The effect of the HPV-16 minor capsid protein L2 on the HPV-16 viral transcription regulator E2

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I dedicate this work to Him who gives me life and strength, my wonderful parents Sir C.E.S & Lady Grace Okoye

and

to my ever loving wife Amaka.

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To my darling wife I say thank you for putting up with me and supporting me for these last three years. I would also like to acknowledge and appreciate my parents Sir (Chief) Sonny and Lady (Lolo) Grace Okoye, my brothers Nnamdi, Ikenna and Amaechi for being there from the beginning; I love you all and could not have done it without your support and care. I would also like to thank my big cousin Chimay Anumba, his wife Clair and his beautiful daughters Nmachi and Kenechi for being an encouragement and a good friend. To my friends who have also supported me over these years, my big brother Ola, Ekene & Ayo, Femi, Jumoke, Eni, Sade & Eniolu Rotimi, the Ekuwezor and Komolafe families, my uncle Chris and my dear aunty Ekwy Madichie.

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ABBREVIATIONS

ATP	Adenosine triphosphate
bp	Base pairs
BPV	bovine papilomavirus
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
CIN	Cervical intraepithelial neoplasia
cm	Centimeters
CRPV	Cottontail rabbit papillomavirus
°C	degree centigrade
DMEM	Dulbecco's modified Eagles medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	3' deoxyribonucleoside 5' triphosphate
E.coli	Escherichia Coli
EBV	Epstein-Barr virus
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediamine tetra-acetic acid
EGTA	Ethylene Glycol-bis(β-aminoethyl Ether) N,N,N',N'-tetra-acetic acid
EtBr	Ethidium bromide
FITC	Flurorescein-isothiocynate
FCS	Foetal calf serum
g	Gram
G418	Geneticin, g418-sulphate
GFP	Green Fluorescent Protein
GST	glutathione-S-transferase

HBV	Hepatitis B virus
HEPES	N-[2-Hyroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HBS	HEPES buffered saline
HPV	Human papillomavirus
HRP	Horseradish peroxidase
HTLV-1	Human T cell leukaemia virus-1
hr	Hours
HSV	Herpes Simplex Virus
IgG	immunoglobulin G
kb	Kilobase pairs
kDa	KiloDalton
kg	Kilogram
l	Litre
LCR	Long control region
µg	microgram
µl	microlitre
M	Molar
mg	milligram
min	minute
ml	millilitre
mM	Millimolar
mRNA	Messenger ribonucleic acid
PAGE	Polyacrylamide gel electrophoresis
OD	Optical density (light absorbance)
ORF	Open reading frame
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline plus 0.1% Tween
PCR	Polymerase chain reaction
pmols	Picomoles
POD	Promonocytic leukaemia protein (PML) oncogenic domains

RNA	Ribonucleic acid
Rnase	Ribonucleasc
RT-PCR	Reverse transcriptase-polymerase chain reaction
rpm	Revolutions per minute
SDS	Sodium dodecyl sulpate
sec	Second
SV40	Simian virus 40
TBP	TATA box binding protein
TBS	Tris-buffered saline
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	Tris (hydroxymethyl) aminomethane
Tween 20	Polyoxyethylene sorbitan monolaurate
tk	Thymidine kinase
UV	Ultraviolet
V	Volts
v/v	Volume per unit volume
w/v	Weight per unit volume
wt	Wild type
µg	Microgram
µl	Microlitre
µM	Micromolar
%	Percentage
^{32}P	Phosphorous isotope 32 atom
^{35}S	Sodium isotope 35 atom

Single letter DNA nitrogenous base

A	Adenine
C	Cytosine
G	Guanine
T	Thymine

Single letter amino acid code

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

DECLARATION

The work presented in this thesis is my own original work except confocal imaging in chapter 5, which was performed by Dr Pablo Cordano in the Department of Veterinary Pathology University of Glasgow. This work has not been previously submitted for any other degree.

ABSTRACT

The nucleus contains a variety of morphologically distinct substructures called nuclear bodies, which include the promyelocytic leukemia oncogenic domains (PODs) also known as PML-ND10. PODs are macromolecular multiprotein complexes that are present in all cultured cell lines as well as in *vivo*. The major component of PODs is the PML protein, which was originally identified as the fusion partner of retinoic acid receptor alpha (RARα) in the chromosomal translocation t(15;17) in patients with acute promyelocytic leukaemia (APL) (Kakizuka *et al.*, 1991; Lavau *et al.*, 1991; Goddard *et al.*, 1992).

The minor capsid proteins L2 of BPV-1, HPV-11 and HPV-33 have been shown to localise to PODs in the absence of other viral components (Day *et al.*, 1998) and co-expression of BPV-1 L2 with BPV-1 E2TA recruits E2 to PODs (Lambert *et al.*, 2000). The presence of L2 in PODs also appears to be associated with the recruitment of the major capsid protein L1. The association of PODs with E2 is dependent on L2 but is independent of L1.The effect of HPV-16 L2 on the functions of HPV-16 E2 and the implications of this interaction to the virus life cycle are discussed.

This study showed that HPV-16 L2 has a selective effect on the functions of HPV-16 E2. L2 was able to down regulate the transcription transactivation function of E2 in HaCaT, U2OS and C33a cells. No effect of L2 on E2 mediated DNA replication was observed. L2 was also able to reduce the level of E2 expression in HaCaT and U2OS cells but not in C33a cells. The effect of L2 on E2 expression in HaCaT cells was further investigated by

examining E2 mRNA levels and protein half-life. No difference in E2 mRNA or protein half-life was detected in the presence of L2.

A series of L2 amino and carboxyl terminal deletion mutants were constructed as GST fusion proteins and GST binding assays were performed which showed that the amino terminus of L2, even just the first 50 amino acids, was capable of binding with E2. GFP fusion forms of each L2 deletion mutant were also constructed and cellular localisation detected by immunofluorescence. GFP-L2 and all C-terminal deletion mutants localised and were retained in the nucleus while the N-terminal deletion mutants localised to both the nucleus and the cytoplasm.

Investigation of the effect of L2 deletion mutants on the transcription transactivation function of E2 showed that mutants expressing 1-200 and 150-473 amino acids of L2 do not down regulate function in HaCaT and C33a cells. In HaCaT cells, mutants expressing amino acids 1-50 and 1-100 also did not inhibit E2 function indicating that binding to E2 did not correlate with down-regulation of transcription transactivation. Furthermore, only full-length L2 was able to reduce the level of E2 expression.

CHAPTER 1: INTRODUCTION

1.1. Viruses and Cancer

Viruses and cancer have a long history in animal studies. Leukemia in chickens was experimentally transmitted by an agent that passed through a filter by Danish researchers in 1908. Soon afterwards Peyton Rous, in New York, showed the same for chicken sarcoma. In 1936, Bittner showed that predisposition to breast cancer in C3H mice was passed through the milk to their offspring; this milk factor was later shown to be mammary tumour virus (Lyons and Moore., 1962; Lyons and Moore., 1965) . In 1964, three scientists Epstein, Achong and Barr working on Burkitt's lymphoma cells discovered the first human tumour virus, today known as the Epstein-Barr virus (Epstein *et al.*, 1964). Since then the link between viral infections and human cancer has been well established. Today, viral infections have been associated with 10 to 15% of all cancers worldwide. In most cases, cancer is an infrequent consequence of infection by the causative virus (usually as a by-product of viral replication) and often occurs years after the initial infection has taken place. As tumour progression is regarded as a multistep process, viruses may be an essential step but alone might not cause cancer. Certain factors must be considered for a virus to be implicated in tumor progression such as the introduction of a viral oncogene into a cell or insertion of viral genome into the genome of the host cell, thus disrupting the normal regulation of cell division. Also the virus should usually persists during the disease, prevent reinfection and be located

appropriately to account for disease. Today a variety of DNA and RNA viruses have now been associated with various malignancies in both humans and animals.

1.1.1. Human T-cell Leukemia virus Type 1 (HTLV-1)

Shortly after its discovery in 1980, HTLV-1 was found to be the etiologic agent of both adult T cell leukamia (ATL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), a neurologic disease characterised by demyelinating lesions in both the brain and the spinal cord. Approximately 5-10% of HTLV-1 infected individuals develop either ATL or HAM/TSP. Recent research has suggested that the route of primary viral infection may dictate the course of disease pathogenesis associated with HTLV-1 infection. Specifically, mucosal exposure to HTLV-1 has been associated with cases of ATL. ATL is an aggressive lymphoproliferative disease that develops in approximately 1-5% of seropositive individuals. The leukemic cells in ATL are almost exclusively $CD4^+$ T cells, likely reflecting the fact that HTLV-1 displays an in vivo cellular tropism for this cell population within the peripheral blood. It has been demonstrated that 90-99% of HTLV-1 provirus segregates with $CD4^+$ T cells within the peripherial blood of ATL patients infected with the virus (Richardson *et al.*, 1990). The genesis of ATL has been linked to the effects of the viral protein TAX (Franchini., 1995; Uchiyama., 1997). Tax is a 40-kDa phosphoprotein essential for both viral replication and cellular transformation. It is a transcriptional transactivator and can dramatically increase viral gene expression through its interaction with cellular factors and the 5' long terminal repeats (LTR) of the proviral genome (Marriott *et al.*, 1989). HTLV-1 has been found primarily in populations in southern Japan, Brazil, the Caribbean, and regions of central Africa. HTLV-2 has been linked with hairy cell leukaemia although its connection

with the disease is weak because it has been identified in cells from only a few patients with the disease (For review) (Barmak *et al.*, 2003; Uchiyama., 1997).

1.1.2. Hepatitis B virus (HBV)

HBV causes hepatitis B and plays a major role in the development of liver cancer known as hepatocellular carcinoma (HCC) (Brechot *et al.*, 2000; Buendia., 1998; Buendia., 2000). HBV is a small-enveloped DNA virus, which replicates in paraenchymal cells of the liver. During acute and chronic infections, the liver produces empty viral envelopes organized in spherical or filamentous particles that carry the HBV surface antigen. In endemic regions, young children are usually infected at birth by their infected mothers or by contact during early years of life. In non-endemic regions, HBV-infected individuals usually develop adequate cellular and humoral immune responses, produce antibodies against HBV and recover. In 5-10% of patients, failure of the immune system to clear the virus results in a chronic HBV carrier state (Hollinger., 1996). These individuals usually develop persistent infections and an increased risk of liver cirrhosis and liver cancer (HCC). Integration of the viral DNA and deregulation of cell growth control by inappropriate expression of viral transactivators have been implicated in HBV induced oncogenesis (Brechot., 1998).

1.1.3. Kaposi's sarcoma-associated herpesvirus (KSHV)

KSHV is a member of the gammaherpesvirus subfamily, Rhadinovirus genera. They are double-stranded DNA viruses approximately 165kbp in size. A characteristic of KSHV and other gammaherpesviruses is the similarity of a large number of the ORFs to known cellular genes suggesting that some of these genes may be pirated from the host

chromosome during the process of viral evolution. KSHV latently infects predominantly B-cells and endothelial cells. Infected cells retain the virus from one generation to the next and can also transform (Ganem., 1997). Strong evidence now exists to establish KSIIV as the etiologic agent of Kaposi sarcoma (KS) (Boshoff *et al.*, 1995; Chang *et al.*, 1994). KS is an unusual multifocal neoplasm characterised by dark purple lesions, which differs from most other common tumours in that the lesions contain multiple cell types (polyclonal), with the dominant cell being the spindle cell derived from endothelial origins. In addition, the KS lesions contain numerous infiltrating inflammatory cells (Verma and Robertson., 2003). KS is more wide spread in patients who are immunocompromised such as people with AIDS. In such cases it can become detrimental to the patient and even fatal.

1.1.4. Epstein-Barr virus (EBV)

EBV is a member of the gammaherpesvirus family that infects 94% of adult populations worldwide and following primary infection the individual remains a lifelong carrier of the virus. The virus is generally spread to and between young children through salivary contact. Cancer usually develops in cases of clinical illness, where primary infection is delayed until adolescence or beyond, or when an intense immunopathological reaction leads to the symptoms of infectious mononucleosis (a lymphoproliferative disorder). The virus is found in nearly all Burkitt's lymphoma tumours from patients in central Africa, where the disease is most common. EBV has a pronounced tropism for human B-lymphocytes, which are readily infected and immortalized *in vitro* (Kaye *et al.*, 1999; Rickinson *et al.*, 1984). By influencing B-cell survival mechanisms, EBV may induce tumours such as B Lymphoproliferative disease and Hodgkins disease. EBV contributes

to the immortalization/transformation of infected cells through the activity of viral proteins that interfere with cellular pathways controlling growth and/or survival. These viral proteins act cooperatively and may induce different biological effects in different cellular backgrounds (Kaye *et al.*, 1993; Wang *et al.*, 1985).

1.1.5. Viruses and Breast Cancer

The mouse mammary tumour virus (MMTV) is a B-type retrovirus. MMTV causes mammary cancer in mice through a process called insertional mutagenesis. Researchers have long sought a human homologue of MMTV. Recent reports from a group in Sydney suggest a link between a new virus and a large percentage of breast cancer cases (Ford *et al.*, 2003). They found that 40% of breast cancer samples taken from Australian-Caucasian women had MMTV-like gene sequences although it was found in only two percent of normal breast samples taken from cosmetic surgery. The virus was found in the cancerous tissue and not in the normal breast tissue from women with breast cancer. It may also play an important role in male breast cancer, with over 50% of male samples testing positive for the virus. Previous studies have also found MMTV-like gene sequences in 38% of breast cancer tissue from United States women (Wang *et al.*, 1995).

PATHOGEN	MALIGNANCY
HTLV-I	Adult T-cell leukaemia/lymphoma
HTLV-II	Hairy cell leukaemia
HBV	Liver cancer
KSHV	Kaposi's sarcoma
EBV	Lymphoproliferative disorders
EBV	Nasopharyngeal carcinomas
EBV	Burkitt's lymphoma
HPV	Anogenital carcinoma, cervical cancer, skin cancer

Table 1.1. Showing some known viral-cancer associations.
HTLV=human T-cell leukaemia/lymphoma virus, HHV= human herpes virus, EBV= Epstein-Barr virus, HBV= hepatitis B virus, HPV= human papillomavirus

1.2. Papillomaviruses

Papillomaviruses are nonenveloped icoshedral, double-stranded DNA viruses that belong to the family of Papoviridae. They are found in many species, including humans, rabbits, cows, and dogs (Campo., 2002). They are highly species specific and are not known to cross the species barriers, with the exception of Bovine papillomaviruses, which can infect horses and induce equine sarcoid (Chambers *et al.*, 2003). There are over 90 different types of Human papillomaviruses (HPVs) identified based upon degree of relatedness of viral genome. Papillomaviruses induce tumours in skin and mucosa, thus explaining the name of this virus group (from the Latin *papilla*, nipple, pustule and the Greek suffix oma to denote tumour). In some tumours, the viruses occur in large amounts (up to 10^{13} physical particles per gram tumour). Certain types of HPVs may cause warts or papillomas, which are benign (non cancerous) tumours that grow on hands and feet and or in the mouth and genital area. HPVs are divided into low-risk viruses such as HPV-6, HPV-11 associated with skin warts and high-risk viruses such as HPV-16, HPV-18 and HPV-31, which are recognised as the major cause of cervical cancer. HPVs that lead to the development of cancer are referred to as "cancer associated types" (Campo., 1998).

1.2.1. Papillomavirus Genome Organisation

HPVs are DNA viruses with double-stranded circular DNA enclosed in a protein coat, the capsid. The protein structural units of the capsid are capsomeres. HPVs contain 72 capsomers and measure 52-55 nm in diameter. The genome of HPVs can be divided into three regions, the early and late open reading frames separated by a long control region (LCR) (Fig 1.1). The LCR is a 1kb sequence containing many of the viral transcriptional

regulatory signals as well as origin of replication (Fig 1.2). The early region contains those ORFs expressed primarily in non-productively infected cells expressing genes designated E1 to E7. The late region contains the ORFs for the capsid proteins L1 and L2 (Fig 1.1).

The E1 ORF encodes a 68-76kDa protein essential for viral DNA replication. The full-length E1 product is a phosphorylated nuclear protein that binds to the origin of replication in the LCR of papillomaviruses. E1 binds to the E2 protein enhancing viral replication. Binding to E2 also strengthens the affinity of E1 for the origin of DNA replication. The E2 ORF encodes a 42-48kDa protein that regulates viral DNA transcription. BPV-1 encodes three E2 proteins, the major transcription transactivator (E2TA) which activates viral promoters by binding to E2 responsive elements. The function of the E2TA is repressed by two other E2 proteins; the E2 transcriptional repressor (E2TR) and E8/E2 transcriptional repressor (E8/E2TR) (Choe *et al.*, 1989; Lambert *et al.*, 1990). The E2 protein is described in further detail in section 1.2.7.

The Human Papillomavirus type 16 Genome

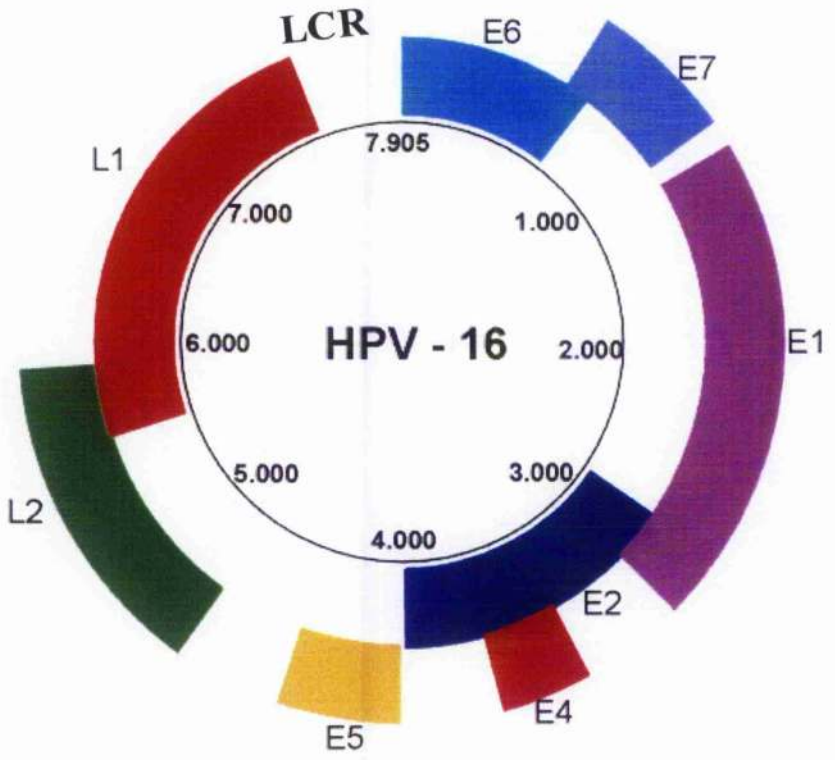


Figure 1.1. Schematic representation of the HPV-16 genome showing the early and late open reading frames. The early region contains those ORFs expressed primarily in non-productively infected cells expressing genes designated E1 to E7. The late region contains the ORFs for the capsid proteins L1 and L2.

The Long Control Region of HPV-16

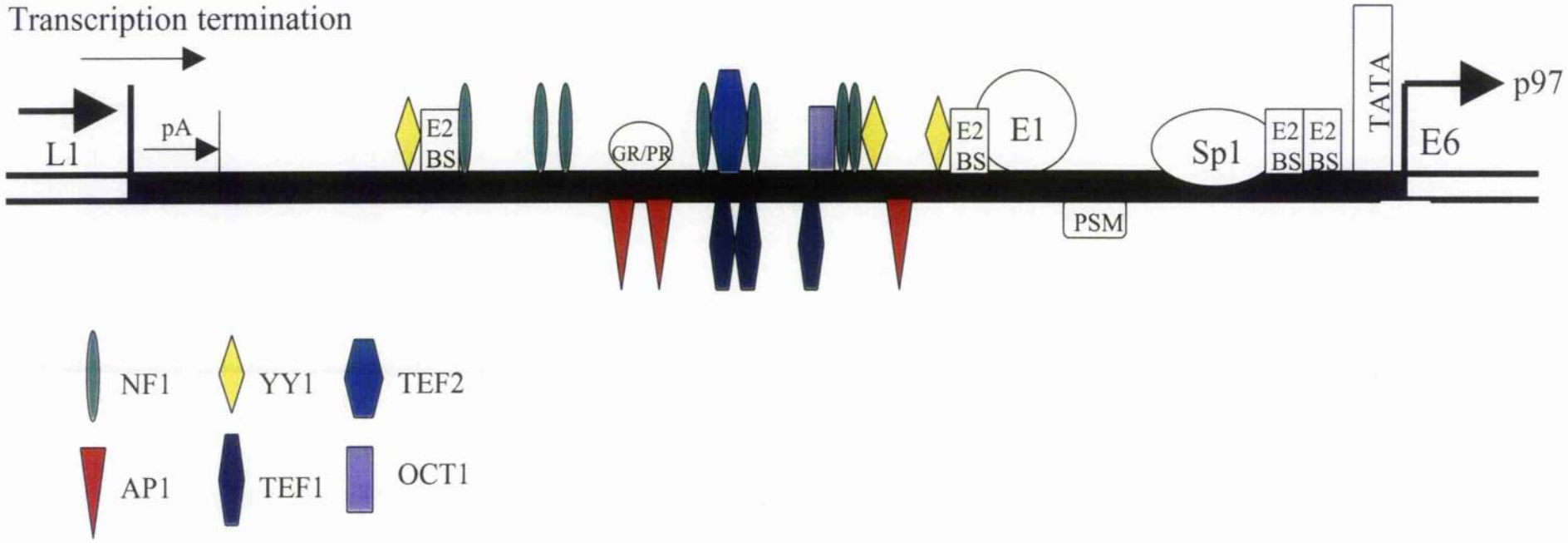


Figure 1.2. Schematic representation of the human papillomavirus 16 long control region. HPV-16 LCR has 4 E2 binding sites (E2BS), two immediately proximal to the TATA box which are E2 repression sites. The E2 site furthest away from the TATA is the major E2 activation site. Transcription factor binding sites are shown by the symbols NF1 (nuclear factor 1), AP1 (activation factor 1), TEF1 and TEF2 (transcription enhancer factor 1 and 2), E1(replication factor E1), PSM (papillomavirus silencing motif) GR/PR (glucocorticoid and progesteron receptor) and OCT1 (octamer binding factor-1).

The E4 gene product is found primarily in the cytoplasm of keratinocytes, where it is extremely abundant. It is expressed from spliced mRNA (E1^E4) transcripts from a differentiation inducible promoter that lies within the E7 open reading frame. It was first detected in naturally occurring lesions in cells in which vegetative viral DNA replication is occurring. It persists during the late stages of infection and is modified by proteolytic processing and phosphorylation. The role of E4 in the viral life cycle is described in more detain section 1.2.3.

The E5 ORF encodes a 4.6-9.2kDa cell-transforming protein. It is one of the more poorly conserved ORFs among the papillomaviruses and often lacks an initiation codon. The E5 gene product is a very small hydrophobic transforming protein that is expressed in the deep layers of the epithelium (Anderson *et al.*, 1997; Burnett *et al.*, 1992), localising in the endomembrane compartments of the endoplasmic reticulum and Golgi apparatus (Burkhardt *et al.*, 1989; Pennie *et al.*, 1993). E5 from both animal and human papillomaviruses can transform mammalian cells with varying degrees of efficiency. The transforming activity of BPV-1 E5 is shared by other fibropapillomviruses; in contrast, E5 proteins of HPVs show only weak transforming activity. E5 cell transformation is brought about by the activation of several kinases, from growth factor receptors to cyclins-cdks. BPV-1 E5 has been shown to stimulate the transforming activity of EGF and CSF-1 receptors associated with the inhibition of receptor degradation and persistence of activated receptors on the cell surface (Martin *et al.*, 1989). E5 interacts with the 16 kDa subunit of the vacuolar H^+-ATPase usually resulting in the inhibition of the acidification of the endosomes (Straight *et al.*, 1995). Failure to acidify endosomes

may be responsible for the inhibition of the EGF receptor degradation, thereby allowing increased receptor recycling to the membrane. BPV E5 also activates the platelet derived growth factor receptor (PDGFR) for the platelet-derived growth factor in cells stably transformed with E5 (Petti *et al.*, 1991).

E5 has also been implicated in viral immune evasion with recent observations that in E5 expressing cells, the major histocompatability complex (MHC I) is not present on the cell surface but is retained in the Golgi apparatus. The treatment of E5-transformed cells with interferon which increases the synthesis of MHC I, did not prevent E5 from inhibiting MHC I transportation to the cell surface (Ashrafi *et al.*, 2002; Marchetti *et al.*, 2002). Preventing MHC I transport to the cell surface would have immense consequences for presentation of viral peptides to the immune system.

The E6 ORF encodes a 16-19kD cell transforming protein. Although small, E6 induces several important changes in the host cell that impact both on the normal viral life cycle and the process of immortalisation. The E6 protein contains four Cys-X-X-Cys motifs, indicating a potential for zinc binding. It may also act as a nucleic acid binding protein. E6 alone is not capable of immortalising primary human foreskin keratinocytes (HFK) but can efficiently immortalise human mammary epithelial cells (HMEC) (Kiyono *et al.*, 1997; Liu *et al.*, 1999). In high-risk HPVs such as HPV-16 however, E6 and E7 proteins are necessary and sufficient to immortalize their host squamous epithelial cells. The E6 proteins of high-risk HPVs have been shown to complex with p53 and to promote its degradation (Scheffner *et al.*, 1990) via the ubiquitin pathway, while low risk HPV E6

protein binds p53 less efficiently. In addition to interacting with p53, E6 may impact on apoptosis through p53-independent pathways. E6 can interact and inhibit Bak, a proapaototic protein expressed at high levels in the upper layers of differentiating epithelium (Thomas and Banks., 1998).

The E7 ORF encodes a 10-14kD cell-transforming protein. E7 proteins are approximately 100 amino acids in size and divided into domains with sequences homologous to the Ad5 E1A sequence with conserved region 1 (CR1) consisting of amino acids 1-20, CR2 containing residues 21-39 and CR3 composed of amino acids 40-100. The protein contains two Cys-X-X-Cys zinc-finger DNA-binding motifs, a pRB-binding domain, a casein kinase II (CKII) phosphorylation site, and regions that show homology to other viral oncoproteins. The role of E6 and E7in cell transformation is described in section 1.2.4.

L1 and L2 late gene products are estimated to be in a molar ratio of 30 to 1. The L1 ORF encodes the 56-60kD major capsid protein. The carboxy-terminus of the L1 protein of HPV-16 contains two basic sequences believed to be responsible for the nuclear localisation of the L1 protein. The minor capsid protein L2 has a molecular weight of 76-78kD. The N-terminal domain of the L2 protein is highly conserved among different papillomaviruses. Differentiation of the epithelium triggers a coordinate increase in the replication of the viral genome and expression of the L1 major and L2 minor structural viral proteins leading to the assembly of infectious viral particles in the nucleus. When L1 alone is expressed in eukaryotic cells by recombinant baculovirus or vaccinia virus

(Hagensee *et al.*, 1993b; Kirnbauer *et al.*, 1993; Rose *et al.*, 1993), it can self-assemble to form icosahedral particles or virus-like particles (VLP). When L2, which is not required for assembly, is coexpressed with L1, both L1 and L2 are incorporated into the particles (Hagensee *et al.*, 1993b; Kirnbauer *et al.*, 1993). Restriction of HPV late gene expression to terminally differentiated cells and differences in level of expression of late gene products from various HPV types may be due to the presence of negative regulatory elements in the HPV late mRNAs (Cumming *et al.*, 2002; Kennedy *et al.*, 1990; Kennedy *et al.*, 1991; Koffa *et al.*, 2000). L2 is described in further detail in section 1.2.8.

1.2.2. Epithelial Differentiation

The complete HPV replication cycle resulting in the production of infectious progeny (virions) is tightly linked to the differentiation state of infected cells (Meyers *et al.*, 1997). Epithelial cells are tightly bound together in sheets called epithelia which line all cavities and free surfaces of the body forming barriers to the movement of water, solutes and cells from one compartment to another. The differentiation of epithelial cells such as in the skin epithelium (i.e. the epidermis) is characterised by a series of coordinated biochemical and morphological changes resulting in a highly organised stratified epithelium. The epidermis is a dynamic epithelium that is constantly renewed throughout life. Its turnover is estimated at about 7days in mice and about 60 days in humans (Ghazizadeh and Taichman., 2001; Potten., 1981). Maintenance of the epidermis is accomplished by the presence of stem cells that self renew and generate progeny, which then undergo terminal differentiation (Watt., 2001).

Stem cells are not the only cells within the epidermis capable of proliferation. Stem cell progeny that are destined to undergo terminal differentiation can divide a small number of times before they irreversibly exit the cell cycle; these are known as transit amplifying cells (Fig 1.3b) (Lavker and Sun., 2003; Watt., 2001). Divisions of transit amplifying cells increase the number of terminally differentiated cells that are produced by each stem cell division. In normal undamaged epidermis, stem cells divide infrequently, whereas transit-amplifying cells are actively recycling but have a low self-renewal capacity and a high probability of undergoing terminal differentiation (Taylor *et al.*, 2000).

The layers of the stratified epithelium begin with a basal layer of cells anchored to a basement membrane. The basal layer contains proliferating cells, which expand by cell division, some cells then detach from the basement membrane and begin to move upwards towards the surface and activate a programme of terminal differentiation. As the cells move up towards the surface, they progress through three distinct stages; spinous, granular and stratum corneum (Fig 1.3a). As basal cells enter the spinous layer, they strengthen their cytoskeletal and intracellular connections, gaining resilience to mechanical stress. Once this task is completed, the cells enter the granular layer, where they produce the epidermal barrier. The barrier precursors consist of two major components: (i) glutamine- and lysine-rich cornified envelope precursor proteins, which are synthesized and deposited beneath the plasma membrane and (ii) lamellar granules, which are filled with lipid bylayers. As the cells enter the final phases of terminal differentiation, a flux of calcium activates the enzyme transglutaminase, which biochemically cross-links the cornified envelope proteins through ε-(γ-glutamyl)lysine

isopeptide bonds and which activates extrusions of the lipid bylayers onto this scaffold. Cell death ensures, leaving dead, flattened squames at the skin surface, the end process of terminal differentiation (Fig 1.3a).

Differentiation Programe of a Stratified Epithelium

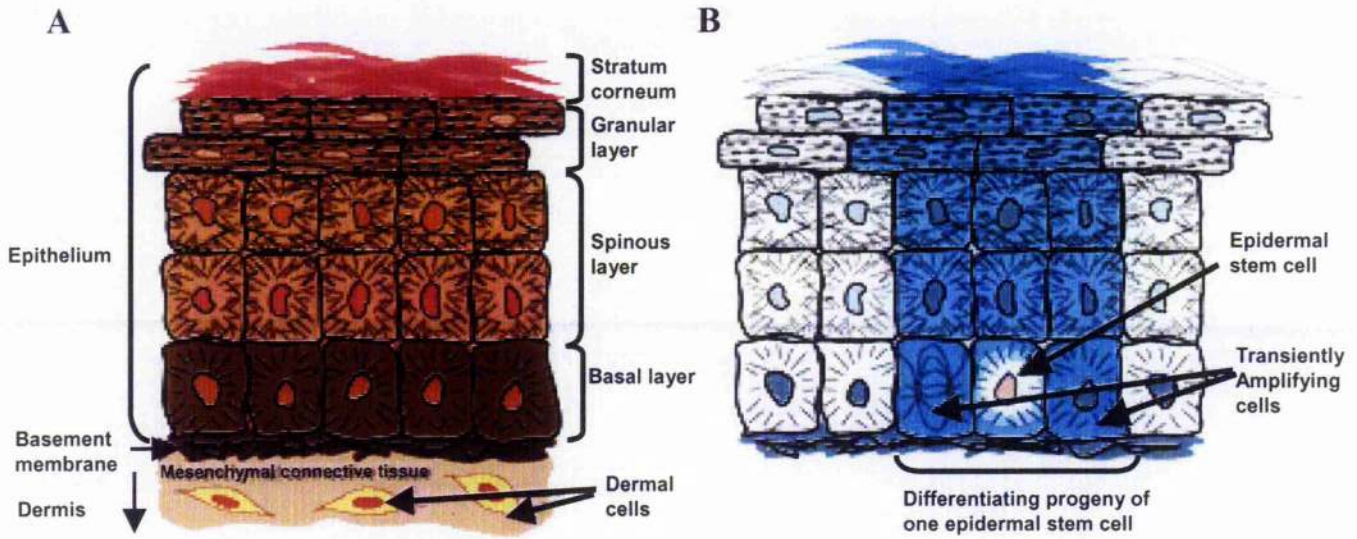


Figure. 1.3. (*A*) Diagrammatic representation of skin epithelial histology. Cells of the basal layer attach to an underlying basement membrane. As basal cells enter the spinous layer, they strengthen their cytoskeletal and intercellular connections, gaining resilience to mechanical stress. The cells then enter the granular layer, where they produce the epidermal barrier. As the cells enter the final phases of terminal differentiation, a flux of calcium activates the enzyme transglutaminase, which biochemically cross-links the cornified envelope proteins. Cell death ensues, leaving dead, flattened squames at the skin surface, the end-process of terminal differentiation. These squames of the stratum corneum eventually slough from the skin surface, to be replenished continually by inner layer cells moving outward. (*B*) Diagram of the epidermal proliferative unit. A putative slow-cycling epidermal stem cell occasionally divides, giving rise to a stem cell daughter and a transiently amplifying daughter. The transiently amplifying cell divides two to four times, and these progeny then leave the basal layer and execute a program of terminal differentiation. *From Alonso and Fuchs, Proc Natl Acad Sci U S A. 2003 Sep 30;100 Suppl 1:11830-5.*

As the cells progress through the different layers of the epithelium, unique sets of genes are turned on and off in a growth and differentiation specific manner. These include genes encoding structural cytoskeletal proteins, transcription factors and products that are necessary for the barrier function of the skin. Basal cells transcribe genes encoding keratins K5 and K14, while cells in the spinous layer switch off these genes and induce K1 and K10, required for the marked mechanical resistance of these cells (Fuchs, 1995). In the granular layer, cells express loricrin, filaggrin and transglutaminase, which are involved in the assembly of the cornified envelope. The cornified envelope plays a critical role in barrier function of the tissue and for the organism. Several other proteins have been identified to be components of the epidermal cornified envelope, they include involucrin, cystatin α, elafin and several small proline rich proteins (SPR1 and SPR2) (Steinert and Marekov., 1995). This structure forms a sac to contain the keratin filaments and to provide a scaffold upon which specialized lipids are extruded and organized (Fuchs., 1993; Fuchs., 1997). Once the barrier is in place, keratinocytes cease their metabolic activity becoming dead squames that are eventually sloghed off the skin surface, continually being replaced by stems cells transiting outward and differentiating.

Several molecular networks and cellular signalling pathways regulate the balance between epithelial growth and differentiation. In cultured epidermal cells, the concentration of extracellular Ca^{2+} can select for a basal or differentiation phenotype (Hennings *et al.*, 1980; Yuspa *et al.*, 1983). Under conditions were Ca^{2+} is <0.1mM, keratinocytes proliferate rapidly, express a basal cell phenotype and do not cornify, while higher Ca^{2+} induces terminal differentiation in basal keratinocytes (Yuspa *et al.*, 1982).

Also physical analyses of tissue sections from mouse and human epidermis have demonstrated the existence of a gradient of Ca^{2+} in which basal layer Ca^{2+} content is low (<0.1mM) while granular layer Ca^{2-} content is much higher (Malmquist *et al.*, 1984; Menon *et al.*, 1985).

Adhesion mediated by β1 integrins have also been known to regulate terminal differentiation of epidermal keratinocytes and when mouse or human keratinocytes are placed in suspension they withdraw rapidly from the cell cycle and initiate terminal differentiation (Watt., 2000). Other regulators of terminal differentiation are 14-3-3σ (Dellambra *et al.*, 2000) and α-catenin (Vasioukhin *et al.*, 2001). α-Catenin links adherens junctions to the actin cytoskeletion, targeted ablation of α-catenin in mouse epidermis results in epidermal hyperproliferation and sustained activation of the Ras/MAPK cascade (Vasioukhin *et al.*, 2001). 14-3-3σ binds to phosphoserine motifs in a variety of cellular proteins and can thus contribute to the regulation of a range of signalling pathways, including Ras/MAPK cascade; expression of 14-3-3σ antisense mRNA blocks exit from the stem cell compartment in cultures of human keratinocytes (Dellambra *et al.*, 2000).

1.2.3. HPV LIFE CYCLE

The productive life cycle of papillomaviruses usually begins in epithelial stem cells located in the basal layers of the stratified epithelium. The virus enters the epithelium through microlesions and infects the basal epithelial cells. As these cells keep dividing, they produce daughter cells, which withdraw from the cell cycle, migrate away from the basal layer and become committed to differentiation. As HPVs infect proliferating undifferentiated keratinocytes, the HPV genome becomes established as extrachromosomal elements in the nucleus and copy number is increased to approximately 50-100 copies per cell. As infected cells divide, viral DNA is distributed between both daughter cells. One of the daughter cells migrates away from the basal layer and initiates a program of differentiation. The other daughter cell continues to divide in the basal layer and provides a reservoir of viral DNA for further cell divisions. Since production of HPV is restricted to suprabasal cells, the cells in the basal layer continue to proliferate (Fig 1.4). This differentiation dependence allows the infected cells to persist in the basal layers for periods as long as several years (For reviews see Desaintes and Demeret., 1996; Phelps *et al.*, 1998; Stubenrauch and Laimins., 1999).

All HPV genomes contain approximately eight open reading frames, which are transcribed as polycistronic messages from a single DNA strand. Regulatory sequences for early viral transcription and DNA replication are concentrated in a small non-coding region termed the long control region (LCR). In basal cells, transcripts from high-risk types are initiated from a promoter in the LCR such as p97 for HPV-16. The maintenance and establishment of HPV genomes are associated with expression of the early HPV transcripts that encode the oncoproteins E6, E7 and E5 as well as the replication proteins

E1 and E2. Viral replication is intimately tied to the differentiation programme of the host tissue. As the cells of the basal layer divide, one of the daughter cells migrates away from the basal layer and initiates a program of differentiation. The other daughter cell continues to divide in the basal layer and provides a reservoir of viral DNA for further cell divisions. Since production of HPV is restricted to suprabasal cells, the cells in the basal layer continue to proliferate (Fig 1.4). This differentiation dependence allows the infected cells to persist in the basal layers for periods as long as several years (For reviews see (Desaintes and Demeret., 1996; Phelps *et al.*, 1998; Stubenrauch and Laimins., 1999).

Cell proliferation poses a problem for the virus which needs the replicative machinery of the cell for viral DNA synthesis, and a terminally differentiated cell will contain little or no replicative enzymes. Therefore the virus needs to stimulate G1 to S phase progression in the face of a cell programmed to terminally differentiate, in order to produce the correct environment for viral DNA amplification. The virus is still dependent on cellular differentiation as the late promoter, which regulates the mRNA coding for the capsid proteins L1 and L2, is only switched on in partially differentiated cells. HPV-infected cells, contrary to normal epithelial cells, continue cycling also in the subrabasal layers (Doorbar *et al.*, 1997b) resulting in amplification of the viral genomes to 1000-10000 copies per cell (Lambert., 1991) (Fig 1.4).

The cycle of human papillomavirus infection of epithelial cells

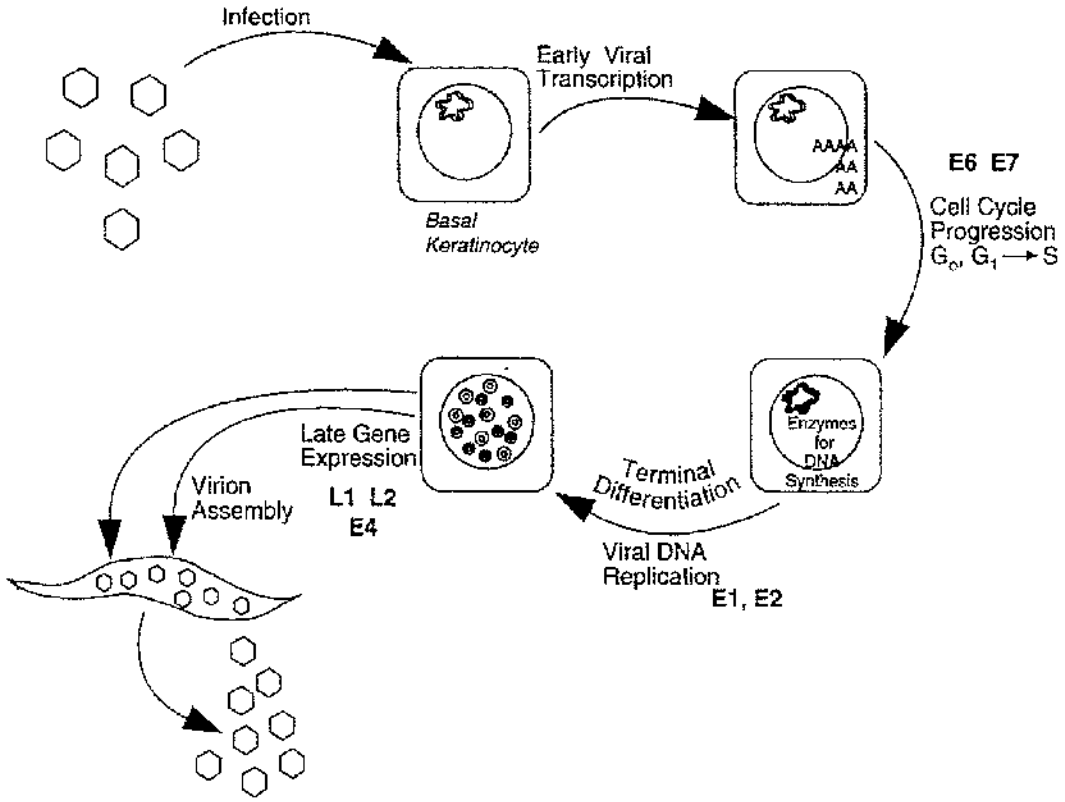


Figure 1.4. The cycle of human papillomavirus infection of epithelial cells. Virus infection is believed to occur within the basal keratinocytes, and early transcription of the viral genome is thought to be regulated by the E2 protein. Cellular proliferation and perturbation of keratinocyte differentiation is induced by the E6 and E7 proteins. Stimulation of cells into the S phase of the cell cycle leads to induction of host enzymes for DNA synthesis. Viral DNA replication is initiated, and double-stranded progeny episomes are accumulated. Capsid proteins, L1 and L2, and a late-associated protein, E4, are synthesized, and virus particles are assembled during terminal stages of keratinocyte differentiation. Progeny virus particles are released as dead keratinocytes are sloughed from the surface of the epithelium. *From Phelps, W. C. P. et. al. Ann Intern Med 1995;123:368-382.*

Transcription of papillomavirus genes is dependent upon epithelial specific transcriptional enhancers present in the viral long control region (LCR). Multiple promoters direct transcription of subsets of viral genes. These promoters are differentially regulated by viral and cellular transcription factors. Promoters directing transcription of early genes are active throughout the epithelial layers, while those directing expression of the late genes are preferentially active in highly differentiated cells. Viral proteins are expressed from differentially spliced mRNA at different times during the migration of the infected cell towards the epithelial surface. With the exception of the virion structural proteins and E4, viral gene products are not readily detected in vivo (Doorbar *et al.*, 1986; Doorbar *et al.*, 1988). The onset of HPV-31b early transcription following epithelial cell infection was examined by detecting newly synthesized, spliced viral transcripts by reverse transcription and PCR (Ozbun., 2002). E1 and E2 transcripts were detected as early a 4 hours post-infection, whereas the other major viral transcripts such as E6, E1^E4 were detected by 8 to 10 hour post-infection.

E7 is expressed in lower epithelial layers as determined by the presence of E2F-activated gene products such as MCMs (Middleton *et al.*, 2003). In HPV-16 infected epithelial raft tissues prepared using NIKS cells harbouring HPV-16 episomes, cells expressing both E4 and E7 were found in the intermediate epithelial layers. Cells expressing E7 in the absence of E4 were occasionally apparent in the upper epithelial layers (Middleton *et al.*, 2003). In lesions caused by HPV 16, E7 is expressed from the early promoter (p97), which also directs expression of E6. Very low E6/E7 transcripts are detected in basal cells in low-grade dysplastic lesions, while an increase in their levels is observed in

differentiated upper layers of the epidermis (Durst *et al.*, 1991). In contrast, high-grade lesions and squamous cell carcinoma (SCC) show high levels of E6/E7 transcripts throughout all layers of the epithelium, including the basal layers (Durst *et al.*, 1991; Stoler 1992). Staining of cervical section for E2 showed detection in CIN I and II grade lesions. E2 expression was confined to the superficial layers of the epithelium, specifically to the koilocytic nuclei with little immunoreactivity observed in the intermediate or basal layers. Weak detection of E2 was observed in CIN III lesions, while no E2 was detected in SCC lesion (Stevenson *et al.*, 2000).

E4 proteins expressed from the spliced mRNA E1^E4 was first detected in naturally occurring lesions in cells in which vegetative viral DNA replication is occurring (Doorbar *et al.*, 1997a). The E4 proteins persist throughout the late stages of infection and are modified by proteolytic processing and phosphorylation (Grand *et al.*, 1989). E4 proteins localise in part to cytoplasmic intermediate filaments in low grade squamous intraepithelial lesions caused by HPV-16 but are also diffusely cytoplasmic and perinuclear. As infected cells near the epithelial surface, E4 proteins localize to perinuclear bundles (Doorbar *et al.*, 1997b). Although its function in the viral life cycle is still unclear, expression of the HPV E1^E4 protein in keratinocytes in monolayer cultures leads to a collapse of cellular intermediate filament network (Doorbar *et al.*, 1991). E4 has been implicated in viral genome amplification with the observation of a correlation between initiation of E4 expression and the onset of viral DNA amplification in suprabasal epithelial cells (Breitburd *et al.*, 1987).

Expression of E4 precedes the synthesis of virus structural proteins and the assembly of infectious particles. The production of HPV late-gene products, L1 and L2 are detected primarily in the terminally differentiated cells in the upper layers of the epithelium (Hagensee *et al.*, 1993a; Turek., 1994). In addition to promoter switch, one reason for the restriction of HPV late gene expression to terminally differentiated cells is due to the presence of negative regulatory elements in the HPV late mRNA identified as an inhibitory sequence in the 3' untranslated region (UTR) which acts by reducing mRNA stability *in vitro* (Kennedy *et al.*, 1990; Kennedy *et al.*, 1991). This post-transcriptional regulation of late mRNA may also be responsible differences observed in the levels of expression of late gene products from various HPV types.

1.2.4. HPV and Cell Transformation

The association of HPV infection and squamous cell precancer lesions of the uterine cervix has been established since the late 1970s. Today, oncogenic HPV types are regarded as the most important etiological factors of cervical squamous cell carcinoma. HPV types cause a variety of lesions in the genital tract of males and females (Schiffman and Brinton., 1995). Some types cause benign warts and low-grade premalignant lesions and are not found in malignant tissues. HPV-6 and HPV-11 are the most common isolates from these lesions. The high-risk viruses such as HPV-16 and HPV-18 are found in premalignant and malignant tissues. Initial infection with high-risk types causes low-grade disease, which is manifested by inhibition of the normal differentiation in the lower third of the epithelium. The lesions may remain low grade, regress, or progress to severe dysplasia. This later stage may persist or may start to penetrate the basement membrane leading to invasive disease. During progression from premalignant to malignant phases of

the disease, the relationship between the HPV genome to the host cell chromosomal DNA may change. Most metaplastic cells have been shown to have integrated HPV sequences (McCance *et al.*, 1986; zur., 2002) with integration occurring randomly in cellular chromosomes. All viral DNA integration events associated with malignant disease allow for the expression of E6 and E7.

Due to their limited coding capacity, HPVs have to use the cellular DNA synthesis machinery in order to replicate their genomes. However, while low-risk HPVs begin replication in cells that are still proliferating, the replicative phase of high-risk HPV infection is confined to more differentiated cells that have already exited the cell cycle and are non-permissive for DNA synthesis (Doorbar *et al.*, 1997a). In order to overcome the problem, the high-risk HPV E7 protein targets a number of cell cycle regulatory proteins, including the pocket protein family of pRb, p107 and p130, thereby upregulating genes required for G1/S transition and DNA synthesis (Fig 1.5a,b). However, the normal response of the host cell to this unscheduled induction of proliferation would be to trigger apoptosis and/or growth arrest. To overcome these obstacles, the high-risk E6 protein targets a variety of cellular proteins involved in regulating these defence mechanisms (Fig 1.6, Table 1.2).

The tumour suppressor protein p53 is a transcription factor that stimulates the expression of genes involved in cell-cycle arrest and apoptosis such as the cyclin-dependent kinase inhibitor p21. E6 binds to p53 and leads to ubiquitin-mediated degradation of the latter (Scheffner *et al.*, 1990). E6 mediated degradation of p53 involves an E3 ubiquitin ligase

E6AP. Under normal circumstances, viral replication would then continue resulting in production of infectious virions. On rare occasions, however, the viral life cycle is interrupted and processes are initiated that lead to immortalization and ultimately to full transformation of the cell. E6 alone is not capable of immortalising primary human foreskin keratinocytes (HFK) but can efficiently immortalise human mammary epithelial cells (HMEC) (Kiyono *et al.*, 1997; Liu *et al.*, 1999). In high-risk HPVs such as HPV-16 however, E6 and E7 proteins are necessary and sufficient to immortalize their host squamous epithelial cells. Both *in vitro* and *in vivo* studies have show that the function of both proteins, particularly the high-risk types are essential for cellular transformation (Herber *et al.*, 1996; Song *et al.*, 1999). E6 and E7 from low risk types are weakly transforming proteins (Mansur and Androphy., 1993). The contribution of E6 and E7 to tumour progression is shown in fig 1.7.

Although targeting p53 for degradation is the major route by which E6 overcomes its effects, several reports indicate that E6 makes use of additional pathways to abrogate p53's growth suppressive activities. The capacity of high-risk E6 proteins to abrogate transactivation of p53 target genes does not depend only on p53 destabilization, as E6 mutants defective for degradation retain the ability to abrogate transcriptional activation by p53 *in vivo* (Pim *et al.*, 1994). This could be due to E6 interfering with binding of p53 to its DNA recognition site (Lechner and Laimins., 1994; Thomas *et al.*, 1995) or due to the interaction of E6 with the transcription coactivator p300/CBP (Patel *et al.*, 1999; Zimmermann *et al.*, 2000). Reports also indicate that other activities other than targeting p53 for degradation are required for the full transformation potential of E6 (Liu *et al.*,

1999; Nakagawa *et al.*, 1995; Pim *et al.*, 1994). Moreover, cutaneous HPVs mainly rely on their E6 proteins, which do not interact with p53, for their transformation ability (Elbel *et al.*, 1997).

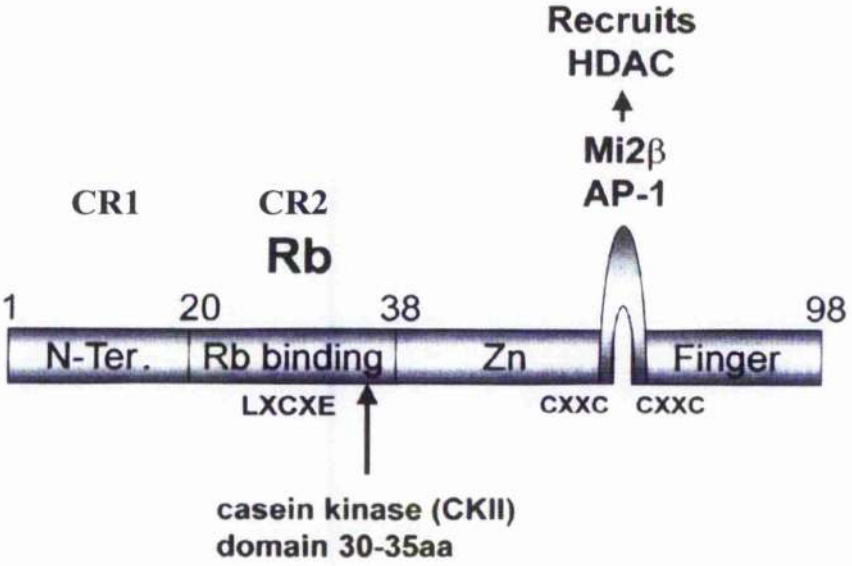
The high-risk HPV E6 oncoproteins have been implicated in activating telomerase, a potential mechanism for HPV-induced immortalisation (Klingelhutz *et al.*, 1996). Telomerase adds repeat sequences to chromosome ends and is believed to play a role in cellular immortalisation. E6-induced telomerase activity alone cannot result in immortalisation without coexpression of E7. E6 protein has also been shown to interact with cellular minichromosome maintenance proteins, which are believed to play a key role in regulating cellular DNA replication (Kuhne and Banks., 1998). E6 can also interact and inhibit Bak, a proapaototic protein expressed at high levels in the upper layers of differentiating epithelium (Thomas and Banks., 1998).

E6 has been shown to interact with a variety of PDZ domain-containing proteins. PDZ domains consist of approximately 90 amino acid long protein-protein interaction units often found within cellular proteins located at areas of cell-cell contact such as synaptic junctions in neurons and tight junctions in epithelial cells (Fanning and Anderson, 1999). PDZ domain containing proteins shown to interact with E6 include hDlg (Kiyono *et al.*, 1997, Lee *et al.*, 1997), MUPP1 (Lee *et al.*, 2000) and hScrib (Nakagawa and Huibregtse. 2000). HPV E6 proteins possess PDZ domain-binding motifs at their extreme carboxyl-termini (Lee *et al.*, 1997) (Fig 1.6). Binding of high-risk HPV E6 proteins to these PDZ proteins results in E6AP mediated ubiquitination and proteolysis (Gardiol *et al.*, 1999;

Nakagawa and Huibregtse, 2000). The cellular targets of E6 have been summarised in table 1.2.

Structure and cellular targets of HPV E7

a



b

Associated Cellular Proteins	*Functional consequences*
pRB, p107, p130	Disruption of E2F transcription factor complexes Degradation
$p21^{cip1}$	Inactivation of cdk and replication inhibitory activity
$p27^{kip1}$	Inactivationof cdk inhibitory activity
TBP	Transcription ?
TAF110	Transcription ?
AP-1	Activation of c-jun transcriptional activity function
Mi2β (HDAC)	Chromatin remodelling ?
IGFBP-3	Inhibition of IGFBP-3 mediated apoptosis
M2 Pyruvate kinase	?
α-Glucosidase	?
hTid-1	?
p48 component of ISGF3	Inhibition of interferon-α stimulation
F-actin	?
Forkhead transcription factor MPP2	Activation of MPP2 transcriptional activity
S4 subunit of proteasome	?

Figure 1.5. (a) A schematic representation of the HPV E7 protein showing CR1 HD, CR2 HD denoting conserved regions of 1 and 2 of the Adenovirus E1A protein respectively. CR2 HD contains the core pRB binding domain (LXCXE) and a consensus sequence for casein kinase II phosphorylation (CKII). The metal binding domain consists of two C-X-X-C sequences in the carboxyl terminus. *From McCance J., ed. Human Papillomaviruses. Perspectives in medical virology 8. Elsevier 2002.* (b) **Table 1.2**. Showing known cellular proteins which interact with E7. *Data complied from Munger and Howley 2002. Virus Res. (8) 9:213-228.*

Schematic Representation of HPV E6 protein

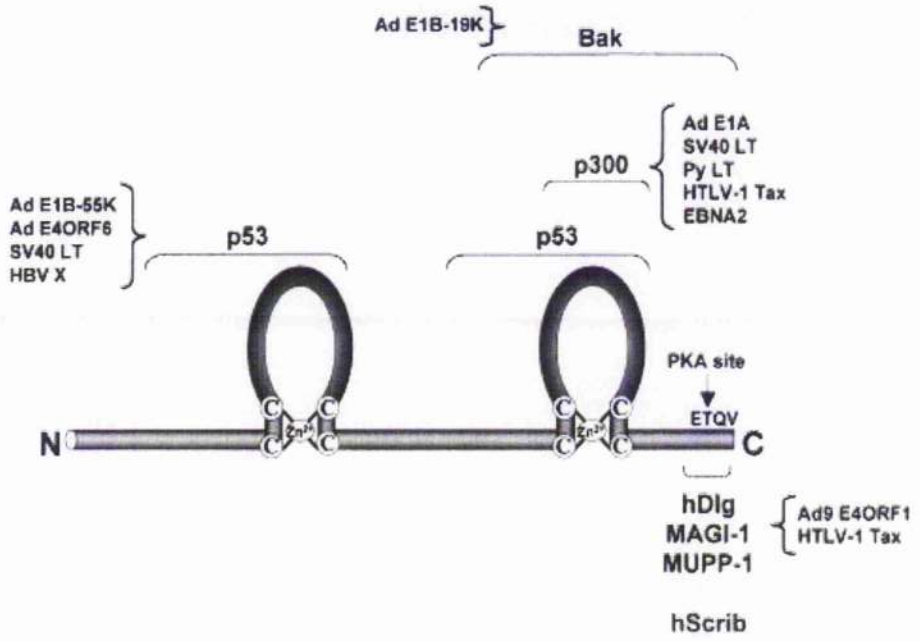


Figure.1.6. The high-risk HPV E6 protein. Schematic diagram of E6 showing the two zinc fingers, together with the regions involved in interactions with cellular proteins (summarised in table 1.2) that are targeted by the oncoproteins of other viruses. Also shown is the C-terminal PDZ binding motif, ETQV, and the overlapping site of PKA phosphorylation is arrowed. *From Mantovani and Banks, Oncogene (2001) 20, 7874-7887.*

Cellular Targets of HPV E6

Target Protein	*High-risk E6*	*Low-risk E6*	*Degradation*	*Cellular function*	*E6AP involved*
P53	yes	yes	yes	Transcription factor, apoptosis inducer, tumour suppressor	yes
E6AP	yes	no	yes	Ubiquitin ligase	yes
hMcm7	yes	yes	yes	DNA replication initiation	yes
E6TP1	yes	yes	yes	Putative GAP protein	yes
Bak	yes	yes	yes	Apoptosis inducer	yes
C-Myc	yes	no	yes	Transcription factor	yes
P300/CBP	yes	yes	yes	Transcriptional coactivators	no
AMF-1/Gps2	yes	yes	yes	Transcriptional coactivator	?
IRF-3	yes (HPV-16)	no	no	Transcription factor	no
E6BP/ERC-55	yes	no	no	Ca^{2+} binding protein	yes
Paxillin	yes	no	no	Signal transduction	no
hDlg	yes	no	yes	Control of cell polarity and growth	no
MAGI-1	yes	no	yes	Putative signal transduction	no
MUPP-1	yes	no	yes	Scafolding protein/putative signal transduction	no
hScrib	yes	no	yes	Control of cell polarity and growth	yes

Table 1.3. Summary of known cellular targets of HPV E6 indicating the mutlifunctional nature of the protein. *Data from Mantovani and Banks, Oncogene (2001) 20, 7874-7887.*

HPV E6, E7 and Tumour Progression

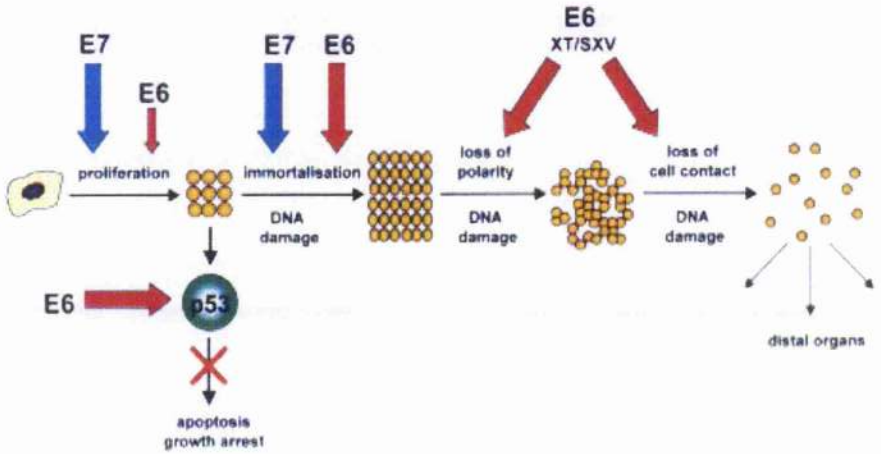


Figure 1.7. Contribution of HPV E6 and E7 to different stages of tumour progression. The role of E6 in tumour promotion is weak compared with that of E7 which actively stimulates cell proliferation. In contrast E6 promotes malignant progression: degradation of p53 overcomes growth arrest and/or apoptosis allowing accumulation of DNA damage, and induction of telomerase contributes towards immortalization. Finally, targeting PDZ-containing proteins through the C-terminal XT/SXV sequence of E6 causes loss of cell polarity and contact, strongly contributing to the malignant phenotype. *From Mantovani and Banks, Oncogene (2001) 20, 7874-7887.*

The ability of E7 to bind to the retinoblastoma (Rb) protein is perhaps the most characterized property of this viral oncoprotein. Evasion of cell cycle arrest by binding Rb is a property common to various viral oncoproteins, such as adenovirus E1A and simian virus SV40 large T antigen (Munger., 1995), this underscores the importance of Rb binding in the natural history of virus infection. Hypophosphorylated Rb binds to E2F transcription factor, repressing the transcription of cell cycle genes. During progression from G0 through G1 to S-phase, Rb gets hyperphosphorylated by G1 cyclin-cyclin dependent kinases, releasing E2F, which in turn activates genes involved in DNA synthesis and cell cycle progression (Dyson., 1998). E7 binds to hypophosphorylated Rb and thereby induces cells to enter into premature S-phase by disrupting Rb-E2F complexes (Huang *et al.*, 1993; Wu *et al.*, 1993).

Binding to Rb is primarily mediated through amino acid sequences contained in the conserved amino acid terminal of CR2 region of E7 (Barbosa *et al.*, 1990). The CR2 region of E7 binds to Rb and its family members p107 and p130, through sequence motifs LXCXE. The LXCXE motif of E7 has been shown to specifically bind to the Rb pocket region, between 649 and 772 amino acids (Huang *et al.*, 1993). The E7 proteins of HPV-16 and HPV-18 bind hypophosphorylated Rb with higher affinity than HPV-6 and HPV-11 (Griffiths and Mellon., 1999). E7 has also been associated with histone deacetylases (HDAC), AP-1 transcription factors, cyclins, cyclin-dependent kinases (cdks), and cdk inhibitors. These associations contribute to the ability of E7 to induce cellular proliferation, immortalisation and transformation.

Cyclin E-cdk2 is essential for initiation of DNA synthesis and can activate cell cycle progression in the absence of cyclin D-cdk4 underlining this complex as a potential target by which E7 propels cell cycle re-entry. E7 expressing cells have been shown to maintain cyclin E-cdk2 activity in the presence of growth arrest signals such as epithelial differentiation, serum deprivation, and anchorage-independent growth (Ruesch and Laimins., 1998). E7 has been detected in complex with p107-cyclin E-cdk2, suggesting that E7 may affect cyclin E-cdk2 through physical interaction (McIntyre *et al.*, 1996).

To determine whether E7 plays a role in the HPV-16 life cycle, Flores *et al.*, (2000), created an E7-deficient HPV-16 genome by inserting a translational termination linker in the E7 gene of the full-length genome (Flores *et al.*, 2000). This DNA was transfected into an immortalized human foreskin keratinocyte cell line, BC1-Ep/SL, which support the full viral life cycle. Cells harbouring the wild type or E7 deficient HPV-16 were grown using raft cultures and also suspended in methylcellulose to induce differentiation, which promotes the productive phase of the HPV-16 life cycle. The loss of E7 resulted in lack of viral DNA amplification and reduced L1 expression. In contrast, cells harbouring wild type HPV-16 genome induced the host cell DNA replication machinery and inhibited the differentiation of the host cell in the suprabasal layers of the raft.Concluding that E7 is important for the full viral life cycle.

The link between disruption of E2F/pRB binding and cell transformation by E7 is still under investigation. Interaction of pRB with E7 is mediated through the amino terminal domain containing the LXCXE pRB binding motif. Although this region of E7 mediates

high affinity binding to pRB, it is not sufficient to displace E2F from pRB (Huang *et al.*, 1993; Wu *et al.*, 1993). The carboxyl region of E7 has been shown to bind pRB and is required for displacement of E2F from pRB. Chimeric E7 proteins with carboxyl terminus replaced with structurally related HPV E6 derived domains were impaired for disruption of E2F/pRB complexes but retained transformation competence (Braspenning *et al.*, 1998; Mavromatis *et al.*, 1997). This suggests that the ability of E7 to disrupt pRB/E2F complexes and cellular transformation are not necessarily linked. This is further supported by evidence that several non-transforming HPV-16 E7 mutants retain the ability to interact with pRB and efficiently activate transcription of E2F-responsive promoters (Banks *et al.*, 1990; Edmonds and Vousden., 1989; Phelps *et al.*, 1992). Jewers *et al* (1992) showed that mutations in HPV-16 E7, which eliminate binding to pRB, do not alter the ability of the constructs to immortalize primary keratinocytes (Jewers *et al.*, 1992). Therefore these results suggest that the transformation ability of E7 is not primarily linked to its interaction with pRB but could result with interaction with other cellular proteins.

1.2.5. HPV and Cervical Cancer

The cervix is the narrow lower portion of the female uterus that extends into the vagina. Cancer of the cervix is the second most common malignant disease among women coming only second to cancer of the breast. In many developing countries, it is the most common cause of cancer and death. The primary cause of cervical cancer is HPV as more than 90% of squamous cervical cancers contain HPV DNA (Herzog., 2003; Waggoner., 2003). Infection is believed to be primarily by sexual activity. Only high risk HPVs such as types 16, 18, 31 and 33 promote the development of cervical cancer to any appreciable

extent (Campo., 1998; Waggoner., 2003). The variation in carcinogenic potential among HPVs is governed by the E6 and E7 proteins; specifically the capacity of these proteins to interact with and alter or destroy key cell cycle regulatory molecules. Factors associated with the development of cervical cancer include the age of onset of sexual activity, number of sexual partners as well as history of genital warts. Immunocompromised patients such as HIV positive ones and smokers are also at increased risk.

Cervical intraepithelial neoplasia (CIN) associated with infections of high-risk HPV types typically begins as flat or inverted condylomas (CIN I). Grades of CIN identify increasing numbers of undifferentiated malignant cells and a decrease in normal epithelial cell differentiation. CIN III therefore morphologically describes severe dysplasia and carcinoma *in situ* (Fig 1.8). In most cases progression of CIN lesions to carcinoma takes a few years of continuous presence of high-risk HPVs. The prognosis for patients with cervical cancer is dependent on the stage of disease at diagnosis. Routine Pap screening is able to detect precancerous lesions in the cervix and carcinoma in situ. Preinvasive disease is generally managed through ablative or excisional procedures. Radical hysterectomy or radiotherapy can be used for early invasive stages while more advanced stages are treated with radiotherapy or combined chemotherapy and radiotherapy (For review see Herzog., 2003).

Diagram of cervical cancer disease progression

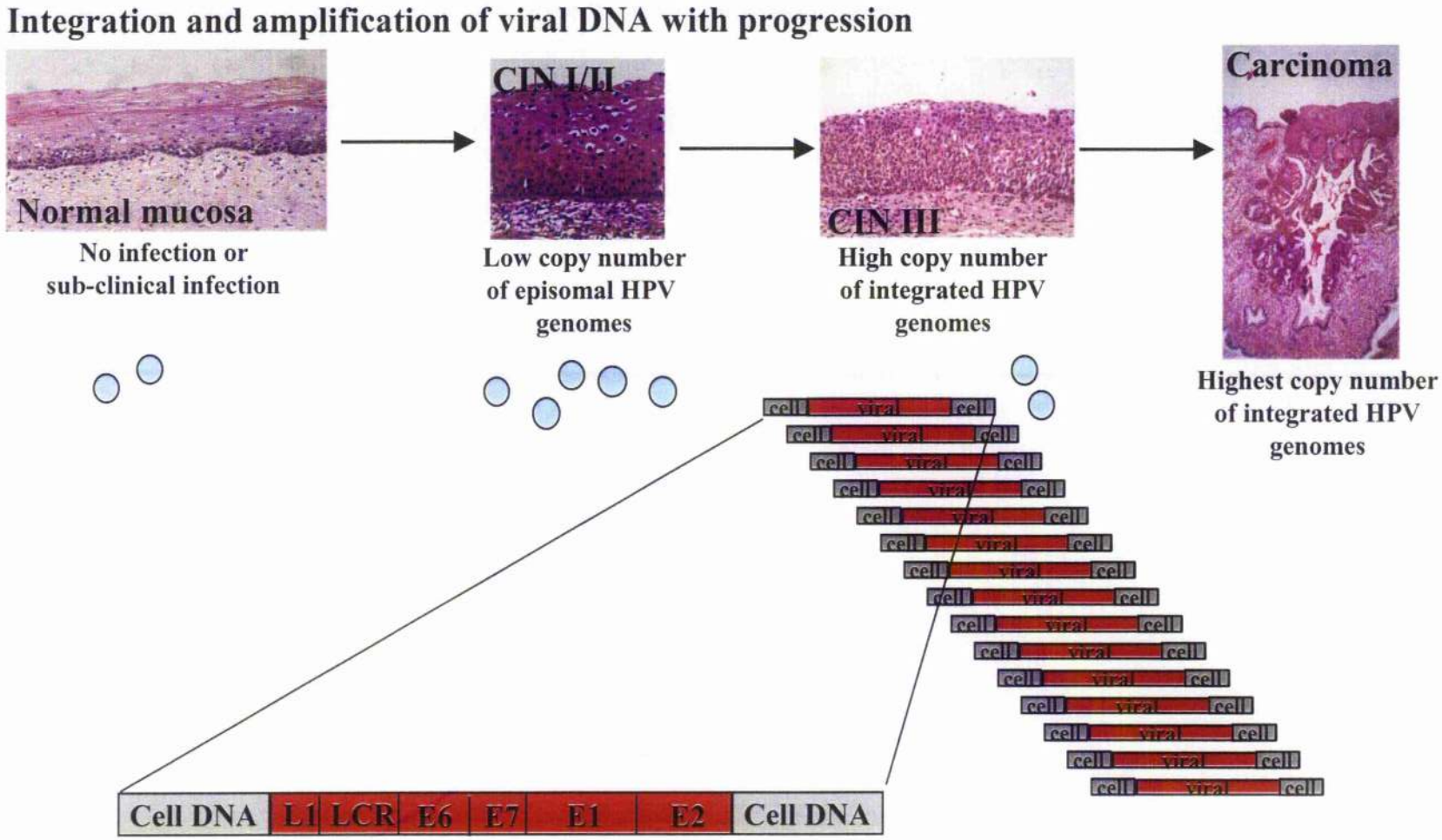


Figure 1.8. Showing progression of HPV viral infection detected by *in situ* hybridization. Cervical carcinoma begins with a noninvasive intraepithelial lesion designated cervical intraepithelial neoplasia (CIN). CIN I/II lesions correlate wit establishment and maintainance HPV DNA. CIN III is associated with integration of viral DNA and replication of HPV DNA to high copy number. CIN III eloves into carcinoma with prolonged persistence. *Cortesy of Campo M S.*.

1.2.6. Other HPV Associated Diseases

A small but significant number of patients present with papillomas of the mucosa of the respiratory tract. Although laryngeal papillomas are associated with low-risk HPV-6 and HPV-11, these usually benign infections can cause life-threatening disease by obstruction of the airways, and from the debilitating effects of frequent treatments for recurrences. Squamous cell carcinoma of some oral and head and neck cancer contain high-risk HPV-16, HPV-18 and HPV-31, and occasionally, low-risk HPV-6 and HPV-11 (Schwartz *et al.*, 1998). Epidemiological and experimental data suggest that some chemicals, nutritional deficiencies, physical factors and infectious agents are associated with the development of oesophageal squamous cell carcinoma (Syrjanen, 2003). One of the earliest evidence for the involvement of papillomaviruses in oesophageal carcinogenesis has been obtained from studies from cattle in Scotland, which have a high incidence of upper alimentary tract papillomas and carcinomas (Campo *et al.*, 1981; Campo, 1987; Campo *et al.*, 1990). Bracken fern is a crucial factor in malignant conversion of these papillomas. Bracken fern contains carcinogenic agent that can induce damage to DNA and immunosuppresants that inhibit the ability of the animal to mount an immune response against the virus (Jackson *et al.*, 1993). Experimental data suggests that similar mechanisms to those that occur in cervical carcinogenesis may also be involved in oesophageal carcinogenesis with both E6 and E7 interfering with cell-cycle regulation (Syrjanen., 2003).

Epidermodysplasia verruciformis (EV) is a rare recessive autosomal disease associated with cutaneous flat papillomas known as macular lesions. Some lesions progress to

malignant squamous cell carcinomas (Jablonska *et al.*, 1972; McGregor and Rustin., 1994). EV carcinomas harbor high copy numbers of episomal HPV genomes (usually HPV5 or occasionally HPV8, 14, 17, 20, or 47) and abundant transcripts of the E6 and E7 open reading frames (Orth., 1986). Patients with EV tend to have depressed cell-mediated immunity. A susceptibility locus for EV has been mapped to chromosome 17q25, with the identification of nonsense mutations in two adjacent genes, EVER1 and EVER2 (Ramoz *et al.*, 2002). The gene products EVER1 and EVER2 have features of integral membrane proteins and are localized in the endoplasmic reticulum.

The viral and cellular molecular mechanisms leading to the early development of skin malignancies in EV patients are still poorly understood. HPV-5 and HPV-8 E7 proteins have been shown to associate with retinoblastoma (Rb) protein, though with reduced binding affinities compared to HPV-16 and HPV-18 E7 proteins (Schmitt *et al.*, 1994; Yamashita *et al.*, 1993). In addition, the E6 proteins of oncogenic EV HPV do not possess the ability to interact with p53 protein and ubiquitin-protein ligase E6-AP, which would promote p53 degradation and inhibit p53-mediated transactivation of cellular genes (Elbel *et al.*, 1997; Kiyono *et al.*, 1994; Steger and Pfister., 1992). Analyses of the p53 status in EV carcinogenesis from lesion specimens from two HPV5-infected EV patients showed a dysfunction of the p53 gene in EV tumours with mutations detected at different stages of tumour progression (Padlewska *et al.*, 2001). The role of p53 in EV carcinogenesis is supported with the report that expression of HPV-5 E6 inhibits ultraviolet (UV) radiation-induced G1 arrest and apoptosis, in spite of increased levels of transcriptionally active p53 protein (Jackson and Storey., 2000). This is particularly

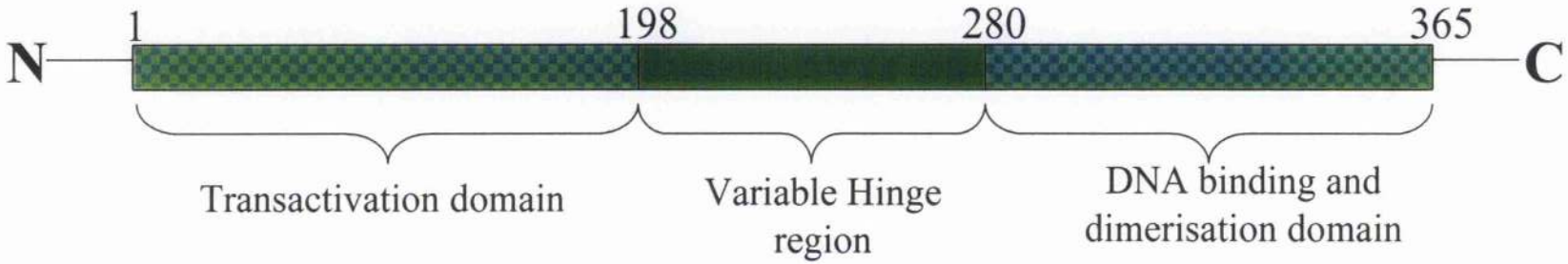
relevant as malignant transformation of EV lesions usually occurs on sun-exposed areas of the skin, suggesting that UV radiation may act as a cofactor in the carcinogenesis process (Majewski and Jablonska., 1995; Orth., 1986).

1.2.7. The Transcription Regulator E2

The E2 protein belongs to a family of proteins that form dimeric β-barrels and use surface α-helices for DNA interaction. The E2 protein has three domains; the well-conserved N-terminal/trans-activating and C-terminal DNA-binding/dimerisation domains are connected through a variable hinge region (Giri and Yaniv., 1988). The activation domain is involved in interactions with the replication protein E1 and the cellular proteins TFIIB and AMF-1. E2 activates transcription in a cell type dependent manner (Morgan *et al.*, 1998; Vance *et al.*, 1999). The DNA-binding domain of E2 has been shown to make functional interactions with the replication protein E1 and the transcription factor TBP (Fig 1.9). E2 facilitates efficient binding of E1 to its target DNA sequence (Seo *et al.*, 1993). The E1 binding site is juxtaposed with an E2 DNA binding site. The transcription and replication functions of E2 can be separated by point mutations within the amino-terminal 200 amino acids (Ferguson and Botchan., 1996; Sakai *et al.*, 1996; Winokur and McBride., 1992). The crystal structure of BPV-1 E2 DNA binding domain revealed that the protein forms a dimeric β-barrel with surface recognition α-helices. BPV-1 E2 undergoes a subtle rearrangement in subunit orientation upon DNA binding while maintaining the overall structure of each monomer (Hegde and Androphy., 1998). The DNA wraps around the E2 β-barrel enclosing both recognition helices in successive major groves (Fig 1.10).

The Papillomavirus E2 protein

a



b

Associated cellular proteins	*Functional consequences*
TATA-binding Protein (TBP)	Enhance transcriptional activation
TFIIB	Enhance transcriptional activation
AMF-1/Gps2	Enhance E2 interaction with p300
p300/CBP	Transcription activation
YY1	Transcription
p53	DNA damage response
TopBP1	DNA damage response
PARP	DNA damage response
RPA	DNA replication

Figure 1.9. Schematic representation of Human papillomavirus type-16 E2 protein. (a) HPV-16 E2 has three domains, an N-terminal trans-activating domain, a C-terminal DNA-binding and dimerisation domain which are connected through a variable hinge region. The transactivation domain is involved in interactions with the replication protein E1 and the cellular proteins TFIIB, Sp1 and AMF-1. DNA-binding domain also interacts with E1 and the transcription factor TBP. (b) Table showing known cellular proteins associated with E2.

The E2-DNA complex of BPV-1 and HPV-18

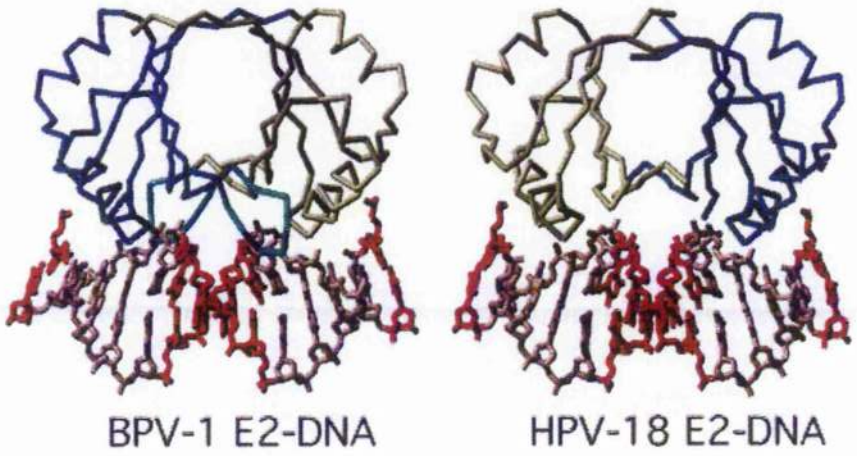


Figure 1.10. The BPV-1 E2-DNA complex (left) and the HPV-18 E2-DNA complex (right). The subunits of both proteins are indicated in blue and yellow. The conserved half-sitesin DNA are indicated in pink. The green loops connecting β2 and β3in each subunit of BPV-1 E2 reach across the molecular dyad to make phosphate contacts. The corresponding loop in HPV-18 E2 remains disordered upon DNA binding not shown in this diagram. *From Hegde, R. S., Annu. Rev. Biophys. Biomol. Struct. 2002. 31:343-360.*

The transcriptional regulation and replication functions of E2 are mediated through its interaction with a palindromic consensus sequence $ACCGN_4CGGT$ in the viral LCR. Multiple E2 binding sites that differ in the sequences of the central N_4 "spacer" nucleotides are present in the viral LCRs (17 in BPV-1 and 4 in HPV-16). Although all E2 proteins recognise the same consensus palindromic DNA sequence, proteins from different viral types differ in their abilities to discriminate among their specific DNA-binding sites. The LCR of HPV-16 as well as other mucosal epitheliotropic papillomaviruses such as HPV-18 or BPV-4 has four E2 binding sites. Two immediately upstream from the TATA box separated by 3 or 4bp, the most proximal of which to the TATA box mediates transcriptional down-regulation at high levels of E2 (Morgan *et al.*, 1998). Further upstream there are two other sites, one juxtaposed with the E1 DNA binding site while the other is some 300 or 400bp further upstream (Fig 1.2).

The role of the hinge domain of E2 is less well defined, although it is generally considered a flexible linker between the two functional domains (Gauthier *et al.*, 1991). In a recent study, Zou *et al.*, (2000) used green fluorescent protein (GFP) as a tracer to delineate the domains of HPV-11 E2 responsible for its nuclear localization (Zou *et al.*, 2000). Mutational analysis identified in the hinge a cluster of basic amino acids critical for nuclear localisation signalling and for nuclear matrix association. Phosphorylation of the hinge domain has been associated with the stability of BPV-1 E2 protein (Lehman *et al.*, 1997; McBride *et al.*, 1989). However studies with HPV-18 have shown that the hinge region is not involved in controlling the stability of the protein (Bellanger *et al.*, 2001).

The major role of E2 in HPV DNA replication is to target E1 to the HPV origin of replication as E1 has a weak affinity for it's binding sequence. Once viral replication has been initiated, E2 is no longer required for DNA synthesis. E2 has been shown to bind the cellular replication factor RPA (Li and Botchan, 1993). It has also been shown that E1-mediated replication of bovine papillomavirus type 1 is repressed by nucleosomal assembly. E2 counteracts this repression, suggesting that E2 might have additional functions during viral replication (Li and Botchan, 1994). Analyses of various deletion mutants of E2 demonstrate that in most cases, an intact transactivation domain and an intact DNA binding domain are required for DNA replication (Ustar and Ustar 1998). However some mutants which lack regions in the C-terminal DNA-binding dimerization domain have been shown to support replication (Winoker and McBride 1992). Mutational analysis has also shown that the role of the hinge region of E2 in replication is to provide a flexible linker function, which connects the N-terminus transactivation domain and the C-terminus DNA binding-dimerization domain. The particular amino acid sequence of the hinge region is not important for the ability of E2 to bind DNA or initiate replication (Allikas *et al.*, 2001).

1.2.8. The Minor Capsid Protein L2

The exact role of the L2 minor capsid protein in the viral life cycle is still unclear although its importance in viral capsid assembly has been well documented. It is speculated to be involved in encapsidation of the viral DNA, generation and infectivity of PV virions as well as interaction with cell surface receptors (Kawana *et al.*, 2001; Okun *et al.*, 2001). The exact location of the L2 in the viral capsid is unclear although the ability of an antibody to L2 to neutralise infectivity demonstrates that a portion of the

protein is exposed on the surface of the capsid (Heino *et al.*, 1993). It is speculated that L2 molecules are located in the centre of the twelve pentavalent capsomeres in the capsid (Trus *et al.*, 1997).

Differentiation of epithelium triggers the expression of L1 and L2, leading to the assembly of infectious viral particles in the nucleus. Studies on the regulation of late gene expression have resulted in the detection of negative regulatory elements in the HPV late mRNA and factors binding to these elements have been identified. Analysis of HPV-16 late poly(A) sites showed the presence of a negative regulatory element (NRE) in the late RNA 3' untranslated region (Dietrich-Goetz *et al.*, 1997).

The L2 protein of HPV-16 is 473 amino acids long with groups of positively charged residues at the N (from amino acids 1-12) and C terminus amino acids (456 to 461) corresponding to classic nuclear localisation signals (NLS). The amino acid sequence of the N terminus is **MRHKRSAKRTKR** with four charged amino acid clusters important for DNA binding (Zhou *et al.*, 1994). Retention of at least one charged amino acid is necessary for DNA binding. Both the N- terminus DNA binding domain and C-terminus NLS sequence of HPV-6b L2 function in nuclear transport and contribute to the L2 translocation into the nucleus (Sun *et al.*, 1995). The sequence 286-306aa which is relatively conserved among HPV types has been found to be responsible for HPV6b L2 nuclear accumulation and retention (Fig 1.11).

BPV-1 L2 is 469 amino acids long, with groups of positively charged residues at the extreme C and N termini. With the goal of assessing the role of these positively charged termini in the generation of infectious virions, Roden *et al* (2001) constructed BPV-1 L2 deletion mutants of amino acids 2 to 9 (L2Δ2-9) and the final nine amino acids (L2Δ461-469) (Roden *et al.*, 2001). Coexpression of L1 and L2 mutants lacking either the eight N-terminal or nine C- terminal amino acids resulted in wild-type levels of viral genome encapsidation; the resulting mutant virions, despite binding to the cell surface with similar efficiency of wild type virions, were not infectious. As L2 interacts *in vitro* through positively charged side chains with DNA independently of nucleotide sequence (Mallon *et al.*, 1987; Zhou *et al.*, 1991), and is associated with viral episomes in vivo (Stauffer *et al.*, 1998), it is possible to speculate that L2 may function in the delivery of the viral genome to the nucleus (Kawana *et al.*, 2001). Characterisation of L2 mutants has identified two L2 domains independently interacting with L1 (Becker *et al.*, 2004; Okun *et al.*, 2001). Wild type L2 is able to interact with L1 in the nucleus and cytoplasm with VLP assembly occurring in the nucleus as the L1-L2 complex is translocated from the cytoplasm to the nucleus.

Recent studies have identified several cellular L2 interacting proteins (Table 1.4). Using the HPV-16 L2 protein as bait, Gornemann *et al.*, (2002) used a yeast two-hybrid system to isolate putative interacting partners. Their work suggests that L2 can interact with several cellular host proteins, has the ability to recruit such proteins to the nucleus and the property of complexing with at least three cellular proteins in specific nuclear domains. Two of the proteins, the previously identified PATZ and a novel protein designated

PLINP, were localized in PODs and colocalised with L2. PATZ in a transcriptional regulator and member of the zinc finger proteins originally identified as an interactor for the androgen receptor, a nuclear hormone receptor (Fedele *et al.*, 2000; Pero *et al.*, 2002). The third protein designated PMSP, is a newly identified cytoplasmic protein, which was recruited to PODs when coexpressed with L2. The functional outcome of these interactions is unknown. The relationship between L2 and PODs is discussed in further detail in section 1.3.2.

L2 has also been shown to bind β-actin, Yang *et al.*, (2003) demonstrated that a conserved domain comprising residues 25-45aa of HPV 16 L2 was sufficient for this interaction. The 25-45 L2 residue fused to green fluorescent protein co-localized with actin and caused cell retraction and disruption of the microfilament network possibly assisting in the release of mature virus.

Cellular proteins shown to interact with HPV 16 L2
PATZ (POZ-AT-Zn-finger
Tip60 (tat interacting protein)
AUP1 (ancient ubiquitous protein)
TIN-Ag-RP
c-myc-modulating protein
Creatine-transporter (CRTR)
Serine-threonine-kinase 11
G-protein, β-subunit
Lysosomal α-Mannosidase
β-actin

Table 1.4. Table showing HPV-16 L2 interacting partners identified using a yeast two-hybrid system. *Data from Gornemann et al., 2002. Virology, 303, 69-78 and, from Yang et al., 2003. J Biol Chem. 278(14) 12546-53*

The Papillomavirus minor capsid protein L2

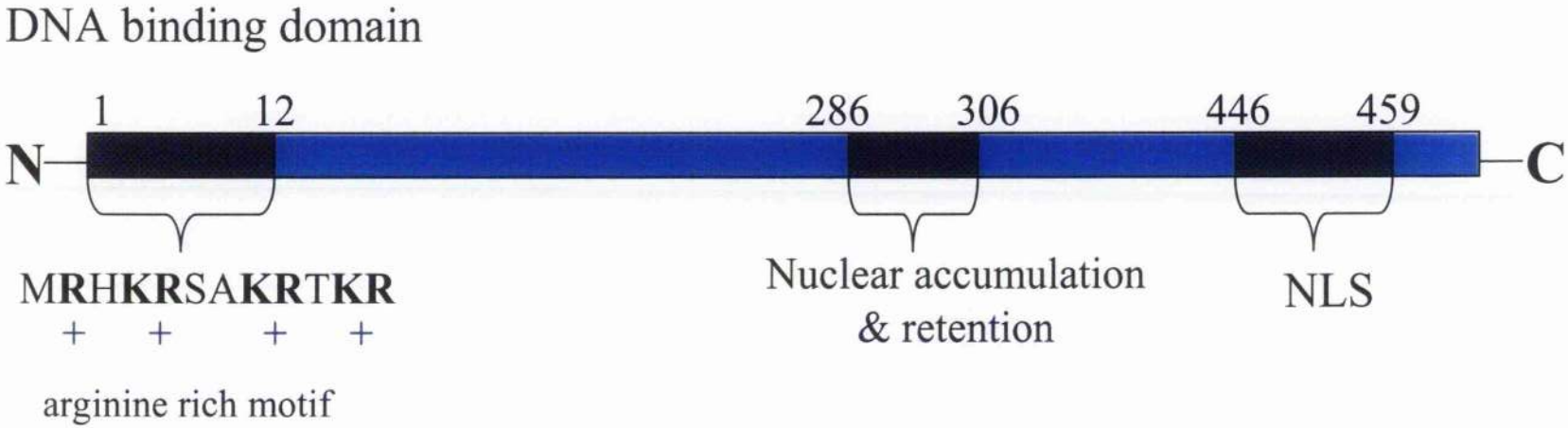


Figure 1.11. Schematic representation of the papillomavirus minor capsid protein L2. HPV-16 L2 protein contains a positively charged residue at the amino terminus (1-12) corresponding to a DNA binding domain. HPV-6b L2 has been shown to contains a positively charged residue at carboxyl terminus (446-459) corresponding to a nuclear localisation signal and a domain responsible for nuclear accumulation and retention (286-306) (Sun et al., 1995).

1.3. Promyelocytic Leukemia (PML) Oncogenic Domains "PODs"

The cell nucleus contains a variety of morphologically distinct substructures called nuclear bodies, which include the sphere organelles, colloid bodies and the PML nuclear bodies or PODs. PODs (also known as PML-ND10) are macromolecular multiprotein complexes that are present in all cultured cell lines as well as in *vivo*. They were initially visualised using sera from autoimmune patients with primary biliary cirrhosis. Immunofluorescence studies have demonstrated that depending on the cell type and other factors such as hormonal exposure and cell cycle (Doucas *et al.*, 1996), the number of PODs per nucleus can vary between 10 and 20. A major component of PODs is the PML protein, which was originally identified as the fusion partner of retinoic acid receptor alpha (RARα) in the chromosomal translocation t(15;17) in patients with acute promyelocytic leukaemia (APL) (de The *et al.*, 1991; Goddard *et al.*, 1991; Kakizuka *et al.*, 1991; Lavau *et al.*, 1995a). In leukemic cells from patients with APL that carry the translocation t(15;17), the expression of the PML-RARα fusion protein disrupts the structural integrity of PODs. In fact PML-RARα forms heterodimers *in vivo*, which results in disruption of normal POD integrity and may contribute to the oncogenic state in APL patients. The POD structure is reformed in leukemic cells following treatment with all-*trans* retinoic acid (RA), a process that is associated with RA therapy (Dyck *et al.*, 1994; Grignani *et al.*, 1993; Weis *et al.*, 1994).

During mitosis, PODs seem to disappear until early G_1 phase where they seem to reappear as small sites of low frequency. A cell-cycle dependent regulation of POD

frequency has been reported (Terris *et al.*, 1995). PML appears to be essential for POD assembly. Using cells that lack Sp100 or PML, Ishov *et al* demonstrated that the lack of Sp100 did not affect the structure of PODs. In contrast, cells lacking PML exhibited dispersion of all POD-associated proteins. PODs could be reconstructed by the introduction of PML into PML -/- cells either by transfection or at more physiological concentrations of PML through fusion with PML containing cells (Ishov *et al.*, 1999). This reconstruction includes the recruitment of all POD proteins including Sp100.

The central function of PML in POD formation seems to suggest that interaction of PML is a key factor in the accumulation of proteins (Fig 1.7 and 1.8). Studies of other POD associated proteins such as Daxx (a DNA-binding protein) showed that its localisation to PODs depended on the presence of a PML interaction domain located at the same region of Daxx as the interaction domain with Fas (Ishov *et al.*, 1999). PML has several SUMO-1 modification sites (Kamitani *et al.*, 1998a; Kamitani *et al.*, 1998b) and it has been observed that Daxx accumulation at PODs is dependent on SUMO-1 modification of PML. SUMO-1 is a small ubiquitin-related modifier, which binds to PML at three lysine residues (Fig 1.7) (Duprez *et al.*, 1999; Muller *et al.*, 1998; Sternsdorf *et al.*, 1997). Testing PML constructs with decreasing numbers of SUMO-1 modification sites suggests less Daxx binding at PODs. Sp100 can also be SUMO-1 modified, but SUMOylation is not essential for its localization to the nuclear body.

The Promeolytic Leukaemia Protein

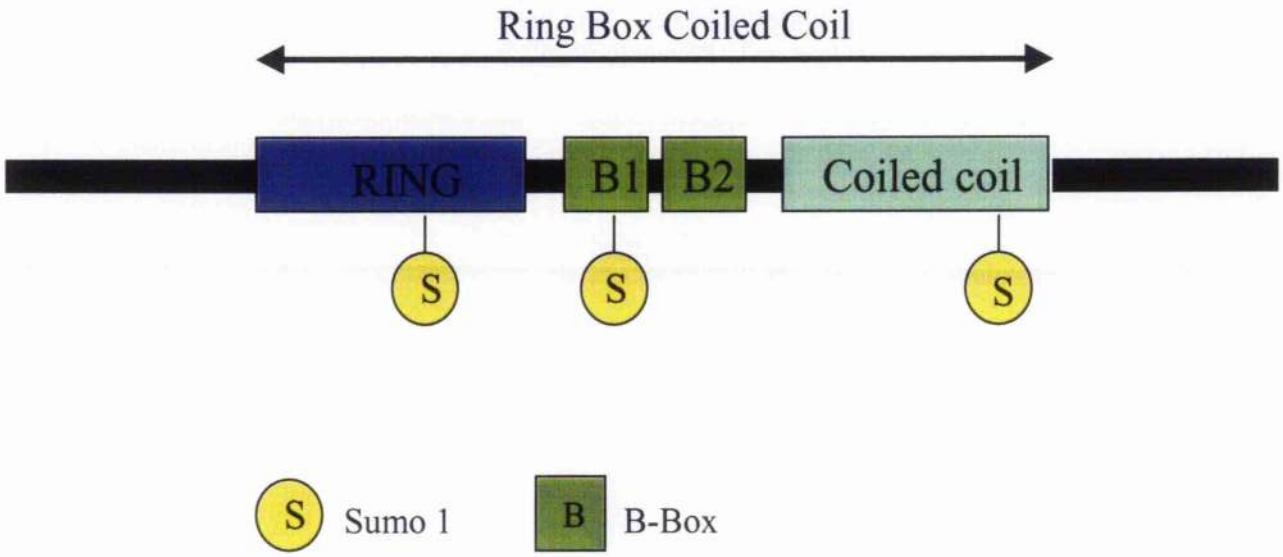


Figure 1.12 Schematic representation of the structure of the promeolytic leukaemia protein (PML) showing the ring box coiled coil motif (RBCC). The RBCC contains a ring finger domain, two B-boxes (zinc binding cystein-rich motifs) and an alpha-helical coiled coil dimerization domain. Alternate splicing of the C-terminal region results in generation of over 15 different isoforms.

Formation of PODs

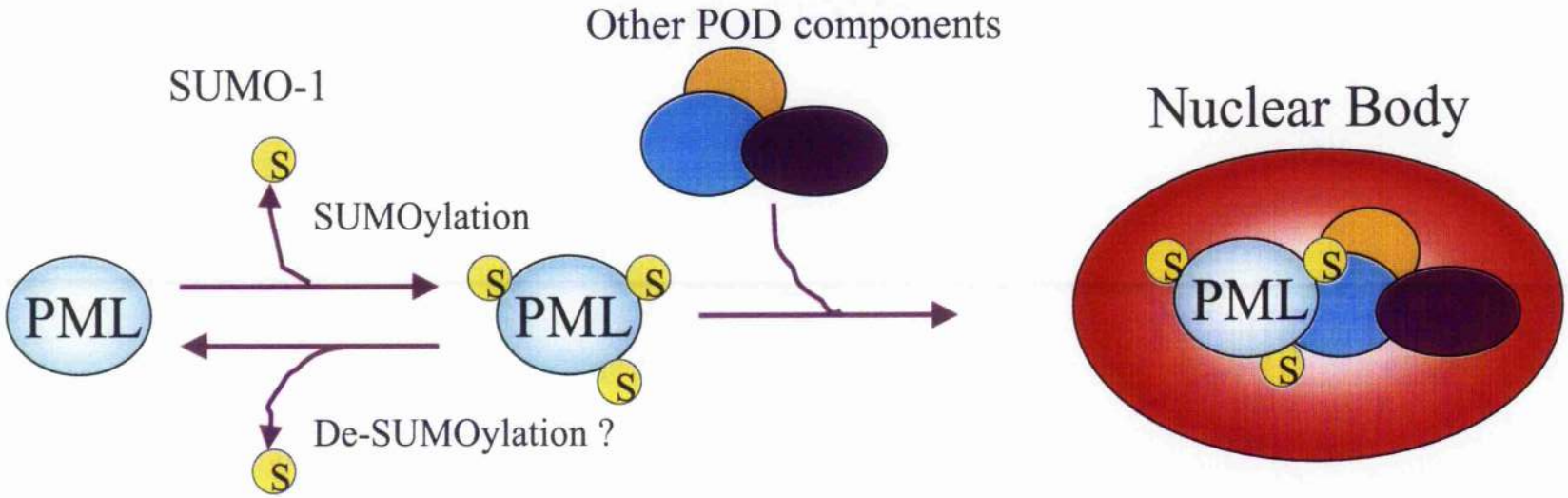


Figure 1.13 Formation of PODs due to SUMOylation of PML. SUMO-1 (s) refers to small ubiquitin-related modifier which binds to PML at three lysine residues. The SUMOylation of PML is crucial to POD assembly.

To date several components of the nuclear body have been identified; these include pRB, p53, Daxx, CBP, Sp100 (transcription regulation), BLM (DNA helicase), eIF-4 (initiation of translation), SUMO-1 (SUMOylation) and HAUSP (De-ubiquitination). An understanding of common biological and biochemical features shared by these molecules may reveal the function of the nuclear body. However the various nuclear body components seem to posses multiple biological functions and as such do not appear to act in a common pathway or share structural features.

Sp100 is an acidic protein with a molecular mass of 54kDa, which has a highly aberrant electrophoretic mobility in SDS-polyacrylamide gel electrophoresis of approximately 100kDa (Szostecki *et al.*, 1990). Sp100 is an autoantigen that was originally identified in patients suffering from the auto-immune disease, primary biliary cirrhosis. The Sp100 gene located on the human chromosome 2 gives rise to a number of alternatively spliced Sp100 variants, some of which contain a domain with high sequence similarity to an interferon-inducible human nuclear phosphoprotein HNPP1/2 (Grotzinger *et al.*, 1996; Guldner *et al.*, 1999; Seeler *et al.*, 1998). Sp100 has been described to exhibit transcriptional modulatory effects under certain experimental conditions possibly due to its interaction with the heterochromatin protein 1 (HP1), which has been shown to interact directly with Sp100 (Lehming *et al.*, 1998; Seeler *et al.*, 1998). Both HP1 and SP100 concentrate in PODs and overexpression of SP100 leads to enhanced accumulation of endogenous HP1 in these structures (Seeler *et al.*, 1998). The molecular domains of Sp100 for interaction with HP1 is located in the C-terminal region and the Sp100 homo-dimerization domain in a rather long sequence at the N-terminal end

(Sternsdorf *et al.*, 1999), suggesting that a three dimensional structure is responsible for the oligomerization of Sp100 at PODs.

1.3.1. Association of Viruses with PODS

The association of POD bodies with viral infection was first explored by Gerd Maul (Maul *et al.*, 1993) when he observed that HSV-1 infection led to the disruption of these nuclear bodies. Since then, there has been a great interest in the field of virology to understand the exact role of POD during viral infection. Today, a large number of viruses have been linked with POD alteration in one form or the other. At present, the functional consequence of this alteration of PODs is still not known. It is possible that this alteration could be a result of nuclear reorganisation in response to infection or a specific viral strategy to block cellular systems that may hamper viral replication.

1.3.1.1. Interferon

Interferons (IFNs) are a large family of multifunctional secreted proteins that regulate cellular antiviral, antitumour and immunological responses through IFN-stimulated gene expression. They consist of two different types, type I IFNs (i.e. IFN-α/β) that are produced in virally infected cells, and type II IFN (i.e. IFN-γ) that is not virus inducible and is restricted to mitogen or cytokine-activated lymphoid cells such as T lymphocytes and natural killer cells.

It has been observed that upon treatment of cells with interferon, PML is induced and the number of nuclear bodies increases dramatically (Gaboli *et al.*, 1998; Lavau *et al.*, 1995b), possibly suggesting a role for PML and the nuclear bodies as part of the anti-viral

defence mechanism. Apart from PML, other POD associated proteins such as Sp100, Sp40, Sp110 and PA28 also have increased expression as a result of IFN treatment. Type I (α,β) and type II (γ) IFNs strongly enhance Sp100 and PML gene expression both at the RNA and protein level (Chelbi-Alix *et al.*, 1995); (Grotzinger *et al.*, 1996; Guldner *et al.*, 1992; Lavau *et al.*, 1995b) with very similar kinetics (Grotzinger *et al.*, 1996).

The IFNs α, β and IFNγ-inducible gene promoters are characterised by the presence of consensus elements known as IFN-stimulated response elements (ISRE) and IFN-γ activation sites (GAS), which mediate primary transcriptional induction in response to IFN-α/β and IFN-γ, respectively (Darnell, Jr. *et al.*, 1994). In the case of Sp100, elevated RNA levels are mainly due to an IFN-enhanced transcription rate of Sp100 gene. Identification and analysis of ISRE and GAS in the Sp100 promoter region (Grotzinger *et al.*, 1996) demonstrated that both elements are necessary for long term transcriptional IFN-α/β induction of the Sp100 gene and that the Sp100-GAS site suffices to render a heterologous promoter IFN-γ-inducible. Since promoters of IFN-inducible genes usually contain either an ISRE or a GAS element, it appears that this seems to be a unique characteristic of the Sp100 promoter.

The PML promoter also contains an ISRE and GAS (Stadler *et al.*, 1995). In contrast to SP100, the PML-ISRE alone is sufficient to mediate type I and II IFN-enhanced gene expression. Expression is usually upregulated with a noticeable swelling of POD bodies. Different types of PML, arising from alternative splicing of a single gene are induced by IFN in different cell lines. Probably due to sequence differences the Sp100- and PML-

ISRE bind transcription factors with strikingly different affinities (Grotzinger *et al.*, 1996; Stadler *et al.*, 1995; Sternsdorf *et al.*, 1997). Deletion of the ISRE motif in the PML promoter abolishes the response to type I and diminishes induction by type II IFN, whereas deletion of the GAS element only modestly alters the response to IFNγ. This is due to the binding of IFN signal transducers and activators of transcription (STATs), which have been shown to be weak for the GAS, but strong for the ISRE, which also seemed to contribute substantially to the IFN-gamma response (Stadler *et al.*, 1995).

1.3.1.2. Herpes Simplex virus type one (HSV-1)

A variety of viruses target the PODs and often cause their disruption. HSV-1 carries out gene expression, DNA replication, and DNA encapsidation in globular nuclear domains designated replication compartments (Puvion-Dutilleul *et al.*, 1995; Quinlan *et al.*, 1984). These domains contain the essential viral DNA replication proteins (the origin-binding protein, the single-stranded DNA–binding protein, the helicase-primase subunits, and the polymerase subunits (Liptak *et al.*, 1996) (Lukonis and Weller., 1997; Quinlan *et al.*, 1984)and are usually visualised by antibodies either against ICP8, the single-stranded DNA-binding protein, or UL42, the polymerase processivity subunit. The formation of replication compartments is mediated by interactions with PODs. HSV-1 DNA accumulates in the periphery of the nuclear bodies (Ishov and Maul., 1996), within minutes of infection, the immediate early protein ICP0 (Vmw110) also localizes to the nuclear bodies and rapidly induces their disruption (Everett *et al.*, 1998; Everett and Maul., 1994). Specifically, the expression of ICP0, which like PML is a RING finger protein, causes a decrease in the amounts of SUMOylated PML and Sp100 (Muller and Dejean., 1999). ICP0 is a potent and promiscuous activator of gene expression and plays

a role in lytic growth of the virus as well as in viral latency. ICP0 is also a co-activator of ICP4 (Vmw175), the major transcriptional regulator of HSV-1. The loss of POD staining after HSV-1 infection seems not to be due to degradation of the corresponding proteins but to relocalisation of POD proteins into virus-induced structures (Maul *et al.*, 1993; Puvion-Dutilleul *et al.*, 1995). The proposal that HSV-1 induces either the degradation of PML and Sp100 or their de-SUMOylation (Chelbi-Alix and de The., 1999) (Everett *et al.*, 1998)is still widely debated. ICP0 has been postulated to be a derepressing agent for HSV-1 viral genome; this suggests that disruption of the nuclear bodies can be a critical event for expression of viral genes (Everett *et al.*, 1998).

Everett *et al* identified a 135kDa protein HAUSP (herpes simplex-associated ubiquitin-specific protease), so named because it binds strongly to the C-terminal region of ICP0 (Everett *et al.*, 1997). HAUSP is a member of a family of proteins that removes ubiquitin adducts from proteins, thus protecting them from degradation by the ubiquitin-protease pathway. It is distributed in the nucleus in a micropunctate pattern with a limited number of larger discrete foci, some of which co-localize with PML in PODs. At early stages of viral infection, the presence of ICP0 increases the proportion of PODs, which contain HAUSP. The interaction of HAUSP and ICP0 implicates ICP0 in a ubiquitin-protease pathway. Deletion of the region of ICP0 which binds to HAUSP reduced significantly its ability to stimulate gene expression and viral lytic growth (Everett *et al.*, 1997; Meredith *et al.*, 1995), and a deletion which overlaps this region eliminates the ability of ICP0 to reactivate latent virus in an *in vitro* latency system (Zhu *et al.*, 1990). Varicella zoster virus, type I bovine and equine herpesviruses as well as pseudorabies virus, all

members of the alpha herpesvirus sub-family express proteins similar to ICP0 which disrupt PODs to varying degrees (Parkinson and Everett., 2000).

1.3.1.3. Epstein-Barr virus

EBV initially replicates within epithelial cells in the oropharynx and subsequently infects B cells trafficking through the pharynx. In B cells, the virus usually converts to a latent form and persists indefinitely in the host (Li *et al.*, 1992; Sixbey *et al.*, 1984). During latency, only a small subset of EBV-encoded proteins is expressed. The first EBV genes expressed during the lytic form of viral replication are the immediate-early genes BZLF-1 and BRLF1 (Kieff *et al.*, 1996; (Adamson and Kenney., 2001). Both proteins function as transcriptional activators and induce expression of the next group of EBV genes, the early genes that encode the viral proteins required for EBV DNA replication (Kieff *et al.*, 1996). Induction of the lytic form of EBV infection using either expression vectors for BZLF-1 or BRLF1 or an adenovirus vector that expresses BZLF-1 leads to dispersion of PODs in EBV-positive cells (Adamson and Kenney., 2001). In EBV negative cells, expression of BZLF-1 alone but not BRLF1 is sufficient to disperse PODs. The first 86 amino acids of BZLF-1, which encode the transcriptional activation domain as well as replication function, are required for PML dispersion (Adamson and Kenney., 2001). EBV also expresses a protein, EBNA-5 that associates with PODs (Szekely *et al.*, 1996). EBNA-5 is one of the earliest of six nuclear proteins expressed by EBV and is nuclear matrix associated. EBNA-5 is homogeneously distributed throughout the nucleus within the early phase of infection but associates with PODs at the end of the first day of infection.

1.3.1.4. Human Cytomegalovirus (HCMV)

Infection by HCMV abolishes POD staining by anti-Sp100 and PML antibodies both during the early and late phase of infection. HCMV early proteins IE1 and IE2 accumulate in the nuclear bodies and induce their disruption (Ishov *et al.*, 1997). Interestingly, IE1, like ICP0, can act as a de-SUMOylating agent for PML (or induce its degradation) (Maul *et al.*, 1996). IE2 interacts with Ubc and SUMO-1,-2 and –3 (probably through a specific SUMO-interaction domain), and can be modified by both SUMO-1 and SUMO-2. Mutation of the two modification sites on IE2 does not affect its interaction with PODs (Ahn and Hayward., 2000). The HCMV protein IE72 includes a potential zinc-binding motif in a region that is also required for its ability to disrupt PODs.

1.3.1.5. Adenovirus

Adenovirus infection also results in the redistribution of PODs into fibrous structures of elongated tracks within 4 hours of infection. This redistribution of PODs into nuclear tracks is mediated by E4orf3 which itself co-localizes with PML in the nuclear tracks. The E1A oncoprotein of Adenovirus also concentrates in PML-containing fibres (Carvalho *et al.*, 1995), which becomes visible after extraction of soluble E1A from cells. Although E4orf3 does not affect SUMO-1 conjugation to exogenous PML in co-transfected cells, there is a gradual change in PML during adenovirus infection that results in an additional modification of many of the endogenous PML isoforms. This is followed by loss of the SUMO-1 modified PML species.

1.3.1.6. Other viruses and PODs

As seen, viral infections appear to target and dramatically alter the composition of PODs. Disruption of the PODs function can in turn favour transformation by oncogenic viruses, or allow expression of viral genes, or aid viral replication by triggering the cell replication machinery though inactivation of the growth/tumour suppressive role of the PML nuclear body. Rabies virus genome encodes a phosphoprotein, which is amino-terminally truncated to give four other products known as P2, P3, P4 and P5. GFP-P3 has been shown to localise to PODs. In Lymphocytic choriomenengitis virus (LCMV) infection, PML is redistributed to the cytoplasm (Borden *et al.*, 1998). In the case of HTLV-1, the tax protein alters POD associated Int-6 protein localisation. The binding of Tax to Int-6 causes its redistribution from PODs (Desbois *et al.*, 1996). Hepatitis delta virus (HDV) large antigen (L-HDAg) colocalises with PODs during infection. PML is found in the rims with L-HDAg and viral RNA while the other POD components Sp100 and Daxx were found in the centre of the PODs (Bell *et al.*, 2000).

1.3.2. Papillomavirus proteins and PODs

It has been observed that the minor capsid protein L2 of BPV has an intrinsic capacity to localize to PODs in the absence of other viral components (Day *et al.*, 1998). The presence of L2 in PODs appeared to be associated with the recruitment of the major capsid protein L1 and E2 (the transcriptional activator). L2 has been shown to also recruit the E2 transcriptional repressor E2TR of BPV 1 to PODs as well as the full-length E2TA (Heino *et al.*, 2000). The association with E2 is dependent on L2 but is independent of L1. The reason why L2 localizes to PODs still remains unclear but one may speculate that E2 (which is capable of binding DNA with high-affinity at multiple sequence–

specific sites) is recruited to PODs by L2, suggesting that L2 may function to facilitate virion production by inducing the co-localization of other components required for virion assembly. Alternatively association with PODs can also be speculated to play a positive role in viral replication. The localisation of L2 might result in an increase of the local concentration of viral products and as such promote viral assembly and packaging. It could also result in access to cellular transcription and/or replication factors promoting processing of viral products.

1.4. Project Aims

The role of PODs during viral infection has not been clearly defined. Viruses have been implicated in a variety of different ways with these discrete nuclear domains within the nucleus. Are they part of the hosts natural defence mechanism to fight off viral infection or are they utilised by the virus as sites of viral replication and virion assembly?

Previous studies have shown that BPV-1, HPV-11 and HPV-33 L2 localises to PODs and co-expression of BPV-1 L2 with BPV-1 E2TA sees the recruitment of E2 by L2 to PODs. The aim of this thesis was to define the relationship between HPV-16 E2 and HPV-16 L2 proteins. As such, the consequence of the co-localisation of E2 and L2 at PODs on viral transcription was studied. A number of HPV-18 LCR derived E2-responsive promoters with mutations in one or more E2 binding sites and two synthetic E2 responsive promoters linked to the tk or HPV-18 TATA were used. The effect of L2 on E2 mediated viral DNA replication in transient replication assays was also studied.

HaCaT, U2OS and C33a cells were used in most experiments. HaCaT are spontaneously immortalised keratinocytes (Boukamp *et al.*, 1988) while U2OS are osteosarcoma cell lines. C33a are cervical carcinoma derived cells, which have no HPV DNA. The effect of L2 on E2 protein expression and stability was examined in all three cell lines.

To further characterise the interaction of L2 with E2, the sequence domains of L2 required for its association with E2 were investigated. Amino and carboxyl terminus deletion mutants of L2 were generated. The L2 mutants were cloned into pCDNA 3.1 expression plasmids and their effect on E2 mediated viral transcription and E2 protein expression was studied. Fusion forms of these mutants were constructed with Glutathione S-transferase (GST) and Green Fluorescence Protein (GFP). Deletion-mutants of L2 fused with GFP were transfected in HaCaT cells to analyse by confocal microscopy their localisation. This enabled correlation between the intracellular localisation of these proteins and their effects on E2 mediated transcription transactivation. GST binding assays were done using *in vitro* translated E2 labelled with ^{35}S-methionine to determine domains of L2 that bind to E2.

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

2.1.1. Antibodies

SUPPLIER	ANTIBODY
CalbioChem- Novabiochem corporation San Diego, USA	Anti-mouse IgM horseradish peroxidase linked whole antibody (raised in goat)
CalbioChem- Novabiochem corporation San Diego, USA	Anti-Actin (Ab-1). Mouse monoclonal IgM antibody (clone JLA20).
Amersham International plc, Amersham, Bucks, England	Anti-rabbit IgG horseradish peroxidase linked whole antibody (raised in sheep)
Amersham Pharmacia Biotech, Amersham, Bucks, England	Anti-mouse IgG horseradish peroxidase linked whole antibody (raised in sheep)
Amersham Pharmacia Biotech UK Ltd. Amersham, Bucks, England	Anti-GST goat polyclonal antibody horseradish peroxidase conjugated
Sigma Chemical Co., Ltd., Poole, Dorset, UK	Anti-Rat IgG (whole molecule) peroxidase conjugate (raised in rabbit)
Sigma Chemical Co., Ltd., Poole, Dorset, UK	Anti-Rabbit IgG (whole molecule) peroxidase conjugate (raised in goat)
Santa Cruz Biotechnology, Inc California, USA	Anti-GFP (FL) rabbit polyclonal IgG antibody (Sc-8334)
Santa Cruz Biotechnology, Inc. California, USA	Anti-HA Rabbit polyclonal IgG antibody (Y-11: Sc-805)
A gift from Dr. M. Hibma, ICRF Tumour Virus Group, University of Cambridge	TVG261, a monoclonal antibody directed against amino acids 2–17 in the amino terminus of HPV-16 E2.
Roche Diagnostics GmbH, Germany	Anti-HA high affinity rat monoclonal antibody (clone 3F10)
A gift from Dr. M. Muller, Deutsches	HPV-16 L2 polyclonal rabbit serum (No. 20)

Krebsforschungszentrum, Angewandte Tumorirologie, Heidelberg, Germany.	

2.1.2. Bacterial Hosts

SUPPLIER	BACTERIAL HOSTS
Invitrogen Life Technologies, Ltd., Paisley, Scotland	*E. coli* DH5α competent cells
Amersham International plc, Amersham, Bucks, England	*E. coli* BL21 competent cells

2.1.3. Buffers

TE	10mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0
10× TBE buffer	900mM Tris base, 900mM boric acid, 25mM EDTA, pH8.0
1 × ligase buffer	50mM Tris HCL pH7.6, 10mM $MgCl_2$, 1mM ATP, 1mM DTT, 5% (w/v) polyethylene glycol-8000
Phosphate buffered saline (PBS)	137mM NaCl, 44mM KCl, 1.4 mM KH_2PO_4, 8.5 mM Na_2HPO_4
10 × loading buffer	0.45% (w/v) Bromophenol blue, 1% (w/v) SDS, 100mM EDTA, 2.5% (w/v) Ficoll 400 in TE
SDS-PAGE Lysis buffer	1M Tris-HCl (pH 6.8), 10% (w/v) SDS, 20% (v/v) glycerol
SDS-PAGE Resolution gel buffer	0.5M Tris (pH8.8), 0.4% (w/v) SDS
Tris-glycine electrophoresis buffer	25mM Tris, 250mM glycine and 0.1% (w/v) SDS
2x SDS gel loading buffer	4% (w/v) SDS, 0.2% (w/v) bromophenol

	blue, 20% (v/v) glycerol and 100mM Tris, pH6.8

2.1.4. Cells

Cell types	Description	Growth Medium
HaCaT	Spontaneously immortalised keratinocytes	Dulbecco's Modified Eagles Medium, 10% Foetal Calf Serum 1mM sodium pyruvate 4mM L-glutamine 100 IU penicillin 100µg streptomycin
HaCaT-E2	HaCaT derived cell line expressing HPV-16 E2 constitutively kindly provided by Dr Pablo Cordano, ICM, Glasgow.	Dulbecco's Modified Eagles Medium, 10% Foetal Calf Serum 1mM sodium pyruvate 4mM L-glutamine 100 IU penicillin 100µg streptomycin Geneticin G418 sulphate
C33a	Human cervical carcinoma cells with no HPV DNA	Dulbecco's Modified Eagles Medium with GlutaMAX-1 4500mg/L D-Glucose with sodium pyruvate 10% Foetal Calf serum 100 IU penicillin 100µg streptomycin
U2OS	Osteosarcoma cell line	Dulbecco's Modified Eagles Medium with GlutaMAX-1 4500mg/L D-Glucose with sodium pyruvate

		10% Foetal Calf serum 100 IU penicillin 100µg streptomycin

2.1.5. Cell Culture Materials

SUPPLIER	MATERIAL
Cadisch and Sons, Finchley, UK	70µm filter nylon gauze
Invitrogen Life Technologies, Ltd., Paisley, Scotland	Foetal Calf Serum
Invitrogen Life Technologies, Ltd., Paisley, Scotland	10% Dulbecco's Modified Eagles Medium 200 mM glutamine Geneticin, G418 sulphate 100 mM sodium pyruvate 2.5% Trypsin

2.1.6. Chemicals and Enzymes

Supplier- Amersham International plc, Amersham, Bucks, England

ECL Western detection agent

ECL Plus Western detection agent

Redivue [α^{32}P]dATP

Amplify™ Fluorographic reagent

Glutathione Sepharose® 4B

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

Calcium chloride

D-glucose

Glycerol

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

Yeast Extract

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

DNase 1, RNase-free

Protease K

Supplier- Calbiochem (Merck)

MG-101; Calpain Inhibitor 1

MG-132; Carbobenzoxt-L-leucyl-L-leucyl-L-leucinal

ALLM; Calpain Inhibitor 11

Lactacystin, synthetic

Leupeptin hemisulfate

Supplier- Difco laboratories, Detroit, Michigan, USA.

Bacto-Agar

Bactotryptone

Supplier- Fisons Scientific Equipment, Loughborough, England.

Acetic acid

Butan-1-ol

Chloroform

di-potasium hydrogen orthophosphate anhydrous

Ethylene diamine tetra acetate (EDTA) disodium salt

Dimethyl sulfoxide (DMSO)

Hydrochloride acid

Magnesium chloride

Magnesium sulphate

Methanol

Potassium chloride

Potassium dihydrogen orthophosphate

Propan-2-ol

Sodium acetate

Sodium carbonate

Sodium chloride

Sodium dodecyl sulphate (SDS)

Sodium hydroxide

Supplier- Invitrogen Life Technologies, Ltd., Paisley, Scotland.

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

Agarose (ultrapure electrophoresis grade)

Tris Base

LIPOFECTAMINE™ Reagent

LipofectAMINE PLUS™ Reagent

SuperScript™ One-Step RT-PCR with PLATINUM® Taq

Deoxyribonuclease 1, Amplification Grade

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

Ethanol

Supplier- NBL Gene Sciences

Alkaline phosphatase

T4 DNA ligase

Supplier- New England Biolabs

NEB buffer 2

Xmn1

Dpn1

Bovine Serum Albumin

Supplier- Novagen, Inc USA

BugBuster™ Protein Extraction Reagent

Supplier- Promega, Southampton, England

Deoxynuclease Triphosphates (dNTPs); dATP, dCTP, dGTP, dTTP

Rnasin ® Ribonuclease Inhibitor

Pfu DNA Polymerase

AMV Reverse transcriptase

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

Arabinose

β-mercaptoethanol

Bicinchoninic Acid solution

Bovine Serum Albumin

Bromophenol Blue

Coomassie Brilliant Blue R

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

DEPC

Dithiothreitol (DTT)

Ethidium Bromide

Kanamycin

Leupeptin

Nonidet P-40 (NP40)

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

PMSF

Ponceau S solution

TEMED (N,N,N',N'-tetramethylethylenediamine)

Tween-20 (Polyoxyethylene sorbitan nonolaurate)

Supplier- Roche Diagnostics GmbH, Germany

Protease Inhibitor Cocktail tablets

Supplier- Stratagene.

QuickHyb® Hybridization Solution

Prime it II Random primer labelling kit

Supplier-Qiagen, Dorking, Surrey, England

Superfect Transfection Reagent™

Supplier- Vector Laboratories, Burlingame, USA.

Vectashield™ Mounting Medium

2.1.7. Equipment and Plasticware

SUPPLIER	EQUIPMENT
Alpha Laboratories Ltd., Eastleigh, Hampshire, England	Microfuge tube Pastettes
Amersham International plc, Amersham, Bucks, England	Hybond-C extra Hybond-N
Becton Dickinson Labware, Plymouth, England	Falcon 1059 polypropylene tubes Falcon 2059 polypropylene tubes Falcon 2097 polypropylene tubes Falcon 2098 polypropylene tubes Sterile Plastipak syringes 18 gauge sterile syringe needles 60, 90 and 140 mm tissue culture dishes
Greiner	Sterile bijou tubes Sterile universal tubes Cell scrapers Filter tips 60 and 100mm bacteriological petri dishes Sterile plastic universal containers
Corning BV, High Wycombe, Bucks, England	25ml, 10ml 5ml pipettes 24 well tissue culture plates 96 well tissue culture plates Cryogenic vials
Eastman Kodak Co., Rochester, New York, USA	X-ray film (XAR-5)

VMR (MERCK/BDH)	Sterile syringes Needles Saran wrap Foil
Ilford Ltd., Mobbrrley, Cheshire, England	Ilford PANF 50 black and white film
Scientific Laboratory Supplies	T25, 80, and 175 cm^2 tissue culture flasks
Sartorius	Sterile 0.2µm filter Sterile 0.45µm filter
Labco	Cin Bins
Whatman International Ltd., Maidstone, Kent, England	Whatman 3MM filter paper

2.1.8. Kits

SUPPLIER	KIT
Perkin Elmer Cetus, Norwalk, USA.	GeneAmp PCR core kit GeneAmp thinwalled reaction tubes BigDye™ Terminator Cycle Sequencing
Promega Ltd., Chilworth Research Centre, Southampton, England	Luciferase Assay System Reporter Lysis 5 X Buffer TNT® Quick Coupled transcription/translation system
Qiagen Ltd., Dorking, Surrey, England	QIA prep Spin plasmid miniprep kit QIA quick gel extraction kit RNeasy Mini RNA purification kit Omniscript RT Kit HotStar Taq PCR Kit
Applied Biosystems	Big dye 3 Big dye 3 buffer III-DI formamide

2.1.9. Molecular Weight Markers

SUPPLIER	MARKER
Invitrogen Life Technologies, Ltd., Paisley, Scotland	Rainbow™ coloured protein molecular weight markers
Invitrogen Life Technologies, Ltd., Paisley, Scotland	Bacteriophage λ DNA (HindIII digested) 100bp DNA ladder 1Kb DNA Ladder

2.1.10. Other Materials

SUPPLIER	MATERIALS
Veterinary Pathology Central Services	LB-Medium (Luria-Bertani Medium) TSS (Transformation storage solution) YTA medium Sterile distilled water Sterile glycerol Sterile phosphate-buffered saline (PBS)
Merck Ltd., Poole, England	Silicone grease
Johnson and Johnson Medical Limited, Berks, UK	PRESEPT* effervescent disinfectant tablets
Premier Beverages., Adbaston, Stafford, UK	Marvel (Dried Skimmed milk)

2.1.11. Plasmids

pCMV is an expression vector which contains the cytomegalovirus (CMV) immediate-early promoter/enhancer. **pCDNA 3.1+** is a 5.4kb vector derived from pcDNA3 and designed for high-level stable and transient expression in mammalian hosts. It also contains CMV promoter and a multiple cloning site, which facilitates cloning.

pCMV-E1$_{16}$ is a construct that consists of the HPV-16 DNA fragment nt 865 to 2813 encoding the E1 protein, amplified by PCR to add the Kozak consensus sequence, inserted into the Xba1-Sma1 sites of pCMV$_4$.

pCMV-E2$_{16}$ contains the HPV-16 DNA fragment from nt 2725 to 3852 encoding the viral transcription regulator E2, excised from pSK-E2$_{16}$ and cloned into the Xba1-Sma1 sites.

pGL3 is a luciferase reporter vector obtained commercially from Promega

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

pBPV-4LCR contains the BPV-4 LCR from nucleotide 6710-331 cloned into the BamH1 site of the p0luc luciferase plasmid.

p18LCR-BS1 is a pGL3 luciferase reporter plasmid that contains the full length HPV-18 LCR with four point mutations in the E2 binding site 1 introduced by PCR.

p18LCR-BSI-3 contains the HPV-18 LCR with mutations in the E2 binding sites 1-3. It was derived from CAT reporter plasmid, generously provided by F. Thierry.

p18-6E2 is a luciferase reporter plasmid which contains six E2 binding sites linked to the HPV-18 TATA box promoter element.

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

ptk6E2 is a ptkluc with six E2 binding sites linked to the tk promoter.

p16ori contains the HPV-16 origin of replication (nucleotides 7838-130 of the HPV-16 genome) cloned into pBSII SK(-)(Stratagene). This plasmid was a gift from Prof. Peter Howley (Harvard University).

p16ori-m, is a p16ori plasmid with a point mutation at nt 115 from C to A to create a DpnI restriction enzyme site.

pCDNA-L2 is a construct that consists of the HPV-16 DNA fragment nt 4135 to 5560 encoding the minor capsid protein L2, amplified by PCR and inserted into the BamH1-EcoR1 sites of pCDNA 3.1 under the control of cytomegalovirus promoter.

p16-HAL2 contains the HPV-16 L2 DNA sequence from nt 4135 to 5656 inserted into pHA1 between the Xho1 and Xba1 sites under the transcriptional control of a CMV promoter. pHA1 was a kind gift from C.W. Lee. It contains three copies of the HA1 epitiope cloned in pcDNA3 (Invitrogen, UK).

pEGFP-C1 is a eukaryotic expression plasmid for the Green Flourescent Protein (GFP). GFP expression is driven by the CMV promoter and the multiple cloning site is between the GFP sequences and SV40 poly A site. It is commercially available from Clontech Lab. This plasmid was a kind gift from Dr M. Roberts (ICM, Glasgow).

pEGFP-L2 is a pEGFP plasmid expressing the GFP protein fused amino terminally to the full-lenght HPV-16 L2 protein.

pGEX-4T-2 contains a GST domain and the recognition sequence for thrombin cleavage. This plasmid is commercially available from Amersham Pharmacia Biotech.

pGEX-L2 expresses the GST protein fused to wild type HPV-16 L2 under the control of the *tac* promoter.

A series of L2 deletion mutants were designed generating amino and carboxyl terminal deletions of the HPV-16 L2. All mutants of L2 were cloned into pCDNA 3.1+, pEGFP-N1 and pGEX-4T-2. A summary of all deletion mutants is given in the table below.

Mutant	
1-400aa	Encoding HPV-16 L2 from amino acids 1-400
1-300aa	Encoding HPV-16 L2 from amino acids 1-300
1-200aa	Encoding HPV-16 L2 from amino acids 1-200

1-100aa	Encoding HPV-16 L2 from amino acids 1-100
1-50aa	Encoding HPV-16 L2 from amino acids 1-50
25-473aa	Encoding HPV-16 L2 from amino acids 25-473
50-473aa	Encoding HPV-16 L2 from amino acids 50-473
150-473aa	Encoding HPV-16 L2 from amino acids 150-473
250-473aa	Encoding HPV-16 L2 from amino acids 250-473
350-473aa	Encoding HPV-16 L2 from amino acids 350-473
390-473aa	Encoding HPV-16 L2 from amino acids 390-473

2.1.12. Water

Distilled water for the preparation of buffer stocks was obtained from a Millipore MilliRQ 15 system, and for protein, enzyme, RNA or recombinant DNA procedures was further purified on a Millipore MilliQ System to 18MΩ/cm.

2.2. Methods

2.2.1. Molecular biology

2.2.1.1. DNA Extraction

DNA samples were purified by extraction with phenol:chloroform in order to remove contaminants, such as residual enzyme activities from a restriction reaction or detergent which might otherwise interfere with subsequent cloning steps. In the first round of extraction the DNA sample was mixed with an equal volume of phenol:chloroform. The aqueous DNA and organic phase were mixed thoroughly by vortexing, and then separated by centrifugation in a microcentrifuge at 14000 rpm for 5 minutes at room temperature. The upper aqueous phase was transferred in a clean eppendorf tube, care was taken not to transfer any of the interphase to the tube, and the extraction process repeated. The aqueous phase was then extracted with an equal volume of chloroform (chloroform:isoamyl alcohol, 24:1 v/v) by vortexing and centrifugation as described above. This was repeated to remove any traces of phenol from the aqueous phase. The aqueous phase was transferred to a fresh eppendorf for ethanol precipitation.

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

of salt and dried under vacuum before resuspension in distilled water at an appropriate concentration. The DNA concentration was determined as described below.

2.2.1.2. Quantification of Nucleic Acids

The concentration of nucleic acid in a solution was determined spectrophotometrically in a WPA UV1101 Biotech spectrophotometer. Samples were diluted in dH_2O and transferred to a quartz cuvette with a pathway of 1cm. The spectrophotometer was initially calibrated using dH_2O only as a blank. The optical density reading were obtained at 260nm and 280nm; an O.D. reading of 1 at 260nm (A_{260} = 1) corresponds approximately to a concentration of 50µg/ml of double stranded DNA, for oligonucleotides an A_{260} of 1 was taken to correspond to ~35µg/ml, and for RNA an A_{260} of 1 was taken to correspond to 40µg/ml. The ratio between readings at 260nm and 280nm (A_{260}:A_{280}) provided an estimate of the sample purity; a ratio of ~1.8 indicated that preparations contained essentially no protein or phenol contamination.

2.2.1.3. Restriction Enzyme Digestion of DNA

Restriction digests were carried out in small reaction volumes using enzymes and their appropriate concentrated buffer solutions according to the manufacturer's instructions. Plasmid DNA was incubated with 5-10 units enzyme/µg DNA in a buffered solution ensuring that the total volume of enzyme added did not exceeded one tenth of the final reaction volume. Small quantities of plasmid DNA (<5µg) were routinely digested in a 20µl reaction volume as specified by the manufacturer for 1 hour at 37°C. Large digests were carried out in proportionally larger reaction volumes. The digestion fragments were analysed by agarose gel electrophoresis as described below.

2.2.1.4. Agarose Gel Electrophoresis

In general, 1% (w/v) agarose gels were used, but smaller fragments (100-400) were separated on 2-4% gels. Low melting point agarose was used at a concentration of 1% (w/v) in order to isolate and purify required DNA restriction fragments. Gel mixes containing the appropriate amount of agarose were dissolved in 0.5 × TBE buffer by heating the solution in a glass conical flask in a microwave until all the particles of agarose gel had dissolved. The gel was poured when the agarose was hand hot and a comb with the required number and size of teeth placed immediately into the gel to form the sample wells. The gel was submerged in 0.5 × TBE buffer. The samples containing 1 × loading buffer were loaded in each well along with an appropriate size marker (e.g. 100b ladder, 1Kb ladder) into the first and/or last well in the gel and run at 70-100 constant voltage usually until the samples' blue dye front was 1-3 cm from the end of the gel. Once run, the DNA fragments were visualised by staining the gel in running buffer containing 0.5µg/ml ethidium bromide with gentle agitation for 10 minutes at room temperature. The separated DNA was visualised by illumination with short wave (312nm) UV light and photographed through a red filter onto video print paper using an UVP gel documentation system.

2.2.1.5. Southern Blotting

The DNA fragment to be used was digested with the appropriate restriction enzymes as described in section 2.2.1.3 and run on an agarose gel with the appropriate DNA size markers (2.2.1.4). The gel was stained with ethidium bromide and photographed with a ruler laid alongside the gel so that band positions can be identified on the membrane. The following procedure was then undertaken:-

1. The gel was rinsed in distilled water and placed in a clean glass dish containing ~10 gel volumes of denaturation solution (1.5M NaCl, 0.5M NaOH) and shaken slowly on a platform shaker for 30 minutes at room temperature. This was done twice.
2. The denaturation solution was poured off and the gel rinsed with distilled water washed twice with ~10 gel volumes of neutralisation solution (1.5M NaCl, 0.5M Tris-HCl pH 7.0) shaking slowly on a platform shaker for 30 minutes at room temperature.
3. To transfer the DNA from the gel to the membrane, a solid support was placed in a glass dish with wicks made out of Whatman 3MM filter paper. Three pieces of Whatman 3MM paper the same size as the gel was then cut and stacked onto the solid support.
4. The gel was rinsed in distilled water and placed on the filter paper stack and air bubbles were removed by rolling a glass pipette over the surface.
5. Four strips of plastic wraps were cut and placed over the edge of the gel so that the buffer flows through rather than around the gel.
6. A piece of Hybond-N nylon membrane just large enough to cover the exposed surface of the gel was immersed in distilled water for ~5 minutes. The wet membrane was then placed on the surface of the gel. Air bubbles were removed by rolling a glass pipette over the surface of the membrane.
7. The surface of the membrane was then flooded with 20 x SSC (3M NaCl, 0.3M $C_6H_5O_7Na_3$ (tri-sodium citrate)). Five sheets of Whatmann 3MM filter paper cut to the same size of the gel were placed on top of the Hybond-N membrane.
8. Paper towels were then cut the same size as the membrane and stacked on the top of the Whatmann 3MM paper to a height of ~4cm.

9. A glass plate was placed on top of the structure and a weight placed on top to hold everything in place and left overnight.

The next day, the paper towels and filter paper were removed and the Hybond-N membrane recovered. The membrane was rinsed in 2 x SSC and placed on a sheet of Whatmann 3MM paper to dry. The membrane was then placed in a Spectrolinker XL1500 (Spectronics Corperation) and irradiated with 1600 J/m^2 of UVC at 254nm to crosslink the DNA. The membrane was then baked for >60 minutes at 80°C in a Hybaid mini-oven MKII hybridisation oven.

2.2.1.6. Isolation and Purification of DNA Restriction Fragment from Agarose Gel

The DNA fragment to be used for cloning was recovered from low melting point agarose gel and visualised as described in section 2.2.1.4. The fragment was cut out of the gel with a clean scalpel blade and the gel slice placed in an eppendorf tube. Extraction of the DNA fragment from the agarose was achieved using a Qiagen Qiaquick gel extraction kit following the manufacturer's instructions.

2.2.1.7. Ligation of DNA Fragments

Both vector DNA and the DNA fragment to be inserted into the vector were separately digested with restriction enzymes and purified as described above and then isolated by gel electrophoresis as described in section 2.2.1.4. The vector DNA was dephosphorylated at its termini to prevent re-ligation. After the vector DNA had been linearized by digestion, the reaction mixture was adjusted by adding dephosphorylation buffer and 1 unit of Calf Intestinal Alkaline Phosphatase (CIAP) was added to the reaction mixture and incubated at 37°C for 1 hour. The reaction was stopped by heating to 90°C for a further 5 minutes.

All enzyme activity in the reaction was finally stopped by heating to 90°C for a further 5 minutes.

The DNA was phenol:chloroform extracted, ethanol precipitated and then resuspended in appropriate volume of distilled water and stored at -20°C. The DNA fragment was incubated with dephosphorylated vector (100ng) at a ratio of 3:1 respectively in a reaction containing 1 × ligase buffer and 1 unit of T4 ligase at 16°C for 1 hour or overnight. Dilutions of this reaction volume were used to transform competent bacterial cells (section 2.2.1.8).

2.2.1.8. Preparation of competent cells

E. coli BL21 (Amersham Pharmacia Biotech) were made competent according to the manufacturers instructions. A sterile streak technique was used to streak bacteria from a glycerol stock onto an LB medium plate and incubated overnight. A single colony was isolated and used to inoculate 50ml of LB broth at 37°C with shaking at 225rpm until cells grew to an absorbance measurement of 0.4-0.5 at 600 nm. Cells were sedimented at approximately 2500xg for 15 minutes at 4°C, then gently resuspended in 1/10 volume (5-10ml) of ice-cold TSS (1.0g tryptone, 0.5g yeast extract, 0.5g NaCl, 10g polyethylene glycol, 5ml dimethylsulfoxide [DMSO], and 5ml $MgCl_2$ [1ml] in 70ml sterile dH_2O) and placed on ice. Cells were transformed within 2-3 hours.

2.2.1.9. Transformation of *E coli* BL21 cells

GST expression plasmids were transformed and expressed in *E coli* BL21 made competent as described above (2.2.1.8). For transformation, 1 ml of freshly prepared competent bacteria was added to pre-chilled 50ml sterile disposable centrifuge tubes and

stored on ice. 1ng of plasmid was added to the cells, and swirled gently to mix and placed on ice for 45 minutes. The cells were heat shocked in a 42°C water bath for 2 minutes then chilled briefly on ice. 100µl of transformed cells were then transferred to 17x100mm tubes (Falcon) containing 900µl of LBG medium (LB + 20mM glucose) pre-warmed to 37°C and incubated for 1hour at 37°C with shaking at 225rpm. 100µl of the diluted transformed cells were plated onto LBAG plates (LBG + 100µg/ml ampicillin). The plate was inverted and incubated overnight at 37°C to allow colony formation. Frozen glycerol stocks cultures were prepared as described below in section 2.2.1.11.

2.2.1.10. Transformation of *E coli* DH5α cells

Plasmids were propagated in commercially available *E. coli* DH5α competent cells supplied as frozen stocks (Invitrogen) kept at -70°C until use. Bacteria were transformed following manufacturer's instructions. Competent cells were thawed slowly on ice, and 20µl of aliquots put into prechilled 1.5ml eppendorf tubes. 1-2 ng of the appropriate plasmid DNA was added to each aliquot and mixed by gently moving the pipette tip trough the cells while dispensing. The cells were then incubated on ice for 30 minutes before being heat shocked for 45 seconds at 42°C. The tube was then immediately placed on ice for 3 minutes. 180 µl of room temperature SOC Media (2% Bactotryptone, 0.3% Yeast Extract, 10mM NaCl, 2.5mM KCl, 20mM Mg^{2+} Stock (equimolar ratio of $MgCl_2.6H_2O$ & $MgSO_4.7H_2O$) and 20mM Glucose) was then added to each transformation reaction. The tube was then transferred to a shaking 37°C incubator (approximately 225rpm) for 1 hour to allow expression of the antibiotic resistant marker.

2.2.1.11. Glycerol Stocks

Host strains, and their derivatives containing useful plasmids, were stored as glycerol stocks for future retrieval. 800 µl of an overnight culture was mixed gently with 200 µl sterile glycerol in a 1.5 ml Nunc Cryotubes and stored at -70°C. A sterile plastic loop was used to retrieve an aliquot of cells as and when required.

2.2.1.12. Small Scale Preparation of Plasmid DNA (Miniprep)

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

2.2.1.13. Large Scale Preparation of Plasmid DNA (Maxiprep)

Bacteria containing the plasmid of interest were streaked onto an L-agar plate containing the appropriate antibiotic and the plate inverted and incubated overnight at 37°C to allow colony formation. A single colony was picked, using a sterile yellow tip, from this plate and used to inoculate a sterile universal tube containing 5 ml of L-Broth medium and the appropriate antibiotic (100µg/ml Ampicillin) which was then put in a shaking incubator at 225rpm overnight at 37°C. This culture was then added to 500 ml of broth, containing 100µg/ml Ampicillin in a 1litre glass conical flask (to allow good aeration), then returned

to the shaking incubator for 48 hours. DNA was prepared using the QIA prep Spin plasmid maxiprep kit following the manufacture's instructions.

2.2.2. Cell Culture and Transfection

2.2.2.1. Cell Culture

All cell culture work was performed following strict aseptic techniques inside a laminar flow hoods (Class II Microbiological safety Cabins; Gelaire BSB4). Cells were incubated in dry 37°C incubators containing 5% (v/v) CO_2 (Napco Model 5410 Genetic Reseach Instrumentation LTD).

2.2.2.2. Maintenance of cells in culture

Cells were fed twice weekly, old medium was aspirated from sub-confluent flasks and fresh medium added. Replating was performed as follows: for T150 cm^2 tissue culture flask medium was aspirated off and the cells washed once with 10 ml phosphate-buffered saline (PBS). The PBS was removed and 1 ml of trypsin solution (0.25% trypsin in 1x PE buffer; PBS with the addition of EDTA to 1 mM), which had been pre-warmed to 37°C, was added to cells. Flasks were transferred to the 37°C incubator until the cells had detached from the flasks. Complete medium was added and the cell suspension transferred to a sterile universal tube. The cells were pelleted by centrifugation at 1000rpm for 5 minutes at room temperature. The pellet was then resuspended in fresh growth medium and the cells reseeded at a 1:10 density.

2.2.2.3. Long Term Cell Storage

To freeze cell stocks for storage, confluent cultures were trypsinised, and pelleted as described above (section 2.2.2.2). The pellet was then resuspended at a concentration of approximately 10^5 cells/ml in growth medium containing 10% (v/v) DMSO. The DMSO in the medium acts as a cryoprotectant but all solution must be chilled, as DMSO is toxic to cells at room temperature. Suspensions were divided into 1 ml aliquots in 1-2 ml Nunc cryotubes and placed in a polystyrene box and frozen, well insulated, at -70°C overnight to ensure a slow rate of cooling. The ampoules were then transferred to a liquid nitrogen bank containing labelled storage rack until required. Frozen stocks were recovered by removing the ampoules from liquid nitrogen and placing them into a small, covered bucket of water at 37°C. Once thawed, the cells were added to 10ml of the appropriate pre-warmed growth medium, centrifuged, resuspended in fresh growth medium and transferred to 175 cm^2 flasks.

2.2.2.4. Transient Transfection of HaCaT and C33a cells

C33a cells were transiently transfected using a standard calcium phosphate precipitation technique. Cells were plated out at $6x10^4$/60mm tissue culture dish. The following day a solution of calcium phosphate containing the DNA was added to the cells. This was carried out as follows, for each 60mm cell monolayer; 250µl of a solution containing the plasmid DNA in 250 mM $CaCl_2$ was added dropwise with gentle mixing to 250µl to 2x HEPES buffered saline (280mM NaCl, 10mM KCl, 1.5mM $Na_2HPO_4.2H_2O$, 50mM HEPES). The mixture was left for 30 minutes to allow a fine precipitation 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. HaCaT cells were transfected using LIPOFECTAMINE PLUS™ Reagent (Invitrogen) according to the manufacturer's instructions.

2.2.2.5. Luciferase Assays

Transfected C33a and HaCaT cells were lysed directly on 60mm tissue culture plates. Cells were washed twice with PBS. The PBS then was completely removed by aspiration and 200µl of 1 × reporter lysis buffer (Promega) added to each plate. Following 10 minutes incubation at room temperature, cells were scraped off the culture plate and each lysate transferred to a 1.5ml eppendorf. Cell debris was pelleted by spinning lysates at 4°C in a microcentrifuge at 14000rpm for 5 minutes. The supernatant was transferred to a second eppendorf tube taking care not to disrupt the cell pellet. The lysate were either assayed for reporter enzyme activity immediately or stored at -20°C.

Luciferase activity was determined using a luminometer with automatic injection (Luminoskan Ascent-Thermo Labsystems). For each sample, 80µl of lysate and 80µl of Luciferase assay buffer (Promega) were used. Luciferase activity was normalised for protein content determined using the BCA assay (Sigma).

2.2.2.6. Transient DNA replication assay

Transient DNA replication assays were performed in C33a cells, as first described by Sakai et al., (1996) and as modified by Boner et al., (2002). Cells were set up in 100mm plates at $6x10^5$ per plate and transfected with 1µg p16ori-m, 5µg pCMV-$E1_{16}$, 10ng or 100ng pCMV-$E2_{16}$ with or without 1µg p16-HAL2 using calcium phosphate precipitation method for C33a cells or LIPOFECTAMINE PLUS™ Reagent method for HaCaT cells, respectively. Low molecular weight DNA was extracted using the Hirt protocol (Hirt B.,

1967) modified as described below. On day two after transfection, cells were washed with PBS and 800µl of Hirt solution (0.6% SDS, 10mM EDTA) was added per plate. After 5 minutes the cell lysate was transfered into 1.5ml tubes and 200µl of 5M NaCl was added to each tube. The tubes were mixed by inverting gently to allow efficient precipitation of genomic DNA and left overnight at 4°C. The next day samples were centrifuged at 15000g for 30 minutes at 4°C and the supernatant, containing low molecular weight DNA, was retained while the pellet was discarded. DNA in the supernatant was phenol-chloroform extracted and ethanol precipitated. To create linearised plasmid, 25µl of each sample were digested with 20U Xmn1 for 3 hours at 37°C. Linearised plasmid was further digested with 20U Dpn1 overnight at 37°C. Xmn1 linearises p16ori-m while Dpn1 digests only unreplicated *dam*-methylated DNA. Replication was assayed after Xmn1 and Dpn1 digestion. DNA was electrophoresed in a 1% agarose gel in 0.5x TBE and transferred to a Hybond-N nylon membrane (Amersham Pharmacia Biotech, UK) by Southern blotting as described in section 2.2.1.5. Hybridisation was done using QuikHyb hybridisation solution (Stratagene, UK) according to the manufacturer's instructions. A Molecular Dynamics Storm 840 phosphoimager (Amersham Pharmacia Biotech, UK) was used to scan the Southern blot and quantify hybridised signals. The extent of replication was calculated by measuring the ratio of double cut/single cut bands. This method of measurement controls for variation in transfection efficiency between experiments.

2.2.2.7. Probe for Hybridisation

The p16ori-m (2µg) plasmid was digested with PvuII. The digestion was electrophoresed in a 1% agarose gel and a band of ~700bp, corresponding to the sequence of HPV 16-ori, was cut out. It was purified using the QIAquick™ Gel Extraction kit (QIAGEN, UK) and eluted in 60µl dH_2O. A Prime-it II Random labelling kit (Stratagene) with [α-P^{32}]dCTP

was used to generate the probe according to the manufacturer's instruction and purified using a NICK Column (Amersham Pharmacia Biotech, UK).

2.2.2.8. Beta-Galactosidase Assay

The plasmid pCH110 was used in all transient transfections as an internal control against which the efficiency of transfection could be normalised. β-galactosidase catalytically converts colourless o-nitrophenyl-β-D-galactopyranoside (ONPG) to yellow o-nitrophenol. The level of activity of this enzyme can be assayed by measuring changes in light absorbance at 420nm. Cells were lysed as detailed in section 2.2.3.7. To 80µl of each cell lysate, 1ml of solution I (60mM Na_2HPO_4, 40mM NaH_2PO_4, 10mM KCl, 1mM $MgCl_2$, 50mM β-mercaptoethanol) and 0.2ml of solution II (60mM Na_2HPO_4, 40mM NaH_2PO_4, 2mg/ml ONPG) was added. After mixing, all samples were incubated at 37°C for 30-60 minutes or until a yellow colour change could be seen. Samples were transferred to plastic disposable cuvettes and the reactions stopped by the addition of 0.5ml 1M sodium carbonate. The absorbance was read at 420nm using a Beckman DU 650 spectrophotometer.

2.2.3. DNA and RNA analysis

2.2.3.1. Total RNA Extraction from Cell Lines

Cells were grown in a 175cm^2 (T175) flask to approximately 80% confluency. Total RNA was then extracted by use of the RNAeasy Kit (Qiagen). The cells were washed once with pre-warmed sterile PBS and trypsinised and counted. 1x10^7 cells of all cell lines were subjected to the RNA extraction method as per manufacturer's details. For all RNA

extractions the concentration of RNA was measured spectrophotometrically as described in section 2.2.1.2. RNA samples were aliquoted and stored at -70°C.

2.2.3.2. Polymerase Chain Reaction (PCR)

2.2.3.2.1. Amplification of DNA

All reagents were provided in the HotStarTaq PCR Kit with the exception of dNTP's, which were obtained from the Perkin-Elmer Core DNA PCR kit. Primer sequences are described in table 2.1.

Table 2.1 Oligonucleotide PCR primers

Primer Name	Primer Nucleotide Sequence	Purity	
HPV-16 L2 BamH1 ATG forward	tgcaggatccatgcgacacaaacgttctgc	T_m	81
		%GC	53
HPV-16 L2 BamH1 forward	tgcaggatcccgacacaaacgttctgc	T_m	77
		%GC	38
HPV-16 L2 EcoR1 reverse	tgcagaattcggcaagtagacagtggc	T_m	81
		%GC	53
25-473atgL2 forward	tgcaggatccatggcaggtacatgtccacct	T_m	82
		%GC	54
50-473atgL2 forward	tgcaggatccatgagtatgggtgtatttttt	T_m	76
		%GC	38
150-473atgL2 forward	tgcaggatccatgaatactgttactactgttact	T_m	76
		%GC	38
250-473atg forward	tgcaggatccatgattacatatgataatcct	T_m	74
		%GC	35
350-473atg forward	tgcaggatccatgactacccccttcacatgca	T_m	81
		%GC	51

390-473atg forward	tgcaggatccatgttatcaggttatattcct	T_m	76
		%GC	38
GST-L2 1-50 reverse	tgcagaattcctaacttccatattgtaatat	T_m	72
		%GC	29
GST-L2 1-100 reverse	tgcagaattcctaagggcccacaggatctac	T_m	81
		%GC	51
GST-L2 1-200 reverse	tgcagaattcctatgtatccataggaatttc	T_m	74
		%GC	35
GST-L2 1-300 reverse	tgcagaattcctagccagtacgcctagaggt	T_m	81
		%GC	51
GST-L2 1-400 reverse	tgcagaattcctaaattgttgtatttgcagg	T_m	74
		%GC	35
GST-L2 25-473 forward	tgcaggatccgcaggtacatgtccacct	T_m	81
		%GC	57
GST-L2 50-473 forward	tgcaggatccagtatgggtgtatttttt	T_m	74
		%GC	39
GST-L2 150-473 forward	tgcaggatccaatactgttactactgtt	T_m	74
		%GC	39
GST-L2 250-473 forward	tgcaggatccattacatatgataatcct	T_m	72
		%GC	35
GST-L2 350-473 forward	tgcaggatccactacccccttcacatgca	T_m	79
		%GC	53
GST-L2 390-473 forward	tgcaggatccttatcaggttatattcct	T_m	74
		%GC	39
RT-L2 forward	caggcggacgcactgggt	T_m	74
		%GC	72
RT-L2 reverse	gtcaggatctggtgctat	T_m	65
		%GC	50
Actin forward	gcgtctggacctggctggccgggacct	T_m	87
		%GC	74
Actin reverse	ggaaggctggaagagtgcctcagggcag	T_m	84
		%GC	64

The reaction mixture comprised 200μM of each dATP, dGTP, dCTP and dTTP, 1 × PCR kit buffer, 0.2μM of each primer, 2.5 units *HotStarTaq* polymerase (a modified thermostable DNA polymerase from *Thermus aquaticus*) and 1μg of DNA sample. It was aliquoted into 0.5ml GeneAmp PCR reaction microfuge tubes in a final volume of 100μl. Each cycle of PCR amplification consists of a number of steps which produce two oligonucleotide-primed single-stranded DNA templates, set up the polymerisation reaction and synthesize a copy of each strand of the template being targeted. The tubes were placed into the PCR machine (MJ Research PTC-200 Peltier Thermal Cycler) and heated to 95°C for 15 seconds to inactivate DNase and ensure all DNA duplexes were melted, in addition to activating the HotStarTaq.

The DNA was amplified for 35 cycles at 94°C for 60 seconds, then between 55-65°C for 60 seconds, to allow the primers to anneal to the template DNA. The sequences of the primers are a major consideration in determining the temperature of the PCR amplification cycles. For primers with a high T_m, a higher annealing temperature is usually advantageous. The higher temperature minimizes non-specific primer annealing, increasing the amount of specific product produced and reducing the amount of primer-dimer formation. The annealing temperatures for all primers were determined by optimising the annealing conditions by performing the reaction at several temperatures starting at 5°C below any calculated T_m. The annealing temperature for each PCR reaction was dependent on the primer with the lowest T_m. The melting temperature for oligonucleotides can be calculated with the formula below.

$$T_m = 81.5 + 16.6 (\log_{10}[Na^+]) + 0.41 (\%G+C) - 675/n$$

Where [Na^+] is the molar salt concentration; [K^+]=[Na^+] and n= number of bases in the oligonucleotide. Primers are usually designed with a 40-60 G+C%.

The extensions of the primers were performed at 72°C for 60 seconds. After completion of the cycles, the reaction was incubated at 72°C for a further 7 minutes to ensure full extension and then cooled to 4°C. 5µl of each sample was analysed by agarose gel electrophoresis (as described in section 2.2.1.6) to check the correct product was amplified.

2.2.3.2.2. Amplification from RNA: Reverse Transcriptase-PCR (RT-PCR)

RNA was prepared (see section 2.2.4.2) and used as the template for reverse transcription and PCR amplification of cDNA. Firstly cDNA was synthesised from RNA by reverse transcription using the Omniscript RT kit (Qiagen). The reaction was carried out according to the manufacturer's instructions, to the following final concentrations: 1× RT buffer, 5mM of each of dATP, dGTP, dTTP, dCTP, 10 unit RNase inhibitor, 1µg RNA, 4 units Omniscript reverse transcriptase, 1µM of Oligo-dT primer, and DEPC-treated water to a final volume of 20 µl. Control reactions using no Omniscript reverse transcriptase were carried out, in addition to assaying suitable negative cell lines for each experiment.

All samples were placed in the thermocycler and further incubated at 37°C for 60 minutes, and then incubated at 4°C for 5 minutes. The above reaction was then stored at -20°C until use. Typically 2µl of the RT reaction was carried forward to the amplification step. PCR reactions were carried out as per manufacturer's guidelines (Qiagen) for HotStarTaq amplification. Briefly final reaction volumes were 100µl consisting of 2µl of RT reaction

mixture combined with a final concentration of 1x PCR Buffer, 200μM of each dNTP, and 0.2μM of the forward and reverse primers, 2.5 Units of the HotStarTaq made up to 98μl with distilled water. To activate the HotStarTaq an initial incubation of 95°C was carried out for 15 minutes. Amplification proceeded for 35 cycles of 94°C for 60 seconds, 59°C for 60 seconds, plus a 60 second extension at 72 °C. PCR was carried out in a MJ Research PTC-200 Peltier Thermal Cycler. The samples were then analysed by agarose gel electrophoresis as described in section 2.2.1.6 to ensure correct amplification.

2.2.3.2.3. Real Time Quantitative PCR.

Cells were seeded and transfected with 4μg plasmid and total RNA was isolated with RNeasy®MiniKit (QIAGEN, UK) and resuspended in RNase-free water following the instructions of the supplier. Thirty-two nanogram total RNA was digested with 0.5U Dnase I (Invitrogen™, UK) according to the manufacturer's protocol. The reverse transcription was primed in duplicate with Random Primers (Promega, UK) at a concentration of 0.5μg in a 25μl reaction mixture with or without 3U AMV-RT (Promega, UK) and 0.5mM each dNTP (Promega, UK), 20U rRNAsin (Promega, UK), and 1X AMV-RT buffer (Promega, UK) following the instructions of the supplier. As a preliminary screening, cDNA was amplified by PCR with 2.5U Taq Polymerase (Gibco BRL®, UK) in a final volume of 50μl containing 1X PCR buffer (Gibco BRL®, UK), 125μM of each dNTP (Promega, UK) and 0.5μM of each primer (sense: 5'CGA TGG AGA CTC TTT GCC AA; antisense: 5'TAT AGA CAT AAA TCC AGT) for 30 cycles at 94°C 1 minute, 50°C 1 minute and 72°C for 2 minutes and 1 cycle at 72°C for 10 minutes and the PCR products were electrophoresed in a 1% agarose gel. Once checked that the RNA was free from DNA contamination, cDNA was amplified in triplicate with the

primers Tq16E2f 5'CCT GAA ATT ATT AGG CAG CAC TTG and Tq16E2r 5'GCG ACG GCT TTG GTA TGG at a concentration of 300mM each in a 50µl reaction that contained in final concentration: 1X PCR Buffer II, 200µM dATP, dCTP, dGTP, and 400µM dUTP, 5.5mM $MgCl_2$ and 1.25U AmpliTaq® DNA Polymerase with GeneAmp®. The reaction also contained the detection probe TaqMan® Probe: 5' CAA CCA CCC CGC CGC GA, at a concentration of 300nM. The thermal cycling conditions were 2 minutes at 95°C, followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 minute. All reactions were performed in the model 7700 Sequence Detector (PE Applied Biosystems), which contains a GeneAmp PCR System 9600. As control cDNA was amplified with primers for actin: β-actin Forward Primer and β-actin Reverse Primer (PE Applied Biosystems, Foster City, Ca, USA) at a concentration of 60nM each in a reaction mix with the same cycling conditions, with β-actin Probe (PE Applied Biosystems, Foster City, Ca, USA) at a concentration of 40nM.

2.2.3.3. DNA Sequencing

The sequence of all new plasmids was checked using Taq terminator sequencing on an Applied Biosystems Prism 3100 Genetic Analyzer DNA sequencer. The region to be sequenced first underwent PCR amplification. The sequencing reaction mix was prepared by adding 500ng of template DNA (~6µl of standard miniprep), 2µl 5x sequencing buffer, 3.2pmoles the appropriate primer, 4µl of BigDye™ Terminator Ready Reaction Premix (Applied Biosystems) made up with distilled H_2O to a total reaction volume of 20µl in 200µl thin walled eppendorf tubes. The samples were placed in a MJ Research PTC-200 Peltier Thermal Cycler and exposed to 25 cycles of 96°C for 10seconds, 50°C for 5seconds and 60°C for 4 minutes. The PCR products were purified using PERFORMA® DTR Gel Filtration Cartridges. The columns were first spun at 3000rpm for 2 minutes to

remove buffer. The columns were then transferred to sample collection tubes. The PCR sequencing reaction mix was then loaded onto the centre of the gel column. The column was spun for 3000rpm for 2 minutes. The sample was then dried under vacuum for 15-20 minutes and then resuspended in 25µl formamide.

Table 2.2 Sequencing primers

Primer Name	Position	Primer Nucleotide Sequence
T7 forward 5'-3'	864-882	5' - attaatacgactcactataggga -3'
BGH reverse 5'-3'	1018-1249	5' - ctagaaggcacagtcgaggc -3'
PGEX forward 5'-3'	869-891	5' - gggctggcaagccacgtttggtg -3'
PGEX reverse 5'-3'	1042-1020	5' - ccgggagctgcatgtgtcagagg -3'
pEGFP-C1 sequencing Primer	679-658	5' - cctaggagcactaagcga -3'

2.2.4. Protein Analysis

2.2.4.1. Protein Preparations from Cells for Western Blot Analysis

Cells were lysed by aspirating the culture medium off, washing the cell monolayer once with ice-cold PBS, the PBS was completely removed by aspiration. For lysis with NP-40 lysis buffer (150mM NaCl, 0.5% NP-40, 50mM Tris-HCl and one tablet protease inhibitor cocktail), 1ml of trypsin was added to cells until they detached. Cells were collected in universal tubes using 6ml of serum containing medium to inactivate trypsin. Cells were pelleted by spinning for 5 minutes at 1000rpm. The pellet was washed once with ice cold PBS and repelleted. The supernatant was removed and 200µl of NP-40 lysis buffer was

added to the pellet and kept on ice for 10 minutes. Cell debris was pelleted at 14000 rpm and the supernatant transferred to a new microcentrifuge tube.

For lysis with SDS-lysis buffer (100mM Tris-HCl pH6.8, 2% SDS, 2% glycerol and one tablet protease inhibitor cocktail), the cells were lysed by aspirating the culture medium off, washing the cell monolayer once with PBS. The PBS was completely removed by aspiration. 200µl of SDS lysis buffer was added to the plates and cell debris was scraped off and transferred to microcentrifuge tubes. The cells were further disrupted using either a sonicator or repeated aspiration with a syringe. The cell debris was pelleted at 14000 rpm and the supernatant transferred to a new microcentrifuge tube.

2.2.4.2. Protein Concentration Assays

The BCA/CuSO4 Protein assay was used to spectrometrically determine the protein concentration of dilute solutions following the manufacturer's instructions. Proteins reduce alkaline Cu(II) to Cu(I) in a concentration-dependent manner. Bicinchoninic acid is a highly specific chromogenic reagent for Cu(I), forming a purple complex with an absorbance maximum at 562nm. 10µl of protein solution was placed in separate wells in 96 well plate. 200µl of developing solution (5ml BCA (Biocinchoninic acid) solution, 100µl of 4% (w/v) CuSO4 (copper II sulphate pentahydrate solution) was added to the protein samples and incubated at 37°C for 30 minutes.

The absorbance of each sample was read at 590nm using a WPA UV1101 Biotech photometer plate reader. The absorbance reading was converted to concentration in µg/ml for each sample using a standard curve generated from a series of control BSA solutions

of known concentration. The actual concentration of each protein sample was calculated after multiplying by the relevant dilution factor.

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

Protein samples were resolved according to the molecular weight using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). All Western blot analysis was performed using Nu PAGE™ pre cast gel system. 10µg of each protein sample were electrophoresed on Nu PAGE™ 4-12% Bis-Tris gel (Invitrogen, UK) under denaturing conditions. Prior to loading, equivalent amount of each protein samples (10µg) was mixed with 5µl of 4x SDS gel loading buffer (Invitrogen, UK) and 2µl of 2x reducing agent (Invitrogen, UK) made up to 20µl with dH_2O. Samples were then boiled for 10minutes at 70°C. The prepared protein samples were then loaded into consecutive wells and 15µl Rainbow™ protein molecular weight marker mix (molecular weight range 6KD-188KD) added to the first and/or last well on the gel. The gel was run by electrophoresis at a constant voltage of 200V for 45minutes. The running buffer (Nu PAGE™ MES buffer, Invitrogen, UK) was used with 500µl antioxidant (Invitrogen, UK) added to 200ml of the running buffer for the inner tank. Once the dye front was approximately 5-10cm from the bottom of the gel, the gel was removed and used for western blot analysis.

2.2.4.4. Western Blotting

Separated protein samples were transferred to a nitro-cellulose membrane using the Nu PAGE™ wet blotting tank. For this purpose the gel was removed from the electrophoresis tank and transferred onto a nitrocellulose membrane (Invitrogen, UK). Before transferring the membrane, the Nu PAGE™ gel sandwich was pre-soaked with transfer buffer with 500µl antioxidant added to 1ml of transfer buffer (with 10% or 20% methanol depending

on the number of gels being transferred). The gel sandwich contains a pre-cut nitrocellulose membrane sandwiched between two filter papers. The gel tank was set up as follows:-

1. The first sheet of filter paper, soaked in transfer buffer was placed neatly on to the gel avoiding any air bubbles.
2. Then the nitro-cellulose membrane was laid on the gel, and then a further sheet of filter paper, all soaked in transfer buffer was added to the top.
3. The sandwich was rolled with a glass pipette to eliminate any air bubbles
4. The sandwich was placed into the transfer tank and run at 30V for approximately 60 minutes, the time taken for the pre-stained marker proteins to be completely transferred.

Once the transfer was completed, the membrane was blocked by shaking for a minimum of 1 hour in 50 ml of block buffer (5% Marvel [dried milk] in PBS-0.01% Tween) at room temperature. The nitro-cellulose filter was washed in wash buffer (PBS-T) for 10 minutes. The filter was then placed in 7ml blocking buffer containing suitable primary antibody and incubated at room temperature for 1 hour with gentle shaking. The primary antibody solution was removed and the filter rinsed in blocking buffer then washed 1x 15 minutes and 2x 5minutes in 100ml volumes of fresh PBS-T. The filter was then incubated in 20 ml blocking buffer containing a 1/5000 dilution of the applicable HRP-linked secondary antibody for 1 hour at room temperature with gentle shaking. The filter was washed 1x for 15 minutes and 4x for 5 minutes with PBS-T buffer. Excess surface liquid was removed from the filter by briefly blotting with a piece of Whatman 3MM paper. The detection consisted of incubating the filter in an equal volume of an Amersham Enhanced

chemilluminescence (ECL) detection reagents 1 and 2 for 1 minute at room temperature. The excess detection solution was drained off the nitro-cellulose filter and this was then wrapped in Saran wrap and exposed to Amersham ECL film for 30 seconds and up to 30 minutes (depending on the strength of the signal).

2.2.4.5. Stripping Western Blot Membranes

If a western blot membrane was required for multiple analyses with different probe antibodies then the initial primary and secondary antibodies were removed from the membrane by stripping. The membrane post exposure to the Amersham ECL film was washed for 10 minutes three times in PBS before being incubated for 1 hour at room temperature in Stripping Buffer (0.2M Glycine pH 2.5, 0.2% SDS). The membrane was then washed with copious amounts of PBS until all traces of the SDS were safely removed. The membrane was then blocked as per protocol for the next probing primary antibody.

2.2.4.6. Immunoprecipitation

For immunoprecipitation assays, 0.1g of protein-A sepharose beads (Sigma) was pre-swollen in NP40 lysis buffer overnight at 4^0C. The next day, the beads were centrifuged at 14000rpm at 4^0C for 1 minute and washed three times with lysis buffer and finally resuspended in 1 volume lysis buffer (~300µl). Then 10µl of beads was added to 50µl protein extract plus 50µl lysis buffer for 1 hour with rotation at 4^0C. The mixture was centrifuged and the supernatant was transferred to new microcentrifuge tubes. The antibody was diluted 1:10 and 1µl added to supernatant and incubated with rotation at 4^0C for 1 hour. Then 10µl of beads was added and further incubated for another 1 hour with rotation at 4^0C. The mix was centrifuged and pellet washed 5x with 0.5ml lysis buffer. To

load onto a gel, traces of lysis buffer were removed and samples prepared for loading onto SDS-PAGE gel as described in section 2.2.4.3 above.

2.2.4.7. Expression and Purification of GST Proteins

Individual bacterial colonies (*E coli* BL21) containing recombinant pGEX-GST fusion expression vectors were used to inoculate 5 ml 2x YTA medium and grown overnight at 37°C with shaking at 225rpm. 1ml of culture was used to inoculate 50ml of pre-warmed 2x YTA medium and grown at 37°C with shaking until an A_{600} of 0.6-0.8 was reached. Expression was induced by the addition of IPTG (final conc. 0.1mM) and the culture was incubated with shaking at 37°C for a further 5 hours. Bacteria were pelleted by centrifugation at 2500g for 30 minutes. The pellets were resuspended in 5ml (1/10 volume) of BugBuster™ Protein Extraction Reagent (Novagen) containing 1 tablet protease inhibitor cocktail (per 10ml reagent) and incubated for 30 minutes with rotation. The cell extracts were centrifuged at 2500g for 30 minutes. 1ml aliquots of the supernatant were transferred to fresh tubes and stored at –70°C. Fusion proteins were purified on glutathionine-Sepharose beads by incubating 1ml supernatant (crude extract) with 50µl beads for 30 minutes at room temperature with rotation. The beads were pelleted in a microfuge (14000 rpm, 20 seconds) and washed 3x 0.5 ml NETN (20mM Tris-HCL, pH 8.0), 100mM NaCl, 1mM EDTA, 0.5% Nonidet P-40 (NP-40) containing 1 tablet protease inhibitor cocktail per 10ml buffer. The beads were resuspended in 50µl NETN and purified proteins were analysed by 10% SDS-PAGE (Section 2.2.4.3) before subsequent manipulations.

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

HPV-16 E2 was subcloned from pCMV-HPV16 E2 into the BamH1 site of pBluescript SKII under control of the T7 promoter using standard molecular biology techniques. HPV-16 E2 was *in-vitro* transcribed-translated using TNT Quick Coupled Transcription/Translation System (Promega) as instructed by the manufacturer to produce ^{35}S labelled E2. The efficiency of transcription-translation was checked using a luciferase control plasmid construct to produce ^{35}S labelled luciferase. 5µl of each reaction was analysed by SDS-PAGE and proteins were fixed in 7% methanol and 7% glacial acetic acid for 15 minutes shaking and 10minutes with Amplify™ Fluorographic reagent (Amersham). The gel was transferred onto filter paper, dried and exposed for autoradiography at –70°C overnight.

GST pull-down assay were performed as follows: pGEX, pGEX-L2 and pGEX-L2 deletion mutants were used to transform competent *E coli* BL21 cells. GST fusion proteins were expressed and purified as described in section 2.2.4.7. The proteins immobilised on beads were pre-washed three times in pull-down buffer (PDB: 50mM Tris pH 7.9, 100mM NaCl, 1mM DTT, 0.5mM EGTA, 0.5% NP-40, 1mM PMSF). The NaCl concentration in PDB can be changed to assess the specificity of binding. 7.5µl ^{35}S labelled *in vitro* translated protein was then incubated with approximately 1µl immobilised fusion protein (approximately 10µl protein on beads) in a total volume of 200µl fresh PDB for 30 minutes at 4°C with rotation. The beads were pelleted in a microfuge (14000 rpm, 10sec) washed four times in PDB. Bound proteins were separated by 10% SDS-PAGE, fixed, the gel was dried and exposed for autoradiography at –70°C overnight. Bands were then analysed by densitometry using a UMAX Powerlook III Flatbed scanner and ImageQuant v5.2 software.

2.2.4.9. Pulse-chase [^{35}S] Methionine labelling for protein Half-Life

Cells were plated out at $1.2x10^6$ cells/100mm dishes (to give ~80% confluency when labelling). Culture medium was aspirated from dishes and cells washed once with PBS and once with 5ml 1x short term labelling (STL) medium. Cells were then incubated with STLM (STL, 5% DFCS, 4mM L-Glutamine) for 15 minutes. The STLM was aspirated and STLM with [^{35}S] methionine (0.2µCi/ml) was added to cells for 1 hour at 37^{0}C, 5% CO_2 to pulse cells. To chase, the dishes were washed with 1x chase medium (10% FCS in DMEM) then incubated with 10ml chase medium. For protein half-life studies, cells were harvested over a described time course. To harvest cells, chase medium was removed and washed 1x with 5ml PBS. 1ml of trypsin was added per dish. Once detached, 4ml of chase medium was added and aspirated and centrifuged at 14000rpm at 4^{0}C for 10 minutes. NP-40 lysis buffer was used to harvest the cells as described in section 2.2.4.1. To analyse for protein half-life, cell lysates were immunoprecipitated with required antibody as described in section 2.2.4.6. The gel was dried and exposed for autoradiography at –70°C overnight. Bands were then analysed by densitometry using a UMAX Powerlook III Flatbed scanner and ImageQuant v5.2 software.

2.2.4.10. Cycloheximide treatment for protein Half-Life

Cells were plated out at $1.2x10^6$ cells in 100mm dishes then left for 24 hours before being treated with cycloheximide (CHX). CHX (Sigma-Aldrich) in DMSO was added to a final concentration of 100µg/ml to each plate and DMSO only was used as a negative control. Cells were harvested in SDS lysis buffer at regular time intervals after CHX/DMSO addition. Lysates were then run on Nu PAGE™ pre cast gel system (Invitrogen), before being transferred to a nitrocellulose membrane for probing. Bands were then analysed by

densitometry using a UMAX Powerlook III Flatbed scanner and ImageQuant v5.2 software.

2.2.5. Immunofluorescence

Round coverslips were first washed in distilled water, air-dried and put into a beaker for autoclaving. The coverslips were placed in 16 well tissue culture plates and cells seeded $1x10^5$ cells per well. All transfections for immunofluorescence studies were performed using LIPOFECTAMINE™ according to the manufacturer's instructions. Twenty-four hours after transfection, the cells were washed twice with PBS and fixed by 10 minute incubation at room temperature with 1.85% formaldehyde (Sigma) diluted in PBS containing 2% sucrose and washed three times with PBS. They were permeablilsed by 10 minute incubation at room temperature with 0.5% NP40 in PBS containing 10% sucrose and washed three times with 1% FCS in PBS After washes with 1% FCS in PBS, coverslips were mounted in AF1 (Citifluor, UK). Fluorescence was analysed using a Leica TCS SP2 true confocal scanner (Leica-microsystems, Heidelberg Germany) with three lasers giving excitation lines at 488nm.

CHAPTER 3: EFFECT OF THE HUMAN PAPILLOMAVIRUS TYPE 16 L2 PROTEIN ON THE FUNCTIONS OF THE E2 PROTEIN

3.1. The Viral Regulatory Protein E2

The viral E2 protein regulates viral transcription through binding as a dimer to its specific recognition sequences (binding sites) contained within the viral long control region (LCR). The LCR of mucosal papillomaviruses has four E2 binding sites (E2BS1-4) (Fig 1.3) (Desaintes and Demeret, 1996). Two of these sites are immediately upstream from the TATA box (E2BS 1,2), separated from each other and from the TATA box by 3 or 4 base pairs. For the other two sites, one is beside the E1 DNA binding site involved in the regulation of viral DNA replication, and one is a further 300-400 bp upstream (Fig 1.3). In general, binding of E2 to E2BS 1, 2 and 3 causes transcriptional repression, while E2 binding to E2BS 4 leads to activation (Demeret *et al.*, 1997). The full-length E2 protein is essential for efficient viral DNA replication. The E1 protein has DNA binding, DNA helicase and ATPase activities and plays a key role in viral DNA replication. Both E1 and E2 proteins bind to sites located within the LCR called the origin of DNA replication (ori) (Chiang *et al.*, 1992), and are the only viral proteins necessary to initiate replication from the ori (Chow and Broker, 1994). The ability of E2 to form a complex with E1 is mediated by the amino-terminal portion of E2.

In BPV-1, co-expression of L2 and E2 causes the redistribution of E2 into PODs, leading to the suggestion that a major redistribution of viral components occurs during virion

assembly (Day *et al.*, 1998; Heino *et al.*, 2000). Despite the observed co-localisation of E2 and L2 to PODs, little is known about the effect of this interaction on E2 function in viral transcription regulation or replication. As such the effect of the interaction of HPV-16 E2 and HPV-16 L2 on the transcription transactivation and replication function of E2 was investigated.

3.1.1. Down-regulation of the transcription transactivation function of HPV-16 E2 by HPV-16 L2

In order to determine the effect of L2 on E2 function in transcription transactivation, the role of E2 in enhancing transcription from the HPV-18 LCR was investigated. The BPV-4 LCR was also used to observe activation of transcription transactivation from a different promoter. C33a and U2OS cells were transfected with wild type HPV-18 LCR or BPV-4 LCR luciferase reporter constructs and pCMV-$E2_{16}$. In both cell lines, E2 activated transcription from the HPV-18 and BPV-4 promoters as described previously (Jackson and Campo, 1995; Morgan *et al.*, 1998; Steger and Corbach, 1997). This activation was approximately one and a half fold to two fold at low and intermediate levels of E2 while high levels of E2 results in down-regulation of transcription transactivation (Fig 3.1) indicating that over expression of E2 resultes in the down-regulation of transcription transactivation from the wild type HPV-18 and BPV-4 LCR promoters. This down-regulation has been shown to occur as a result of E2 binding to E2BS 1 interfering with TBP binding to the TATA-box (Steger *et al.*, 1995) or by competing with the cellular transcription factor Sp1, which binds to a site overlapping E2BS 3 (Demeret *et al.*, 1994; Demeret *et al.*, 1997; Tan *et al.*, 1994). This is further

elucidated with the observation that overexpression of E2 increases transcription activation of an SV40 promoter (Morgan *et al.*, 1998) and mutations in the E2BS 1-3 have been shown to lead to an increase in transcription activation eliminating trans-repression (Morgan *et al.*, 1998; Phelps and Howley, 1987; Vance *et al.*, 1999), indicating that down-regulation is mediated through E2 binding to its E2BS. E2BS 4 has been shown to be the major site for E2 activation on the LCR with which it has its highest binding affinity (Steger and Corbach, 1997).

In this study, E2 responsive reporter constructs derived from the HPV-18 LCR with mutations in E2BS 1 (p18LCR-BS1) or E2BS 1-3 (p18LCR-BS1-3) to prevent E2 binding these sites (Fig 3.2) and two synthetic promoters consisting of six multimerized E2BS 4 fused to the HPV-18 TATA (p18-6E2) or tk promoter (ptk6E2) were used. These reporter constructs were chosen, as transcription is not repressed by E2 as observed with wild type LCR. Any activity difference between these plasmids in a reporter assay is attributed to the binding of E2 to the E2BS, inducing transcription of the reporter gene. HaCaT and C33a cells were transfected with each plasmid and increasing amounts of pCMV-$E2_{16}$. The results indicated that luciferase expression increased with increasing amounts of E2 in both cell lines and no trans-repression was observed even at the highest amounts of E2 used. In HaCaT cells, the LCR was five to ten times more transcriptionally active when the E2BS 1 was mutated. Mutation of E2BS 1-3 similarly activated transcription about 12 fold. The use of synthetic promoters further increased transcription, with ptk6E2 showing the highest activation (Fig 3.3). In C33a cells, mutation of E2BS 1 or 1-3 gave activations of three to six fold while p18-6E2 and ptk6E2 gave 15 and 35 fold activation respectively (Fig 3.4). In summary, overexpression of E2

increased the transcription transactivation of all reporter constructs in a dose dependent manner with complete abrogation of down-regulation. Although the kinetics of activation differed slightly between promoters and between different cell lines, a similar pattern of activation was observed for all promoters.

As the down-regulation of E2 transcription transactivation is abrogated using the E2 responsive reporter constructs described above, it provided a system to study any change in E2 function in the activation of transcription. To study the effect of HPV-16 L2 on the transactivation function of HPV-16 E2, HaCaT and C33a cells were transfected with p18LCR-BS1, p18LCR-BS1-3, p18-6E2, ptk6E2, pCMV-$E2_{16}$ and increasing amounts of p16-HAL2, a HPV-16 L2 expressing plasmid. The amount of E2 was kept sub-optimal for E2-mediated transactivation to allow for detection of any effect of L2. The addition of L2 led to an inhibition of E2-mediated transactivation in a dose-dependent manner in both cell lines (Fig. 3.5 and Fig. 3.6). This was observed for all reporter plasmids used although the kinetics of inhibition varied slightly from reporter to reporter. All reporters were however fully inhibited at the highest amounts of L2 used in both cell lines. The addition of L2 in the absence of E2 did not affect the constitutive activity of reporters (Fig 3.5 and Fig 3.6). To determine if L2 had any effect on a promoter not responsive to E2, C33a cells were transfected with ptkluc (the tk promoter reporter plasmid with no E2 binding sites), and increasing amounts of p16-HAL2. The results indicate that L2 and E2 co-expression has no effect on the tk promoter activity (Fig. 3.7).

3.1.2. HPV-16 L2 does not affect the replication function of HPV-16 E2

E2 binds to E1 and promotes origin-dependent viral replication. The effect of HPV-16 L2 on the ability of HPV-16 E2 to co-operate with HPV-16 E1 in a transient DNA replication assay was investigated. The DNA replication assays were performed in C33a cells, because replication assays did not work in HaCaT cells, perhaps due to a decrease in cdk2/cyclin E activity as described for HeLa cells (Lin *et al.*, 2000). C33a cells were transfected with p16ori-m (a plasmid which contains the HPV16 origin of replication cloned into pSKII(-), with a point mutation at 115 from C to A to create a DpnI restriction enzyme site), pCMV-$E1_{16}$, and pCMV-$E2_{16}$ with or without p16-HAL2 and assayed for E2 mediated DNA replication. DNA replication was dependent on the presence of p16ori-m, E1 and E2. Forty-eight hours post transfection, low molecular weight DNA was extracted using the Hirt protocol (Hirt, 1967) and linearised by digestion with Xmn1. The linearised plasmid was further digested with Dpn1, which cleaves only when its recognition site is methylated. Xmn1 linearises p16ori-m while Dpn1 digests only *dam* methylated unreplicated DNA. Replication was assayed after Xmn1 and Dpn1 digestion by detection of an undigested but linearised p16ori-m band. In the absence of E2, no replication took place, while at 10ng of E2, a replicated band was detected, increasing with E2 concentrations (Fig. 3.8a). There was no replication in the presence of L2 and absence of E2. The results indicate that L2 had little effect on the replication activity of E2 (Fig. 3.8a and b). Altogether, these results indicate that L2 has a selective effect on the functions of E2. L2 drastically inhibits the transcriptional function of E2 but has little effect on replication.

Figure 3.1. Transactivation of HPV-18 LCR and BPV-4 LCR luciferase reporter constructs by HPV-16 E2 in C33a and U2OS cells respectively. C33a and U2OS cells were transiently transfected with 0.1μg of each luciferase reporter construct and with increasing amounts of the E2 expression plasmid pCMV-$E2_{16}$, (0.01μg, 0.1μg and 1μg). The total amount of DNA was kept constant by adding appropriate amounts of the parental plasmid pCMV. Each experiment was performed in triplicate. Transactivation is shown as fold activation of luciferase expression over that of the reporter plasmids without E2, taken arbitarily as 1.

Transactivation of wild type BPV-4 LCR in U2Os cells & HPV-18 LCR in C33a cells by HPV-16 E2

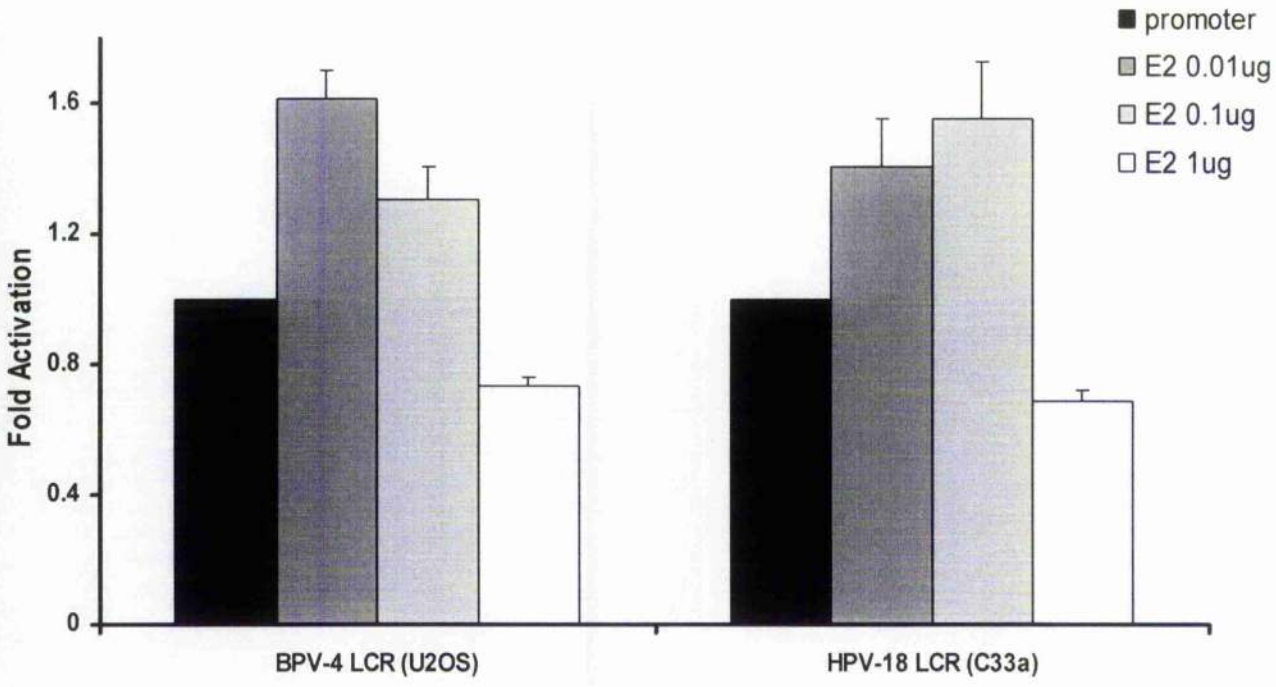


Figure 3.2. Schematic representation of luciferase reporter plasmids. The first three plasmids contain respectively the wild type LCR of HPV-18 (pHPV18-LCR), the LCR with four point mutations in the E2 binding site 1 (pHPV18-LCR-BS1) or four point mutations in each of sites 1 to 3 (pHPV18-LCR-BS1-3). The other two plasmids are p18-6E2 containing six E2 binding sites upstream of the HPV-18 TATA and the E2-minimal promoter constructs ptk6E2, containing six E2 binding sites upstream of the tk promoter.

E2 Responsive Luciferase Reporter Constructs

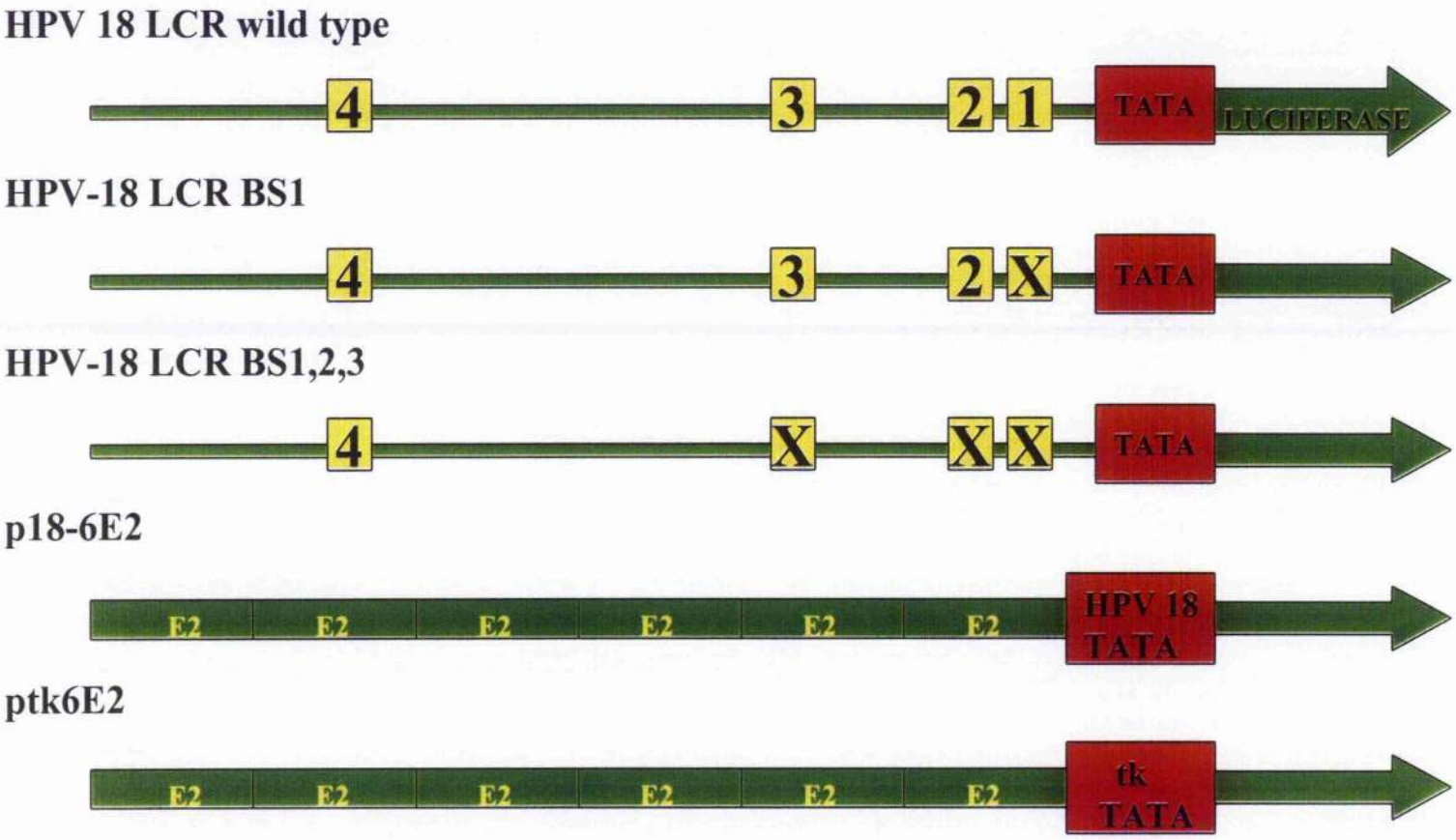


Figure 3.3. Transactivation of HPV-18 LCR BS mutants and E2 minimal promoter constructs by HPV-16 E2 in HaCaT cells. HaCaT cells were transiently transfected with 0.1μg of each luciferase reporter construct (BS1, BS1,2,3, p18-6E2, and ptk6E2) and with increasing amounts of the E2 expression plasmid pCMV-$E2_{16}$ (0.01ng, 0.1ng, 1ng and 10ng). Shown are representative results from each promoter in HaCaT cells. The total amount of DNA was kept constant by adding appropriate amounts of the parental plasmid pCMV. Each experiment was performed in triplicate. Transactivation is shown as fold activation of luciferase expression over that of the reporter plasmids without E2, taken arbitarily as 1.

Dose dependent transactivation of HPV-18 LCR BS mutants and E2 minimal promoter constructs by HPV-16 E2 in HaCaT cells

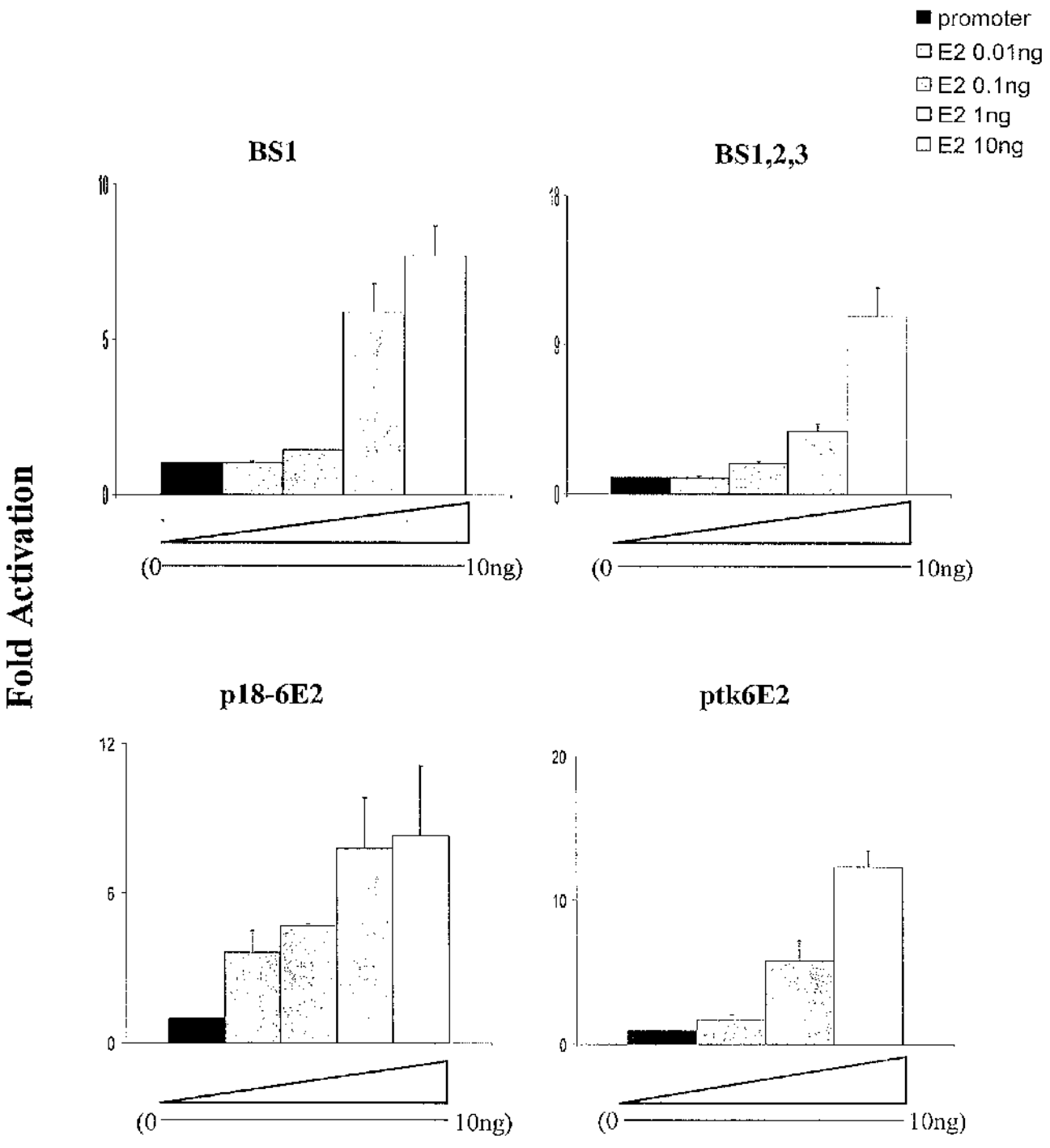


Figure 3.4. Transactivation of HPV-18 LCR BS mutants and E2 minimal promoter constructs by HPV-16 E2 in C33a cells. C33a cells were transiently transfected with 0.1μg of each luciferase reporter construct (BS1, BS1,2,3, p18-6E2, and ptk6E2) and with increasing amounts of the E2 expression plasmid pCMV-$E2_{16}$, (0.01ng, 0.1ng, 1ng and 10ng). Shown are representative results from each promoter in C33a cells. The total amount of DNA was kept constant by adding appropriate amounts of the parental plasmid pCMV. Each experiment was performed in triplicate. Transactivation is shown as fold activation of luciferase expression over that of the reporter plasmids without E2, taken arbitarily as 1.

Dose dependent transactivation of HPV-18 LCR BS mutants and E2 minimal promoter constructs by HPV-16 E2 in C33a cells

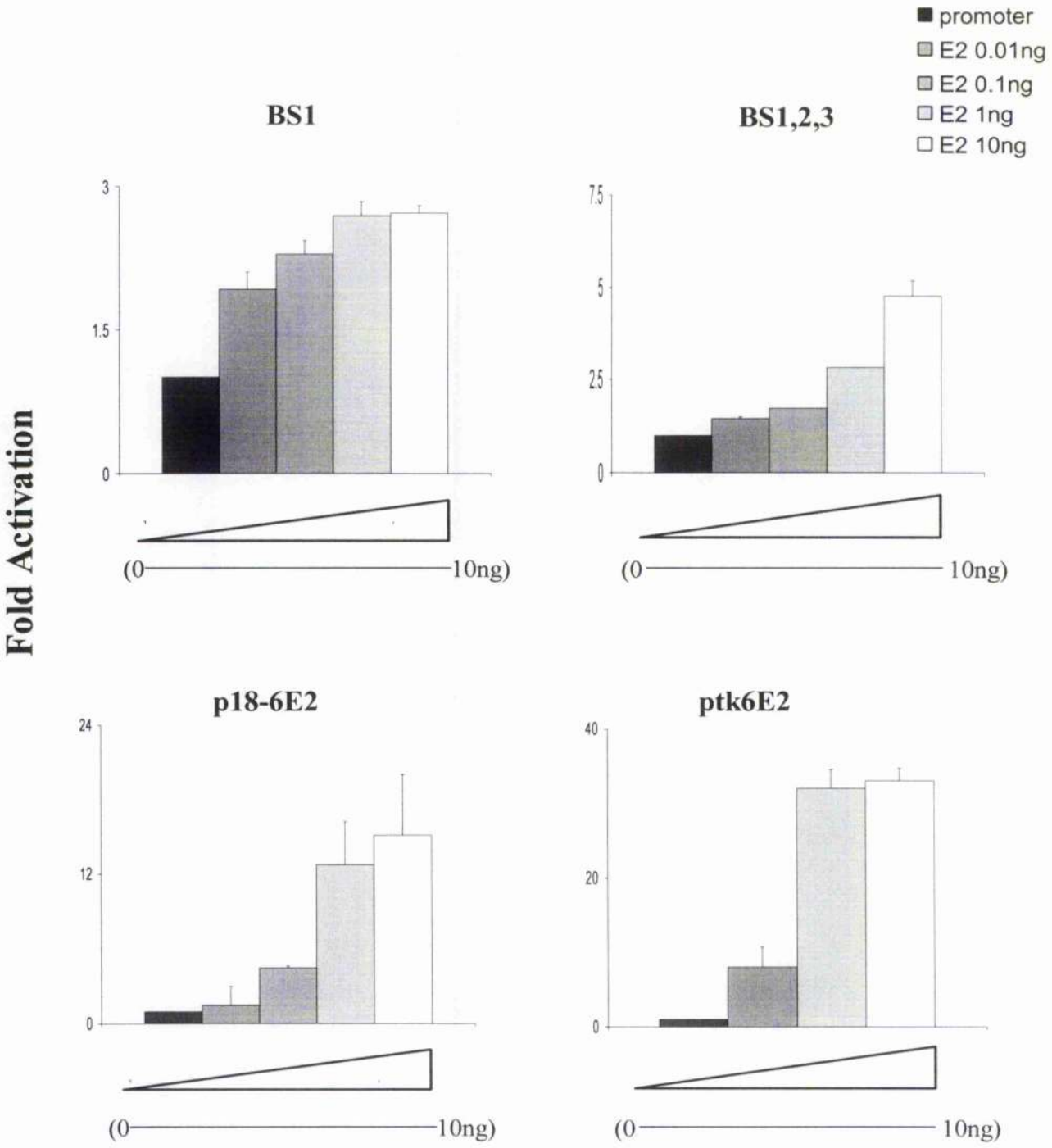


Figure 3.5. HPV-16 L2 down-regulates the transcription transactivation function of E2 in HaCaT cells. HaCaT cells were transiently co-transfected with 0.1μg of each luciferase reporter construct and 1ng pCMV-$E2_{16}$ with increasing amounts of p16-HAL2, (0.05μg, 0.1μg, 0.5μg and 1μg). Each experiment was adjusted for total DNA by co-transfecting with the parental plasmid pCMV. Shown are representative experiments for each promoter (a) BS1, (b) BS1,2,3, (c) p18-6E2 and (d) ptk6E2 in HaCaT cells. Each experiment was performed in triplicate. Transactivation is shown as fold activation of luciferase expression over that of the reporter plasmids without E2, taken arbitrarily as 1.

HPV-16 L2 down-regulates the transcription transactivation function of HPV-16 E2 in HaCaT cells

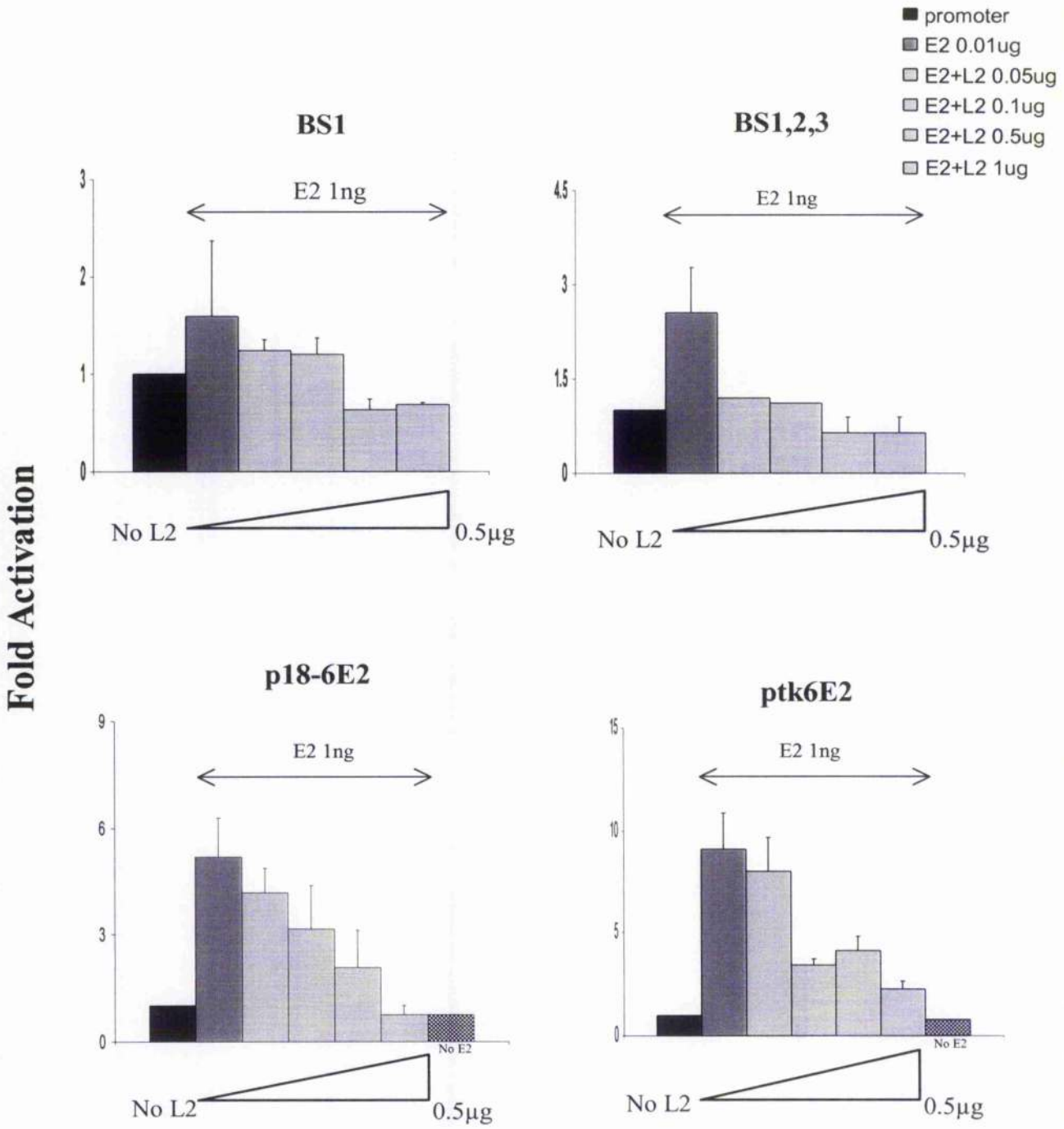


Figure 3.6. HPV-16 L2 down-regulates the transcription transactivation function of E2 in C33a cells. C33a cells were transiently co-transfected with 0.1μg of each luciferase reporter construct and 1ng pCMV-$E2_{16}$ with increasing amounts of p16-HAL2, (0.05μg, 0.1μg, 0.5μg and 1μg). Each experiment was adjusted for total DNA by co-transfecting with the parental plasmid pCMV. Shown are representative experiments for each promoter (a) BS1, (b) BS1,2,3, (c) p18-6E2 and (d) ptk6E2 in C33a cells. Each experiment was performed in triplicate. Transactivation is shown as fold activation of luciferase expression over that of the reporter plasmids without E2, taken arbitrarily as 1.

HPV-16 L2 down-regulates the transcription transactivation function of HPV-16 E2 in C33a cells

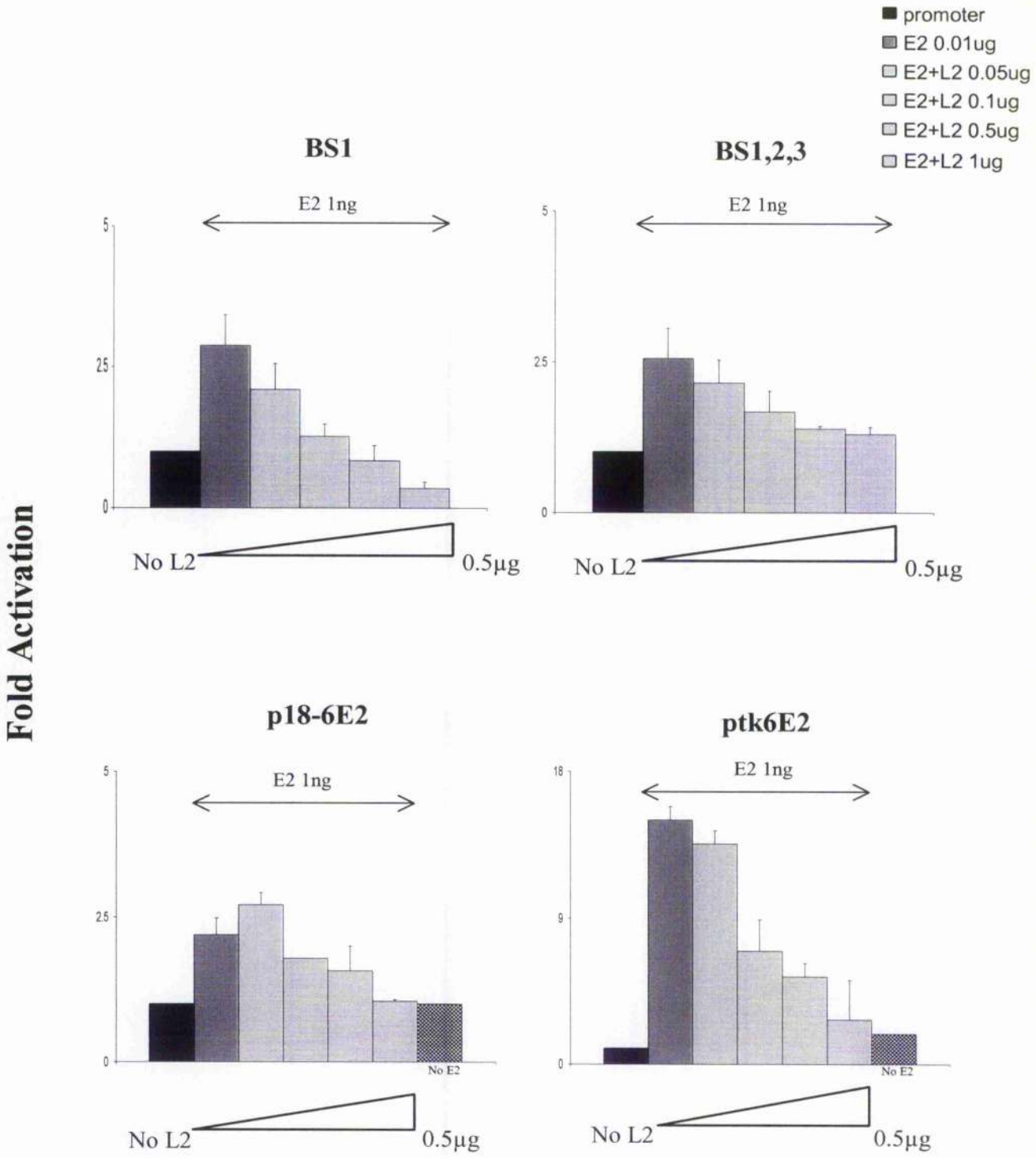


Figure 3.7. Effect of HPV-16 L2 on the transactivation of tk and tk6E2 promoters by HPV-16 E2 in C33a cells. Cells were transiently co-transfected with 0.1μg of each luciferase reporter construct and 1ng pCMV-$E2_{16}$ with increasing amounts of p16-HA L2, (0.1μg, 0.5μg and 1μg). The total amount of DNA was kept constant by adding appropriate amounts of the parental plasmid pCMV. Each experiment was performed in triplicate. Transactivation is shown as fold activation of luciferase expression over that of the reporter plasmids without E2, taken arbitarily as 1.

Co-expression of HPV-16 L2 and HPV-16 E2 does not affect the activity of the tk promoter

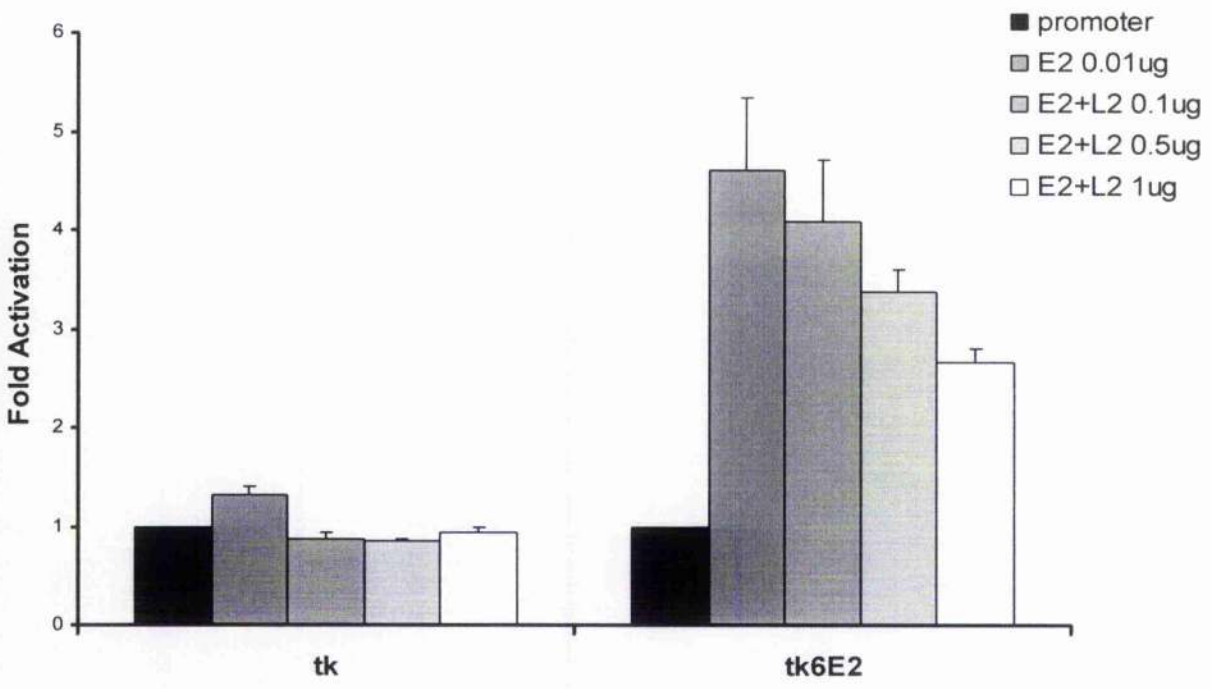
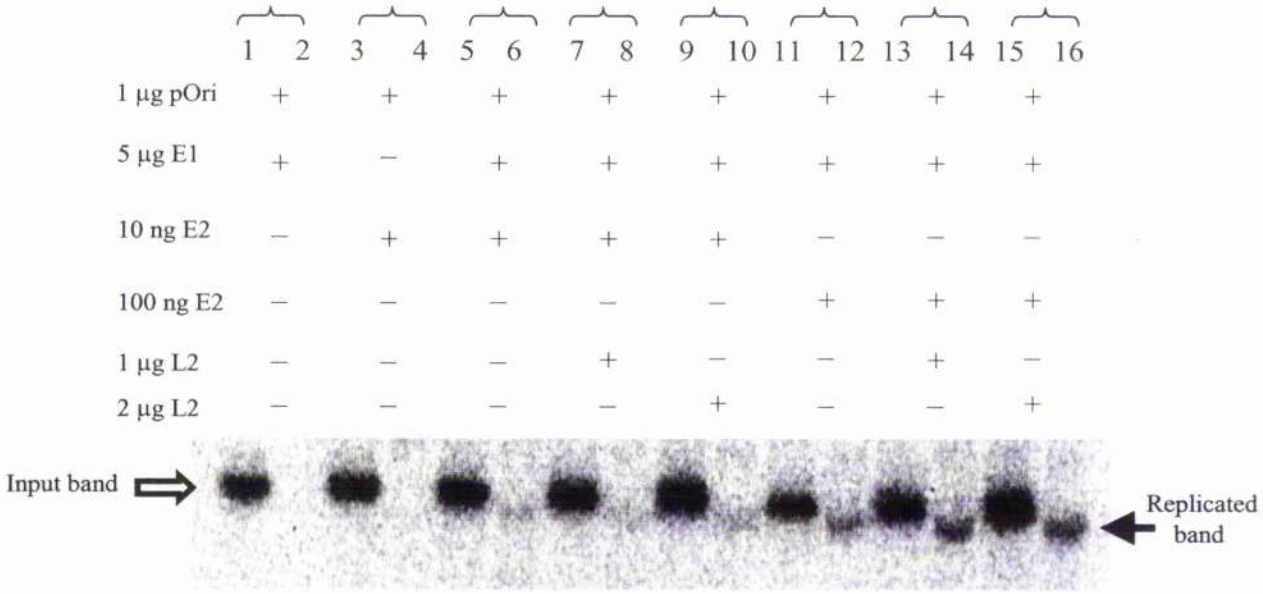


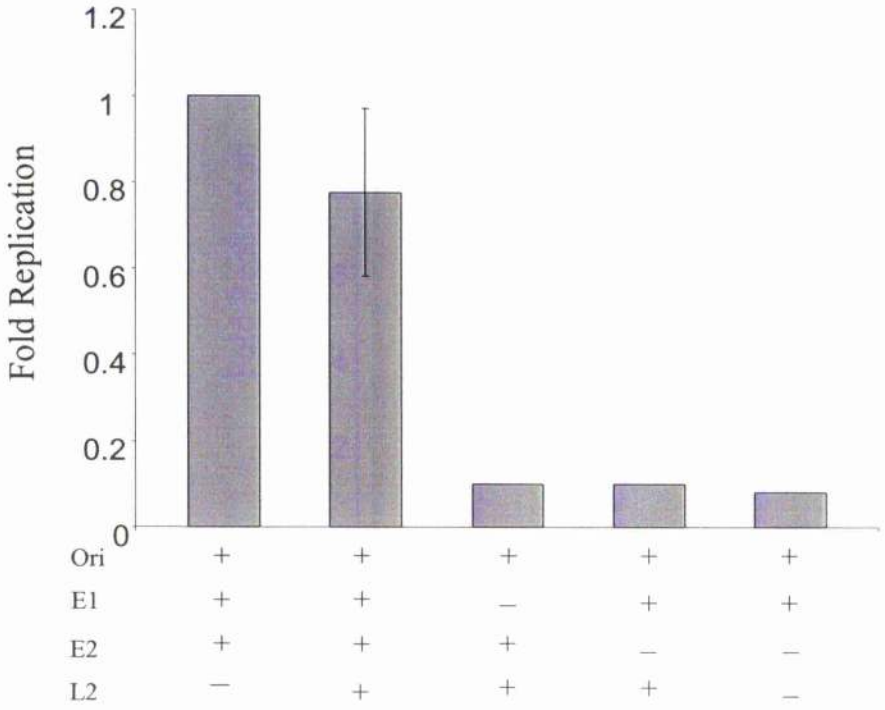
Figure 3.8. Effect of HPV-16 L2 on the transient replication function of HPV-16 E2 in C33a cells. (A) Phosphoimage of Southern blot hybridised with ^{32}P-labelled HPV-16 ori plasmid. Cells were transfected with 1μg of p16ori-m, +/- 5μg pCMV-E1$_{16}$, +/- 10ng or 100ng pCMV-E2$_{16}$ and +/- 1μg or 2μg p16-HAL2. For each reaction, Xnm1 was used to linearise p16ori-m (odd-numbered lanes), and then Dpn1 was used to digest replicated DNA (even-numbered lanes). The open arrow on the left indicates unreplicated plasmid and the closed arrow on the right indicates replicated plasmid. Replication occurred in the presence of 10ng or 100ng of pCMV-E2$_{16}$ co-transfected with 1μg p16ori-m and 5μg pCMV-E1$_{16}$ (lanes 6 and 12). The absence of either E1 (lane 4) or E2 (lane 2) resulted in no detectable replication. The experiment was carried out three times with essentially the same results. (B) Quantification of DNA replication. Southern blots were scanned and extent of DNA replication was calculated by measuring the ratio of double cut/single cut bands, thus controlling for variations between experiments. The graph represents a summary of three independent experiments +/- standard deviation.

HPV-16 L2 does not affect the DNA replication function of HPV-16 E2

3.8a



3.8b



CHAPTER 4: THE EFFECT OF L2 ON E2 PROTEIN EXPRESSION AND STABILITY

4.1. E2 expression and stability

Proteins utilize a variety of protease pathways for their destruction. These include non-specific lysosymal protease; proteases involved with apoptosis such as caspase and calpains; and the ubiquitin-dependent proteasome pathway. The degradation of BPV-1 E2 is regulated by phosphorylation, which is required for efficient ubiquitination and subsequent degradation of E2 by the ubiquitin-proteasome pathway (Penrose and McBride, 2000). The ubiquitin-proteasome degradation pathway also controls the stability of HPV-18 E2 mediated through its amino-terminal transactivation domain (Bellanger *et al.*, 2001). The full-length or transactivation domain of E2 is efficiently ubiquinated and proteasome inhibition in cells expressing E2 increases its half-life about sevenfold. As the transcription transactivation function of E2 is down regulated in the presence of L2, the effect of L2 on E2 expression was investigated.

4.1.1. Down-regulation of HPV-16E2 protein expression by HPV-16 L2 in HaCaT and U2OS but not C33a cells

HPV-16 L2 down regulates HPV-16 E2-mediated transcription transactivation in a dose dependent manner. To determine whether the effect of L2 on E2 function in transcription transactivation was due to the degradation of E2, HaCaT, U2OS and C33a cell lines were transfected with 4μg pCMV-$E2_{16}$ alone or 4μg of pCMV-$E2_{16}$ and 4μg p16-HAL2 expression plasmids and detection of E2 levels was accomplished by Western blotting. In

HaCaT cells, the presence of L2 lead to a marked reduction in the level of the E2 protein (Fig 4.1a). In U2OS cells, the level of E2 expression in the presence of L2 was also drastically reduced when compared to cells expressing E2 alone (Fig 4.1c). In contrast, co-expression of E2 and L2 in C33a cells resulted in no marked reduction in the level of the E2 protein (Fig 4.1d). The effect of L2 on E2 protein levels in HaCaT cells was further investigated using a stable E2 expression system established in HaCaT cells. This system provided a functional and detectable level of E2 in HaCaT cells. Transfection of HaCaT-E2 cells with 4μg p16-HAL2 lead to a marked reduction in the level of the E2 with actin levels remaining unchanged (Fig 4.1b). These results indicate that the effect of L2 on E2 levels is a cell type dependent effect.

As the degradation of E2 is proteasome mediated (Bellanger *et al.*, 2001; Penrose and McBride, 2000), the down-regulation of E2 protein expression by L2 was further investigated to determine whether this effect was proteasome dependent. A variety of proteasome inhibitors were used, which have been shown to inhibit proteasome-mediated degradation of E2 (Penrose and McBride, 2000). HaCaT and C33a cells were transfected with 4μg pCMV-$E2_{16}$ alone or 4μg of pCMV-$E2_{16}$ and 4μg p16-HAL2. The cells were then treated 24h post transfection with each proteasome inhibitor for 8h. The inhibitors used were 5μM MG132, 12.5μM Lactacystin, 100μM ALLN (calpain inhibitor I) and 100μM ALLM (calpain inhibitor II) and detection of E2 levels was accomplished by Western blotting. In untreated HaCaT cells, the presence of L2 lead to an ~75% reduction in E2 level while no reduction was observed in C33a cells. Treatment with all proteaome inhibitors led a 30% increase in E2 protein levels in both cell lines transfected with E2

alone. In the presence of L2, treatment with all proteasome inhibitors did not restore the level of E2 in HaCaT cells expressing both E2 and L2 (Fig 4.2). In contrast, in C33a cells, the inhibitors increased the amount of E2 present in cells expressing both E2 and L2 to similar levels as cells expressing E2 alone (Fig 4.2). These results conclude that the proteasome degradation pathway is not involved in L2 mediated decrease in E2 protein levels as proteasome inhibition fails to restore E2 levels in the presence of L2. This leads to the possibility that the effect of L2 could be acting at the level of protein transcription.

4.1.2 L2 does not alter E2 mRNA transcription.

To ascertain whether the decrease in E2 protein in HaCaT cells expressing L2 was due at least in part to inhibition of E2 mRNA sysnthesis, HaCaT cells were transfected with 4µg pCMV-$E2_{16}$ +/- 4µg p16-HAL2 and total RNA isolated. The RNA preparations were analysed by comparing RNA extracted from cells expressing E2 or co-expressing E2 and L2 after RT-PCR. The results indicated that there was no difference in the level of E2 in cells expressing E2 alone compared with cells expressing both E2 and L2 indicating that L2-induced decrease in E2 protein level is a post-transcriptional process (Fig 4.3a).

A Real Time Quantitative PCR was also used to investigate differences in E2 mRNA synthesis due to co-expression of E2 with L2. HaCaT cells were seeded and transfected with 4µg pCMV-$E2_{16}$ +/- 4µg p16-HAL2, and total RNA was isolated and resuspended in RNase-free water. The results indicated that there were no differences in E2 mRNA levels in cells transfected with pCMV-$E2_{16}$ or pCMV-$E2_{16}$ and p16-HAL2, as all the amplification curves were coincident (Fig 4.3b). As E2 mRNA levels remain the same in

the presence or absence of L2, these results indicate that the effect of L2 on E2 protein expression is not mediated by transcription inhibition.

4.1.3. L2 down-regulation of the transcriptional activity of E2 is proteasome independent

To further investigate if the effect of L2 on the transcription transactivation function of E2 could be overcome by inhibition of E2 degradation mediated through the proteasome, C33a cells were transfected with p18-6E2, ptk6E2, pCMV-$E2_{16}$ and increasing amounts of p16-HAL2 and treated with the proteasome inhibitor MG132. The amount of pCMV-$E2_{16}$ was kept sub-optimal for E2-mediated transactivation. For both promoters used, inhibition of E2 degradation had no effect on the down-regulation of E2 transcription transactivation by L2 as the presence or absence of MG132 resulted in similar kinetics of inhibition of transcription transactivation (Fig 4.4). This further confirms that the down regulation of transcription transactivation function of E2 by L2 is not due to E2 protein degradation.

4.2. L2 does not affect E2 half-life/stability

Biosynthetic labelling techniques are commonly used in the study of biochemical properties, synthesis, processing, intracellular transport, secretion and degradation of proteins. To investigate the effect of L2 on E2 protein turnover, the half-life of E2 was measured by pulse-chase analysis in HaCaT cells. HaCaT cells were transfected with pCMV-$E2_{16}$, +/- p16-HAL2 and labelled with [^{35}S]methionine for 1hr. The labelling medium was removed and samples extracted at hourly time points up to 6h. E2 was immunoprecipitated and analysed by SDS PAGE and autoradiography. A graphical

representation of the experiment shown in Fig 4.5, illustrates that E2 had a half-life of ~75 minutes with ~60% degradation observed after 2 hour. In HaCaT cells co-transfected with both pCMV-$E2_{16}$ and p16-HAL2, the level of residual E2 was lower than in cells transfected with only pCMV-$E2_{16}$ but the half-life remained similar (Fig 4.5). The use of pulse-chase analysis to observe E2 half-life in the presence of L2, results in ^{35}S labelled E2 being affected by a continuous expression of L2. The presence of L2 did not affect the level of E2 in cells expressing both E2 and L2 when compared to cells expressing E2 alone.

To further investigate the effect of L2 on E2 half-life, cycloheximide (CHX), which shuts down protein translation, was used to treat HaCaT cells. As CHX shuts down protein translation, the effect of L2 on E2 will be as a result of the presence of residual L2 left after protein translation has been shut off. HaCaT cells were transfected with pCMV-$E2_{16}$ or pCMV-$E2_{16}$ and p16-HAL2. CHX was added and cells were harvested over an hourly time course up to 8 hours. The E2 protein was detected using Western blotting analysed by SDS PAGE. Dimethyl sulfoxide (DMSO) the solvent for CHX was used as a control. A graphical representation of the experiment shown in Fig 4.6, illustrates that E2 had a half-life of ~110 minutes with ~60% degradation also observed after 2 hour (Fig 4.6). The half-life of E2 was slightly longer than the half-life seen with the pulse-chase experiment. In both experiments, the results also did not indicate a difference in E2 half-life in the presence of L2.

Although there were slight differences in the measurement of protein turnover, both experiments indicated that L2 had no effect on the half-life of E2 in HaCaT cells. The reason why no difference in E2 half-life was detected is unclear. In both experiments L2 reduced the level of E2 expressed and although less E2 was present, this residual E2 had no change in its half-life. These results suggest that L2 may act on E2 mRNA translation.

Figure 4.1. HPV-16 L2 down-regulates E2 in HaCaT but not in C33a cells. (A), HaCaT cells were transfected with 4μg pCMV-$E2_{16}$ (lane 1), or 4μg pCMV-$E2_{16}$ plus 4μg p16-HAL2 (lane 2), or with 4μg p16-HAL2 alone (lane 3). (B), HaCaT cells stably expressing E2 (lane 1) were transfected with 4μg p16-HAL2 (lane 2). (C) U2OS cells were transfected with 4μg pCMV-$E2_{16}$ (lane 1), or 4μg pCMV-$E2_{16}$ plus 4μg p16-HAL2 (lane 2). (D), C33a cells were transfected with 4μg pCMV-$E2_{16}$ (lane 1), or 4μg pCMV-$E2_{16}$ plus 4μg p16-HAL2 (lane 2). Cell were harvested using SDS-PAGE lysis buffer and extracts were analysed in independent Western blots with mouse anti-HA monoclonal antibody (HA.11) to probe for HA-L2, anti-E2 mouse monoclonal antibody TVG261 and mouse anti-actin antibodies.

HPV-16 L2 down-regulates E2 in HaCaT and U2OS but not C33a cells.

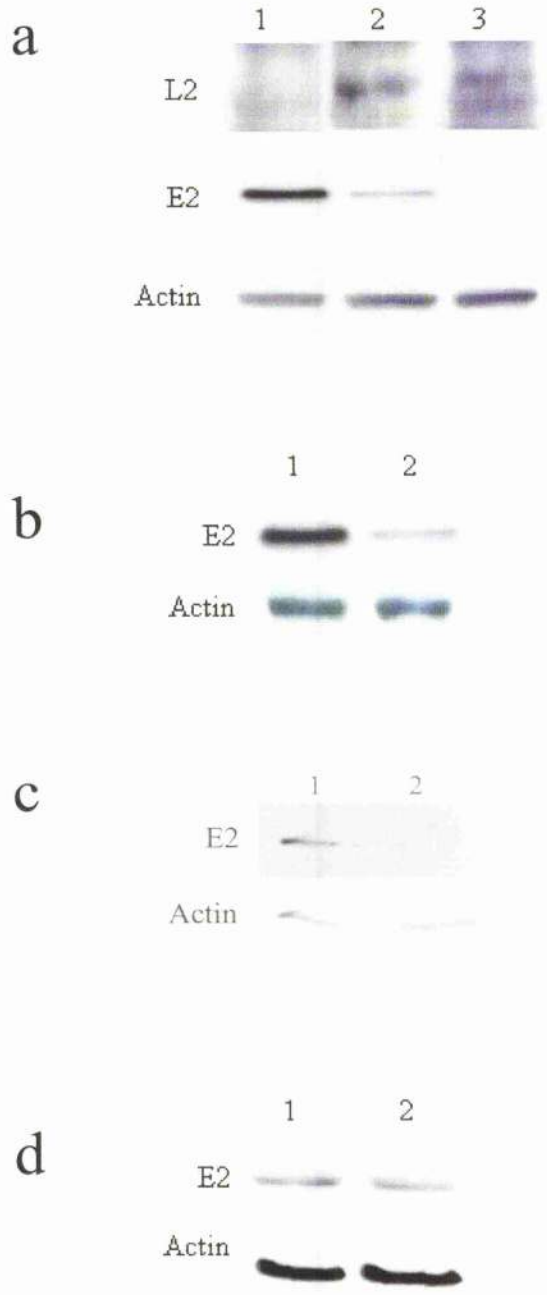


Figure 4.2. HPV-16 L2 decreases the level of E2 in HaCaT cells in a proteasome independent manner. HaCaT cells or C33a cells were transiently transfected with 4µg pCMV-$E2_{16}$ alone ("-" lanes) or with 4µg pCMV-$E2_{16}$ and 4µg p16-HAL2 ("+" lanes). After 24 hours the transfected cells were either treated for 8 h with the indicated proteasome inhibitors or kept untreated. All cells were harvested using SDS-PAGE lysis buffer and the different cell extracts were analysed by Western blot and revealed by anti-E2 mouse monoclonal antibody TVG261 and mouse anti-actin antibodies.

HPV-16 L2 decreases the level of E2 in HaCaT cells in a proteasome independent manner.

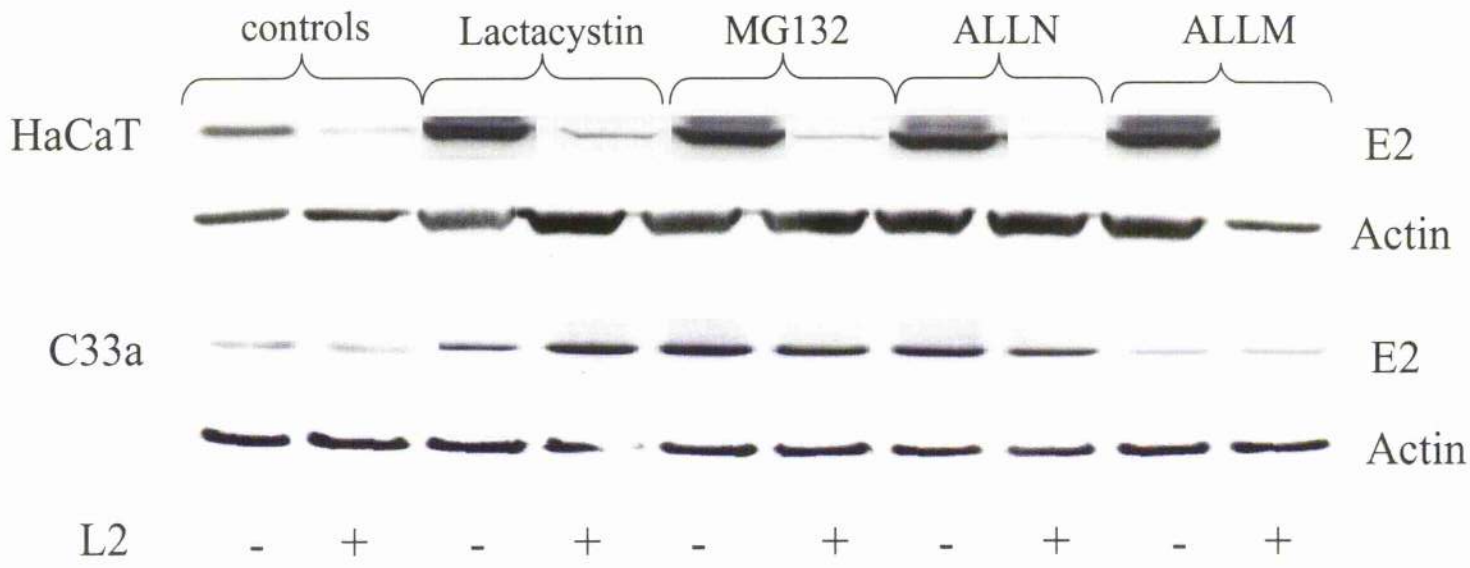
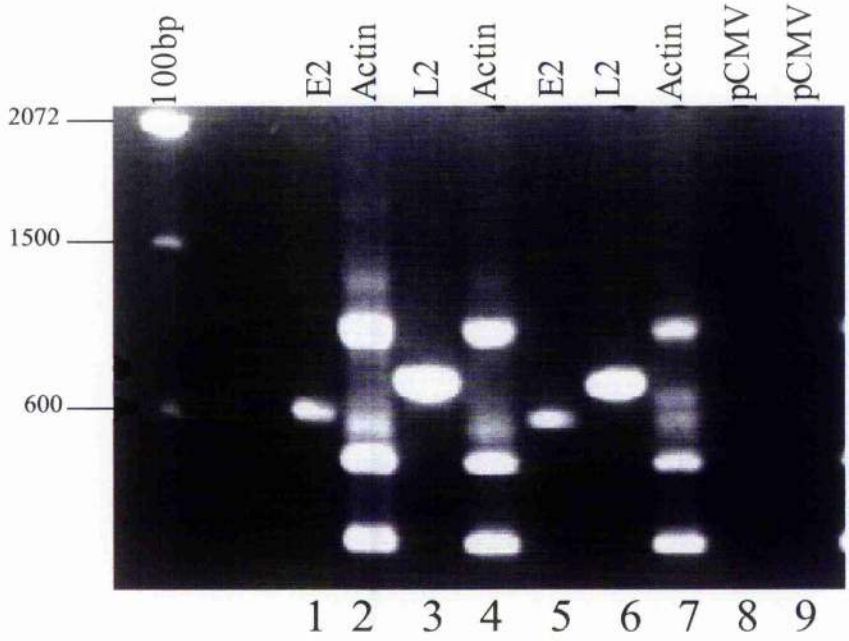


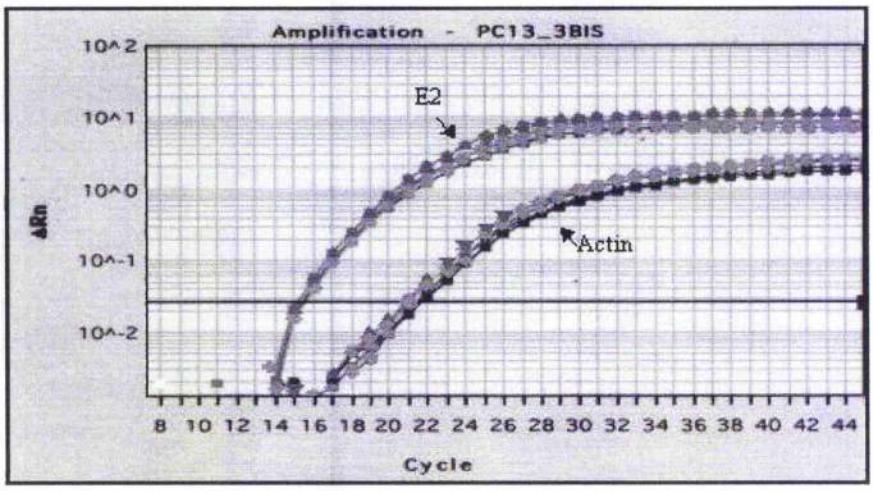
Figure 4.3. HPV-16 L2 does not alter E2 mRNA transcription. (A) Total RNA from HaCaT cells transfected with pCMV-$E2_{16}$ alone or with pCMV-$E2_{16}$ and p16-HAL2 After RNA extraction and DNase treament, the extracted RNA was reverse transcribed to cDNA and PCR was performed using E2 and L2 primers. Lanes 1 and 2 show RNA from HaCaT transfected with E2 alone, and actin control respectively. Lanes 3 and 4 show RNA from cells transfected with L2 alone and actin control respectively. Lanes 5, 6 and 7 show RNA from cells transfected with E2 and L2 as well as the actin control respectively. Lanes 8 and 9 show RNA from cells transfected with pCMV, which did not yield cDNA using the E2 and L2 primers. The actin control in lanes 2, 4 and 7 show the three actin isotypes (alpha, beta and gamma). All three isotypes were detected with the actin primers used. **(B)** Total RNA from HaCaT cells transfected with pCMV-$E2_{16}$ alone or with pCMV-$E2_{16}$ and p16-HAL2 was digested with DNAse and reverse transcribed with random primers. Real time PCR was performed in triplicate with primers and probes for E2 and actin. There was no difference in E2 transcription in the presence or absence of L2, and all the amplification curves were coincident.

HPV-16 L2 does not alter HPV-16 E2 mRNA transcription.

a



b



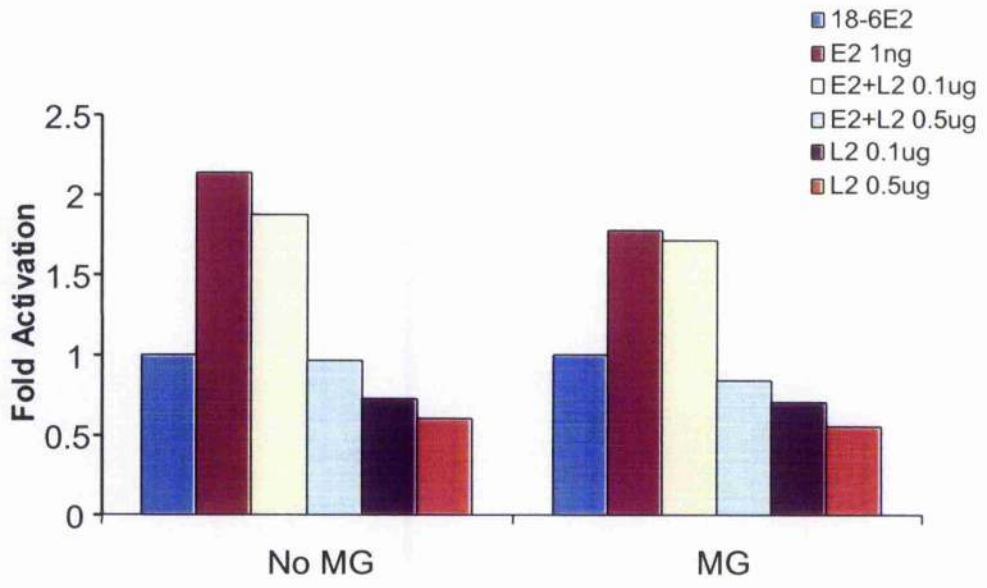
▼, ▲, ● : HaCat cells transfected with E2

■, ◆, ◆ : HaCat cells transfected with E2+L2

Figure 4.4. HPV-16 L2 down-regulates the transcription transactivation function of E2 in cells is independent of E2 degradation. C33a cells were transiently co-transfected with 0.1μg of luciferase reporter construct and 1ng pCMV-$E2_{16}$ with increasing amounts of p16-HAL2, (0.05μg, 0.1μg, 0.5μg and 1μg). Each experiment was adjusted for total DNA by co-transfecting with the parental plasmid pCMV. The experiment was conducted in the presence or absence of MG132 treatment for 8 hours post-transfection. Shown are representative experiments for each promoter (a) p18-6E2 (b) ptk6E2 in C33a cells. Transactivation is shown as fold activation of luciferase expression over that of the reporter plasmids without E2, taken arbitrarily as 1.

MG132 does not alter HPV-16 L2 down regulation of HPV-16 E2 transactivation

a



b

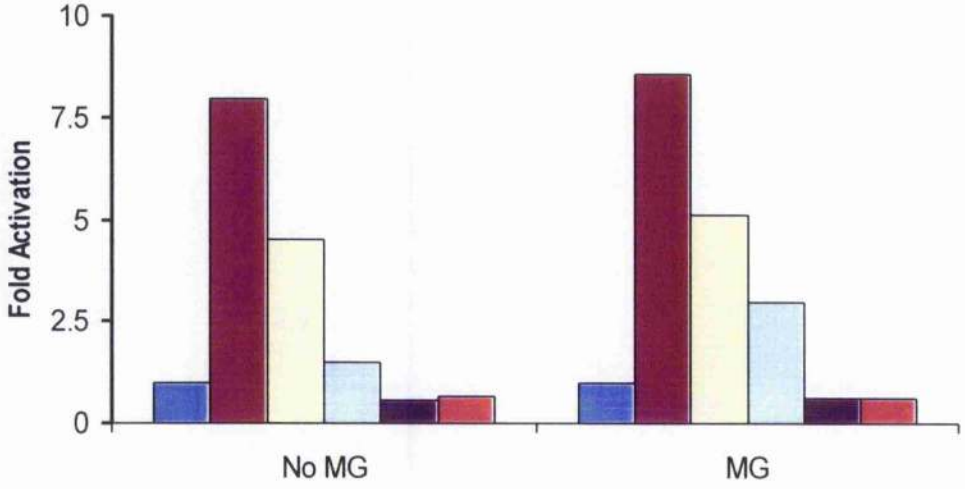
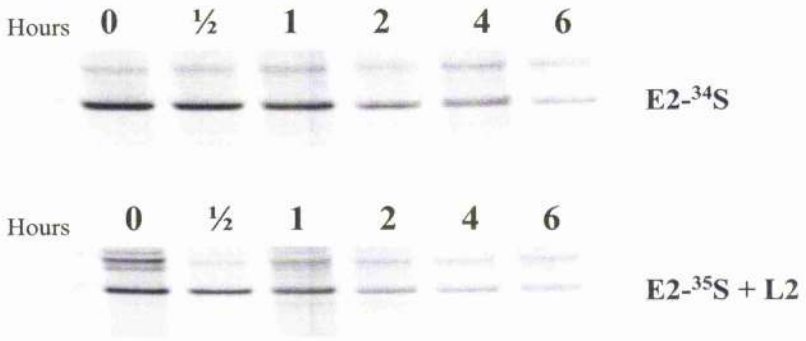


Figure 4.5. The effect of HPV-16 L2 on the stability of HPV-16 E2 using ^{35}S methionine labelling in HaCaT cells. HaCaT cells were transiently co-transfected with 4μg of pCMV-$E2_{16}$ or 4μg of pCMV-$E2_{16}$ and 4μg of p16-HAL2. Twenty-four hours post transfection, the cells were incubated with [^{35}S] methionine (0.2μCi/ml) for 1 hour at 37^0C, 5% CO_2 to pulse cells. To detect protein half-life, cells were harvested using NP-40 lysis buffer over a six-hour time course. Detection of ^{35}S-labelled E2 was achieved by immunoprecipitation with anti-E2 polyclonal antibody and exposed by autoradiography (a). Densitometry was carried out on bands and shown by graphical representation (b).

HPV-16 L2 does not alter the stability of HPV-16 E2 after 35^S-methionine labelling

a



b

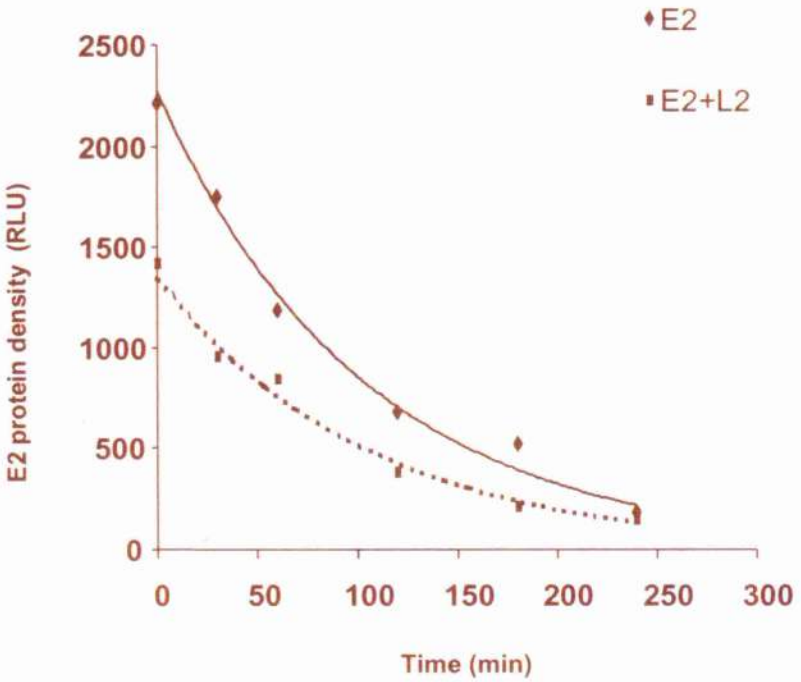
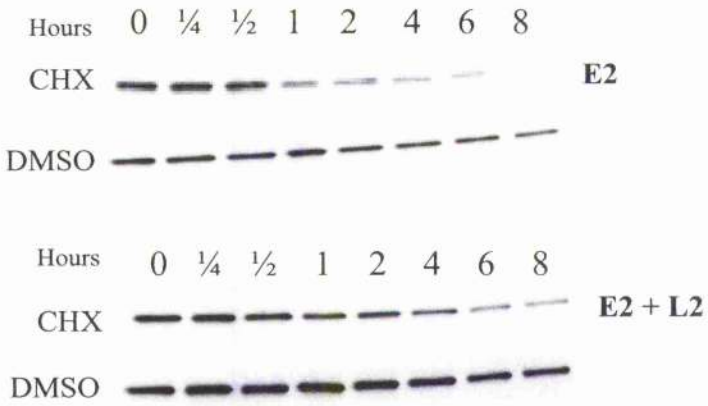


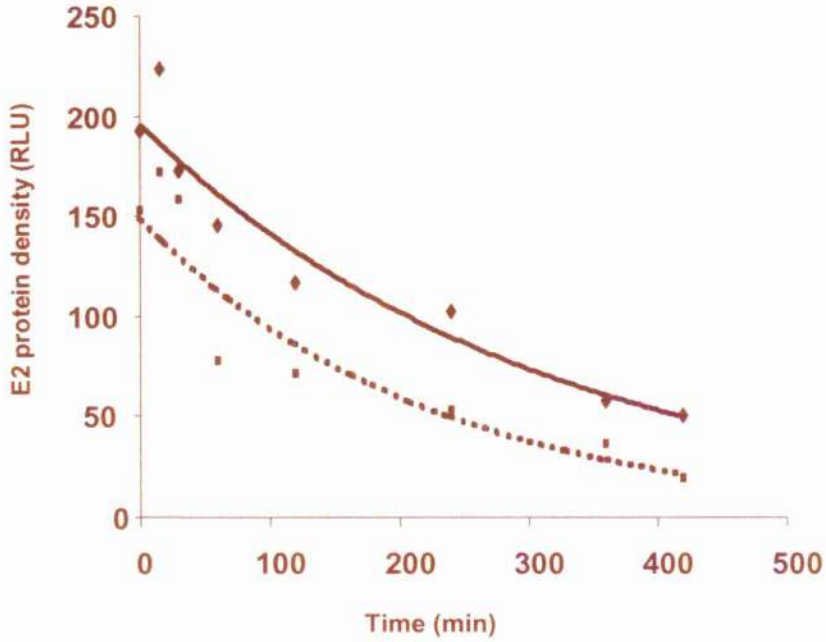
Figure 4.6. The effect of HPV-16 L2 on the stability of HPV-16 E2 after cycloheximide treatment in HaCaT cells. HaCaT cells were transiently co-transfected with 4μg of pCMV-$E2_{16}$ or 4μg of pCMV-$E2_{16}$ and 4μg of p16-HAL2. Twenty four hours post transfection, cells were treated with 100μg/ml CHX in DMSO and DMSO only used as a negative control. Cells were harvested using SDS-PAGE lysis buffer at regular time intervals after CHX/DMSO addition for up to 8h. Samples were immunoblotted and samples probed for E2 by anti-E2 mouse monoclonal antibody TVG261 (a). Densitometry was carried out on bands and shown by graphical representation (b).

HPV-16 L2 does not alter the stability of HPV-16 E2 after cyclohexamide treatment

a



b



CHAPTER 5: ANALYSIS OF HPV-16 L2 DELETION MUTANTS

5.1 Introduction

The interaction of HPV-16 L2 with HPV-16 E2 has been shown to result in the down regulation of the transcription transactivation function of E2 as well as a proteasome independent down-regulation of E2 expression in a cell dependent manner, with no effect on E2 mRNA synthesis. To determine the domain or domains of L2 responsible for the alteration of E2 transcription transactivation or expression, deletion mutants of L2 (Fig 5.1) were constructed. An *in vitro* protein-protein association assay was used to determine the ability of L2 and L2 deletion mutants to bind with E2. GFP fusion forms of each L2 deletion mutant were also constructed to determine cellular localisation and luciferase transcription assays were performed to identify domain or domains of L2 responsible for the down-regulation of E2 transcription transactivation.

5.2. Identification of E2-binding domains of L2

5.2.1. GST Gene Fusion System

Glutathione S-transferase is a naturally occurring enzyme that can be expressed in *E. coli* with full enzymatic activity. The system has been widely used for the expression, purification and detection of fusion proteins expressed in bacteria. Fusion proteins with the GST moiety at the amino terminus and the protein of interest at the carboxyl terminus are expressed in bacteria. The protein accumulates within the cytoplasm and can be purified by affinity chromatography using immobilised glutathione. The HPV-16 L2 N-

and C- terminus deletion mutants were constructed by inserting the deleted L2 sequences as a BamHI-EcoR1 insert within the multiple cloning site of the pGEX-4T-2 vector. Expression of the fused protein is under the control of the tac promoter which can be induced by the addition of isopropyl β-D thiogalactosidase (IPTG). The bacterial host strain used was *E. coli* BL21, a strain defective in OmpT and Lon protease production, designed to maximize expression of full-length fusion proteins by minimizing the effects of proteolytic degradation.

5.2.2. Construction of GST-L2 deletion mutants

The PCR amplified inserts from HPV-16 L2 deletion mutants were cloned into pGEX-4T-2 expression plasmid (Fig 5.2). L2 was amplified by PCR as a BamH1-EcoR1 fragment and inserted into pGEX-4T-2. The restriction sites chosen ensured that all proteins would be inserted in frame. All mutants were expressed as fusion proteins with the GST moiety at the amino terminus and L2 or its deletion mutants at the carboxyl terminus. For the C-terminus L2 deletion mutants, the primer *HPV-16 L2 BamH1 forward* was used for all forward reactions while the primers *GST L2 1-50 (or 1-100, 1-200,1-300,1-400) reverse* (Table 2.1) were used for the reverse reaction. For cloning of the amino terminus deletion mutants, the primer *HPV-16 L2 EcoR1 reverse* was used for all reverse reactions while the primers *GST-L2 25-473 (or 50-473,150-473,250-473,350-473,390-473) forward* (Table 2.1) were used for all forward reactions. For sequencing of GST-L2 deletion mutants, the following sequencing primers 5'-GGGCTGGCAAGCCACGTTTGGTG-3' forward corresponding to position 869-891 on the pGEX-4T-2 plasmid and 5'-CCGGGAGCTGCATGTGTCAGAGG-3' reverse corresponding to position 1042-1020 were designed. Sequencing showed L2 and the L2

deletion mutants all fused in frame with the GST protein. Restriction digest were also performed to confirm that all L2 deletion inserts were of the correct size (Fig 5.3). The amino acid sequences of each L2 deletion mutant (Fig 5.1) were as follows:-

1. HPV-16 L2 amino acids 1-50
2. HPV-16 L2 amino acids 1-100
3. HPV-16 L2 amino acids 1-200
4. HPV-16 L2 amino acids 1-300
5. HPV-16 L2 amino acids 1-400
6. HPV-16 L2 amino acids 25-473
7. HPV-16 L2 amino acids 50-473
8. HPV-16 L2 amino acids 150-473
9. HPV-16 L2 amino acids 250-473
10. HPV-16 L2 amino acids 350-473
11. HPV-16 L2 amino acids 390-473

5.2.3. The amino terminus of L2 mediates binding with E2

GST pull-down assays were performed to determine the domain or domains of L2 that mediate binding to E2. L2 and L2 deletion mutants were cloned into pGEX-4T-2 as described above. Bacteria were transformed with GST-L2 and each GST-L2 deletion mutants and grown at 37°C for 5 hours after induction with 0.5mM IPTG. Bacteria were harvested, lysed and cell extracts were incubated with glutathione beads, which immobilises GST fusion proteins. Expression of GST-L2 and GST-L2 deletion mutants was analysed by SDS-PAGE and Western blotting. Immunoblotting with anti-GST

antibody to detect expression levels of the GST-L2 deletion mutants showed that GST-L2 and GST-L2 C-terminal deletion mutants all gave the expected migration on polyacrylamide gels (Fig 5.4). N-terminal deletion mutants were also detected and the expected migration was also observed, although with multiple banding indicating increased instability of mutants with deletions in the N terminus.

Bacterially expressed GST-L2 and GST-L2 deletion mutants were immobilised onto glutathione beads and incubated with ^{35}S-methionine labelled HPV-16 E2. The result indicated that E2 interacts with GST-L2, with approximately 5% of input E2 bound to GST-L2 (Fig 5.4). The binding observed between E2 and GST alone was negligible when compared to binding of E2 with GST-L2. GST-L2 N-terminal and C-terminal deletion mutants were also bacterially expressed, immobilised on glutathione beads and incubated with ^{35}S labelled E2. The mutant GST-L2 1-50 showed strong binding with E2 (Fig 5.4a), all C-terminal mutants also showed binding with E2. The N-terminal deletion mutants all showed weak interaction with E2 indicating the loss of possible binding domains in the amino terminal portion of L2. The specificity of the interaction between E2 and L2 was confirmed the binding of ^{35}S labelled L2 with GST-E2 immobilised on beads. ^{35}S-L2 did not bind to bead immobilised GST. An unrelated protein ^{35}S labelled luciferase did not bind to bead immobilised GST-E2 (Fig 5.4c).

To quantify the binding of GST-L2 and its deletion mutants with E2, the amount of ^{35}-S E2 bound to each fusion protein immobilised onto beads was measured by autoradiography. These values were then normalised by dividing them by the mount of

GST-L2 proteins immobilised on beads. The latter was measured with the use of image analysis software by measuring bands of each fusion protein after SDS-PAGE and immunoblotting. The results indicated that binding of the first 50 N-terminal amino acids of L2 with E2 gave binding at levels observed for full-length L2. Mutant GST-L2 1-300 showed a weak interaction with E2 probably as a result of conformational changes in the protein (Fig 5.5). The GST-L2 C-terminal deletion mutants did not bind E2 to levels observed with binding with the full-length or N-terminal deletion mutants possibly as a result of loss of E2 binding domains.

The analysis of the expression of GST-L2 deletion mutants by SDS-PAGE western blotting with anti-GST antibody showed that GST-L2 and GST-L2 deletion mutants had undergone degradation, as several bands were present, in addition to the expected bands. This degradation prevents accurate quantification of the amount of E2 bound by the L2 mutants, as the quantification method used does not take into consideration any E2 binding by the GST-L2 breakdown product. Therefore the amount of E2 bound by L2 in fig 5.5 may be an underestimate. Nevertheless, the use of GST-pull down assays does give an indication of HPV-16 E2-L2 interaction in vitro as has previously been observed for BPV-1 (Heino *et al.*, 2000). The results lead us to conclude that the interaction of L2 with E2 is mediated through the amino terminus of L2.

Figure 5.1. Schematic representation of HPV-16 L2 deletion mutants. L2 N-terminus and C-terminus deletion mutants were generated by PCR from a HPV-16 genome containing plasmid.

Structure of HPV-16 L2 deletion mutants

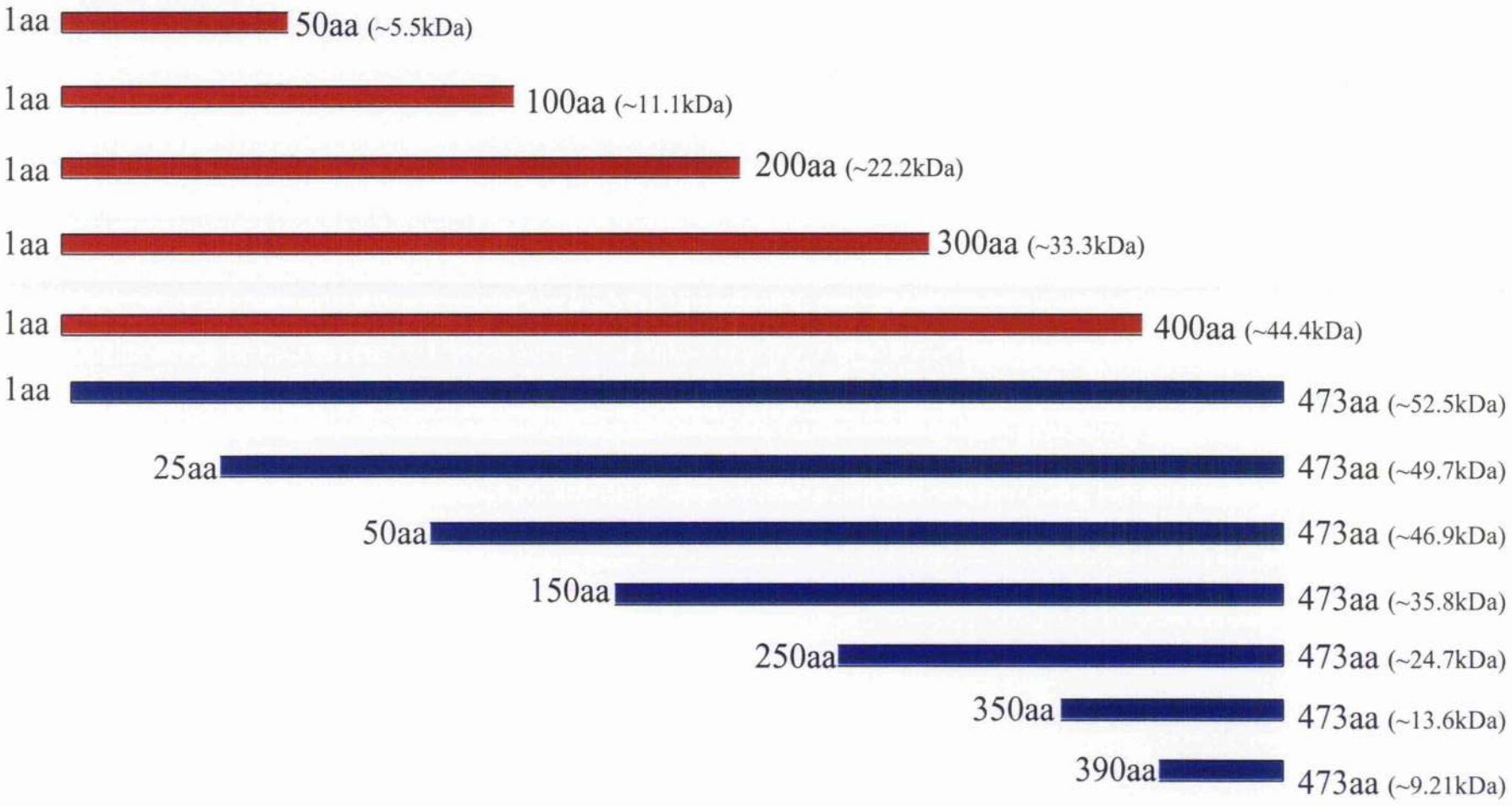


Figure 5.2. Graphical representation of pGEX-4T-2. The gene of interest is inserted into the multiple cloning site (MCS) of the vector and expressed as a fusion to the carboxyl terminus of GST. Expression of the fused protein is under the control of the tac promoter which can be induced by the addition of IPTG.

Graphical representation of pGEX-4T-2 vector and multiple cloning site.

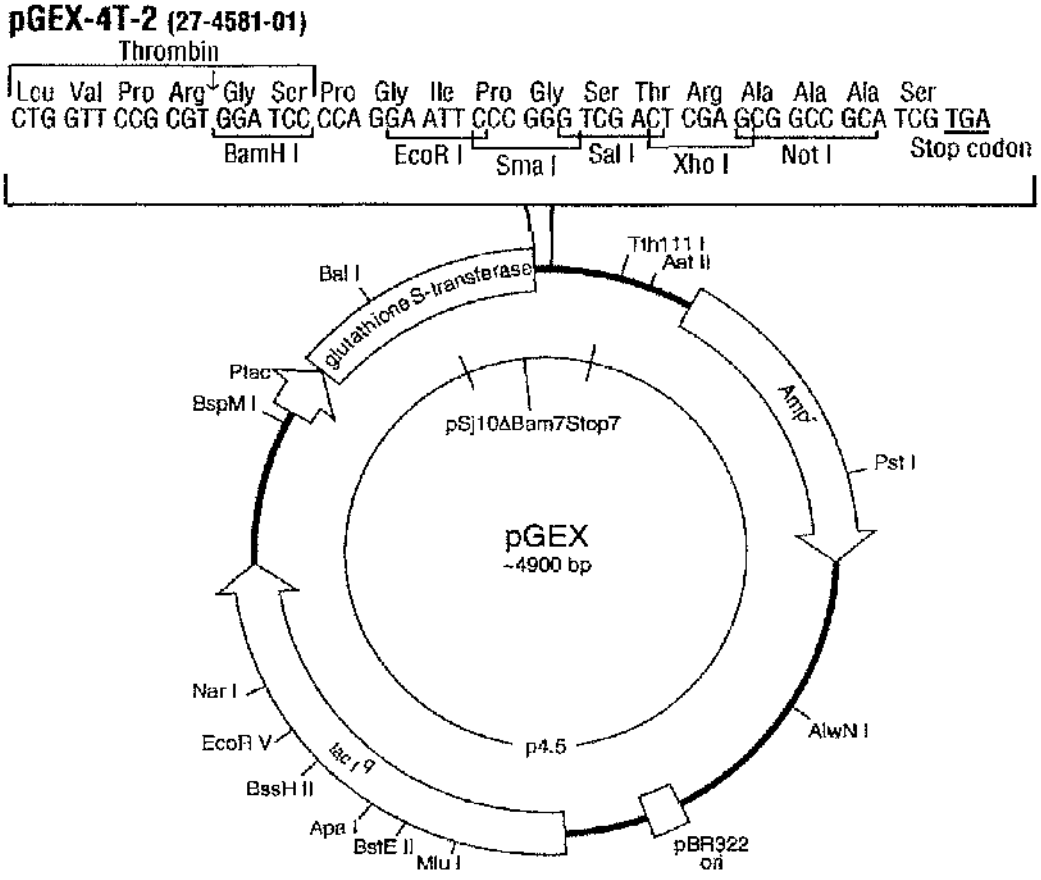


Figure 5.3. Digestion of GST-L2 N-terminal and C-terminal deletion mutants. 1μg of each plasmid was digested for 1h with BamH1-EcoR1 and analysed using agarose gel electrophoresis and observed under UV light. All inserts gave bands at the expected mobility when compared with 1kb and 100bp ladders.

Digestion of GST-L2 deletion mutant plasmids with BamH1-EcoR1

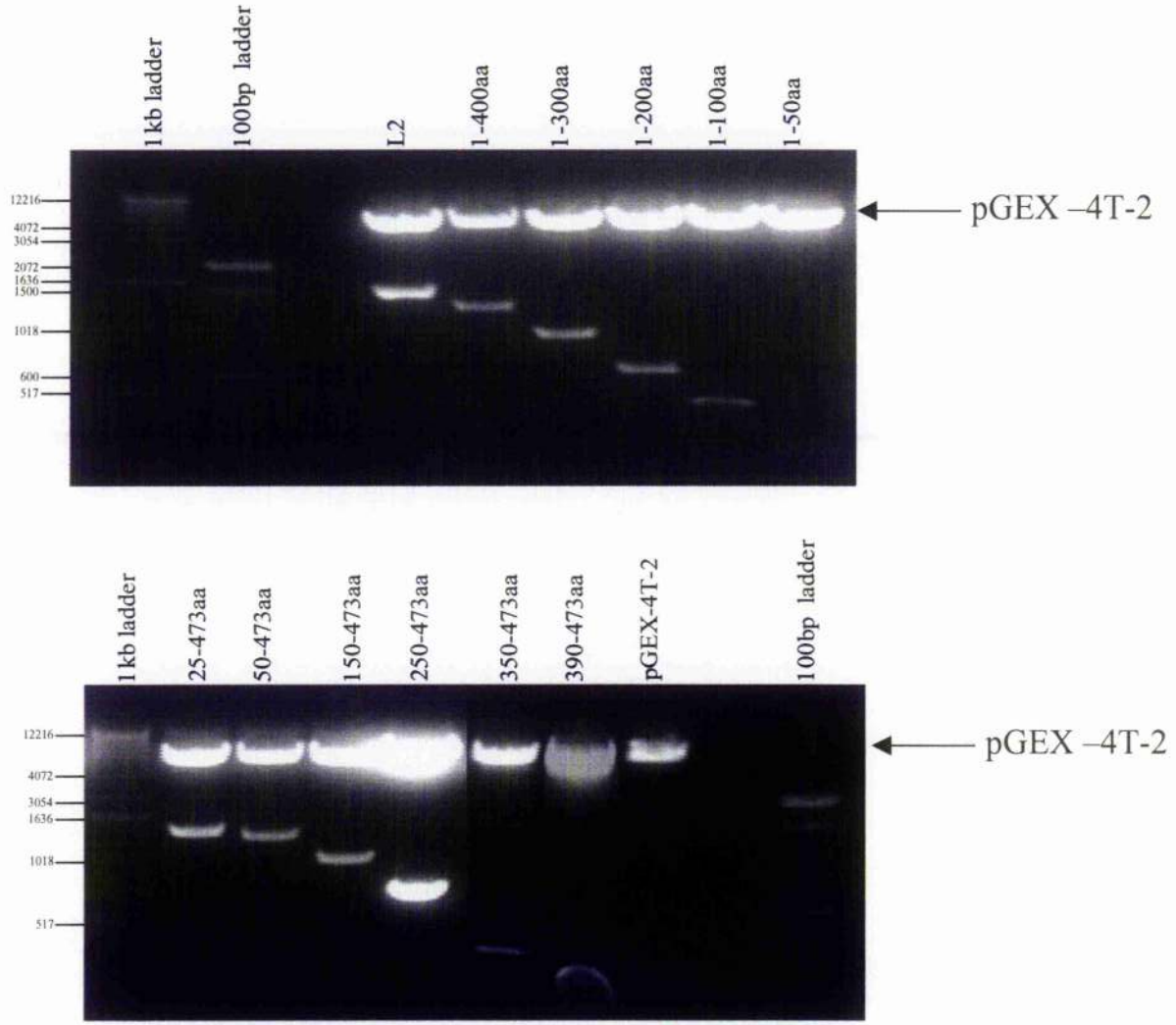


Figure 5.4. The N-terminal domain of L2 mediates binding with E2. Bacterially expressed GST and GST-L2 (a) C-terminal or (a) N-terminal deletion mutants immobilised on glutathione beads. 10µl of immobilised beads was incubated with 5µl ^{35}S labelled in vitro translated E2. (c) Bacterially expressed GST and GST-E2 were immobilised on glutathione beads. 10µl of immobilised beads was incubated with 5µl ^{35}S labelled in vitro translated L2 or 10µl ^{35}S labelled in vitro translated luciferase. Bounds proteins were separated by SDS-PAGE and visualised by autoradigraphy.

The N-terminal domain of HPV-16 L2 mediates binding with HPV-16 E2

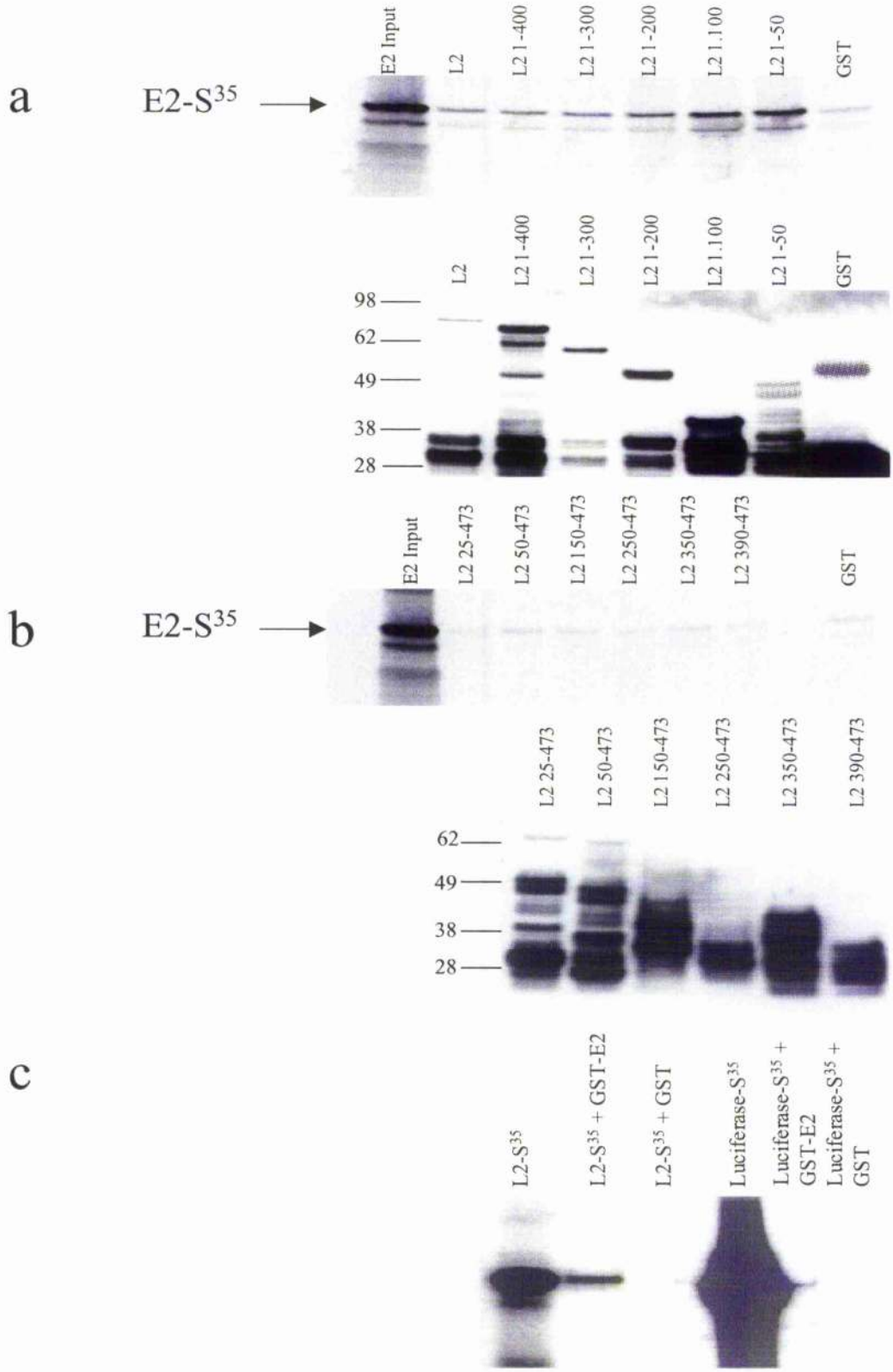
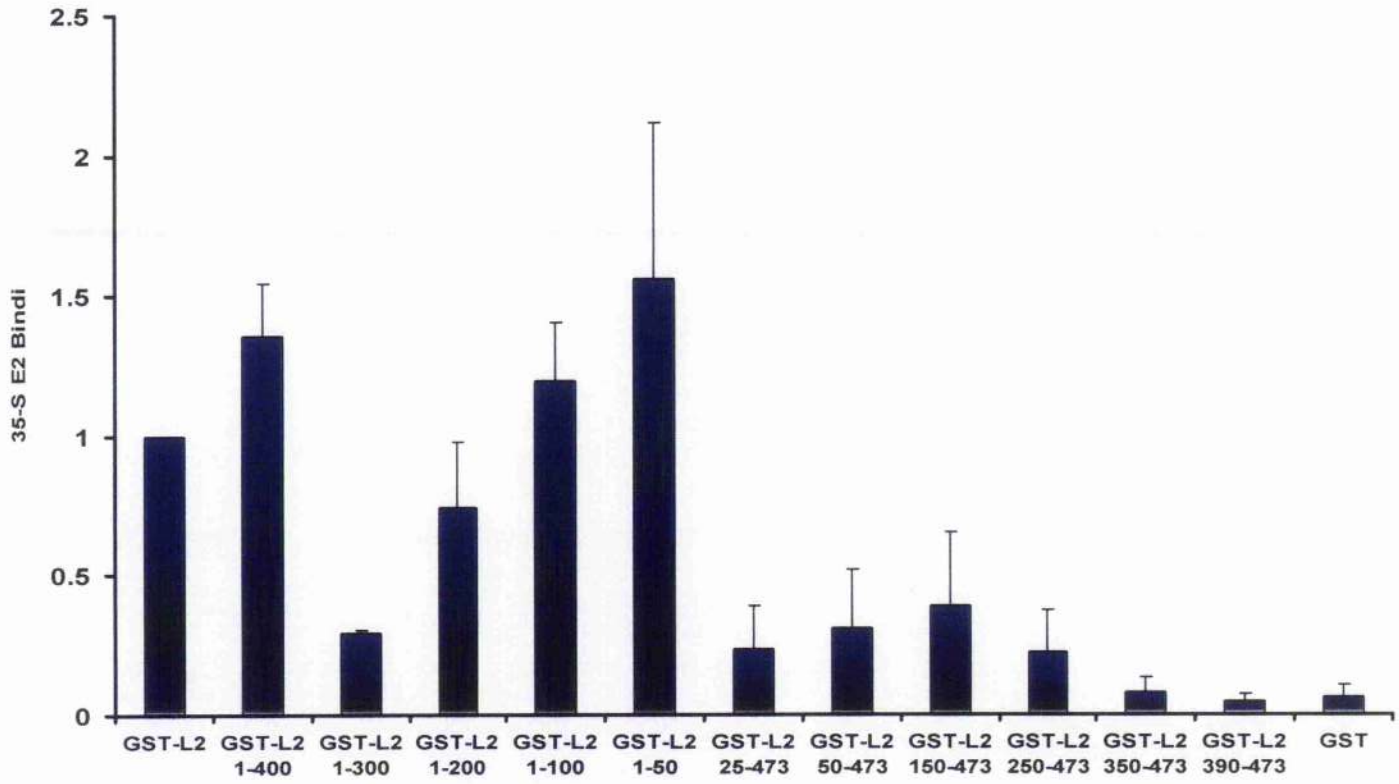


Figure 5.5. Then N-terminal domain of L2 mediates binding with E2. Bacterially expressed GST and GST-L2 carboxyl terminal or amino terminal deletion mutants immobilised on glutathione beads were incubated with ^{35}S labelled in vitro translated E2. Bounds proteins were separated by SDS-PAGE and visualised by autoradigraphy. Graph represents binding of ^{35}S-methionine labelled E2 to immobilised GST or GST-L2 deletion mutants normalised for amount of GST-L2 protein.

The N-terminal domain of HPV-16 L2 mediates binding with HPV-16 E2



5.3. Visualisation of GFP-L2 deletion mutants

5.3.1. The Green Fluorescent Protein

The green fluorescent protein, GFP (Fig 5.6a), is a spontaneously fluorescent protein isolated from the Pacific jellyfish, *Aequoria Victoria* which transduces, by energy transfer, the blue chemiluminescence of another protein, aequorin, into green fluorescent light. The molecular cloning of GFP cDNA and the demonstration by Chalfie *et al* (1994) that GFP can be expressed as a functional transgene has opened exciting new avenues of investigation in cell, developmental and molecular biology. Fluorescent GFP has been expressed in bacteria, yeast, plants, Drosophila, and mammalian cells. GFP can function as a protein tag, as it tolerates N- and C-terminal fusion to a broad variety of proteins many of which have been shown to retain native function. When expressed in mammalian cells, fluorescence from wild type GFP is typically distributed throughout the cytoplasm and nucleus. The flexibility of GFP as a non-invasive marker in living cells allows for its use as a reporter of gene expression. The cDNA of GFP encodes a protein of 238 amino acids with the molecular weight of 26.9kDa. Only three amino acids serine 65,tyrosine 66 and glycine 67 that make up the chromophore are responsible for the fluorescence. Its wild-type absorbance/ excitation peak is at 395 nm with a minor peak at 475 nm with extinction coefficients of roughly 30,000 and 7,000 M^{-1} cm^{-1}, respectively. The emission peak is at 508 nm.

5.3.2. Construction of GFP-L2 deletion mutants

To obtain a fusion form of HPV-16 L2 with GFP (Fig 5.6b), the L2 sequence was cloned into pEGFP-C1 vector (Fig 5.7) at the C-terminus of GFP using PCR. L2 cDNA was

PCR amplified as a BamH1-EcoR1 fragment from HPV-16 genome. The resulting plasmid was sequenced to confirm its identity and called pGFP-L2. A series of L2 N-terminus and C- terminus deletions of HPV-16 L2 were also designed and cloned into pEGFP-C1 vector at the C-terminus of GFP. Primers were designed with BamH1-EcoR1 restriction sites. For cloning into the pEGFP-C1 vector, the restriction sites Bgl II-EcoR1 were used to clone each insert into the pEGFP-C1 plasmid.

5.3.3. Primer design and cloning

For the C-terminus L2 deletion mutants, the primer *HPV-16 L2 BamH1 forward* was used for all forward reactions while the primers *GFP L2 1-50 (or 1-100, 1-200,1-300,1-400) reverse* (Table 2.1) were used for the reverse reaction. For cloning of the N-terminus deletion mutants, the primer *HPV-16 L2 EcoR1 reverse* was used for all reverse reactions while the primers *GFP-L2 25-473 (or 50-473,150-473,250-473,350-473,390-473) forward* (Table 2.1) were used for all forward reactions. For sequencing GFP-L2 and its deletion mutants, the sequencing primer 5'-CCTAGGAGCACTAAGCGA-3' corresponding to position 679-658 of the pEGFP-C1 plasmid was used. All plasmids sequences had the L2 deletion fused to the GFP protein as a Bgl II (BamH1)-EcoR1 insert. Sequences showed HPV-16 L2 inserts all fused in frame with the GFP fusion protein and designated pGFP-L2 1-400, pGFP-L2 1-300, pGFP-L2 1-200, pGFP-L2 1-100, pGFP-L2 1-50, pGFP-L2 25-473, pGFP-L2 50-473, pGFP-L2 150-473, pGFP-L2 250-473, pGFP-L2 350-473 and pGFP-L2 390-473. Restriction digest were also performed to confirm that all L2 deletion inserts were of the correct size (Fig 5.8).

5.3.4. Detection of GFP-L2 and its deletion mutants

To determine the localisation of GFP-L2 and its deletion mutants, HaCaT cells were first transiently transfected with varying amounts of GFP-L2 and the vector expressing GFP alone. Cells were seeded onto n° 01 coverslips in 24-well plates at a density of 10^5 cells/well and cultured overnight. When 80% confluent, cells were transfected with 5ng, 10ng, 25ng, 50ng 100ng, 0.5µg, 1µg or 5µg of pGFP-L2 or pEGFP (GFP-L2 or GFP alone expressing plasmids). A DNA concentration of 100ng was chosen for subsequent transfections as it gave best results for efficiency of transfection.

Twenty-four hours after transfection, the cells were washed twice with PBS and fixed by 10-min incubation at room temperature with 1.85% formaldehyde diluted in PBS containing 2% sucrose, and washed three times with PBS. Staining for DNA was done using 4'6'-Diamino-2-phenylindole (DAPI) that stains nucleic acids in general and fluoresces in the deep blue spectrum. Cells were incubated with a DAPI staining solution (a 1/100 dilution from a 10µg/ml stock of DAPI in washing buffer) for 10mins, washed in PBS-FCS and then distilled water (to remove traces of FCS) and dried before the coverslips were mounted in AF1 (Citifluor, UK) and visualised using the confocal microscope as described in section 2.2.5. Cells transfected with GFP showed a green fluorescence uniformly distributed in the whole cell (Fig 5.9a) while GFP-L2 expressing cells resulted in a well-defined localisation to the nucleus (Fig 5.9b) suggesting that the nuclear localisation of GFP-L2 was exclusively due to L2 sequence. All cells even with the weakest fluorescence presented a nuclear localisation of GFP-L2 excluding the possibility that the cellular localisation of GFP-L2 was due to its over expression.

In order to determine the localisation of GFP-L2 C-terminal deletion mutants, HaCaT cells were transfected with 100ng pGFP-L2 1-400, pGFP-L2 1-300, pGFP-L2 1-200, pGFP-L2 1-100 and pGFP-L2 1-50. Cells were fixed as described above, mounted and visualised using the confocal microscope. Cells expressing all C-terminal deletion mutants showed clear localisation to the nucleus (Fig 5.9c-g). Nuclear localisation results from the presence of an arginine rich DNA binding domain (Zhou *et al.*, 1994) which has been shown to function as NLS sequences with N-terminal amino acids 1-60 of HPV-6b L2 being able to localise beta-galactosidase to the nucleus (Sun *et al.*, 1995).

Transfection of 100ng of pGFP-L2 25-473, pGFP-L2 50-473, pGFP-L2 150-473, pGFP-L2 250-473, pGFP-L2 350-473 and pGFP-L2 390-473 in HaCaT cells all showed localisation to both the nucleus and the cytoplasm. In contrast to cells transfected with GFP alone with green fluorescence uniformly distributed in the whole cell when transfected, with the N-terminal deletion mutants, clearly defined nucleus and cytoplasm could be identified (Fig 5.9h-m). Localisation to the nucleus results from the presence of NLS sequences at the C-terminus of L2. Deletion of sequences within the N terminal protein of L2 appears to affect effective translocation or prevent retention of L2 within the nucleus. Both the N-terminal DNA binding domain and the C-terminal NLS of HPV-6b L2 have been shown to be functional in protein nuclear transport and contribute to the translocation of L2 into the nucleus (Sun *et al.*, 1995). In summary, GFP-L2 and GFP-L2 C-terminal deletion mutants all gave a localisation to the nucleus while the GFP-L2 N-terminal deletion mutants gave a staining pattern that was both nuclear and cytoplasmic.

5.3.5. Expression of GFP-L2 deletion mutants

To detect GFP-L2 and GFP-L2 deletion mutants by immunoblotting, HaCaT cells were transfected with 4μ pGFP-L2 or 4μ of each pGFP-L2 deletion mutants. Cells were harvested 24 hours post transfection and detection of GFP fusion proteins was accomplished by SDS-PAGE and Western blotting. Probing with HPV-16 L2 polyclonal rabbit serum detected a high steady state level of expression in cells expressing GFP-L2 1-400, GFP-L2 1.300 and GFP-L2 1-200 (Fig 5.10a). Cells transfected with pGFP-L2 showed a lower expression level with multiple banding observed indicating a high level of protein instability. A low level of GFP-L2 1-100 could be detected while GFP-L2 1-50 could not be detected using the L2 polyclonal rabbit serum possibly due to a loss of the L2 epitope. Mutants pGFP-L2 25-473 and pGFP-L2 50-473 showed a lower steady state level of expression while mutants pGFP-L2 150-473, 250-473, 350-473 and 390-473 could not be detected using the L2 serum (Fig 5.10a). Differences in detection between the N-terminal and C-terminal deletion mutants could result from deletion of instability sequences located within the 3'-end of the L2 mRNA (Sokolowski *et al.*, 1998) leading to increased expression of C-terminal deletion mutants. All GFP-L2 C-terminal deletion mutants were also detected using an anti-GFP rabbit polyclonal antibody (Santa Cruz) including mutants GFP-L2 1-50 and GFP-L2 1-100, which could not be detected using the HPV-16 L2 polyclonal rabbit serum (Fig 5.10b). GFP-L2 as well as all N-terminal GFP-L2 deletion mutants could not be detected using the anti-GFP antibody.

Expression of papillomavirus structural proteins L1 and L2 is tightly regulated resulting in difficulties in detection of L2 in mammalian cells by means of DNA transfections. This regulation has been linked to the differentiation status of cells, polyadenylation

signals terminating transcripts before reaching the late region (Baker and Howley, 1987; Terhune *et al.*, 2001) and mRNA encoding negative regulatory sequences that prevent nuclear export or destabilize the message (Kennedy *et al.*, 1990; Kennedy *et al.*, 1991; Sokolowski *et al.*, 1998). Transient expression of L2 in non-differentiated human cells has been shown to be highly improved by changing the RNA coding sequences (Leder *et al.*, 2001; Zhou *et al.*, 1999). Considering that GFP-L2 and N-terminal GFP-L2 deletion mutants could be visualised by immunofluorescence but not by Western blotting indicates that the process of protein extraction and SDS-PAGE analysis could contribute to the increased instability and reduced detection of L2 by immunoblotting.

Figure 5.6. Schematic representation of the backbone of the green fluorescent protein. (**A**) GFP consists of eleven anti-parallel beta-sheets, which surround a co-axial helix enclosing the chromophor. The structure gives high stability to the protein, making it resistant to denaturing chemicals. (**B**) Schematic representation of HPV-16 L2 fused to the carboxyl terminus of GFP.

The Green Fluorescent Protein and HPV-16 L2 fused to the C-terminus of GFP

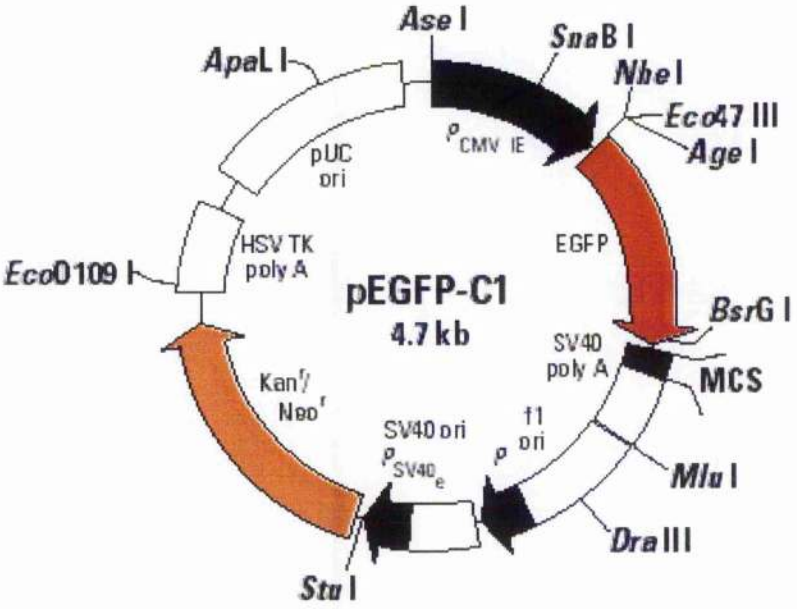
a

b

pEGFP-C1 *(Bgl II, EcoR1)*

Figure 5.7. Graphical representation of pEGFP-C1. pEGFP-C1 is a GFP expression plasmid designed to generate fusion proteins for expression and localization studies, or expression of GFP in mammalian cells. The gene of interest is inserted into the multiple cloning site (MCS) of the vector and expressed as a fusion to the C-terminus of GFP.

Graphical representation of pEGFP-C1 vector and its multiple cloning site.



MCS

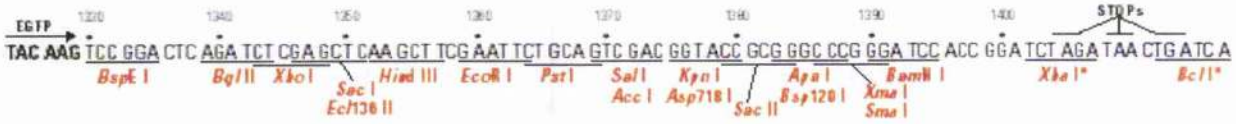


Figure 5.8. Digestion of pEGFP-L2 N-terminal and C-terminal deletion mutants. 1µg of each plasmid was digested for 1h with Nhe1-EcoR1, analysed using agarose gel electrophoresis and observed under UV light. All inserts gave bands at the expected mobility when compared with 1kb and 100bp ladders.

Digestion of GFP-L2 deletion mutant plasmids with Nhe1-EcoR1

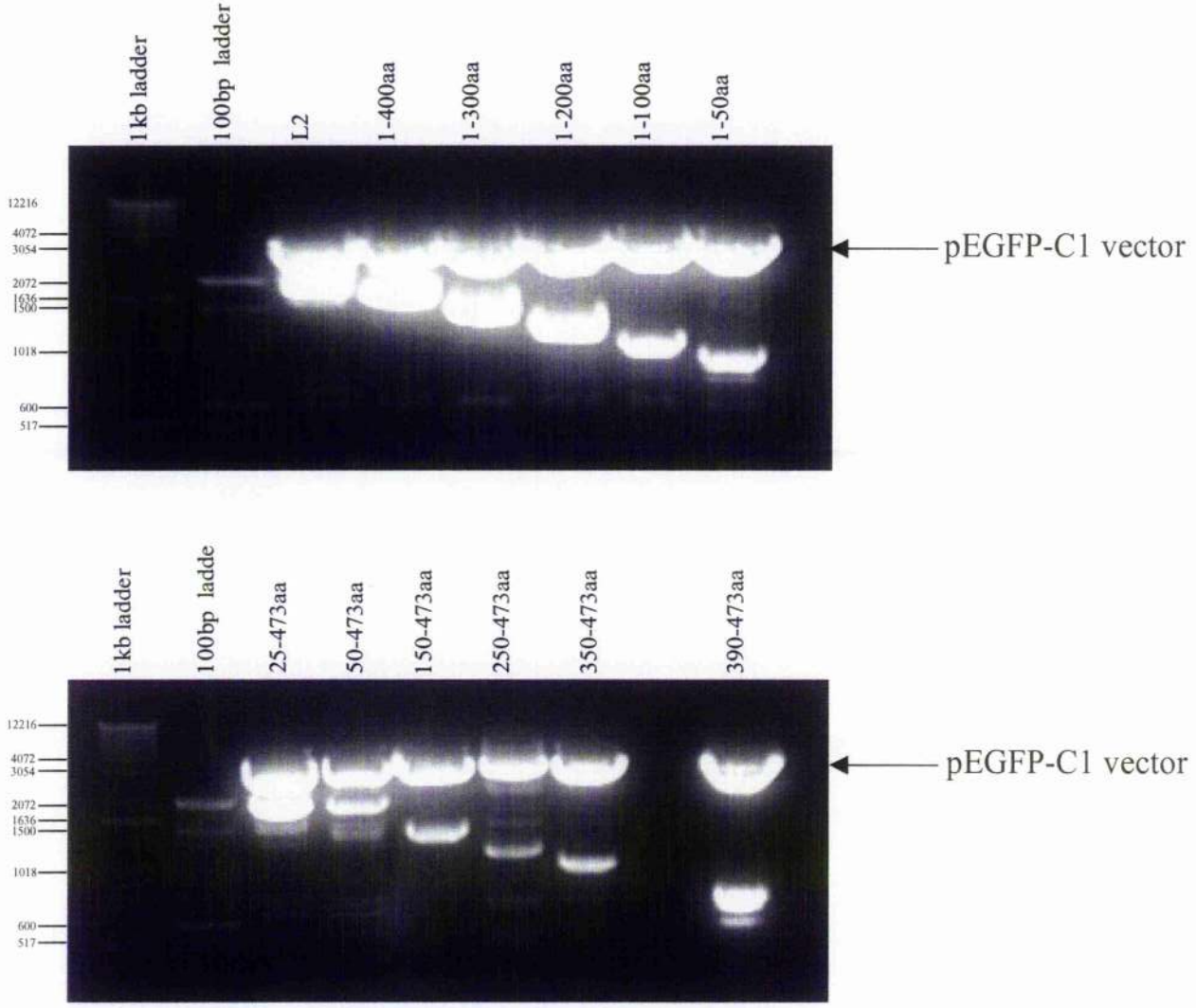
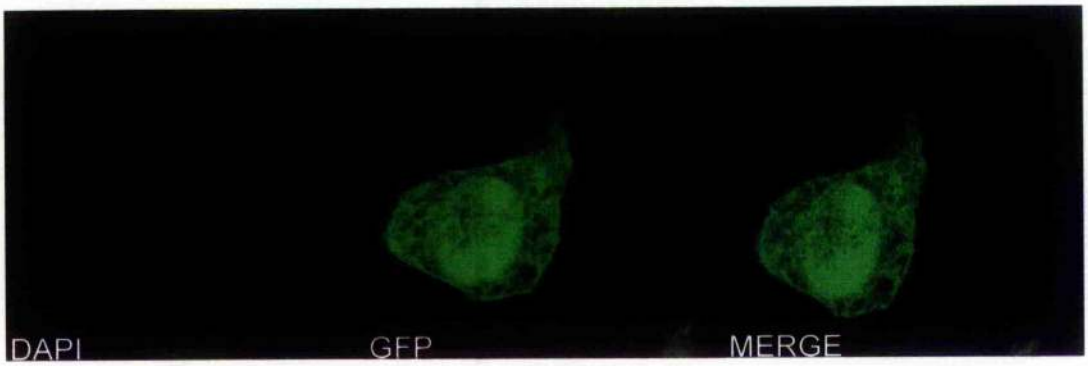


Figure 5.9. Visualisation of GFP-L2 and GFP-L2 deletion mutants. Confocal images of HaCaT cells transfected with 100ng of (a) pEGFP, (b) pGFP-L2 (c) pGFP-L2 1-400, (d) pGFP-L2 1-300, (e) pGFP-L2 1-200, (f) pGFP-L2 1-100, (g) pGFP-L2 1-50, (h) pGFP-L2 25-473, (i) pGFP-L2 50-473, (j) pGFP-L2 150-473, (k) pGFP-L2 250-473, (l) pGFP-L2 350-473 and (m) pGFP-L2 390-473, fixed after 24h and stained for DNA using DAPI. GFP alone showed a uniform distribution in the cell. GFP-L2 showed a well-defined localisation to the nucleus. The C-terminal deletion mutants also showed well-defined localisation to the nucleus. The N-terminal GFP-L2 deletion mutants showed a disperse localisation in both the cytoplasm and nucleus.

Localization of GFP and GFP-L2 in HaCaT cells

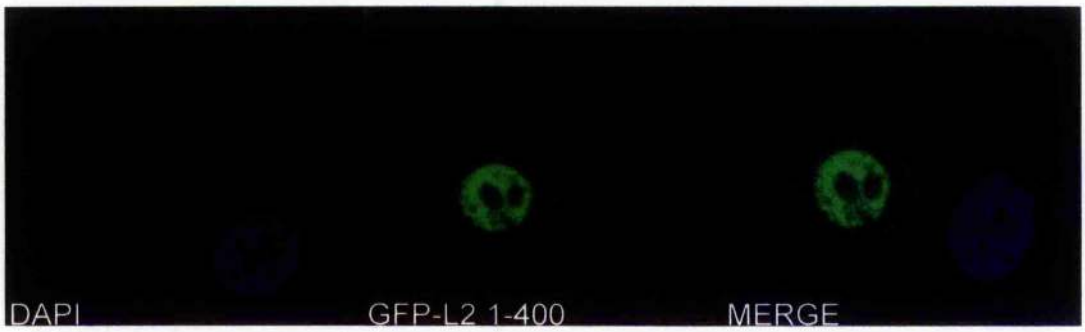
a



b

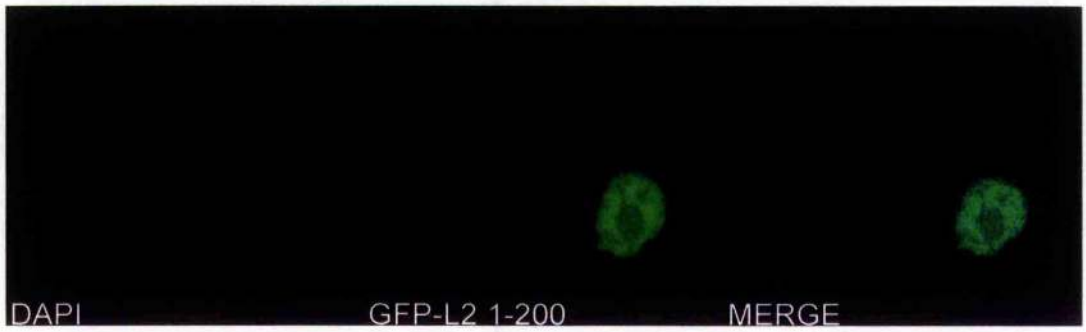
Localization of GFP-L2 C-terminal deletion mutants in HaCaT cells

c



d

e



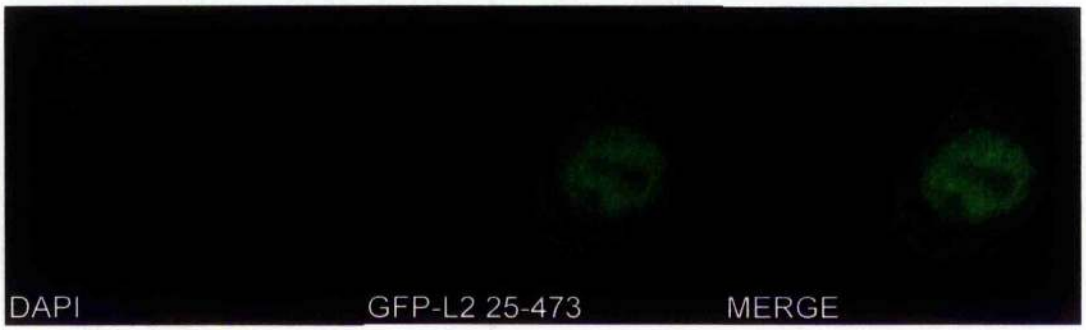
Localization of GFP-L2 C-terminal deletion mutants in HaCaT cells

f

g

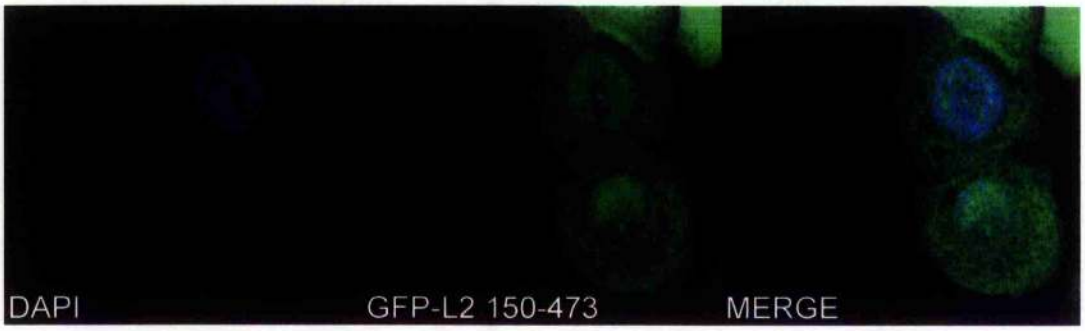
Localization of GFP-L2 N-terminal deletion mutants in HaCaT cells

h



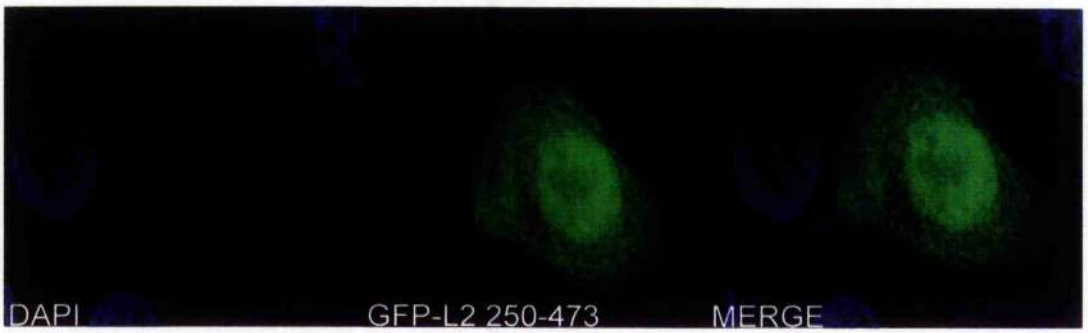
i

j



Localization of GFP-L2 N-terminal deletion mutants in HaCaT cells

k



l

m

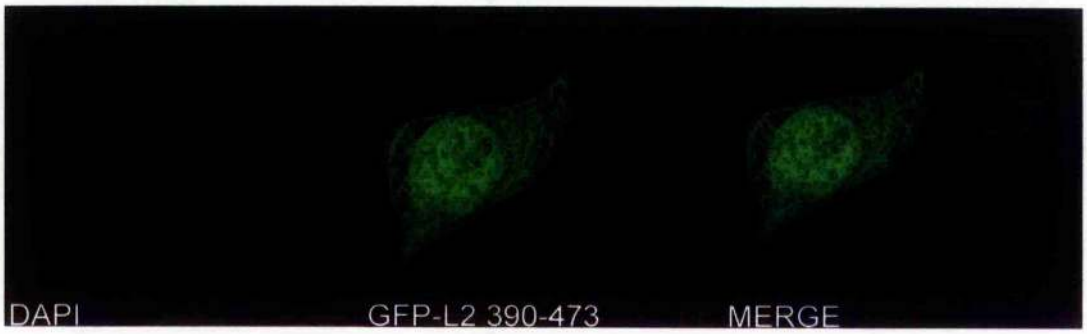
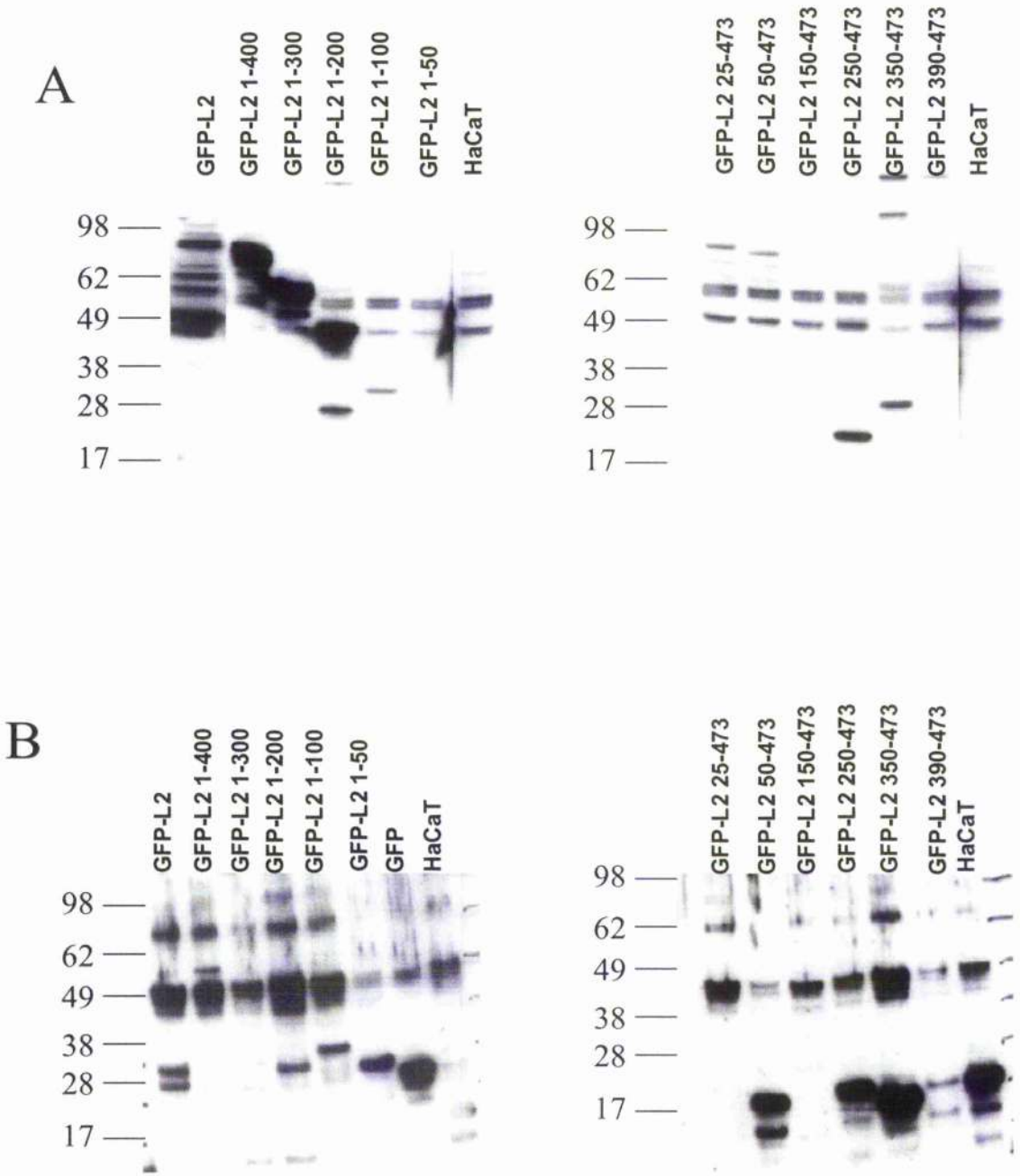


Figure 5.10. Expression of GFP-L2 and GFP-L2 deletion mutants. HaCaT cells were transfected with 4μg pGFP-L2, or 4μg pGFP-L2 deletion mutants. Cell were harvested using SDS-PAGE lysis buffer and extracts were analysed by Western blotting with (A) HPV-16 L2 polyclonal rabbit serum (B) Anti-rabbit polyclonal antibody to probe for GFP fusion proteins.

Detection of GFP-L2 deletion mutants



5.4. Analysis of the effect of L2 deletion mutants on E2 function

5.4.1. Construction of L2-deletion mutants in pCDNA expression vector

To investigate the domain or domains of L2 that mediate the down-regulation of E2 transcription transactivation as well as E2 expression, the series of L2 deletion mutants was cloned into pCDNA3.1(+) expression vector. The pCDNA3.1(+) plasmid is a 5.4kb vector derived from pCDNA3 and designed for high-level stable and transient expression in mammalian hosts. It contains the CMV promoter, neomycin resistance gene and a multiple cloning site, which facilitates cloning. A series of primers were designed for cloning of both the N-terminal and the C-terminal deletions of HPV-16 L2 (Fig 5.1).

5.4.2 Primer design and cloning

The following primers were used to PCR amplify inserts for cloning into pCDNA 3.1+ vector. For the carboxyl terminus L2 deletion mutants, the primer *HPV-16 L2atg BamH1 forward* was used for all forward reactions while the primers *L2 1-50 (or 1-100, 1-200,1-300,1-400) reverse* (Table 2.1) were used for the reverse reaction. For cloning of the amino terminus deletion mutants, the primer *HPV-16 L2 EcoR1 reverse* was used for all reverse reactions while the primers *L2 25-473atg (or 50-473atg, 150-473atg, 250-473atg, 350-473atg, 390-473atg) forward* (Table 2.1) were used for all forward reactions. For sequencing of pCDNA-L2 deletion mutants, the forward sequencing primer '5-ATTAATACGACTCACTATAGGGA-3' corresponding to position 864-882 in pCDNA3.1(+) and the reverse primer 5'-CTAGAAGGCACAGTCGAGGC-3' corresponding to position 1249-1018 were designed. All plasmid sequences had the L2 deletion as a BamH1-EcoR1 insert with the ATG start site and designated pCDNAL2,

pCDNAL2 1-400, pCDNAL2 1-300, pCDNAL2 1-200, pCDNAL2 1-100, pCDNAL2 1-50, pCDNAL2 25-473, pCDNAL2 50-473, pCDNAL2 150-473, pCDNAL2 250-473, pCDNAL2 350-473 and pCDNAL2 390-473. Restriction digests were also performed to confirm that all L2 deletion inserts were of the correct size (Fig 5.10).

5.4.3. Effect of L2 and L2 deletion mutants on the transcription transactivation function of E2

To determine the domain or domains of L2 that mediate the down regulation of the transcription transactivation function of E2, C33a and HaCaT cells were transfected with ptk6E2, pCMV-$E2_{16}$ and increasing amounts (0.1-1μg) of p16-HAL2, pCDNAL2 or L2 deletion mutants (pCDNA1-400, 1-300, 1-200, 1-100, 1-50, 25-473, 50-473, 150-473, 250-473, 350-473 and 390-473). The amount of E2 was kept sub-optimal for E2-mediated transactivation. In C33a cells transfection with p16-HAL2, pCDNAL2 or mutants pCDNAL2 1-400 and pCDNAL2 1-300, down-regulated E2 transcription transactivation in a dose dependent manner, completely repressing E2 activation to levels observed with the base promoter in cells transfected with 1μg of plasmid (Fig 5.11a). Mutant 1-200 did not inhibit E2 activity while mutants 1-100 and 1-50 inhibited E2 activity but not to levels observed with full-length L2. For the N-terminus L2 deletion mutants, all mutants with the exception of 150-473 were able to fully inhibit E2 transcription transactivation (Fig 5.11b). In HaCaT cells, mutants 1-200, 1-100 and 1-50 did not inhibit E2 activity. The N-terminus deletion mutants 25-473, 350-473 and 390-473 inhibited E2 transcription transactivation at the highest amount of plasmid transfected while mutants 50-473, 150-473 and 250-473 showed no inhibition of E2 activity (Fig 5.12b). In C33a cells, it appears that sequences between amino acids 150-

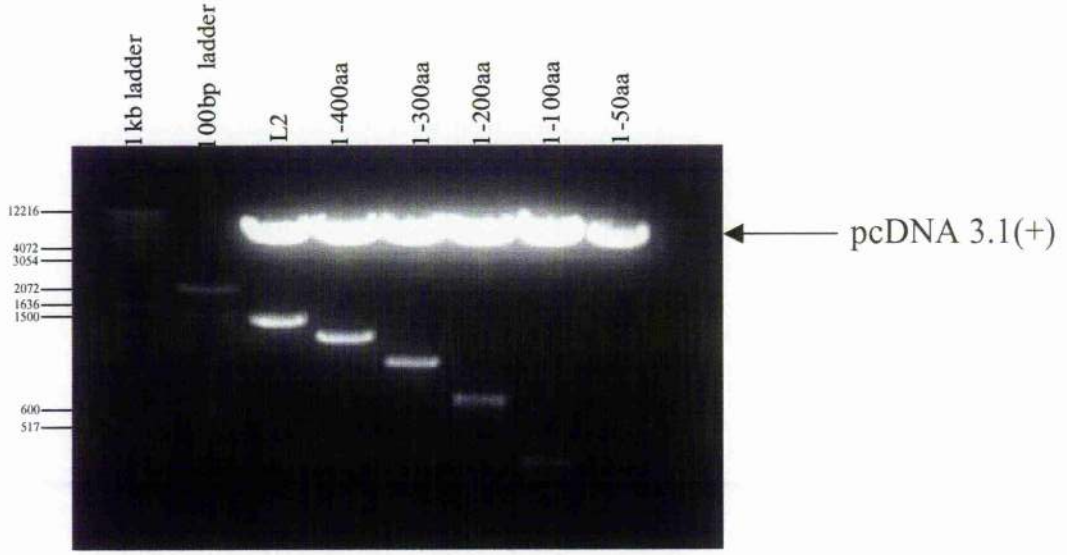
200 inhibits repression of E2 transactivation function. It also appears that deletion of the C-terminus of L2 from amino acid 200 also alleviates repression of E2 transcription transactivation which is better elucidated with results observed in HaCaT than in C33a cells. In HaCaT cells, transfection of the N-terminal deletion mutants did not give levels of repression of E2 transactivation as observed in C33a cells although results with mutant 150-473 remained similar. Differences in the mode of transfection could contribute to differences in results obtained from both cell lines, as calcium phosphate precipitation is used to transfect C33a cells while LIPOFECTAMINE is used for transfection with HaCaT.

5.4.4. Effect of L2 and L2 deletion mutants on E2 expression

HPV-16 L2 down-regulates the expression level of HPV-16 E2 in HaCaT and U2OS but not C33a cells. To determine the domain or domains of L2 which mediate this down regulation of E2 expression, HaCaT cells were transiently transfected with 4μg pCMV-$E2_{16}$ alone or 4μg of pCMV-$E2_{16}$ and 4μg p16-HAL2, pCDNAL2 or pCDNAL2 deletion mutants and detection of E2 levels was accomplished by Western blotting. In HaCaT cells, the expression of HA-L2 or wild type L2 lead to a marked reduction in the level of the E2 protein (Fig 5.13). In cells expressing E2 and L2 deletion mutants, the level of E2 did not appear to be inhibited to levels comparable with the full-length L2 protein, indicating that the full-length protein is required for the down-regulation of E2 expression.

Figure 5.11. Digestion of pCDNA-L2 and pCDNA-L2 N-terminal and C-terminal deletion mutants. 1μg of each plasmid was digested for 1h with BamH1-EcoR1 and analysed using agarose gel electrophoresis and observed under a UV light. All inserts gave bands at the expected mobility when compared with 1kb and 100bp ladders.

Digestion of pcDNA-L2 deletion mutant plasmids with BamH1-EcoR1



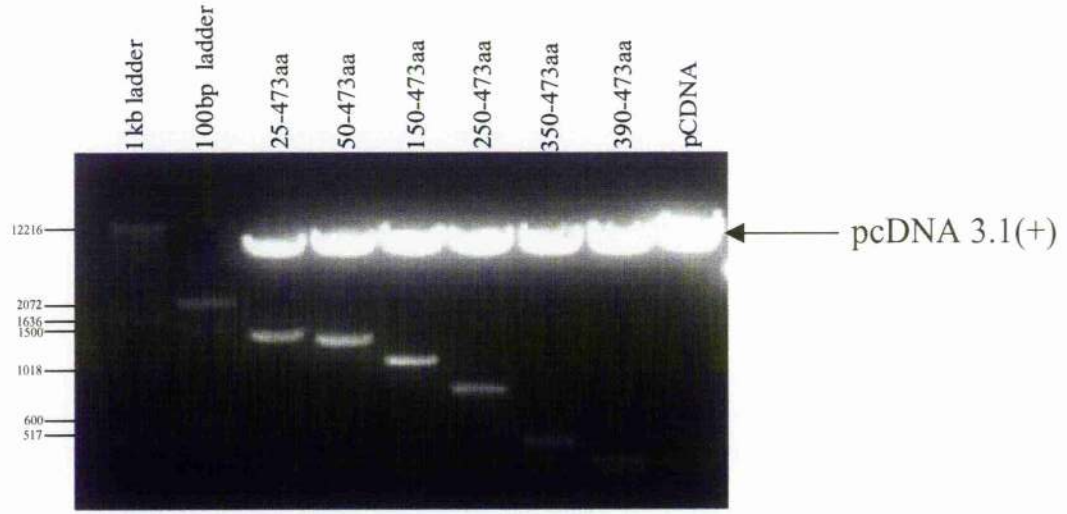
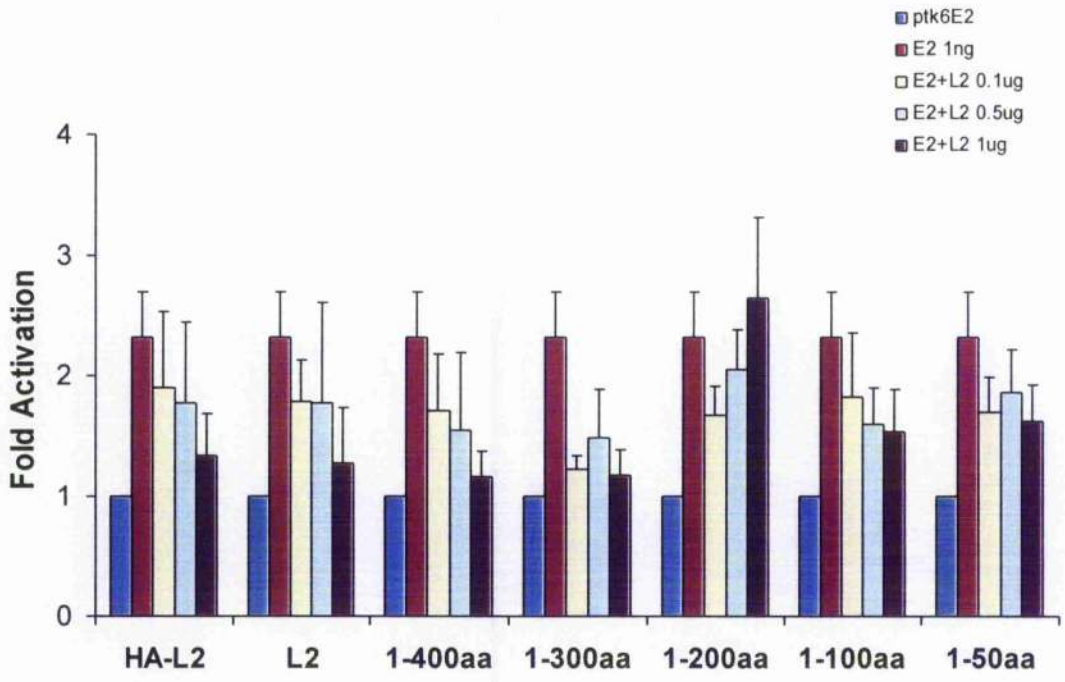


Figure 5.12. Effect of L2 and L2 deletion mutants on the transcription transactivation function of E2 in C33a cells. C33a cells were transiently co-transfected with 0.1μg of ptk6E2 luciferase reporter construct and 1ng pCMV-$E2_{16}$ with increasing concentrations (0.1μg, 0.5μg and1μg) of pCDNA16-L2 or (a) C-terminus and (b) N-terminus deletion mutants. Shown is the average of three consecutive experiments. Transactivation is shown as fold activation of luciferase expression over that of the reporter plasmids without E2, taken arbitrarily as 1.

Effect of HPV-16 L2 N- and C- terminus deletion mutants on HPV-16 E2 mediated transcription transactivation in C33a cells

a



b

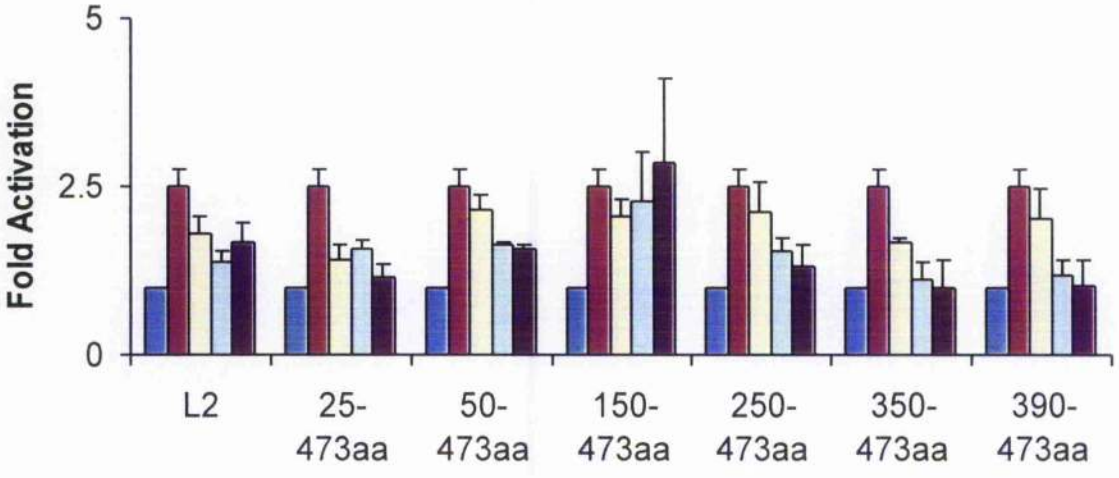
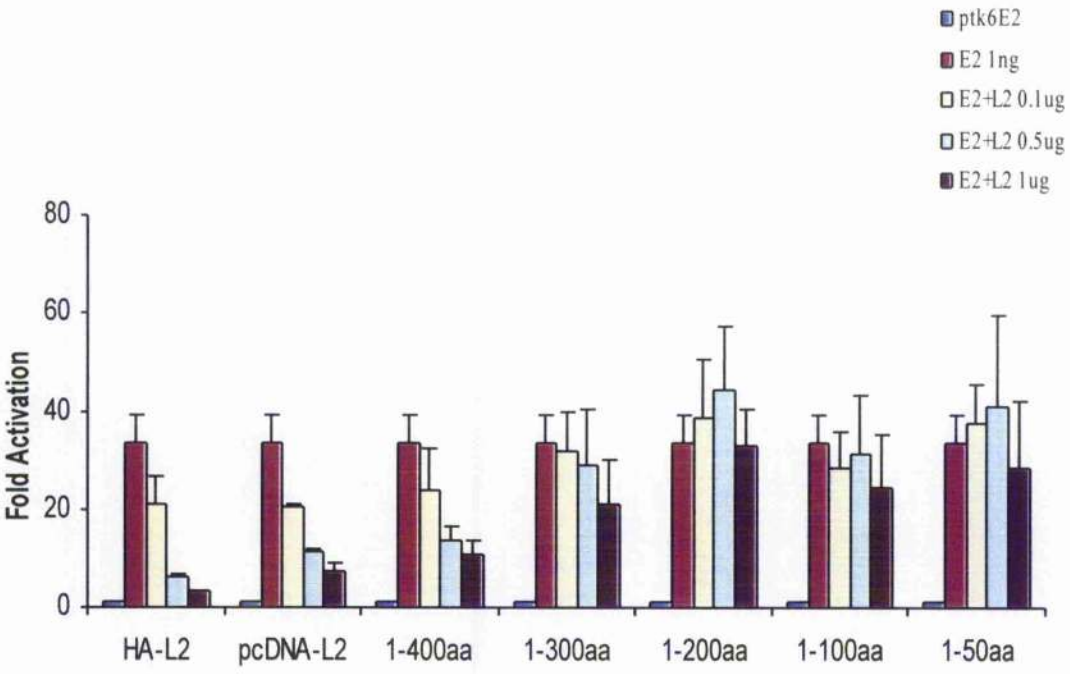


Figure 5.13. Effect of L2 and L2 deletion mutants on the transcription transactivation function of E2 in HaCaT cells. HaCaT cells were transiently co-transfected with 0.1μg of ptk6E2 luciferase reporter construct and 1ng pCMV-$E2_{16}$ with increasing concentrations (0.1μg, 0.5μg and1μg) of pCDNA16-L2 or (a) C-terminus and (b) N-terminus deletion mutants. Shown is the average of three consecutive experiments. Transactivation is shown as fold activation of luciferase expression over that of the reporter plasmids without E2, taken arbitrarily as 1.

Effect of HPV-16 L2 N- and C- terminus deletion mutants on HPV-16 E2 mediated transcription transactivation in HaCaT cells

a



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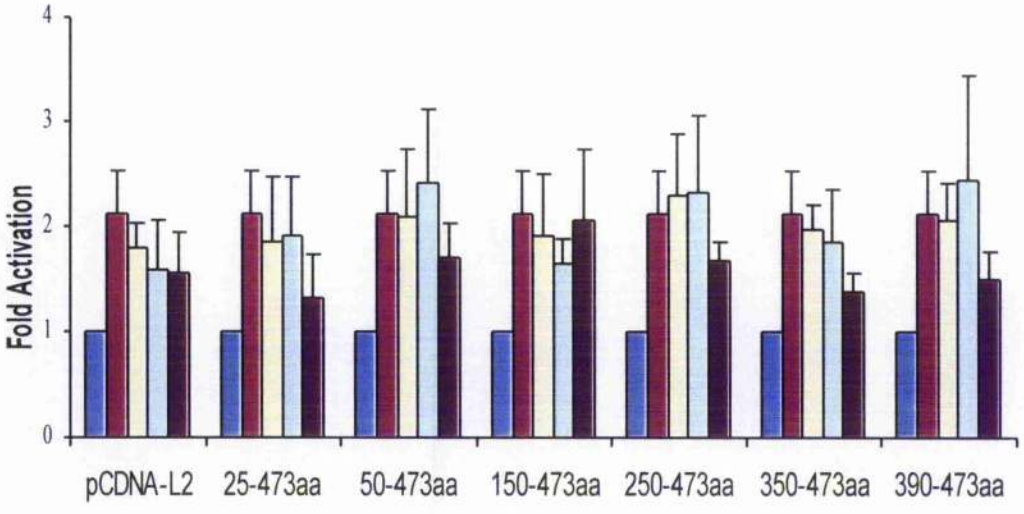
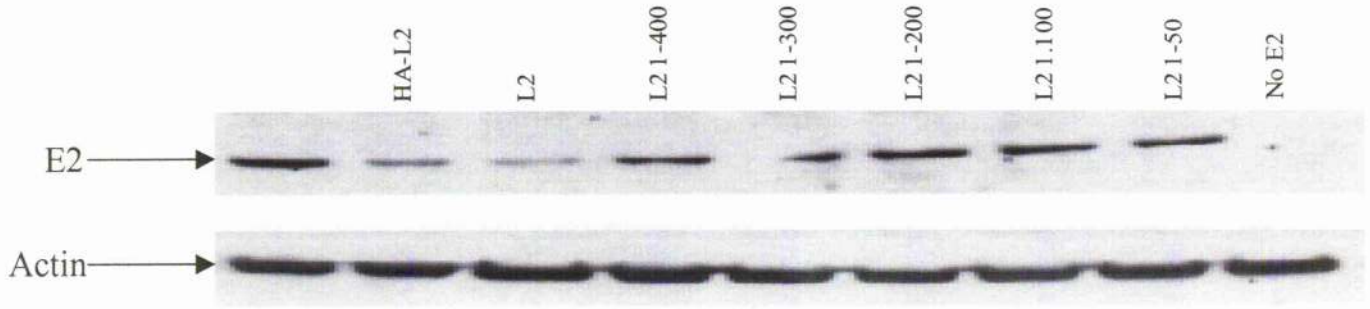


Figure 5.14. Full length HPV-16 L2 is required to down-regulate E2 in HaCaT cells. HaCaT cells were transfected with 4μg pCMV-$E2_{16}$, or 4μg pCMV-$E2_{16}$ plus 4μg p16-HAL2, pCDNAL2 or pCDNAL2 (a) C-terminus and (b) N-terminus deletion mutants. Cell were harvested using SDS-PAGE lysis buffer and extracts were analysed in independent Western blots with anti-E2 mouse monoclonal antibody TVG261 and mouse anti-actin antibodies.

Full-length HPV-16 L2 is required for the down regulation of HPV-16 E2 gene expression in HaCaT cells

a



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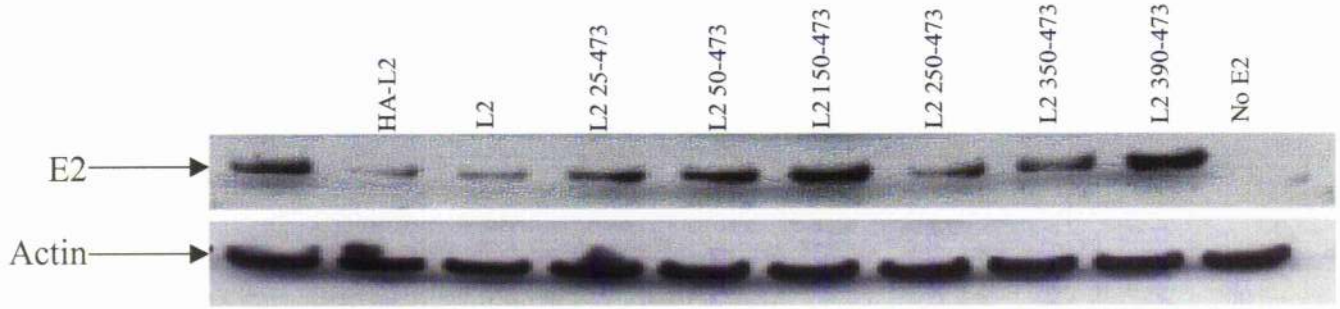


CHAPTER 6: DISCUSSION

6.1. Introduction

The viral minor structural protein L2 of BPV-1, HPV-16 and HPV-33 has been shown to localise to dynamic nuclear substructures associated with the nuclear matrix called PODs (Day *et al.*, 1998; Florin *et al.*, 2002; Gornemann *et al.*, 2002). Co-expression of BPV-1 L2 and E2 causes the redistribution of E2 into PODs (Day *et al.*, 1998; Heino *et al.*, 2000). Besides the co-localisation of E2 and L2 to PODs, little is known about the effect of this interaction on E2 function. As a result, the aim of this thesis was to address the question of whether L2 plays a role in regulating E2 functions during the viral life cycle. The study presents evidence in a HPV-16 system that L2 was able to down regulate the transcription transactivation function of L2 in a dose dependent manner but had no effect on E2 function in DNA replication. Although no effect of L2 on E2 mRNA levels was observed, L2 was able to reduce the level of E2 expression in a cell type dependent manner and this reduction was not proteasome dependent. Using L2 deletion mutants, the domain of L2 required for binding with E2 was identified. Binding of L2 did not correlate with the down regulation of E2 transcription transactivation function or a reduction of E2 protein expression levels.

6.2. Functional interaction between HPV-16 E2 and HPV-16 L2

6.2.1. Regulation of transcription

The viral E2 gene product regulates papillomavirus gene expression. E2 is a sequence specific DNA-binding protein, which interacts directly with E2-dependent enhancer elements, located within the viral genomes through E2 binding sites (E2BS) and plays an essential role in the activation of viral gene expression. There are four E2BS on the LCR, which mediate the activity of E2 in transcriptional activation and repression. E2BS 1-3, mediate the ability of E2 to repress transcription (Demeret *et al.*, 1997; Dong *et al.*, 1994; Romanczuk *et al.*, 1990; Thierry and Howley, 1991). This is thought to result from the ability of E2 to prevent TATA box binding protein (TBP) from binding to the TATA box thus preventing transcriptional initiation when E2 is bound to E2BS 1 (Dostatni *et al.*, 1991). E2BS 3 is thought to mediate E2 repression by competing with the cellular transcription factor Sp1, which binds to a site overlapping it (Demeret *et al.*, 1997; Tan *et al.*, 1994). E2BS 4 mediates activation of transcription by E2, especially under conditions where the other binding sites are disrupted (Demeret *et al.*, 1997). During the productive stage of the viral life cycle, E2 regulates transcription of the viral E6 and E7 oncogenes. Malignant transformation usually occurs as a result of disruption of the E2 gene and consequent deregulated expression of E6 and E7 (Fig 1.7). Reintroduction of HPV-18 E2 to cervical carcinoma cell lines by transient transfection leads to repression of the endogenous E6/E7 transcription, inducing cell cycle arrest in G_1 mediated by the stabilization of p53 (Desaintes *et al.*, 1997; Dowhanick *et al.*, 1995).

The role of E2 in regulating expression of the E6/E7 promoter of HPV-18 has been analysed using an *in vitro* transcription system to determine the dose response of E2 on the long control region (LCR) (Steger and Corbach, 1997). E2 was shown to activate transcription at low concentrations, while increasing the concentration of E2 resulted in complete promoter repression. BPV-4 E2 has also been shown to activate transcription from the BPV-4 LCR, which was up-regulated at low E2 concentrations while high levels of E2 resulted in down regulation of transcriptional activation (Jackson and Campo, 1995; Morgan *et al.*, 1998).

The effect of increasing amounts of E2 on the activity of the LCR was further elucidated in this study. E2 regulated transcription from the wild type HPV-18 and BPV-4 promoters in a dose dependent manner with low levels of E2 stimulating transcription. Activation of transcription has been shown to result from E2 binding to E2BS 4 with which it has its highest binding efficiency (Steger and Corbach, 1997). An increase in the amount of E2 resulted in repression of transcription transactivation. This repression is mediated by E2 binding E2BS 1-3 with which it has been shown to bind with lower efficiency (Steger and Corbach, 1997). Elimination of repression of E2 transcription activation was achieved with the use of a variety of E2 responsive reporter constructs derived from the HPV-18 LCR with mutations in E2BS 1 or E2BS 1-3 to prevent E2 from binding these sites. Two synthetic promoters consisting of multimerized E2BS fused to the HPV-18 TATA or tk promoter were also used. E2 strongly activated transcription in a dose dependent manner with no repression observed even at the highest amounts of E2, as previously shown (Morgan *et al.*, 1998; Phelps and Howley, 1987;

Vance *et al.*, 1999). The use of HPV-18-derived and synthetic E2 responsive promoters showed that the transcriptional transactivation activity of E2 was drastically inhibited by L2 in a dose dependent manner. These observations are in agreement with observations using a bovine system that showed repression of BPV-1 E2TA-dependent luciferase expression by increasing amounts of BPV-1 L2 in CV-1 3T3 cells (Heino *et al.*, 2000). In this study, two human cell lines, HaCaT and C33a were used representing two different human models of carcinogenesis. In both cases, similar results were obtained showing repression of HPV-16 E2 dependent luciferase expression by increasing amounts of HPV-16 L2. These results suggest that this aspect of the interaction between E2 and L2 may be a common feature of papillomaviruses of different types.

How L2 inhibits E2 transcriptional transactivation activity is not clear. Recruitment of E2 to PODs by L2 may be the underlying mechanism. PODs are sites of protein modification and as a result, post-translational modifications of E2 may interfere with some of its activities. PML has been shown to regulate transcription of certain genes in either a positive or negative manner. PML interacts with Sp1 and inhibits Sp1-mediated transcriptional activity of the epidermal growth factor receptor promoter by disrupting Sp1-DNA binding (Vallian *et al.*, 1998). Recruitment of E2 to PODs could result in PML interference with Sp1 binding to the viral LCR, which could result in repression of the viral promoter (Tan *et al.*, 1994).

Another possible mechanism for E2 transcription down regulation could result from L2 acting as a transcription repressor with the ability to actively target E2 transcription

initiation factors or indirectly by displacing the binding of E2 responsive transcription factors by competing for their binding sites. The N-terminus of L2 binds viral DNA in a sequence independent process (Zhou *et al.*, 1994). Binding of L2 to viral DNA could interfere with the activity of transcription factors that modulate E2 mediated transcription initiation such as TBP, TFIIB or Sp1 (Li *et al.*, 1991; Rank and Lambert, 1995). E2 binds TBP and has been shown to act synergistically with exogenous TBP to facilitate high-level transcription from a minimal promoter (Ham *et al.*, 1991; Rank and Lambert, 1995; Steger *et al.*, 1995). L2 transcription repression could involve interference with the TBP-TATA box interaction affecting E2 mediated transcription initiation.

L2 has been shown to associate with the transcription repressor PATZ (Gornemann *et al.*, 2002) (Table 1.4). PATZ (POZ-AT hook-zinc finger protein) is a member of the BTB/POZ family (for Broad complex, tramtrack, and bric à brac and POZ for poxvirus and zinc finger), which associates with RNF4 RING finger protein, a mediator of androgen receptor activity and acts as a transcription repressor (Fedele *et al.*, 2000). POZ/ZF (zinc finger) proteins display a transrepressive activity in a variety of cell types and on various promoters (Huynh and Bardwell, 1998). As with other POZ proteins, the repressor activity of PATZ is mediated by its POZ domain (Fedele *et al.*, 2000). The mechanisms by which transrepression by PATZ occurs still remains unclear but its interaction with L2 could be implicated with repression of E2 transcription transactivation. PATZ has been shown to localise to nuclear dot and to colocalise with L2. The presence of PATZ and the recruitment of E2 to PODs by L2 could result in PATZ transrepression of E2 activation.

The question arises as to why L2 should act to repress the transcription transactivation function of E2 during the viral life cycle. Expression of L2 represents a shift from the maintenance stage of the viral life cycle to the productive stage as cellular differentiation is initiated resulting in expression of the structural proteins. At this stage, the expression of E6/E7 needs to be down-regulated to enable cellular differentiation to proceed. Although over-expression of E2 down-regulates transcription of E6/E7, E2 is also needed to enhance genome replication. Expression of L2 could assist in down-regulating E2 transcription transactivation from the viral LCR with E2 still being able to associate with E1 in genome replication. To further enhance this hypothesis, the effect of L2 on E2 function in DNA replication was investigated.

6.2.2. DNA replication

Papillomaviruses replicate their genomes as episomal DNA using E2 and E1 (LaPorta and Taichman, 1982). E2 binds to E1 and promotes origin-dependent viral DNA replication. In this study, transient replication assays were used to investigate the effect of L2 on E2 function in viral replication. These assays allowed for the detection of low-level replication corresponding to the maintenance stage of the viral life cycle. At this stage, HPV-16 DNA has been shown to replicate bidirectionally via theta structures (Flores and Lambert, 1997). With every round of replication, theta structures require initiation involving E2 tethering E1 to the origin and allowing for the recruitment of the rest of the DNA replication machinery. In differentiated epithelial cells HPV-16 DNA has been shown to replicate by a rolling cycle mode of replication which is unidirectional and

requires one initiation event leading to amplification of PV DNA resulting in multiple copies of the genome (Flores and Lambert, 1997) consistent with the productive phase of the viral life cycle.

The effect of L2 on the ability of E2 to co-operate with E1 in a transient DNA replication assay was studied. It was shown that expression of L2 had no effect on the ability of E2 to enhance DNA replication in C33a cells as seen in figure 3.8. This is in agreement with previous studies showing that BPV-1 L2 does not interfere with the replication function of BPV-1 E2TA (Heino *et al.*, 2000). These results indicate that L2 preferentially inhibits the transcription transactivation function of E2 in both bovine and human papillomavirus systems with no effect on E2 function in replication. The loss of E2 function in transcription transactivation but not replication as a result of expression of L2 during productive stage of the viral life cycle is supported by data showing that E2 mutants which had no transcription transactivation function but retained replication activity did not significantly interfere with the stable maintenance of episomes, the expression of late genes, or the differentiation-dependent amplification of the viral genome (Stubenrauch *et al.*, 1998). Altogether, these results suggest a novel function for L2 as a possible negative regulator of E2 transcription transactivation during cell differentiation and the viral genome amplification stage of the viral life cycle.

In this study a transient replication system corresponding to the maintenance stage of the viral life cycle was used which requires one initiation event with every round of replication possibly requiring more E2. In this system, no effect of L2 on E2 function in

replication was detected. As one initiation event is expected to occur during the productive stage of the viral life cycle, less E2 would be required suggesting that L2 should not affect viral DNA amplification at this stage.

HPV-11 E1, E2 and replicating DNA have been shown to associate with PODs after transient co-transfection of the ori plasmid and expression plasmids for E1 and E2 (Swindle *et al.*, 1999), indicating that PODs could be sites of HPV DNA replication as observed with Herpes Simplex virus (HSV), which form replication compartments juxtaposed to PODs (Lukonis and Weller, 1997). SV40 DNA replication also occurs in similar POD associated foci (Ishov and Maul, 1996) suggesting that recruitment of viral proteins to PODs with subsequent association with viral replication could be a common feature among DNA viruses. Although L2 does not affect E2 function in replication, L2 could assist in the translocation of the necessary viral proteins required for replication and subsequently packaging.

6.2.3. Protein Expression

E2 degradation is mediated through the ubiquitin-proteasome pathway (Bellanger *et al.*, 2001; Penrose and McBride, 2000). L2 down-regulates the expression of E2 in HaCaT, and U2OS cells as well as in HaCaT cells stably expressing E2 as shown in figure 4.1. This effect did not occur in C33a cells suggesting a cell type dependence for L2 induced down-regulation of E2 expression. The presence of proteasome inhibitors failed to restore E2 to normal amounts leading to the conclusion that proteasome mediated degradation is not the only mechanism implicated in L2-induced decrease of E2 levels. As E2 mRNA levels were shown not to change in cells with or without L2 expression (section 4.1.2),

down-regulation of transcription of the E2 gene by L2 can be discounted. How L2 leads to such a decrease in E2 levels remains unclear, particularly as this down-regulation does not take place in C33a cells. As E2 gene transcription is not affected by L2, a possible mechanism for the down regulation of E2 repression could be the inhibition of E2 protein translation.

Interestingly L2 had no effect on E2 protein turnover in HaCaT cells (section 4.2). Even though both systems used to analyse E2 stability in the presence of L2 differed with respect to the measurement of protein turnover, the half-life of E2 remained unchanged. In pulse chase experiments, the degradation of E2 results from a continuous expression of L2 as protein translation is not switched off. Treatment of cells with cycloheximide (CHX) on the other hand efficiently shuts down protein translation. As a result, the observed E2 half-life is due to the effect of any residual L2 present.

In both systems, L2 did not appear to change the half-life of E2. The reason why no change in E2 half-life in the presence of L2 was observed even though L2 is capable of reducing E2 levels is unclear. The experimental protocol used allows for the measurement of E2 half-life only after the effect of L2 on E2 protein levels has taken place. As a result, residual E2 protein not affected by L2 expression has similar kinetics of half-life with the wild type E2. A system whereby L2 could be induced instead of co-transfecting with E2 could result in an improved analysis of the effect of L2 on E2 half-life.

The down regulation of E2 protein expression by L2 is interesting as this effect occurs in HaCaT and U2OS cells but not in C33a cells. HaCaT are spontaneously immortalised keratinocytes (Boukamp *et al.*, 1988) while U2OS are osteosarcoma cell lines. C33a are cervical carcinoma derived cells, which have no HPV DNA. The difference in E2 expression in the presence of L2 could represent differences in the model of carcinogenesis represented by the different cell lines used. HaCaT cells represent an *in vitro* carcinogenesis model of human skin keratinocytes while U2OS cells represent a model for carcinogenesis of human osteocytes. C33a cells represent an *in vitro* model for cervical carcinogenesis. The mechanism for L2 down regulation of E2 expression in one cell line but not the other requires further investigation.

6.3. HPV-16 L2 deletion mutants and E2 function

The role of L2 during the life cycle of papillomaviruses is still unclear, particularly as it has been shown that the major structural protein L1 can self assemble into capsid-like particles (Kirnbauer *et al.*, 1993; Zhou *et al.*, 1993), suggesting that L2 is not required in the assembly of the viral capsid. Recent studies have begun to enhance our understanding of the function of L2 with reports that L2 is able to increase the efficiency of DNA encapsidation (Zhao *et al.*, 1998) and infectivity of HPV-33 peudovirions generated in COS-7 cells (Unckell *et al.*, 1997). The observation that co-expression of BPV-1 L2 and E2 causes the redistribution of E2 into PODs (Day *et al.*, 1998; Heino *et al.*, 2000) opens up the possibility that L2 could posses non-structural functions.

To further characterise the effect of L2 on E2 function, deletion mutants of L2 were constructed to identify domain or domains of L2 responsible for regulating E2 function in transcription transactivation and the modulation of E2 expression. The domain or domains responsible for direct physical interaction between E2 and L2 was also investigated.

6.3.1. L2-E2 Association

L2 is able to affect the transcription transactivation but not replication function of E2 as well as reduce the level of E2 expression, as a result, the ability of L2 to physically interact with E2 was investigated to determine if binding of L2 with E2 correlated with an effect on transcription transactivation and E2 protein expression levels. In this study

Glutathione S-transferase (GST) pull-down assays were performed using GST-L2 and GST-L2 deletion mutants.

To generate GST-L2 deletion mutants, both N-terminal and C-terminal deletions of HPV-16 L2 were constructed to yield fusion proteins with the GST moiety at the amino terminus. All mutants were cloned as BamHI-EcoR1 inserts into the multiple cloning site of the pGEX-4T-2 plasmid (Amersham) in which, expression of GST is under the control of the *tac* promoter which can be induced using isopropyl β-D thiogalactoside (IPTG). All mutants were subsequently expressed in BL21 *E. coli*, which are protease deficient and designed to maximize expression of full-length fusion proteins. Once all mutants were sequenced to confirm that the inserts were in the correct orientation, expression studies were performed to optimise fusion protein expression for each mutant. Optimisation of expression involved growing bacteria transformed with GST-L2 or GST-L2 deletion mutants at various temperatures (30-37°C), for different times (3-24 hours) and induction with different amounts of IPTG (0.1-1mM) in order to determine the best growth conditions for each mutant. Expression of full-length GST-L2 and its deletion mutants proved quite difficult, as L2 appeared to be very unstable. As a result, when bacteria were lysed and the GST fusions were analysed by SDS-PAGE and Western blotting, multiple bands were often seen. With subsequent manipulation of the growth conditions, a temperature of 37°C and an incubation time of 5-6 hours after induction with 0.5mM IPTG was chosen as the best expected migration of each fusion protein analysed after SDS-PAGE and Western blotting was observed in these conditions.

GST pull-down assays were performed by incubating beads bound to GST-L2 or each GST-L2 deletion mutant with *in vitro* translated ^{35}S-methionine labelled HPV-16 E2. The results indicated that GST L2 deletion mutants expressing 1-50 and 1-100 amino acids of L2 fused to GST showed strong binding to E2, comparable to binding with full length L2. This indicated that the N-terminus of L2 mediates binding with E2. BPV-1 E2 contains two BPV-1 L2 interaction domains (Heino *et al.*, 2000). No E2 interaction domains on L2 have previously been identified. A 30 amino acid sequence of HPV-33 L2 between amino acids 390-420 is required for localisation of L2 to PODs (Becker *et al.*, 2003). As a result, we could hypothesise that L2 localises to PODs via its C-terminal portion and binds E2 via its N-terminus recruiting it to PODs. Recruitment of E2 subsequently results in the down regulation of E2 transcription transactivation leading to the down regulation of E6/E7 expression. Recruitment of E2 to PODs by L2 also allows for the recruitment of the viral replication machinery facilitating genome amplification and subsequent packaging and release of the mature virion (Fig 6.1). This is confirmed by studies, which have shown that E2 enhances packaging of full-length plasmid DNA in BPV-1 pseudovirions (Zhao *et al.*, 2000). E2 increased the number of VLPs incorporating a circular plasmid supporting the role of L2 and E2 interaction in facilitating packaging of the circular PV genome in to the viral capsid.

6.3.2. Cellular localisation of GFP-L2 and GFP-L2 deletion mutants

Papillomavirus late genes are inefficiently expressed in undifferentiated cells possibly due to the presence of codons that are rarely used in mammalian cells. This could help in restricting late gene expression to terminally differentiating cells. Consequently, detection of L2 with eucaryotic expression plasmids designed to produce L2 in

mammalian cells is not very efficient. L2 mRNA has negative regulatory sequences in the 3' end, which reduces protein levels approximately 10 fold (Sokolowski *et al.*, 1998). In an attempt to obtain an efficient detection system for visualising L2 expressed from transiently transfected expression plasmids, fusion forms of L2 and L2 N-terminal and C-terminal deletion mutants fused to the green florescent protein (GFP) were constructed.

Fluorescence microscopy revealed HPV-16 GFP-L2 localised to the nucleus (Fig 5.5b). In this study no localisation to PODs was observed, in contrast to HPV-33 GFP L2, HPV-6b L2 or BPV-1 L2, which have all been shown to localise to PODs (Becker *et al.*, 2003; Roden *et al.*, 2001; Sun *et al.*, 1995). Differences in localisation could be a result of differences in the mode of transfection. The use of recombinant vaccinia viruses or baculoviruses is commonly used (Xi and Banks, 1991; Zhou *et al.*, 1991) which appears to result in expression levels of L2, which permit visualisation of L2 localisation to PODs (Becker *et al.*, 2003; Roden *et al.*, 2001; Sun *et al.*, 1995). Codon modification of L2 has also been used to detect localisation by immunofluorescence (Heino *et al.*, 2000). In this study, transient transfections with plasmids expressing wild type L2 or L2 deletion mutants were used resulting in high levels of protein expression not permitting visualisation of co-localisation of L2 with PODs although localisation to the nucleus was observed.

The C-terminal deletion mutants all localised specifically to the nucleus with their distribution not differing from wild type GFP-L2. Expression of the first 50 amino acids of L2 fused to GFP was sufficient for localisation to the nucleus suggesting the presence

of a nuclear localisation signal (NLS) at the N-terminus of HPV-16 L2. In contrast, the N-terminal deletion mutants localised to both the nucleus and the cytoplasm. As the mutant GFP-L2 1-50 localised to the nucleus and N-terminal deletion mutants with sequences either completely or partially lacking the first 50 amino acids of L2 localised to both the nucleus and the cytoplasm, these results indicate that the sequences contained within the first 50 amino acids of L2 are sufficient to localise and retain GFP in the nucleus. Disruption of this sequence inhibits the effective retention of GFP to the nucleus as observed with the N-terminal deletion mutants. HPV-6b L2 contains three potential NLS, localised at the N-terminus, the C-terminus and around amino acids 286 to 306 (Sun *et al.*, 1995). HPV-33 L2 also has two NLS located within the central and C-terminal part of L2, homologous to HPV-6b L2 NLS (Becker *et al.*, 2003). From this study, HPV-16 L2 has been shown to have an NLS at both the N and C-terminus.

6.3.3. Expression of L2 and L2 deletion mutants in HaCaT

GFP-L2 and GFP-L2 deletion mutant expression plasmids were transfected into HaCaT cells and protein extracts were analysed by SDS-PAGE and Western blotting to determine the levels of expression of each mutant. Detection of GFP-L2 and its deletion mutants was performed using an anti-HPV-16 L2 polyclonal rabbit serum and anti-GFP polyclonal antibody. GFP-L2 was detected at the expected migration but multiple bands were also detected indicating breakdown of the fusion protein. GFP-L2 mutants 1-400, 1-300 and 1-200 all gave quite a high steady state levels of expression. The L2 serum could not efficiently detect GFP-mutants 1-100 and 1-50 possibly due to loss of the L2 epitope. This is confirmed by the observation that both could be detected using an anti-GFP antibody. Detection of the GFP-L2 N-terminal deletion mutants 25-473 and 50-473 could

be achieved with the L2 serum but a lower steady state of expression was observed. The L2 serum could not detect mutants 150-473, 350-473 and 390-473. This loss of detection could be as a result of the presence of negative regulatory sequences present at the C-terminus of L2 mRNA, which has been shown to reduce protein levels (Sokolowski *et al.*, 1998). No detection of bands at the expected migration for all N-terminus deletion mutants was observed using an anti-GFP antibody. Although the GFP-L2 N-terminus deletion mutants could not be observed by Western blotting, they could be efficiently detected by immunofluorescence. As such it is possible that the process of cell extraction and SDS polyacrylamide gel electrophoresis significantly reduces the ability to detect the mutants by immunoblotting

6.3.4. E2 function and L2 deletion mutants

The domain or domains of L2 that mediate the down-regulation of the transcription transactivation function of E2 were investigated. HPV-16 L2 and L2 deletion mutants were cloned into pCDNA 3.1 expression plasmid. All plasmids were sequenced to determine that all inserts were in the correct orientation and subsequently transfected into HaCaT and C33a cells to determine the effect of each deletion mutant on the transcription transactivation function of E2 in order to correlate binding of L2 and E2 with the down-regulation of E2 mediated transcription transactivation. In C33a cells, most mutants lead to a down regulation of transcription transactivation at the highest amount of L2 used except for mutants 1-200 and 150-473, suggesting the presence of an overlapping sequence between amino acids 150-200 which seems to alleviate L2 induced inhibition of E2 transcription transactivation. Mutants 1-50 and 1-100 had less of an effect on E2 transcription transactivation than full length L2. Plasmids expressing 350-473 and 390-

473 amino acids of L2 gave strong repression of transcription transactivation at the highest amount of L2 transfected. In HaCaT cells, mutant 1-200 did not repress transcription as observed with C33a cells. Mutants 1-100 and 1-50 also did not repress transcription transactivation at least not to levels comparable with full length L2. This appears to indicate that in HaCaT cells, deletion of the C-terminus of L2 alleviates the repression of E2 mediated transcription transactivation. The N-terminal deletion mutants of L2 in HaCaT cells gave slightly less repression of E2 transcription transactivation, with mutants 50-473 and 150-473 showing no repression even at the highest level of L2.

The difficulty in using deletion mutants for such studies is the role of the protein three-dimensional structure to determine protein function. As a result, linear deletions could provide results difficult to interpret with regard to the full-length protein although it may give us an understanding of the role of certain sequences within the protein. An alternative method would be to use site directed mutagenesis to construct mutants of L2 which retain their three dimensional structure but with mutations in sites implicated in protein-DNA or protein-protein interaction.

6.3.5. L2 deletion mutants and E2 protein expression

Following on from studies examining the effect of L2 deletion mutants on the transcription transactivation function of L2, the effect on E2 protein expression levels was also examined to determine a correlation between binding and inhibition of transcription transactivation and reduction of protein level. Full-length L2 and all L2 deletion mutants cloned into pCDNA 3.1 were co-expressed with E2 in HaCaT cells.

Using SDS-PAGE and Western blotting, the levels of E2 after co-expression of L2 or each deletion mutant were analysed. It was observed that the full length L2 is required for the down regulation of E2 expression as seen in figure 5.8. In cells co-expressing E2 and L2 deletion mutants, the level of E2 was not inhibited indicating that the three dimensional structure of the protein is required for this effect.

6.4. Summary

In summary, this thesis has been able to elucidate that HPV-16 L2 has a selective effect on the functions of HPV-16 E2. L2 is able to down regulate the transcription transactivation function of E2 in a cell line independent manner. No effect of L2 on E2 mediated DNA replication was observed. L2 was also able to reduce the level of E2 expression in HaCaT and U20S cells but not in C33a cells indicating that the down-regulation of transcription transactivation is not due to the reduction in E2 expression levels. The effect of L2 on E2 expression in HaCaT cells was further investigated by examining the effect of L2 on E2 mRNA levels and protein half-life. No difference in E2 mRNA or protein half-life was detected indicating that the effect of L2 on E2 protein expression could be an effect on E2 protein translation and not transcription.

To further investigate the effect of L2 on E2 function, a series of L2 deletion mutants were constructed and GST binding assays were performed to determine the domains of L2 required for direct physical interaction with E2. The first 50 amino acids of L2 was shown to bind in vitro translated E2 comparable to binding with full-length L2 indicating that the N-terminus of L2 mediates the binding of L2 with E2. To determine the cellular

localisation of L2 and its deletion mutants, GFP fusion proteins were constructed and detected by immunofluorescence. GFP-L2 and all C-terminal deletion mutants localised and were retained in the nucleus. All N-terminus deletion mutants localised to both the nucleus and the cytoplasm indicating the presence of two NLS in the C-terminus and N-terminus of HPV-16 L2. Each mutant was analysed for expression by immunoblotting which showed the detection of GFP-L2 and all C-terminal deletion mutants with either anti-L2 serum or anti-GFP antibodies. Detection of N-terminal deletion mutants was difficult although mutants 25-473 and 50-473 could be detected with anti-L2 serum.

The effect of L2 deletion mutants on the transcription transactivation function of E2 was investigated and mutants 1-200 and 150-473 did not inhibit the transcription transactivation function of E2. In HaCaT cells, mutants 1-100 and 1-50 also did not inhibit E2 transcription transactivation indicating that the C-terminal domain of L2 could be responsible for the down regulation of E2 function in transcription transactivation. Deletion of the carboxyl and amino terminus of L2 did not result in the reduction of E2 expression levels, only full length L2 was able to reduce the level of E2 expression indicating the requirement of the secondary structure of the protein for this effect.

The full-length L2 protein, which localises to the nucleus binds E2, down regulates its transcription transactivation function but not replication and is able to reduce the level of E2 protein expression as summarised in Table 6.1. Binding with E2 does not correlate with the down regulation of transcription transactivation as L2 mutants 1-50, 1-100 and 1-200 all bind E2 but do not (especially in the case of HaCaT cells) down regulate E2

transcription transactivation. Binding also does not correlate with down-regulation of E2 expression as only full length L2 is able to reduce E2 expression levels while mutants 1-50, 1-100, 1-200 and 1-400 which all bind E2 do not affect E2 protein expression.

Table 6.1. Summary of the characterisation of L2 deletion mutants. Table shows the summary of the characterisation of L2 and the L2 deletion mutants indicating domains involved with binding of L2 with E2, cellular localisation, mutants involved with down-regulating E2 transcription transactivation and the effect of each mutant on E2 protein expression levels.

Summary of L2 deletion mutants

HPV-16 L2 amino acid sequence	Binding with E2	Cellular Localisation	Down regulation of HPV-16 E2 transcription transactivation	Down regulation of HPV-16 E2 expression
L2	Yes	Nuclear	Yes	Yes
1-50aa	Yes	Nuclear	Yes/No	No
1-100aa	Yes	Nuclear	Yes/No	No
1-200aa	Yes	Nuclear	No	No
1-300aa	Yes (Low)	Nuclear	Yes	No
1-400aa	Yes	Nuclear	Yes	No
25-473aa	No	Nuclear/cytoplasmic	Yes	No
50-473aa	No	Nuclear/cytoplasmic	Yes	No
150-473aa	No	Nuclear/cytoplasmic	No	No
250-473aa	No	Nuclear/cytoplasmic	Yes	No
350-473aa	No	Nuclear/cytoplasmic	Yes	No
390-473aa	No	Nuclear/cytoplasmic	Yes	No

6.5. E2, L2, PODs and the viral life cycle

The life cycle of papillomavirus is intimately tied to the differentiation of the keratinocyte (Fig 1.4). The early stages of the virus life cycle take place in the lower layers of the epithelium and, according to a widely accepted paradigm, include maintenance of the viral DNA as an episome. This requires the expression of low levels of E1 and E2. In addition to its involvement in episomal DNA maintenance, E2 transactivates the early viral transcriptional promoter (Bouvard *et al.*, 1994) and initiates the expression of the transforming genes E6/E7, responsible for the induction of unscheduled cell proliferation (Fig 1.7). No L2 is expressed in these epithelial layers, as terminal differentiation is not initiated. The inhibition of terminal differentiation of promyleocytes has been observed to be associated with the disruption of PODs as a result of the fusion of PML with the retinoic acid receptor α (Grignani *et al.*, 1993). A similar disruption of PODs in papillomavirus infected keratinocytes would, in conjunction with the expression of the transforming proteins, delay cell differentiation and favour cell proliferation, as in myeloid cells (Wang *et al.*, 1998a), thus helping the virus replicate its DNA.

As the infected keratinocytes progress from the lower layers to the more superficial ones, the expression levels of E2 increase up to the stratum spinosum, above which E2 is no longer detected (Penrose and McBride, 2000). High levels of E2 repress the early promoter (Bouvard *et al.*, 1994; Steger and Corbach, 1997), thus down-regulating expression of the transforming genes and favouring cell differentiation and expression of the structural proteins. In the differentiating superficial layers, L2 is expressed and

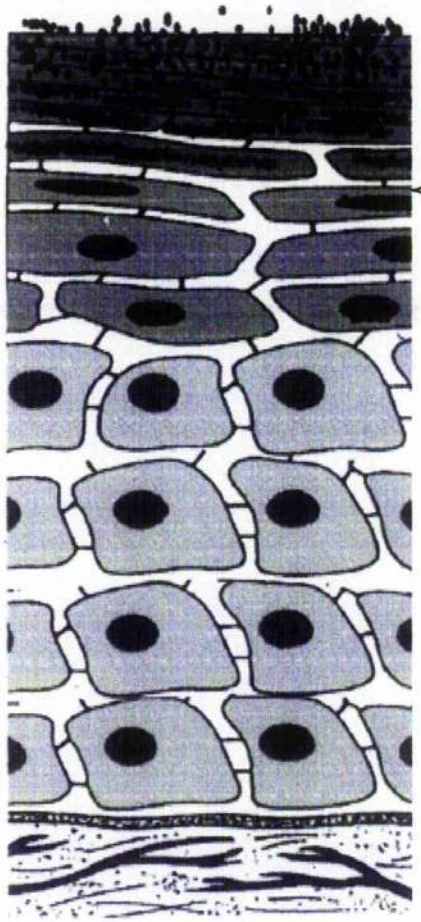
contributes to the reduction in E2 transcriptional control function while the maintenance of its replication functions would help the production of mature virus. The recruitment of E2 to PODs by L2 could represent a switch in the virus life cycle from the non-productive to the virion productive phase as suggested by Heino *et al.*, (2000). E2 has a high affinity for the viral DNA, and it has been proposed that the recruitment of E2 into PODs directs the viral genomes into regions of high concentrations of structural proteins, so helping DNA encapsidation and virion assembly (Day *et al.*, 1998). The set of hypotheses outlined above requires that, although believed to function at different stages of the viral life cycle and in different layers of the epithelium, E2 and L2 are co-expressed and interacting during keratinocyte differentiation. The proposed outcome of the L2-E2 interaction is shown diagrammatically in Fig 6.1. In conclusion, the interaction between E2 and L2 observed for BPV-1 and now for HPV-16 elucidating the effect of L2 on E2 function in transcription transactivation and replication, suggests that this may be part of a common function or phase in papillomavirus life cycle, and therefore not merely a peculiarity of cultured cells

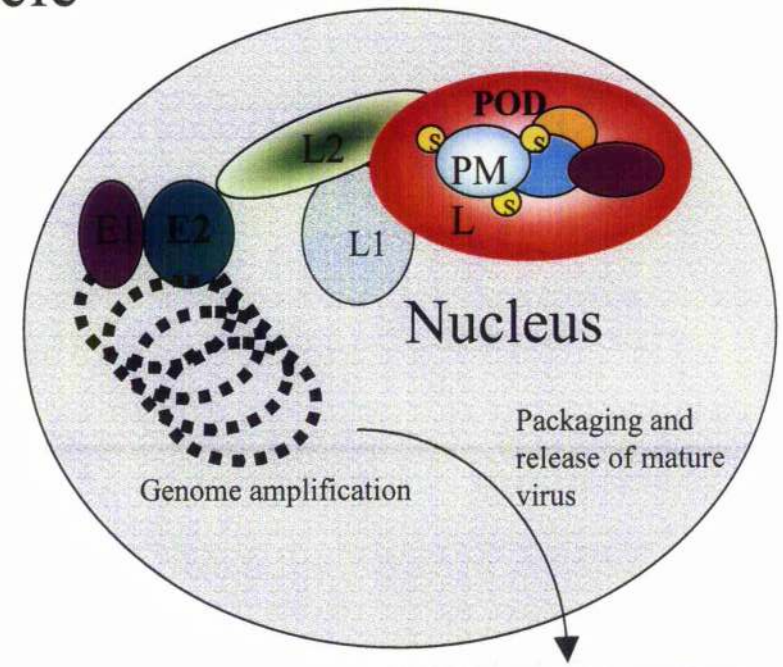
Figure 6.1. Cartoon showing the possible role for L2-E2 interaction during the viral life cycle. Recruitment of E2 to PODs by L2 leads to a down regulation of transcription of E6 and E7 and the possible recruitment of the cellular DNA replication machinery as E2 mediated DNA replication is not affected. Subsequently recruitment of L1 to PODs by L2 would allow for packaging of replicated viral DNA resulting in the release of mature virus.

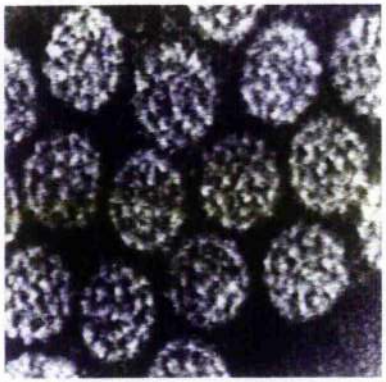
E2, L2 PODs and the papillomavirus Life Cycle

Expression of L2 leading to E2 recruitment, down regulation of transcription, viral genome amplification and subsequent packaging

Expression of E2, up regulation of transcription of E6/E7and low level viral replication







6.6. Future Work

The effect of L2 on the functions of E2 raises some interesting questions that would be of immense interest for future experiments. Firstly, the ability of L2 to down regulate the transcription transactivation function of E2 in transient transcription assays requires further investigation to elucidate the mechanism of action of L2 in this process. As L2 has been shown to associate with a known transcription repressor PATZ, the effect of PATZ in this process is of interest. Transient transcription assays would be used, co-transfecting E2 with PATZ to study the effect of PATZ on E2 transcription transactivation. This has been observed for co-expression of PATZ and RNF4 resulting in a strong repression of RNF4-mediated activation (Fedele *et al.*, 2000). The effect of PATZ on the activity of the LCR would also be of interest. The association of PATZ with L2 may be further investigated to determine if an increase in L2 expression results in a subsequent rise in PATZ levels. As L2 associates with PATZ at PODs (Gornemann *et al.*, 2002), the correlation between L2 localisation to PODs and down-regulation of transcription transactivation would also be examined to determine if the effect of a possible L2-PATZ interaction is dependent on localisation to PODs.

In this study, deletion mutants of L2 were used to analyse the domain or domains of L2 required for the modulation of E2 function or for mapping sites required for the physical interaction of L2 with E2. Although this provided useful information for understanding L2 effect on E2 function, it failed to provide a full-length mutant of L2 that does not bind E2. Such a mutant would provide a useful tool to further understand the role of L2 on E2 function in transcription transactivation, replication and E2 protein expression. This could

be achieved by site directed mutagenesis of the N-terminal domain of L2 responsible for binding to E2.

In this study, the effect of L2 on E2 function was observed in monolayers with the use of HaCaT, C33a and U2OS cell lines, a system which will facilitate the study of the effect of L2-E2 localisation to PODs on viral transcription during cell differentiation will provide an effective tool to observe this interaction in an *in vivo* situation. W12, which are, immortalised keratinocytes derived from low-grade cervical intraepithelial neoplasia biopsy, containing episomal HPV-16 genomes (Sterling *et al.*, 1990) will be used to establish organotypic raft cultures. W12 cells can differentiate and form ordered layers of differentiating cells in organotypic raft cultures which can be assessed by production of differentiation markers, late viral proteins and viral capsids (Sterling *et al.*, 1990). In raft cultures, keratinocytes are grown submerged on a bed of collagen containing fibroblasts and when the culture is raised to the liquid-air interface, the keratinocytes undergo differentiation. This will provide the possibility to follow events in the individual layers of a differentiating epithelium by histological techniques

To study the effect of L2 on E2 activity during differentiation, cells expressing E2 and L2 will be transfected with the LCR reporter constructs driving expression of green fluorescent protein (GFP) or β-galactosidase markers and grown on raft cultures. The use of a GFP reporter will allow analysis by confocal microscopy of regulated viral gene expression in the layers of the raft culture. These studies will be complemented by analysis of transactivation of the LCR by staining sections of raft cultures for β-

galactosidase. Serial sections will be analysed for expression of the differentiation markers keratin 1, involucrin and filaggrin. The results should establish if an interaction between an early and a late protein changes LCR activity. This would suggest that *in vivo* the interplay between early and late proteins could change the viral gene expression program, perhaps helping to sustain the switch from early promoter transcription to late promoter transcription during keratinocyte differentiation.

The loss of PML from PODs leads to loss of PML functions (Zhong *et al.*, 2000) and possibly preventing differentiation of keratinocytes in analogy to maturation arrest of myeloid cells in acute promyelocytic leukaemia (APL). In APL cells, the PML gene is disabled by a translocation with the gene for the retinoic acid receptor (Sternsdorf *et al.*, 1997). It has been shown that fibroblasts from $PML^{-/-}$ mice lacking PODs exhibit an increased proportion of cells in S phase and display a marked growth advantage compared to $PML^{+/+}$ cells (Wang *et al.*, 1998b). The loss of PML will be of immense benefit to certain viruses such as papillomaviruses, which require a delay in cell differentiation supporting the maintenance stage of the viral life cycle. To determine the role of PML in the effect of L2 on E2 function in transcription transactivation, $PML^{-/-}$ and $PML^{+/+}$ cells will be used in E2 dependent transient transcription assays, co-transfecting E2 with increasing concentrations of L2. PML has been shown to be a tumour suppressor with $PML^{-/-}$ mice giving rise to more papillomas than $PML^{+/+}$ mice, with papillomas occasionally progressing to carcinomas after several months (Wang *et al.*, 1998b). The level of E2 expression in $PML^{-/-}$ and $PML^{+/+}$ cells co-expressing E2 and L2 will be examined to determine if PML is required for L2 mediated reduction in E2 protein

expression levels. Also transient DNA replication assays will be performed in $PML^{-/-}$ and $PML^{+/+}$ cells, which will be able to further elucidate the role of PML and PODs to papillomavirus DNA replication with the hypothesis that the presence of PML and hence PODs will enhance the level of viral DNA replication.

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