E2 responsive chimaeric promoter constructs suggest that the BPV-4 promoter region upstream of the TATA box determines the cell type selective response of the BPV-4 promoter. Deletion analysis identifies two novel repressor elements, PRE-1 and PRE-2, that are at least in part responsible for mediating the enhanced epithelial response of this promoter. These elements function in cis to repress the basal activity of the

SV40 promoter. PRE-2 binds a specific protein complex in both PalK and PalF cells. The active DNA binding protein in this complex is a major species of approximately 50 kDa. PRE-2 mutants that do not compete for binding in band shift assays do not repress transcription when multimerised upstream of the SV40 promoter. Also, non-binding mutations introduced into the BPV-4 promoter show that the PRE-2 binding factor represses the transcriptional response of the BPV-4 promoter to E2. The PRE-2 binding protein seems to represent a novel transcriptional repressor and regulator of papillomavirus transcription.

The E2 amino terminal transactivation domain is essential for regulating transcription and replication of the viral genome. Far western blot analysis shows that the HPV-16 E2 amino terminus is able to interact with different cellular factors in keratinocytes and fibroblasts. The isolation of cDNAs encoding proteins interacting with this region of E2 in HeLa cells (an immortalised keratinocyte cell line) is described. One of the cDNAs encodes the full length L31 ribosomal protein. L31 specifically interacts with the HPV-16 E2 transactivation domain *in vitro*. Also, an *in* vitro interaction is demonstrated between E2 and a truncated version of a protein previously shown to bind the cytoplasmic domain of integrin β_4. These E2 interacting factors may represent potential regulators of transcription, replication or cellular growth control.

Chapter 1 – Introduction

1.1. Papillomaviruses

1.1.1. Human papillomavirus life cycle

Papillomaviruses are a family of small double stranded DNA viruses that infect a wide range of animals inducing benign proliferative lesions, or warts. Papillomaviruses exhibit both host species and tissue specificity. Over 90 types of human papillomaviruses (HPVs) have been identified, based on different degrees of sequence homology. HPVs are strictly epitheliotropic and can be divided into two subsets depending on the tropism for the target cell type, infecting either the cutaneous epithelium of the skin or mucosal epithelium at specific body locations (Chan et al., 1995). Productive infection by HPV is absolutely dependent upon the differentiation of the host cell (for reviews see (Howley, 1996; Stubenrauch and Laimins, 1999)). HPVs are believed to infect proliferating undifferentiated keratinocytes in the basal layers of the stratified epithelium. Following infection, the HPV genomes are established as autonomous replicating nuclear plamids, copy number is amplified to approximately 50-100 copies per cell and a low level of HPV early gene expression occurs. As infected cells migrate upwards from the basal layer and undergo differentiation productive viral DNA replication and expression of the viral late genes is induced resulting in the assembly and release of progeny virion. In normal epithelia, differentiating suprabasal cells exit the cell cycle after leaving the basal layer. However, papillomaviruses require a proliferating host cell to replicate their genomes. One major consequence of papillomavirus infection is therefore a blockage of cell cycle exit and induction of S-phase, allowing HPV genomes to be

replicated to high levels in the intermediate layers of the epithelium. Early gene transcription increases concomitantly with the onset of vegetative viral DNA replication, while transcripts encoding the late genes are restricted to the terminally differentiated layers of the epithelium where virions are being assembled and released (Durst et al., 1992).

1.1.2. HPVs are causative agents of human cancers

Papillomavirus infection usually results in self-limiting, productive proliferative lesions which spontaneously regress. However, a subset of the mucosal epitheliotropic HPVs, termed high risk, are causally involved in the pathogenesis of various anogenital cancers (zur Hausen, 1989). Over 90% of human cervical cancers contain viral sequences from HPV-16 and –18, often integrated into the host genome (zur Hausen, 1991). Despite the identification of these HPV types as causative agents of human cancers, this is not part of the normal viral life cycle as poorly differentiated cancer cells are not permissive for production of viral progeny. Primary HPV-16 and –18 lesions are common and frequently inconspicuous. Cells infected by high risk HPV types may acquire a transformed phenotype, over a prolonged period of time, due to additional genetic and epigenetic events and develop into malignant tumours. Low risk mucosal epitheliotropic HPVs, for example, HPV-6 and –11 induce pronounced, benign proliferative warts known as genital warts or condylomata acuminata and very rarely associate with malignancies (Gissmann, 1992). Cutaneous HPVs can also be classified as high risk or low risk depending on the association with skin cancers *in vivo*. Infection by high risk cutaneous HPVs, for example HPV-5 and –8, is associated with the development of squamous cell carcinoma, particularly in immunosuppressed patients. Patients with

epidermodysplasis verruciformis (EV), a rare hereditary lifelong disease, are susceptible to persistent infection by HPV types –5 and –8, and have a high risk of developing skin cancer (Pfister, 1992). Low risk cutaneous HPVs, such as HPV-1 and –2, induce warts on the skin which show limited growth and normally regress. HPV types –16 and -18 were officially declared a human carcinogen by the World Health Organisation in 1995 (IARC/WHO, 1995).

1.1.3. Animal model systems

The differentiation dependence of the HPV life cycle, the ethics of studies in humans and the lack of suitable *in vitro* culture systems has hindered the study of the complete virus life cycle. Animal model systems have proved an invaluable tool to study the interaction of a papillomavirus with its natural host. The two most extensively studied animal papillomaviruses are the cottontail rabbit (CRPV) and bovine (BPV) papillomaviruses. CRPV induces papillomas in domestic rabbit skin that are histologically similar to cutaneous human papillomas, while the natural history of CRPV induced disease parallels that of high risk HPV infection (Kreider and Bartlett, 1981; Wettstein, 1987). The CRPV system has been a valuable model to study the molecular basis for papillomavirus latency and how reactivation of latent infections occurs at the molecular level (Amella et al., 1994). There are two groups of BPVs (Jarrett et al., 1984): subgroup A comprises BPV-1, -2 and –5 and subgroup B contains BPV-3, -4 and –6. The viruses of subgroup A induce fibropapillomas, with both a fibroblastic and epithelial component, as infection leads to an initial transformation of the sub-epithelial fibroblasts followed by papillomatosis. The subgroup B viruses are epitheliotropic, showing a similar tissue tropism to the HPVs, inducing epithelial papillomas with no dermal involvement.

1.1.4. Papillomavirus genomic organisation

Bovine papillomavirus-1 (BPV-1) is perhaps the most extensively studied papillomavirus and has long been the paradigm of all papillomaviruses. The ease with which BPV-1 could be isolated from warts and its ability to transform cells in culture led to the sequencing of the complete BPV-1 genome and the assignment of functions to the individual open reading frames (ORFs) (Law et al., 1981). The BPV-1 genome is a closed circle of approximately 7.8 kb that exhibits an organisation similar to all papillomaviruses (Iftner, 1990). It can be divided into three regions: regions encoding the early, E1-E8, and late, L1 and L2 gene products, separated by a non-coding region of approximately 1000 bp called the long control region (LCR) (Fig. 1.1.). All the ORFs are present on the same DNA strand, and only this strand is expressed as a mRNA (for a review see (Baker, 1990)). A polyadenylation site is present at the end of each coding region. The early region encodes the proteins essential for transcription and replication of the viral genome and the viral transforming proteins, while the late region encodes the proteins necessary for viral DNA encapsidation. Although the early genes are expressed in all layers of the epithelium, expression of the late genes is restricted to the terminally differentiated keratinocytes, in the upper layers of the epithelium. Numerous polycistronic BPV-1 transcripts, which show a complex pattern of splicing between the ORFs, have been identified (Baker, 1990). In BPV-1 transcripts from the early region are initiated from multiple promoters and are polyadenylated at the early polyadenylation site. However, transcripts encoding the E6, E7, E1 and E2 ORFs of the high risk HPV types originate at the early promoter located upstream of the E6 ORF. This promoter is designated p105 in HPV-18 and p97 in HPV-16 and –31 (Stubenrauch and

Laimins, 1999). BPV-1 late mRNAs are transcribed from a single late promoter (P_L), located within the E7 ORF, and are polyadenylated at the late polyadenylation site (Baker and Howley, 1987). A differentiation dependent late promoter has also been identified for HPV-16 (p846) and HPV–31 (p742). Expression of the late region mRNAs therefore requires a differentiation dependent shift in polyadenylation site usage and activation of the late promoter (Barksdale and Baker, 1993).

1.1.5. Papillomavirus protein function

The gene products of the major papillomavirus ORFs have been well characterised for BPV-1 and many HPVs. The **E1** protein is essential for viral DNA replication. The involvement of E1 in replication was originally predicted by sequence similarities to SV40 large T antigen (Tag) (Clertant and Seif, 1984). Papillomaviruses utilise a number of cellular factors to replicate the viral genome. These include DNA polymerase α-primase (Bonne-Andrea et al., 1995b; Park et al., 1994) and δ which synthesise the viral DNA, replication protein A (RPA), a single-stranded DNA binding protein which stabilises the open duplex region of the DNA at the replication fork, proliferating cell nuclear antigen (PCNA), replication factor C (RFC) and topoisomerases I and II (Kuo et al., 1994; Melendy et al., 1995). E1 is a 68-72 kDa nuclear phosphoprotein with ATPase and helicase activities (Sun et al., 1990; Yang et al., 1993). E1 specifically binds an 18 bp inverted repeat (E1BS) in the viral origin of replication located in the 3' region of the LCR (Holt et al., 1994). Functional origins of replication have been defined for BPV-1 and several HPVs including types –16 and –18 (for a review see (Ustav and Ustav, 1998)). The E1BS is flanked by an A/T rich region and two E2 DNA binding sites. Initial binding of E1 leads to a structural distortion of the origin, DNA unwinding and the formation of a

hexameric E1 complex with ATPase and DNA helicase activities (Gillette et al., 1994; Sedman and Stenlund, 1998).

Efficient viral DNA replication requires the formation of a specific complex between E1 and the viral E2 protein, targeting E1 to the origin of replication (Sedman and Stenlund, 1995). *In vitro* replication of BPV-1 DNA has suggested that the BPV-1 E2 protein is not required for elongation and is released from the origin after the initial binding step (Bonne-Andrea et al., 1995a). Although biochemical analysis of HPV E1 proteins has been much more limiting than that of BPV-1 E1, the HPV-11 and –16 E1 and E2 proteins have also been shown to associate (Bream et al., 1993; Storey et al., 1995). The E1-E2 interaction has been mapped to both the N-terminal and C-terminal regions of both BPV-1 and HPV E1 and E2 proteins suggesting several functional interactions. The minimal DNA binding domain in the amino terminus of BPV-1 E1 binds the E1BS in an E2 dependent manner. Only the E2 DNA binding domain interacts with this E1 fragment suggesting that the DNA binding domains of E1 and E2 cooperate in the process of binding DNA (Chen and Stenlund, 1998). The C-terminal helicase domain of HPV-16 E1 binds the E2 transactivation domain and the DNA polymerase α-primase p68 subunit in a mutually exclusive manner (Masterson et al., 1998). The HPV E1 proteins can also interact with histone H1 (Swindle and Engler, 1998) and hSNF5, a component of the SWI/SNF ATP dependent chromatin remodelling complex (Lee et al., 1999). E1 is also a tight-binding substrate of cyclin E-cyclin dependent kinase-2 (Cdk-2) *in vitro*, a key cell cycle regulator of S-phase. Phosphorylation of E1 by cyclin E/Cdk complexes is required for efficient viral DNA replication *in vitro* and *in vivo*. This interaction may

be a crucial determinant of cell-cycle regulation of papillomavirus DNA replication (Ma et al., 1999).

The **E2** protein is the major regulator of viral transcription, is essential for the initiation of viral DNA replication and has a number of pleiotropic effects within the cell. The function of E2 will be described in detail later. No function has been assigned to the **E3** ORF. Only a few papillomavirus types possess it.

The role of the **E4** protein in the viral life cycle is still subject to speculation. E4, first identified in HPV-1 induced warts, is highly divergent between papillomavirus types. E4 is expressed as a 10 kDa polypeptide fused to the five amino-terminal residues of E1 (E1^E4) (Doorbar et al., 1988). In HPV-11 and –31 the mRNA encoding E1^E4 is the most abundant viral RNA transcript in the intermediate layers of the epithelium (Desaintes and Demeret, 1996). Although this transcript is initiated from the differentiation dependent late promoter E1^E4 is not a component of the virus particle. E1^E4 is also not needed for transformation *in vitro*. The HPV-16 E1^E4 protein, when expressed in human epithelial cells, associates with the intermediate filament network. This results in the total collapse of the keratin cytoskeleton suggesting a possible involvement in virus release by disturbing the integrity of the infected cells (Doorbar et al., 1991).

The product of the **E5** ORF is the major transforming protein of BPV-1 (Schiller et al., 1986). BPV-1 E5 is a 44 amino acid, disulphide linked, homodimeric transmembrane protein, localised largely in the membranes of the endoplasmic reticulum and Golgi apparatus (Schlegel and Wade-Glass, 1987). BPV-1 E5 can transform established mouse fibroblasts in the absence of other viral gene products,

and has been shown to induce cellular DNA synthesis (Leptak et al., 1991). BPV-1 E5 exhibits a short region of sequence homology with platelet derived growth factor (PDGF). BPV-1 E5 directly binds the PDGF receptor, inducing ligand independent oligomerisation and stimulation of signal transduction in the absence of mitogens. Amino acids in the transmembrane and juxtamembrane domains of the PDGF receptor, not the ligand binding domain, are required for the interaction with E5 (Meyer et al., 1994). BPV-1 E5 also indirectly activates the epidermal growth factor (EGF) and colony stimulating factor-1 (CSF-1) receptors (Martin et al., 1989). BPV-1 E5 can bind the 16 kDa protein of the ductin family of proteolipids, a major structural component of gap junctions and a subunit of the vacuolar H^+-ATPase (Finbow et al., 1991). It is proposed that the interaction with ductin indirectly potentiates mitogenic signalling by inhibiting the vacuolar ATPase, a proton pump responsible for the acidification of cytoplasmic organelles, inhibiting receptor down regulation. However, mutations in the E5 transmembrane domain, which retain the ability to interact with ductin, are not transforming (Sparkowski et al., 1996). HPV-16 E5, although not as powerful a tranforming agent as BPV-1 E5, can transform mouse keratinocytes, stimulate the transforming activity of the EGF receptor, bind 16 kDa ductin and down regulate the function of cellular gap junctions (Oelze et al., 1995).

In high risk mucosal HPV types, the blockage of cell cycle exit and induction of S-phase in differentiated suprabasal cells is mediated by the products of the **E6** and **E7** ORFs (Cheng et al., 1995). E6 and E7 are the major transforming proteins of these papillomaviruses. Co-expression of high risk HPV E6 and E7 can immortalise primary human genital epithelial cells (Vousden, 1991). The low risk HPV E6 and

E7 proteins, expressed from the same strong promoters, function very inefficiently in cell transformation and immortalisation assays, reflecting biochemical differences in the activities of these viral gene products (Storey et al., 1988). The HPV E6 gene product is an approximately 18 kDa protein that contains two zinc binding CXXC motifs (Barbosa et al., 1989). A series of alternatively spliced transcripts lacking the E6 C-terminus also exist, but the encoded proteins are rapidly turned over and are hardly detectable *in vivo* (Schneider-Gadicke and Schwartz, 1986). High risk HPV E6 proteins function, at least in part, by binding and specifically stimulating the ATP dependent degradation of the p53 tumour suppressor protein (Scheffner et al., 1990; Werness et al., 1990). The N-terminus of E6 simultaneously interacts with E6AP, a cellular ubiquitin ligase, and the core region of p53 targeting p53 for ubiquitin dependent proteolysis (Huibregtse et al., 1991). E6AP does not interact with p53 in the absence of E6. E6 proteins from low risk genital HPVs, cutaneous HPVs and animal papillomaviruses either do not, or only weakly associate with E6AP or p53, and do not affect p53 stability (Elbel et al., 1997). The binding affinity of different E6 proteins for the p53 core region correlates with their efficiency to stimulate p53 degradation. Although, the C-terminal domain of E6 from both high and low risk HPV types is able to bind the p53 C-terminus, it is only the interaction of E6 with the p53 core region that induces degradation (Li and Coffino, 1996). The E6 protein may also use additional pathways to disrupt p53 mediated growth suppression. HPV-16 E6 can block p53 binding to its DNA recognition site, probably through the interaction with the C-terminal domain of p53 (Lechner and Laimins, 1994), and can downregulate p53 activity by targeting the transcriptional coactivator p300/CBP (Zimmermann et al., 1999). p53 independent functions of E6 may also contribute to growth regulation. High risk HPV E6 proteins can bind the focal adhesion protein

paxillin (Tong and Howley, 1997), the pro-apoptotic protein bak (Thomas and Banks, 1999), the c-myc oncoprotein (Gross-Mesilaty et al., 1998), the putative human tumour suppressor protein disc large (hDCG) (Kiyono et al., 1997) and can stimulate telomerase activity during keratinocyte immortalisation (Kiyono et al., 1998).

The product of the E7 ORF from high risk HPV types is an approximately 17 kDa protein present in both the nucleus and cytoplasm of infected cells (for a review see (Zwerschke and Jansen-Durr, 2000)). The E7 amino terminus shows structural and functional homologies to a portion of conserved region 1 (CR1) and the entire CR2 domain of the adenovirus E1a and SV40 large Tag oncoproteins (Vousden, 1991). The CR2 domain contains a consensus casein kinase II (CKII) phosphorylation motif and a LXCXE retinoblastoma protein (pRb) family binding site. pRb and the related proteins, p107 and p130, can mediate the association of E7 with cyclin A/cdk-2 and cyclin E/cdk-2 complexes (Davies et al., 1993; Dyson et al., 1992; Dyson et al., 1989). The E7 carboxy terminus contains two zinc binding CXXC motifs that function as a dimerisation domain (McIntyre et al., 1993). Mutations in the CXXC or LXCXE motifs drastically reduce or abolish the transforming activity of E7. HPV-16 E7 preferentially binds the hypophosphorylated, tumour suppressor form of pRb releasing transcriptionally active E2F family members. The E2F transcription factor family regulates expression of many genes responsible for progression into S-phase, for example, cyclin E, cyclin A and b-myb (Weinberg, 1995). E7 proteins from low risk HPV types bind pRb with a reduced affinity compared to high risk HPV types correlating with transforming capacity (Munger and Phelps, 1993). However, the ability of E7 to interact with pRb family members is not the only determinant of oncogenic potential. The C-terminus of HPV-16 E7 can interact with and block the

activities of the $p27^{Kip1}$ and $p21^{WAF1}$ cyclin dependent kinase inhibitors (Jones et al., 1997; Zerfass-Thome et al., 1996). These inhibitors negatively regulate cell cycle progression and play a role in inhibiting cellular proliferation during epithelial cell differentiation. E7 can regulate gene expression through interacting with E2F unrelated transcription factors. HPV-16 E7 interacts with the Jun component of the AP-1 transcription factor through one of its CXXC motifs (Antinore et al., 1996). This interaction activates transcription of AP-1 responsive genes suggesting that E7 can promote G_0/G_1 progression under conditions where external proliferative signals are reduced or absent. HPV-16 E7 has also been shown to interact with TATA box binding protein (TBP), the affinity of which is increased by CKII phosphorylation (Massimi et al., 1997). The interaction of E7 with TBP may mediate transcriptional repression or activation at certain promoters to promote cell cycle progression. The first cytoplasmic target of E7 has recently been identified. HPV-16 E7 interacts with enzyme type M2 pyruvate kinase (M2-PK) increasing the rate of glycolysis, suggesting that E7 can directly alter the carbohydrate metabolism of the host cell to allow sustained proliferation (Zwerschke et al., 1999).

The function of the putative BPV-1 **E8** has not yet been established.

The products of the **L1** and **L2** ORFs are only expressed in the terminally differentiated layers of the epithelium late in the virus life cycle. L1 is the major capsid protein and L2 is the minor capsid protein of the virion. These two proteins encapsidate a histone associated, closed circular, double stranded DNA minichromosome. X-ray crystallography shows that the icosahedral outer shell of the

virion contains 72 pentamers of L1 (Chen et al., 2000). L2, a largely internal protein, is present at roughly 1/30 the abundance of L1 (Kirnbauer et al., 1993).

1.1.6. Bovine papillomavirus type 4 (BPV-4)

BPV-4 is a mucosal epitheliotropic papillomavirus that infects the upper alimentary canal of cattle causing benign papillomas with a high risk of progressing to carcinoma in cattle feeding on bracken fern (Campo et al., 1980; Campo et al., 1994). The BPV-4 genome is a double stranded closed circle of 7265 nucleotides. BPV-4 has a genetic organisation similar to all other papillomaviruses: regions encoding the early and late gene products separated by the non-coding LCR (Fig. 1.2.). Numerous BPV-4 transcripts, initiated from two major promoters, have been identified: the early promoter (P_E), maps to the TATA box at nucleotide 283 (Jackson and Campo, 1995) and the late promoter (P_L) maps between nucleotides 777 and 902 (Stamps and Campo, 1988). The BPV-4 E1, E2 and E7 proteins show a high degree of sequence homology to the equivalent proteins in other papillomaviruses. BPV-4 also encodes a polypeptide analogous to the E1^E4 fusion protein described for HPV-1. The BPV-4 E8 ORF, located at the 5' end of the early region, encodes a protein homologous to the E5 protein of BPV-1. Although BPV-4 E8 and BPV-1 E5 have different genomic locations BPV-4 E8 has recently been renamed E5 because of the many functional similarities between these proteins (see below) (Morgan and Campo, In press). The L3 and L4 ORFs do have translation start codons but their function, if any, is not known (Campo et al., 1996). The main difference between the BPV-4 genome to that of most papillomaviruses is the absence of the E6 ORF, which is also deleted in the related epitheliotropic papillomaviruses, BPV-3 and –6.

The ability of BPV-4 to transform cells in culture provides an excellent model for the identification of genetic and epigenetic events which contribute to the multiple steps of tumourigenic progression. Although BPV-4 DNA alone can transform established NIH3T3 mouse fibroblasts (Campo and Spandidos, 1983), BPV-4 requires the cooperation of active ras to morphologically transform primary bovine fibroblasts (PalF) derived from a foetal palate (Jaggar et al., 1990). The transformed cells have an extended life span and are capable of anchorage independent growth but are not immortal or tumourigenic in nude mice. This indicates that viral infection represents only one event during malignant progression and that additional events are needed for the development to carcinoma. E7, expressed in all layers of papillomas at all stages of development, and E8, expressed only in the basal and suprabasal layers of papillomas, are the major transforming proteins of BPV-4 (Anderson et al., 1997). In cooperation with ras, BPV-4 E7 induces morphological transformation of PalF cells in the absence of all other viral genes (Pennie et al., 1993). BPV-4 E7 contains the two zinc binding CXXC motifs and the pRb family binding domain, but does not possess the CKII phosphorylation site conserved in the E7 proteins of high risk HPV types. BPV-4 E8 encodes a 42 amino acid hydrophobic peptide that is localised to cellular membranes. BPV-4 E8 alone does not cooperate with an active ras gene to confer any growth advantage to PalF cells. However, BPV-4 E8 contributes to cellular transformation by conferring an anchorage independent growth phenotype on PalF cells co-transfected with E7 and ras (Pennie et al., 1993). Also, NIH3T3 cells expressing E8 alone are capable of anchorage independent growth and escape growth arrest after serum withdrawal. Like BPV-1 E5, BPV-4 E8 interacts with ductin and induces down regulation of gap junctional intercellular communication in PalF cells (Faccini et al., 1996). Also, E8 upregulates expression of cyclin A increasing the

activity of the cyclin A/cdk-2 complex, and deregulates expression of the cell cycle inhibitor $p27^{KIP}$, contributing to transformation (O'Brien and Campo, 1998).

Experimental reproduction of the progression of papillomas to carcinomas in cattle has identified the immunosuppressants and mutagens present in bracken fern as cofactors necessary for the development of BPV-4 associated alimentary canal cancer (Campo et al., 1994). Immunosuppressed cattle are subjected to life long papillomatosis. The persistant papillomas are at a high risk of progressing to cancer as they are continually exposed to the mutagens present in the fern (for a review see (Jackson et al., 1996)). Also, the *in vitro* transformation system has demonstrated that the flavinoid quercetin, one of the most potent mutagens found in bracken, can synergise with BPV-4 E7 to confer a fully malignant phenotype to PalF cells (Cairney and Campo, 1995; Pennie and Campo, 1992). Quercetin induces mutations in DNA through the generation of single stranded breaks (Fazal et al., 1990), can act as an initiator of tumour progresion (Sakai et al., 1990) and disrupts normal cell signalling pathways by interfering with kinases and phosphatases (Van Wart-Hood et al., 1989). Quercetin also induces growth arrest of PalF cells in both the G1 and G2/M phases of the cell cycle and can upregulate the transcriptional activity of the BPV-4 LCR (Connolly et al., 1998). Papillomas induced by BPV-4 can therefore progress to carcinoma even though BPV-4 does not have an E6 ORF or encode E6-like functions. If disruption of p53 function is important in BPV-4 cell transformation, it may occur by alteration of the cellular gene. Indeed, the *in vitro* bovine cell transformation model has revealed a correlation between transcriptionally inactivating p53 mutations and tumourigenicity. In PalF cells, p53 protein is elevated and transcriptionally activated in response to quercetin. However, in tumourigenic

cells although p53 protein levels increase in response to quercetin, p53 is transcriptionally inactive. Sequencing of the bovine p53 gene in tumourigenic cell lines shows a glycine to valine substitution in conserved region V in the p53 DNA binding domain (Beniston, 1999).

1.2. Control of papillomavirus transcription

HPV-16, -18 and BPV-4 are mucosal epitheliotropic papillomaviruses. This may reflect a restricted distribution of an epithelial cell receptor for the virus or the cellular factors necessary for viral DNA replication or gene expression may be limited to the mucosal epithelium. However, the receptor utilised by papillomaviruses for the initial step of infection appears to be ubiquitously expressed as both artificial virions and virus-like particles can bind to a variety of cell lines (Muller et al., 1995; Sibbet et al., 2000). Also, HPV genomes can replicate in various undifferentiated cell lines regardless of their natural permissiveness to infection (Desaintes and Demeret, 1996). As viral gene expression is limited to the host cell type, the tropism for the mucosal epithelium and the differentiation dependence of the viral life cycle might be dictated at the transcriptional level. The level of transcription of the viral genome is also a major determinant of the transformation potential of the virus. Enhanced transcription of both HPV-16 and BPV-4 has been shown to lead to increased transformation efficiency *in vitro* (Jaggar et al., 1990; Lees et al., 1990). Most human cervical tumours contain physical integration of the viral DNA into the host genome. Integration is a terminal event for the virus resulting in disruption of the E1 and E2 ORFs (zur Hausen, 1991). However, specific transcripts originating from the E6 and E7 ORFs, expressed under aberrant control of an intact LCR, are consistently found in HPV associated tumours (Schwartz et al.,

1985) and are required for maintenance of the transformed phenotype (zur Hausen, 1991). Infection by high risk HPV types is frequently detected in women with cervical intraepithelial neoplasia (CIN), benign cervical lesions that are the progenitors of cervical carcinomas (Nelson et al., 1984). CIN is graded from I to III depending on the extent to which epithelial differentiation has been disrupted. Integration of viral DNA has been proposed as an activation mechanism for progression from advanced pre-invasive lesions to cervical cancers. Viral copy number is negatively correlated with the clinical stage of the tumour and directly associated with the degree of histological differentiation (Berumen et al., 1994). Integration leads to the loss of papillomavirus control of E6 and E7 expression, due to disruption of the E2 gene, resulting in a selective growth advantage of cells (Jeon et al., 1995).

1.2.1. The papillomavirus E2 protein

The E2 ORF, essential for the viral life cycle, encodes a 42-48 kDa protein (depending on the papillomavirus) consisting of three modular domains: a conserved amino terminal transactivation domain, a central flexible hinge region and a conserved carboxy terminal DNA binding and dimerisation domain (Fig. 1.3.). The E2 protein binds to the 12 bp palindromic DNA sequence –ACCGNNNNCGGT- as a dimer (for reviews see (Ham et al., 1991b; McBride et al., 1991)). The BPV-1 genome contains 17 E2 DNA binding sites (Li et al., 1989) while the mucosal epitheliotropic HPVs have 4 (McBride et al., 1991). Genetic analysis of BPV-1 first demonstrated that the E2 protein is a transcriptional activator. However, BPV-1 also encodes two truncated versions of E2 which act as transcriptional repressors by antagonising the function of the full length protein (Lambert et al., 1987; Lambert et

al., 1989). A 31 kDa E2 polypeptide translated from an internal methionine lacking a region of the amino terminus and a 28 kDa E8-E2 fusion, generated by alternate splicing, containing 11 amino acids of E8 linked to the E2 carboxy terminus, have also been described. HPV types encode an E2 protein homologous to the BPV-1 transactivator. The E2 protein can activate transcription from heterologous promoters in a variety of mammalian cell lines and in yeast (Cripe et al., 1987; Morrissey et al., 1989; Romanczuk et al., 1990; Thierry and Yaniv, 1987). This indicates that the interactions of E2 with the transcription machinery may be conserved. One E2 site inserted upstream from a heterologous promoter only activates transcription weakly, whereas two constitute a strong inducible enhancer suggesting that a complex of two E2 dimers is the functional form of E2 (Gauthier et al., 1991). Indeed, one E2 dimer cannot cooperate with cellular factors such as AP-1 to activate transcription whereas two E2 dimers can. Also, the strong cooperative activation between E2 sites can occur over a large distance suggesting that the interaction between E2 dimers and cellular factors necessary for activation may be facilitated by DNA looping, bringing E2 molecules close to the promoter (Thierry et al., 1990).

The carboxy terminal region of E2, consisting of approximately 90 amino acids, is necessary and sufficient for dimerisation and sequence specific DNA binding (Prakash et al., 1992). This domain does not contain any known eukaryotic DNA binding motifs, for example, the zinc finger or leucine zipper. The crystal structure of the E2 carboxy terminus bound to its DNA target reveals a novel antiparallel β-barrel structure responsible for dimerisation. Also, two α-helices from each E2 C-terminal molecule in the dimer interact with the major groove of the DNA. The E2 carboxy terminus dimer induces a substantial bend ($42\text{-}44^0$) in the DNA (Hegde et al., 1992).

The C-terminal domain contacts the guanosines on either strand of the consensus motif, which are on the same face on the DNA. An internal A-T rich core increases the affinity of the interaction between E2 and the DNA (Sanders and Maitland, 1994). Direct protein-protein interactions between the E2 C-terminus and both viral and cellular proteins have been demonstrated. The E2 C-terminus can bind the viral E1 protein (Chen and Stenlund, 1998) and the cellular p53 tumour suppressor protein (Massimi et al., 1999). Also, the E2 C-terminus has been shown to bind two components of the cellular basal transcription machinery, TBP and TFIIB (Rank and Lambert, 1995). However, the significance of these interactions have yet to be determined as the E2 transactivation domain is able to function independently of the C-terminus to activate transcription when tethered to the heterologous GAL4 or LexA DNA binding domains (McBride et al., 1989b).

The non-conserved internal linker region of the E2 protein is not required for DNA binding or transcriptional activation. This region varies in length between different papillomaviruses and is rich in proline residues suggesting that it acts as a flexible hinge. Also, the hinge region contains the two major phosphorylation sites of the BPV-1 E2 protein (McBride et al., 1989a). E1 binds preferentially to the underphosphorylated form of E2 suggesting that phosphorylation at these sites may regulate stable replication of the viral genome (Lehman et al., 1997). In addition, the hinge region of HPV-11 E2 has been shown to contain the determinants for nuclear localisation and nuclear matrix association (Zou et al., 2000).

The amino terminal transactivation domain of E2, consisting of approximately 200 amino acids, is highly conserved among papillomavirus types and is essential for

transcription and replication of the viral genome (for a review see (Desaintes and Demeret, 1996)). This region mediates protein-protein interactions with cellular factors involved in transcription, replication and growth control and binds E1, the papillomavirus replication factor, targeting it to the origin of replication. X-ray crystallography reveals that the HPV-16 E2 amino terminus can form a dimer in solution and consists of two domains (Antson et al., 2000). Domain 1, residues 1-92, is composed of three long, antiparallel α-helices rich in acidic amino acids. Domain 2, residues 110-201, has an antiparallel β sheet structure. It is proposed that the amino terminal domains from distantly bound E2 dimers interact with each other to stabilise the formation of DNA loops. There are 17 amino acids in the E2 transactivation domain that are identical between paillomavirus E2 proteins, indicating that they may be crucial for protein structure and viral function (Brokaw et al., 1996). Many conserved amino acids are clustered at the dimer interface, reflecting the functional importance of amino terminal dimer formation, or are exposed to the solvent indicating a role mediating important protein-protein interactions. Targeted mutational analysis of these amino acids, introducing alanine substitutions to minimise structural disruption, has demonstrated that the ability of E2 to regulate transcription and replication can be separated (Brokaw et al., 1996; Ferguson and Botchan, 1996; Sakai et al., 1996). Mutation at amino acid 73 (I73A) results in an E2 protein that fails to activate transcription but still supports viral DNA replication. Also, E2 with a mutation at position 39 (E39A) retains wild type transcriptional activity but fails to interact with E1 and is defective in transient replication assays. There is a good correlation between the ability of mutant E2 proteins to bind E1 and to support replication, demonstating the functional importance of the E1-E2 interaction for efficient viral DNA replication *in vivo*.

However, two mutant E2 proteins (R37A and D122A) have been identified that retain E1 and DNA binding but are unable to stimulate replication. This suggests that E2 also plays an additional role in viral DNA replication. Indeed, E2 has been shown to alleviate nucleosome mediated repression of BPV-1 DNA replication suggesting a role in chromatin modification (Li and Botchan, 1994).

E2 regulates viral gene expression through contacting components of the cellular transcription machinery. However, the cellular factors involved in E2 mediated transcriptional regulation and the mechanism by which E2 modulates viral gene expression remains relatively unclear. The E2 transactivation domain has been shown to functionally interact with the cellular transcription factors TFIIB, Sp1 and AMF-1 (activation domain modulating factor 1) (Breiding et al., 1997; Li et al., 1991; Yao et al., 1998). The interaction between E2 and TFIIB, an essential component of the RNA polymerase II transcription machinery, has been mapped to residues 74-134 while the AMF-1 interaction is mediated by E2 amino acids 134-216. AMF-1 binds p300/CBP and enhances its interaction with E2 (Peng et al., 2000). A direct interaction between E2 and p300/CBP has also been demonstrated (Lee et al., 2000). p300/CBP is a multi-functional transcriptional co-activator involved in mediating protein-protein interactions between activator proteins and the basal machinery (Nakajima et al., 1997). p300/CBP also possesses intrinsic histone acetyltransferase (HAT) activity and has been implicated in chromatin remodelling (Bannister and Kouzarides, 1996).

E2 requires the co-operation of at least one additional DNA binding proximal promoter factor, such as Sp1, or other factors that interact with papillomavirus

promoters, such as AP-1, Oct-1, NF-1/CTF, or USF for the activation of a minimal tk TATA box promoter (Ham et al., 1991a; Ushikai et al., 1994). The inability of E2 to activate a minimal tk TATA box promoter can also be overcome by TBP overexpression suggesting that recruitment of TFIID, a multisubunit complex composed of TBP and TBP-associated factors (TAFs), is normally a rate limiting step for activation by E2 (Ham et al., 1994). E2 and TBP bind co-operatively to DNA through a direct interaction mediated by the carboxy terminal domain of E2. E2 does not affect the on rate of association but reduces the off rate increasing the amount of TBP bound to the TATA box (Steger et al., 1995). An E2 mutant lacking the transactivation domain but containing the C-terminal TBP binding domain does not activate promoters containing a TATA box and Sp1 site. This suggests that the E2 transactivation domain affects another step in the assembly of the pre-initiation complex after TBP has bound the DNA.

As well as regulating viral gene expression and being required for viral DNA replication E2 possesses other functions essential for the viral life cycle. Long term episomal maintenance of viral genomes requires expression of the E1 and E2 proteins (Piirsoo et al., 1996). Within replicating cells E2 and BPV-1 genomes are found associated with mitotic chromosomes (Skiadopoulos and McBride, 1998). It is proposed that E2 links the genomes to mitotic chromosomes to ensure viral genomes are segregated to daughter cells in approximately equal numbers. This interaction is mediated by the E2 amino terminus (Bastien and McBride, 2000). Recently, it has been shown that phosphorylation of BPV-1 E2, at sites located in the hinge region, can also modulate viral genome copy number by regulating E2 protein levels (Penrose and McBride, 2000). Furthermore, BPV-1 E2 is directed to promonocytic

leukaemia protein (PML) oncogenic domains (PODs) by the papillomavirus L2 protein (Day et al., 1998) and BPV-1 has also been shown to facilitate the packaging of plasmid DNA into pseudovirions (Zhao et al., 2000).

E2 can also disrupt cellular growth control in certain HPV transformed and HPV negative cell lines. The exact mechanism of how E2 induces apoptosis and cell cycle arrest remains unclear and may differ between papillomavirus E2 molecules despite the many similarities. Overexpression of BPV-1 E2 or HPV-18 E2 proteins in HeLa cells leads to growth arrest in the G1 phase of the cell cycle and p53 dependent induction of apoptosis (Dowhanick et al., 1995; Hwang et al., 1996). HeLa cells contain integrated copies of the HPV-18 genome that have a disrupted E2 gene but actively express E6 and E7. Re-introduction of E2 causes a decrease in E6 and E7 expression, reactivation of p53 and the p21 cyclin dependent kinase inhibitor, accumulation of hypo-phosphorylated pRb and decreased E2F expression. The growth suppressive effect of E2 is at least in part mediated by transcriptional repression of the p105 promoter and depends on a specific function of the E2 transactivation domain, not shared by other acidic transactivators such as VP16 (Goodwin et al., 1998). However, a truncated E2 protein lacking the amino terminus also represses E6 and E7 expression in HeLa cells, but fails to induce apoptosis. HPV-16 E2 can also inhibit cell cycle progression in HPV negative cells (Webster et al., 2000) and BPV-1 and HPV-18 E2 proteins have been shown to upregulate p53 mediated transcriptional activation in transformed cell lines (Desaintes et al., 1997). Therefore, E2 can induce apoptosis through mechanisms that are unrelated to its inhibitory effect on viral oncogene expression. BPV-1 E2 has also been shown to induce G1 arrest through p53 independent mechanisms. BPV-1 E2 inhibits

proliferation of HT-3 cells, a p53 negative cervical carcinoma cell line containing integrated HPV-30 DNA (Naeger et al., 1999). Overexpression of E2 leads to repression of E6 and E7 expression, a reduction in cdk-2 activity and an accumulation of hypo-phosphorylated pRb. E2 does not induce p21 but represses expression of cyclin A, which regulates cdk-2 activity, and cdc25 phosphatases which activate cdk-2. Overexpression of HPV E2 proteins has also been shown to cause a growth arrest in the G2/M phase of the cell cycle in both yeast and mammalian cells (Fournier et al., 1999; Frattini et al., 1997).

1.2.2. Organisation of the papillomavirus LCR

All papillomaviruses have a non-coding region of 500-1000 bp called the LCR. The LCR is the transcriptional control unit of the virus regulating expression of the viral transforming proteins and of the proteins essential for the viral life cycle. Mucosal epitheliotropic papillomaviruses, for example, BPV-4 and HPV-16 and –18, have a similar LCR organisation (Fig. 1.4.): a promoter region, an epithelial specific enhancer and an identical distribution of DNA binding sites for the virally encoded E2 protein (Morgan et al., 1998). Immediately upstream from the TATA box are two E2 DNA binding sites separated from each other and the TATA box by 3 or 4 bp. Two additional upstream sites flank the enhancer region: one beside the E1 DNA binding site involved in the regulation of viral DNA replication and one a further 300-400 bp upstream. The conservation of the organisation of E2 DNA binding sites, which is not observed in the cutaneous HPV LCRs, strongly suggests that the mechanism E2 uses to regulate transcription from mucosal epitheliotropic LCRs is conserved.

1.2.3. Transcriptional regulation of the LCR by E2

Transient transfections using various cell lines and E2 expression systems have demonstrated that HPV-16 and –18 E2 proteins can both activate and repress transcription from their respective LCRs (Bernard et al., 1989; Bouvard et al., 1994). The precise position of the E2 DNA binding site determines whether E2 functions as an activator or repressor. Mutational analysis has demonstrated that binding of E2 to the promoter distal sites enhances transcription while repression is mediated through the TATA proximal E2 DNA binding site (Romanczuk et al., 1990). *In vitro* transcription studies have shown that E2 regulates transcription from the HPV-18 LCR in a dose dependent manner (Steger and Corbach, 1997). Low levels of E2 stimulate the LCR promoter, whereas increasing amounts result in promoter repression. E2 therefore autoregulates its own expression levels. Binding assays have demonstrated that E2 binds E2 BS4, the most distal site to the promoter, with the highest affinity. The TATA box proximal site, E2 BS1, has the lowest affinity for E2 suggesting that this site only becomes occupied at high levels of E2 (Steger and Corbach, 1997). It is hypothesised that repression of the HPV-16 and –18 LCR promoters by E2 is mediated by E2 interacting with BS1 and disrupting assembly of the basal transcription machinery, resulting in a down-regulation of transcription initiation (Steger et al., 1995). Disruption of the E2 ORF by integration of the viral genome into the host chromosome would relieve repression of LCR promoter activity by high levels of E2. The overall effect of the E2 protein on LCR promoter activity is therefore dependent on the relative affinity of E2 for each binding site, the number and arrangement of the binding sites and the level of expression of E2 within the cell.

When compared to the HPV-16 LCR, the BPV-4 LCR has a higher transcriptional activity in its target cell type, primary bovine palate keratinocytes (PalK). Also, PalK and primary bovine palate fibroblasts (PalF) are easily maintained and transfected in tissue culture. Low to intermediate levels of HPV-16 and BPV-4 E2 upregulate transcription efficiently from the BPV-4 LCR in PalK cells but do so only poorly in PalF (Morgan et al., 1998). The BPV-4 LCR and primary bovine cell system therefore presents a model to study the mechanisms of E2 mediated transcriptional regulation of mucosal epitheliotropic papillomaviruses and the cell type specificity of this regulation.

1.2.4. Epithelial specific regulation of papillomavirus transcription by cellular factors

DNAse I footprinting has demonstrated that the BPV-4 LCR (Fig. 1.4.) contains numerous binding sites for potential cellular transcription factors while deletion analysis of the BPV-4 LCR has identified several positive and negative E2 independent control elements that regulate transcription from the heterologous tk promoter in established mouse fibroblasts (Jackson and Campo, 1991). Each of the three positive regulatory elements seems to be paired with a negative control element which modulates its activity. However, cellular factors upregulate transcription from the BPV-4 LCR preferentially in epithelial cells (Morgan et al., 1999). An epithelial specific enhancer of approximately 200 bp in the central portion of the LCR, that fails to activate transcription from the SV40 promoter in fibroblasts, accounts for 99% of the transcriptional activity of the LCR. Two main regions, Site1 and Site 2, contribute to enhancer activity. The cellular factors binding to the BPV-4 enhancer still require characterisation. However, the BPV-4, HPV-16 and –18 enhancers are of

a similar size and position, located in the central portion of the LCR. A 400 bp enhancer in the HPV-16 LCR, and a 200 bp enhancer region in the HPV-18 LCR, critical for efficient transcription from these promoters, have been identified (for a review see (Hoppe-Seyler and Butz, 1994)). These constitutive enhancers are epithelial specific as they fail to activate transcription from heterologous promoters in non-epithelial cell types. Tissue specific gene expression can be determined by factors present exclusively in the target cell type, for example, the basic helix-loop-helix (bHLH) transcription factor MyoD in striated muscle (Weintraub et al., 1991) and the POU domain protein Pit-1/GHF-1 in the anterior pituitary (Bodner et al., 1988). However, no one factor has been identified, present exclusively in epithelial cells, that determines the epithelial specific nature of the papillomavirus enhancer elements. It has been proposed that epithelial specificity is brought about by the co-operative interactions of ubiquitously expressed transcription factors. The mechanism of this activation may involve synergism or antagonism between DNA bound factors that are differentially expressed, or modified in a cell type dependent manner. Epithelial specificity might also be established by non-DNA bound co-factors that are expressed in a cell type specific manner or interact only with a particular state of a DNA bound factor. Binding sites for the ubiquitously expressed cellular transcription factors AP-1, NF-1, Oct-1, PEF-1, TEF-1 and the glucocorticoid receptor are commonly found in papillomavirus enhancer regions, though at different locations and in varying numbers.

Activator protein-1 (AP-1) factors play an important role in the activation of HPV transcription. The HPV-16 LCR contains three AP-1 binding sites within the enhancer region while the HPV-18 LCR contains two AP-1 recognition elements,

one within the enhancer and the other located in the proximal promoter region (Chong et al., 1991; Thierry et al., 1992). Mutational inactivation of these AP-1 sites within the context of the complete LCR severely reduces promoter activity. AP-1 factors, encoded by the jun and fos proto-oncogenes, consist of either jun-jun homodimers or jun-fos heterodimers. Both jun and fos constitute multi-gene families: three jun family members, c-jun, junB, and junD, and four fos family members, c-fos, fosB, fra1, and fra2, have been identified. AP-1 proteins can confer a high degree of transcriptional variability to a gene as different jun and fos family members have similar DNA binding specificities but different tissue distributions and activation potentials. Also, subtle sequence variations from the consensus AP1 binding motif are preferentially recognised by specific subsets of AP-1 proteins. Both jun and fos family members have been shown to be differentially expressed during epithelial differentiation (Wilkinson et al., 1989). In nuclear extracts prepared from human keratinocytes, junB, expressed predominantly in the terminally differentiated layers of the epidermis during mouse organogenesis, is the major jun component recognising the HPV-18 AP-1 elements (Thierry et al., 1992). However, AP-1 factors are necessary but not sufficient for activation of HPV-16 and –18 transcription in keratinocytes as LCR transcriptional activity could not be detected in jun/fos co-transfected fibroblasts suggesting that additional factors must be involved in determining epithelial specificity. Consistent with this, a putative keratinocyte specific transcription factor, KRF-1, has been shown to interact with AP-1 to activate HPV-18 gene expression in squamous epithelial cells (Mack and Laimins, 1991). However, KRF-1 binding sites are not present in the HPV-16 LCR and KRF-1 does not appear to contribute to cell type specific activation of other genital HPV types.

Nuclear factor 1 (NF1) proteins are a family of transcription factors encoded by four genes, NF1-A, -B, -C and –X, whose diversity is further increased by differential RNA splicing (Apt et al., 1993). NF1 proteins bind their consensus motif as dimers. The amino terminal DNA binding and dimerisation domains of all NF1 proteins are highly conserved but the carboxy terminal, proline rich transactivation domains are heterogeneous. Seven NF1 sites within the HPV-16 LCR and three sites within the HPV-18 enhancer have been identified (Gloss et al., 1989). Most of these sites only contain half the consensus binding motif and bind NF1 with low affinity. Mutational analysis of the HPV-16 LCR has demonstrated that NF1 and AP-1 can functionally co-operate to regulate papillomavirus transcription (Chong et al., 1990). Epithelial cells contain proteins derived from the NF1-C gene (NF1/CTF), but in fibroblasts where the viral enhancer is inactive, high levels of NF1 from the NF1-X gene is expressed (Apt et al., 1994). Overexpression of NF1-X in epithelial cells downregulates the activity of the HPV-16 enhancer, demonstrating a crucial role for the NF1 binding sites in the epithelial specific function of the viral enhancer.

POU domain proteins, for example, the ubiquitous Oct-1, are characterised by a 150-160 amino acid stretch containing highly conserved amino (POU specific) and carboxy (POU homeodomain) terminal regions separated by a variable linker (for a review see (Wegner et al., 1993)). The POU specific and the POU homoedomains form a high affinity, sequence specific DNA binding domain. Although POU domain proteins generally have weak, intrinsic, amino terminal transactivation domains, the POU domain provides an interface for functionally important interactions with other proteins, for example, Oct-1 binds to a related octomer motif in the HSV early promoter recruiting the multi-subunit host cell factor HCF/C1 and the virally

encoded VP16 acidic transactivator (O'Hare et al., 1988). The resultant complex allows a strong activation of transcription of HSV immediate early genes. Also, the interaction of POU domains with cell type specific co-activators might be an important determinant of cell type specific gene expression, for example, a B-cell specific coactivator, OCA-B, potentiates Oct-1 mediated activation of the immunoglobulin heavy chain promoter (Luo et al., 1992). The enhancer in the HPV-16 LCR contains a degenerate octomer binding site overlapping a binding site for a novel 110 kDa protein, PEF-1 (papillomavirus enhancer binding factor 1). PEF-1 binding to this element upregulates, while Oct-1 binding downregulates enhancer activity (Sibbet et al., 1995). However, Oct-1 has also been shown to activate the HPV-16 enhancer via a synergistic interaction with NF-1 (O'Connor and Bernard, 1995). Two degenerate octomer binding sites are present in the HPV-18 enhancer. Overexpression of Oct-1 represses HPV-18 transcription (Hoppe-Seyler and Butz, 1994). A DNA binding defective Oct-1 mutant with a triple amino acid substitution in the homeodomain indicates that repression does not require direct DNA binding by Oct-1 but involves protein-protein interactions. However, the HPV octomer elements can activate transcription when inserted upstream of a heterologous promoter (Morris et al., 1993). Epoc-1/skn-1a, a POU domain protein expressed almost exclusively in the skin, binds and activates the HPV-16 and –18 promoters (Yukawa et al., 1996). Epoc-1/skn-1a transcripts show a similar differentiation dependent distribution of expression as HPV transcripts during the viral life cycle.

Binding sites for the transcriptional enhancer factor (TEF) proteins 1 and 2, previously shown to bind and co-operatively activate the SV40 enhancer, have been identified in the HPV-16 enhancer (Chong et al., 1991; Ishiji et al., 1992).

Squelching experiments have indicated that transcriptional activation by TEF-1 requires a limiting, cell specific co-activator. Mutational analysis suggests that TEF-1 is a major determinant of HPV-16 enhancer activity.

Several additional factors have been shown to contribute to enhancer activity. The HPV-16 and –18 enhancer is responsive to certain steroid hormones through binding sites for the glucocorticoid and progesterone receptors (Chan et al., 1989). The HPV-16 LCR contains three different glucocorticoid responsive elements (GREs) while one is present in the HPV-18 LCR. HPV-16 and –18 transcriptional activity is also suppressed by overexpression of wild type p53 (Chan et al., 1989). Deletion analysis has mapped the p53 responsive domain to the epithelial specific enhancer. The HPV-16 and –18 enhancers also contain CpG dinucleotide repeats and are downregulated by site specific methylation (List et al., 1994). *In vivo* footprinting has suggested that these enhancer regions are more densely occupied than previously thought and suggest that additional novel factors contribute to HPV enhancer activity (Bednarek et al., 1998). The activity of many of the factors that interact with papillomavirus enhancers can also be regulated by post translational mechanisms, for example, phosphorylation and glycosylation, allowing the integration of information carried by different signal transduction pathways.

Deletion of the LCR region upstream from the constitutive enhancer has no significant effect on transcriptional activity. This region contains the late polyadenylation site and is presumed to be involved in regulating the stability of late mRNAs. The 3' promoter region of mucosal epitheliotropic LCRs contains the origin of DNA replication, a TATA box and three binding sites for virally encoded E2.

Binding sites for the cellular factors Sp1, YY-1 and C/EBP are also commonly found in HPV promoters.

In HPV gene expression Sp1 binds to a single GC rich box within the proximal promoter (Hoppe-Seyler and Butz, 1992). Sp1 bound to this site mediates promoter activation by the epithelial specific enhancer. Also, Sp1 can functionally interact with the viral E2 protein (Li et al., 1991). Sp1 is one of at least four ubiquitously expressed transcription factors derived from a multigene family. All these factors contain a highly conserved zinc finger DNA binding domain and a glutamine rich activation domain. Sp1 is a transcriptional activator while Sp3 acts as a repressor, probably due to competition with Sp1 for the same binding sites (Hagen et al., 1994). In various epithelial and fibroblast cell lines high levels of Sp1 compared to Sp3 are consistently found where the HPV-16 promoter is active and low levels are found where it is inactive (Apt et al., 1996).

Yin-yang 1 (YY1) is a zinc finger protein that functions either as a transcriptional repressor, activator or initiator binding protein, depending on the sequence context. YY1, bound to sites in the HPV-16 and –18 proximal promoter region, mediates transcriptional repression (Bauknecht et al., 1992). These sites are often found mutated in primary tumours or metastases containing non-integrated HPV-16 episomes suggesting an important role for YY1 mediated negative regulation of HPV gene expression (May et al., 1994). CCAAT/enhancer binding protein (C/EBP) factors, a family of conserved, leucine zipper (bZip) DNA binding proteins, have also been implicated as both positive and negative regulators of HPV gene expression (Bauknecht and Shi, 1998). C/EBP factors are linked to cellular differentiation and

proliferation control in a variety of tissues including adipose and skin (Muller et al., 1999), suggesting a role in differentiation dependent regulation of HPV transcription.

The overall LCR sequence homology between BPV-4 and HPV-16 and –18 LCRs is low. BPV-4 therefore uses distinct cellular factors from the HPVs to achieve epithelial specificity, for example, there is no BPV-4 promoter proximal Sp1 site and no AP-1 sites in the BPV-4 LCR (Morgan et al., 1999). However, C/EBP family members have been implicated as negative regulators of BPV-4 transcription (McCaffery and Jackson, 1994) and there are several putative NF1 sites in the BPV-4 LCR and octomer like sequences in the BPV-4 enhancer region. The BPV-4 LCR and primary bovine cell system therefore presents another opportunity to study the mechanisms mucosal epitheliotropic papillomaviruses use to achieve epithelial specific transcriptional regulation.

THE BPV-1 GENOME

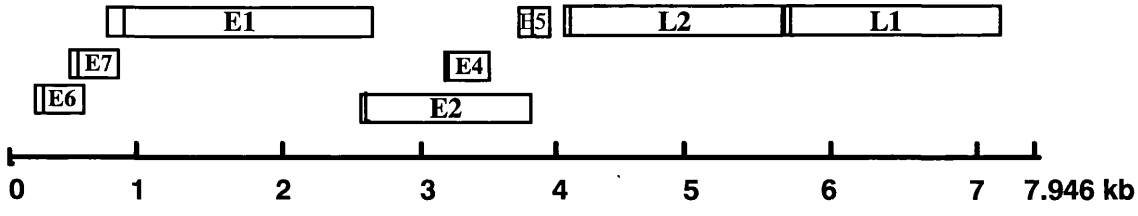


Fig. 1.1. Organisation of the BPV-1 genome. The BPV-1 genome is a closed circle of approximately 7.9 kb that exhibits an organisation similar to most papillomaviruses. The open reading frames (ORFs) are shown as boxes. E represents early and L late ORFs.

THE BPV-4 GENOME

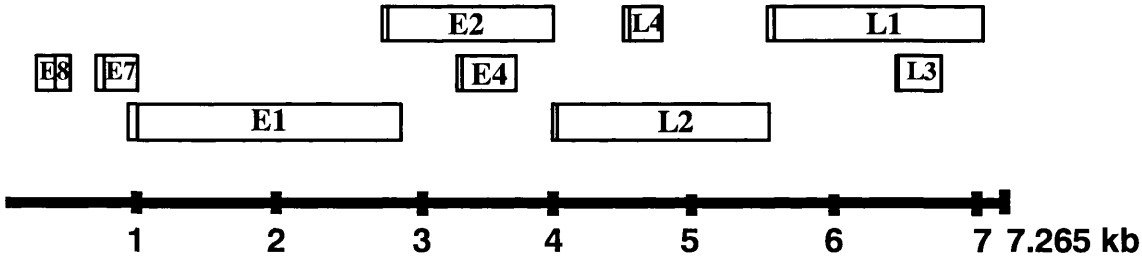


Fig. 1.2. Representation of the BPV-4 genome. The BPV-4 genome (approximately 7.2 kb) is smaller than that of BPV-1. The main difference of the BPV-4 genome to that of most papillomavirus is the absence of the E6 ORF.

The papillomavirus E2 protein

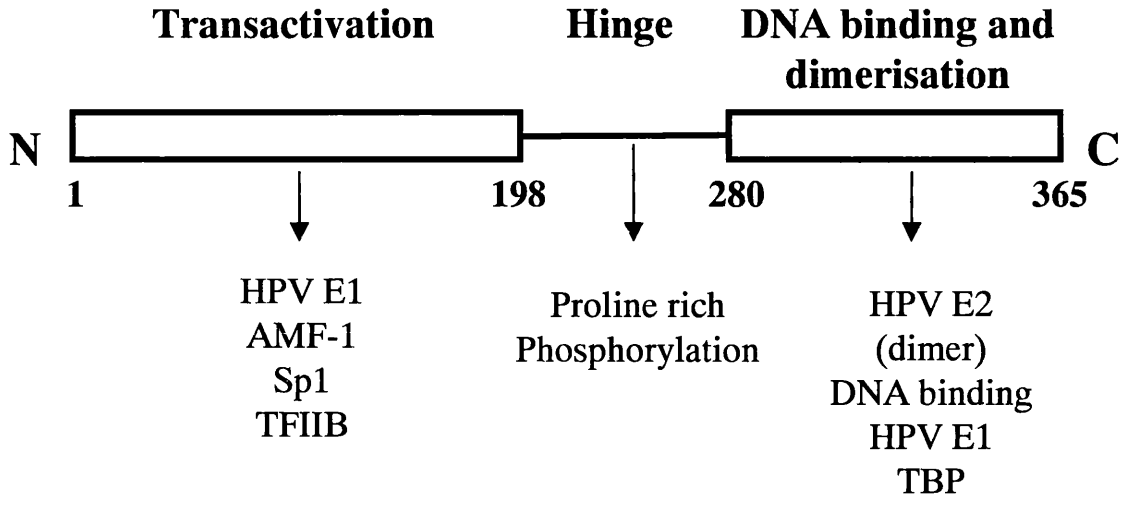
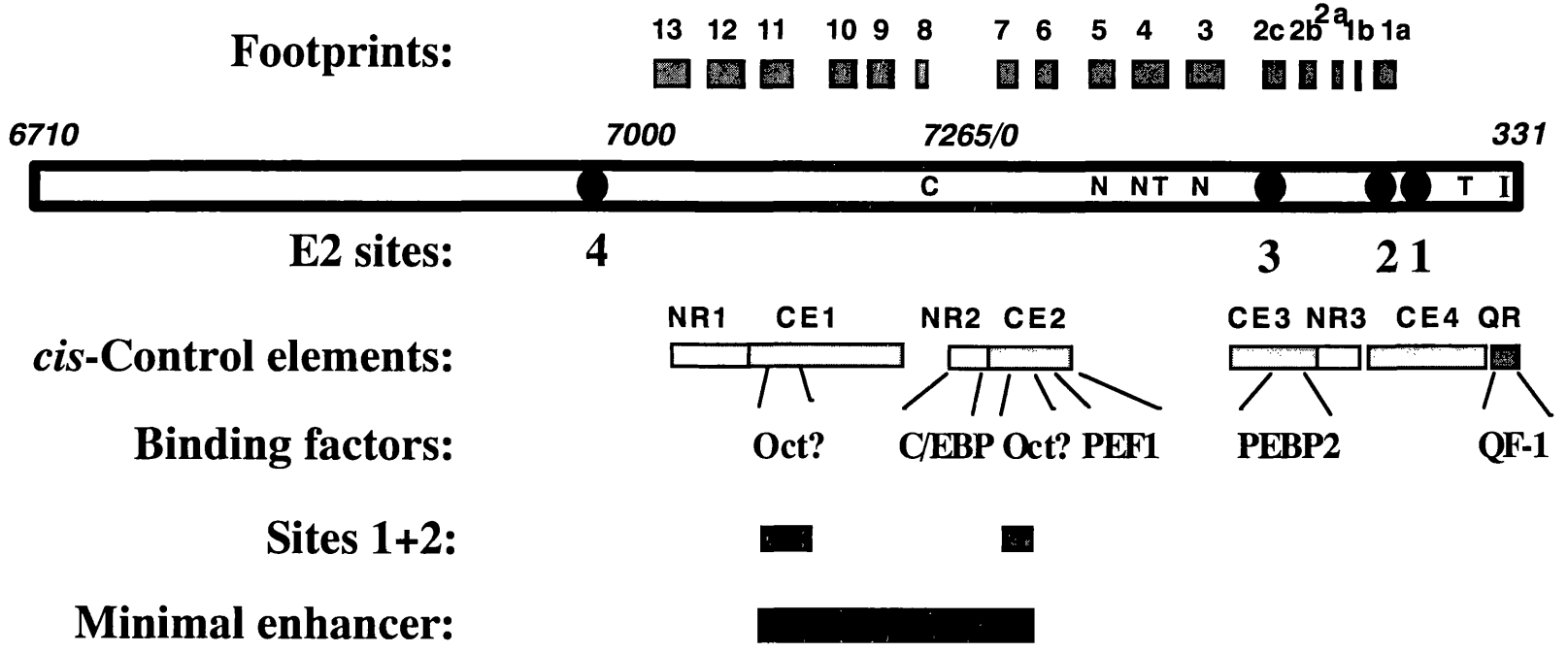


Fig. 1.3. Diagrammatic representation of the papillomavirus E2 protein. The E2 protein consists of three modular domains: a conserved amino terminal domain, a central flexible hinge and a conserved carboxy terminal domain. The function of each domain is shown and the interactions with viral and cellular factors are indicated. The amino acid numbers refer to HPV-16 E2.

Fig. 1.4. Organisation of the BPV-4 LCR from nucleotide 6710 to 331. Four binding sites for the viral E2 protein are shown as circles. This organisation of E2 DNA binding sites is identical in the HPV-16 and –18 LCRs. Binding sites for potential transcription factors as identified by footprinting studies are shown (boxes 1-13). Nuclear factors interacting with the LCR are indicated. Sequence elements are also shown: I indicates the putative initiator element, T the TATA box, C the CAAT box and N indicates NF-1 like elements. The BPV-4 LCR contains three positive control elements each of which seems to be paired with a negative element. CE boxes represent positive control elements and NR boxes represent negative regulatory elements. QR indicates the quercetin repsonsive element. The epithelial specific enhancer is also shown. This is of a similar size and position to the enhancer in the HPV-16 and –18 LCRs. The two main regions contributing to enhancer activity, Sites 1 and 2, are depicted.

Organisation of the BPV-4



Adapted from Jackson and Campo., 1995

Chapter 2 – Materials and Methods

2.1. Materials

2.1.1. Antibodies

Amersham International plc

Anti-mouse IgG horseradish peroxidase linked whole antibody (raised in sheep)

TVG261, a monoclonal antibody directed against amino acids 2-17 in the amino terminus of HPV-16 E2, was a kind gift from Dr. M. Hibma, ICRF Tumour Virus Group, University of Cambridge.

2.1.2. Bacteriology

Beatson Institute Central Services

L-broth

Becton Dickinson Labware

Falcon 1059 polypropylene tubes

Falcon 2059 polypropylene tubes

Beta laboratories

Yeast extract

Bibby Sterilin Ltd

90mm and 150mm bacteriological petri dishes

Clontech

E.Coli BM25.8 cells

E.Coli XL1-Blue

Difco Laboratories

Bacto-Agar

Bactotryptone

Fisons Scientific Equipment

Glycerol

Gibco BRL Europe Life Technologies Ltd

E. Coli DH5α competent cells

Nunc

Sterile disposable inoculating loops

Sigma Chemical Co. Ltd

Ampicillin

Betaine

Carbenicillin

Chloroamphenicol

Isopropyl-thio-β-D-galactosidase (IPTG)

Lysozyme

Maltose

Sorbitol

Tetracycline

2.1.3. Cell lines

PalK cells are primary bovine palate keratinocytes derived from foetal biopsies.

PalF cells are primary bovine palate fibroblasts derived from foetal biopsies.

Swiss 3T3 cells are an immortalised mouse fibroblast cell line.

HeLa cells are an HPV-18 positive keratinocyte cell line derived from an invasive cervical carcinoma (ECACC Ref. No. 8609201).

2.1.4. Chemicals and Reagents

Amersham International plc

Enhanced Chemiluminescence (ECL) Western detection agent

BDH Analar Laboratory Supplies

Ammonium persulfate (APS)

D-glucose

Boehringer Mannhein Ltd

Caesium chloride

Calbiochem

Guanidine hydrochloride

Fisons Scientific Equipment

Acetic acid

Ammonium acetate

Butan-2-ol

Chloroform

di-sodium hydrogen orthophosphate (anhydrous)

Dimethyl sulfoxide (DMSO)

Ethylene diamine tetra acetate (EDTA) disodium salt

EGTA

Lithium chloride

Hydrochloric acid

Magnesium acetate

Magnesium chloride

Magnesium sulphate

Methanol

Potassium chloride

Potassium hydroxide

Propan-2-ol

Sodium acetate

Sodium chloride

Sodium dihydrogen orthophosphate

Sodium dodecyl sulphate (SDS)

Sodium pyrophosphate

Gibco BRL Europe Life Technologies Ltd

Agarose (ultrapure electrophoresis grade)

Glycine

Tris base

James Burrough Ltd

Ethanol

Pharmacia Biotech Ltd

ATP

Poly (dI-dC)

Promega

Nuclease free H_2O

RNasin Ribonuclease Inhibitor

Severn Biotech Ltd

30% (w/v) acrylamide: 0.8% (w/v) bis-acrylamide

40% (w/v) acrylamide: 2.1% (w/v) bis-acrylamide

Sigma Chemical Co. Ltd

β-mercaptoethanol

Bicinchonoinic Acid (BCA) solution

Bovine Serum Albumen (BSA)

Bromophenol Blue

Coomassie Brilliant Blue R

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

Dithiothreitol (DTT)

Ethidium bromide

Ficoll (type 400)

Glutathione (reduced)

HEPES

Leupeptin

Nonidet P-40 (NP-40)

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

PMSF

Ponceau S solution

TEMED (N,N,N',N' –tetraethylethylenediamine)

Tween-20 (Polyoxyethylene sorbitan nonolaurate)

2.1.5. Enzymes and Kits

Gibco BRL Europe Life Technologies Ltd

All restriction enzymes and reaction buffers were obtained from Gibco BRL.

NBL Gene Sciences

Alkaline phosphatase

T4 DNA ligase

T4 polynucleotide kinase

Perkin Elmer Corporation

GeneAmp PCR Core Kit

Promega Ltd

Luciferase Assay System

Pfu polymerase

Reporter Lysis 5x Buffer

TNT T7/T3 Coupled Reticulate Lysate System

TNT T7 Quick Coupled Transcription kit

Quiagen Ltd

QIAprep Spin plasmid miniprep kit

QIAquick gel extraction kit

Sigma Chemical Co. Ltd

Protein Kinase A, catalytic subunit

2.1.6. Miscellaneous

Amersham International plc

Hybond-C extra

Alpha Laboratories Ltd

Microcentrifuge tubes

Pastettes

Cadisch and Sons

70 μm filter nylon gauze

Canberra Packard Ltd

Superpolyethylene scintillation vials

Costar Corporation

96 well plates

Cruachem Ltd

Cruachem oligonucleotide purification (COP) cartridges

Decon Laboratories Ltd

Decon 75

Du Pont (UK) Ltd

Polyallomer ultracentrifuge tubes

Gelman Sciences Ltd

Sterile 0.2 μm acrodisc filters

Premier Beverages

Marvel (dried skimmed milk)

Satorius AG

Collodion Bags

Sigma Chemical Co. Ltd

Kodak X-OMAT AR X-ray film

GST beads

Technical Photo Systems

Fuji RX medical X-ray film

Whatman International Ltd

Whatman 3MM filter paper

2.1.7. Molecular Weight Markers

Amersham International plc

Rainbow™ coloured protein molecular weight markers

Gibco BRL Europe Life Technologies Ltd

λ DNA/ HindIII digested 100 bp ladder

2.1.8. Phage Libraries

Clontech

λTriplex human HeLa 5' STRETCH PLUS cDNA library

2.1.9. Plasmids

pGL3 is a luciferase reporter vector obtained commercially from Promega.

pGL3 PRO, also available commercially from Promega, contains the SV40 promoter driving expression of the luciferase gene.

pGL3 CONT contains the SV40 enhancer-promoter driving expression of the luciferase gene. This reporter plasmid is commercially available from Promega.

pLCR contains the wild type BPV-4 LCR from nucleotide 6710-331 cloned into the BamHI site of the p0luc luciferase plasmid. pLCR was a gift from Dr Maria Jackson (Beatson Institute).

pLCR-E2(3)mt1 contains the BPV-4 LCR from nucleotide 6710-331 and has the TATA box proximal E2 BS1 mutated to prevent E2 binding. This plasmid was a gift from Dr Maria Jackson (Beatson Institute).

pLCR-E2(d/3) contains the full length BPV-4 LCR with both the TATA box proximal E2 DNA binding sites mutated. Again, a gift from Dr Maria Jackson (Beatson Institute).

pBluescript SKII, available commercially from Stratagene, contains the T3 and T7 promoters for *in vitro* transcription and translation.

pCMV and **pCG** are mammalian expression vectors.

pCMV HPV-16 E2 expresses the wild type HPV-16 E2 protein under control of the cytomeglovirus promoter. A kind gift from Dr Lawrence Banks (International Centre for Genetic Engineering and Biotechnology, Italy).

pCG BPV-1 E2 expresses wild type BPV-1 E2 under control of the cytomeglovirus promoter. This plasmid was a gift from Dr Mart Ustav (Estonian Biocentre).

pCG VP16-E2 expresses a chimaeric protein which has the VP16 transactivation domain fused to the BPV-1 E2 DNA binding domain under control of the cytomeglovirus promoter. This plasmid was a gift from Dr Mart Ustav (Estonian Biocentre).

pVP16-LexA expresses the VP16 transactivation domain and the LexA DNA binding domain as a fusion protein. This vector was a gift from Dr Chris Batholomew (Glasgow Caledonian University).

phTBP expresses human TBP from the cytomeglovirus promoter. phTBP was a kind gift from Dr Moshe Yaniv (Unite des Virus Oncogenes, Paris).

ptkluc contains the tk promoter from HSV-1 cloned into the pGL2 luciferase vector.

pGEX-2TK contains a GST domain and the recognition sequence for the catalytic subunit of protein kinase A. This plasmid is commercially available from Pharmacia Biotech.

pGADT7 contains the GAL4 activation domain, the T7 promoter and an HA epitope tag. pGADT7 is available commercially from Clontech.

pGADGH is also available commercially from Clontech. This yeast expression vector generates a hybrid protein containing the GAL4 activation domain.

2.1.10. Radiochemicals

Amersham International plc

Redivue [γ ^{32}P] dATP

Redivue [^{35}S] methionine

2.1.11. Tissue Culture

Beatson Institute Central Services

Sterile distilled water

Sterile glycerol

Sterile phosphate buffered saline (PBS)

Sterile phosphate buffered saline + EDTA (PE)

Becton Dickinson Labware

18 gauge sterile syringe needles

60, 90 and 140 mm tissue culture dishes

Falcon 2097 polypropylene tubes

Falcon 2098 polypropylene tubes

Serological plastic pipettes

Sterile Plastipak syringes

Bibby Sterilin Ltd

Sterile plastic bijoux and universal containers

Costar Corporation

Disposable cell scrapers

Gibco BRL Europe Life Technologies Ltd

Special Liquid medium

10x Dulbecco's Modified Eagles Medium

10x Nutrient Mixture F-10 (HAM)

2.5% Trypsin

200 mM L-glutamine

7.5% sodium bicarbonate

100 mM sodium pyruvate

Globepharm

Foetal calf serum

Harlan Sera-Lab Ltd

Foetal calf serum

Nunc

Cryotubes

T25, T75 and T175 cm^2 tissue culture flasks

Sigma Chemical Co. Ltd

Adenine

Cholera enterotoxin

Epidermal growth factor (EGF)

Hydrocortisone

Insulin

Polybrene (Hexadimethrine bromide)

2.2. Methods

2.2.1. Tissue culture

All cell culture work was performed using strict aseptic techniques inside a laminar flow hood (Class II Microbiological Safety Cabinets, Medical Air technology Ltd., Manchester, UK). Cells were incubated at 37^0C in a dry atmosphere containing 5% (v/v) CO_2 (Heraeus, Essex, UK).

2.2.1.1. Swiss 3T3 Feeder cells

Swiss 3T3 feeders were grown in Special Liquid Medium containing 10% (v/v) foetal calf serum and 2mM glutamine. Cells were seeded at approximately 10^6 cells/T175 tissue culture flask and grown until confluency. Confluent cells were washed twice with PBS (phosphate buffered saline) and trypsinised using a solution of 0.25% (w/v) trypsin in PE buffer (PBS containing 1mM EDTA). Flasks were incubated at 37^0C until the cells detached, the cells were resuspended in growth medium, transferred to a sterile universal tube and pelleted by centrifugation at 1000 rpm for 5 min at room temperature. The cell pellet was resuspended in fresh growth medium and the concentration determined using a haemocytometer. Swiss 3T3 cells were either passaged 1 in 10 or lethally irradiated with 60 grays of γ-irradiation using a ^{60}Cobalt source prior to use as feeders. Feeder cells, incapable of further cell division, were plated out at a density of approximately $2x10^6$ cells/T175 tissue culture flask before the addition of keratinocytes.

2.2.1.2. PalK cells

PalK cells, prepared from bovine foetal biopsies as described for human cervical keratinocytes (Cuthill et al., 1993), were routinely cultured on irradiated Swiss 3T3 feeders in Special Liquid Medium supplemented with 10% (v/v) foetal calf serum, 2mM glutamine, 2% (w/v) 10x Nutrient Mixture F-10, 0.31% (w/v) sodium bicarbonate, 10 ng/ml cholera enterotoxin, 10 ng/ml EGF, 0.5 μg/ml hydrocortisone, 5 μg/ml insulin and 180 μM adenine. PalK cells were grown to approximately 80-90% confluency and then passaged (split 1 in 4) or seeded into the appropiate tissue culture dish. The feeder cells were removed from the PalK cells by incubating with 5 ml PE at 37^0C prior to trypsinisation as they are less firmly attached to the tissue culture flask compared to the keratinocytes.

2.2.1.3. PalF cells

PalF cells, prepared as described (Jaggar et al., 1990) from the same foetal biopsy used to prepare the PalK cells, were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal calf serum, 2 mM glutamine, 1 mM sodium pyruvate and 0.375% sodium bicarbonate. PalF cells were grown until just subconfluent and then passaged 1 in 2 or seeded into the appropiate tissue culture dish.

2.2.1.4. Transient transfection of PalK cells

PalK cells were transfected using the polybrene-DMSO technique. $5x10^5$ cells were seeded on a 60 mm tissue culture dish without feeders. 18 hours later the medium was replaced with 2 ml growth medium containing 10 μg/ml polybrene and the

plasmid DNA. After 5-6 hours the DNA-polybrene containing medium was removed and 5 ml of Special Liquid Medium containing 35% DMSO was added to the cells for 3 min. Following this incubation the cells were washed twice with PBS and then re-fed with 5 ml fresh growth medium. The cells were harvested 42-48 hours later.

2.2.1.5. Transient transfection of PalF cells

PalF cells were transiently transfected using a standard calcium phosphate precipitation technique. Cells were plated out at $2x10^5$/60 mm tissue culture dish. The following day a calcium phosphate precipitate containing the DNA was added to the cells. This was carried out as follows for each 60 mm cell monolayer: 250 µl of a solution containing the plasmid DNA in 250 mM $CaCl_2$ was added dropwise with gentle mixing to 250 µl of 2x HEPES buffered saline (280 mM NaCl, 10 mM KCl, 1.5 mM $Na_2HPO_4.2H_2O$, 50 mM HEPES). The mixture was left for 30 min to allow a fine precipitate to form and added directly into the medium above the cell monolayer. 16-18 hours later the cells were washed twice with PBS and refed with fresh growth medium. The cells were harvested 24-32 hours later.

2.2.1.6. Luciferase assay

PalK and PalF cells were lysed directly on the tissue culture plates. The medium was removed and the cells washed twice with PBS. 300 µl of Reporter Lysis Buffer (Promega) was added to the plate and left for 10 minutes. The cell lysate was then scraped from the dish and placed in a 1.5 ml centrifuge tube. The lysate was cleared by centrifuging the sample for 10 min and removing the supernatant to a fresh tube. An aliquot of the supernatant was then assayed for luciferase activity using the Luciferase Assay System (Promega) with a BioOrbit 1251 (80 µl supernatant) or a

Tropix TR717 Microplate luminometer (10 µl). To standardize for cell number, the protein concentration was determined using the BCA/$CuSO_4$ assay (Section 2.2.3.5.). pGL3CONT (which contains the SV40 promoter and enhancer driving expression of the luciferase gene) was transfected in parallel to confirm efficient transfection. This construct demonstrates high levels of transcriptional activity in both keratinocytes and fibroblasts. All transfections were repeated at least three times in duplicate.

2.2.1.7. Nuclear extract preparation

PalK and PalF nuclear extracts were prepared as follows. Approximately $1x10^7$ cells were washed twice with 5 ml ice-cold PBS, removed from the tissue culture dish by scraping and transferred to a sterile universal container. The cells were pelleted by centrifugation at 2000 rpm for 5 min, re-suspended in 1.5 ml ice-cold PBS and transferred to a clean eppendorf tube. The cells were pelleted again by spinning in a microfuge at 14 000 rpm for 2 min at 4^0C and re-suspended in 400 µl hypotonic lysis buffer (10 mM HEPES/KOH pH. 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT and 0.2 mM PMSF). Samples were incubated on ice for 10 min, vortexed for 10 sec, pelleted and the supernatant was removed. The pellet was re-suspended in 50 µl high salt buffer (20 mM HEPES/KOH pH. 7.9, 25% (v/v) glycerol, 420 mM NaCl. 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT and 0.2 mM PMSF) and incubated on ice for 20 min. The cellular debris was removed by centrifugation (14000 rpm, 15 min, 4^0C). The supernatant was removed, frozen on dry ice and stored at –70 °C until use.

2.2.1.8. Whole cell extract preparation

PalK, PalF and HeLa whole cell extracts were prepared in the same manner. Transiently tranfected cells were washed twice with ice-cold PBS. Cells were lysed

on ice in 50 µl SDS-PAGE lysis buffer (100mM Tris HCl pH6.8, 2% SDS, 20% glycerol), removed from the tissue culture dish and transferred to a clean eppendorf tube. The cells were sonicated (2x 10 sec) and clarified by centrifugation (14 000 rpm, 10 min, 4^0C). The supernatant was removed, frozen on dry ice and stored at – 70^0C until use. The protein concentration was determined by absorbance measurement at 280nm using a Beckman DU 650 spectrophotometer. An OD_{280} reading of 1 corresponds to approximately 1 µg/µl protein.

2.2.2. Molecular biology

2.2.2.1. Oligonuceotide synthesis

Oligonucleotides were synthesised on an Applied Biosystems Model 381A DNA synthesiser using the manufacturers protocols and Cruachem reagents by Beatson Institute technical services staff. The oligonucleotides were synthesised with or without trityl group protection. "Trityl off" oligonucleotides, obtained as a pellet, were dissolved in 1ml sterile distilled water. "Trityl on" oligonucleotides, obtained in ammonia and routinely used as primers for PCR amplification, were deprotected after synthesis by incubation in a 55^0C water bath overnight. The oligonucleotides were detritylated using a Cruachem oligonucleotide purification (COP) cartridge according to the manufacturers instructions. The final oligonucleotide was eluted from the COP cartridge in 1-2 ml acetonitrile, precipitated using ethanol and sodium acetate (Section 2.2.2.5.) and resuspended in 0.5-1 ml sterile distilled water. The final concentration of oligonucleotide was determined by absorbance measurement at 260 nm and 280 nm using a Beckman DU 650 spectrophotometer. An OD_{260} reading of 1 corresponds approximately to 33 µg/ml single stranded DNA or 50 µg/ml double

stranded DNA. The OD_{260}/OD_{280} ratio provided an estimate of the DNA sample purity.

2.2.2.2. Agarose gel electrophoresis

Agarose gel electrophoresis was performed using horizontal gel cast apparatus (Promega). 1% agarose gels were routinely used unless otherwise stated. The appropiate amount of ultrapure electrophoretic grade agarose was dissolved in 0.5x TBE buffer (5x TBE: 40mM Tris base, 16 mM acetic acid, 1 mM EDTA, pH 8.0) by heating the solution in a glass conical flask in a microwave. The gel cast apparatus with a comb containing the appropiate number and size of teeth to form the sample wells was assembled and the gel was poured. The solidified gel was placed in the gel tank and submerged in 0.5x TBE buffer containing 0.5 μg/ml ethidium bromide. Samples containing 1x loading buffer (10x loading buffer: 0.45% (w/v) bromophenol blue, 1% (w/v) SDS, 100 mM EDTA, 2.5% (w/v) Ficoll 400) were loaded into individual wells. An appropiate sized DNA ladder was loaded into the first and/or last well in the gel and the DNA was separated by running at 70-100 constant voltage until the dye front was 1-4 cm from the end of the gel. Separated DNA was visualised by illumination on a short wave (312 nm) UV light box and photographed using an Appligene Imager.

2.2.2.3. Polyacrylamide gel electrophoresis

In general, 6 or 8% polyacrylamide gels were set up. This was carried out by adding the appropiate volume of 40% (w/v) acrylamide: 2:1% (w/v) bisacrylamide solution to 0.5x TBE buffer. Polymerisation of the gel was catalysed by the addition of 0.07% APS and 0.08% TEMED. The solution was mixed thoroughly and poured between

two glasss plates (1 or 2 mm) clamped together in the gel forming apparatus (ATTO). A comb to make the sample wells was placed in the top of the gel immediately after pouring. The gel was then left to polymerise in the vertical position. After the gel had set, the spacer was removed and the gel, formed between the two glass plates, was placed vertically into the electrophoresis tank (ATTO). The tank was filled with 0.5x TBE buffer, the comb was removed and each well was washed out with 0.5x TBE using a syringe. Polyacrylamide gel electrophoresis (PAGE) was carried out at 100-150 constant voltage.

2.2.2.4. Restriction Enzyme Digests

Restriction digests were performed using the appropiate enzymes and reaction buffers according to the manufacturers instructions. Typically, 5-10 units of restriction enzyme/μg DNA was used. In general, small quantities of plasmid DNA (<5 μg) were digested in a 30 μl reaction volume for 2-3 hrs at 37^0C whereas PCR fragments were digested overnight at 37^0C.

2.2.2.5. DNA purification

DNA samples were purified by phenol chloroform extraction after each manipulation to remove contaminants such as residual enzyme activities. An equal volume of phenol:chloroform:isoamyl alcohol (24:24:1 v/v/v) was added to the DNA solution. The aqueous DNA and organic phases were mixed by vortexing and separated by centrifugation in a microfuge (14 000 rpm, 5 min, room temp). The upper aqueous phase was carefully removed making sure none of the interphase was taken and transferred to a clean eppendorf tube. DNA samples were then precipitated using organic solvents to remove contaminants such as salt. Double stranded DNA was

precipitated by adding 1/10 volume of 3M sodium acetate and 2.5 volumes ethanol. The sample was mixed and left at -20^0C for 1 hour to facilitate precipitation. The DNA was pelleted in a microfuge (14 000 rpm, 20 min, 4^0C), washed with 70% ethanol to remove any traces of salt and pelleted again. After the ethanol was removed, the purified DNA was air dried and resuspended in the appropiate volume of sterile distilled water. PCR fragments were routinely precipitated by the addition of 4/5 volume 10M ammonium acetate and 2.5 volumes ethanol. The DNA was pelleted and washed as described above and the process was repeated. Single stranded oligonucleotides were precipitated by the addition of 1/10 volume lithium chloride and 3 volumes of ethanol. The samples were left on dry ice for 20 min, pelleted by centrifugation in a microfuge (14 000 rpm, 30 min, 4^0C) and washed with 70% ethanol as before. Alternatively or in combination with organic solvent precipitation, DNA fragments to be used in cloning were purified by agarose gel electophoresis and DNA extraction using a QIAquick gel extraction kit according to the manufacturer's instructions.

2.2.2.6. DNA ligation

Plasmid DNA and the DNA fragment to be inserted into the vector were separately digested using the appropiate restriction enzymes (Section 2.2.2.4). The 5' phosphate residues of linearised vector DNA were dephosphorylated to prevent vector religation. 1 µl (1 unit) of alkaline phosphatase was added at the end of a restriction digest reaction. The reaction mixture was incubated for 30 min at 37^0C followed by a second incubation at 70^0C for 10 min to stop all enzyme activity. The plasmid DNA and DNA fragment were then purified as detailed above (Section 2.2.2.5.). The DNA fragment was ligated into the cut vector using 1 µl (4 units) of T4 DNA ligase in 1x

ligase buffer in a 20 µl reaction volume. This was carried out according to the manufacturer's instruction. An excess of DNA fragment compared to vector was used for ligation reactions. Reactions were routinely incubated overnight at 11^0C.

2.2.2.7. Transformation of Competent Bacterial Cells

E. Coli DH5α competent cells were used for the propagation of plasmid DNA unless stated otherwise. Stocks of competent cells were stored at -70^0C until use when they were thawed on ice. 1 µl of purified ligation reaction or 1-2 ng plasmid DNA was added to a chilled polypropylene tube (Falcon 2059). 20 µl of competent cells were then aliquoted into each tube and left on ice for 30 min. Cells were heat shocked at 42^0C for 1 min and then returned to ice for 5 min. 100 µl sterile SOC medium (25 bactotryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added to each sample and the tubes were incubated at 37^0C for 1 hour with shaking. The transformation mixes were spread on L-agar plates containing the appropiate antibiotic, plates were inverted and incubated overnight at 37^0C to allow colony formation.

2.2.2.8. Small Scale Preparation of Plasmid DNA (Miniprep)

Small amounts of plasmid DNA were obtained from transformed bacterial colonies to allow the identification of positive transformants. A single bacterial colony was used to inoculate 3 ml of L-Broth (1% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl) containing antibiotic (100 µg/ml ampicillin unless stated otherwise) and was grown overnight at 37^0C with shaking. 1.5 ml of bacterial culture was pelleted by cenrifugation in a microfuge (14 000 rpm, 1 min). Plasmid DNA was

prepared from the colony using the QIAprep Spin plasmid miniprep kit according to the manufacturer's instructions.

2.2.2.9. Large Scale Preparation of Plasmid DNA (Maxiprep)

1 ml of a 3 ml overnight bacterial culture was used to inoculate 500 ml of L-broth containing the appropiate antibiotic in a 2 litre glass conical flask. The culture was incubated overnight at 37^0C with shaking. Bacterial cells were pelleted by centrifugation (8000g, 10 min, 4^0C) using a Sorvall rotor. The bacterial pellet was resuspended in 10 ml TGE (50 mM glucose, 25 mM Tris-Cl (pH 8.0), 10 mM EDTA) containing 5 mg/ml lysozyme and allowed to stand at room temperature for 5 minutes. 20 ml of freshly prepared alkaline SDS (0.2M NaOH, 1% SDS) was added, the contents were mixed and the solution was incubated on ice for 10 minutes. 15 ml of an ice cold 5M potassium acetate solution was added and the contents were mixed by inverting the tube sharply several times. The solution was incubated on ice for 10 min and then centrifuged at 8000g for 20 min at 4^0C. The bacterial debris forms a tight pellet on the bottom of the tube. The supernatant was filtered through nylon gauze and 0.6 volumes of isopropanol added. The solution was mixed and left to stand at room temperature for 15 minutes. Nucleic acid was pelleted by centrifugation as before, the supernatant was removed and the pellet was allowed to air dry. The pellet was resuspended in 9 ml distilled H_2O, 10g of caesium chloride was added and allowed to dissolve. 0.5 ml 10 mg/ml ethidium bromide was added and the solution was transferred to a 10 ml polyallomer ultracentrifuge tube. Samples were balanced carefully with CsCl solution, each tube was sealed and placed in a T1270 Sorvall rotor. A protective cap was placed over the top of each tube and the

plasmid DNA was banded by centrifuging at 55000 rpm for 24 hours at 20^0C in a Sorvall OTD Combi 80 ultracentrifuge.

The tube was carefully removed from the centrifuge rotor and placed securely in a clamp on a retort stand. An 18 gauge needle was first inserted into the top of the tube to act as a air inlet and then a second needle attached to a 2 ml syringe was inserted into the side of the ultracentrifuge tube just below the plasmid band. The plasmid DNA was removed using the syringe and transferred to a clean ultracentrifuge tube. A second round of centrifugation was carried out as before. The plasmid DNA was removed again and transferred to a 5 ml bijoux tube. Eithidium bromide in the solution was extracted by adding an equal volume of water saturated butanol. The solution was mixed causing the ethidium bromide to separate with the organic phase. This was removed and discarded and the process repeated until the lower aqueous phase became colourless. CsCl was removed by transferring the plasmid DNA solution into a dialysis tube (Collodion Bag), placing the tube in a large beaker filled with water and dialysing overnight with stirring at 4^0C. The plasmid DNA was precipitated using sodium acetate and ethanol as described in Section 2.2.2.5.

2.2.2.10. Polymerase Chain Reaction (PCR)

Unless stated otherwise, PCR reactions containing 1 ng of DNA template, 20 pmol of each primer, 200 μM each dNTP, 1 μl (2.5 units) Pfu polymerase in a total volume of 50 μl containing 1x Pfu reaction buffer were set up in 0.5 ml sterile eppendorf tubes. A negative control containing no template and a positive control were always included with each set of PCR reactions. One drop of paraffin oil was added to the top of the each reaction mixture to prevent evaporation. PCR amplification was

carried out using a Perkin Elmer Cetus type 480 DNA thermal cycler. Unless otherwise stated, samples were heated to 94^0C for 1 min (denaturing step), 50^0C for 1 min (annealing step) and 72^0C for 1 min (elongation step). This cycle of denaturing, annealing and elongation was repeated 25 times. 5 μl each PCR reaction was analysed by agarose gel electophoresis and the PCR products were purified using the techniques described in Section 2.2.2.5.

To generate the BPV-4 promoter constructs the corresponding BPV-4 promoter regions were PCR amplified as BglII-HindIII fragments. Primer sequences are shown in Table 2.1. with the restriction sites in bold. The PCR fragments were cloned into the pGL3 luciferase vector.

Primer	Nucleotide sequence
5' PV pro	5' acgt**agatct**ggtcgaaactctcacgct 3'
5' 80bpTATA	5' acgt**agatct**aggtaagtgttgtacctaac 3'
5' 80bp mt1	5' acgt**agatct**aggtccttgttgtacctaac 3'
5' 66bp TATA	5' acgt**agatct**cctaacaactgtttacctag 3'
5' 41bp TATA	5' acgt**agatct**atatcagttgcaaaccattc 3'
5' 19bp TATA	5' acgt**agatct**tcgtaaagaatcgaatgcat 3'
5' 3bp TATA	5' acgt**agatct**gcatatataaggagagcagt 3'
3' PV pro	5' tgcc**aagctt**caccaaatccgcactgctctc 3'

Table 2.1. Primers for PCR amplification of the BPV-4 promoter

Oligonucleotides used to PCR amplify L31 and EIF3 are shown in Table 2.2. L31 was PCR amplified as a EcoRI-XhoI fragment from pTriplEx-L31 and inserted into pGADT7. EIF3 was PCR cloned as a XmaI-XhoI fragment from pGADGH-EIF3 into pGADT7.

Primer	Nucleotide Sequence
5'L31	5' acgt**gaattc**atggctcccgcaaagaaggt 3'
3'TriplExAmp	5' atacgactcactatagggcgaattggcc 3'
5'GAL4AD	5' ctattcgatgaagataccccaccaaaccc 3'
3'GAL4AD	5' agatggtgcacgatgcacag 3'

Table 2.2. Oligonucleotides to PCR amplify L31 and EIF3

2.2.2.11. Splicing by overlap extension PCR

Splicing by overlap extension PCR can be used to join two halves of the same gene with a sequence change incorporated in the middle (site directed mutagenesis) or to join two completely unrelated sequences. The first round of PCR was set up as described (Section 2.2.2.10.). Primers were designed so that the products of the two primary PCR reactions contained 20 bp overlapping complementary ends. By using the primers annealing at the non-overlapping ends a second PCR reaction was carried out using the products of the first reaction as a template. It should be noted that it is necessary to purify the primary PCR products before the second step. This resulted in the primary products priming on each other and extending to yield a hybrid product. The 5'lcr-tkTATA and 3'lcrTATA primers were used in conjunction with the 5'RV3 and 3'GL3 external primers to generate the lcr/tk hybrid promoter. The 5' RV3, 3'GL3, 5'tk-lcrTATA and 3'tkTATA primers were used to generate the tk/lcr hybrid. These promoters were cloned into pGL3 as BglII-HindIII fragments. The I73A mutant E2 transactivation domain was constructed using 5'E2AD and 3'E2AD with the 5'I73A and 3'I73A internal primers and inserted into pGEX2TK as a BamHI-EcoRI fragment. Primer sequences are shown in Table 2.3.

Primer	Nucleotide Sequence
5' RV3	5' ctagcaaaataggctgtccc 3'
3' GL3	5' ctttatgtttttggcgtcttcca 3'
5' lcr-tkTATA	5' tcaatcgtaaagaatcgaatgcatattaaggtgacgcgtg 3'
3' lcrTATA	5' attcgattctttacgattgaatggtttgcaactg 3'
5' tk-lcrTATA	5' cgcggtccgaggtccacttcgcatatataaggagagcagt 3'
3' tkTATA	5' gaagtggacctcggaccgcgccgccccgactgca 3'
5' E2AD	5' tgca**ggatcc**gagactctttgccaacgt 3'
3' E2AD	5' tgca**gaattc**cggcgacggctttggtatgggtcgcggcggg 3'
5' I73A	5' cattacaagcagctgaactgcaactaacgttagaaacaat 3'
3' I73A	5' cagttcagctgcttgtaatgctttattctttgatacagcc 3'

Table 2.3. Primer sequences used for splicing by overlap extension PCR

2.2.2.12. Direct PCR screening of Bacterial Colonies

Transformed bacterial colonies were directly PCR screened to determine if the plasmid DNA contained an insert of the correct size. Commercially available primers complementary to the appropiate region in the plasmid were used for PCR screening. A reaction mix using reagents from a Perkin Elmer GeneAmp PCR kit was set up in bulk. Each 20 µl reaction volume contained 2 µl 10x Taq buffer, 2 µl $MgCl_2$ solution (2.5 mM final conc.), 0.4 µl each dNTP (200 mM), 1 µl each primer (10 pmol), 0.2 µl Taq polymerase and 11.2 µl sterile distilled water. The bulk reaction mix was aliquoted into 0.5 ml sterile eppendorf tubes. The edge of the colony to be screened was picked using a sterile yellow pipette tip. The pipette tip was then swirled in the reaction mix. Each reaction mix was covered with a single drop of paraffin to prevent evaporation. Samples were first heated to 95^0C for 5 min in the PCR machine to help lyse the bacteria. Samples were then heated to 95^0C for 30 sec, 55^0C for 30 sec and 72^0C for 30 sec. After this cycle was repeated 25 times the temperature was held at

72^0C for 10 min. 5 µl of each PCR product was analysed by agarose gel electrophoresis.

2.2.2.13. Generation of Concatenated Oligonucleotides

Concatenated oligonucleotides to be used in cloning were generated as follows. The oligonucleotdes containing potential transcription factor binding motifs were synthesised without the trityl moiety (Table 2.4.). The single stranded oligonucleotides were separated by polyacrylamide gel electrophoresis, excised, eluted overnight in a minimal volume of elution buffer (0.1% SDS, 0.5M NH_4OAc, 10mM MgOAc) and purified by LiCl and EtOH precipitation (Section 2.2.2.5.). Equimolar amounts of complementary oligonucleotides were annealed together by heating to 95^0C and then allowing to cool slowly to room temperature. This generated a double stranded oligonucleotide containing the appropiate binding motif with BamH1/BglII compatible ends. The oligonucleotides were end- labelled with T4 kinase according to the manufacturer's instructions, ligated together and restriction digested with BamH1/BglII. This ensured that only concatamers of binding sites facing in the same orientation were generated, as ligation in the wrong orientation reconstituted a restriction site. The oligonucleotides were separated on a 6% polyacrylamide gel, the bands corresponding to the correct number of binding sites were excised, eluted overnight in minimal volume of elution buffer and purified by phenol chloroform extraction and ethanol precipitation. E2 and LexA binding sites were inserted into the BglII site upstream of the BPV-4 promoter region in the pGL3 luciferase vector. E2 sites were also inserted into the BglII site upstream of the pGL3 tk and hybrid promoter constructs. Binding sites for PRE-1, PRE-2 and PRE-2mt1

were cloned into the BglII site upstream of the SV40 promoter in the pGL3 PRO luciferase vector.

Primer	Nucleotide sequence
E2 upper strand	5' gatccaccgaaaacggtcgggaccgaaaacggta 3'
E2 lower strand	5' gatctaccgttttcggtccgaccgttttcggtg 3'
LexA upper strand	5' gatccttgctatataaaaccagtggttatatgtacagtaa 3'
LexA lower strand	5' gatcttactgtacatataaccactggttttatatacagcag 3'
PRE-1 upper strand	5' gatcccaatcgtaaagaatcgaatgca 3'
PRE-1 lower strand	5' gatcttgcattcgattctttacgattgg 3'
PRE-2 upper strand	5' gatccgctaggtaagtgttgtaccta 3'
PRE-2 lower strand	5' gatctaggtacaacacttacctagcg 3'
PRE-2mt1 upper strand	5' gatccgctaggtccttgttgtaccta 3'
PRE-2mt1 lower strand	5' gatctaggtacaacaaggacctagcg 3'

Table 2.4. Oligonucleotides for the generation of transcription factor binding sites

2.2.2.14. DNA sequencing

The fidelity of all plasmid constructions was verified using an Applied Biosystems 373A automated sequencer. The region to be sequenced was initially PCR amplifying using commercially available primers complementary to the appropiate region of the vector DNA. For example, RV3 and GL3 primers (Promega) were routinely used to sequence constructs in pGL3. PCR reactions containing 0.5 μg plasmid DNA, 3.2 pmol primer and 8 μl Big Dye Terminator Reaction premix were made up to 20 μl with RQ grade H_2O. 250 μl thin walled eppendorf tubes were used for all sequencing PCR reactions. Samples were heated to 95^0C for 30 sec, 50^0C for 30 sec and 60^0C for 4 min. This cycle was repeated 25 times. PCR products were precipitated using sodium acetate and ethanol as shown in Section 2.2.2.5. and dried under vacuum

using a speedivac. Sequencing gel electrophoresis was performed by members of the Beatson Institute technical services staff.

2.2.2.15. End Labelling Double Stranded Oligonucleotides

Single stranded oligonucleotides to be used in band shift assays (Section 2.2.2.16.) were synthesised "trityl off" as described in Section 2.2.2.1. Oligonucleotides were separated by polyacrylamide gel electrophoresis, precipitated, and annealed together as shown in Section 2.2.2.13. The double stranded oligonucleotide was ^{32}P labelled with T4 polynucleotide kinase. Kinase reactions contained 3 μl ds oligonucleotide (1pmol/ μl), 2 μl [γ ^{32}P] ATP, 2 μl 10x T4 kinase buffer and 1 μl T4 kinase in a total volume of 20 μl. Reactions were incubated at 37^0C for 30 min. The ^{32}P labelled oligonucleotide was separated on a 8% polyacrylamide gel. No loading buffer was added to the sample. To monitor the progress of the gel loading dye was added to the first and last wells. When the dye front was approximately 2/3 down the gel the position of the oligonucleotide was determined by exposing the gel, covered with cling film, to X-ray film for approximately 45 sec. The labelled probe was excised from the gel using a sterile scalpel and eluted overnight at 37^0C in distilled water. The specific activity of a ^{32}P labelled probe was determined using a scintillation counter.

2.2.2.16. Band Shift Assays

Nuclear extracts were prepared from cells in culture as described in Section 2.1.7. Band shift assays were carried out as follows. 10-15 μg nuclear extract (or 5 μl *in vitro* translated HPV-16 E2) was added to 3 μg poly(dI-dC) in a final volume of 30

μl binding buffer. For E2 band shifts the binding buffer was 10 mM Tris-Cl (pH 7.0), 100 mM KCl, 1 mM $MgCl_2$, 1mM DTT, 1mM EDTA and 5% glycerol. For PRE-2 band shifts the binding buffer contained 20 mM HEPES pH 7.9, 4% ficoll, 2 mM $MgCl_2$, 40 mM KCl, 0.1 mM EGTA and 0.5 mM DTT. After 15 min pre-incubation at room temperature, approximately 5 fmol of ^{32}P labelled probe was added. The binding reaction was incubated for a further 15 min at room temperature and then electrophoresed on a 6% polyacrylamide gel (100 volts, room temperature). All polyarylamide gels used in band shift assays were pre-run for 1 hr prior to use to equilibrate the gel. Competition band shifts were performed under the same conditions except that a 100- or 500-fold excess non-radioactive self oligonucleotide or AP-1 oligonucleotide was added were indicated. After electrophoresis, the gel was transferred onto Whatman 3MM filter paper and dried using a Biorad 583 gel drier (80^0C for 2 hours). The dried gel was placed in a lead, light tight cassette with intensifying screens and exposed to X-ray film at -70^0C overnight.

2.2.2.17. Phage Based Expression Cloning

E. Coli XL1-Blue cells were infected with a λTiplexEx HeLa cDNA library and plated out according to the manufacturer's instructions. The library was initially titered to give an estimate of the number of independent clones. XL1-Blue cells were streaked out onto an LB-agar plate containing 10 mM $MgSO_4$ and 15 μg/ml tetracycline (LB/$MgSO_4$/tet). An isolated XL1-Blue colony was used to inoculate 50 ml of L-broth containing 10 mM $MgSO_4$ and 0.2% maltose. Mg^{2+} and maltose are required for optimal adsorption of phage onto bacteria. The integrity of the λ phage particles requires the Mg^{2+} and maltose induces expression of the phage receptor. The culture was incubated overnight at 37^0C with shaking. The cells were pelleted by

centrifugation (3 000 rpm, 5 min), the supernatant poured off and the pellet resuspended in 25 ml (1/2 volume) 10 mM $MgSO_4$. Serial dilutions of the phage library was made in 1x lambda dilution buffer (0.1 M NaCl, 10 mM $MgSO_4.7H_20$, 35 mM Tris-HCl pH 7.5). 10 µl λ lysate was added to 90 µl dilution buffer. 10 µl of this dilution was diluted 1 in 10 again. This process was repeated two more times. 10 µl each dilution was added to 400 µl XL1-Blue overnight culture and allowed to adsorb at 37^0C for 15 min. 2 ml melted LB/$MgSO_4$ top agar (100 ml L-broth, 1 ml 1M $MgSO_4$, 0.72 g agar) was added, the samples were mixed quickly by inverting and immediately poured onto 90 mm LB/$MgSO_4$/tet plates pre-warmed to 37^0C. The plates were swirled to allow an even distribution of the top agar, cooled at room temperature for 10 min to allow the top agar to set, inverted and incubated overnight at 37^0C to allow plaques to develop. The following morning plaques were counted and the phage titer calculated (pfu/ml: plaque forming units/ml).

$1x10^6$ independent clones were initially screened. As λTriplEx expresses each cDNA in all three open reading frames 2-3 times this amount of phage plaques were plated out. 50 150 mm plates at $5x10^4$ plaques/plate were set up. This was carried out as described above except 500 µl XL1-Blue and 7 ml top agar was used for the larger sized plate. Once the plaques had developed each plate was overlayed with a nitrocellulose filter that had been pre-soaked in 10 mM IPTG for 15 min and allowed to air dry. The plates were then incubated for 6-8 hours at 37^0C to transfer plaques to the nitrocellulose and induce expression of the library encoded proteins. Following this, each plate was chilled at 4^0C for at least 30 min, the orientation of the filter to plate was marked by piercing a 20-G needle dipped in India ink through the filter into the agar. The back of the agar plate was also marked. The filters were then removed

from the plates and washed 3x 10 min in TBS-T (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% Triton X-100). Filters were blocked for at least 2 hours at 4^0C in HB buffer plus 5% dried milk (Section 2.2.3.8.) and probed with 150 000 cpm/ml ^{32}P labelled GST-E2 (see Sections 2.2.3.6-7.) in 30 ml binding buffer (Section 2.2.3.8.) overnight at 4^0C. Labelled probe was removed, the filters were washed 4 times in binding buffer, dried and exposed for autoradiography at -70^0C (2 hours and overnight). Filters were orientated with the plates, agarose plugs of positive plaques were picked using a sterile pasteur pipette and stored in 1 ml lambda dilution buffer containing a drop a chloroform (4^0C). Positive plaques were purified through two more rounds of screening. The secondary screen contained approximately 2000 pfu/150 mm plate whereas the tertiary screen contained approximately 400 pfu/150 mm plate.

Plasmid DNA was rescued from the positive plaques by transduction into *E. Coli* BM25.8 cells. These cells express cre-recombinase when grown at 31^0C excising the plasmid automatically. BM25.8 cells were streaked onto an LB-agar plate containing 50 μg/ml kanamycin and 150 μg/ml chloroamphenicol (LB/kan/cam). An isolated colony was used to innoculate 10 ml LB/$MgSO_4$ broth and incubated overnight at 31^0C with shaking. 100 μl 1M $MgCl_2$ (10 mM final conc) was then added to the overnight culture. Following this, 200 μl of the overnight culture was mixed with 150 μl of the eluted positive plaque and incubated at 31^0C for 30 min. 400 μl L-broth was added and the samples incubated for 1 hour at 31^0C with shaking. 100 μl of each infected cell suspension was spread out on an LB/carbenicillin plate and grown overnight at 31^0C to obtain isolated colonies. Colonies were directly PCR screened using the 5'TriplEx and 3'TriplEx primers (Clontech) to determine the size of the

insert (Section 2.2.2.12.). Positive cDNAs were sequenced (Section 2.2.2.14.) and compared with those in databases using basic logic search alignment (BLAST).

2.2.3. Protein Manipulation

2.2.3.1. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

10% SDS-polyacrylamide gels were routinely used to resolve protein samples. The resolving gel was made by adding the appropiate volume of 30% (w/v) acrylamide: 0.8% (w/v) bis-acrylamide to 1x resolving gel buffer (4xRGB: 1.5 M Tris-HCl (pH 8.8), 0.4% SDS). 0.08% TEMED and 0.1% APS were then added to catalyse polymerisation. This solution was poured between 2 glass plates as described in Section 2.2.3. The top of the gel was covered with isopropanol and left to polymerise. Once the gel had set, the isopropanol was poured off, washed with water and blotted dry. A 5% stacking gel was poured on top of the resolving gel, a comb was inserted and the gel was left to polymerise. Stacking gels consist of the appropiate volume of 30% (w/v) acrylamide: 0.8% (w/v) bis-acrylamide in 1x stacking gel buffer (4xSGB: 0.5 M Tris-HCl (pH 6.8), 0.4% SDS) with 0.08% TEMED and 0.1 % APS to catalyse polymerisation.

Protein samples were mixed with an equal volume of 2x SDS gel loading buffer (100mM Tris-HCl (pH 6.8), 2% (v/v) β-mercaptoethanol, 4% SDS, 0.2% bromophenol blue, 20% glycerol), boiled for 5 min and loaded into the individual sample wells. 5 μl Rainbow^{TM} protein molecular weight marker (14.3-200 kD) was added to the first and last well in the gel. Protein gels were run at approximately 40 mA constant current in 1x SDS-PAGE running buffer (25mM Tris, 250 mM glycine,

0.1% (w/v) SDS). After electrophoresis, proteins were either transferred to nitrocellulose (Section 2.2.3.2.) or stained for 20 min using a solution containing 0.25% Coomassie Brilliant Blue dissolved in 45% (v/v) methanol, 45% (v/v) H_2O and 10% (v/v) glasial acetic acid. To visualise the protein bands the gel was destained in the same solution minus the Coomassie Blue.

2.2.3.2. Semi-Dry Electrophoretic Transfer

Separated protein samples were transferred to nitrocellulose by semi-dry electrophoretic transfer. After SDS-PAGE, one of the glass plates was removed and the stacking gel was cut away. Six sheets of Whatmann 3MM paper and a piece of nitrocellulose membrane (Hybond C^{extra}) were cut to the size of the gel. The nitrocellulose membrane was soaked in distilled water and the blotting paper was soaked in transfer buffer (48 mM Tris base, 39 mM glycine, 0.037% (v/v) SDS and 20% methanol). Three layers of absorbent paper were placed on the bottom plate (anode) of the blotting apparatus, followed by the nitrocellulose membrane, the gel and another three layers of absorbent paper. Any air bubbles were removed and the transfer was performed at 180 mA for 1 hour. The fidelity of transfer was checked by staining the nitrocellulose with Ponceau S solution.

2.2.3.3. Western Blot Analysis of HPV-16 E2 Expression Levels

PalK and PalF cells were transiently transfected with increasing amounts of pCMV HPV-16 E2 expression vector. Equal amounts of whole cell extract were separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was incubated in blocking buffer (5% (w/v) dried milk in PBS-T (0.1% (v/v)

Tween in PBS)) overnight at 4^0C with shaking. The nitrocellulose filter was washed 3x 5 min in PBS-T and then incubated in 10ml of a 1:50 dilution of the TVG261 monoclonal antibody in blocking buffer (2 hours, 4^0C with shaking). The primary antibody solution was removed and the filter was washed 4x 5 min in PBS-T. The membrane was then incubated for 1 hour at room temperature in 20ml of a 1:5000 dilution of anti-mouse IgG HRP linked whole antibody in blocking buffer. The secondary antibody solution was removed and the membrane was washed 3x 5 min in PBS-T. Excess surface liquid was removed from the membrane and the western blot developed by enhanced chemiluminescence (ECL).

2.2.3.4. UV Crosslinking

A derivative of the PRE-2 oligonucleotide was synthesised with bromodeoxyuridine (BrdU) in place of thymine to enhance UV induced protein/DNA crosslinking. The single stranded oligonucleotides were purified, annealed and ^{32}P labelled with T4 kinase as described in Section 2.2.2.15. Radiolabelled ds BrdU-PRE-2 was used to probe PalK and PalF nuclear extracts in a band shift assay (Section 2.2.2.16.). After electrophoresis, the gel was UV irradiated (304 nm) for 45 min at 4^0C to covalently crosslink the protein to the DNA. This was carried out by removing one of the gel plates, covering the gel in cling film, placing the gel directly on a transilluminator and covering with a bag of ice. The gel was visualised using a phoshor imager and the bands corresponding to the retarded complexes excised using a scalpel. The gel pieces were soaked in SDS-PAGE sample buffer for 15 min at 37^0C and loaded directly onto a 10% SDS gel. Following electrophoretic separation, the gel was transferred onto Whatmann 3MM filter paper, dried, exposed to film (-70^0C) and visualised by autoradiography.

2.2.3.5. BCA/$CuSO_4$ Protein Assay

The protein concentration of a sample was determined using the BCA/$CuSO_4$ assay. Protein reduces alkaline Cu(II) to Cu(I) in a concentration dependent manner. Bicinchoninic acid (BCA) forms a purple complex with Cu(I) with an absorbance maximum at 562 nm. 10 μl of each protein sample (or dilution) was added to a 96 well plate. 200 μl of developing solution (5ml BCA and 100 μl $CuSO_4$) was added to each well and the plate was incubated at 37^0C for 30-60 min. The absorbance of each sample was measured at 590 nm using a Dynatech MR7000 automatic plate reader. The protein concentration was obtained using a standard curve generated from the absorbance measurements of a series of BSA solutions of known concentration.

2.2.3.6. Expression and Purification of GST Proteins

Individual bacterial colonies transformed with GST fusion protein expression vectors were used to innoculate 10 ml L-broth plus 100 μg/ml ampicillin (LB-amp) and grown overnight at 37^0C with shaking. 1 ml of overnight culture was used to innoculate 50 ml LB-amp containing 0.5 M sorbitol and 2.5 mM betaine. Cultures were grown at 37^0C with shaking until an absorbance measurement at 600 nm of 0.6-0.8 was obtained. Expression was induced by the addition of IPTG (final conc. 0.3 mM) and the culture was incubated overnight with shaking at room temperature. Bacteria were pelleted by centrifugation at 2500 g for 10 min. The pellet was resuspended in 5 ml (1/10 volume) of NETN (20 mM Tis-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40 (NP40)) containing protease inhibitors (2 mM PMSF and 5 μg/ml leupeptin). Samples were sonicated (3x 30 sec), centrifuged at 12000 g for 10 min using a Sorvall rotor and the supernatant was removed. Fusion

proteins were purified on glutathione-Sepharose beads by incubating 1 ml supernatant (crude extract) with 50 µl beads for 30 min at room temperature with rotation. The beads were pelleted in a microfuge (14000 rpm, 10 sec) and washed 3x 0.5 ml NETN. The beads were resuspended in 30 µl NETN and purified proteins were analysed by 10% SDS-PAGE (Section 2.2.3.1.) before subsequent manipulations.

2.2.3.7. ^{32}P labelling of GST Fusion Protein Probes

Recombinant plasmids allowing the expression of chimaeric proteins containing the E2 amino terminal wild type and mutant sequences fused to the GST domain and a recognition site for protein kinase A were constructed using standard molecular biology techniques (Section 2.2.2.). Proteins were bacterially expressied and purified as shown above (Section 2.2.3.6.). Following purification the beads were washed with 30 µl HMK buffer (20 mM Tris (pH 7.5), 100 mM NaCl, 12 mM $MgCl_2$). Proteins were then ^{32}P labelled on the beads in 30 µl HMK buffer containing 1 mM DTT and 10 units of the catalytic subunit of protein kinase A (30 min, 37^{0}C). The reaction was stopped by adding 1 ml stop buffer (10 mM sodium phosphate (pH 8.0), 10 mM sodium pyrophosphate, 10 mM EDTA and 10 mg/ml BSA) and the beads were washed 3x in NETN. Labelled probes were eluted from the beads using 50 µl elution buffer (20 mM glutathione (reduced), Tris pH8.0, 120 mM NaCl). Elution was repeated and incorporated radioactivity was quantified using a scintillation counter. Aliquots of labelled protein were analysed by 10% SDS-PAGE (Section 2.2.3.1.), the gel was transferred onto filter paper, dried, exposed to film for 2 min and visualised by autoradiography (Section 2.2.2.16.).

2.2.3.8. Far Western Blot Analysis

PalK, PalF and HeLa whole cell extracts were prepared from cells in culture as described in Section 2.2.1.8. 60 μg each sample was separated by SDS-PAGE and transferred to nitrocellulose (Section 2.2.3.1. and 2.2.3.2.). Nitrocellulose filters were denatured/renatured in 6 M to 0.187 M guanidine hydrochloride as follows: the membrane was submerged in 250 ml 6 M guanidine hyrochloride in HB buffer (25 mM HEPES pH 7.7, 25 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT) for 10 min at 4^0C with shaking. The solution was removed, replaced with the same buffer and the incubation repeated. The solution was removed again, 125 ml was decanted into a measuring cylinder and an equal volume of HB buffer (without guanidine hydrochloride) added. The membrane was incubated in 250 ml of the 1:2 dilution for 5 min at 4^0C with shaking. This dilution step was repeated four times. After the final dilution the membrane was washed 2x 5 min in HB buffer at 4^0C. Membranes were then blocked in HB buffer containing 5% dried milk and 0.05% NP-40 followed by 1% milk plus 0.05% NP-40. Each incubation step was carried out for 2 hours at 4^0C with shaking. The membranes were then incubated with 200 000 cpm/ml of ^{32}P labelled probes (Section 2.2.3.7.). This was performed overnight in 10 ml binding buffer (20 mM HEPES pH 7.7, 75 mM KCl, 0.1 mM EDTA, 2.5 mM $MgCl_2$, 0.05% NP-40, 1% dried milk, 1mM DTT) at 4^0C with shaking. Membranes were washed 3x 10 min in binding buffer alone, dried and exposed for autoradiography at -70^0C (1-3 days).

2.2.3.9. *In vitro* Transcription-Translation and GST-Pull Down Assays

HPV-16 E2 was subcloned from pCMV-HPV-16 E2 into the BamHI site of pBluescript SKII under control of the T3 promoter using standard molecular biology

techniques (Section 2.2.2.). HPV-16 E2 was *in vitro* transcribed-translated using the TNT T7/T3 Coupled Reticulate Lysate kit (Promega) as instructed by the manufacturer. The efficiency of transcription-translation was checked using a luciferase control plasmid and assaying for luciferase activity. *In vitro* transcribed-translated HPV-16 E2 was used for band shift assays (Section 2.2.2.16). *In vitro* transcription-translation of pGADT7 constructs to produce ^{35}S labelled proteins was carried out using the TNT T7 Quick Coupled Transcription kit (Promega) according to the manufacturer's instructions. 1 µl each reaction was analysed by 10% SDS-PAGE and proteins were fixed in 2.5% methanol and 7.5% glacial acetic acid for 30 min with shaking. The gel was transferred onto filter paper, dried and exposed for autoradiography at -70^0C overnight.

GST pull down assays were performed as follows: pGEX and pGEX-E2AD (cloned by Iain Morgan) were used to transform competent bacterial cells. GST fusion proteins were expressed and purified as described in Section 2.2.3.6. The proteins immobilised on beads were pre-washed three times in pull down buffer (PDB: 50 mM Tris pH 7.9, 100mM NaCl, 1mM DTT, 0.5 mM EDTA, 0.5 mM EGTA, 0.5% NP-40, 1 mM PMSF). The NaCl concentration in PDB can be changed to assess the specificity of binding. 7.5 µl ^{35}S labelled *in vitro* translated protein was then incubated with approximately 1 µg immobilised fusion protein (approximately 10 µl protein on beads) in a total volume of 200 µl fresh PDB for 30 min at 4^0C with rotation. The beads were pelleted in a microfuge (14 000 rpm, 10 sec) and washed four times in PDB. Bound proteins were separated by 10% SDS-PAGE, fixed, the gel was transferred onto filter paper, dried and exposed for autoradiography at -70^0C overnight.

Chapter 3 – Results

3.1. Transcriptional analysis of the BPV-4 LCR

All papillomaviruses have a non-coding region of 500-1000 bp called the long control region (LCR). The LCR is the transcriptional control unit of the virus and contains a number of binding sites for transcription factors including virally encoded E2 (for a review see (Ishiji, 2000)). The LCR of mucosal epitheliotropic papillomaviruses, for example, HPV-16, -18 and BPV-4, have a similar organisation: a promoter region, an enhancer region and a highly conserved distribution of E2 DNA binding sites (Fig. 3.1.1a). One of the restrictions of these viruses to the epithelial cell type is at the transcriptional level. The enhancer of these papillomaviruses is epithelial specific as it fails to activate transcription from heterologous promoters in non-epithelial cell types (Cripe et al., 1987; Gloss et al., 1987). The BPV-4, HPV-16 and -18 enhancers are of a similar size and position. There are two main regions contributing to the BPV-4 LCR enhancer activity (Morgan et al., 1999). One of these sites, Site 2, is 100% conserved in the HPV-16 LCR. There are four E2 DNA binding sites located in the HPV-16, -18 and BPV-4 LCRs. Immediately upstream of the TATA box are two E2 DNA binding sites separated from each other and the TATA box by 3 or 4 base pairs. Two additional sites flank the epithelial specific enhancer: one beside the E1 DNA binding site involved in the regulation of viral DNA replication and one a further 300-400 bp upstream. This conservation of the organisation of E2 DNA binding sites between the HPV-16, -18 and BPV-4 LCRs strongly suggests that the mechanism E2 uses to regulate transcription from mucosal epitheliotropic LCRs is conserved. This

organisation of E2 DNA binding sites is lost in the cutaneous HPV and subgroup A BPV LCRs.

Studies on E2 transcriptional regulation of the human mucosal epitheliotropic papillomaviruses have been hindered by poor access to the natural target cell type and by the observation that some of the HPV promoters, including HPV-16, are repressed in immortalised epithelial cells. The BPV-4 LCR and primary bovine palate keratinocytes (PalK) and palate fibroblasts (PalF) present a model system to study the mechanism of E2 transcriptional regulation of mucosal epitheliotropic papillomaviruses and the cell type specificity of this regulation. Comparisons are made between the results in PalK cells, the natural target cell type for transformation by BPV-4, with PalF cells from the same source. Several E2 molecules, including BPV-1 E2, BPV-4 E2 and HPV-16 E2, activate transcription efficiently from the BPV-4 LCR in PalK cells but do so only poorly in PalF (Morgan et al., 1998). Low to intermediate levels of E2 upregulate transcription in keratinocytes, while at high levels transcription is down-regulated. Mutation of E2 binding site 1 (BS1), which is 3 bp upstream from the TATA box, abrogates down-regulation of transcription by high levels of E2 (Jackson and Campo, 1995; Morgan et al., 1998). The inability of E2 to upregulate transcription in fibroblasts is promoter specific as E2 can activate transcription from heterologous promoters in a variety of cell types. A chimaeric molecule that has the VP16 transactivation domain fused to the BPV-1 DNA binding domain upregulates transcription from the BPV-4 LCR in a cell type independent manner suggesting that the epithelial specific transcriptional regulation of the BPV-4 LCR is mediated by the E2 transactivation domain (Morgan et al., 1998). However, the BPV-4 LCR is approximately 40-fold more active in PalK than in PalF cells in

the absence of E2 due to the presence of the epithelial specific enhancer (Morgan et al., 1999).

3.1.1. Generation of E2 responsive BPV-4 promoter constructs

Epithelial specific transcriptional regulation of the BPV-4 LCR promoter by E2 may be mediated at three levels: the E2 protein might function more efficiently in epithelial cells, E2 may interact with the epithelial specific enhancer to regulate transcription or the LCR promoter may show an enhanced epithelial response to transcriptional activators. To determine which of these possibilities are responsible for the enhanced epithelial response of the BPV-4 LCR to E2, the epithelial specific enhancer was removed and a series of concatamers of increasing numbers of E2 DNA binding sites were cloned upstream from the BPV-4 promoter in the position of E2 BS3 (Fig. 3.1.1b). Reporter constructs were generated by PCR amplifying the BPV-4 LCR promoter from nucleotide 184-310 as a BglII-HindIII fragment from pLCR, pLCR-E2(3)mt1 and pLCR-E2(d/3). These plasmids contain the BPV-4 LCR from nucleotides 6710 to 331 cloned into the BamHI site of p0luc. pLCR contains the wild type BPV-4 LCR sequence while pLCR-E2(3)mt1 and pLCR-E2(d/3) contain the TATA box proximal E2 DNA binding site mutations preventing E2 binding (Jackson and Campo, 1995). The BPV-4 promoter fragments were then cloned into the pGL3 luciferase vector to generate a series of plasmids with the following nomenclature: the PV series represents the wild type LCR promoter sequence, the PV1 series has the TATA proximal E2 DNA binding site mutated whereas the PV2 series has both the TATA proximal and the adjacent E2 site mutated (Fig. 3.1.1b). Increasing numbers of E2 DNA binding sites were then inserted upstream from these promoters. Oligonuleotides were designed which when

annealed together generate a pair of E2 sites separated by 4 base pairs with BamHI/BglII compatible ends. After end-labelling with T4 kinase and ligation, the double stranded oligonucleotides were restriction digested with BamHI/BglII. This ensured that only concatamers of E2 binding sites facing in the same orientation were obtained as ligation in the wrong orientation reconstituted a restriction site. After separation on a 6% polyacrylamide gel, the bands corresponding to 2, 4, 6 and 8 E2 sites were excised, purified and cloned into the BglII site immediately upstream of the BPV-4 promoter region. The E2 site sequence corresponding to E2 BS4 in the BPV-4 LCR was used to generate these constructs. BPV-1 E2 has previously been shown to bind this site with high affinity binding (Sanders and Maitland, 1994). Also, BS4 mediates transactivation of the BPV-4 LCR by BPV-1 E2 and forms a more stable E2-oligonucleotide complex *in vitro* than the TATA box proximal E2 DNA binding sites (Jackson and Campo, 1995). Consistent with this, Fig. 3.1.2. demonstrates that *in vitro* translated HPV-16 E2 specifically binds the synthetic double stranded E2 site in a band shift assay. Binding to the –ACCGAAAACGGT- sequence was competed by 100-fold excess non-radioactive self oligonucleotide but not by the related AP-1 oligonucleotide. No binding was detected when *in vitro* translated luciferase protein was used as a non-specific control (Fig. 3.1.2).

3.1.2. E2 upregulates transcription from the BPV-4 promoter preferentially in epithelial cells

PalK and PalF cells were co-transfected with the E2 responsive promoter constructs and HPV-16 E2, which functions in an identical manner to BPV-4 E2 on the BPV-4 LCR, to study the mechanism of E2 mediated transcriptional regulation of the BPV-4 promoter. In contrast to the full length LCR, the BPV-4 promoter constructs have

similar background transcriptional activity in both PalK and PalF cells. Also, in the absence of E2 protein the E2 sites do not enhance transcription and without the BPV-4 promoter sequences E2 does not activate transcription indicating there is no cryptic promoter in the pGL3 reporter plasmid that is responsive to E2 (data not shown). In PalK cells, HPV-16 E2 upregulates transcription from the BPV-4 promoter in an additive manner increasing as the number of E2 binding sites increases (Fig 3.1.3). A maximum of 20-fold transcriptional activation (relative to the luciferase activity in the absence of E2) is observed with the PV1 8E2 and PV2 8E2 constructs. PV1 6E2 and PV2 6E2, which both have the TATA proximal E2 site (BS1) mutated, have a 2- to 3-fold elevated response over the PV 6E2 construct (Fig. 3.1.3). This is in agreement with previous studies showing that BS1 is responsible for mediating down-regulation of transcription by elevated levels of E2.

To determine if the epithelial specific response of the full length LCR is retained by the promoter region, the ability of increasing concentrations of HPV-16 E2 to activate transcription from the PV 6E2 construct was assayed in both PalK and PalF cells. In keratinocytes transcription is upregulated a maximum of 5-fold at a 1:1 ratio of E2 expression vector to reporter plasmid, while over a range of increasing E2 concentrations transcription is not activated more than 2-fold in fibroblasts (Fig. 3.1.4a). To determine the effect of the TATA box proximal E2 binding site mutations on epithelial specific transcriptional regulation, the transcriptional response to HPV-16 E2 of the PV1 6E2 and PV2 6E2 constructs were assayed in both PalK and PalF cells. Low levels of E2 are sufficient to upregulate transcription from the PV1 and PV2 promoters in PalK cells. A 1:1 ratio of E2 expression vector to reporter plasmid activates transcription 12-fold from the PV1 promoter (Fig. 3.1.4b) and 10-fold from

the PV2 promoter (Fig. 3.1.4c). Over a range of E2 concentrations the PV1 (Fig. 3.1.4b) and PV2 (Fig. 3.1.4c) promoters are not upregulated more than 2-fold in PalF cells. However, previous studies have shown that E2 activates transcription in fibroblasts when the BPV-4 LCR is cloned upstream of a heterologous promoter (Jackson and Campo, 1991; Morgan et al., 1998). Western blot analysis was therefore carried out to determine whether E2 fails to activate transcription from the BPV-4 promoter constructs in PalF cells because of a lack of expression. PalK and PalF cells were transiently transfected with increasing amounts of pCMV HPV-16 E2 expression vector. Equal amounts of whole cell extract were separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane. The level of E2 expression was monitored by probing the membranes with a monoclonal antibody directed against an epitope in the amino terminal region of HPV-16 E2. Fig. 3.1.5 shows that failure of E2 to function in fibroblasts is not due to lack of expression as E2 is being expressed at similar levels as a doublet of approximately 42 kDa in both PalK and PalF cells.

These results demonstrate that the BPV-4 promoter mimics the response of the full length LCR to transcriptional up-regulation by E2, that is, the response is much enhanced in PalK cells when compared with PalF. The results confirm that the TATA box proximal E2 DNA binding sites BS1 and BS2 mediate down-regulation of transcription by E2. Mutation of these sites to prevent E2 binding does not affect the epithelial specific response of the BPV-4 promoter to E2. The results also show that the epithelial specific enhancer element of the BPV-4 LCR is not required for the enhanced activity of E2 in PalK cells.

3.1.3. The BPV-4 promoter shows an enhanced epithelial response to transcriptional activators

It has been proposed that the epithelial specific transcriptional regulation of the BPV-4 LCR by E2 might be mediated by the E2 transactivation domain (Morgan et al., 1998). To test this hypothesis the ability of a VP16-E2 chimaera, the VP16 transactivation domain fused to the BPV-1 E2 DNA binding domain, and of wild type BPV-1 E2 to activate transcription from the PV2 6E2 construct was assayed. VP16 is a strong acidic transactivator from the Herpes Simplex Virus (HSV). The PV2 6E2 construct was used in these experiments as the PV2 promoter contains the TATA box proximal E2 DNA binding site mutations eliminating down-regulation of transcription at elevated levels of E2 as a complicating factor. Fig. 3.1.6a. shows that BPV-1 E2 activates transcription 18-fold in PalK cells but no more than 2-fold in PalF cells. This may not represent an accurate value of the background activity in PalF cells due to the sensitivity of the BioOrbit luminometer used in this experiment (see Fig. 3.1.13 measured using a Tropix TR717 luminometer). However, this result demonstrates that the preferential activation in epithelial cells is retained with BPV-1 E2. VP16-E2 also activates transcription from PV2 6E2 better in PalK cells than in PalF (Fig. 3.1.6b). At low levels VP16-E2 activates transcription 180-fold in fibroblasts and 900-fold in keratinocytes. At high levels of VP16-E2 transcription is down-regulated in both cell types. This may be due to squelching where excess VP16-E2 proteins not bound to DNA sequester factors required for transcriptional activation. The PV2 promoter has a 8- to 9-fold enhanced epithelial response to activation by BPV-1 E2 and a 5-fold enhanced response to activation by VP16-E2 in epithelial cells, demonstrating that the E2 transactivation domain is not solely

responsible for the enhanced function of E2 on the BPV-4 promoter in epithelial cells.

Two components of the cellular basal transcription initiation complex, TBP and TFIIB, have been shown to interact directly with the carboxy terminal DNA binding domain of E2 (Rank and Lambert, 1995). However, the functional significance of these interactions are not known. To analyse the contribution of the DNA binding domain of E2 to cell type specific transcriptional activation four copies of the LexA site from the colE1 promoter were cloned upstream of the PV2 promoter which contains both TATA box proximal E2 binding sites mutated (Fig. 3.1.7a). Concatamers of LexA binding sites were generated in the same manner as the E2 DNA binding sites. The ability of a chimaeric molecule which has the VP16 transactivation domain fused to the bacterial LexA DNA binding domain to activate transcription from this construct was assayed in PalK and PalF cells. At low to intermediate levels VP16-LexA activates transcription to a similar degree in both cell types. However, the BPV-4 promoter shows a 5-fold enhanced epithelial response to activation by high levels of VP16-LexA. A 1:1 ratio of expression vector to reporter plasmid upregulates transcription approximately 200-fold in fibroblasts and 1000-fold in keratinocytes (Fig. 3.1.7b). This difference is similar to that observed with VP16-E2. These results suggest that the C-terminal region of E2 is not involved in mediating epithelial specific transcriptional regulation of the LCR promoter but functions to localise active E2 dimers to the target promoter.

To address the possibility that E2 binding to its target sequences was being blocked by cellular factors in fibroblasts and that the enhanced epithelial response to

transcriptional activators was a specific property of the BPV-4 promoter an E2 responsive tk promoter construct was generated (Fig. 3.1.8a). The HSV tk promoter from nucleotide 75-199 was PCR amplified as a BglII-HindIII fragment. This tk promoter fragment containing the tk TATA box, the initiator element and a binding site for the Sp1 transcription factor was cloned into pGL3 6E2. pGL3 6E2 contains six E2 DNA binding sites inserted into the BglII site of the pGL3 luciferase vector. The tk promoter has similar background transcriptional activity in both PalK and PalF cells in the absence of E2 (data not shown) and is approximately the same length as the PV promoter so that the E2 molecules are operating from a similar distance. The ability of VP16-E2 to upregulate transcription from the tk 6E2 construct was assayed in PalK and PalF cells. It is clear that the VP16-E2 chimaera is being expressed, binding to its target sites and activating transcription in both PalK and PalF cells (Fig. 3.1.8b). At low levels VP16-E2 activates transcription from the tk promoter preferentially in PalF cells. At intermediate levels VP16-E2 activates transcription to a similar degree in both cell types while at high levels of VP16-E2 transcriptional activity is down-regulated probably due to a squelching mechanism. To further investigate the contribution of the E2 transactivation domain to epithelial specificity the ability of HPV-16 E2 to upregulate transcription from the tk 6E2 construct was assayed in PalK and PalF cells. E2 upregulates transcription from the tk promoter in a cell type independent manner (Fig. 3.1.9). A maximum of approximately 90-fold activation is observed with a 0.1:1 ratio of expression vector to reporter plasmid in both cell types. These results demonstrate that the tk promoter does not show an epithelial preference to activation by either VP16-E2 or HPV-16 E2.

Taken together, these results show that a level of epithelial specificity resides in the BPV-4 promoter. The BPV-4 promoter has an enhanced epithelial response to activation, not only by HPV-16 and BPV-1 E2, but also by VP16-E2 and VP16-LexA. The enhanced epithelial response is a promoter specific effect as the tk promoter shows no such epithelial preference to activation by VP16-E2 and E2 itself. The E2 transactivation domain does not contribute towards an enhanced epithelial response of the tk promoter although it still remains a possibility that the E2 transactivation domain contributes towards epithelial specific transcriptional regulation of the BPV-4 promoter.

3.1.4. The ability of TBP overexpression to enhance E2 mediated transcriptional activation depends on promoter structure

Activators are believed to function, at least in part, through contacting components of the cellular transcription machinery and affecting the formation and/or stability of the preinitiation complex. The interaction between TBP and the TATA box, the first step in preinitiation complex assembly, is often a rate limiting step for transcription initiation (Chatterjee and Struhl, 1995). Many transcriptional activators, including VP16 and E1a bind TBP *in vitro* (Boyer and Berk, 1993; Ingles et al., 1991). Mutations that decrease binding to TBP decrease transcriptional activation, demonstrating functional significance. The C-terminal E2 DNA binding domain has also been shown to interact with TBP (Rank and Lambert, 1995). However, the ability of activators to stimulate TBP binding *in vivo* is a tightly controlled, cooperative process involving multiple transcription factors (Li et al., 1999). Also, the arrangement of recognition elements in a core promoter can determine the differential response to upstream activators (Das et al., 1995). To determine whether

TBP overexpression can enhance E2 mediated transactivation of the BPV-4 promoter increasing concentrations of TBP were cotransfected with a 0.01:1 ratio of E2 expression vector to PV2 6E2 reporter construct. This ratio is sub-optimal for E2 mediated activation of the BPV-4 LCR. Fig. 3.1.10a shows that TBP overexpression enhances E2 mediated transcriptional activation of the BPV-4 promoter in a dose dependent manner in both PalK and PalF cells. Low levels of E2 (0.01 μg) upregulate transcription from the PV2 promoter 6-fold in PalK and 4-fold in PalF cells. It should be noted that from this point luciferase activity was monitored on a Tropix TR717 luminometer. This is more sensitive than the previous BioOrbit model accounting for the higher level of E2 activation in PalF cells. High ratios of TBP to E2 (1:0.01) increase E2 activity approximately 20-fold in PalK and 12-fold in PalF cells. However, TBP overexpression preferentially upregulates basal BPV-4 promoter activity in keratinocytes (Fig. 3.1.10b). High levels of TBP upregulate transcription from the PV2 6E2 construct 17-fold in PalK and 4-fold in PalF cells. This result suggests that TBP is recruited more efficiently to the BPV-4 promoter in the absence of E2 in PalK cells than in PalF. The tk promoter has a different response to TBP overexpression (Fig. 3.1.11). Low levels of E2 (0.01μg) upregulate transcription preferentially in fibroblasts. A 0.01:1 ratio E2 expression vector to tk 6E2 reporter construct activates transcription 10-fold in PalK and 50-fold in PalF cells. TBP overexpression potentiates E2 mediated activation of the tk promoter in a dose dependent manner in PalK cells. Cotransfection of 0.01:1 ratio of E2 to TBP enhances E2 activity approximately 6-fold in keratinocytes. Low levels of TBP increase the ability of E2 to activate transcription from the tk promoter in PalF cells while high levels of TBP downregulate E2 mediated transcription (Fig. 3.1.11a). However, TBP overexpression has a moderate effect on basal tk promoter activity

(Fig. 3.1.11b). High levels of TBP upregulate basal tk promoter activity 5.5-fold in PalK and 3-fold in PalF cells. Taken together, these results suggest that core promoter elements play an important role in determining the effect of TBP overexpression on basal and activated promoter activity.

3.1.5. Identification of the promoter region responsible for the enhanced epithelial response of the BPV-4 promoter to upstream activators

The enhanced epithelial response of the BPV-4 promoter may be determined by DNA bound proximal promoter factors that are either cell type specific, differentially expressed, alternatively spliced, or modified in a cell type dependent manner. Also, as shown for the Pit-1/GHF-1 gene, which contains a 15 bp minimal TATA containing pituitary specific promoter (McCormick et al., 1991), the TATA box and surrounding sequences can dictate cell type specific transcriptional regulation. To identify the BPV-4 promoter region responsible for the differential response of this promoter in keratinocytes and fibroblasts, two chimaeric promoters with six E2 DNA binding sites inserted upstream were generated by 'splicing by overlap extension' PCR. The region of the heterologous tk promoter containing the tk TATA box and initiator was exchanged with the corresponding region in the BPV-4 promoter to generate the lcr/tk hybrid promoter (Fig. 3.1.12a). The PV2 promoter from nucleotide 184-279 and the tk promoter from nucleotide 120-199 were PCR amplified. The internal primers used were designed so that the primary PCR products contained 20 bp overlapping complementary ends. A second PCR reaction using primers annealing at the non-overlapping ends was performed to generate the lcr/tk hybrid promoter as a BglII/HindIII fragment. This fragment was then cloned into

pGL3 6E2. The tk/lcr hybrid, generated in the same manner, contains the BPV-4 TATA box and surrounding sequence (nucleotides 279-310) fused to the upstream tk promoter region from nucleotide 75-119 containing the Sp1 site (Fig. 3.1.12b).

The ability of E2 and VP16-E2 to upregulate transcription from these constructs was assayed in PalK and PalF cells. Fig. 3.1.13a shows the enhanced epithelial response of the BPV-4 promoter to upstream activators. E2 upregulates transcription a maximum of 7-fold in PalF and 60-fold in PalK cells, while VP16-E2 transactivates the PV2 promoter a maximum of 700-fold in PalF and 2500-fold in PalK cells. Fold activation is higher than that observed previously due to luciferase activity now being assayed on a Tropix TR717 luminometer. The lcr/tk hybrid promoter retains the enhanced epithelial response of the BPV-4 promoter to activation by E2 and VP16-E2 (Fig. 3.1.13b). E2 activates transcription a maximum of 2.5-fold in PalF and 11-fold in PalK cells. VP16-E2 upregulates transcription a maximum of 130-fold in fibroblasts and 300-fold in keratinocytes. The overall level of response of the lcr/tk 6E2 construct is reduced when compared with the PV2 promoter suggesting that the BPV-4 TATA box is more responsive than that of the tk promoter. The tk/lcr chimaeric promoter shows no such epithelial preference (Fig. 3.1.14a). E2 transactivates the tk/lcr hybrid promoter a maximum of 320-fold in PalK and 250-fold in PalF cells. VP16-E2 upregulates transcription from the tk/lcr promoter preferentially in fibroblasts. A maximum of 850-fold activation in PalK and 2600-fold activation in PalF cells is observed. Fig. 3.1.14b shows the cell type independent response of the tk promoter to activation by E2 and the preferential response to activation by VP16-E2 in fibroblasts. E2 upregulates transcription a maximum of 90-fold in both cell types whereas VP16-E2 activates transcription a maximum of 1000-

fold in PalK and 2500-fold in PalF cells. These results suggest that it is the upstream BPV-4 LCR promoter region and not the core TATA box and adjacent sequence that dictates the enhanced epithelial response of the BPV-4 promoter to upstream activators.

3.1.6. Identification of the DNA elements responsible for the differential response of the BPV-4 promoter to upstream activators in fibroblasts and keratinocytes

The chimaeric promoter constructs have identified the upstream BPV-4 promoter region as an important determinant of cell type specific transcription. A series of 5' deletions of the PV2 promoter were therefore generated to identify the specific LCR promoter elements that either co-operate or antagonise with E2 to regulate transcription preferentially in keratinocytes (Fig. 3.1.15). Promoter deletions were PCR amplified as BglII-HindIII fragments and cloned into pGL3 6E2. The ability of HPV-16 E2 to upregulate transcription from the E2 responsive promoter deletion constructs was assayed in PalK and PalF cells. It should be noted that in each construct the E2 DNA binding sites are an extra 6bp upstream from the TATA box due to the BglII restriction enzyme site used for cloning. Deletion analysis of the papillomavirus promoter identifies two novel repressor elements that are, at least in part, responsible for mediating the differential response of the BPV-4 promoter to upstream activators in fibroblasts and keratinocytes (presented graphically in Fig. 3.1.16 and numerically in Table 3.1.1.). Deletion of the region from 19bp to 3bp TATA results in a 3.5-fold increase in transcriptional activation by E2 in PalK and a 7-fold increase in PalF cells. This region defines Promoter Repressor Element-1 (PRE-1). The PRE-1 element spans the TATA box proximal E2 binding sites in the

BPV-4 LCR that have been mutated to prevent E2 binding. Although this is not the wild type BPV-4 sequence, these mutations do not affect the epithelial specific response of the BPV-4 promoter to E2 (Fig. 3.1.4.). Deletion from 80bp to 66bp TATA (Promoter Repressor Element-2 (PRE-2)) results in a 3-fold increase in transactivation in PalK and a 6.5-fold increase in PalF cells. Deletion of either of these regions does not have a significant effect on basal promoter activity (data not shown).

A core tk promoter must contain at least two elements to be able to respond to E2 and these elements, the TATA box, the initiator element, or a binding site for an upstream promoter factor are interchangeable (Ham et al., 1991a). In contrast to a minimal tk TATA box containing promoter E2 efficiently activates a minimal BPV-4 TATA promoter that contains neither an initiator element nor a binding site for an upstream factor. E2 upregulates transcription approximately 560-fold in keratinocytes and 280-fold in fibroblasts from the 3bp TATA construct (Fig. 3.1.16 and Table 3.1.1.). This minimal promoter contains only 32 bp of BPV-4 promoter sequence. This result suggests that the general transcription machinery assembled at the BPV-4 and tk TATA boxes contain distinct coactivator complexes. Also, the minimal BPV-4 TATA promoter has an elevated epithelial response to E2 suggesting that the BPV-4 TATA box and surrounding sequence may play an important role in determining cell type specific transcription or that the E2 protein contributes to activating transcription from the BPV-4 promoter preferentially in keratinocytes.

To address these possibilities the ability of increasing concentrations of VP16-E2 and HPV-16 E2 to activate transcription from the 3bpTATA construct was assayed (Fig.

3.1.17). At low to high levels VP16-E2 activates transcription preferentially in fibroblasts. A maximum of approximately 3500-fold in PalF and 1500-fold activation in PalK cells is observed at a 1:1 ratio of expression vector to reporter plasmid (Fig. 3.1.17a). The 5-fold enhanced epithelial response of the PV2 promoter to activation by VP16-E2 shown in Fig. 3.1.6b is lost with the minimal BPV-4 TATA containing construct demonstrating that the BPV-4 TATA box is not involved in dictating the enhanced epithelial response of the BPV-4 promoter to activation by VP16-E2. This is in agreement with the results obtained with the chimaeric promoter constructs (Fig. 3.1.13. and Fig. 3.1.14.). Low to intermediate levels of E2 activate transcription from the 3bp TATA construct to a similar degree in both PalK and PalF cells. At these levels of E2 the activation of the PV2 promoter is 8- to 9-fold enhanced in epithelial cells (Fig. 3.1.6a) confirming the involvement of PRE-1 and PRE-2 in mediating the cell type selective response of the BPV-4 promoter to upstream activators. However, at high levels of E2 (1μg) transcription is downregulated from the 3bp TATA construct in fibroblasts while in keratinocytes activation remains elevated. A maximum of approximately 300-fold in fibroblasts and 450-fold activation in keratinocytes is observed (Fig. 3.1.17b). As E2 is expressed at similar levels in both PalK and PalF cells (Fig. 3.1.5) these results suggest that certain cell type specific co-activators may interact with the E2 transactivation domain in epithelial cells but not in fibroblasts enabling E2 to retain the ability to activate transcription at elevated levels in PalK cells.

3.1.7. PRE-1 and PRE-2 can repress the basal activity of a strong heterologous promoter

Two functional types of repressor elements exist. Negative regulatory elements (NRE) that are promoter specific and silencer elements that are able to repress heterologous promoter activity out of context of the native promoter. To further characterise the functional properties of BPV-4 promoter repressor elements, PRE-1 and PRE-2, oligomers corresponding to these sequences were multimerised upstream of the SV40 promoter in the pGL3 luciferase vector in both a positive and negative orientation (Fig. 3.1.18). These potential repressor binding sites were generated in the same manner as shown for the E2 sites. The ability of these elements to direct repression of SV40 promoter activity was assayed in both PalK and PalF cells. Fig. 3.1.19 shows that over a range of concentrations one copy and four copies of PRE-1 strongly represses the SV40 promoter by about 50-80% in both cell types. As the extent of repression does not depend on the number of copies of PRE-1, this suggests that the cellular factor interacting with PRE-1 acts independently to repress transcription. In fibroblasts repression is orientation independent. However, in keratinocytes repression is alleviated at high levels of reporter construct when PRE-1 is cloned in the reverse orientation. Although this result is reproducible there is no obvious explanation for the loss of repression under these conditions. Over a range of concentrations four copies of PRE-2 represses SV40 promoter activity approximately 40-80% in PalK and 60-80% in PalF cells (Fig. 3.1.20). Orientation of PRE-2 had no effect on the extent of repression in both cell types. These results demonstrate that PRE-1 and PRE-2 can repress the basal activity of a strong heterologous constitutive promoter.

3.1.8. PRE-2 binds a single protein complex in both PalK and PalF cells

Extensive database searches revealed that PRE-1 and PRE-2 do not contain any known transcription factor binding sites. The PRE-2 motif is conserved in position and sequence in the related mucosal epitheliotropic papillomaviruses, BPV-3 and BPV-6 (Fig. 3.1.18). Also, a YY-1 independent silencer in roughly the same position as PRE-2 in the HPV-16 LCR has recently been identified (O'Connor et al., 1998). As functional analysis has identified PRE-2 as a transcriptional silencer band shift assays were performed to determine whether PRE-2 could bind a nuclear protein. A double stranded synthetic oligonucleotide containing the 20 base pair PRE-2 motif detected a single protein complex with both PalK and PalF nuclear extracts (Fig. 3.1.21). Binding to PRE-2 was confirmed to be specific as it was competed by 100-fold excess non-radioactive self oligonucleotide but not by the unrelated AP-1 oligonucleotide. No reproducible specific complex binding to PRE-1 could be detected by EMSA analysis. This may reflect the sensitivity and conditions used in the band shift assay.

3.1.9. Functional characterisation of the PRE-2 binding complex

In vitro footprinting shows that PRE-2 contains a DNA binding site for a potential transcription factor (Jackson and Campo, 1991). To identify the exact nucleotides necessary for *in vitro* binding two double stranded oligonucleotides each containing a single PRE-2 motif with a 3 base pair substitution in the footprint were generated (Fig. 3.1.22a.). The mutant PRE-2 oligonucleotides were tested for competition using the band shift assay. Fig. 3.1.22b demonstrates that binding of the detected protein complex is not competed by either of the two mutant sequences identifying these

mutated residues to be important for sequence specific binding in both PalK and PalF cells.

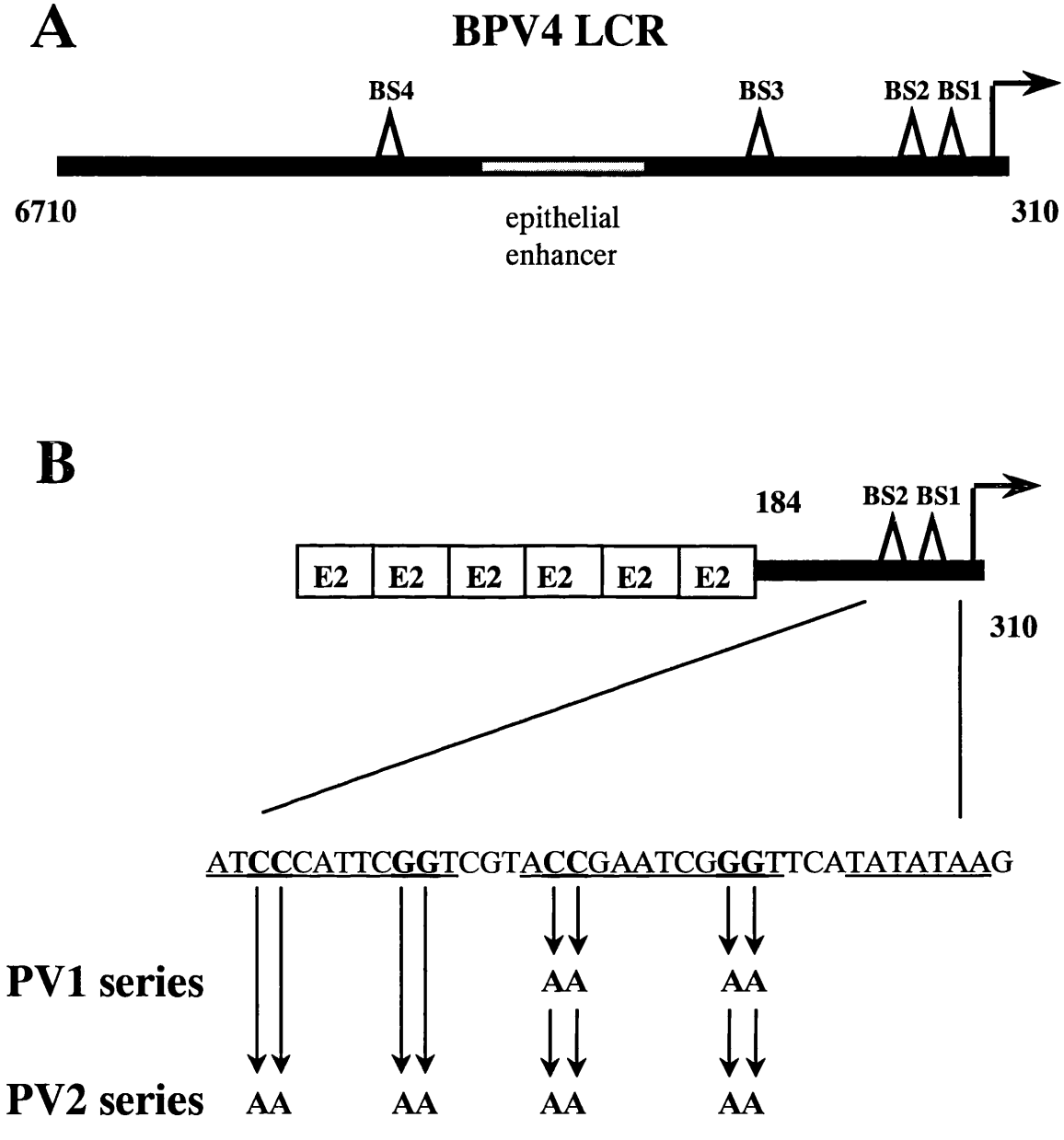
To determine the functional consequences of loss of binding four copies of PRE-2 mt1 were inserted upstream of the SV40 promoter in the pGL3PRO reporter vector generating PRO4Xmt1. Again, this was performed as described for the multimerisation of E2 sites. The ability of this construct to relieve PRE-2 mediated repression of SV40 promoter activity was assayed in both PalK and PalF cells. Fig. 3.1.23 shows that loss of factor binding correlates with loss of transcriptional repression. Over a range of concentrations the activity of the PRO4xmt1 construct is similar to that of the SV40 promoter alone in both PalF and PalK cells. To determine the effect of loss of binding on cell type specific transcriptional regulation of the BPV-4 promoter, PRE-2 mt1 was introduced into the 80bp TATA promoter construct by PCR amplification using a 5' primer containing the mutation. Six E2 sites were inserted upstream of this mutated promoter fragment generating 80bpmt1. The ability of E2 to upregulate transcription from 80bpmt1 was assayed in both PalK and PalF cells (Fig. 3.1.24). The 80bpmt1 construct has a two-fold increase in transcriptional activation by E2 in PalF cells and a 0.5-fold increase in PalK relative to the 80bpTATA promoter deletion. This result confirms that the PRE-2 binding protein represses the transcriptional response of the BPV-4 promoter to E2 and suggests that PRE-2 is one component involved in mediating the cell type selective response of the BPV-4 promoter to activation by E2 in PalF and PalK cells.

3.1.10. PRE-2 specifically binds a 50 kDa cellular protein

UV cross-linking was performed to determine the molecular weight of the active DNA binding form of the detected protein complex. A single radiolabelled bromodeoxyuridine (BrdU) substituted PRE-2 motif was used to probe PalK and PalF nuclear extracts in a band shift assay. No difference in binding was observed when the wild type and BrdU substituted PRE motifs were used as probes in band shift assays. After electrophoresis, the gel was UV irradiated (304 nm) to cross-link the protein to the DNA. The retarded complexes were visualised using a phosphor imager, the bands of interest excised and the protein complexes resolved by 10% SDS-PAGE. A non-labelled BrdU PRE-2 motif and the unrelated AP-1 oligonucleotide were used to assess the specificity of binding. Fig. 3.1.25 shows that BrdU PRE-2 binds a major species of approximately 50 kDa in both PalK and PalF nuclear extracts. Binding of this factor was confirmed to be specific as it was competed by excess non-labelled BrdU PRE-2 but was not competed by AP-1 in both cell types. A minor species was also detected just above the 50 kDa band. This suggests that the PRE-2 binding protein may be post translationally modified or that a heterodimeric protein complex interacts with the DNA.

Fig. 3.1.1. (a) Organisation of the BPV-4 LCR. The LCR can be divided roughly into two regions, the promoter region and an epithelial specific enhancer. The LCR contains four binding sites for the viral transcription factor, E2 (BS1-BS4). BS1 and BS2 are separated from each other and the TATA box by 3 bp. BS3 is 77 bp upstream from BS2 and BS4 is a further 409 bp upstream. **(b) E2 responsive promoter constructs.** The BPV-4 promoter region from nucleotide 184 to 310 was PCR amplified as a BglII-HindIII fragment. This region contains the TATA box, E2 BS1 and BS2 but no initiator element. Mutations were introduced into the TATA proximal E2 sites preventing E2 binding to generate PV1 and PV2. A series of concatamers of E2 binding sites were inserted into the BglII site immediately upstream in the position of E2BS3.

E2 responsive promoter constructs



In vitro binding of HPV-16 E2 to the E2 BS4 sequence

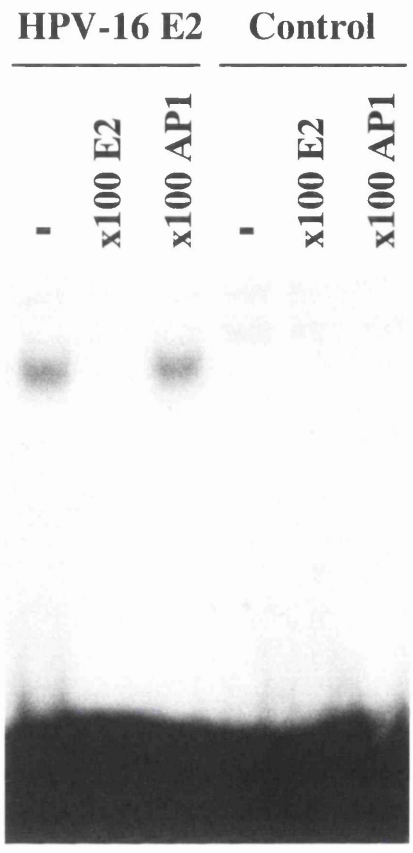


Fig. 3.1.2. *In vitro* translated HPV-16 E2 specifically binds the -ACCGAAAACGGT- sequence used in cloning. Radiolabelled synthetic E2 BS was incubated with either *in vitro* translated HPV-16 E2 or control luciferase protein in a band shift assay. 100-fold excess unlabelled E2 BS and AP-1 oligonucleotides were used as indicated to assess the specificity of binding. Retarded complexes were separated on a 6% polyacrylamide gel and visualised by autoradiography.

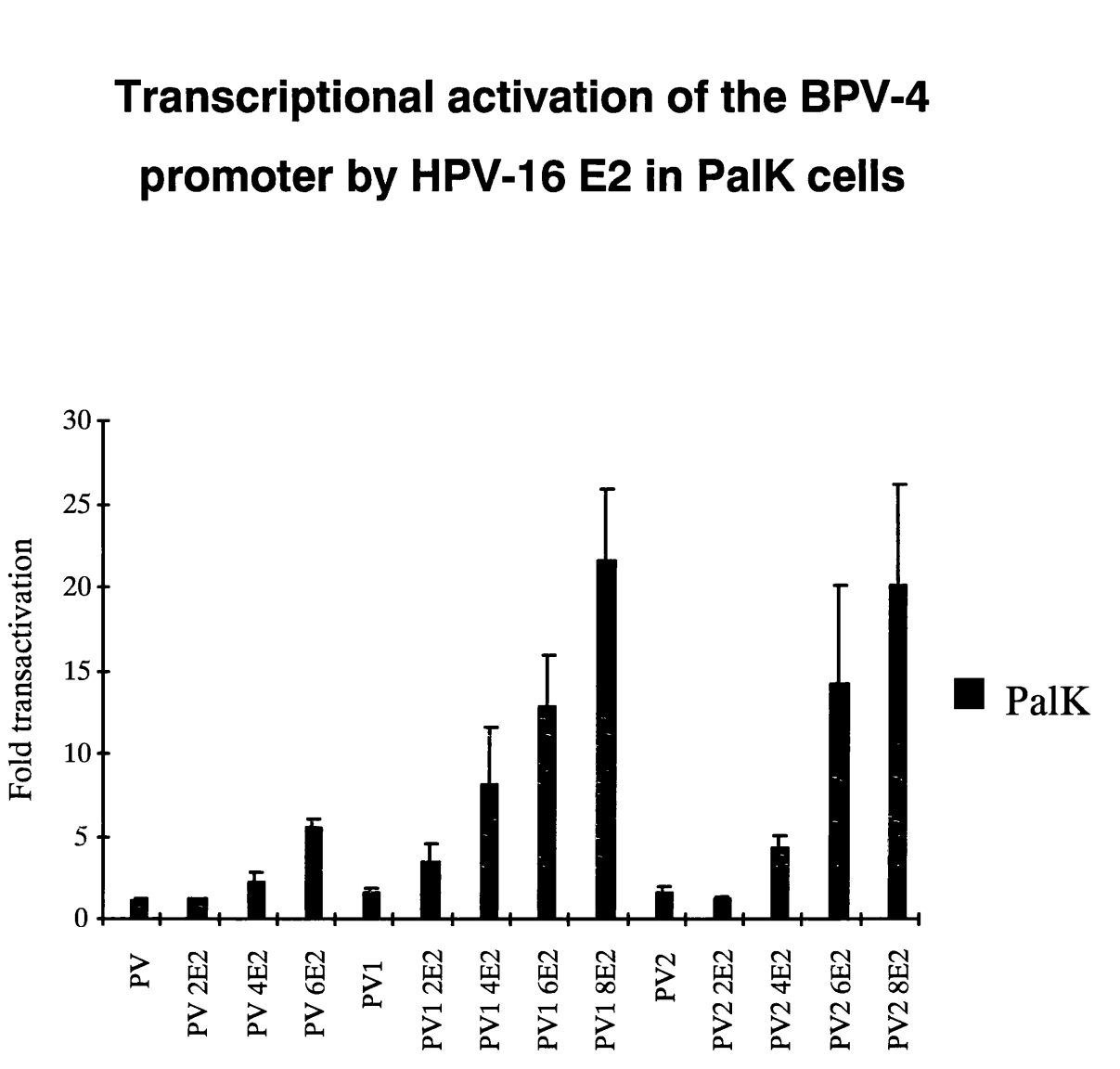
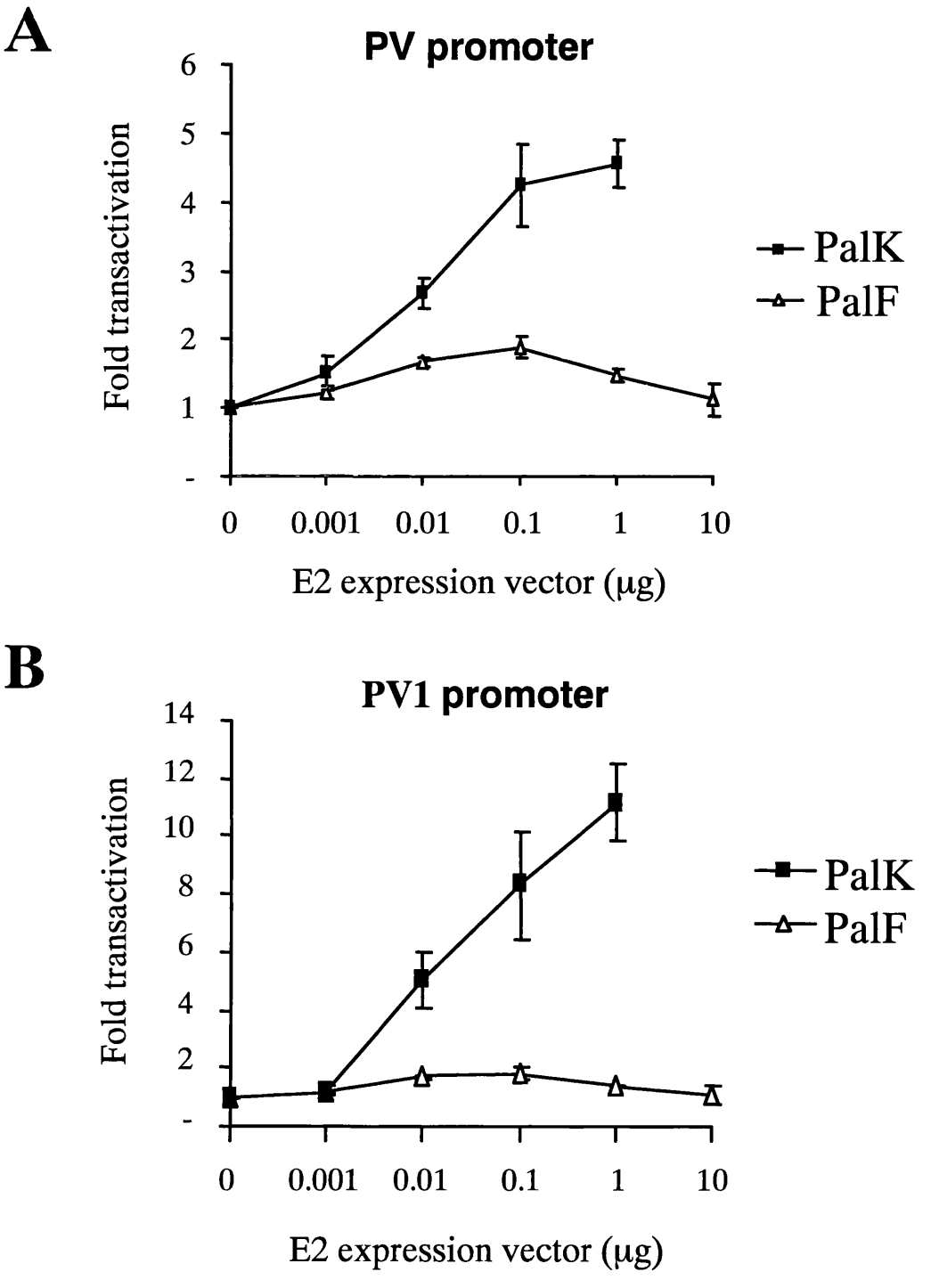


Fig. 3.1.3. E2 upregulates transcription from the BPV-4 promoter in additive manner. 1 μg reporter plasmid was cotransfected with 0.1 μg pCMV HPV-16 E2 expression vector into PalK cells. This ratio has previously been shown to be optimal for maximal activation of the LCR promoter by E2. Results are expressed as fold transactivation relative to the luciferase activity of each reporter in the absence of E2 (normalised to 1). Each transfection was repeated at least three times in duplicate.

Epithelial specific transcriptional regulation of the BPV-4 promoter by HPV-16 E2



Epithelial specific transcriptional regulation of the BPV-4 promoter by HPV-16 E2

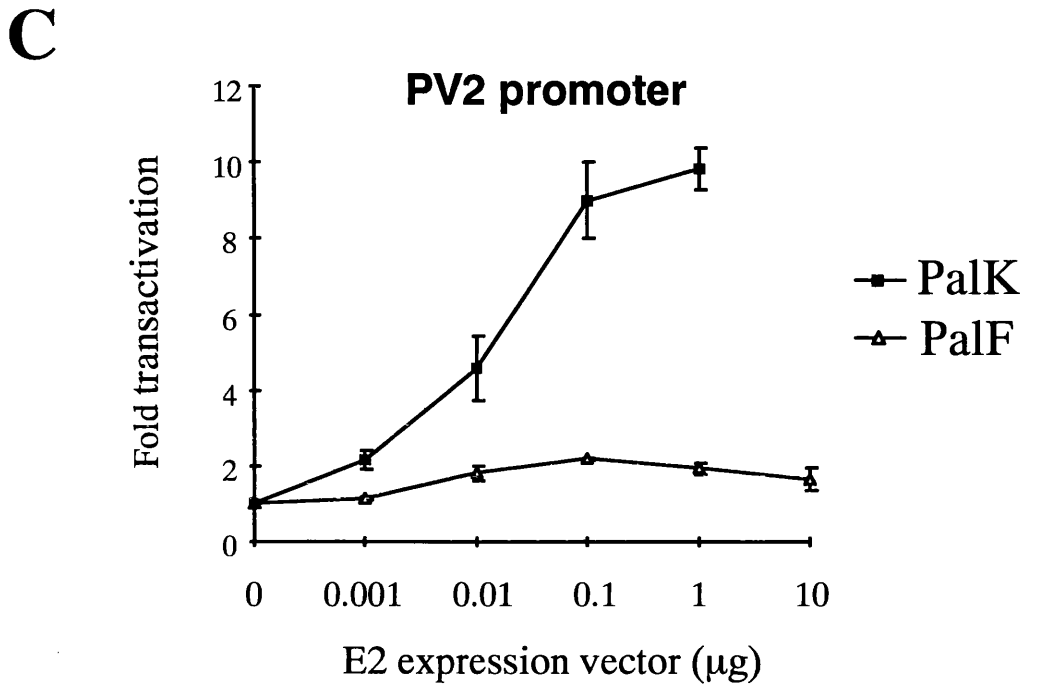


Fig. 3.1.4. Epithelial specific transcriptional regulation of the BPV-4 promoter by HPV-16 E2. 1 μg of either **(a)** PV 6E2 or **(b)** PV1 6E2 or **(c)** PV2 6E2 reporter construct was cotransfected with increasing amounts of pCMV HPV-16 E2 expression vector into both PalK and PalF cells. pCMV was used to make the total amount of DNA transfected equal in all cases. A control vector with the SV40 enhancer and promoter driving expression of the luciferase gene (pGL3 CONT) gave similar activity in both cell types.

Western blot analysis of HPV-16 E2 expression levels

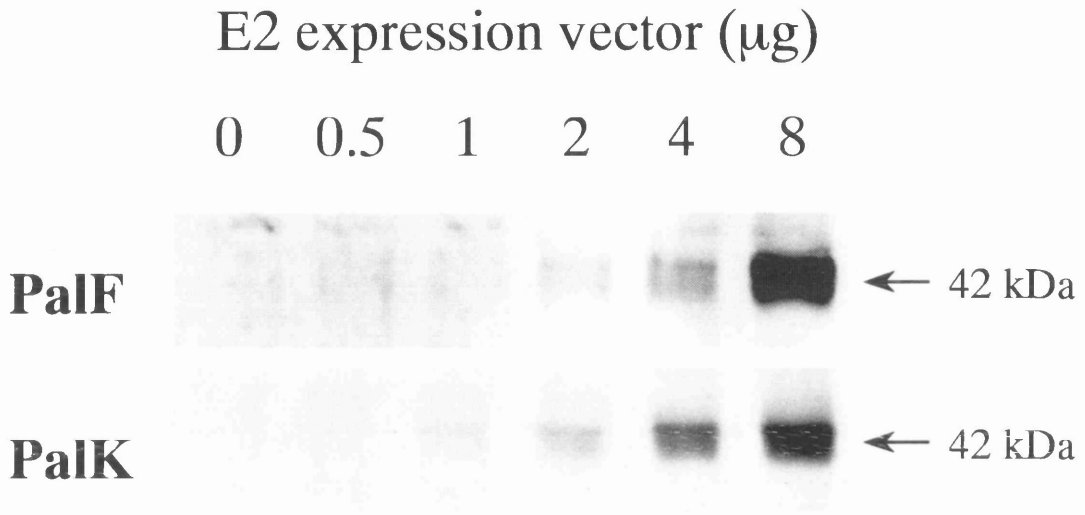


Fig. 3.1.5. HPV-16 E2 is expressed at similar levels as a doublet of approximately 42 kDa in both PalK and PalF cells. PalK and PalF cells were transfected with increasing amounts of pCMV HPV-16 E2 expression vector. 50 mg whole cell extracts were separated by 10% SDS-PAGE, transferred to nitrocellullose and probed with a TVG261 monocloned antibody directed against amino acids 2 to 17 in the amino terminus of HPV-16 E2.

Fig. 3.1.6. The PV2 promoter shows an enhanced epithelial response to transcriptional activators. PalK and PalF cells were cotransfected with 1 μg PV2 6E2, which has both the TATA box proximal E2 BS1 and BS2 mutated, and the indicated amounts of pCG BPV-1 E2 and pCG VP16 E2 expression vectors. pCG was used to make the total amount of DNA used 2 μg in each case. Results are expressed as fold transactivation over the luciferase activity in the absence of expression vector.

Transcriptional activation of the PV2 promoter by BPV-1 E2 and VP16-E2

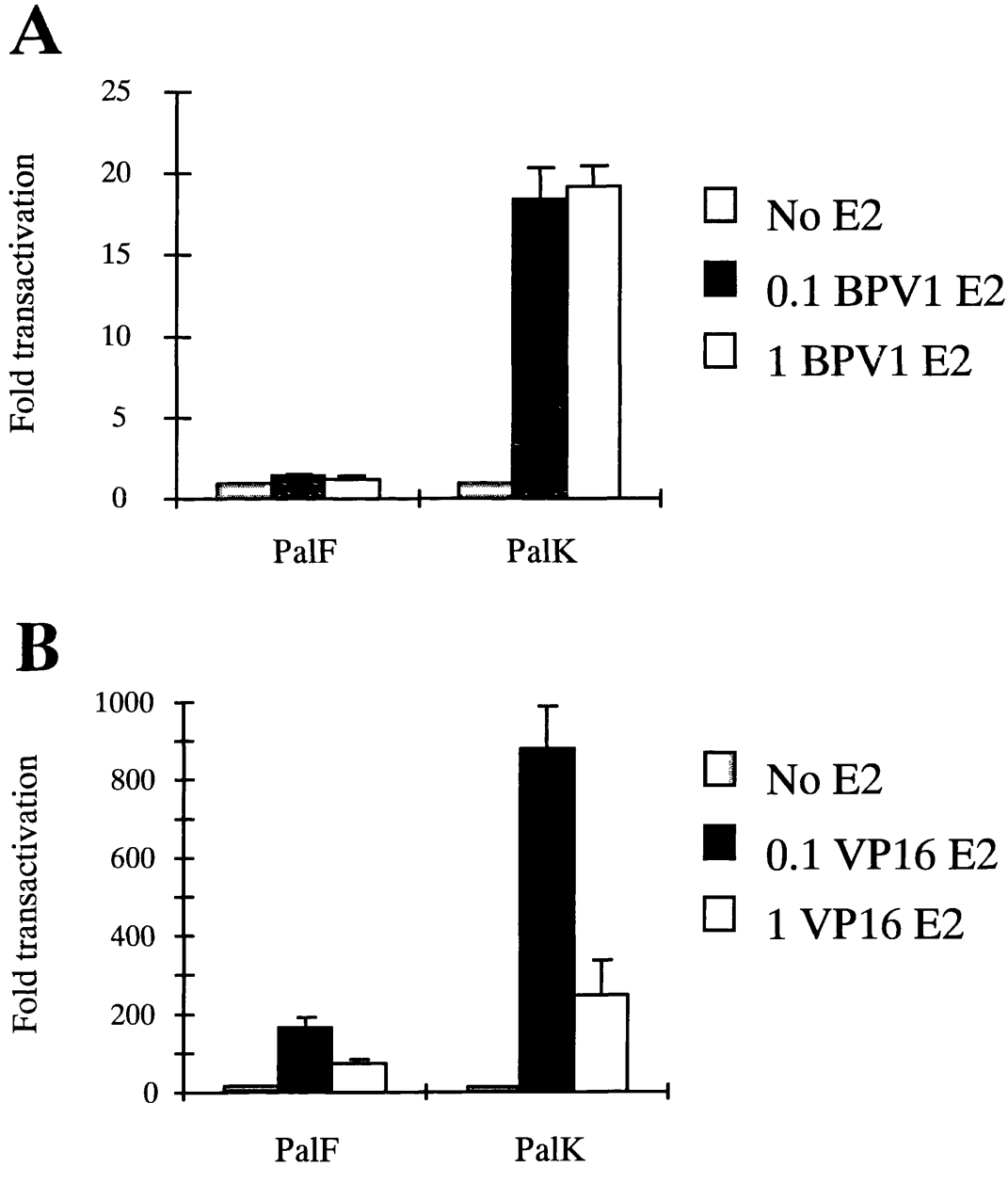


Fig. 3.1.7. (a) PV2 4LexA reporter construct. Four copies of the LexA site from the colE1 promoter were cloned into the BglII site immediately upstream of the PV2 promoter. **(b) The PV2 promoter shows an enhanced epithelial response to activation by VP16-LexA.** PalK and PalF cells were cotransfected with 1 μg PV2 4LexA reporter construct and the indicated amounts of pCGVP16-LexA expression vector. The total amount of DNA transfected was made equal in each case. Results are expressed as fold transactivation over the luciferase activity in the absence of VP16-LexA expression vector (set at 1).

Functional analysis of the E2 C-terminus

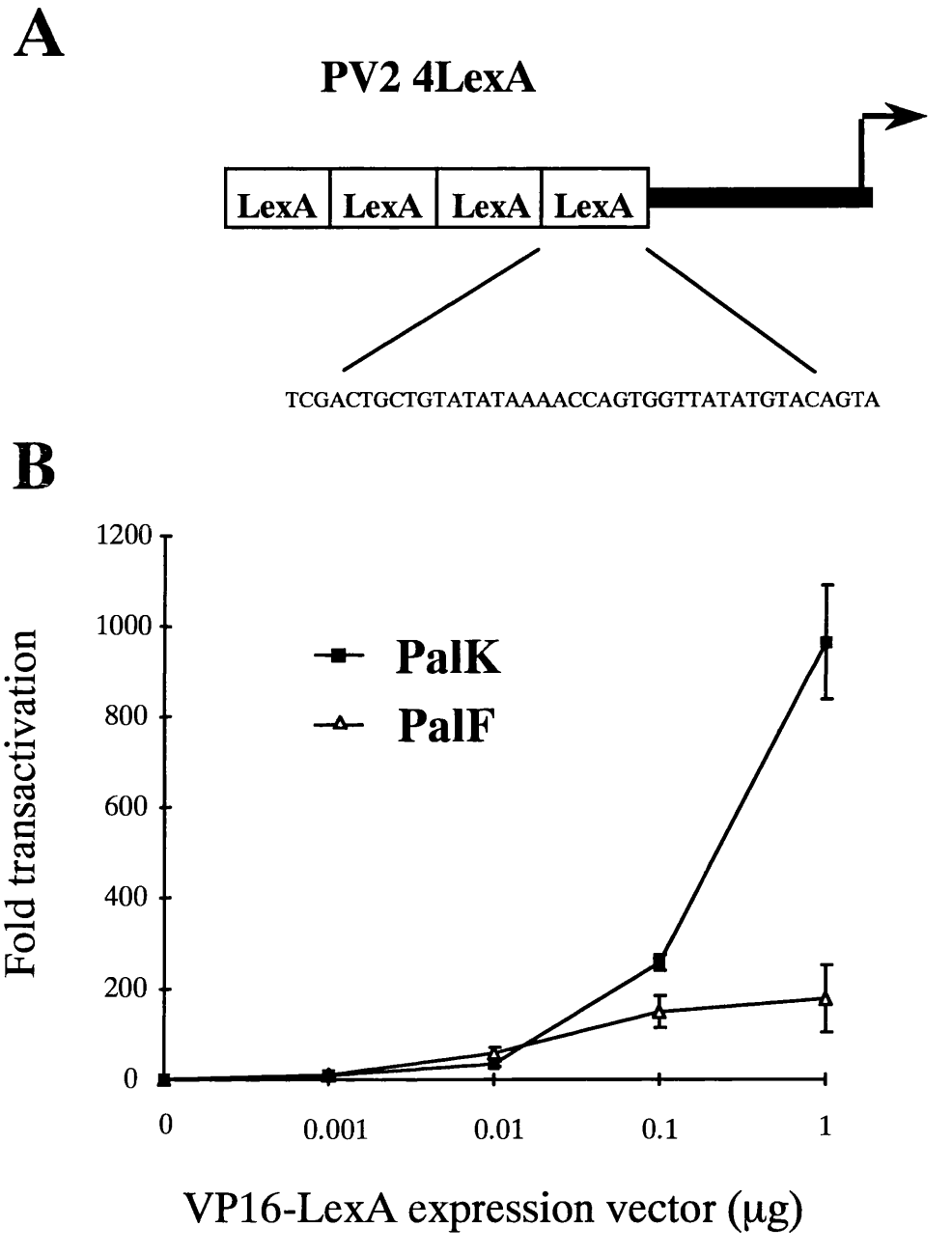
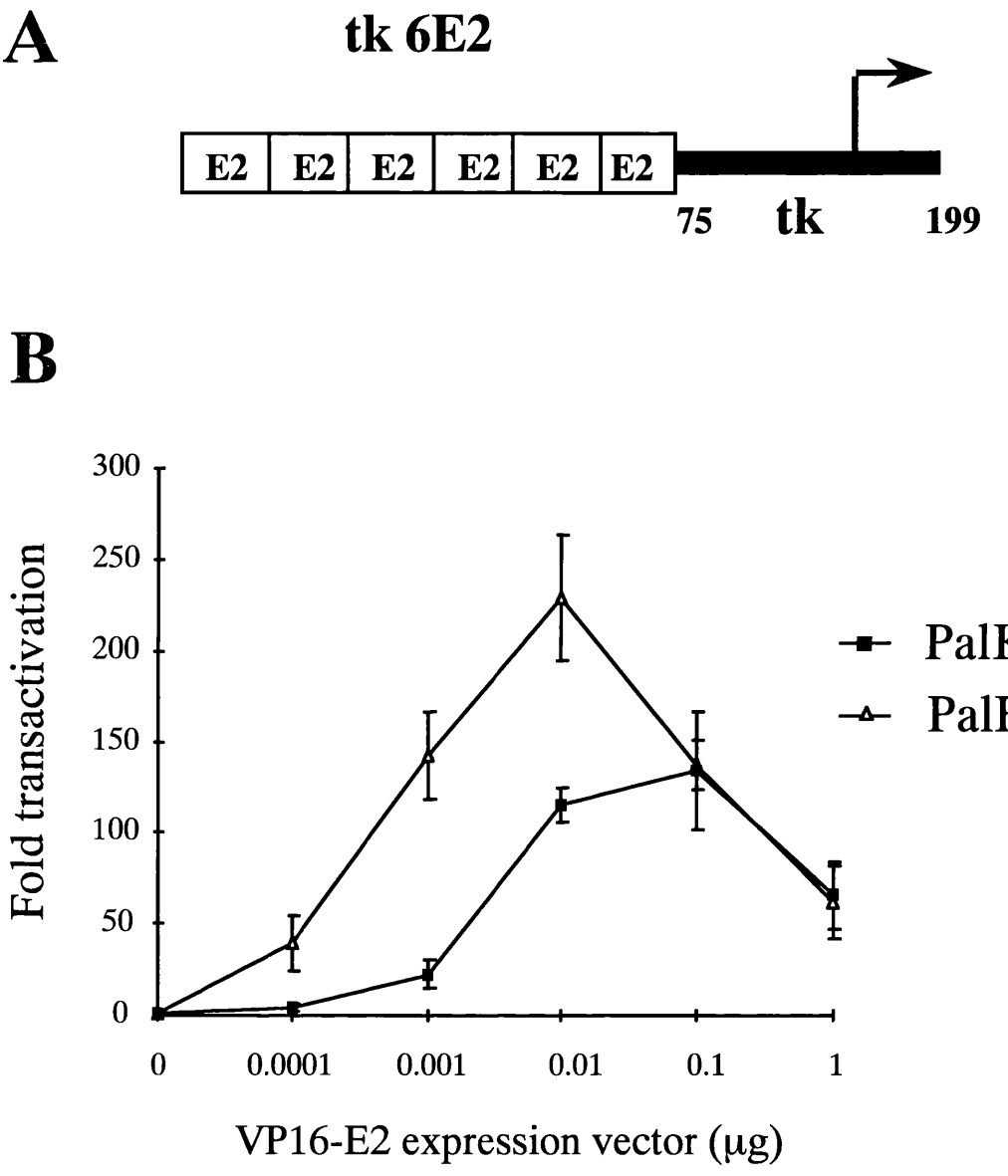


Fig. 3.1.8. (a) E2 responsive tk promoter construct. The tk promoter from nucleotide 75 to 199 containing the TATA box, initiator element and a GC rich box was PCR cloned as a BglII-HindIII fragment. Six E2 binding sites were inserted into the BglII site immediately upstream to generate tk 6E2. **(b) The tk promoter does not show an epithelial preference to activation by VP16-E2.** PalK and PalF cells were cotransfected with 1 μg tk 6E2 reporter plasmid and the indicated amounts of pCGVP16-E2 expression vector. PCG was added to make the total amount of DNA transfected 2 μg in each case. Results are expressed as fold transactivation over the luciferase activity in the absence of VP16-E2.

Cell type independent activation of the tk 6E2 construct by VP16-E2



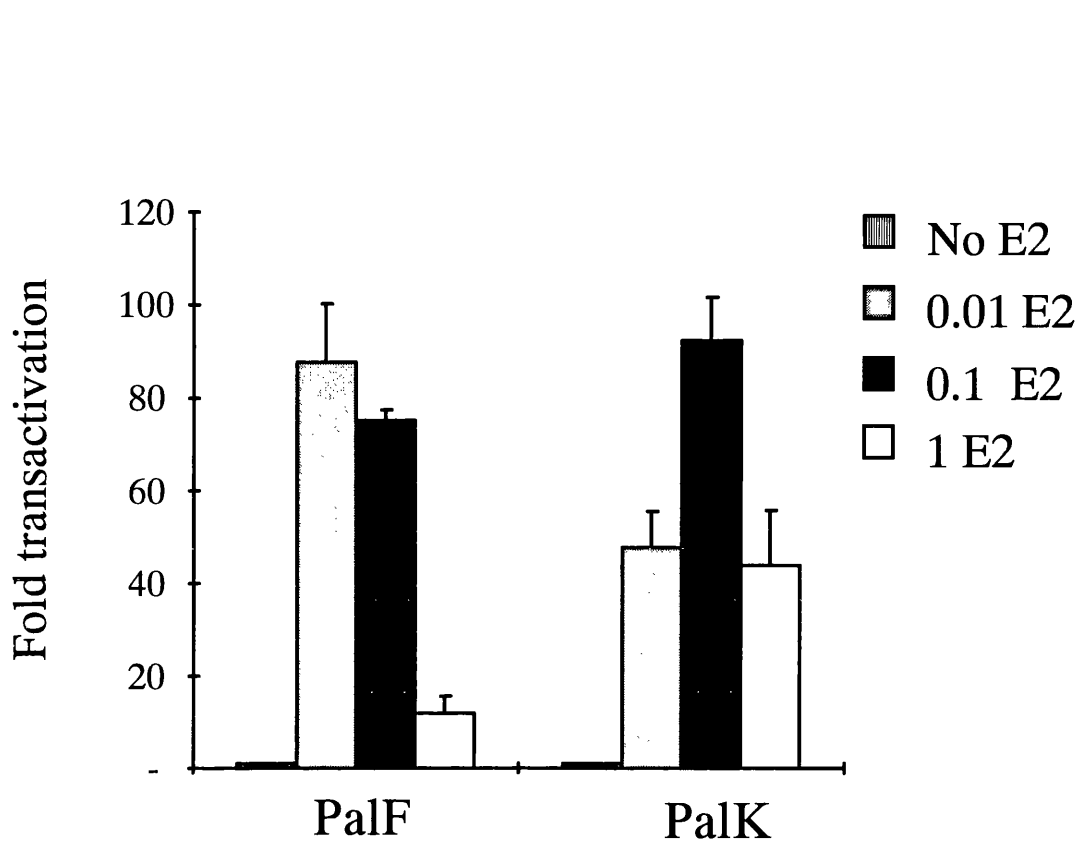


Fig. 3.1.9. HPV-16 E2 activates transcription from the tk promoter in a cell type independent manner. PalK and PalF cells were cotransfected with tk 6E2 and the indicated amounts of pCMV HPV-16 E2 expression vector. 1 μg reporter construct was used in each assay and the total amount of DNA transfected was made equal by the addition of pCMV. Results are expressed as fold transactivation over the luciferase activity in the absence of E2.

Effect of TBP overexpression on PV2 promoter activity

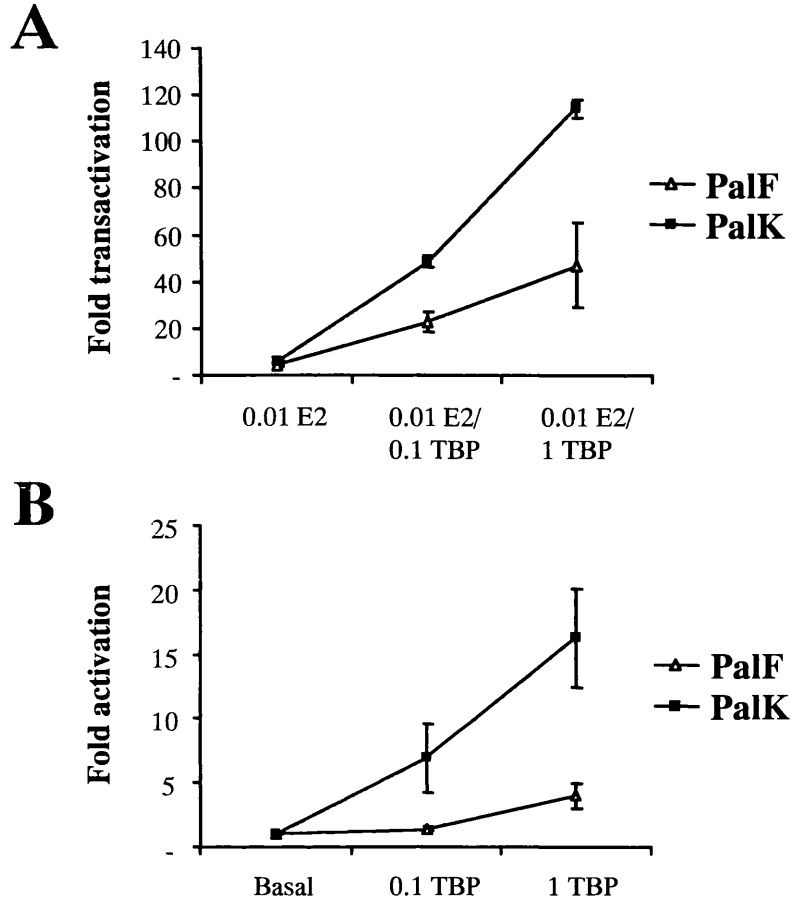


Fig. 3.1.10. TBP overexpression upregulates basal and E2 activated PV2 promoter activity preferentially in PalK cells. PalK and PalF cells were co-transfected with 1 μg of PV2 6E2 reporter construct and the indicated amounts of hTBP expression vector in the **(a)** presence or **(b)** absence of 0.01 μg of pCMV HPV-16 E2. A 0.01:1 ratio of E2 expression plasmid to reporter construct is sub-optimal for E2 mediated activation of the BPV-4 LCR. The total amount of DNA transfected was made equal in all cases. Results are expressed as fold activation relative to the luciferase activity of the PV2 6E2 reporter construct alone (set at 1).

TBP potentiation of activated and basal tk promoter activity

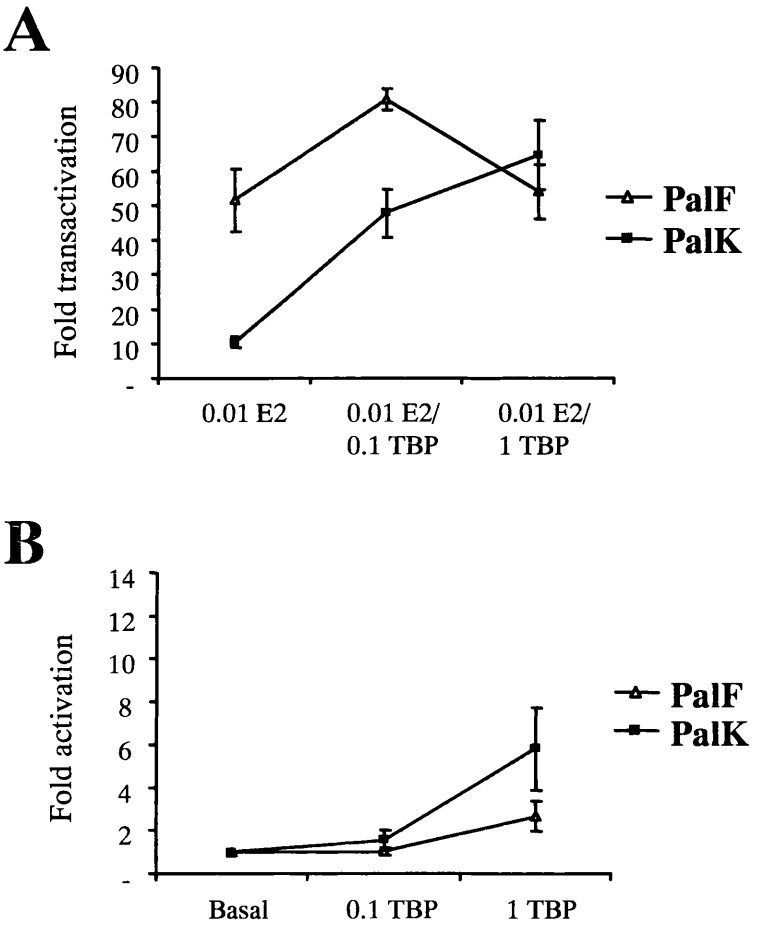


Fig. 3.1.11. The tk promoter has a different response to TBP overexpression. PalK and PalF cells were co-transfected with 1 μg of tk 6E2 reporter construct and the indicated amounts of hTBP expression vector in the **(a)** presence or **(b)** absence of 0.01 μg of pCMV HPV-16 E2. The total amount of DNA transfected was made equal in all cases. Results are expressed as fold activation relative to the luciferase activity of the PV2 6E2 reporter construct alone (set at 1).

Chimaeric promoter constructs

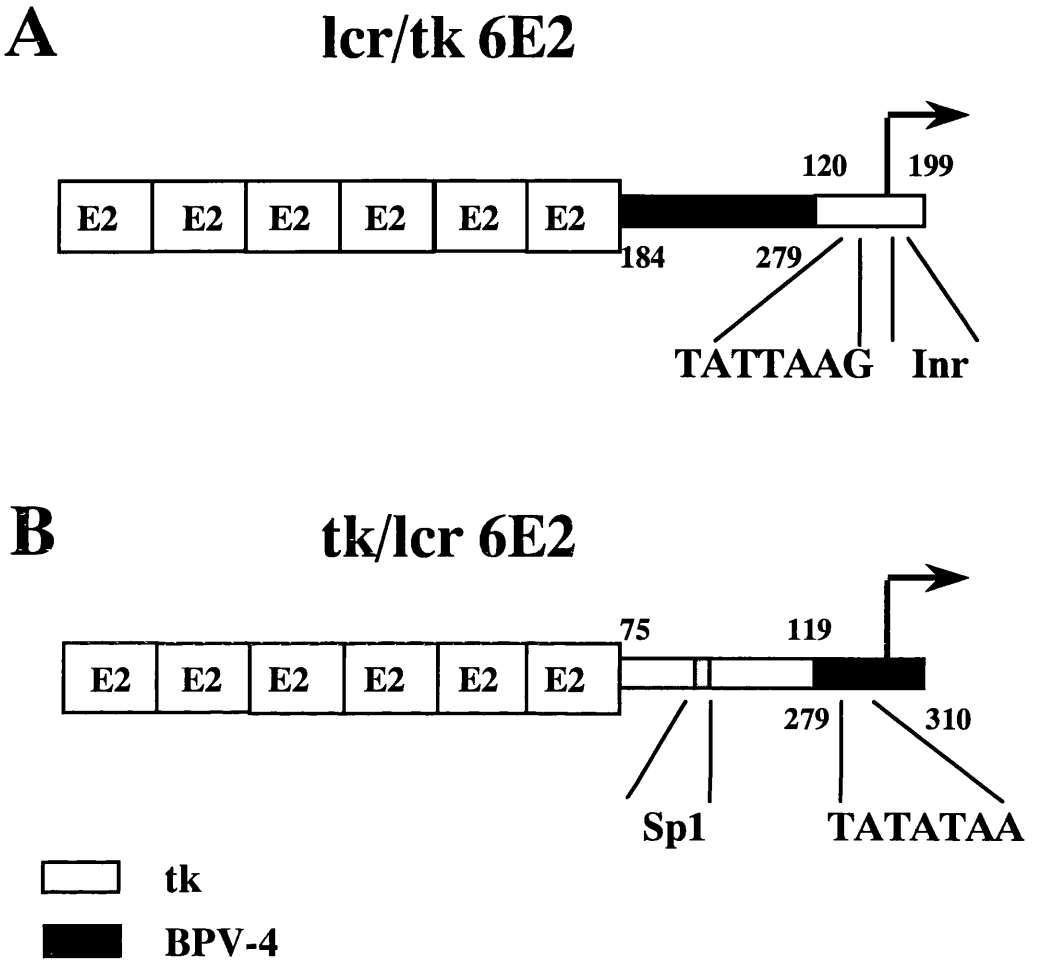


Fig. 3.1.12. E2 responsive chimaeric promoter constructs were generated by splicing by overlap extension PCR **(a)** The lcr/tk hybrid promoter contains the BPV-4 upstream promoter region from nucleotide 184-279 fused to the tk promoter region from nucleotide 120-199. This region of the tk promoter contains the TATA box and initiator element. **(b)** The tk/lcr hybrid promoter contains the tk upstream promoter region from nucleotide 75-119 containing the Sp1 site fused to the BPV-4 TATA box and surrounding sequence (nucleotides 279-310).

Fig. 3.1.13. The lcr/tk hybrid promoter retains the enhanced epithelial response of the BPV-4 promoter to upstream activators. PalK and PalF cells were co-transfected with the indicated amounts of pCMV HPV-16 E2 and pCG VP16 E2 expression vectors and either 1μg of **(a)** PV2 6E2 or **(b)** lcr/tk 6E2 reporter constructs. The total amount of DNA transfected was made equal in each case. Results are expressed as fold transactivation relative to the luciferase activity in the absence of expression vector.

Activation the lcr/tk hybrid promoter by HPV-16 E2 and VP16-E2

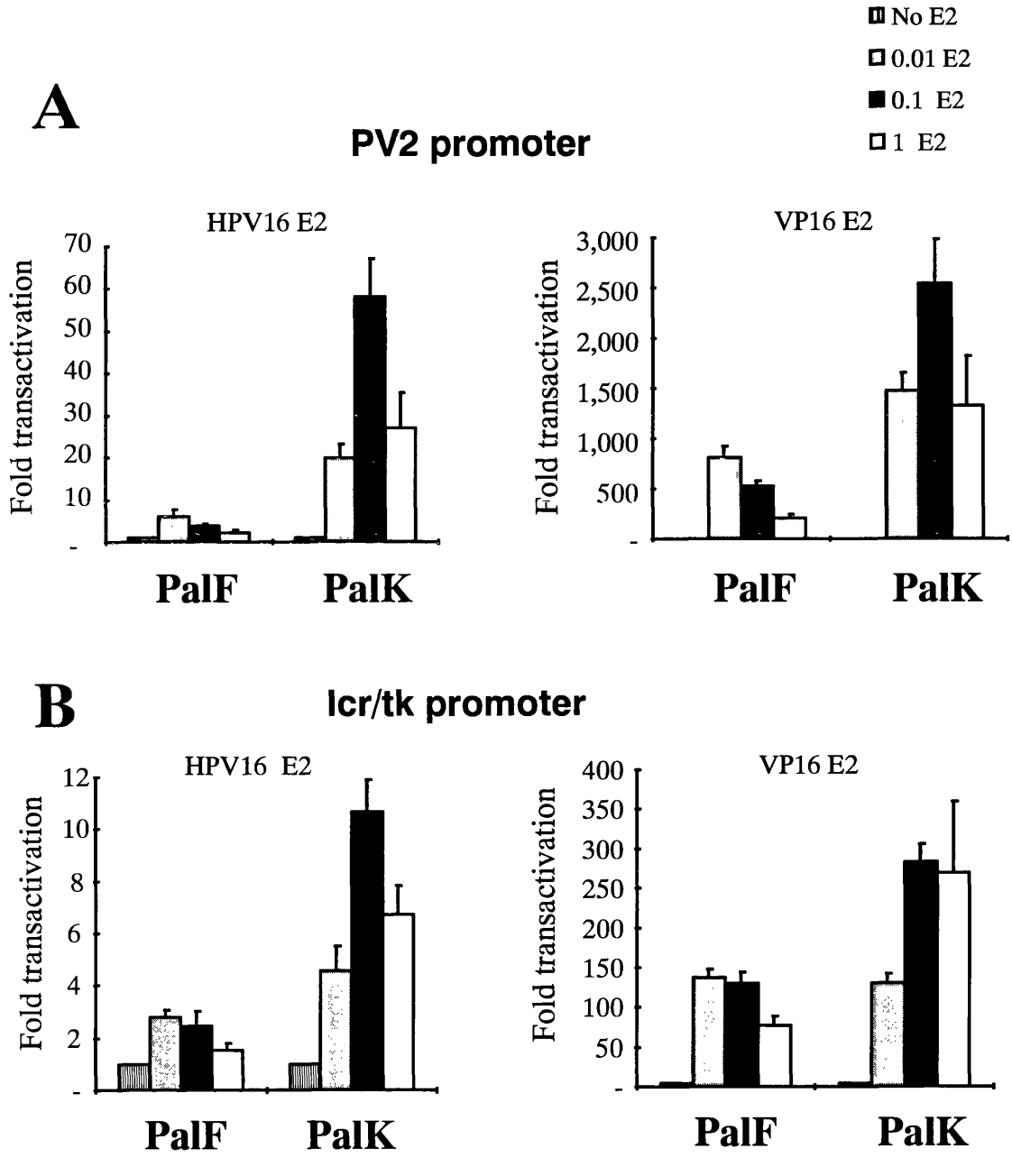


Fig. 3.1.14. The tk/lcr hybrid promoter shows no epithelial preference to activation by HPV-16 E2 and VP16-E2. PalK and PalF cells were co-transfected with the indicated amounts of pCMV HPV-16 E2 and pCG VP16 E2 expression vectors and 1μg of either **(a)** tk/lcr 6E2 or **(b)** tk 6E2 reporter constructs. Empty expression vector was used to make the total amount of DNA transfected 2 μg in each case. Results are expressed as fold transactivation relative to the luciferase activity in the absence of expression vector (set at 1).

Transcriptional response of the tk/lcr hybrid promoter to upstream activators

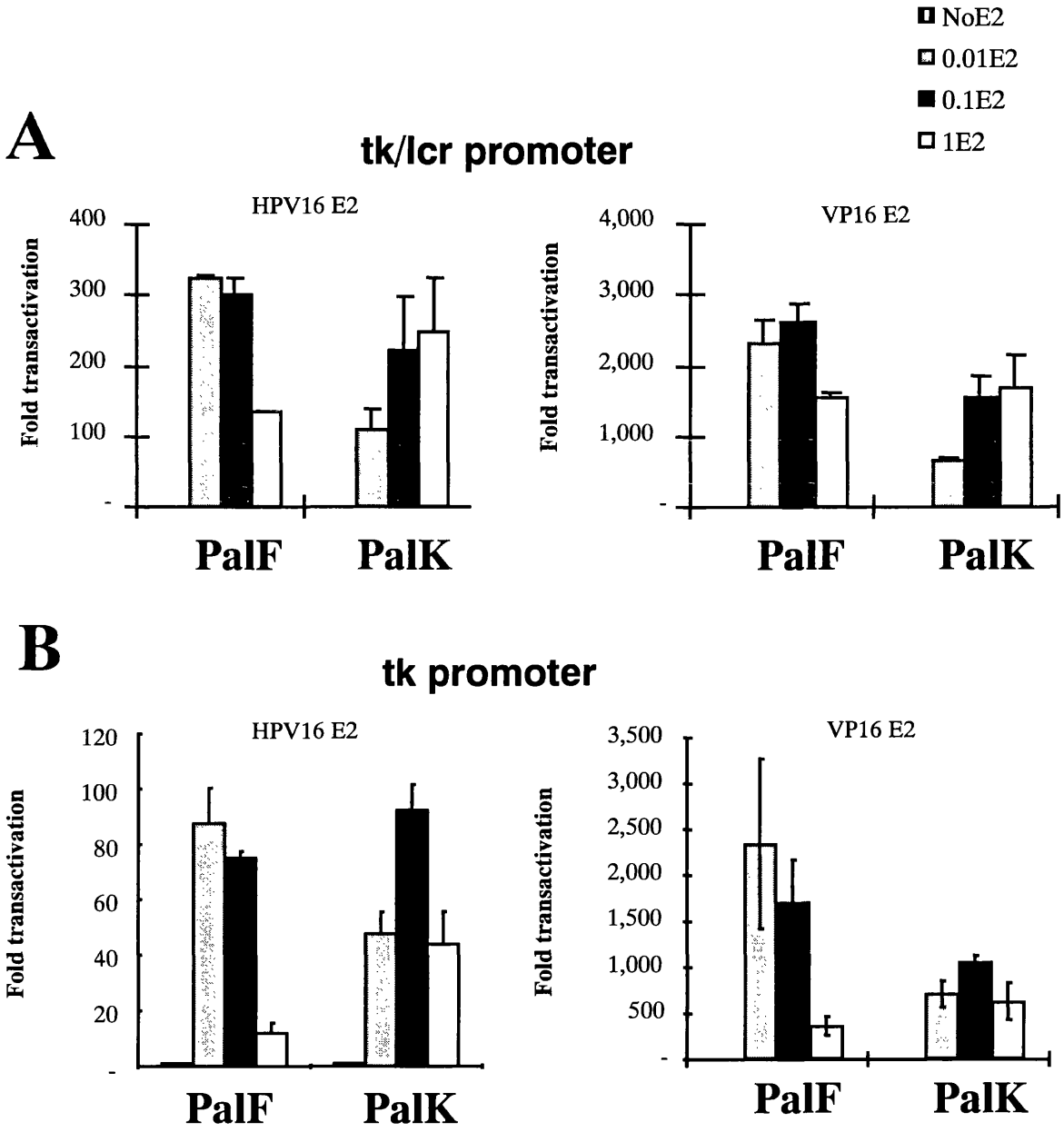
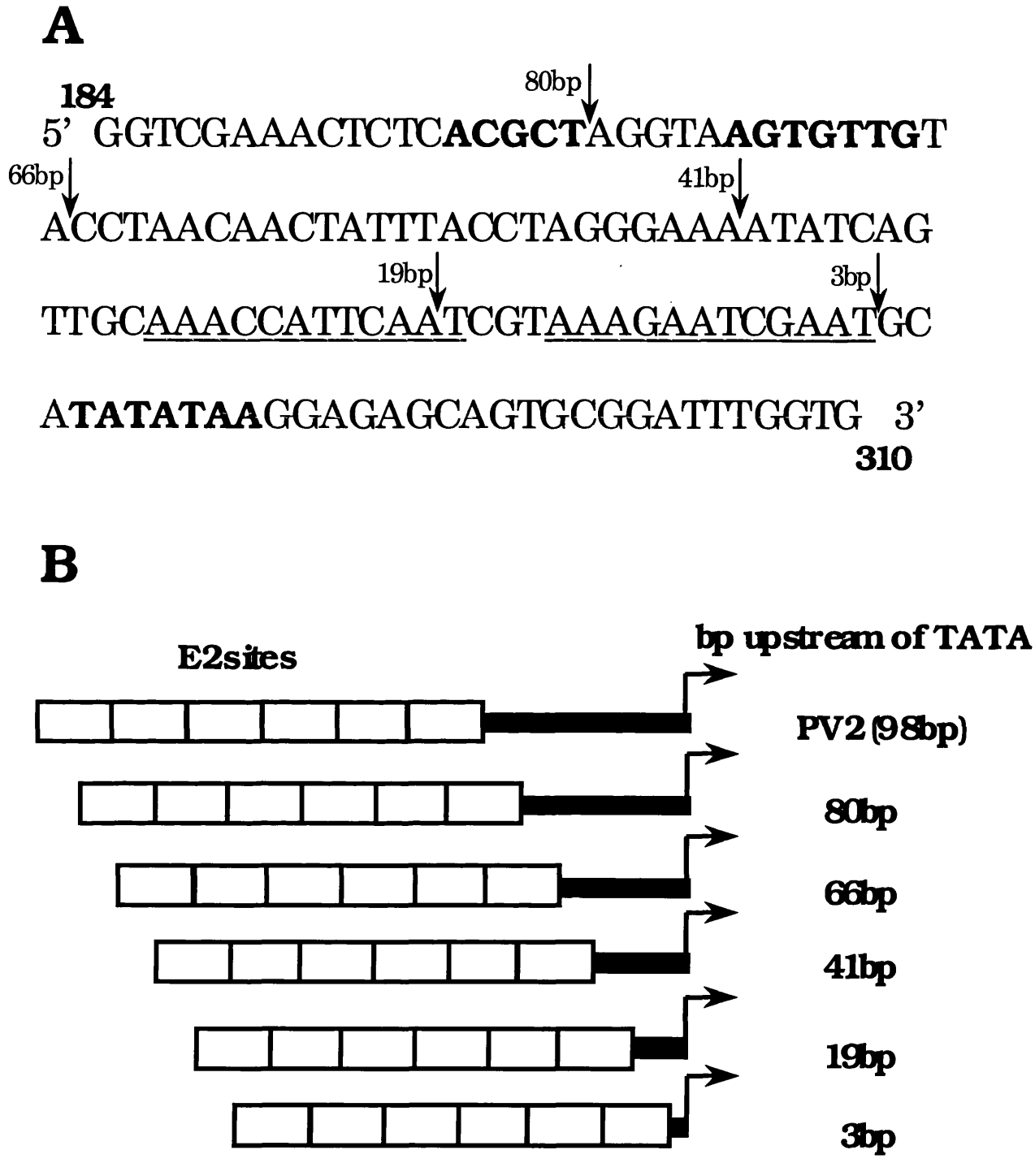


Fig. 3.1.15. (a) Sequence of the BPV-4 promoter from nucleotide 184 to 310. The TATA box and potential binding sites for transcription factors identified by footprinting studies are shown in bold. The TATA box proximal E2 binding sites are shown underlined. Mutations have been introduced into these sites preventing E2 binding as these have been shown to mediate down-regulation of transcription at high levels of E2. Promoter deletions are indicated by arrows. **(b) E2 responsive promoter deletion constructs**. A series of 5' deletions of the BPV-4 promoter were PCR amplified as BglII-HindIII fragments. These fragments were cloned into pGL36E2 which contains six E2 DNA binding sites inserted into the BglII site of pGL3.

Characterisation of the BPV-4 promoter deletion mutants



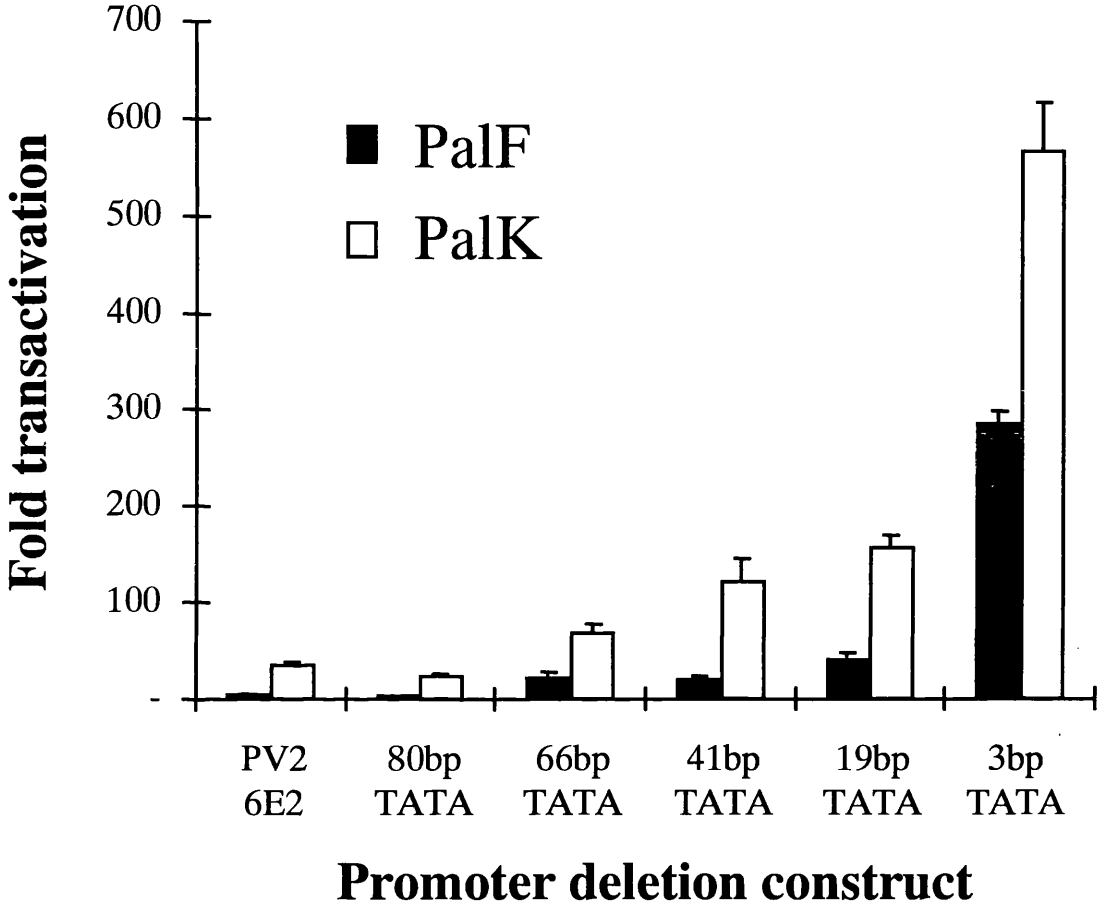


Fig. 3.1.16. Deletion analysis of the BPV-4 promoter identifies two repressor elements. PalK and PalF cells were cotransfected with1 μg of the indicated reporter plasmid and 0.1 μg of pCMV HPV-16 E2 expression vector. This ratio has previously been shown to be optimal for maximal activation of the LCR promoter by E2. Results are expressed as fold activation relative to the luciferase activity of each reporter in the absence of E2.

Transcriptional activation of the BPV-4 promoter deletions by HPV-16 E2

Promoter deletion	PalF		PalK	
	Fold TA	se	Fold TA	se
PV2 6E2	5.06	0.97	36.06	2.97
80bp TATA	3.54	0.82	23.88	1.44
66bp TATA	23.09	4.92	68.83	9.06
41bp TATA	20.86	3.30	122.40	24.15
19bp TATA	40.52	6.85	157.68	12.77
3bp TATA	286.22	12.01	566.55	50.27

Table 3.1.1. Numerical representation of the results shown in Fig. 3.1.16.

Transcriptional activation of the minimal BPV-4 TATA containing promoter

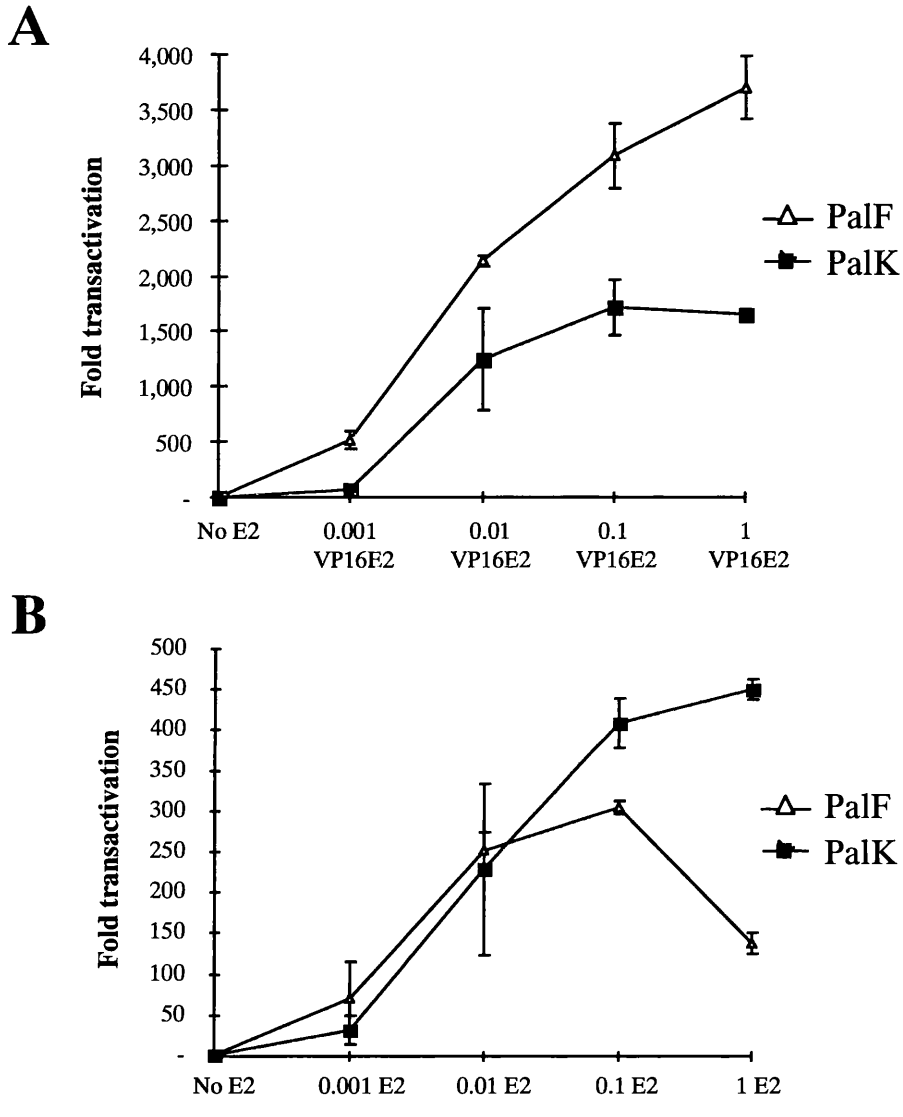


Fig. 3.1.17. PalK and PalF cells were cotransfected with 1 μg of the 3bpTATA construct, which contains neither an initiator element nor a binding site for an upstream factor, and the indicated amounts of either **(a)** pCG VP16 E2 or **(b)** pCMV HPV-16 E2 expression vectors. PCMV or pCG was used to make the total amount of DNA transfected equal in all cases. Results are expressed as fold transactivation over the luciferase activity in the absence of activator.

Repressor constructs

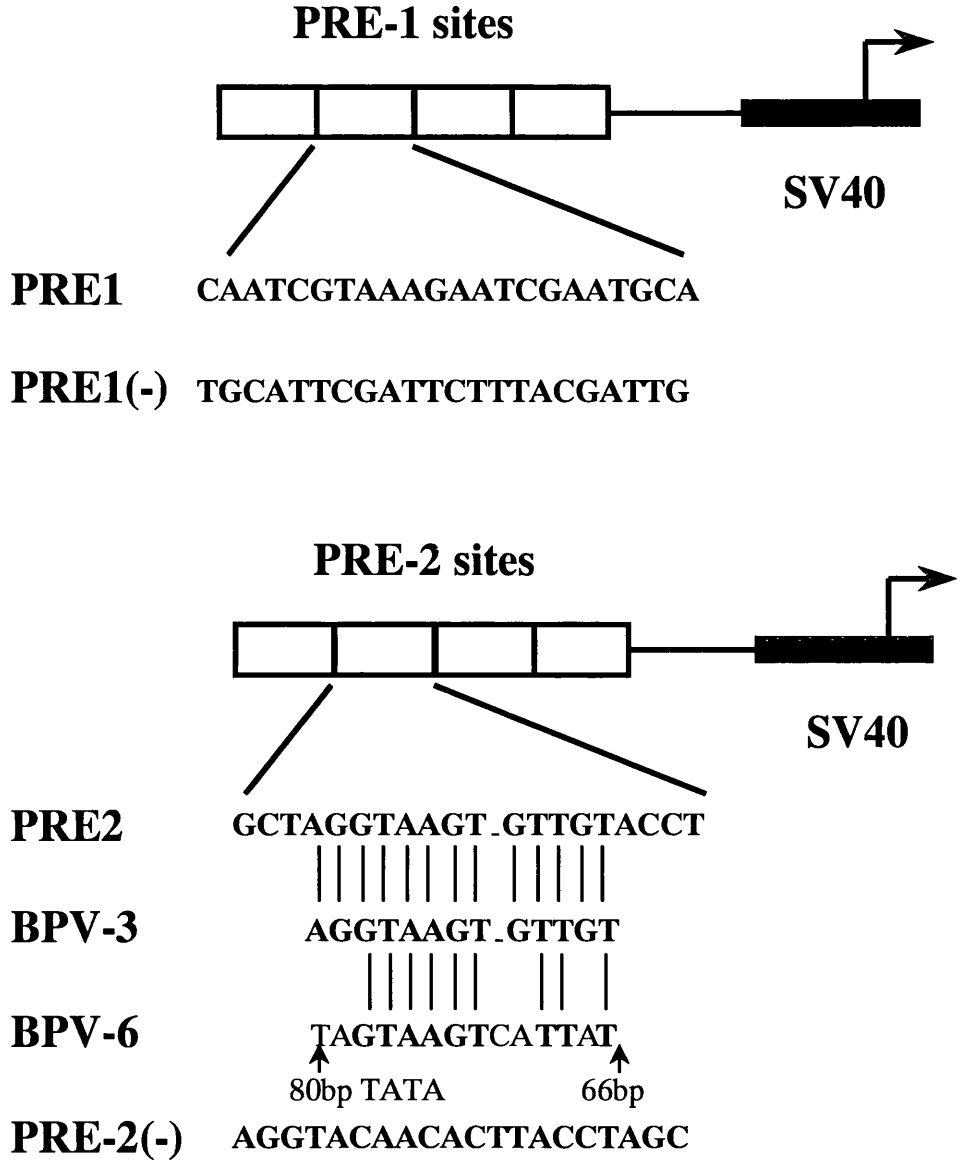


Fig. 3.1.18. PRE SV40 constructs. The PRE-1 and PRE-2 elements were multimerised and inserted into the BglII site, upstream of the SV40 promoter in the pGL3 luciferase vector, in both a positive and negative orientation. The position and sequence of PRE-2 is conserved between the mucosal epitheliotropic papillomaviruses BPV-4, BPV-3 and BPV-6.

PRE-1 mediated repression of SV40 promoter activity

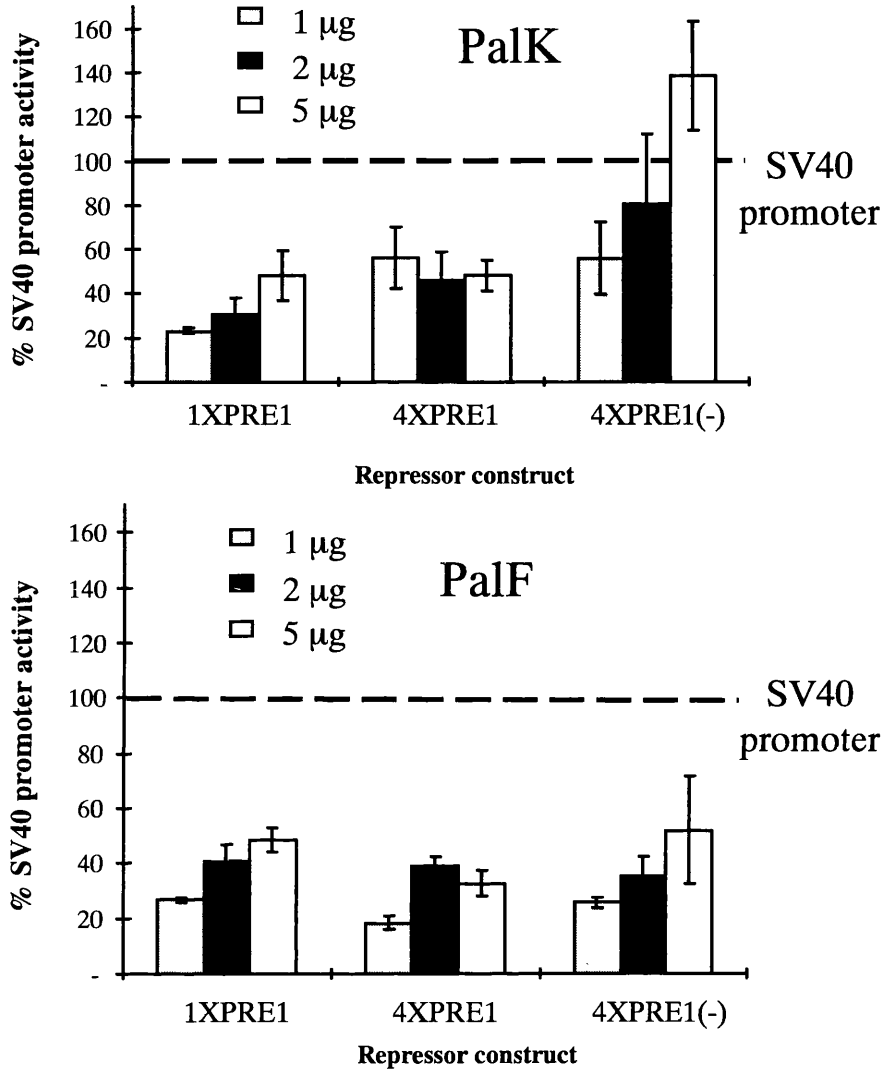


Fig. 3.1.19. PRE-1 strongly represses SV40 promoter activity in a cell type independent manner. PalK and PalF cells were transfected with the indicated amounts of pGL3PRO, PRO1XPRE1, PRO4XPRE1 and PRO4XPRE1(-). Results are expressed relative to the lucifierase activity of pGL3PRO (set arbitrarily at 100%) which contains only the SV40 promoter.

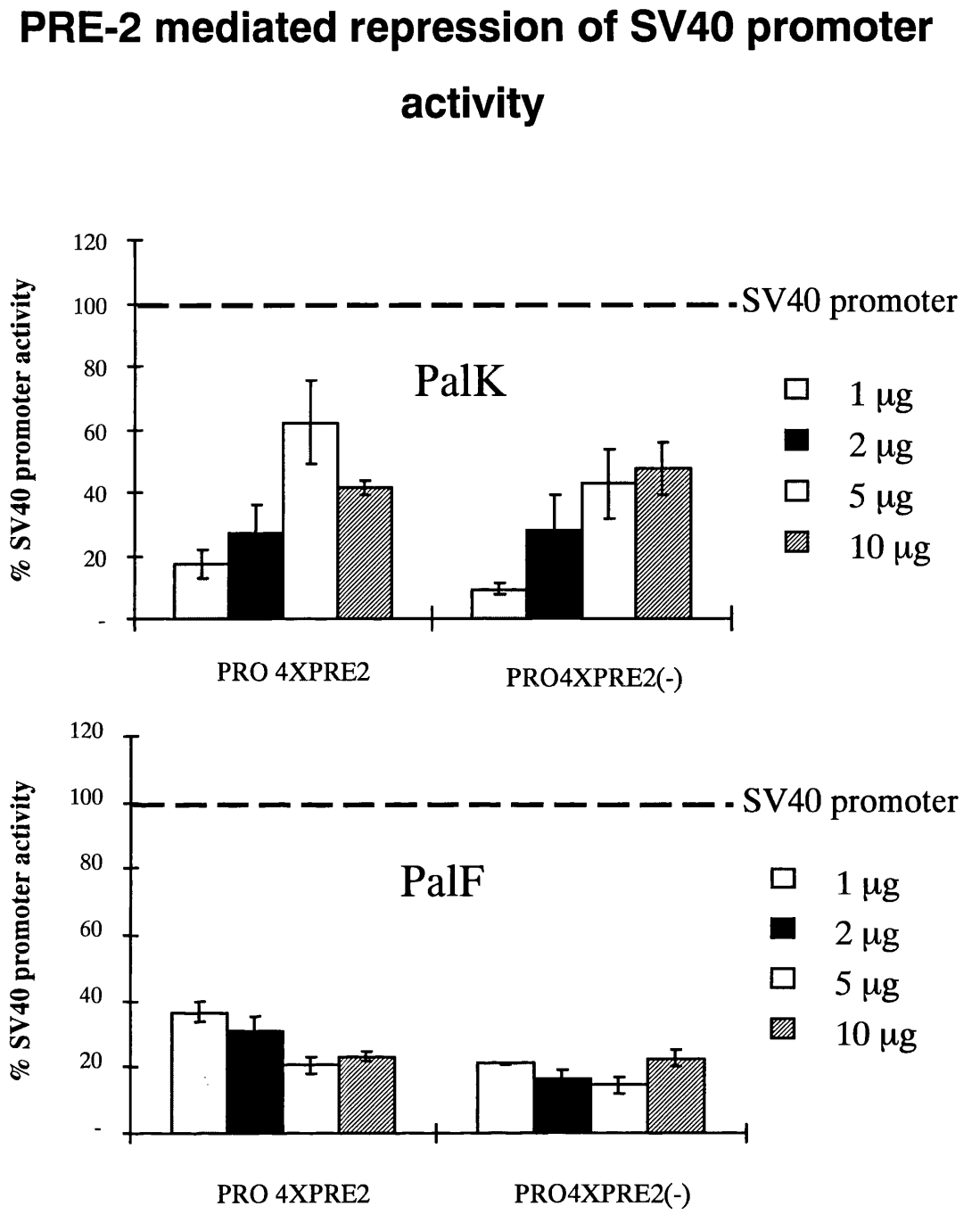


Fig. 3.1.20. PRE-2 strongly represses SV40 promoter activity in an orientation independent manner. PalK and PalF cells were transfected with the indicated amounts of pGL3PRO, PRO4XPRE2 and PRO4XPRE2(-). Results are expressed relative to the luciferase activity of the SV40 promoter alone (set at 100%).

EMSA analysis to detect nuclear proteins interacting with PRE-2

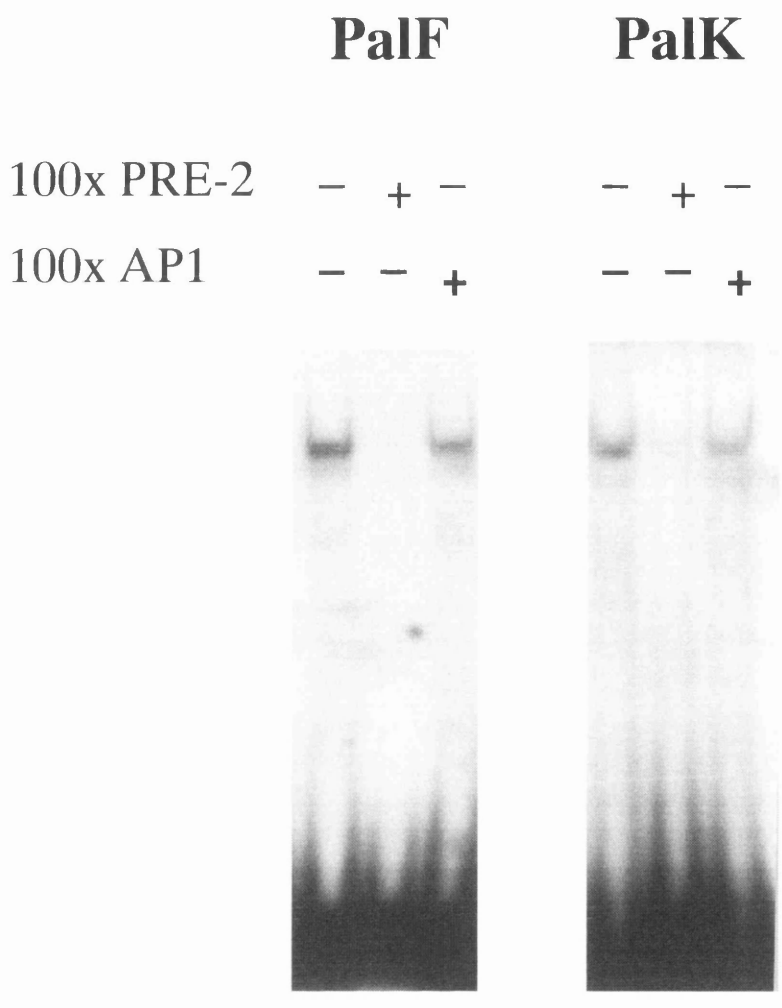


Fig. 3.1.21. PRE-2 binds a specific protein complex in both PalK and PalF cells. A single radiolabelled PRE-2 motif was used to probe PalK and PalF nuclear extracts in a band shift assay. 100-fold excess unlabelled PRE-2 and AP-1 oligonucleotides were used as indicated to assess the specificity of binding. Retarded complexes were resolved on a 6% polyacrylamide gel and visualised by autoradiography.

Fig. 3.1.22. (a) Sequence of PRE-2 mutants. Footprinting studies demonstrate PRE-2 contains a potential binding site for a transcription factor (shown underlined). Two double stranded oligonucleotides, each containing a single PRE-2 motif with a three base pair substitution in this footprint, were generated and tested for competition in the band shift assay. **(b) PRE-2mt1 and PRE-2mt2 do not compete for binding to the detected protein complex.** A single radiolabelled PRE-2 motif was used in a band shift assay to probe PalK and PalF nuclear extracts. Cold competitors were added as indicated at either 100-fold (+) or 500-fold (++) molar excess as shown. Binding reactions were electrophoresed on a 6% polyacrylamide gel, the gel was dried and visualised by autoradiography.

Generation of PRE-2 non-binding mutants

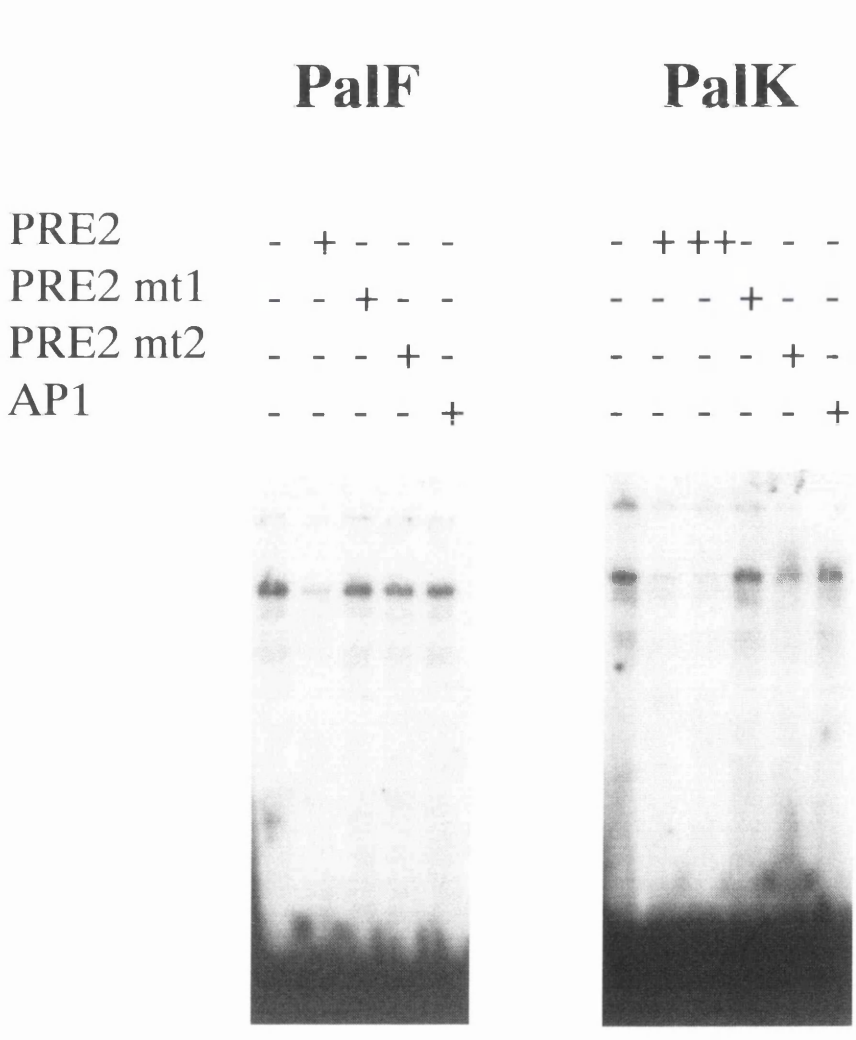
A

PRE-2 5' gctaggtaagtgttgtacct 3'

PRE-2 mt1 5' gctaggt**cct**tgttgtacct 3'

PRE-2 mt2 5' gctaggtaagt**agg**gtacct 3'

B



Functional consequences of loss of binding on PRE-2 mediated repression

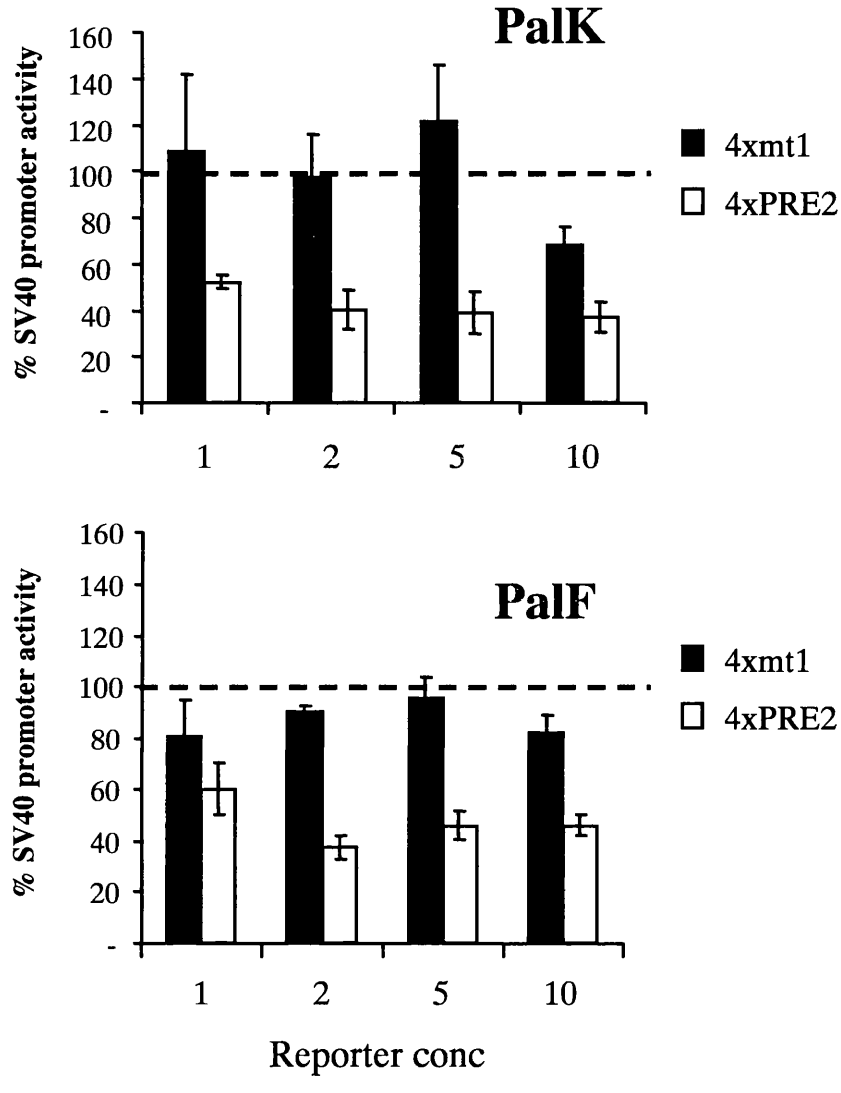


Fig. 3.1.23. Loss of complex binding to PRE-2 correlates with loss of transcriptional repression. Four copies of mt1 were inserted into the BglII site, upstream of the SV40 promoter in the pGL3PRO luciferase vector, generating PRO4Xmt1. PalK and PalF cells were transfected with the indicated amounts of pGL3PRO, PRO4XPRE-2 and PRO4Xmt1. Results are expressed relative to the luciferase activity of pGL3PRO (set arbitrarily at 100%) which contains only the SV40 promoter.

Transcriptional response of the 80bpmt1 deletion construct to HPV-16 E2

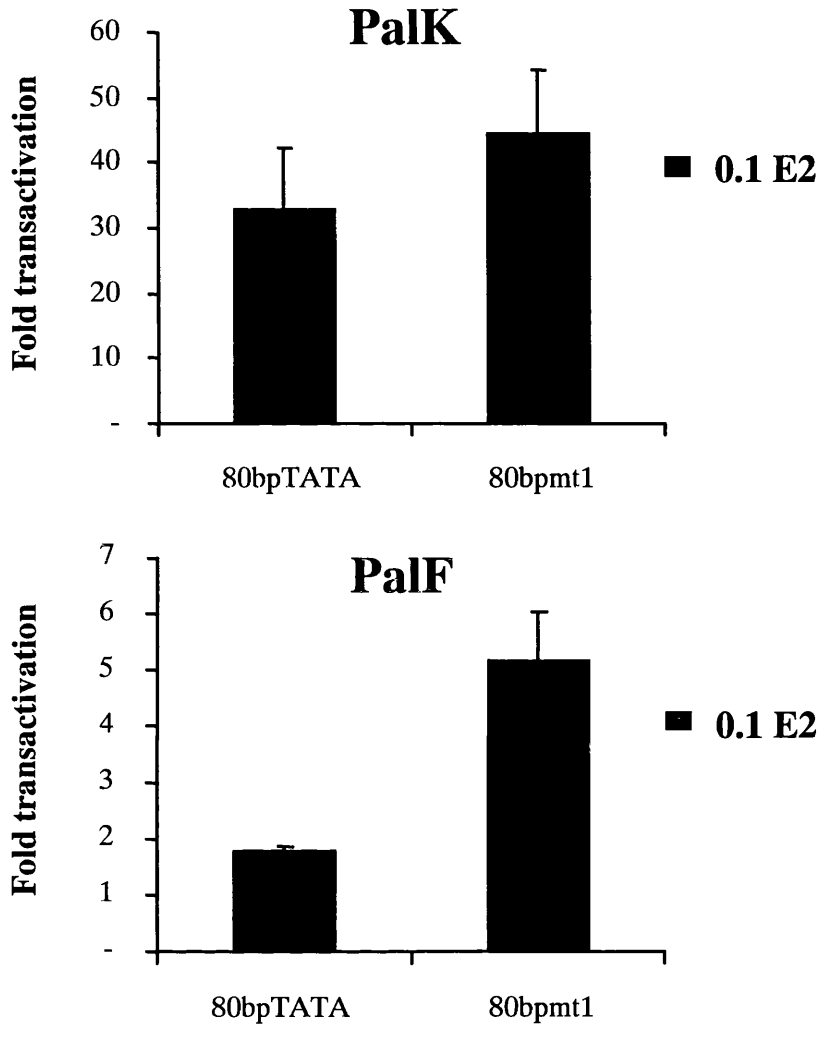


Fig. 3.1.24. The PRE-2 binding protein represses the transcriptional response to HPV-16 E2. PRE-2 mt1 was introduced into the 80bp TATA promoter construct generating 80bpmt1. 1 μg 80bpTATA and 80bpmt1 reporter constructs were cotransfected into PalK and PalF cells with 0.1 μg pCMV HPV-16 E2 expression vector. A 0.1:1 ratio of E2 to reporter has previously been shown to be optimal for maximal activation of the BPV-4 LCR by E2. Results are expressed as fold transactivation relative to the luciferase activity of each reporter in the absence of E2.

UV crosslinking of a nuclear protein to PRE-2

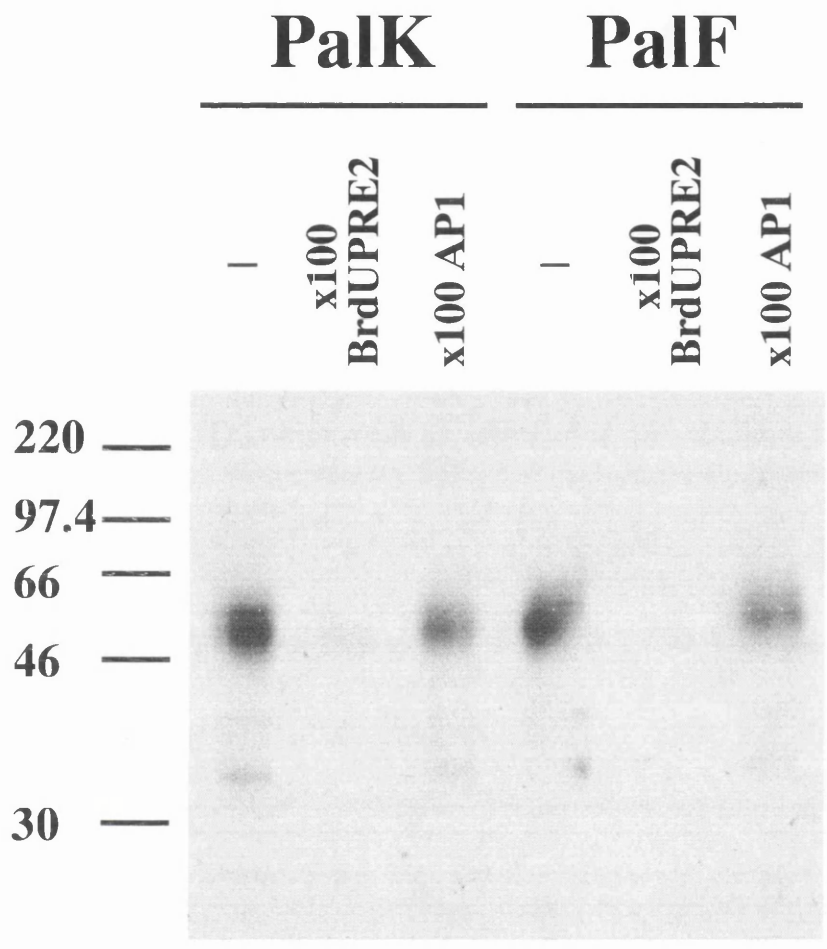


Fig. 3.1.25. Molecular weight determination of the PRE-2 binding factor. PalK and PalF nuclear extracts were probed with a radiolabelled BrdU substituted PRE-2 motif in a band shift assay. After polyacrylamide gel electrophoresis and UV exposure the PRE-2 crosslinked protein complexes were excised and separated on a 10% SDS gel. Competition band shift reactions with either 100-fold non-labelled BrdU or AP-1 were performed as indicated to assess the specificity of binding.

3.2. Analysis of the interactions between the HPV-16 E2 transactivation domain and cellular proteins

The product of the papillomavirus E2 ORF is required for viral transcriptional regulation and DNA replication and is therefore essential for the viral life cycle (for a review see (Desaintes and Demeret, 1996)). The E2 protein can be divided into three functional domains: an amino terminal transactivation domain, a central flexible hinge region and a carboxy terminal dimerisation and DNA binding domain (see (Ham et al., 1991b) for a review). The structure and function of the amino and carboxy terminal domains is relatively conserved among human and animal papillomavirus, while the hinge region is of indeterminant function and is highly variable in sequence and length. The E2 protein binds to the 12bp palindromic DNA sequence -ACCGNNNNCGGT- as a dimer. All papillomavirus LCRs contain DNA binding sites for E2. The E2 carboxy terminus localises active E2 dimers at the target promoter while the E2 transactivation domain is essential for transcription and replication of the viral genome. Mutational analysis of the BPV-1 and HPV-16 E2 amino terminus has demonstrated that the ability of E2 to regulate transcription and replication can be separated, indicating that the proteins with which E2 interacts to carry out these two functions are different (Brokaw et al., 1996; Ferguson and Botchan, 1996; Grossel et al., 1996; Sakai et al., 1996)].

E2 can also disrupt cellular growth control in certain mammalian cell lines and in yeast. Overexpression of different papillomavirus E2 proteins can induce a growth arrest in both the G1 and G2/M phases of the cell cycle, and can induce apoptosis through both p53 dependent and independent mechanisms (see (Massimi et al., 1999) and references therein). The exact mechanism of how E2 functions to disrupt cellular

growth control remains unclear and seems to differ between E2 proteins. For example, induction of apoptosis by BPV-1 E2 seems to be p53 independent (Desaintes et al., 1999) whereas HPV-16 E2 can induce apoptosis in certain HPV transformed and non-HPV transformed cell lines through p53 dependent mechanisms (Webster et al., 2000). Mutations that block the DNA binding activity of HPV-16 E2 do not impair the ability of HPV-16 E2 to induce apoptosis, while removal of both amino terminal domains of the E2 dimer completely blocks HPV-16 E2-induced cell death (Webster et al., 2000).

3.2.1. Interaction of proteins with the HPV-16 E2 transactivation domain

To identify proteins that interact with the HPV-16 E2 transactivation domain, the cDNA encoding amino acid 2 to 229 was PCR cloned as a BamHI-EcoRI fragment into the pGEX-2TK expression vector immediately upstream of the glutathione-S-transferase (GST) and protein kinase A recognition site (RRASV) (Fig. 3.2.1a). To assess the potential significance of any interaction in transcriptional activation a mutant E2 fusion protein, which has amino acid 73 mutated from an isoleucine to an alanine, was generated by 'splicing by overlap extension' PCR (Fig. 3.2.1a). This mutant E2 protein retains the ability to support viral DNA replication but fails to activate transcription (Sakai et al., 1996). Internal primers containing the att to gct mutation were designed for the primary PCR reaction. Two PCR products containing 20 bp overlapping ends were generated and used as template for a further round of PCR. The second PCR reaction using primers annealing at the non-overlapping ends was used to amplify the I73A mutant transactivation domain as a BamHI-EcoRI fragment. This was again inserted into pGEX-2TK.

The GST-E2 and GST-I73A proteins were bacterially expressed, purified with glutathione beads (Fig. 3.2.1b) and *in vitro* ^{32}P labelled using the catalytic subunit of protein kinase A (Fig. 3.2.1c). Both GST-E2 and GST-I73A were expressed to similar levels in *E. coli* and appeared to be identically labelled *in vitro*. Equal amounts of the ^{32}P labelled probes were then incubated with immobilized, renatured PalK, PalF and HeLa whole cell extracts in a Far Western blot assay to detect proteins that directly interact with the E2 transactivation domain. Fig. 3.2.2 shows that the E2 transactivation domain can directly interact with at least 12 cellular proteins in PalK cells and at least 8 in PalF cells. There are obvious differences in the pattern of cellular proteins with which E2 interacts in these two cell types. Although the functional significance of these interactions are unknown they may represent proteins involved in mediating some of the epithelial specific functions of E2. At least 8 cellular proteins interact with the wild type E2 transactivation domain in HeLa cells (an HPV-18 immortalised keratinocyte cell line). There are no obvious differences between the factors interacting with the I73A mutant transactivation domain, which is defective for transcriptional activation, compared to wild type E2. This result suggests that there are additional factors, necessary for transcriptional activation by E2, that interact with the wild type amino terminus but not with the I73A mutant that cannot be detected using this assay. Radiolabelled GST alone does not interact with any cellular factors when used as a probe under the same conditions (data not shown).

3.2.2. Expression cloning to identify proteins interacting with the HPV-16 E2 amino terminus

To isolate cDNAs encoding cellular proteins that interact directly with the E2 amino terminus, an oligo-dT primed, λTriplex HeLa cDNA expression library was screened with bacterially expressed, ^{32}P labelled GST-E2. λTriplex expresses each cDNA in all three open reading frames increasing the likelihood that a recombinant vector containing the target cDNA will be detected by expression screening. The λTriplex multiple cloning site is located within an embedded plasmid, which is flanked by loxP sites at the λ junctions. This plasmid (pTriplEx) can be released automatically by cre-recombinase-mediated recombination at the loxP sites when transduced into bacteria expressing cre-recombinase. $2x10^6$ independent clones were initially screened (the first round of screening was carried out by W. Boner and I. Morgan). This number is representative of about one third of the cDNA population present in the library. Seven positive clones were obtained after the primary screen. The proteins produced in positive plaques were purified by subsequent rounds of screening. Amplification of positive plaques was observed with each successive round. After the tertiary screen the plasmid clone was excised from the positive phage by transduction into *E.coli* BM25.8 cells. Colonies were directly PCR screened to determine the size of the insert. The sequence of the positive cDNAs were compared with those available in databases using basic logic search alignment (BLAST).

Clone	Homology	Comments
1	L31 ribosomal protein complete open reading frame	Human ribosomal protein mRNA with increased expression in colorectal tumours
2	Triplex cloning vector	False positive
3		Two inserts of different sizes obtained- plaques not purified
4	Unknown cDNA	Identical to clone 5
5	Unknown cDNA	Identical to clone 4
6		Two inserts of different sizes obtained- plaques not purified
7	Partial human immunoglobulin superfamily 4 gene (ISFG4) sequence	ISFG4 spans the tumour suppressor gene locus 11q23.2

Table 3.2.1. Sequence analysis of cDNAs interacting with the HPV-16 E2 transactivation domain

The sequence analysis of cDNAs interacting with the E2 transactivation domain is shown in Table 3.2.1. Although, no proteins previously shown to interact with the E2 transactivation domain, such as Sp1 (Li et al., 1991), TFIIB (Yao et al., 1998) and AMF-1 (Breiding et al., 1997) have been identified, verification that the screen has been successful comes from clones 4 and 5 which represent the same cDNA independently isolated. BLAST searches reveal that this cDNA contains no extensive homologies to any known genes. PCR screening shows that clones 3 and 6 contain inserts of different sizes (data not shown) and require subsequent screening steps to purify the positive plaque. Clone 2 represents the λTriplex cloning vector and is a false positive. This is probably due to misalignment of the nitrocellulose filter and

the agar plate whilst picking a positive plaque in the tertiary screen. Clone 7 contains the partial sequence of the immunoglobulin superfamily 4 gene (ISFG4). ISFG4 is transcribed into a 1.6 or 4.4 kb RNA encoding a 442 amino acid protein (Gomyo et al., 1999). IGSF4 spans the tumour suppressor gene locus 11q23.2. Deletion of this region has been associated with cancer of the lung and breast and with neuroblastoma (Gomyo et al., 1999). Clone 1 encodes the human L31 ribosomal protein (accession number NM_000993), a component of the 60S large ribosomal subunit. The L31 ribosomal protein cDNA and complete open reading frame is shown in figure 3.2.3. The signal peptide responsible for nucleolar localisation, RLSRKR, is shown underlined (Quaye et al., 1996).

3.2.3. L31 ribosomal protein and EIF3 (E2 interacting factor 3) bind the HPV-16 E2 transactivation domain *in vitro*

L31 was identified from a normal colon cDNA library on the basis of overexpression in familial adenomatous polyposis (Chester et al., 1989). L31 is also expressed at abnormally high levels in various haematopoietic malignant tumour cells and differentiation of the K562 erythroleukaemia cell line is associated with a coordinate decrease in expression of L31 mRNA (Lin et al., 1994; Shimbara et al., 1993). EIF3 was isolated using a yeast two hybrid screen to identify cellular proteins that interact with the E2 amino terminus (Boner and Morgan, unpublished). An E2 mutant transactivation domain which has amino acid 39 mutated from a glutamic acid to an alanine (E39A) was used in this screen. The E39A mutant protein fails to activate transcription in yeast but still activates transcription in mammalian cells. This mutation abolishes the interaction between E2 and E1, the viral replication factor,

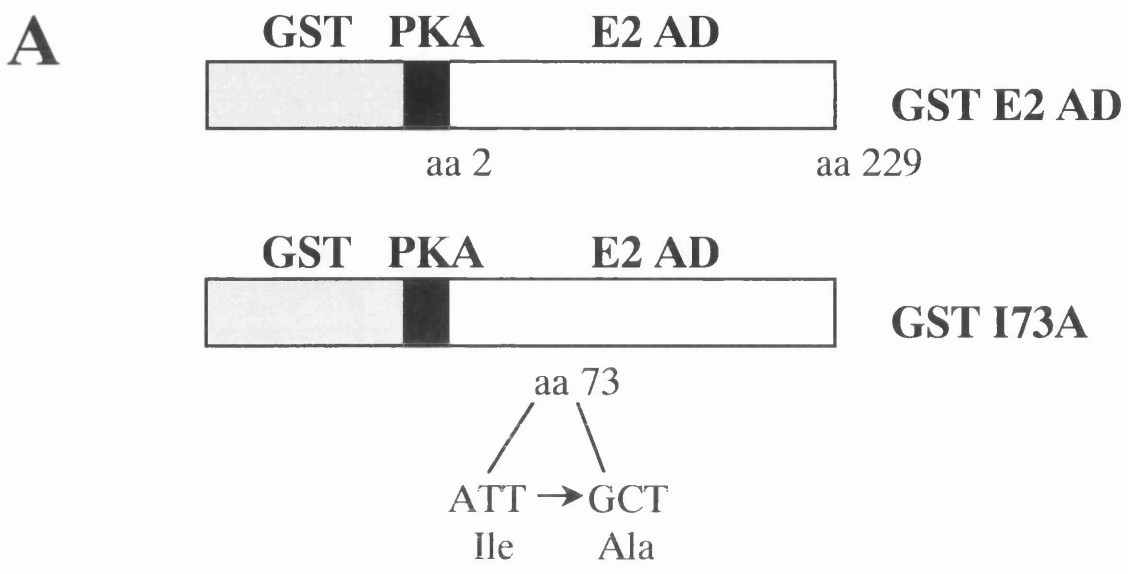
inhibiting the ability of E2 to enhance viral DNA replication (Sakai et al., 1996). EIF3 encodes a truncated version of the $p27^{BBP}$ protein (accession number Y11435), which has been shown to bind the cytoplasmic domain of integrin β_4 (Biffo et al., 1997). $p27^{BBP}$ is induced in mast cells by allergic reaction (Cho et al., 1998) and has also been identified as the putative eukaryotic translation initiation factor (eIF6) based on its *in vitro* ability to inhibit the association between the 40S and 60S ribosomal subunits (Si et al., 1997). $p27^{BBP}$ is present in both in the nucleolus and associated with the nuclear matrix in all cells analysed, and is also in the cytoplasm enriched at the basal membrane of integrin β_4 expressing cells (Sanvito et al., 1999).

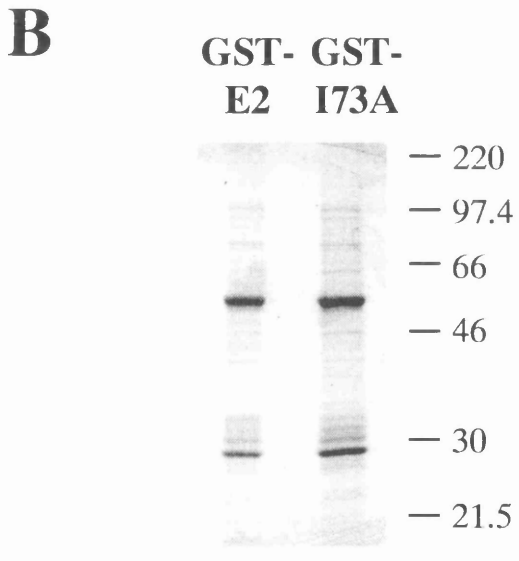
GST pull down assays were performed to determine whether L31 and EIF3 directly interact with the wild type E2 transactivation domain *in vitro*. The L31 and EIF3 cDNAs were cloned into the pGADT7 expression plasmid. L31 was PCR amplified as a EcoRI-XhoI fragment from pTriplEx-L31 whereas EIF3 was PCR amplified as a XmaI-XhoI fragment from pGADGH-EIF3. These fragments were inserted into pGADT7. The restriction sites chosen ensured that each protein would be expressed in frame under control of the T7 promoter. Bacterially expressed GST-E2, the HPV-16 E2 amino terminus from amino acids 2-229 fused to GST, was immobilised on glutathione beads and incubated with ^{35}S labelled *in vitro* translated L31 and EIF3. Fig. 3.2.4 shows that L31 interacts with GST-E2 *in vitro*. Approximately 5% input L31 bound to GST-E2. The binding observed between L31 and GST alone was strongly reduced when compared to L31 and GST-E2. *In vitro* translated ^{35}S labelled EIF3 interacts with both GST-E2 and GST at low salt concentration (Fig. 3.2.5a.). EIF3 was isolated using the GAL4 DNA binding domain-E2 E39A mutant amino terminus fusion protein as a bait in a yeast two hybrid screen. EIF3 does not interact

with the GAL4 DNA binding domain alone (Boner and Morgan, unpublished). To confirm that EIF3 specifically interacts with the wild type E2 transactivation domain *in vitro* GST pull down assays were performed with increasing salt concentrations. Binding of GST-E2 to EIF3 was detected at high salt concentrations (800 mM NaCl) demonstrating a high affinity interaction while the binding of GST to EIF3 decreased as the salt concentration increased.

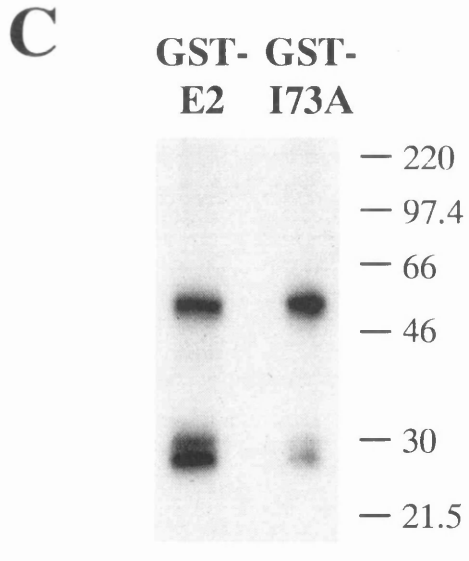
Fig. 3.2.1. (a) Schematic representation of the GST-E2 fusion proteins. The wild type E2 and I73A mutant transactivation domain from amino acid 2 to 229 were PCR amplified as BamHI-EcoRI fragments and inserted into the pGEX-2TK expression vector. The expressed chimaeric proteins contain GST and a recognition site for the catalytic subunit of protein kinase A fused to either the wild type or mutant E2 amino terminal domain. **(b) Coomassie blue staining of GST fusion proteins.** GST-E2 and GST-I73A were expressed in bacteria. Equal amounts of cell lysates were purified with glutathione beads and separated by 10% SDS-PAGE. Proteins were visualised by Coomassie blue staining. **(c) *In vitro* ^{32}P labelled probes.** The fusion proteins, expressed in bacteria, were radiolabelled on glutathione beads using the catalytic subunit of protein kinase A. Labelled proteins were eluted from the beads, equal volumes were separated by 10% SDS-PAGE and visualised by autoradiography.

Characterisation of GST-E2 fusion proteins





GST purification



^{32}P labelling

Far Western blot analysis of interactions between GST-E2, GST-I73A and cellular proteins

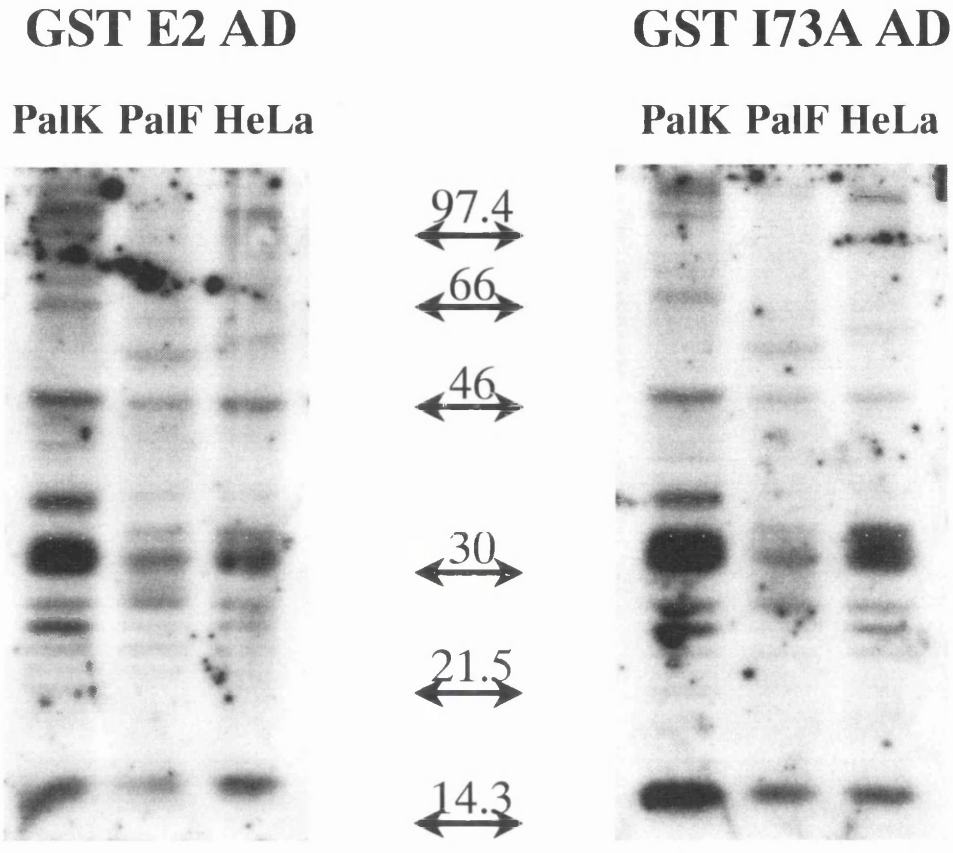


Fig. 3.2.2. Direct protein-protein interactions between ^{32}P labelled GST-E2, GST-I73A and cellular proteins. 60 μg PalK, PalF and HeLa whole cell extracts were separated by 10% SDS-PAGE, transferred to nitrocellulose and denatured/renatured in 6M to 0.187M guanidine hydrochloride. Membranes were probed with 200 000 cpm/ml of either ^{32}P labelled **(a)** GST-E2 or **(b)** GST-I73A and visualised by autoradiography.

L31 ribosomal protein sequence analysis

A

1 ccgcaga**atg** gctcccgcaa agaagggtgg cgagaagaaa aagggccgtt

51 ctgccatcaa cgaagtggta acccgagaat acaccatcaa cattcacaag

101 cgcatccatg gagtgggctt caagaagcgt gcacctcggg cactcaaaga

151 gattcggaaa tttgccatga aggagatggg aactccagat gtgcgcattg

201 acaccaggct caacaaagct gtctgggcca aaggaataag gaatgtgcca

251 taccgaatcc gtgtgcggct gtccagaaaa cgtaatgagg atgaagattc

301 accaaataag ctatatactt tggttaccta tgtacctgtt accactttca

351 aaaatctaca gacagtcaat gtggatgaga ac**taa**

B

1 MAPAKKGGEK KKGRSAINEV VTREYTINIH KRIHGVGFKK RAPRALKEIR

51 KFAMKEMGTP DVRIDTRLNK AVWAKGIRNV PYRIRVRLSR KRNEDEDSPN

101 KLYTLVTYVP VTTFKNLQTV NVDEN

Fig. 3.2.3. (a) L31 complete cDNA. Start and stop codons are shown in bold. **(b) L31 protein sequence.** Amino acids are shown using the single letter code. The peptide sequence responsible for nucleolar localisation is underlined.

In vitro binding of L31 and GST-E2

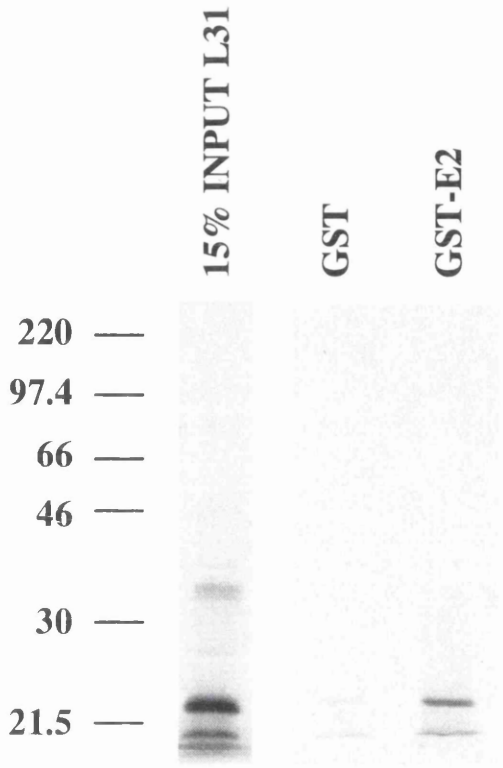


Fig. 3.2.4. E2 transactivation domain specifically interacts with L31 *in vitro*. ^{35}S labelled *in vitro* translated L31 was incubated with bacterially expressed GST or GST-E2 immobilised on glutathione beads. 15% of the input L31 is also shown. Bound proteins were separated by 10% SDS-PAGE and visualised by autoradiography.

In vitro binding of EIF3 and GST-E2

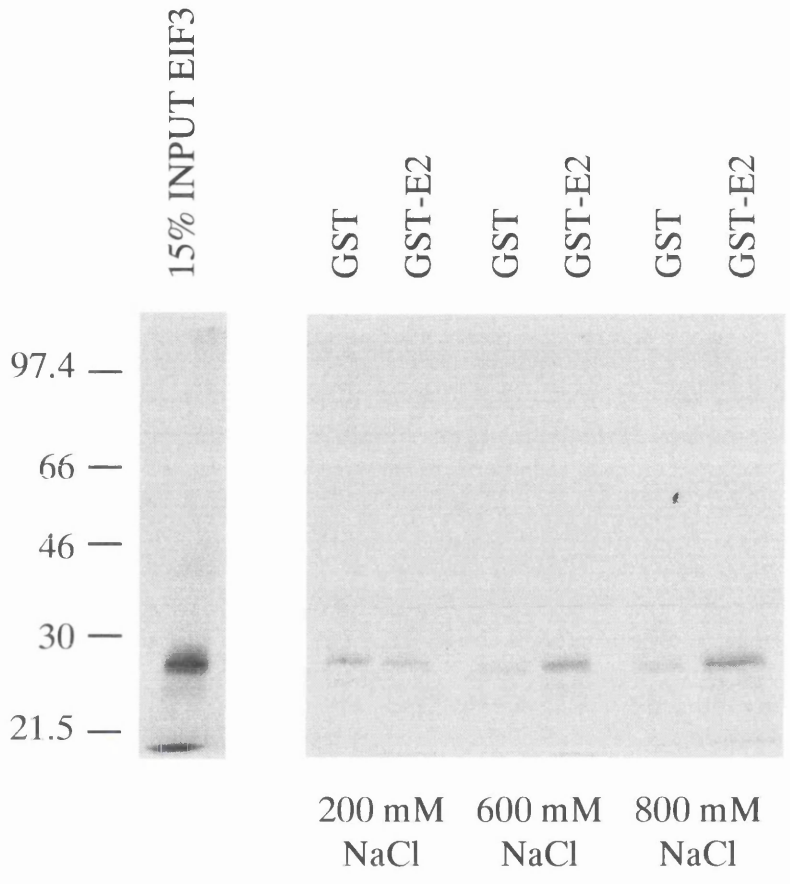


Fig. 3.2.5. E2 transactivation domain specifically binds EIF3 at high salt concentrations. GST and GST-E2 immobilised on glutathione beads were incubated with ^{35}S labelled *in vitro* translated EIF3 in the indicated salt concentrations. 15% input EIF3 is also shown. Bound proteins were separated by 10% SDS-PAGE and visualised by autoradiography.

Chapter 4 – Discussion

4.1. Cell type specific transcriptional regulation of the BPV-4 LCR

4.1.1. Transcriptional characterisation of the BPV-4 promoter

The rate of transcription initiation by RNA polymerase II (pol II) is controlled by cis acting DNA elements that are bound by specific transcription factors. The basal level of transcription of a gene and precise site at which transcription starts is determined by the general transcription factors that assemble at the core promoter to form the pre-initiation complex (for a review see (Roeder, 1996)). Recognition elements in the core promoter include the TATA box, located 25 to 30 bp upstream of the start site, and the initiator (Inr) element which spans the start site. The rate of transcription initiation is regulated by proximal and distal DNA elements that are bound by activator or repressor proteins. In general, this involves the promotion or inhibition of rate limiting steps in pre-initiation complex formation or function through interactions with one or more of the general transcription factors. Furthermore, eukaryotic DNA does not exist as naked DNA *in vivo* but is packaged into chromatin. Nucleosomes, which organise the structure of chromosomal DNA, negatively regulating gene expression. Activation of transcription by RNA pol II therefore requires the modification of chromatin structure to allow access of transcription factors to the DNA.

The genomes of double stranded DNA viruses, such as papillomaviruses, are also organised in the form of nucleosomes both in the viral capsids and in the nuclei of

infected cells (Stunkel and Bernard, 1999). The papillomavirus E2 protein, of which there is no human homologue, functions as the major papillomavirus encoded transcription factor and regulates viral gene expression through contacting components of the cellular transcription machinery. To study the mechanisms of E2 mediated transcriptional regulation of mucosal epitheliotropic papillomaviruses a series of increasing numbers of concatamers of E2 DNA binding sites were inserted upstream of the BPV-4 promoter (Fig. 3.1.1.). In PalK cells, the natural target cell type for transformation by BPV-4, HPV-16 E2 up-regulates transcription from the BPV-4 promoter in an additive manner increasing as the number of E2 DNA binding sites increases (Fig. 3.1.3.). Such an additive effect of pairs of E2 DNA binding sites has been observed previously (Gauthier et al., 1991; Ham et al., 1991a; Thierry et al., 1990). However, many transcriptional activators have been shown to function synergistically. Synergism is thought to be mediated by an activation domain contacting multiple components of the general transcription machinery. E2 has also been shown to synergistically activate transcription when E2 sites are inserted upstream from heterologous promoters (Hawley-Nelson et al., 1988). One E2 DNA binding site activates transcription weakly whereas two or more form a strong E2 inducible enhancer. Multiple E2 sites inserted upstream from the yeast CYC1 promoter has demonstrated that E2 mediated synergistic activation of transcription is accompanied by a modification of chromatin structure around the promoter (Lefebvre et al., 1997). Synergy does not result from cooperative DNA binding as E2 interacts with both a single and multiple copies of the strong E2 site used in this study with the same affinity *in vivo*. E2 does not upregulate transcription from the BPV-4 promoter in a synergistic manner when multiple E2 sites are inserted upstream raising the

possibility that the chromatin organisation of the BPV-4 promoter may be assembled into a transcriptionally active conformation in the absence of E2 in PalK cells.

Mutation of the TATA box proximal E2 DNA binding sites in the BPV-4 promoter results in an elevated response to transcriptional upregulation by E2 (Fig. 3.1.3.). This is in agreement with previous studies on the BPV-4 and HPV LCRs showing that the E2 interaction with BS1 and BS2 mediates down regulation of transcription by elevated levels of E2 (Jackson and Campo, 1995; Morgan et al., 1998; Romanczuk et al., 1990; Steger and Corbach, 1997). The LCR of all genital HPVs contain a conserved SP1 site separated from E2 BS2 by 1 bp. Binding of Sp1 to this element is displaced by increasing amounts of E2 (Demeret et al., 1994; Tan et al., 1992). A 3 bp insertion between the adjacent Sp1 and E2 BS2 sites allows both Sp1 and E2 to simultaneously bind the DNA resulting in the loss of E2 BS2 mediated repression (Dong et al., 1994a). However, there is no Sp1 site in the BPV-4 proximal promoter. As E2 can interact with TBP and E2 BS1 is separated from the TATA box by 3 bp it has been suggested that the interaction of E2 with BS1 displaces TBP from the TATA box resulting in a down-regulation of transcription initiation (Dostatni et al., 1991; Tan et al., 1994). Band shift assays suggest that E2 and purified human TBP cannot bind E2 BS1 and the TATA box simultaneously but when the distance between the E2 sites and TATA box is increased these two factors interact cooperatively with the DNA. In addition to steric hindrance of TBP binding E2 has also been suggested to function as an active repressor under certain conditions by preventing the assembly of a functional pre-initiation complex (Dostatni et al., 1991). *In vitro* experiments using an E2 dependent cell free transcription system reconstituted with purified factors demonstrates that HPV-11 E2 represses

transcription by directly targeting components of the basal transcription machinery after TBP has bound the DNA (Hou et al., 2000). Repression can be alleviated by pre-incubation of a minimal TFIID-TFIIB-RNA pol II-TFIIF pre-initiation complex but not by pre-incubation with TBP or TFIID alone.

E2 upregulates transcription efficiently from the BPV-4 LCR in PalK cells but does so only poorly in PalF (Morgan et al., 1998). This epithelial specific response to E2 is retained by the BPV-4 promoter region as over a range of increasing HPV-16 E2 concentrations the PV 6E2 construct is not transactivated more than two-fold in fibroblasts (Fig. 3.1.4a.). Mutation of the TATA box proximal E2 sites to prevent E2 binding does not affect the epithelial specific response of the BPV-4 promoter to E2 (Fig. 3.1.4 b,c.). However, the competition between E2 and cellular factors may represent an important control point in the regulation of viral gene expression during the papillomavirus life cycle. Differential effects on transcription would be observed depending on the concentration of E2 in the cell. Cellular nuclear factors have been demonstrated to produce DNAse I footprints over E2 BS2 and BS3 in the BPV-4 LCR. Although the factor interacting with E2 BS2 remains to be characterised, the cellular factor PEBP2 binds E2 BS3 and upregulates BPV-4 LCR promoter activity (Jackson and Campo, 1995). The PEBP2 transcription factor family, which includes the human AML1 gene, are heterodimers composed of two groups of subunits, α and β. The α subunit is expressed in a cell type specific manner while the β subunit appears to be ubiquitous (Ogawa et al., 1993). The PEBP2 family appears to be involved in developmental regulation and differentiation, suggesting that PEBP2 may be one of the cellular factors that couple the BPV-4 life cycle to keratinocyte

differentiation. Unknown positively acting cellular factors have also been shown to bind E2 BS1 and BS3 within the HPV-16 LCR (Lewis et al., 1999).

Failure of E2 to function in fibroblasts is not due to lack of expression, as western blot analysis shows that E2 is being expressed, under control of the cytomeglovirus promoter, at similar levels as a doublet of approximately 42 kDa in both PalK and PalF cells (Fig. 3.1.5). This suggests that HPV-16 E2 is post-translationally modified, for example, by phosphorylation or glycosylation, when expressed in PalK and PalF cells. BPV-1 E2 is phosphorylated at two major sites, ser 298 and 301, located in the hinge region (McBride et al., 1989a). Phosphorylation at these sites has been implicated in long term episomal maintenance of BPV-1 viral genomes (Lehman et al., 1997). Also, BPV-1 E2 proteins with a serine to alanine mutation at position 301 have an increased half life compared with wild type E2. It is suggested that phosphorylation at this position regulates E2 protein levels by targeting E2 for ubiquitin mediated degradation by the proteosome (Bastien and McBride, 2000). HPV-16 E2, which is a smaller protein than full length BPV-1 E2 due to a shorter hinge, is phosphorylated when expressed from recombinant baculoviruses in insect cells (Sanders et al., 1995). It therefore remains possible that post-translational modification of E2 may facilitate the interaction between E2 and cellular proteins in a cell type dependent manner resulting in an increased transcriptional activation in epithelial cells.

Chimaeric E2 molecules were used to assess the contribution of the E2 functional domains to epithelial specificity. It has been suggested that the E2 transactivation domain interacts with a specific cellular protein(s) to restrict activation of the BPV-4

LCR to epithelial cells as a VP16-E2 chimaera upregulates transcription from the BPV-4 LCR in a cell type independent manner (Morgan et al., 1998). However, the BPV-4 promoter shows an enhanced epithelial response to activation, not only by HPV-16 E2 (Fig. 3.1.4.) and BPV-1 E2 (Fig. 3.1.6a.), but also by VP16-E2 (Fig. 3.1.6b.) and VP16-LexA (Fig. 3.1.7b.). The difference in response of the BPV-4 LCR and BPV-4 promoter to activation by VP16-E2 can be explained by the observation that the BPV-4 promoter constructs have similar background transcriptional activity in PalK and PalF cells in the absence of E2. The full length LCR is approximately 40-fold more active in keratinocytes than fibroblasts in the absence of E2 due to the presence of the epithelial specific enhancer making the interpretation of changes in fold activation more complex. The enhanced epithelial response of the BPV-4 LCR to E2 is therefore more of a property of the BPV-4 promoter region which responds better to transcriptional activators in epithelial cells. This enhanced activation in epithelial cells is a promoter specific effect as the tk promoter shows no such epithelial preference to activation by VP16-E2 (Fig. 3.1.8b.) and HPV-16 E2 (Fig. 3.1.9.).

Like many other transcriptional activators E2 interacts with TBP (Rank and Lambert, 1995). However, the ability of activators to stimulate TBP recruitment to the promoter, the first step in the assembly of the pre-initiation complex, is a tightly controlled cooperative process involving multiple transcription factors (Li et al., 1999). TBP overexpression upregulates basal activity and potentiates E2 mediated activation of the PV2 promoter preferentially in PalK cells when compared with PalF (Fig. 3.1.10.). It is therefore possible that TBP is recruited more efficiently to the BPV-4 promoter in the absence of E2 in keratinocytes than in fibroblasts. These

results also suggest that E2 and TBP either cooperate with positively acting BPV-4 promoter factors to activate transcription preferentially in keratinocytes or that negatively acting factors, such as Dr1 which directly associates with TBP (for a review see (Maldonado et al., 1999)), block the transcriptional response to E2 in fibroblasts. As the basal and activated PV2 promoter activity are both upregulated by TBP overexpression these results are consistent with the hypothesis that the E2 transactivation domain functions by affecting a step in the assembly of the pre-initiation complex after TBP has bound the DNA (Steger et al., 1995). In agreement with previous studies demonstrating that cooperativity between E2 and TBP depends on core promoter structure (Ham et al., 1994), the tk promoter shows a different response than the PV2 promoter to TBP overexpression (Fig. 3.1.11.). Overexpression of TBP increases E2 mediated activation of the tk promoter in PalK cells while in PalF low amounts of TBP increase the ability of E2 to activate transcription while at high levels E2 mediated transcription is downregulated. TBP overexpression has a moderate effect on basal tk promoter activity in both cell types. The tk promoter, which is activated by E2 to similar levels in both PalK and PalF cells, does not show any significant cell type preference to TBP overexpression. Therefore, it could be suggested that the ability of a promoter to respond to TBP overexpression may reflect the differential response to upstream activators.

4.1.2. Possible mechanisms of cell type specific gene expression

The 127 bp BPV-4 promoter region contains the TATA box and presumably as yet unidentified upstream promoter elements but does not have the putative initiator element identified in the BPV-4 LCR. The enhanced epithelial response of the BPV-

4 promoter may be determined by DNA bound positively acting proximal promoter factors that are either cell type specific, differentially expressed, or modified in a cell type dependent manner. Proximal promoter factors conferring cell type specificity have been described previously, for example, hepatic nuclear factor 1 (HNF-1), a liver specific transcription factor, regulates liver specific expression of the albumen promoter. Also, epithelial specific functions of transcription factors interacting with HPV promoter regions, such as NF1-C/NF1-X and Sp1/Sp3, also exist (Apt et al., 1994; Apt et al., 1996). Cell type specific gene expression can also be determined by cell type specific components of the basal transcription machinery assembled at the TATA box or by cell type specific adaptor proteins mediating the interaction between the basal machinery and upstream factors. An involvement of the core TATA box region in cell type specific transcription has been described for melanocyte specific expression of the human tyrosinase promoter (Bentley et al., 1994) and brain specific transcription of the mouse myelin basic protein promoter (Tamura et al., 1990). Also, a TBP associated factor highly expressed in B lymphocytes, $TAF_{II}105$, has been identified (Dikstein et al., 1996). In addition to positively acting factors, sequence specific transcriptional repressors may play a role in restricting the expression of genes to specific cell types. For example, MyoD and E12 are basic helix loop helix proteins that recognise similar E-box motifs in the regulatory regions of target genes. MyoD is a muscle-specific transcriptional activator while E12 is a B-cell activator. The immunoglobulin heavy (IgH) chain enhancer, which contains the same E-boxes as the myogenic muscle creatine kinase (MCK) enhancer, is activated by E12 but not by MyoD. The IgH enhancer is able to discriminate between MyoD and E12 through a cis acting negative element flanking one of the E-boxes that specifically targets MyoD. This suggests that MyoD only activates myogenic genes, not only because its

expression is restricted to muscle, but also because non-muscle enhancers that contain E-boxes also contain negative elements that prevent MyoD activity (Weintraub et al., 1994).

4.1.3. The upstream BPV-4 promoter region is an important determinant of cell type specific transcription

E2 responsive chimaeric promoters, made by exchanging regions of the heterologous tk promoter with the corresponding regions in the BPV-4 promoter, suggest that the upstream BPV-4 promoter region determines the cell type selective response of this promoter to upstream activators. The lcr/tk hybrid, the upstream BPV-4 promoter sequence from nucleotide 184-279 fused to the core tk TATA box containing region, retains the enhanced epithelial response of the BPV-4 promoter to activation by E2 and VP16-E2 (Fig. 3.1.13). The tk/lcr hybrid, the upstream tk promoter sequence containing the Sp1 element fused to the core BPV-4 TATA region from nucleotide 279-310, shows no epithelial preference to activation by E2 and VP16-E2 (Fig. 3.1.14). Deletion analysis of the BPV-4 promoter confirms a role for the upstream promoter region in mediating epithelial specificity. The results identify two repressor elements that are, at least in part, responsible for the differential response of the BPV-4 promoter to upstream activators in fibroblasts and keratinocytes (Fig. 3.1.16 and Table 3.1.1). Database searches reveal that these elements show no homology to any of the published transcription factor binding sites. The PRE-1 region spans the TATA box proximal E2 binding sites that have been mutated to prevent E2 binding. These mutations do not affect the epithelial specific response of the BPV-4 promoter to E2 (Fig. 3.1.4). The PRE-2 element is conserved in position and sequence in the related mucosal epitheliotropic papillomaviruses, BPV-3 and BPV-6, suggesting

functional significance (Fig. 3.1.18). A silencer element in roughly the same position as PRE-2 in the HPV-16 LCR has been identified (O'Connor et al., 1998). This element, which binds the transcriptional repressor CCAAT displacement protein (CDP/Cut), represses the transcriptional activity of the HPV-16 enhancer. Subsequently, it has been shown that six other genital HPV types contain binding sites for CDP/Cut (O'Connor et al., 2000). Also, CDP/Cut is highly expressed in basal epithelial cells but not in differentiated primary keratinocytes suggesting that it is involved in coupling HPV gene expression to epithelial cell differentiation (Ai et al., 1999).

4.1.4. Analysis of the BPV-4 TATA region

Several lines of evidence suggest that the BPV-4 and tk TATA regions are functionally distinct. The overall levels of response of the lcr/tk hybrid are reduced compared with the PV2 promoter (Fig. 3.1.13.) while the levels of activation of the tk/lcr hybrid are higher than that of the tk promoter (Fig. 3.1.14.). This suggests that the BPV-4 TATA region is stronger than that of the tk promoter. Consistent with this, an *in vitro* transcription study using promoters containing different TATA sequences in the context of the adenovirus major late promoter has demonstrated that the TATATAA sequence, which corresponds to the BPV-4 TATA box, represents a strong TATA sequence. TBP binds this sequence with high affinity forming a stable TBP-TATA complex with a relatively slow rate of dissociation. It was suggested that the corresponding high rate of transcription initiation from the TATATAA containing promoter was due to the conformation of the TBP-TATA complex favouring recruitment of the general transcription factors. The interaction of TBP

with a weak TATA sequence such as TATTAAA, which differs only from the tk TATA sequence by the 3' A, forms a complex with a significantly faster rate of dissociation. Transcription from the TATTAAA containing promoter was less efficient and it was suggested that the TBP-TATA complex adopted a different conformation as measured by footprinting studies and DNA bend (Hoopes et al., 1998).

Also, a minimal BPV-4 TATA promoter containing neither an initiator element nor a binding site for an upstream factor is sufficient to support activated transcription by E2 (Fig. 3.1.16. and Table 3.1.1). In contrast, E2 requires the co-operation of at least one additional DNA binding proximal promoter factor, such as Sp1, for the activation of a minimal tk TATA box promoter (Ham et al., 1991a; Ushikai et al., 1994). This suggests that the general transcription machinery assembled at the BPV-4 and tk TATA boxes contain distinct coactivator complexes. Quanitative differences between the factors interacting with the HPV-16 and SV40 TATA elements and surrounding sequences have also been observed (Smits et al., 1993). The HPV-16 enhancer-promoter is virtually inactive in normal human diploid fibroblasts, but active in human fibroblasts with a deletion in the short arm of one chromosome 11 (del-11 cells). Del-11 cells are susceptible to transformation by HPV-16. Mutation of the HPV-16 TATAAAA box to the SV40 TATTTAT sequence reduces the activity of the HPV-16 enhancer-promoter in del-11 cells. DNA-protein complexes formed with an HPV-16 promoter fragment are quantitatively different in del-11 cells and diploid fibroblasts. This difference disappears upon mutation of the HPV-16 TATA to the SV40 TATA sequence indicating specificity of the HPV-16 TATA box

sequence. This raises the possibility that mucosal epitheliotropic papillomavirus promoter regions may be recognised by a distinct subset of TFIID complexes.

Deletion analysis of the BPV-4 promoter also demonstrates that the 8- to 9-fold enhanced epithelial response of the BPV-4 promoter to activation by E2 has been reduced to two-fold with a minimal BPV-4 TATA containing promoter. This suggests that the BPV-4 TATA box and surrounding sequence and/or the E2 protein may also play an important role in determining cell type specific transcription. This is in contrast to the results of the tk/lcr hybrid promoter which is activated in a cell type independent manner by E2. However, the high level of activation of this promoter due to the functional co-operation of E2 and Sp1 may mask any cell type specificity. Increasing concentrations of VP16-E2, which upregulate transcription from the minimal BPV-4 TATA containing promoter preferentially in fibroblasts, suggests that the BPV-4 TATA region does not contribute towards cell type specific transcription. This result also confirms an important role for the promoter repressor elements in mediating the differential response of the BPV-4 promoter to upstream activators in fibroblasts and keratinocytes. However, low levels of E2 activate transcription from the minimal BPV-4 TATA containing promoter to similar levels in both cell types while at high levels transcription is downregulated in fibroblasts but remains elevated in keratinocytes. As E2 is expressed to similar levels in both PalK and PalF cells this suggests that certain cell type specific coactivators assembled at the BPV-4 TATA region specifically interact with the E2 transactivation domain enabling E2 to activate transcription at elevated levels in keratinocytes.

The involvement of differential interactions between factors bound to specific upstream elements and components of the basal machinery in mediating cell type specific transcription has been described previously. A muscle specific enhancer was shown to function with the core promoter elements of the myoglobin gene but when the TATA box sequence was changed to that of the SV40 promoter responsiveness to the muscle specific enhancer was abolished (Wefald et al., 1990). Also, the B cell enriched TFIID subunit, $TAF_{II}105$, is involved in Oct dependent B cell specific transcriptional activation. The B cell specific cofactor, OCA B, has been shown to form a molecular bridge between both the ubiquitous Oct-1 and the B cell specific Oct-2 proteins and $TAF_{II}105$ (Wolstein et al., 2000).

4.1.5. Characterisation of the papillomavirus promoter repressor elements

The papillomavirus repressor elements, PRE-1 and PRE-2, function as autonomous cis acting elements to repress the basal activity of the SV40 promoter in a cell type independent manner (Fig. 3.1.19 and Fig. 3.1.20.). Band shift assays demonstrate that PRE-2 binds a specific protein complex in both PalK and PalF cells (Fig. 3.1.21.). However, no reproducible specific complex binding to PRE-1 could be detected by EMSA analysis. This may be due to the sensitivity and conditions used in the band shift assay. *In vitro* footprinting of the BPV-4 LCR using an immortalised fibroblast nuclear extract has shown previously that the PRE-2 region contains a DNA binding site for a potential transcription factor (Jackson and Campo, 1991). Base pair substitutions in this footprint confirm that these nucleotides are necessary for sequence specific binding of a nuclear protein in PalK and PalF cells (Fig. 3.1.22). PRE-2 mutants that do not compete for binding in band shift assays do not repress

transcription when multimerised upstream of the SV40 promoter (Fig. 3.1.23). Non-binding mutations introduced into the BPV-4 promoter result in a differential increase in response to transcriptional activation by E2 in fibroblasts and keratinocytes (Fig. 3.1.24). UV crosslinking using a BrdU substituted PRE-2 motif demonstrates that the PRE-2 binding form of the protein complex has a molecular weight of approximately 50 kDa in both PalK and PalF cells (Fig. 3.1.25). Of the transcriptional repressors known to bind papillomavirus promoters, such as YY1 (Bauknecht et al., 1992), C/EBPβ (Bauknecht and Shi, 1998), Sp3 (Apt et al., 1996) and CDP/Cut (O'Connor et al., 2000), none of them are this size. Taken together, these results suggest that the PRE-2 binding protein is a novel transcriptional repressor and regulator of mucosal epitheliotropic papillomavirus transcription. The results also suggest that the context of the repressor elements within the BPV-4 promoter is important for mediating cell type selectivity. A model could therefore be proposed in which the interplay between upstream bound activators, repressor proteins interacting with the PRE elements, and the basal transcription machinery assembled at the BPV-4 TATA region, determines the differential response of the BPV-4 promoter in fibroblasts and keratinocytes.

4.1.6. Mechanisms of transcriptional repression

In general, transcriptional repressors can work either passively to antagonise activator function or actively to target components of the general transcription machinery in such a way as to decrease the frequency of transcriptional initiation (for a review see (Ogbourne and Antalis, 1998)). Negative regulatory elements that direct a passive repression mechanism are position dependent within the context of the native promoter. Passive repressors generally function by displacing the binding of a

positively acting factor, either by interference of overlapping or neighbouring activator binding sites, such as E2 mediated repression of the HPV-16 promoter by displacing Sp1 from a proximal promoter element (Tan et al., 1992), or by direct competition for the same binding site, for example, the Sp1/Sp3 antagonism (Apt et al., 1996). In certain cases passive repressors have also been shown to function by masking the transactivation domain of a positively acting factor or by titrating out limiting cofactors necessary for activator function. For example, the MDM2 oncoprotein can inhibit the ability of p53 to stimulate transcription by binding p53 and disrupting interactions with the basal transcription machinery (Oliner et al., 1993). Also, the glucocorticoid receptor has been shown to inhibit AP-1 transcriptional activity through competition for limiting amounts of p300/CBP in within the cell (Kamei et al., 1996). Active repressors have been shown to function, with or without the recruitment of a corepressor, to inhibit different steps during pre-initiation complex assembly (for a review see (Maldonado et al., 1999)). Examples include the ability of pRb to repress E2F mediated transcriptional activation by targeting the recruitment of TFIIA and TFIID to the promoter (Ross et al., 1999), and the repression of transcription by the unliganded thyroid hormone receptor α by directly binding TBP (Fondell et al., 1996).

The protein interacting with the PRE-2 element does not simply displace the binding of a positively acting factor as PRE-2 can repress the basal activity of the SV40 promoter out of context of the BPV-4 LCR. The extent of repression does not depend on the cell type or on the orientation of the PRE-2 element. This suggests that the PRE-2 binding factor directs an active repression mechanism through interacting with a component of the general transcription machinery. However, it is also possible

that the PRE-2 binding protein functions through distinct promoter specific mechanisms. In contrast to the SV40 promoter, deletion of the PRE-2 region of the BPV-4 LCR has no significant effect on basal promoter activity. This suggests that the PRE-2 binding protein is repressing activator dependent transcription, perhaps by disrupting the interaction between the E2 transactivation domain and the basal transcription machinery assembled at the BPV-4 TATA region.

The chromatin organisation of the HPV-16 and –18 genomes also suggest important regulatory roles of nucleosomes during the viral life cycle (Stunkel and Bernard, 1999; Stunkel et al., 2000). Two nucleosomes are precisely positioned on the HPV-16 LCR: one overlaps the centre of the epithelial specific enhancer, while a second nucleosome overlaps the proximal promoter region. The HPV-18 LCR shows specific assembly of a nucleosome over the replication origin and the proximal promoter, positioned approximately 90 bp upstream of the homologous region of the HPV-16 LCR. There is accumulating evidence suggesting a link between papillomavirus transcription and chromatin modification. E2 has been shown to interact with the transcriptional coactivator p300/CBP (Lee et al., 2000). p300 has intrinsic histone acetyltransferase (HAT) activity and has been implicated in chromatin remodelling (Bannister and Kouzarides, 1996). Acetylase activity stimulates transcription by weakening the interaction between the highly positively charged tails of histones H3 and H4 and the phosphate backbone of DNA within the nucleosomes. Conversely, a number of transcriptional repressors have been shown to recruit histone deacetylase activity as part of multi-protein complexes. Deacetylation of histone tails promotes nucleosome assembly thereby inhibiting the ability of transcription factors to gain access to the DNA. Using an epithelial raft culture

system, Trichostatin A (TSA), a specific inhibitor of histone deacetylase activity, has been shown to upregulate the HPV-11 LCR promoter predominantly in the basal layers of the epithelium (Zhao et al., 1999). This effect was promoter specific but showed no cell type specificity. This suggests that histone deacetylases contribute towards maintaining the relatively low level of HPV gene expression in the lower layers of the epithelium. Also, the differentiation specific factor, CDP/Cut, binds to a silencer element just upstream of the promoter proximal positioned nucleosome in the HPV-16 LCR and represses transcription by a histone mediated mechanism (O'Connor et al., 2000). It therefore seems possible that PRE-2 may function by recruiting histone deacetylases to repress transcription.

The importance of cellular repressors of papillomavirus transcription has also been highlighted by a of number studies. It has been proposed that a class of cellular genes called cellular interference factors (CIF) exist in normal cells (zur Hausen, 1989). The inactivation of these genes, which negatively regulate viral gene expression, is necessary to release the papillomavirus transforming genes from cellular repression *in vivo*, contributing to the development of carcinoma. Consistent with this, studies have shown that HPV positive primary tumours or metastases contain episomal HPV-16 DNA with a prevalence of mutated or deleted YY1 transcription factor binding sites (Dong et al., 1994b; May et al., 1994). Negative regulators of papillomavirus transcription may therefore play an important role, not only in limiting expression of viral proteins to the host cell type, but also in regulating the appropiate levels of viral gene expression during the differentiation dependent viral life cycle.

4.1.7. Future Work

To further understand the function of the PRE-2 binding factor (PBF-2) in transcriptional regulation it will be necessary to first isolate a cDNA clone. UV crosslinking has demonstrated that the PRE-2 element interacts with a major protein species of approximately 50 kDa. However, due to the presence of a minor species just above the 50 kDa band it is not clear whether the active DNA binding form of the protein is a monomer or higher order structure or requires a post-translational modification to bind its target site. South Western blot analysis would initially be performed to determine the subunit composition and the method of purification of PBF-2. A successful South Western blot would allow the direct screening of an expression library using radiolabelled PRE-2 to isolate the cDNA clone encoding PBF-2. The cDNA clone may be incomplete as only sufficient information to generate a complete DNA binding domain is needed for a positive screen. A full length clone would be isolated either using EST database searches or further screening steps using the initial clone as a hybridisation probe. Alternatively, affinity chromatography using a biotinylated PRE-2 element immobilised onto a streptavidin column would be used to purify PBF-2 to homogeneity. Increasing salt concentrations would be used to elute the protein and the different fractions would be monitored for sequence specific DNA binding using the band shift assay. The partial DNA sequence would be determined and used to identify a full length cDNA clone by screening an expression library with the appropiate oligonucleotides.

Once the full length clone has been isolated it would be inserted into the appropiate vectors for further biochemical and functional analysis. Initially, band shift assays and UV crosslinking using *in vitro* translated PBF-2 and radiolabelled PRE-2 would

be carried out to determine that the DNA binding specificity of the isolated clone mimics that of the detected cellular protein. Overexpression studies, using a suitable mammalian cell line that either lacks or has low endogenous levels of PBF-2, would also be performed to demonstrate that PBF-2 functions as a transcriptional repressor. These studies would aim to show both repression of basal SV40 promoter activity and repression of the transcriptional response of the BPV-4 promoter to E2. The minimal domain required for transcriptional repression would also be mapped by constructing PBF-2 deletion mutants.

Eventually, the isolation of factors interacting with the PBF-2 repression domain would allow the mechanism of repression to be elucidated, whether this is through a direct interaction with the basal machinery, or via a co-repressor, or by recruitment of histone deacetylase activity. Initially, overexpression studies in the presence of TSA would be used to determine whether PBF-2 represses transcription from the SV40 and BPV-4 promoters by a histone mediated mechanism. As TSA is toxic and may have non-specific effects on transcription co-transfection of HDAC expression vectors in the functional assays would be used to assess specificity. *In vitro* and *in vivo* assays using GST-PBF-2 fusion proteins and tagged PBF-2 constructs would then be performed to try and detect a specific interaction with a HDAC. Interactions between PBF-2 and components of the basal transcription machinery would first be tested using GST-PBF-2 proteins and *in vitro* translated general factors in a pull down assay. Mutations in the PBF-2 repression domain that abolish binding would be identified and tested in the functional assays to determine the effect on repression. Ideally, PRE-2 mediated repression of basal promoter activity would also be reconstituted using a cell free *in vitro* transcription system. By staging pre-initiation

complex assembly *in vitro* the targets of the PBF-2 repression domain could be identified and a detailed biochemical analysis could be performed.

These experiments would extend on the functional and biochemical analysis of the PRE-2 silencer element identified in the BPV-4 promoter. They would lead to the identification and characterisation of an apparently novel transcriptional repressor and regulator of mucosal epitheliotropic papillomavirus transcription.

4.2. Interaction of cellular proteins with the E2 amino terminus

4.2.1. Detection and identification of E2 interacting proteins

The papillomavirus E2 protein is the major regulator of viral transcription and is essential for viral DNA replication (for a review see (Desaintes and Demeret, 1996)). When overexpressed E2 can disrupt cellular growth control in certain HPV transformed and non-HPV transformed cell lines (see (Massimi et al., 1999) and references therein). A chimaeric E2 protein in which the N-terminal E2 transactivation domain is fused to the GAL4 or LexA DNA binding domain efficiently activates transcription in yeast and mammalian cells (Breiding et al., 1996; Winokur and McBride, 1996). This indicates that the E2 N-terminus is able to function independently of the C-terminal DNA binding and dimerisation domain to activate transcription. By analogy to other acidic transactivators the E2 transactivation domain is believed to function, at least in part, through contacting components of the cellular transcription machinery and affecting the formation and/or stability of the pre-initiation complex. E2 has previously been shown to interact with the cellular transcription factors TBP (Rank and Lambert, 1995), TFIIB (Yao et al., 1998), Sp1 (Li et al., 1991), p300 and AMF-1 (Breiding et al., 1997). However, as E2 can function in a core promoter specific manner it is likely that there are additional, as yet unidentified, E2 interacting cellular factors. For example, E2 efficiently activates a minimal BPV-4 TATA promoter containing neither an initiator element nor a binding site for an upstream factor (Fig. 3.1.16.) but is unable to activate a minimal tk TATA box promoter (Ham et al., 1991a). Also, E2 mutant proteins exist that activate transcription in mammalian cells but fail to do so in yeast

(Breiding et al., 1996), suggesting the presence of additional mammalian specific transcriptional regulatory pathways. In order to gain a greater understanding of how E2 regulates transcription the additional cellular proteins with which it interacts must therefore be identified.

Far Western blot analysis using a GST-E2 fusion protein to probe PalK, PalF and HeLa whole cell extracts demonstrates that the E2 amino terminus interacts with at least 12 cellular proteins in PalK and at least 8 in PalF cells (Fig. 3.2.2.). There are obvious differences in the pattern of interacting proteins in PalK and PalF cells. Although the functional significance of these interactions are unknown and the proteins remain to be characterised they may represent proteins involved in mediating some of the epithelial specific functions of E2. Consistent with this, Fig. 3.1.17. suggests that certain cell type specific transcriptional co-activators may interact with the E2 transactivation domain in keratinocytes and not in fibroblasts enabling E2 to activate transcription at elevated levels in epithelial cells. There are no obvious differences between the factors interacting with wild type E2 and a mutant transactivation domain which has amino acid 73 mutated from an isoleucine to an alanine. This mutant protein retains the ability to support viral DNA replication but fails to activate transcription (Ferguson and Botchan, 1996; Sakai et al., 1996). The crystal structure of the E2 amino terminus showed that amino acid 73 is exposed to the solvent indicating that it might be involved in intermolecular interactions (Antson et al., 2000). Taken together, these results suggest that there are additional cellular factors, necessary for transcriptional activation by E2, that interact with the wild type transactivation domain but not with the I73A mutant, that cannot be detected using this assay. The detection of direct protein-protein interactions in HeLa cells by Far

Western blotting demonstrates that the cDNAs encoding E2 interacting proteins can be isolated by expression screening of a HeLa cDNA library using radiolabelled GST-E2. In contrast, a traditional yeast two hybrid screen cannot be used to identify E2 interacting proteins as the wild type E2 transactivation domain activates transcription in yeast.

Seven independent cDNAs encoding polypeptides interacting with the E2 amino terminus were isolated by expression screening (Table 3.2.1.). However, no proteins previously shown to interact with E2 were identified. This probably reflects differences in the assays used to detect these interactions. Clone 2 represents a false positive probably due to misalignment of the nitrocellulose filter and agar plate whilst picking a positive plaque in the tertiary screen. Clones 3 and 6 were not sufficiently purified after three rounds of screening and clone 7 contains the partial sequence of the ISFG-4 gene. These four clones were not chosen for further analysis. Clones 4 and 5 represent the same cDNA independently isolated. This cDNA was approximately 1.5 kb in length and showed no extensive homologies to any known genes. Analysis of the predicted amino acid sequence showed multiple stop codons in all three open reading frames (data not shown). This suggests that the potential E2 interacting polypeptide has a low molecular weight. Attempts to *in vitro* transcribe and translate a ^{35}S labelled polypeptide from this cDNA in all three open reading frames were unsuccessful. However, failure to detect a product may be due to a lack of ^{35}S incorporation because of a low level of methionine residues in the encoded small polypeptide.

Clone 1 encodes the full length human L31 ribosomal protein. GST pull down assays demonstrate that *in vitro* translated L31 specifically interacts with the E2 amino terminus (Fig. 3.2.4). Immunofluorescence has demonstrated that L31 is targeted to the nucleolus, the site of ribosomal RNA synthesis and ribosome assembly (Quaye et al., 1996). Due to the high level of conservation with the yeast L34 protein L31 is believed to play an important role in ribosome biosynthesis (Nobori et al., 1989). After processing and targeting of the ribosomal subunits to the cytoplasm the L31 protein is believed to form a component of the mature 60S large ribosomal subunit. It is also suggested that L31 might be able to interact with nucleic acid due an unusually high content of basic amino acids compared with other ribosomal proteins.

Ribosomal proteins are potential mediators of growth regulation as rapidly proliferating cells with a high protein synthesising activity have a high ribosome content. Indeed, L31 is expressed at abnormally high levels in colorectal tumours and various haematopoietic malignant tumour cells (Chester et al., 1989; Shimbara et al., 1993). Also, the *in vitro* terminal differentiation of several immature leukaemia cell lines is associated with a decrease in expression of L31 mRNA (Lin et al., 1994). It is therefore possible that the interaction of E2 with L31 may be involved in E2 mediated disruption of cellular growth control. It is also possible that L31 may function downstream of transcription initiation to alter the levels of viral gene expression.

Papillomaviruses exploit several post-transcriptional levels of regulation during the viral life cycle such as the differentiation specific alternative splicing of BPV-1 late mRNAs (Barksdale and Baker, 1993). Functional interactions between E2 and RNA

binding proteins have been demonstrated previously. BPV-1 E2 can bind the product of the survival motor neuron (SMN) gene (Strasswimmer et al., 1999). SMN is the determining gene for spinal muscular atrophy, an inherited neuromuscular disease. The SMN protein functions in the distribution and regeneration of the pre-mRNA splicing machinery and in snRNA biosynthesis. When tethered to a DNA binding domain SMN does not activate transcription. It is therefore suggested that the interaction between E2 and SMN stimulates viral gene expression through an RNA transport or processing step. Also, the arginine/serine rich hinge of HPV-5 E2 has been shown to interact with a set of cellular splicing factors including 4 SR proteins which are involved in the modulation of splice site choice and 2 snRNP associated proteins (Lai et al., 1999). snRNPs are required for RNA processing. Functional assays have demonstrated that the HPV-5 E2 hinge can facilitate the splicing of a primary transcript transactivated by E2 itself in a distant dependent manner. This work suggested that HPV-5 E2 can mechanistically couple transcription and pre-mRNA splicing. It also seems possible that E2 may be able to control the nuclear export of processed RNA or even translation of the protein.

The interaction between E2 and EIF3 was also characterised. EIF3 was isolated using a yeast two hybrid screen with a mutant E2 amino terminus that activates transcription in mammalian cells but fails to do so in yeast (Boner and Morgan, unpublished). Fig. 3.2.5. shows that EIF3 specifically interacts with the wild type E2 transactivation domain at high salt concentrations *in vitro*. EIF3 has many interesting characteristics that suggest it might be a physiologically relevant target for E2. EIF3 encodes a truncated version of $p27^{BBP}$, a protein highly expressed in epithelial cells, which was initially isolated based on its ability to bind the cytoplasmic domain of

integrin β_4 (Biffo et al., 1997). In cells expressing integrin β_4, $p27^{BBP}$ is present both in the cytoplasm enriched at the basal membrane and in the nucleus, present in the nucleolus and associated with the nuclear matrix (Sanvito et al., 1999). Integrins are involved in the control of cell growth, apoptosis and differentiation through the recruitment of several signal transduction and adaptor molecules. Recently, the JAB-1 (jun activation domain binding protein 1) co-activator for c-jun transcription has been shown to interact with the β_2 subunit of the integrin LFA-1. JAB-1 is found in two pools in the cell: one cytoplasmic pool and one in the nucleus. Binding of ligand to LFA-1 increases the JAB-1 nuclear pool resulting in enhanced DNA binding activity of c-jun containing AP-1 complexes and an increase in transcription from an AP-1 dependent promoter (Bianchi et al., 2000). By analogy, EIF3 may represent a co-factor for E2 mediated transcriptional activation.

However, it is also proposed that β_4 independent functions of $p27^{BBP}$ also exist as $p27^{BBP}$ is present in the nucleolus and associated with the nuclear matrix in the absence of β_4 (Sanvito et al., 1999). Also, a yeast homolgue of $p27^{BBP}$, 80% identical to the human protein, has been identified (Sanvito et al., 1999). Yeast have no β_4 integrin. It therefore seems likely that $p27^{BBP}$ performs different roles in each subcellular compartment. Indeed, $p27^{BBP}$ has also been identified as eIF6, a putative translation initiation factor based on its *in vitro* ability to inhibit the association between the 40S and 60S ribosomal subunit (Si et al., 1997) and has subsequently been shown to function in ribosome biosynthesis (Sanvito et al., 1999). Interestingly, both L31 and EIF3 are present in the nucleolus and are implicated in ribosome biogenesis. It could be suggested that E2 might target the processing of the 60S large ribosomal subunit to suppress cellular proliferation. Indeed, the yeast homologue of

$p27^{BBP}$ is essential for cell viability as deletion of the $p27^{BBP}$ yeast gene is lethal (Sanvito et al., 1999). In addition, reduction of $p27^{BBP}$ protein levels in yeast leads to a huge decrease in the levels of free 60S ribosomal subunit and growth arrest in the G1 phase of the cell cycle. This lethal effect can be rescued by expression of human $p27^{BBP}$. Furthermore, in the gut epithelium $p27^{BBP}$ expression levels are high in rapidly cycling cells and low in villous cells committed to apoptotic cell death (Sanvito et al., 2000).

It should also be noted that a pool of EIF3 exists associated with the nuclear matrix (Sanvito et al., 1999). It is therefore possible that EIF3 may tether E2 to the nuclear matrix. Nuclear matrix attachment regions (MAR) are DNA segments with a high affinity for the nuclear matrix and may play a role regulating transcription by bringing cis-responsive DNA elements close to matrix bound transcriptional complexes. Two MARs in the HPV-16 genome are positioned close to the epithelial specific enhancer and the E6 promoter and origin of replication (Tan et al., 1998). These MARs are involved in the regulation of HPV-16 transcription (Stunkel et al., 2000). In transient transfections, the MARs repress HPV-16 transcription but after integration of the viral DNA into cellular chromosomes the MARs function as enhancers (Stunkel et al., 2000). Sequence analysis suggests that these MARs are conserved among genital papillomaviruses suggesting that the nuclear matrix, and perhaps EIF3, might regulate an important process of the HPV life cycle.

The results in this chapter therefore demonstrate the detection of direct protein-protein interactions between the wild type E2 transactivation domain and cellular proteins by Far Western blotting. The isolation of cDNAs encoding proteins

interacting with the region of E2 involved in regulating transcription, replication and growth control by expression cloning is described. Also, the results demonstrate an *in vitro* interaction between E2 and two previously unidentified E2 interacting factors, the L31 ribosomal protein and EIF3, a truncated version of a protein previously shown to bind the cytoplasmic domain of integrin β_4.

4.2.2. Future work

The functional characterisation of the interaction between E2 and the cellular factors, L31 and EIF3, would initially be the main focus of the continuation of this work. Overexpression studies would be performed to look specifically at the involvement of these factors in E2 mediated transcriptional regulation. Initially, the full length EIF3 clone would be isolated. Attempts to obtain the 5' portion of the EIF3 cDNA missing from the yeast 2 hybrid clone by PCR amplification of a HeLa cDNA library have been unsuccessful. A full length clone would therefore be isolated by screening a HeLa cDNA library using a radiolabelled 5' EIF3 oligonucleotide fragment as a probe. The full length L31 and EIF3 clones would then be inserted into mammalian expression vectors. L31 and EIF3 would be co-expressed with E2 in HeLa cells to determine whether these factors can enhance the ability of E2 to activate transcription from both the BPV-4 and tk promoters. Antisense L31 and EIF3 expression vectors would also be constructed for further functional analysis. If L31 and EIF3 are necessary for E2 mediated transcriptional regulation, then expression of antisense RNA should inhibit transcription by blocking synthesis of endogeneous L31 and EIF3. Western blot analysis would also be carried out to determine if the antisense expression vectors affect the level of E2 protein. Eventually, a mutant of E2 that fails

to interact with L31 or EIF3 *in vitro* would be tested for transcriptional activity. Also, mutants of L31 and EIF3 that fail to interact with E2 in a GST pull down assay would be overexpressed to try and squelch E2 mediated transactivation. These experiments would determine whether L31 or EIF3 are involved in E2 mediated transcriptional regulation. However, the contribution of these factors to E2 mediated regulation of viral DNA replication and cellular growth control would also have to be examined.

It would also be important to demonstrate an interaction between E2 and L31 and EIF3 *in vivo*. E2 and HA tagged L31 and EIF3 proteins would be co-expressed in mammalian cells. Immunoprecipitation of L31 and EIF3 using an HA antibody along with an antibody that specifically recognises E2 would determine whether E2 and these factors associate in transfected cells. Immunofluorescent staining and confocal microscopy would be used to examine the subcellular localisation of E2 and L31 and EIF3. It has previously been shown that E2 has a diffuse nuclear staining. However, when co-expressed with the papillomavirus L2 protein E2 is recruited to POD domains (Day et al., 1998). Immunofluorescent staining of cells transfected with HA tagged L31 and EIF3 proteins and E2 would be performed to detect if E2 co-localises with these factors *in vivo*. The effect of E2 expression on the sub-cellular localisation of L31 and EIF3 would also be examined. It would also be essential to develop antibodies against L31 and EIF3 to look at the endogeneous proteins. These antibodies would also be used for in immunoprecipitation experiments. The results of the *in vivo* analysis would provide further evidence to whether the E2 and L31 and EIF3 interaction is biologically significant.

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