The role of DNA damage proteins and signaling pathways in the regulatory functions of the Human Papillomavirus 16 E2 protein

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Disclaimer:

'Unless stated otherwise all work contained within this thesis has been completed personally'

Ewan R. Taylor

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For my Mum and Dad

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For my Aunt Mary.

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Abbreviations

Abbreviation	Definition
A	Angstrom
AMF-1	Activation domain modulating factor
AP1	Activator protein-1
ATP	Adenosine triphosphate
ARS	Autonomous replication sequence
bp	Base pair
BPV	Bovine papillomavirus
BSA	Bovine serum albumin
BRCT	BRCA1 Carboxy-terminal
CBP	CREB binding protein
CKII	Casein Kinase II
CPD	Cyclobutane Pyrimidine Dimer
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DLG	Disc Large Protein
E.coli	Escherichia coli
ECL	Enhanced chemiluminescence
FCS	Foetal calf serum
g	Gram

HAT	Histone Acetyl Transferase
HPV	Human papillomavirus
HSV	Herpes simplex virus
kb	Kilobase pairs
kD	KiloDalton
L	Litre
LCR	Long control region
LDS	lithium dodecyl sulfate
µg	Microgram
µl	Microlitre
M	Molar
MHC	Major Histocompatability
MF	Mutation Frequency
rMF	Rearrangement Mutation Frequency
mg	Milligram
ml	Mililitre
mM	Milimolar
mRNA	Messenger ribonucleic acid
MW	Molecular Weight
NER	Nucleotide Excision Repair
nm	Nanometres
^{o}C	Degree centigrade
OD	Optical Density (light absorbance)

ORC	Origin of replication complex
Ori	Origin of replication
ORF	Open reading frame
PARP	Poly(ADP-ribose) Polymerase
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
P.C.	Personal Computer
PCR	Polymerase Chain Reaction
pmol	Picomole
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SV40	Simian Virus 40
TBP	TATA box binding protein
TLS	Translesion Synthesis
TopBP1	Topoisomerase binding protein 1
tk	Thymidine kinase
UV	Ultraviolet
UVB	Ultraviolet wave length B (312nm)
UVC	Ultraviolet wave length C (254nm)
v/v	Volume per unit volume
WT	Wild Type
w/v	Weight per unit volume

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

Abstract

Human papillomavirus type 16 (HPV16) is a causative agent of cervical cancer. The HPV16 viral transcription/replication factor E2 is essential for the viral life cycle. The function of E2 is dependenton the interaction with cellular partner proteins. The amino-terminal domain of E2 is an acidic protein-protein interaction domain essential for all of the functions of E2. A yeast-2-hybrid screen using the amino-terminal domain of HPV16 E2 as bait identified multiple possible partner proteins for E2 function (Boner & Morgan 2002) including the DNA replication/repair protein TopBP1. E2 molecules from HPV16 and HPV18 interact with multiple proteins involved in the cellular response to DNA damage (e.g. p53, BRCA1, PARP and TopBP1). The role of TopBP1 in E2 function and the functional response of E2 to DNA damage stimuli were investigated. Overexpression of TopBP1 enhances the transcription and replication activation functions of E2. Overexpression of an amino-terminal truncation mutant of TopBP1 has no effect on the transcription/replication functions of E2. During this study a novel method for the detection of E1/E2 DNA replication function by real-time PCR was developed. The UVB irradiation of cells resulted in the significant reduction of both E2 transactivation function and E2 protein amount. These results demonstrated that E2 function is altered by cellular DNA damage response proteins and signaling pathways.

In many HPV induced cancers the HPV genome is either present integrated into the cellular chromosomes or is maintained episomally with large DNA deletions/rearrangements. Therefore HPV genome stability is a significant risk factor for

the development of HPV induced cancer. Thus the fidelity of DNA replication activated by the HPV16 E1/E2 replication factors was investigated on undamaged and UVC damaged templates in a variety of genetic backgrounds. On undamaged DNA templates there were a significant amount of mutations due to DNA deletions/rearrangements and the frequency of mutation increased when the template was damaged. This increase on damaged templates was marked in cells with defects in key DNA replication or repair proteins. These results highlight the instability of HPV16 genome replication. The interaction of E2 with DNA damage response proteins and the reduction of E2 function in response to DNA damage may be an evolutionary response by the virus to ensure genetic integrity and host cell viability.

Introduction

1.1 Human Papilloma Virus

Papilloma viruses are non-enveloped icosahedral double stranded DNA viruses that have been identified in a wide range of animals from birds to rabbits, cattle and several primates including humans (Campo 2002). These small DNA tumour viruses generally selectively infect cutaneous and mucosal epithelia and a few also infect fibroblasts (e.g. Bovine papillomavirus 1). All human papillomaviruses (HPVs) are specifically epitheliotropic. HPVs are thought to infect the undifferentiated keratinocytes in the basal layer of stratified epithelium. The life cycle of HPVs is subsequently dependent of the differentiation of the epithelium. Additionally the ability of HPVs to modulate cellular differentiation processes results in the wide variety of lesions caused by HPV infection (see review Burd 2003).

1.2 HPVs and Disease

Papilloma viruses cause a wide variety of diseases ranging from skin and anogenital warts to laryngeal papillomas and anogenital intraepithelial neoplasias that often progress to malignancy (Croissant *et al* 1985). There are currently over 100 types of human papilloma virus identified. HPVs are termed "low risk" or "high risk" depending on their prevalence in cancers (Munoz *et al* 2003). Low risk HPVs commonly cause benign lesions that regress and do not progress to cancer. High risk HPVs are commonly detected in many epithelial derived cancers and are found in more than 99.7% of all cervical carcinomas (Walboomers *et al* 1999). HPV infection is thought to be a major risk factor for the malignant transformation of epithelium. A summary of the characteristics of a selection of HPV viruses is shown in table 1.1.

The main HPV subtype studied in this project was HPV16.

TABLE 1.1 The characteristics of a selection of HPV types

HPV type	Target tissue	Disease Caused (Risk classification)
HPV1	Cutaneous epithelium	Plantar warts (Low risk)
HPV6	Mucosal eplithelium	Genital warts, Laryngeal papillomatosis (Low/high risk)
HPV8	Cutaneous epithelium	Epidermodysplasia Verruciformis (High risk to immunocomprimised patients and UV exposed skin sites)
HPV16	Mucosal epithelium	Cervical cancer, mouth and throat cancer. (High risk)
HPV18	Mucosal epithelium	Cervical cancer (High risk)

Table adapted from Burd 2003.

1.3 HPV16

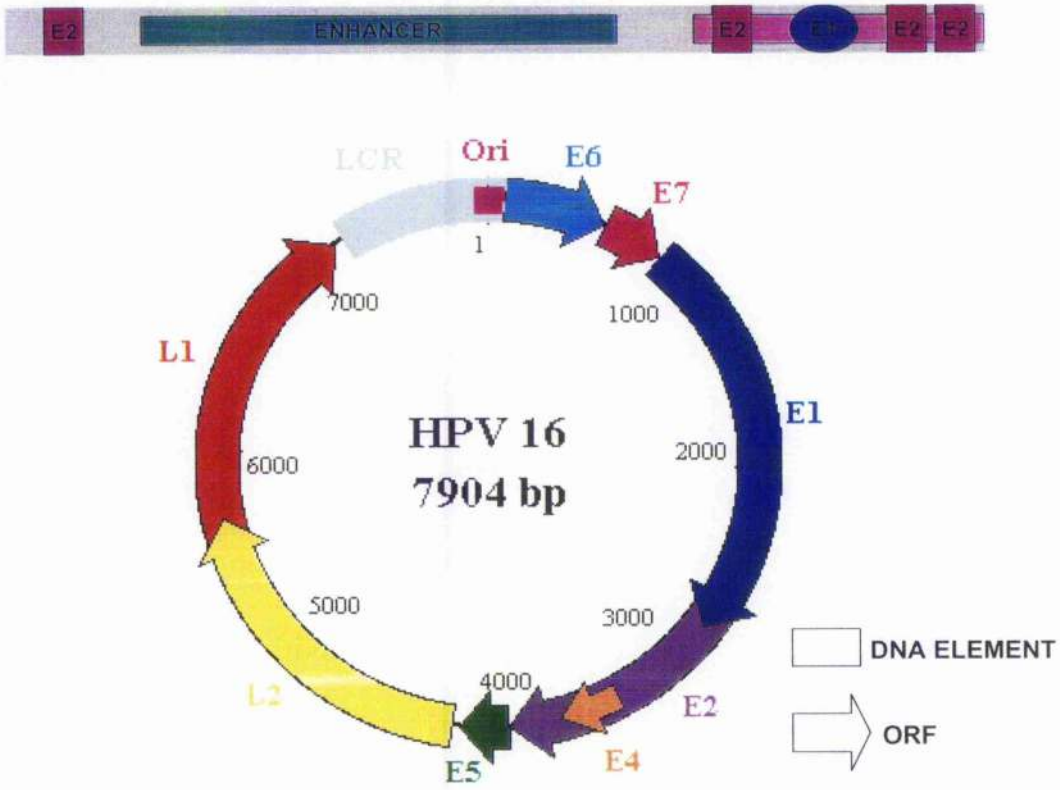
HPV-16 is the most prevalent HPV subtype detected in cervical cancers and it is also found in cancer of the tonsils (Clifford *et al* 2003, Klussmann *et al* 2001). HPV16 has a 7.9kb circular double stranded DNA genome that can be split into early open reading frames (ORFs) (E1-E7) and late ORFs (L1-L2, see figure 1.1). "Early" proteins modulate viral transcription, facilitate viral genome replication, modulate the cell cycle and impede cellular antigen presentation. "Late" proteins are the viral capsid proteins.

The HPV16 genome (accession number NC 001526) can be separated into three regions; an "early" region containing ORFs E1-E7, a "late" region containing ORFs

L1 and L2, and a non-coding region rich in transcription factor binding sites called the long control region (LCR). At the end of each coding region there are polyadenylation sites. HPVs express many different polycystronic transcripts throughout the viral life cycle thus co-ordinating the expression of the viral gene products with the differentiation of the host cell. Additionally transcription initiates from several different promoters during keratinocyte differentiation (Doorbar *et al* 1990, Baker & Calef 1996). Viral transcription is controlled by cellular transcription factors binding the LCR and by the virally encoded E2 protein (Bouvard *et al* 1994, Romanczuk *et al* 1990, Steger & Corbach 1997). The epithelial specificity of HPV infection is thought to be due in part to specific control elements in the enhancer and promoter sequences in the viral LCR (Vance *et al* 1999). Viral replication is initiated within the viral origin of replication (Ori) and is dependent on the virally encoded replication factors E1 and E2 (see review Desaintes and Demeret 1996 for details on transcription and replication control).

Figure 1.1

Map of the HPV16 genome



Map of the HPV16 genome. The above map highlights the significant features within the HPV16 genome. The major open reading frames (ORFs) are drawn as arrows and DNA elements that control viral transcription and replication are drawn as boxes. The long control region (LCR) is boxed in grey and at the top this region is expanded out to demonstrate the position of the E1 binding site and the E2 binding sites within the LCR. A 200 base pair region at the 3' end of this sequence is the minimal region of sequence required for efficient viral DNA replication and is called the origin of replication (Ori). The area highlighted in dark green within the LCR is the epithelial specific enhancer that is rich in transcription factor binding sites.

1.4 HPV16 Life Cycle

Following infection the HPV genome is amplified then maintained at a low level (approx 50 genome copies/cell) in the basal layers of the epithelium. Expression of the "early" ORF facilitates the establishment of the infection. The early proteins E5, E6 and E7 modulate the cellular control of differentiation and the cell cycle (see Fehrmann & Laimins 2003 for review). This results in the proliferation of infected cells and a drive into S-phase that allows the replication of the viral genome and its segregation into the daughter cells. The HPV genome replication is dependent on E1 and E2 and the cellular replication apparatus and E2 also controls viral transcription (see sections 1.5.2 & 1.6). As an infected cell migrates upwards from the basal layers the cell undergoes differentiation. Amplification of the genome to high levels prior to encapsidation is thought to occur with HPV16 in cells of the suprabasal layer. E1^E4 expression is first seen in these cells (Middleton *et al* 2003) and cells in this layer are thought to be held in a pseudo-S-phase due to the co-expression of E6, E7 and the E1^E4 protein (see section 1.5.2). Subsequently the virus expresses the "late" proteins L1 and L2 and the viral genome is encapsidated. Infectious virions are released from the most terminally differentiated keratinocytes as they are sloughed off the top layers of the epithelium. The majority of HPV infections have a productive life cycle as described, however in many HPV induced cancers the viral genome is found integrated into the host genome (zur Hausen 1987). This integration usually deletes E2 coding sequences and results in the elevated expression of the viral oncoproteins, thus driving the cell towards transformation (Jeon & Lambert 1995, Jeon *et al* 1995). In some tumours the viral genome can be found both integrated and episomally, and often some of the episomal genomes contain deletions or genomic rearrangements (Kasher & Roman 1988, Deau *et al* 1991, Hall *et al* 1997, Kalantari *et al* 2001, Mellin

et al 2002). Therefore the loss of viral genome integrity is an important step on the road to HPV induced carcinogenisis.

1.5 HPV Proteins

1.5.1 Transforming proteins

The major transforming proteins of HPV16 are E6 and E7, also E5 is thought to have a minor role in cellular transformation. HPV **E6** protein disrupts the function of p53 to release the cell from p53 mediated growth control mechanisms (see Mantovani & Banks 2001 for review). "High risk" E6 proteins target p53 for ubiquitin mediated degradation (Li & Coffino 1996), some "low risk" E6 proteins are able to bind p53 *in vitro* yet do not target p53 for proteolysis *in vivo* (Elbel *et al* 1997). Elucidation of how the binding to E6 and proteolysis of p53 occurs has helped the understanding of the ubiquitin proteolysis machinery with the identification of E6-AP (E6 Associated Protein) and related proteins (Scheffner *et al* 1994).

Additional mechanisms of p53 functional down regulation have also been identified with both high risk and low risk E6 proteins demonstrating the ability to repress p53 mediated transcription. This is due to E6 binding p53 through its carboxy-terminus and *in vitro* E6 can prevent p53 from binding its DNA recognition sites (Crook *et al* 1994). E6 also binds the transcriptional coactivator p300/CBP therefore hypothetically downregulating p53 dependent transcription by competing for this co-factor (Zimmermann *et al* 1999).

E6 mediates the ubiquitin degradation of the protein disc large (DLG), a protein localized at cell junctions (Kiyono *et al* 1997). The disruption of cell junction cell-cell communication results in loss of contact inhibition of growth and a subsequent increased probability for an invasive malignant phenotype. Also E6 interacts with

Paxillin, a component of focal adhesions resulting in the loss of cell adhesion to the extra cellular matrix (Tong & Howley 1997). Other targets for E6 interaction include c-Myc, bak, the calcium binding protein E6BP and the AP1 component of clathrin coated vesicles (Chen & Defendi 1992, Thomas & Banks 1999). Additionally E6 can stimulate telomerase activity during keratinocyte immortalisation (Klingelhutz *et al* 1996). Telomerase is the terminal chromosomal DNA polymerase, a protein often expressed in malignant tumours that can release cells from senescence by re-synthesizing their telomeres.

E7 binds the tumour suppressor pRb and targets it for ubiquitin mediated proteasome degradation (Hickman *et al* 1994, Hwang *et al* 2002). E7 also binds and inactivates p107 and p130, two pRb related proteins. pRb in its hyperphosphorylated form binds multiple E2F family transcription factors which control the expression of cyclin E, cyclin A and cdc25A which are all G1/S phase boundary control proteins (Stevaux & Dyson 2002). E7 also inactivates the cyclin-dependent kinase inhibitors $p21^{WAF-1}$ and $p27^{KIP-1}$ resulting in further enhancement of the proliferative signal (Jones *et al* 1997, Funk *et al* 1997, Sherr & Roberts 1999). Additionally E7 is able to bind transcription factor AP-1 and activate its activity and therefore hypothetically alter the differentiation of the cell (Antinore *et al* 1996).

The loss of pRb function alone doesn't allow uncontrolled proliferation due to negative feedback exerted by p53 on growth control. The major functions of p53 are to arrest the cell cycle (G1 or apoptosis) in response to DNA damage (e.g. radiation, oxidative stress, chemotheraputics) or improper growth signaling (e.g. oncogenic ras). To escape this effect tumours are often null for functional p53 and viruses that require a proliferating cellular environment often disrupt p53 function. Therefore the

combination of E6 and E7 in cells strongly drives cells to proliferate and escape cellular growth controls.

E5 is a small hydrophobic transmembrane dimeric protein localized in the golgi apparatus and the plasma membrane. E5 from HPV16 and BPV1 both interact with the 16 kDa ductin subunit of the vacuolar H+ ATPase resulting in inhibition of the acidification of the golgi and downstream endomembrane compartments (Straight *et al* 1995, Schapiro *et al* 2000). With BPV1 and BPV4 E5 expressing cells the interaction with 16 kDa results in the loss of endomembrane compartment structural regulation, characterized by the swelling and fragmentation of the golgi apparatus (Ashrafi *et al* 2002). One consequence of this is thought to be the inhibition of MHC class I molecule transport to the plasma membrane, thus preventing virally infected cells from being recognized by the immune system (Marchetti *et al* 2002). Additionally BPV4 E5 reduces MHC I protein levels. HPV16 E5 expression does not lead to endomembrane disorder however it does significantly inhibit MHCI transport (M.Haghshenas unpublished results). Using the epithelia cell raft culture system to study the HPV life cycle, HPV genomes with a deletion in the E5 ORF are able to establish a complete viral life cycle (Genther *et al* 2003). Therefore evasion from the immune system may be the primary function of E5, however there are several other effects of E5. The E5-16kDa subunit association is thought to be responsible for the down regulation of gap junction communication between HPV16 infected cells as 16kDa is an integral component of these structures (Oelze *et al* 1995). HPV16 E5 also activates the epidermal growth factor receptor EGF-R thus inducing a proliferative signal (Pim *et al* 1992). E5 therefore facilitates evasion from the immune system, causes a mobile invasive phenotype within expressing cells and induces proliferation

signal transduction pathways.

1.5.2 Viral DNA replication proteins

HPVs replicate their DNA using the cellular DNA replication machinery. Efficient HPV DNA replication requires the two HPV coded proteins E1 and E2. Together these proteins recruit the cellular proteins required for HPV DNA replication. Also the viral E1^E4 protein has recently been identified as having a significant role in the vegetative replication of the viral genome.

The HPV16 **E1** protein is a nuclear phosphoprotein of 649 amino acids. The C-terminal amino acids (aa 371 to 623 in HPV16) have significant homology with SF3 DNA helicase domains found in proteins like the SV40 replication protein large T antigen (Fouts *et al* 1999). E1 is an ATP dependent DNA helicase that is essential for viral DNA replication (Hughes & Romanos 1993). E1 binds to an AT rich region within the Ori as a hexamer where it hydrolyses ATP to unwind the DNA (Sedman & Stenlund 1998). HPV16 E1 is able to initiate DNA replication *in vitro* in cellular extracts at a low level however for efficient DNA replication E2 is required (Seo *et al* 1991, Kuo *et al* 1994, Liu *et al* 1995). E1 binds E2 and at the viral replication origin this interaction enhances the binding of E1 to the viral replication origin and thus allows efficient HPV DNA replication (discussed in detail later). E1 is essential for both the initiation of viral replication and the elongation of the replication fork (Liu *et al* 1995). To accomplish this function E1 interacts with a variety of cellular proteins. The E1 amino terminus binds multiple sub-units of the polα-primase (Masterson *et al* 1998, Conger *et al* 1999). Polα-primase is the only polymerase able to initiate DNA synthesis therefore it is essential at the replication fork. The p68 binding domain is in

a region of the E1 C-terminal where E2 binds therefore it is possible that the initiation of DNA synthesis by E1 is after the function of E2 in HPV replication has been fulfilled.

The SWI/SNF chromatin altering complex has been identified as a HPV18 E1 partner by yeast 2 hybrid (Lee *et al* 1999). This multiprotein complex relaxes chromatin structure to aid access and binding of transcription and replication factors to DNA. Through additive titration and antisense experiments the complex isolated, Ini1/hSNF5, has been demonstrated to be vital for HPV replication.

HPV11 E1 can bind and functionally interact with the DNA histone H1 protein (Swindle & Engler 1998). H1 histone is the protein that links adjacent nucleosomes thus ordering the condensation of chromatin to a higher order. It is thought that E1 can displace histone H1 from HPV genomic DNA. HPV11 E1 also interacts with the chaperone proteins Hsp70 and Hsp40, this interaction enhances E1 loading onto DNA and increases HPV replication (Liu *et al* 1998, Lin *et al* 2002).

Like SV40 large T antigen and cellular DNA helicases, E1 interacts with the single stranded DNA binding protein complex RPA (Han *et al* 1999). The RPA complex is thought to have multiple roles during DNA replication and it is essential for the correct initiation and the elongation of DNA replication forks.

HPV11 E1 protein is a substrate for the cyclinE/cyclin dependent kinase (cdk). The cyclin E/cdk complex can phosphorylate E1 and it is thought that this phosphorylation is essential for the function of E1 (Ma *et al* 1999). Therefore like the cellular Mcm2-7 DNA helicase complex, the E1 hexamer requires cdk dependent phosphorylation to initiate replication.

E4 is a 10 kDa protein that is expressed from a spliced mRNA that includes part of the E1 ORF fused to the E4 ORF hence is often referred to as E1^E4. E1^E4 is the only viral early protein that can be easily detected in papilloma infected lesions. HPV16 E1^E4 expression is first detected in the parabasal layers. In many papillomavirus lesions vegetative DNA replication co-localises with the layers when E1^E4 expression is first detected (Doorbar *et al* 1997, Middleton *et al* 2003). Although a direct role for E1^E4 in viral replication has not yet been demonstrated several observations suggest how it may be able to significantly enhance viral replication. Many HPV E1^E4 proteins, including HPV16 E1^E4, induce a cell cycle arrest at the G2/M boundary (Davy *et al* 2002, Nakahara *et al* 2002). HPV16 E1^E4 and the DNA replication marker PCNA co-localise in a thin layer when E1^E4 is first expressed (Middleton *et al* 2003). This presents the hypothesis that cells co-expressing E1^E4 and the viral oncoproteins E5, E6 and E7 are held in a G2/pseudo S-phase cell cycle arrest. E5, E6 and E7 activate genes responsible for DNA replication, and during this E1^E4 mediated G2/pseudo S-phase HPV DNA replication will have free access to cellular DNA replication proteins. The cell line W12 is derived from a HPV16 infected patient and early passages of this cell line contain almost exclusively episomal copies of the HPV genome (Alazawi *et al* 2002). In undifferentiated W12 cells the replication of the HPV16 genome is initiated bidirectionally (Flores & Lambert 1997). Induction of differentiation in W12 cells causes a change in HPV genome replication mode to a unidirectional rolling circle mechanism which is thought to be more efficient at producing multiple genome copies (Flores & Lambert 1997). As E1^E4 expression is upregulated by epithelial differentiation (Baker & Calef 1996) this collection of circumstantial evidence indicates that E1^E4 has a significant role in the expansion of HPV genome number.

Therefore the exact role of E1^E4 in HPV genome amplification is a good target for future study.

HPV16 E1^E4 associates with intermediate filaments resulting in the total collapse in the cytokeratin matrix within the cell (Doorbar *et al* 1991, Roberts *et al* 1997). The consequence of this is speculated to be the destruction of the influence of the cytokeratin matrix at desmosomes resulting in efficient virion release through easier sloughing off the infected epidermal layer. Also HPV16 E1^E4 binds a RNA helicase, E4-DBP, a function that only HPV 16 E4 demonstrates with HPV 1 and HPV 6 exhibiting no binding (Doorbar *et al* 2000). E4-DBP is also thought to have a role in pre-mRNA stability and ribosome biogenesis therefore it is thought that E4 may play some role in late gene expression (Doorbar *et al* 2000).

1.5.3 Capsid proteins

The "late" proteins L1 and L2 are expressed in the terminally differentiated layers of the epithelium and encapsidate the viral genome (Firzlaff *et al* 1988, Okun *et al* 2001). L1 is the major capsid protein in the virion. L2 is the minor capsid protein and is found mainly on the inside of the virion. L2 is a small protein that is able to bind both L1 and E2 (discussed 1.6.1). On encapsidation of the double stranded circular viral genome the virion conformationally changes into an icosahedral structure with 72 pentamers of L1.

1.6 HPV16 E2 protein

1.6.1 Structure and functions

HPV16 E2 is the major protein of interest in this study. The HPV E2 protein has three major functions, it activates transcription, it activates viral replication and it induces cell cycle arrest/apoptosis (Dell & Gaston 2001 for review). HPV E2 proteins have a conserved structure of three domains; an amino-terminal activation domain, a central hinge domain and a carboxy-terminal DNA binding domain (see figure 1.2). The carboxy-terminal DNA binding domain of E2 binds as a homodimer to 12bp palindromic sequences in the viral LCR (Hirochika *et al* 1987, McBride *et al* 1991). This DNA binding ability is essential for the E2 transactivation and replication functions (Hirochika *et al* 1988, Ustav *et al* 1993).

The central hinge domain of E2 is a flexible region that is rich in potential phosphorylation sites (Gauthier *et al* 1991). The HPV16 protein is a phosphoprotein, however the phosphorylation sites of HPV16 E2 have not yet been mapped (Sanders *et al* 1995). BPV1 E2 is phosphorylated on both serine and threonine residues, the major phosphorylation sites have been mapped in the hinge domain and are serines 289 and 301 (McBride *et al* 1989, Penrose & McBride 2000). Additionally the hinge region is enriched with proline and arginine residues that are thought to aid the flexibility of this region. The hinge region of HPV E2 is highly divergent between different HPV subtypes.

The amino-terminal activation domain is essential for the transactivation, replication and apoptotic functions of HPV16 E2. Point mutational analysis of highly conserved residues in the E2 amino-terminal of several different papillomaviruses has identified several critical residues for the transactivation and replication functions (Ferguson &

Botchan 1996, Abroi *et al* 1996, Brokaw *et al* 1996, Saki *et al* 1996). Alanine mutation of either R7, R37, I73, E74 or Q76 renders HPV16 E2 null for transactivation function but almost wild type for replication functions. Combining this data and the crystal structure of the HPV16 amino terminus has led to the hypothesis that E2 can homodimerise at its amino terminus and this homodimerisation is essential for transactivation function (Antson et al 2000). Alanine mutation of either Q12 or E39 renders the protein null for activating replication and binding E1, highlighting the pocket where E1 binds E2. However these two mutations have a minimal effect on transactivation functions (Saki et al 1996). Interaction between E1 and E2 is essential for replication of the viral genome, E2 binds to DNA binding sites that flank the E1 binding site and the amino-terminal of E2 interacts with E1 and aids E1 recruitment to the origin of replication (Chao *et al* 1999, Titolo *et al* 1999). Replacement of the E2 DNA binding domain with either the Gal4 or LexA DNA binding domains creates a chimeric E2 molecule able to activate transcription (Breiding *et al* 1996). Additionally replacement of the BPV1 E2 DNA binding domain with the EBNA1 DNA binding domain allows E1/E2 mediated DNA replication from a BPV replication origin with the E2 binding sites replaced with EBNA1 binding sites (Kivimae et al 2001).

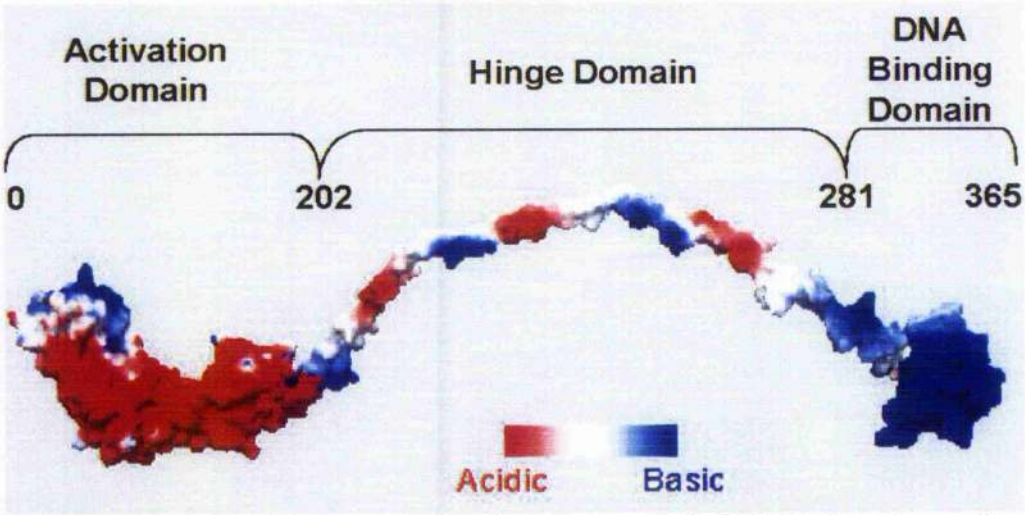
The BPV1 E2 protein is thought to tether the BPV genome to cellular chromosomes during mitosis thus ensuring efficient segregation of the papillomavirus genome into the daughter cells. BPV1 E2 binds to the cellular condensed chromosomes during mitosis and this interaction is mediated through attachment at the amino-terminal of E2 (Skiadopoulos & McBride 1998, Bastien & McBride 2000). While the interaction of HPV16 E2 with mitotic chromosomes has not yet been investigated it would be a beneficial function for HPV16 E2 and the HPV16 life cycle.

Multiple "high risk" HPV E2 proteins modulate the cell cycle and/or induce apoptosis in a variety of different genetic backgrounds. This is discussed below in 1.6.3.

An interaction between the papillomavirus E2 and L2 proteins has been identified for both BPV1 (Day *et al* 1998, Heino *et al* 2000) and HPV16 (Pablo Cordano & Afam Okoye unpublished results). Both the BPV1 and HPV16 L2 proteins are localised in punctate nuclear foci known as PODs (PML onocogenic domain). These structures are defined by the presence of the PML protein and other integral POD proteins arranged in ~10 foci within the nucleus. Other viral proteins associate with these structures and this common association of viral proteins with PODs is also observed in SV40, HSV1 and Ad5 viruses (Maul 1998 for review). POD domains are thought to be cellular organisation centres of various proteins involved in DNA replication, repair and cell cycle control (Negrorev & Maul 2001 for review). BPV1 L2 localises L1 and E2 to these POD organisation centres (Day *et al* 1998). These are hypothesised to be viral genome packaging centres, where E2 through binding the genome at its carboxy-terminal recruits HPV genomes to L1 and L2. To support this idea it has been demonstrated that BPV1 E2 enhances the encapsidation of viral DNA into pseudovirion virus like particles generated in cell culture (Zhao *et al* 2000). Additionally BPV1 or HPV16 L2 expression results in the reduction of the E2 transactivation function (Heino *et al* 2000, Afam Okoye personal communication). The consequence of this during the HPV life cycle is hypothesised to result in the reduction of E6/E7 expression thus allowing the keratinocytes to further differentiate.

Figure 1.2

Structure of HPV16 E2



Computer generated image of the 3D structure of HPV16 E2. The surface electrostatic potential is illustrated and was generated using the Swiss-PdbViewer programme. At the amino-terminal is the acidic activation domain, the central portion is the flexible hinge and at the carboxy-terminal is the largely basic DNA binding domain. The activation domain crystal structure was solved using X-ray diffraction by (Antson *et al* 2000), and the "1dto.pdb" entry in the protein data bank was used in the image above. This structure highlights the role of the acidic transactivation domain in exposing hydrophobic residues at surfaces and highlights the surfaces for E1 interaction and E2 amino-terminal homodimerisation (see figure 3.1.14). The central region is not predicted to have a set secondary structure and is thought to be a flexible hinge between the amino and carboxy-terminal domains. The DNA binding domain crystal structure was solved using X-ray diffraction (Hegde and Androphy 1998) and the "1by9.pdb" entry in the protein data bank is used in the above image. This domain binds the E2 DNA binding site therefore has predominantly basic surface electrostatic potential.

1.6.2 E2 cellular partner proteins

The E2 protein contains no enzymatic activity therefore to function E2 interacts with multiple cellular partner proteins. Trying to understand the role of each of these proteins in the function of E2 is currently central to E2 research.

BPV1 HPV11 and HPV16 E2 are known to bind TFIIB (Rank and Lambert 1995, Yao *et al* 1998, Hou *et al* 2000, Ushikai *et al* 1994), also BPV1 and HPV11 are known to bind TBP. These cellular proteins are two essential components of the RNA polymerase II transcription apparatus. This interaction is likely to be central to the transactivation function of E2.

E2 from HPV16, HPV18 and HPV8 all interact with the cellular co-activator protein p300 and/or its homologue CREB binding protein, CBP (Marcello *et al* 2000, Lee *et al* 2000, Muller *et al* 2002). p300/CBP synergistically enhance E2 mediated transactivation. p300/CBP molecules contain intrinsic histone acetyltransferase activity, activate transcription due to its chromatin remodelling activity and p300/CBP is an intrinsic part of the RNA polymerase II holoenzyme. Additionally p300/CBP interacts with partner proteins that contain HAT activity like p/CAF. HPV18 binds p/CAF and p/CAF synergistically activates E2 mediated transactivation (Lee *et al* 2002). For each of these proteins the HAT activity is essential for the activation of E2 transactivation. BPV1 E2 also binds to the p300 interacting AMF1 protein (Activation domain modulating factor). These three proteins are thought to complex together to activate transcription (Breiding *et al* 1997, Peng *et al* 2000). Additionally mutants of E2 that fail to bind AMF1 fail to activate transcription, and are defective for replication even though they can still interact with E1. Therefore all these results

suggest that the ability to modulate and relax chromatin structure is central for E2 mediated transactivation and replication.

BPV1 E2 interacts with the SMN nuclear protein (Strasswimmer *et al* 1999). SMN is involved with the assembly of splicosomes and the transcriptosome. SMN can interact with the RNA helicase A. Deletion mutants of SMN inhibit E2 mediated transactivation. The HPV5 E2 protein also interacts with SR an snRNP protein involved in pre-RNA splicing through RS/RG/SRG repeats in the hinge region (Lai *et al* 1999).

1.6.3 E2 cellular partner proteins: DNA damage proteins

The transactivation function of HPV16 E2 is enhanced when BRCA1 is overexpressed (Kim *et al* 2003). BRCA1 interacts with the carboxy-terminus of E2. E2 interacts with both the amino-terminal domain and the carboxy-terminal BRCT domains in BRCA1. Point mutation of the BRCT domains in BRCA1 disrupts the enhancement of E2 transactivation function. Additionally chromatin immunoprecipitation (ChIP) assays demonstrate that E2 recruits BRCA1 to E2 dependent promoters *in vivo*. BRCA1 is a protein intimately involved in DNA damage responses, cell cycle checkpoints, and transcription control. However the biological function of the E2-BRCA1 interaction is unknown.

The transactivation function of HPV18 E2 is enhanced by the overexpression of poly (ADP-ribose) polymerase (PARP) (Lee *et al* 2002). E2 co-localises with PARP and PARP binds the carboxy-terminal of E2. E2 binds to the amino-terminal domain containing the Zn finger and BRCT motif. Additionally PARP enhances E2 DNA binding *in vitro* and E2 recruits PARP to E2 dependent promoters *in vivo* as determined by ChIP. PARP is an enzyme that modifies nuclear proteins by the

attachment of poly(ADP)-ribose side chains, this activity is stimulated in response to DNA damage. PARP binds to DNA structures containing either nicks, gaps, cruciforms, and bent DNA structures. PARP has several functions involved in the regulation of cellular transcription, replication and DNA repair (Herceg & Wang 2001 for review). However the biological role of the E2-PARP interaction is unclear.

The p53 tumour suppressor protein binds to the carboxy-terminal of E2 and modulates several of E2 functions. The over-expression of p53 in p53 defective cells results in a significant decrease in E1/E2 mediated replication (Lepik *et al* 1998). This repression is thought to be dependent on p53 binding E2. Replication by SV40 large T antigen is also repressed by p53 over-expression (Lepik & Ustav 2000). In contrast Epstein-Barr virus replication mediated by EBNA1 is unaffected (Lepik *et al* 1998). p53 is found at sites of viral DNA replication in SV40, Herpes viruses and in Adenoviruses. What remains to be determined is the role of this interaction in HPV replication. Is this a genuine repression of E2 function or does p53 play a fundamental role in the HPV DNA replication process?

In cell lines derived from HPV associated cervical cancers with an integrated HPV genome (e.g. HeLa, CaSki) expression of HPV16 or HPV18 E2 induces apoptosis (Dowhanick *et al* 1995, Sanchez-Perez *et al* 1997, Desaintes *et al* 1999). In HPV associated cancer cell lines a common event is the deletion of E2 coding sequences during viral integration. This results in very high levels of expression of the viral oncogenes E6 and E7. Expression of either HPV16 or 18 E2 in these cells represses the expression of E6 and E7 resulting in the induction of apoptosis.

In HPV negative cell lines two studies present differing information about the role of p53 in the induction of apoptosis by HPV16 and HPV18 E2. HPV16 E2 induces

apoptosis in HPV negative cells by a p53 dependent mechanism (Webster *et al* 2000). In cells with a disrupted p53 (e.g. C33a and SaOs2) E2 fails to induce apoptosis. Further expression of a trans dominant negative p53 or expression of HPV16 E6 blocks the induction of apoptosis by E2. In HeLa cells (HPV positive) the induction of apoptosis is not dependent on E2 regulating transcription and the amino-terminal domain of E2 is essential for the induction of apoptosis. However the role of the amino-terminal or a protein-protein interaction at the amino-terminal responsible for apoptosis has yet to be identified.

In contrast to HPV16 E2, HPV18 E2 induces apoptosis in both p53 wild type and compromised cell types through activation of caspase 8 (Demeret *et al* 2003). The induction of this apoptosis is dependent on the amino-terminal transactivation domain of E2. The amino terminus of HPV18 E2 is a target of caspase processing and contains a XEXD caspase cleavage site. HPV16 E2 also contains this motif. There is therefore a complex relationship between E2 and p53 and the viral life cycle that still requires clarification.

1.6.4 Identification of TopBP1 as a potential E2 partner protein

In a yeast 2 hybrid screen using the amino-terminal of HPV16 E2 as the bait several new potential cellular partner proteins for E2 mediated function were identified and described in detail in Boner and Morgan 2002. From this screen TopBP1 was identified as a potential E2 functional partner protein and this interaction is investigated in section 3.1 (see also Boner *et al* 2002). TopBP1 is a large (1435aa) nuclear protein that is implicated in many nuclear processes such as DNA replication initiation, DNA repair and cell cycle control. TopBP1 was first cloned from a screen to identify proteins that interact with the regulatory domain of Topoisomerase IIβ and

it is the carboxy-terminal of TopBP1 that mediates this interaction (Yamane *et al* 1997). The carboxy-terminal of TopBP1 is where HPV16 E2 binds TopBP1 (Boner *et al* 2002).

TopBP1 contains eight BRCT (i.e. BRCA1 Carboxy-terminal) domains and has a carboxy-terminal nuclear localisation signal (figure 3.1.1). BRCT domains are conserved protein interaction domains found in proteins from yeast to humans that function in cellular DNA replication, repair and transcription processes (Huyton *et al* 2000). TopBP1 is a phosphoprotein and contains a potential poly(ADP)-ribosylation modification site within BRCT domain six (Yamane *et al* 1997, Makiniemi *et al* 2001).

Homologous proteins to TopBP1 are found in all eukaryotes. In fission and budding yeast the TopBP1 homologues, Cut5 and Dpb11 respectively, control multiple DNA damage and cell cycle responses (Verkade *et al* 1998, Harris *et al* 2003, Arakai *et al* 1995, Masumoto *et al* 2000). Cut5/Dpb11 is essential for activation of S-phase checkpoint responses and DNA replication. Dpb11 is found at yeast ARS sequences and can bind both DNA polymerase alpha and epsilon at these sequences. The disruption of the Drosophila homologue of TopBP1, Mus101, results in defective DNA synthesis, genetic instability and the failure to condense chromatin (Yamamoto *et al* 2000). The failure to condense chromatin in Mus101 defective cells indicates that Mus101 may be an integral part of the chromosome scaffold and therefore TopBP1 is a good target protein for the attachment of E2 to condensed chromosomes during mitosis (Bastien & McBride 2000).

The BRCT domains of TopBP1 are able to bind single stranded DNA, double stranded DNA and DNA ends (Yamane & Tsuruo 1999). TopBP1 interacts with multiple proteins involved in DNA replication and DNA damage responses. TopBP1

interacts with DNA polymerase epsilon and TopBP1 is demonstrated to be essential for DNA replication *in vitro*. TopBP1 co-localises with BRCA1 during S-phase at sites distant from replication forks. In response to replication blockage by UV irradiation TopBP1 and BRCA1 co-localise with PCNA at replication forks (Makineimi *et al* 2001). In cells where double-stranded breaks are induced TopBP1 redistributes to nuclear foci with the DNA damage response proteins BRCA1, PML, NBS, 53BP1, BLM, γH2AX and Rad9 (Greer *et al* 2003, Yamane *et al* 2003). TopBP1 binds the cellular checkpoint protein Rad9 (Makineimi *et al* 2001). Both TopBP1 and Rad9 are thought to be early DNA damage sensor/signalling molecules and the localisation of TopBP1 to double stranded DNA break sites is dependent on Rad9 and Rad9 phosphorylation (Greer *et al* 2003, St Onge *et al* 2003). Overexpression of Rad9 phosphorylation or TopBP1 interaction mutants results in a prolonged G2/M arrest. This is proposed to be due to defects in the S-phase checkpoint in a manner similar to that found with ATM, BRCA1 and NBS defective cells (Xu *et al* 2002, Xu *et al* 2003). Additionally cells that are compromised for both TopBP1 and BRCA1 function are null for the G2/M checkpoint in response to gamma irradiation DNA damage (Yamane *et al* 2003). This is due to an abolition of the regulation of Chk1 kinase by this double knockout. TopBP1 interacts with E2F1, a transcription factor that has an integral role in the control of the cell cycle (Liu *et al* 2003). In response to adriamycin induced DNA damage TopBP1 is responsible for the co-localisation of E2F1 to punctate nuclear foci containing TopBP1 and BRCA1. Therefore TopBP1 is hypothesised to be responsible for the repression of E2F1 activities including, the activation of transcription and the entry into S-phase. Finally, TopBP1 interacts with the transcription factor Miz-1, a protein that modulates the transcription of multiple anti-mitogenic proteins (Herold *et al* 2002). TopBP1 is

thought to negatively regulate the expression of Miz-1 responsive genes. This repression is alleviated in response to UV irradiation due to the degradation of TopBP1 and is characterised by the activation of p21Cip1 expression. The studies summarised above demonstrate that TopBP1 has an integral role in DNA damage responses and cell cycle control.

The Xenopus homologue of TopBP1, Xmus101, has been used with *in vitro* assays to show that Xmus101 is essential for the loading of the initiation factor Cdc45 to the DNA replication initiation complex (Van Hatten *et al* 2002). The loading of Xmus101 at these complexes is dependent on the origin recognition complex, ORC. Additionally the action of S-phase cyclin dependent kinases (CDKs) at the preinitiation complex is dependent on Xmus101 (Hashimoto & Takisawa 2003). In fission yeast the TopBP1 homologue Dpb11 is essential for the replication checkpoint response. Dpb11 is essential to prevent the loading of DNA polymerase epsilon to late replication origins when the early replication forks are blocked by hydroxyurea treatment (Masumoto *et al* 2000).

Therefore TopBP1 has a wide range of activities from co-ordinating DNA replication initiation to sensing DNA damage/stalled replication and subsequently exerting checkpoint responses. It is therefore possible that TopBP1 could have several roles in the function of E2 ranging from mitotic chromosome attachment, transcription modulation, replication modulation, to mediating E2 induced apoptosis. The role of the TopBP1-E2 interaction to the transcription and replication functions of E2 is investigated in section 3.1.

1.7 DNA replication and DNA damage checkpoint control

The E2 interacting proteins p53, BRCA1, PARP and TopBP1 have many common features about their cellular function. Each of these proteins is part of parallel signalling pathways involved in DNA damage responses and replication control as outlined in the simplified diagram in figure 1.3. At sites of DNA damage BRCA1, TopBP1 and PARP serve as both sensors and signaller thus co-ordinating the cellular response to DNA damage therefore ensuring genomic integrity (Jasin 2002, Greer *et al* 2003, Herzeg & Wang 2001). Downstream signalling by the ATM/ATR/DNA-PK and Chk1/2 kinase network, by p53, and by PARP modulate the cell cycle to induce cell cycle delay/arrest or apoptosis (see Pasero *et al* 2003, Melo & Toczyski 2002, Zachos *et al* 2002, McGowan 2002, Bernstein *et al* 2002 for reviews). This network ensures faithful repair of damaged DNA thus maintaining the integrity of the genome. The complexity of this network ensures the appropriate cellular response to the relevant type of DNA damage. ATM and DNA-PK are activated by double stranded DNA breaks throughout the cell cycle and ATR is activated by the inhibition of the progress of the DNA replication fork during S-phase. However this classification is a generalisation and there are exclusions to the rule currently being identified. ATM/ATR/DNA-PK phosphorylate downstream targets including Chk1/2, p53, BRCA1 and TopBP1 resulting in the activation of the appropriate cell cycle delay and DNA repair response.

The biological role of the p53, BRCA1, PARP and TopBP1 interactions with E2 is not clear. p53, BRCA1, PARP and TopBP1 may interact with E2 to function as scaffolds that enhance the recruitment of cellular transcription and replication complexes to E2. Alternatively E2 may induce apoptosis through interacting with one

or more of these proteins. However it is possible the hard wiring of E2 into these DNA damage response pathways may have a fundamental role to play for the successful completion of the viral life cycle.

This thesis has three research aims;

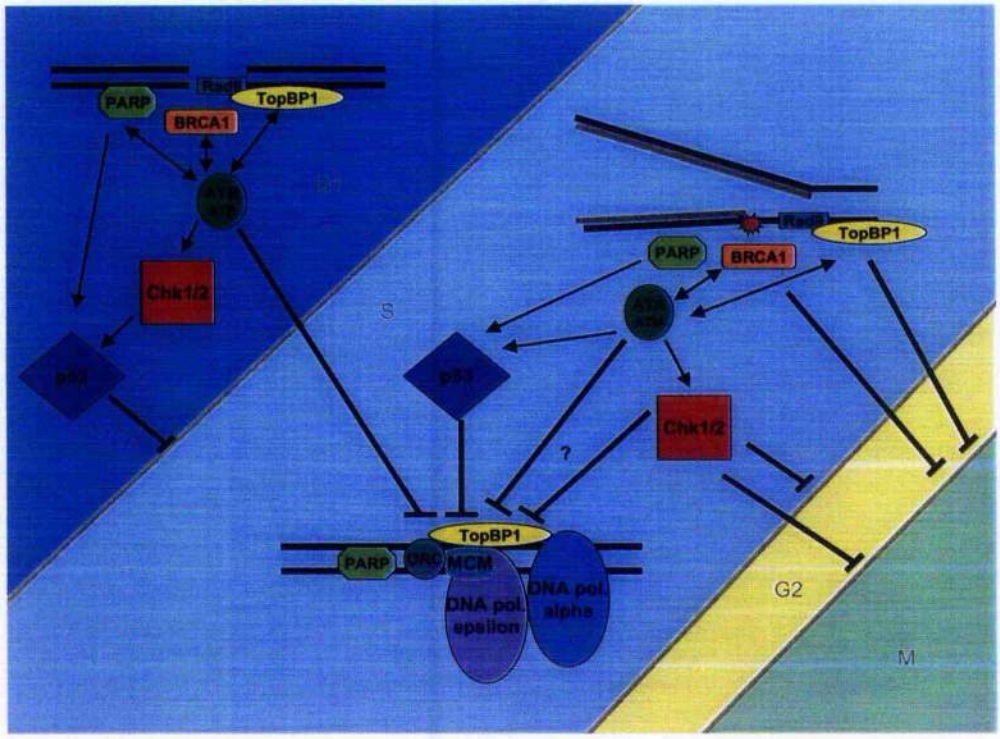
(i) To characterise the functional interaction between TopBP1 and E2.

(ii) To evaluate the consequence of DNA damage stimuli to E2 function.

(iii) To measure the fidelity of HPV E1/E2 mediated DNA replication.

Figure 1.3

Summary of checkpoint controls



Simplified view of the DNA damage response network. The proteins discussed in section 1.7 form a complex network of signal transduction pathways that control the cell cycle, DNA replication, and are activated in response to DNA damage. A simplified interpretation of the roles of each of these proteins throughout the cell cycle and in response to DNA damage stimuli is illustrated above.

Materials and Methods

2.1 Materials

2.1.1. Antibodies

Amersham International plc (Buckinghamshire, U.K.)

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

BD Transduction Laboratories (Oxford, U.K.)

A murine monoclonal antibody raised against amino acids 204-416 of TopBP1 was purchased from BD Transduction Laboratories (T10620).

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

A polyclonal antibody directed against the amino acids 861-1287 of TopBP1 was raised in rabbit. Epitope preparation is described in Boner *et al* 2002. Pre-immune and immune serums are used for the selective immunoprecipitation of TopBP1 in figure 3.1.18.

2.1.2. Bacteriology

Institute of Comparative Medicine Central Services

L-broth

LB-agar

Becton Dickinson Labware (Oxford, U.K.)

Falcon 1059 polypropylene tubes

Falcon 2059 polypropylene tubes

Bibby Sterilin Ltd (Staffordshire, U.K.)

90mm and 150mm bacteriological petri dishes

Fisons Scientific Equipment (Leicestershire, U.K.)

Glycerol

Invitrogen Ltd (Paisley, U.K.)

E. coli DH5α competent cells

E. coli DH10B electro-competent cells

E.coli Inv10 chemically competent cells

Nunc (Hereford, U.K.)

Sterile disposable inoculating loops

Sigma Chemical Co. Ltd (Dorset, U.K.)

Ampicillin

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

Kanamycin

X-Gal

2.1.3. Cell lines

C33a cells are derived from a HPV negative cervical carcinoma and are defective for both p53 and pRb function. C33a cells were obtained from the ATCC.

U2OS cells are derived from a p53 wild type osteosarcoma. U2OS cells were obtained from the CRUK.

MRC5 cells are a SV40 transformed, foetal fibroblast derived cell line. MRC5 cells were obtained from the CRUK.

XP12 cells are a SV40 transformed cell line derived from fibroblasts obtained from a Xeroderma Pigmentosum patient. The cells are deficient in the XPA protein an essential component for nucleotide excision repair of damaged DNA. XP12 cells have been previously described (Kannouche *et al* 2001). XP12 cells were a kind gift from Dr Alan Lehmann.

XP30 cells are a SV40 transformed cell line derived from fibroblasts obtained from a Xeroderma Pigmentosum patient. The cells are defective for the DNA polymerase η protein, a protein that ensures faithful translesion DNA synthesis past DNA lesions. XP30 cells were a kind gift from Dr Alan Lehmann.

XP30η cells are a SV40 transformed cell line derived from fibroblasts obtained from a Xeroderma Pigmentosum patient. The cells are identical to XP30 cells except the DNA polymerase η function is complemented by the stable expression of wild type DNA polymerase η from pcDNA3.1-polη (Kannouche *et al* 2001). XP30η cells were a kind gift from Dr Alan Lehmann.

2.1.4. Chemicals and Reagents

Amersham International plc (Buckinghamshire, U.K.)

Enhanced Chemiluminescence (ECL-plus) Western detection agent

Invitrogen Ltd (Paisley, U.K.)

Agarose (ultrapure electrophoresis grade)

Sigma Chemical Co. Ltd (Dorset, U.K.)

β-mercaptoethanol

Bicinchonoinic Acid (BCA) solution

Bovine Serum Albumen (BSA)

Bromophenol Blue

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

Dithiothreitol (DTT)

Ethidium bromide

HEPES

Nonidet P-40 (NP-40)

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

Ponceau S solution

Tween-20 (Polyoxyethylene sorbitan nonolaurate)

Xylene Cyanol

Roche (Lewes, U.K.)

dATP

dCTP

dGTP

dTTP

dUTP

University of Glasgow (Glasgow, U.K.)

Crude Ethanol

VWR International (Dorset, U.K.)

Acetic acid

di-sodium hydrogen orthophosphate (anhydrous)

Dimethyl sulfoxide (DMSO)

100% pure ethanol

Ethylene diamine tetra acetate (EDTA) disodium salt

Hydrochloric acid

Magnesium chloride

Methanol

Propan-2-ol

Sodium acetate

Sodium chloride

Sodium dihydrogen orthophosphate

Sodium dodecyl sulphate (SDS)

Tri-Sodium Citrate

Tris base

2.1.5. Enzymes and Kits

Applied Biosystems (Warrington, U.K.)

Amplitaq® DNA polymerase with Geneamp® buffer

Bigdye v3.1 DNA sequencing kit

Hi-Di formamide

TaqMan® EZ RT-PCR® kit

TaqMan® β-actin Detection Reagents

Amersham (Buckinghamshire, U.K.)

Nick Column for purification of ^{32}P labelled DNA probe.

Cambio (Cambridge, U.K.)

T4 Endonuclease V. The restriction buffer used is detailed in section 2.2.2.10.

Invitrogen Ltd (Paisley, U.K.)

The restriction enzymes BamHI, BglII, EcoRI and PvuII and their respective reaction buffers.

Alkaline phosphatase

T4 DNA ligase

New England Biolabs (Herts, U.K.)

The restriction enzymes DpnI, MboI and XmnI and their buffers. React buffer 2 was used for each enzyme.

Exonuclease III and its reaction buffer.

Promega Ltd (Southampton, U.K.)

Luciferase Assay System

Pfu polymerase and its buffer

Reporter Lysis 5x Buffer

Qiagen Ltd (Crawley, U.K.)

QIAquick PCR purification kit

QIAprep plasmid megaprep kit

QIAprep Spin plasmid miniprep kit

Stratagene (Amsterdam, Netherlands)

QuickHyb Hybridisation solution

Pfu DNA polymerase

2.1.6. Miscellaneous

Amersham International plc (Buckinghamshire, U.K.)

Hybond-N

Hyperfilm ECL

Applied Biosystems Ltd (Warrington, U.K.)

Real-time PCR 96 well plates and their caps.

Costar Corporation (Bucks, U.K.)

96 well plates

Decon Laboratories Ltd (Hove, U.K.)

Decon 75

Elkay International (Basingstoke, U.K.)

Microcentrifuge tubes

Pastettes

Equibio (Kent, U.K.)

2mm electroporation cuvettes

Safeway Supermarkets (Anniesland, U.K.)

Marvel (dried skimmed milk)

Sigma Chemical Co. Ltd (Dorset, U.K.)

Protein A immobilised on agarose beads

Whatman International Ltd (Maidstone, U.K.)

Whatman 3MM filter paper

2.1.7. Molecular Weight Markers

Invitrogen Ltd (Paisley, U.K.)

1kb ladder

See-Blue 2 protein markers

2.1.8. Plasmids

pBluescript SKII, available commercially from Stratagene.

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

pCMV HPV16 E1 expresses the wild type HPV16 E1 protein under the control of the cytomegalovirus promoter. A kind gift from Prof. Peter Howley (Harvard University, Boston, U.S.A)

pCMV HPV16 E2a expresses the wild type HPV16 E2 protein under the control of the cytomegalovirus promoter. A kind gift from Prof. Peter Howley (Harvard University, Boston, U.S.A). This plasmid is used during E1/E2 dependent DNA replication assays.

pCMV HPV-16 E2b expresses the wild type HPV-16 E2 protein under the control of the cytomeglovirus promoter. A kind gift from Dr Lawrence Banks (International Centre for Genetic Engineering and Biotechnology, Italy). This plasmid is used for transactivation assays

pHPV8 E2 expresses the wild type HPV-8 E2 protein under the control of the cytomegalovirus promoter. A kind gift from Dr Pawel Fuchs (Institute of Virology, University of Koeln) and the late Prof. Herbert Pfister.

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

pTopBP1 expresses the full length human TopBP1 protein under the control of the cytomegalovirus promoter. This plasmid was a kind gift from Dr Kazuhiko Yamane.

pΔTopBP1 expresses the carboxy-terminal half of TopBP1, amino acids 776-1435, under the control of the cytomegalovirus promoter (see Boner *et al* 2002).

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

ptk6E2 contains six consecutive E2 binding sites upstream of the tk promoter from HSV-1 cloned into the pGL2 luciferase vector.

pfos-Luc expresses the firefly luciferase reporter gene under the control of the murine c-fos promoter.

p53fos-Luc expresses the firefly luciferase reporter gene under the control of the murine c-fos promoter. Upstream from the promoter there are two p53-binding sites from the ribosomal gene cluster.

pSKII(-) is a standard cloning vector.

pOri16 contains the HPV16 origin of replication (nucleotides 7838-130 of the HPV16 genome) cloned into pSKII(-). This plasmid was a gift from Prof. Peter Howley (Harvard University).

pOri16M contains the HPV16 origin of replication cloned into pSKII(-). The origin spans nucleotides 7830-138 of the HPV16 genome, with a point mutation at 115 from C to A to create a DpnI restriction enzyme site.

pCR2.1 is a plasmid generated from the commercially available TA cloning vector pCR2.1-Topo (Invitrogen Ltd). This linear vector with a covalently bound topoisomerase was incubated alone and plasmids were screened for LacZ. The pCR2.1 vector created contains an intact LacZ gene, ampicillin and kanamycin antibiotic resistance genes.

pOri16Lac contains the HPV16 origin of replication cloned into BglII site in pCR2.1. The origin spans nucleotides 7830-138 of the HPV16 genome, with a point mutation at 115 from C to A to create a DpnI restriction enzyme site.

2.1.9. Radiochemicals

Amersham International plc (Buckinghamshire, U.K.)

Redivue [α^{32}P] dCTP

2.1.10. Tissue Culture

Institute of Comparative Medicine Central Services

Sterile phosphate buffered saline (PBS)

Becton Dickinson Labware (Oxford, U.K.)

60, 90 and 140 mm tissue culture dishes

Falcon 2097 polypropylene tubes

Falcon 2098 polypropylene tubes

Serological plastic pipettes

Sterile Plastipak syringes

Bibby Sterilin Ltd (Staffordshire, U.K.)

Sterile plastic bijoux and universal containers

Costar Corporation (Bucks, U.K)

Disposable cell scrapers

Invitrogen Ltd (Paisley, U.K.)

Special Liquid medium

10x Dulbecco's Modified Eagles Medium

2.5% Trypsin

Foetal calf serum

Nunc (Hereford, U.K.)

Cryotubes

T25, T75 and T175 cm^2 tissue culture flasks

2.2 Methods

2.2.1 Human cell culture

2.2.1.1 Tissue culture

All cell culture work was performed using strict aseptic techniques inside a laminar flow hood (Class II Microbiological Safety Cabinets, Gelaire BSB4). Cells were incubated at 37^0C in 5% (v/v) CO_2 (Nopco Scientific).

2.2.1.2 Transient transfection

All cells were transiently transfected using a standard calcium phosphate precipitation technique. For luciferase assays cells were plated out at $6x10^5$ cells/60 mm tissue culture dish for C33a cells and $3x10^5$ cells/60 mm tissue culture dish for U2OS cells. The following day a calcium phosphate precipitate containing the DNA was added to the cells. This was carried out as follows for each 60 mm cell monolayer: 250 µl of a solution containing the plasmid DNA in 250 mM $CaCl_2$ was added dropwise with gentle mixing to 250 µl of 2x HEPES buffered saline (280 mM NaCl, 1.5 mM $Na_2HPO_4.2H_2O$, 50 mM HEPES, to pH 7.05 with NaOH). The mixture was left for 30 min to allow a fine precipitate to form and added directly into the medium above the cell monolayer. Sixteen to eighteen hours later the cells were washed twice with PBS and refed with fresh growth medium. For transient replication assays C33a cells were plated out at $6x10^5$ cells/100 mm tissue culture dish, U2OS, MRC5, XP30, XP30η and XP12 cells were all plated out at $3x10^5$ cells/100 mm tissue culture. Transfection was carried out as above except 500µl 250 mM $CaCl_2$ with DNA was added to 500µl of 2x HBS.

2.2.1.3 Luciferase assay

C33a and U2OS cells were lysed directly on the tissue culture plates. The medium was removed and the cells washed twice with PBS. 300 µl of Reporter Lysis Buffer (Promega) was added to the plate and left for 10 minutes. The cell lysate was then scraped from the dish and placed in a 1.5 ml centrifuge tube. The lysate was cleared by centrifuging the sample for 10 min (14000 g-force) and removing the supernatant to a fresh tube. Two 80 µl aliquots of the supernatant were then assayed for luciferase activity using the Luciferase Assay System (Promega) with a Luminoskan Ascent plate reader (Thermo Labsystems). To standardize for cell number, the protein concentration was determined using the BCA/$CuSO_4$ assay. All transfections were repeated at least three times in duplicate.

The BCA/$CuSO_4$ assay is a spectrometric assay to calculate protein concentration. Into a 96 well plate 10µl of protein solution was placed. 200µl of developing solution (5ml BCA(Bicinchoninic acid) solution, 100µl of 4% (w/v) $CuSO_4$ (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 562nm using a Labsystems Multiskan Ascent automatic plate reader. The absorbance reading was converted to concentration in µg/µl using a standard curve generated from a series of control BSA solutions (2µg/µl to 0.08µg/µl) and the Microsoft Excel computer program.

2.2.1.4 Transient replication assay

Replication assays were carried out as a modification of a previously published technique (Sakai *et al* 1996). $6x10^5$ C33a or $3x10^5$ U2OS, MRC5, XP12, XP30 or XP30η cells were plated out in 100 mm dishes and the following day transfected

using the calcium phosphate method. Three days post transfection low molecular weight DNA was extracted using the Hirt method. 800 µl of Hirt solution (0.6% SDS, 10mM EDTA) was directly put on the cells for five minutes to lyse them, then the sample was scraped into a 1.5 ml microcentrifuge tube. 200 µl of 5M NaCl was added and the samples were then left at 4°C overnight. After centrifugation they were extracted once with phenol-chloroform-isoamyl alcohol and precipitated with ethanol (see section 2.2.2.11). Following centrifugation the DNA pellet was washed with 0.5ml 70% ethanol and dried then resuspended in H_2O. For each replication assay different levels of DNA was digested with a combination of restriction enzymes depending on the assay or the detection method.

For each cell line in section 3.1 every transfection was done on one 100mm^2 plate, and the DNA from each plate was dissolved in 100µl of dH2O. For the Southern blots each sample of 25µl DNA was digested with XmnI or XmnI/DpnI, as indicated, in a total volume of 50µl. For the real-time PCR assays 25µl of DNA were digested with either DpnI or MboI as indicated in a total volume of 50µl. The next morning the DNA was further digested in 100units of Endonuclease III for 30 minutes. Subsequently the enzymes were deactivated through heating to 70°C for 30 minutes. 10µl of each digest were used for each individual real-time PCR reaction (see also section 2.2.2.19). For each cell line in section 3.3 every transfection was done in duplicate using 100mm^2 plates. DNA from the two plates was resuspended in a total volume of 100µl. For Southern blots 50µl of DNA were digested with XmnI/DpnI overnight. For the recovery of pOri16Lac plasmid DNA into bacteria 42µl of DNA were digested with DpnI overnight. All digests described above were done in 1x New England Biolabs buffer 2 (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol pH 7.9) supplemented with 100 µg/ml BSA in a total volume of 60µl.

2.2.1.5 Whole cell extract preparation

The cells were washed twice with PBS, then trypsinised, and washed two times with 10ml of ice cold PBS. The cell pellet was then resuspended in 1ml of ice cold PBS and transferred to a 1.5ml microfuge tube and pelleted. They were then resuspended in 100µl of lysis buffer (0.5% NP40, 50mM Tris pH7.8, 150mM NaCl with a protease inhibitor cocktail (Roche, Lewes, UK) dissolved in the lysis buffer). The extracts were then incubated on ice for 30 minutes with occasional mixing. Following this they were centrifuged in a refrigerated microfuge for 10 minutes at maximum speed at 4°C. The supernatant was then removed to another tube and the cell debris discarded. The protein concentration was then determined using the BCA/$CuSO_4$ assay.

2.2.1.6 RNA preparation

RNA extraction from cells was done using a Qiagen RNeasy kit with QIAshredder columns. Cells were washed twice with 1x PBS, then lysed by addition of 600µl of RTL buffer. Cells were then scraped into a QIAshredder spin column then centrifuged to homogenise the sample. Next 600µl of 70% ethanol was added to the lysate and the sample was added to a RNeasy mini column by centrifugation. Subsequently the column was washed with 700µl of RW1 once, and with 500µl RPE twice by centrifugation. The purified RNA was eluted from the column in 30µl of RNase-free water.

2.2.2 Molecular biology

2.2.2.1 Oligonucleotide synthesis

Oligonucleotides used as primers in PCR reactions were synthesized by Invitrogen Ltd. Oligonucleotides were purified by Invitrogen Ltd under desalt conditions. Briefly, this method involves deprotecting the primer under gas phase conditions, and normal phase desalted. The organic products are subsequently removed with 90% Acetonitrile water wash and products are then eluted under aqueous conditions then lyophilized. On receipt of the oligonucleotides they were resuspended in dH_2O to a concentration of 100pmol/µl.

Dual labeled (5'FAM, 3'TAMRA) oligonucleotide probes used for real-time PCR were purchased from Cruachem Ltd. Oligonucleotides were HPLC purified and the lyophilised DNA was resuspended in dH_2O to 20µM.

2.2.2.2 DNA concentration and purity determination

The concentration of either plasmid or DNA oligonucleotides was determined by absorbance measurement at 260 nm and 280 nm using a Biotech spectrophotometer model UV1101. An OD_{260} reading of 1 corresponds approximately to 33 µg/ml single stranded DNA or 50 µg/ml double stranded DNA. The OD_{260}/OD_{280} ratio provided an estimate of the DNA sample purity.

2.2.2.3 Agarose gel electrophoresis

Agarose gel electrophoresis was performed using horizontal gel cast apparatus (Biorad). 1% agarose gels were routinely used. The appropriate amount of ultrapure electrophoretic grade agarose was dissolved in 0.5x TBE buffer (10x TBE: 900mM

Tris base, 900mM boric acid, 25mM EDTA, pH 8.0) by heating the solution in a glass conical flask in a microwave. The gel cast apparatus with a comb containing the appropriate number and size of teeth to form the sample wells was assembled and the gel was poured. The solidified gel was placed in the gel tank and submerged in 0.5x TBE buffer containing 0.5 µg/ml ethidium bromide. Samples containing 1x loading buffer (10x loading buffer is 65% (w/v) sucrose, 10mM Tris-HCl pH7.5, 10 mM EDTA, 0.3% (w/v) Bromophenol Blue) were loaded into individual wells. An appropriate sized DNA ladder was loaded into the first and/or last well in the gel and the DNA was separated by running at 70-100 constant voltage until the dye front was 1-4 cm from the end of the gel. Separated DNA was visualised by illumination on a short wave (312 nm) UV light box and photographed using UVP Gel Documentation System. Agarose gel images presented were captured on a P.C.

2.2.2.4 Southern transfer of DNA

Agarose gels that have separated digested Hirt extract DNA from DNA replication assays (see section 2.2.1.4) are subjected to Southern Transfer (modified from current protocols) to transfer the DNA to nylon membrane (Hybond-N). After electrophoresis the agarose gel was washed in distilled water then placed in denaturation solution (1.5M NaCl, 0.5M NaOH) and gently agitated twice for 30 minutes. Next the gel was washed in distilled water and agitated in neutralisation solution (1.5M NaCl, 0.5 Tris-HCl pH7.0) twice for 30 minutes. Finally the gel was rinsed twice in distilled water.

The transfer of the DNA onto the nylon membrane relies on capillary action. 20x SSC solution (3M NaCl, 0.3M $C_6H_5O_7Na_3$ (tri-sodium citrate)) was placed in a glass dish. On a square platform above the level of the SSC was placed two wicks made of 3MM filter paper to cover the platform and draw SSC upward. Three squares of 3MM filter

paper soaked in 20x SSC the size of gel were placed on top of the wick, followed by the gel, then a square of prewetted Hybond-N nylon membrane 5minutes in distilled water), then five squares of 3MM filter paper, and finally a stack of folded paper towels ~4cm thick. The transfer tower was very gently compressed overnight by a 200g weight to allow efficient DNA transfer.

The following morning the tower was disassembled and the Hybond-N membrane was briefly washed in 2x SSC. The DNA was crosslinked to the DNA membrane through irradiation with 1600 J/m^2 of UVC at 254nm using a Spectrolinker XL1500 (Spectronics Corperation). Next the membrane was baked for >1h at 80°C in a Hybaid mini-oven MKII hybridisation oven.

2.2.2.5 Radio-labelled DNA probe preparation and purification

Radiolabelled probes were generated using the Stratagene Prime-it II® random primer labelling kit as per the manufacturers instructions. For the majority of Southern blots the probe used to detect the replicated pOri16 or pOri16Lac plasmids was a 700bp PvuII fragment released from pOri16M by PvuII restriction digest. For the XP30 and XP30η blots in figure 3.3.4 a smaller 200bp BglII fragment released from pOri16Lac was used for the probe. The longer 700bp probe has two benefits over the 200bp probe; it gives a slightly stronger hybridisation signal and it cross-reacts with the transfected E1 and E2 expression plasmids thus demonstrating if the DpnI digest has been completed, if it was incomplete then there would be a signal from these plasmids at ~5-6kb. However with the XP30η cells the probe cross-reacts with the plasmid sequences in the integrated copies of the DNA polymerase η expression plasmid, therefore the more specific 200bp probe derived from HPV16 Ori sequence allows the detection of the pOri16Lac without interference.

25ng of the DNA to be labelled was mixed with 10µl of 9-mer random oligonucleotide primers and boiled in a total volume of 34µl for 5 minutes. Next 10µl of the 5x dCTP primer buffer was added (contains dATP, dGTP and dTTP), with 5µl of Redivue ^{32}P dCTP (Amersham) and 5U of Exo(-) Klenow DNA polymerase. The reaction was left for 10 minutes at 37°C in a water bath.

Next the probe was purified using a NICK™ Column from Amersham Biosciences as per manufacturers instructions. The column was equilibriated in buffer (10mM Tris-HCl pH7.5 with 1mM EDTA) then the probe reaction was loaded to the column with 400µl of TE buffer, and finally the purified probe was eluted with 400µl of TE buffer and stored at -20 °C in a lead lined pot.

2.2.2.6 Nucleic acid hybridisation and visualisation

The nucleic acid hybridisation of the Hybond-N nylon membrane was using QuikHyb hybridisation solution purchased from Stratagene. The nylon membrane, with 15ml of QuickHyb hybridisation solution, in a conical hybridisation tube, was revolved in a hybridisation oven for 1 hour at 68°C to reduce non-specific nucleic acid hybridisation. The probe generated in section 2.2.2.5 was boiled for five minutes and 100µl of the probe was mixed with 1ml of warm QuikHyb solution and this mixture was then added to the hybridisation tube. The radio-labelled probe was then hybridised at 68°C for an hour. Next the membrane was washed twice for 15 minutes at room temperature with a 2x SSC and 0.1% (w/v) SDS wash solution. Further the membrane was washed once for 40 minutes at 60°C with a 0.1x SSC and 0.1% (w/v) SDS solution. The membrane was then sealed in Saran wrap and exposed to a Phosphor screen for ~16 hours. The image stored on the screen was developed using a Storm™ image system and visualised using the ImageQuant™ software on a P.C.

Densitometry was performed as per the instructions for the ImageQuant™ software. Strength of replication was calculated by measuring the ratio of double cut XmnI/DpnI versus the XmnI single cut bands. This method of measurement controls for variation in transfection efficiency.

2.2.2.7 Polyacrylamide gel electrophoresis

The gels used to separate protein were NuPAGE® 4-12% Bis-Tris-HCl buffered (pH 6.4) poly-acrylamide gels purchased from Invitrogen Ltd. Protein samples were mixed with 4x NuPAGE® LDS Sample buffer (40% Glycerol, 500mM Tris-HCl (pH 6.8), 8% LDS, 0.075% Serva Blue G250, 0.025% Phenol Red), and 10x NuPAGE® Reducing Agent (0.5M DTT), at a ratio of 40:10:4 for sample:sample-buffer:reducing agent, respectively. Subsequently the sample was heated at 70°C for 10 minutes prior to loading.

The gel tanks used to separate the proteins were the Xcell *Surelock* Mini-cell tanks purchased from Invitrogen Ltd and the electrophoresis was performed as per the manufacturer's instructions. Each sample was loaded into the wells of the gel and on each gel at least one lane contains the *See-Blue2* coloured protein markers purchased from Invitrogen. 20x MOPS SDS Running Buffer (1M MOPS, 1M Tris Base, 69.3mM SDS and 20.5mM EDTA) was purchased from Invitrogen Ltd. The electrophoresis was performed in 1x MOPS SDS Running Buffer at a constant voltage of 200V for ~50 minutes.

2.2.2.8 Wet Electrophoretic Transfer of Proteins

Separated protein samples were transferred to nitrocellulose by wet electrophoretic transfer using a Xcell II™ blotting apparatus purchased from Invitrogen Ltd. Blotting

protocol was as manufacturers instructions. After SDS-PAGE, one of the gel cassette plates was removed and the wells were cut away. Two sheets of Whatmann 3MM paper, a piece of nitrocellulose membrane (Hybond C^{extra}), and blotting pads cut to the size of the gel were purchased from Invitrogen Ltd. Each were soaked in 1x NuPAGE® transfer buffer with 10% methanol. 20x NuPAGE® transfer buffer was purchased from Invitrogen Ltd (0.5M Bicine, 0.5M Bis-Tris, 20.5mM EDTA, 1mM Chlorobutanol). Two blotting pads were placed on the bottom plate (cathode) of the blotting apparatus, followed by a sheet of filter paper, then the gel, then the nitrocellulose membrane, then a sheet of filter paper and finally two blotting pads. Any air bubbles were removed and the transfer was performed at 30V constant for 1 hour in 1x NuPAGE® transfer buffer with 10% methanol. The fidelity of transfer was checked by staining the nitrocellulose with Ponceau S solution.

2.2.2.9 Immunoprecipitation

100µg of Protein A sepharose beads (Sigma) were swollen overnight in 1ml of lysis buffer (0.5% NP40, 50mM Tris pH7.8, 150mM NaCl with a protease inhibitor cocktail (Roche Lewes, UK) dissolved in 10 ml of the lysis buffer) at 4°C with continual rotation. Next day the beads were washed three times in lysis buffer and resuspended in 300µl lysis buffer.

$3x10^5$ U2OS cells were plated out on a 6cm plate and the next day were transfected with 2µg of E2 expression plasmid, either WT or mutant as indicated. Two days later the cells were harvested and the whole cell extract was prepared as described in section 2.2.1.5. 60µl of cell extract was mixed with 40µl lysis buffer and 10µl protein A beads in a 1.5ml eppendorf and incubated rotating for 1h at 4°C to pre-clear the extract of proteins that non-specifically interact with the beads. Next the beads were

pelleted out and the supernatant was incubated rotating for 1h at 4°C with 1µl of antibody as indicated. Next 10µl of protein A beads were added to the mixture and the samples were incubated rotating for 1h at 4°C. The complexes containing the antibody-protein A bead complexes were pelleted and they were washed five times with 0.5ml of lysis buffer. The proteins pulled down were resuspended in LDS-polyacrylamide gel sample buffer and the proteins were eluted of the beads through heating at 75 °C for 20 minutes. The beads were then pelleted and the supernatant was electrophoresed using a SDS-PAGE system and subjected to western blot.

2.2.2.10 Restriction enzyme digests

Restriction digests were performed using the appropriate enzymes and reaction buffers according to the manufacturer instructions. Typically, 5-10 units of restriction enzyme/µg DNA was used. In general, small quantities of plasmid DNA (<5 µg) were digested in a 30 µl reaction volume for 2-3 hrs at 37°C. Hirt DNA extracts used for replication assays were typically digested in a 50µl reaction volume overnight at 37°C (see section 2.2.1.4) unless otherwise stated.

Exonuclease III digest was done in the same buffer as the prior restriction enzyme digest (i.e. NEB buffer 2 with mg/ml BSA). See section 2.2.1.4.

T4 Endonuclease V digestion was done on both DNA *in vitro* and using DNA harvested from transiently transfected cells. The samples were first put in 1x T4 Endonuclease V buffer (1mM EDTA, 50mM NaCl, Tris-HCl pH 7.9, 1mM DTT and 100 µg/ml BSA) and digested with 20U of T4 Endonuclease V at 37°C for 12 hours in a total volume of 50µl. Next 5µl of 100mM $MgCl_2$ was added to the reaction to make a final concentration of ~10mM $MgCl_2$. Next 20U of XmnI was added to linearise the DNA and the restriction digest was carried out at 37°C for 12 hours.

2.2.2.11 DNA purification

DNA samples were purified by phenol chloroform extraction after each manipulation to remove contaminants such as residual enzyme activities. An equal volume of phenol:chloroform:isoamyl alcohol (24:24:1 v/v/v) was added to the DNA solution. The aqueous DNA and organic phases were mixed by vortexing and separated by centrifugation in a microfuge (14 000 rpm, 5 min, room temp). The upper aqueous phase was carefully removed making sure none of the interphase was taken and transferred to a clean eppendorf tube. Double stranded DNA was precipitated by adding 1/10 volume of 3M sodium acetate and 2.5 volumes ethanol. The sample was mixed and left at -20^0C for 1 hour to facilitate precipitation. The DNA was pelleted in a microfuge (14 000 rpm, 20 min, 4^0C), washed with 70% ethanol to remove any traces of salt and pelleted again. After the ethanol was removed, the purified DNA was air dried and resuspended in the appropriate volume of sterile distilled water.
PCR fragments used for cloning were routinely purified using the Qiagen QIAquick® PCR Purification Kit as per the manufacturers instructions.

2.2.2.12 DNA ligation

Plasmid DNA and the DNA fragment to be inserted into the vector were separately digested using the appropriate restriction enzymes (Section 2.2.2.10). The 5' phosphate residues of linearised vector DNA were dephosphorylated to prevent vector religation. 1 µl (1 unit) of alkaline phosphatase was added at the end of a restriction digest reaction. The reaction mixture was incubated for 30 min at 37^0C followed by a second incubation at 70^0C for 10 min to stop all enzyme activity. The plasmid DNA and DNA fragment were then purified as detailed above (Section 2.2.2.11). The DNA fragment was ligated into the cut vector using 1 µl (4 units) of T4 DNA ligase in 1x

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

2.2.2.13 Transformation of Competent Bacterial Cells

E. coli DH5α competent cells were used for the propagation of plasmid DNA unless stated otherwise. Stocks of competent cells were stored at –70^{0}C until use when they were thawed on ice. 1 µl of purified ligation reaction or 1-2 ng plasmid DNA was added to a chilled 1.5ml microfuge tube. 20 µl of competent cells were then aliquoted into each tube and left on ice for 30 min. Cells were heat shocked at 42^{0}C for 1 min and then returned to ice for 5 min. 100 µl sterile SOC medium (25 bactotryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added to each sample and the tubes were incubated at 37^{0}C for 1 hour with shaking. The transformation mixes were spread on L-agar plates containing the appropriate antibiotic, plates were inverted and incubated overnight at 37^{0}C to allow colony formation.

The rescue of pOri16Lac plasmid DNA replicated by E1/E2 is described in detail in section 2.2.2.21.

2.2.2.14 Small Scale Preparation of Plasmid DNA (Miniprep)

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

centrifugation in a microfuge (14 000 rpm, 1 min). Plasmid DNA was prepared from the colony using the QIAprep Spin plasmid miniprep kit according to the manufacturer's instructions.

2.2.2.15 Large Scale Preparation of Plasmid DNA (Megaprep)

1 ml of a 3 ml overnight bacterial culture was used to inoculate 500 ml of L-broth containing the appropriate antibiotic in a 2 litre glass conical flask. The culture was incubated overnight at 37^0C with shaking. Bacterial cells were pelleted by centrifugation (6000g, 10 min, 4^0C) using a Sorvall rotor. The bacterial pellet was resuspended in 50ml P1 (50 mM Tris-Cl (pH 8.0), 10 mM EDTA, 100µg/ml RNase A) then gently mixed with 50ml P2 (200 mM NaOH, 1% SDS(w/v)) and allowed to stand at room temperature for 5 minutes to ensure efficient lysis. 50ml of ice cold P3 (3M potassium acetate pH5.5) was added and the contents were mixed by inverting the tube sharply several times. The solution was incubated on ice for 30 min and then centrifuged at 8000g for 20 min at 4^0C. The bacterial debris forms a tight pellet on the bottom of the tube. The supernatant was filtered through nylon gauze and was loaded onto a QIAfilter Mega Cartridge. The column was washed twice with 100ml of QC (1M NaCl, 50mM MOPS pH7, 15% isopropanol (v/v), 0.15% Triton (v/v)). Plasmid DNA was eluted by 35ml of QF (1.25M NaCL, 50mM Tris-Cl pH 8.5, 15% isopropanol (v/v)). DNA was precipitated by the addition of 24.5ml isopropanol. Nucleic acid was pelleted by centrifugation as before, the supernatant was removed and the pellet was washed with 70% ethanol (v/v). Nucleic acid was pelleted by centrifugation as before, the supernatant was removed and the pellet was allowed to air dry. The pellet was resuspended in 1 ml distilled H_2O

2.2.2.16 Polymerase Chain Reaction (PCR)

Unless stated otherwise, PCR reactions containing 1 ng of DNA template, 20 pmol of each primer, 200 μM each dNTP, 1 μl (2.5 units) Pfu polymerase in a total volume of 50 μl containing 1x Pfu reaction buffer were set up in 0.5 ml sterile tubes. A negative control containing no template and a positive control were always included with each set of PCR reactions. PCR amplification was carried out using a MJ Research PTC-200 gradient cycler. Unless otherwise stated, samples were heated to 94^0C for 1 min (denaturing step), 50^0C for 1 min (annealing step) and 72^0C for 1 min (elongation step). This cycle of denaturing, annealing and elongation was repeated 25 times. Ten microlitres of each PCR reaction were analysed by agarose gel electophoresis and the PCR products were purified using the techniques described in Section 2.2.2.11.

Using p16ori as a template and primers 5'-Oritaq and 3'-Oritaq an *Eco*RI-*Bam*HI fragment was cloned into pSKII(-) to generate pOri16M (see table 2.1). This fragment represents nucleotides 7838-139 from the HPV16 genome and contains the HPV16 minimal origin of replication. Additionally there is a point mutation of base 115 from C to A to create a *Dpn*I restriction site so that this plasmid could be used in a TaqMan® real time PCR-based protocol to detect viral DNA replication. The point mutation is highlighted in bold italic for both 3'-Oritaq and 3'-Ori BglII.

Using pOri16M as a template primers 5'-Ori BglII and 3'-Ori BglII generated a BglII fragment which was cloned into the BglII site in pCR2.1-Topo. This fragment represents nucleotides 7838-139 from the HPV16 genome as was inserted into the pOri16M plasmid.

Using pGl3 Cont as a template primers 5'-SV40Ori and 3'-SV40Ori generated a BglII fragment which was cloned into the BglII site in pCR2.1-Topo. This fragment represents the SV40 minimal origin of replication.

Table 2.1 Oligonucleotides to PCR amplify HPV16 Ori and SV40 Ori

Primer	Nucleotide Sequence
5'-Oritaq	5'GTACGGATCCTGCACATGGGTGTGTGCAA 3'
3'-Oritaq	5'GTACGAATTCTAACTTTCTGGGTCGCTCCTGTGATCCTG 3'
5'Ori BglII	5' GTACAGATCTTGCACATGGGTGTGTGCAA 3'
3'Ori BglII	5' GTACAGATCTTAACTTTCTGGGTCGCTCCTGTGATCCTG 3'
5'SV40Ori	5' GTACAGATCTCCAGGCAGGCAGAAGTATGCAAAGC 3'
3'SV40Ori	5' GTACAGATCTCGAAAATGGATATACAAGCTCC 3'

2.2.2.17 Site directed mutagenesis

The site directed point mutagenesis was done using a QuikChange® II Site-Directed Mutagenesis Kit as per manufacturers instructions. The primers used to make point mutations in the HPV16 E2 ORF are listed in table 2.2. Primers were designed to be 25-45 nucleotides in length, and to have a Tm greater than 78°C as figured using the equation; Tm=81.5 + 0.41(%GC) - 675/N - %mismatch, when N equals the primer length. For each mutation generated 25ng of pCMV HPV16 E2 was mixed with 125ng of each 5' and 3' primer, with 1x *Pfu* buffer, 2.5U *PfuUltra* HF DNA polymerase and dNTPs in a total volume of 50µl. PCR amplification was carried out using a MJ Research PTC-200 gradient cycler. Samples were heated to 94°C for 30 seconds (denaturing step), 55°C for 1 min (annealing step) and 68°C for 5 min (elongation step). This cycle of denaturing, annealing and elongation was repeated 25 times. After PCR amplification 10U of DpnI was added to the reaction to digest the template plasmid and the sample was incubated for 1 hour at 37°C. The mutated

plasmids were transformed into XL-Blue supercompetent cells as directed by the manufacturers instructions. The sequence of the plasmids was confirmed by DNA sequencing (see section 2.2.2.18).

Table 2.2 Oligonucleotide sequences for site directed mutagenesis

Primer	Nucleotide Sequence
5'-Y44A	5' CTAGAATGTGCTATTTATGCCAAGGCCAGAGAAATGG 3'
3'-Y44A	5' CCATTTCTCTGGCCTTGGCATAAATAGCACATTCTAG 3'
5'-R47A	5' CGCCTAGAATGTGCTATTTATTACAAGGCCGCAGAAATGGGA3'
3'-R47A	5' TCCCATTTCTGCGGCCTTGTAATAAATAGCACATTCTAGGCG 3'
5'-K68A	5' CACTGGCTGTATCAAAGAATGCAGCATTACAAGCAATTGAAC 3'
3'-K68A	5' GTTCAATTGCTTGTAATGCTGCATTCTTTGATACAGCCAGTG 3'
5'-Y102A	5' GACGTTAGCCTTGAAGTGGCTTTAACTGCACCAACAGGATG 3'
3'-Y102A	5' CATCCTGTTGGTGCAGTTAAAGCCACTTCAACGCTAACGTC 3'

2.2.2.18 DNA sequencing

The fidelity of all plasmid constructions was verified using an Applied Biosystems 3100 automated sequencer. The region to be sequenced was sequenced using a primer complementary to the appropiate region of the vector DNA (see table 2.3). Reactions containing 0.5 µg plasmid DNA, 3.2 pmol primer and 8 µl Big Dye Terminator Reaction premix were made up to 20 µl with dH_2O. 250 µl thin walled eppendorf tubes were used for all sequencing PCR reactions. Samples were heated to 95^0C for 30 sec, 50^0C for 30 sec and 60^0C for 4 min. This cycle was repeated 25 times. PCR

products were precipitated using sodium acetate and ethanol as shown in Section 2.2.2.11 and dried under vacuum using a speedivac. Next the sample was resuspended in 25µl Hi-Di formamide. Subsequently the sample was analysed using an ABI3100 genetic analyser.

Table 2.3 Oligonucleotides used for sequencing

Primer	Nucleotide Sequence	Plasmid Sequenced
5'seqCMV	5' TGTACGGTGGGAGGTCTATA 3'	pCMV-E2
3'seqCMV	5' AACAGACTGATCCACAGGAG 3'	pCMV-E2
5'E2seq200	5' GCAGCAACGAAGTATCCTCTCC 3'	pCMV-E2
5'E2seq300	5' GTGTCGTCTACATGGCATTGGAC 3'	pCMV-E2
3'E2seq300	5' GTCCAATGCCATGTAGACGACAC 3'	pCMV-E2
FwdseqInsert	5'ATGGACAGCAAGCGAAC 3'	pOri16Lac, pOriSV40Lac
RevseqInsert	5'CATCAGAGCAGCCGATTGT 3'	pOri16Lac, pOriSV40Lac
T7 primer	5' GTAATACGACTCACTATAGGGC 3'	pOri16M

2.2.2.19 Real-time PCR detection of E1/E2 dependent transient DNA replication

The design of primers and probes was carried out using Primer Express software (Applied Biosystems). The primer set chosen amplifies a 99bp region of the HPV16 Ori cloned into pOri16M and has the DpnI site at the 3' end of the probe binding site. PCR conditions were optimized and were found to be 5.5mM $MgCl_2$; 200µM dATP, dCTP and dGTP; 400µM dUTP; 900nM of each primer; 100nM probe and 1U Amplitaq® per reaction with 1x geneamp® buffer. PCR reactions were performed on

a sequence detection system (ABI Prism 7700, Applied Biosystems) according to the manufacturer's instructions by using universal PCR conditions (95°C for 30 sec and 60°C for 1 min, in 40 cycles). Primers and probes used for the detection of pOri16M were; Fwd Primer 5' ATCGGTTGAACCGAAACCG 3', Rev Primer 5'TAACTTCTGGGTCGCTCCTG 3', Probe 5'FAM-ACCAAAAGAGAACTGCAATGTTTCAGGATCC-TAMRA 3'.

To differentiate between replicated and input molecules of pOri16M, harvested DNA was digested with either Dpn I or Mbo I. Dpn I will only digest the GATC site when both strands are dam methylated and Mbo I digestion is only possible when both strands are unmethylated. Therefore Dpn I will not digest the replicated DNA and Mbo I will not digest the input DNA.

To detect replicated pOri16M 25µl of sample was digested with Dpn I or to detect input pOri16M 25µl of sample was digested with Mbo I, each digested overnight. Treatment of each sample with exonuclease III for 30min reduces the background that is seen with real-time PCR due to incompletely digested DNA. Exonuclease III is then heat inactivated by heating each sample to 70^0C for 30min. 10µl of each treated sample was run in triplicate using real-time PCR. Quantitation was performed using a 12 step standard curve from a pOri16M dilution series of 100pg to 10^{-5}pg.

2.2.2.20 Real-time PCR detection of mRNA quantity

Probe and primers for the HPV16 E2 were designed using the Primer Express Software (Perkin-Elmer), those for β-actin were purchased from Applied Biosystems and conditions used as per manufacturer. Real-time RT-PCR was performed using the TaqMan® EZ RT-PCR kit (Applied Biosystems), PCR conditions were as per manufacturers recommendations; 3mM MnAc; 300µM dATP, dCTP and dGTP;

600µM dUTP; 200nM of each primer; 100nM probe and 5U rTth polymerase per reaction. PCR reactions were performed on a sequence detection system (ABI Prism 7700, Applied Biosystems) according to the manufacturer's instructions by using universal PCR conditions (60°C for 30 min, 95°C for 10 min, then 94°C for 20 sec and 62°C for 1 min, in 40 cycles). Quantitation of E2 was performed using a 12 step standard curve from a pCMV-HPV16E2b dilution series of 100pg to 10^{-5}pg. All samples were run in triplicate. Primers and probe for HPV16 E2 were; Fwd Primer 5'CCTGAAATTATTAGGCAGCACTTG3', Rev Primer 5'GCGACGGCTTTGGTATGG3', Probe 5'FAM-CAACCACCCCGCCGCGA-TAMRA3'. The β- actin was performed using the endogenous control TaqMan® β-actin Detection Reagents from Applied Biosystems.

To ensure no DNA contamination was in the RNA sample an equivalent aliquot of DNase-I treated RNA was used in a real-time PCR reaction using taq polymease (Applied Biosystems) and similar amplification conditions to the real-time RT-PCR reaction, no DNA was detected.

2.2.2.21 Detection of mutation frequency

42µl of sample was digested overnight with DpnI to remove unreplicated pOri16Lac, the sample was then extracted once with phenol-chloroform-isoamyl alcohol, precipitated with ethanol and washed with 70% ethanol. The DNA was resuspended in 3µl water and electroporated into 25µl DH10B *E.coli* bacteria (Invitrogen). Electroporation was done in a 2mm electroporation cuvette (Equibio) using a Biorad Genepulser II. The conditions used were 2.5kV, 200Ω, and 25µF. The bacteria were subsequently incubated for 1 hour at 37°C in 1ml of SOC media in a 1.5ml eppendorf. Next the bacteria were plated onto two 10cm diameter kanamycin LB agar plates

containing 100µg/ml X-gal (Sigma). The α–complementation between the LacZ' gene on pOri16Lac and the deleted LacZ in DH10B means that DH10B carrying pOri16Lac with a wild type LacZ are blue, and those pOri16Lac with mutations in LacZ are light blue/white. Selected colonies were picked and grown overnight and restreaked on the same medium to confirm the phenotype. Plasmid DNA was prepared using small-scale alkali lysis miniprep system (Quiagen) and samples eluted in 50µl. 5µl of DNA was digested with BamHI, then subjected to 1% agarose gel electrophoresis to highlight plasmid rearrangements.

2.2.3 DNA damage treatments

2.2.3.1 UVB irradiation of cells in culture

UVB irradiation of cells was performed using a Bio-link BLX-312 (Flowgen) that emits UVB at a wavelength of 312nm. The media was briefly removed from all plates and the cells were irradiated with the dose of UVB as indicated in each figure legend, the media from each plate was then returned to each plate.

2.2.3.2 UVC irradiation of plasmid DNA

pOri16Lac DNA was diluted to 50ng/µl and placed on a plastic petri dish. The DNA was then either mock irradiated or irradiated with 1600 J/m^2 of UVC at 254nm using a Spectrolinker XL1500 (Spectronics Corperation).

2.2.4 Bioinformatics

2.2.4.1 Protein images

All protein images were generated using the Swiss-Pdb Viewer v3.7 (downloaded for free from http://www.expasy.org/spdbv). The image of a full length HPV16 E2 protein was generated by displaying the amino-terminal crystal structure (1dto.pdb) with the carboxy-terminal crystal structure (1by9.pdb) and a random structure of the hinge region of HPV16 E2 was generated using Swiss-Pdb Viewer and placed in between the amino-terminal and carboxy-terminal domains.

The Swiss-Pdb Viewer program generated the molecular surface displayed in figures 1.2 and 3.1.14. The electrostatic potential of the surface of the each molecule in the two figures was calculated using the Poisson-Bolzmann computational method using the Swiss-Pdb Viewer program.

2.2.4.2 E2 amino-terminal homodimerisation modelling

The prediction of the homodimerisation of the amino-terminal of HPV16 E2 was done using the Chemera/Bigger molecular graphics and modelling suit obtained from Bio Technol, Portugal. This software package was developed by Nuno Palma & Ludwig Krippahl at the Universidade Nova de Lisboa, Portugal, with the support of Bio Technol, Portugal. The Bigger module is a protein docking prediction program and the Chemera module is a molecular graphics program used to evaluate the data generated with the Bigger program. Using this package I screened different conformations of HPV16 E2 amino-terminal for a homodimerisation interaction and assessed the most likely good candidates as models. The final conformation chosen was due to very high scoring for the electrostatic interactions, high scoring for hydrophobic interactions, side chain interaction and surface contact. Also there was a

very high score for the role of R7, R37 and I73 in the homodimerisation with the conformation chosen.

2.2.4.2 Phosphorylation site prediction

The prediction of possible phosphorylation sites in HPV16 E2 was done using the online-based NetPhos 2.0 program (http://www.cbs.dtu.dk/services/NetPhos/) developed by Blom *et al* 1999. Only those phosphorylation sites predicted with over 90% confidence are shown.

2.2.4.3 PEST sequence prediction

The prediction of possible PEST protein degradation control sequences was done using the online-based prediction program PESTFIND (http://bioweb.pasteur.fr/seqanal/interfaces/pestfind.html) hosted by the Pasteur Institute, Paris, France. This program is written by Michael K. Schuster and Martin Grabner and is a modification of the original PESTFIND program by Scott Rogers and Martin Rechsteiner ©1986.

Results

3.1

Characterisation of the E2-TopBP1 interaction

3.1.1

Identification of TopBP1 as a potential partner protein for E2 function

The amino terminal transactivation domain is essential for the various functions of HPV16 E2. Identification of potential cellular interacting proteins for the amino-terminal domain of E2 was done by Dr W.Boner using a yeast 2 hybrid system (Boner *et al* 2002, Boner & Morgan 2002). The screen was done using a cDNA library made from HeLa cells, and a total of 5×10^6 cDNAs were screened using the mating technique. HeLa cells are derived from a HPV18 positive cervical cancer. From this screen 20 colonies grew on selective media. Three of the colonies that grew coded for the domain in HPV18 E1 that interacts with E2. This result indicates that the yeast 2 hybrid screen is able to identify biologically relevant E2 binding partners.

Six of the clones isolated coded for the carboxy-terminus of TopBP1, a large 1435 amino acid 171kD protein. TopBP1 was cloned when the carboxy-terminus of TopBP1 was pulled out of a HeLa cDNA based yeast 2 hybrid screen that was looking for proteins that interact with the carboxy-terminal regulatory domain of Topoisomerase IIβ (Yamane *et al* 1997). Structurally TopBP1 contains eight BRCT domains and has a carboxy-terminal nuclear localisation signal (figure 3.1.1). BRCT domains are conserved protein interaction domains found in proteins from yeast to humans that function in cellular DNA replication, repair and transcription processes. The cellular functions of TopBP1 and the possible role for the E2-TopBP1 interaction are discussed in section 1.6.4.

Figure 3.1.1

Diagrammatic representation of TopBP1

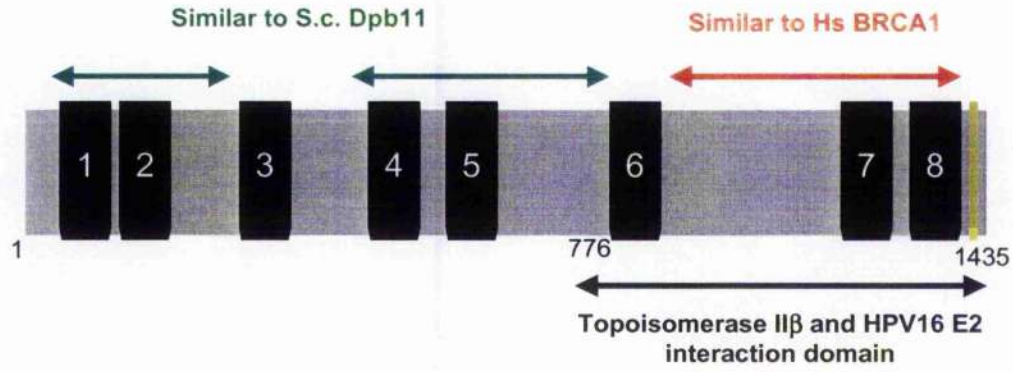


Diagram of the important features in TopBP1. The black boxes represent each of the eight numbered BRCT domains. The yellow box highlights the nuclear localisation signal. The carboxy-terminal region of TopBP1, which binds to both Topoisomerase IIβ and HPV16 E2, is highlighted in black. Two regions of homology are highlighted by the green and red arrows (adapted from Makeneimi *et al* 2001). The green arrows highlight the amino-terminal regions of TopBP1 that have significant homology with the Saccharomyces cerevisiae Dpb11 protein. The red arrow highlights the caboxy-terminal region that has significant to human BRCA1 protein.

3.1.2

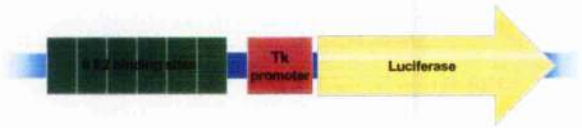
Enhancement of E2 transactivation function

To assess the effect of TopBP1 on the transactivation potential of E2, the transactivation function of E2 was measured alone, and with the overexpression of TopBP1. Transient transfection of the cervical carcinoma cell line C33a with ptk6E2 and the HPV16 E2 expression plasmid pCMV-E2b allows the measurement of E2 transactivation potential. The C33a cell line was used as it is a cervical carcinoma epithelial derived cell line that contains no HPV sequences, and therefore does not contain any viral proteins that may alter E2 function. The ptk6E2 plasmid has luciferase reporter gene expression driven by the HSV tk promoter with six consecutive E2 binding sites. E2 binds here and drives the expression of the luciferase reporter (Vance *et al* 1999). E2 alone efficiently transactivates the tk6E2 promoter in ptk6E2. When TopBP1 is overexpressed this E2 dependent transactivation is enhanced 2-4 fold. To test if this enhancement is due to an indirect effect on the tk promoter a parallel experiment was done using a reporter plasmid that lacks the six E2 binding sites (ptk). E2 expression led to no increase in tk activity, and with all E2 levels overexpression of TopBP1 led to a 20-40% reduction in the tk promoter activity. These results demonstrate that TopBP1 enhances E2 mediated transactivation (see figure 3.1.2).

Figure 3.1.2.

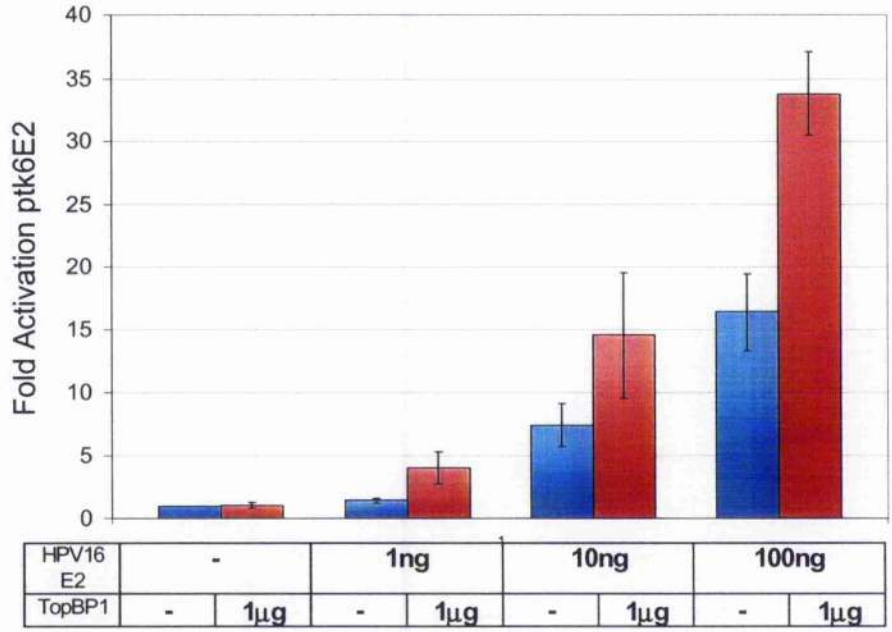
Enhancement of HPV16 E2 transactivation function

(a) ptk6E2

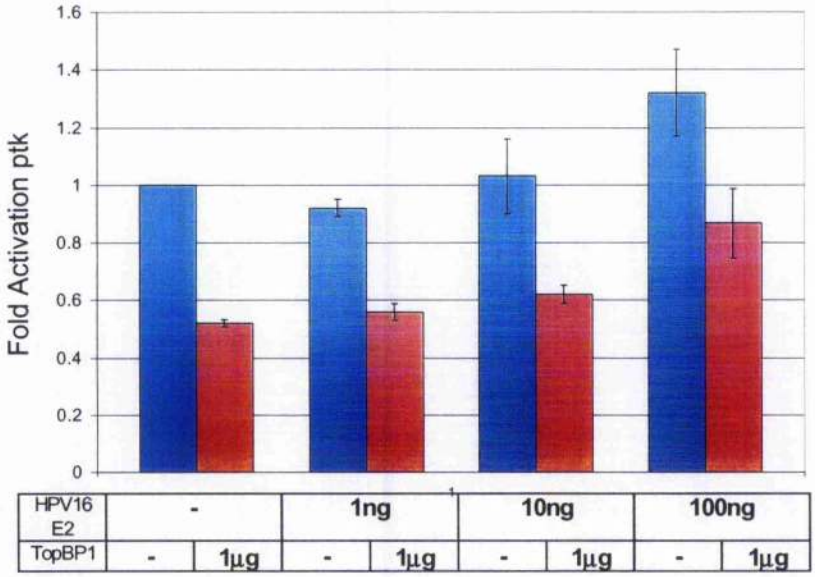


(b) ptk

(c) Activation of ptk6E2 by HPV16 E2 and TopBP1



(d) Activation of ptk by HPV16 E2 and TopBP1



(a) and **(b)** are both a diagrammatic representation of the reporter constructs used. Red box is the tk promoter from HSV1, yellow arrow is the luciferase gene and the green boxes are the E2 binding sites.

(c) C33a cells were transfected with 100ng ptk6E2 with the indicated amounts of HPV16 E2 expression plasmid with or without 1µg TopBP1 expression plasmid. Results are graphed as the fold activation of transcription relative to the level of transcription in the absence of E2.

(d) C33a cells were transfected with 100ng ptk with the indicated amounts of HPV16 E2 expression plasmid with or without 1µg TopBP1 expression plasmid. Results are graphed as the fold activation of transcription relative to the basal level of transcription.

3.1.3

HPV E1/E2 mediated DNA replication.

A transient viral DNA replication assay allows the measurement of the ability of E1/E2 to use the cellular replication machinery to replicate a plasmid containing the HPV16 viral origin of replication, pOri16. Briefly, plasmids expressing the viral E1 and E2 proteins are transfected along with pOri16 into the target cell line (pOri16 is a plasmid containing the HPV16 origin of replication). E1 and E2 mediate the replication of pOri16 and at three days post-transfection the low molecular weight DNA was harvested. The plasmid DNA harvested was linearised using the *XmnI* endonuclease, then the input plasmid DNA is selectively digested with the *DpnI* endonuclease. *DpnI* will only digest DNA that contains the bacterial *dam* adenine methylation, therefore only DNA transfected will be digested because DNA replicated in mammalian cells lack *dam* methylation. Subsequent agarose gel electrophoresis and Southern blotting allows the visualisation and quantitation of the level of DNA replication mediated by E1 and E2 (see figure 3.1.3)

To initially set-up and characterise the transient replication assay system the level of transfected E2 that allows maximal and sub-maximal rates of DNA replication was first established. E2 was titrated into the system, with pOri16 and E1 levels kept constant. Figure 3.1.4a is a representative Southern blot from these experiments. No replication was detectable when pOri16M was transfected alone, or with pE1 or pE2 individually. When E1 and E2 are co-expressed significant levels of replication are visible with 10ng of pE2, replication levels peak at 1μg and are significantly reduced at 5μg of pE2. When results from three experiments are graphed it is clear that some

replication can be detected at 1ng pE2 on occasions, and that maximal levels of replication occur between 100ng and 1μg pE2 (see figure 3.1.4b).

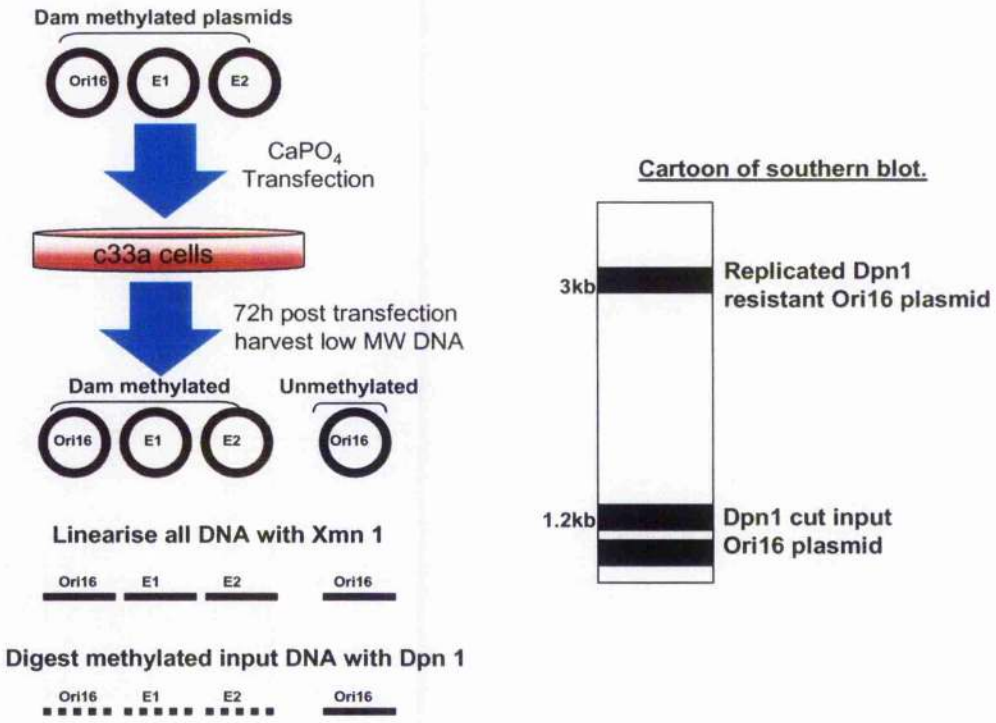
3.1.4

Effect of TopBP1 on HPV DNA replication.

To test if TopBP1 effects E1/E2 mediated replication, C33a cells were transfected with pOri16M, E1, E2 and TopBP1. Levels of replication are summarised in figure 3.1.5a. In C33a cells overexpression of TopBP1 at both submaximal and maximal levels of E2 had no significant effect on the level of replication. To further investigate if TopBP1 has any effect, similar assays were done in U2OS cells. U2OS cells demonstrated greater activation of E2 transcription function by TopBP1 overexpression when compared to C33a cells (I. Morgan, Boner *et al* 2002). In U2OS cells the submaximal level of replication is at 100ng pE2 and maximal is 1μg pE2 (data not shown). At submaximal levels of pE2 (10ng) in U2OS cells the overexpression of TopBP1 enhances E1/E2 mediated replication by 2-3 fold, with no significant enhancement at maximal levels of pE2 100ng (see figures 3.1.5 b and c).

Figure 3.1.3

Outline of transient DNA replication assay

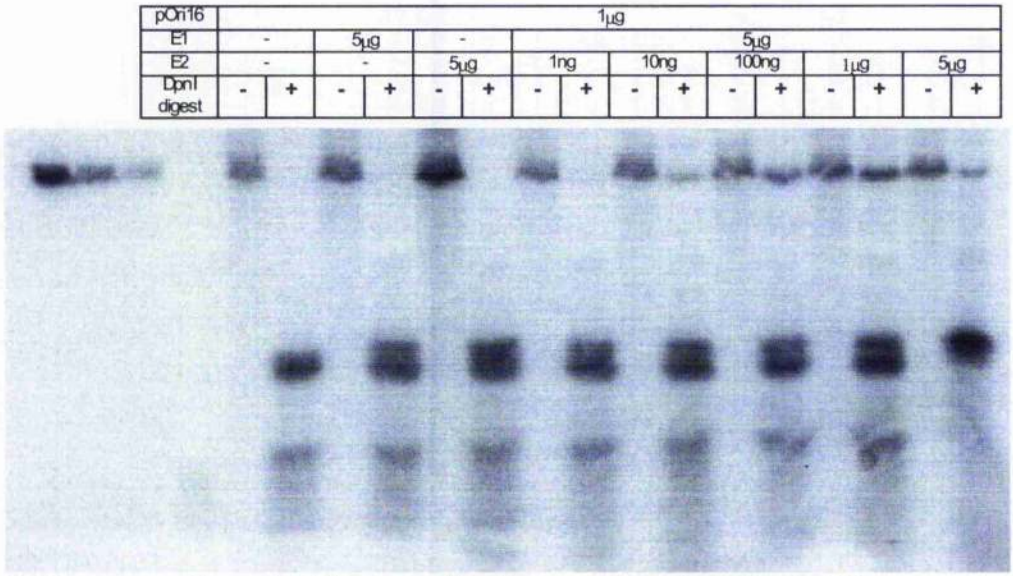


Schematic diagram of a transient DNA replication assay. The HPV16 Ori containing plasmid (pOri16), and the E1 and E2 expression plasmids are transfected into the target cell line. Three days post transfection low molecular weight DNA is harvested. In the DNA harvested there are the bacterially dam methylated plasmids (pOri16, pCMV-E1 and pCMV-E2a) that were initially transfected, and the freshly replicated unmethylated pOri16 plasmid DNA. All plasmid DNA is linearised by *XmnI* digestion. Digestion with *DpnI* multiply digests and therefore fragments the transfected dam methylated DNA, the unmethylated replicated DNA is *DpnI* resistant. Agarose gel electrophoresis and subsequent Southern blotting using a pOri16 derived probe allows the detection of replicated pOri16 at 3kb, and input pOri16 at ~1.2kb.

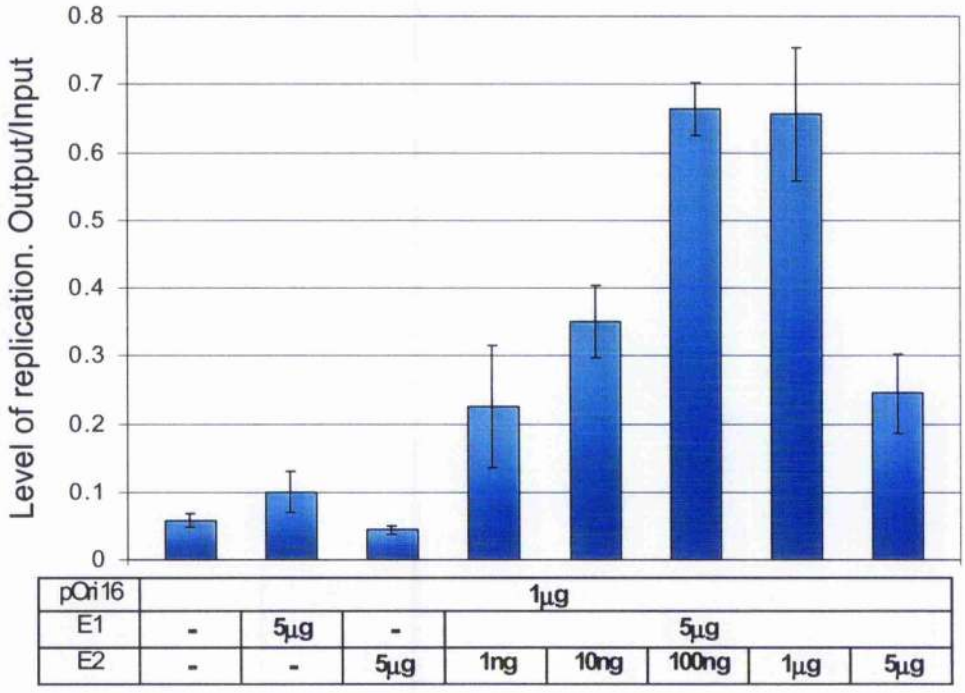
Figure 3.1.4

Transient replication of pOri16 in C33a cells

(a) Southern blot of E1/E2 dependent DNA replication in C33a cells



(b) Summary of results of three replication experiments with pOri16



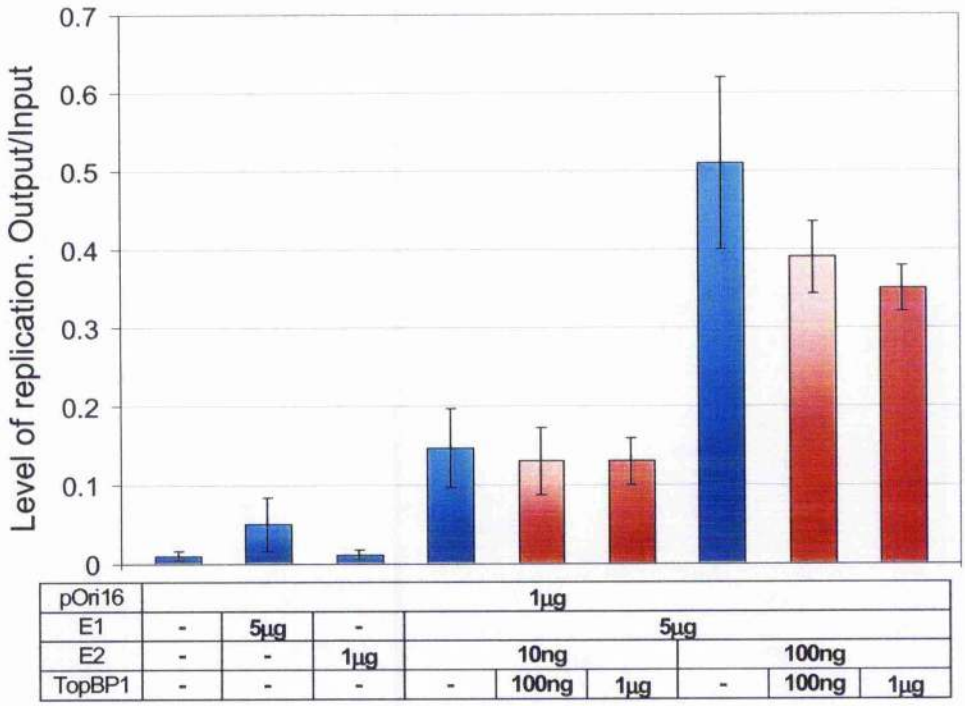
(a) Southern blot of E1/E2 dependent DNA replication in C33a cells. The determination of the effects of various levels of E2 expression on DNA replication. The replication template, pOri16, and the E1 and E2 expression vectors were transfected as indicated. Samples were first linearised by *XmnI* digestion, then digested with *DpnI* as indicated to reveal the replicated band at 3kb. Linearised pOri16 loaded at 40pg, 10pg and 4pg are the markers in the left three lanes respectively.

(b) Summary of results of three replication experiments with pOri16. Levels of the pOri16 replication template, and the E1 and E2 expression plasmids transfected is indicated. Quantitation was done using a phosphor-imager. The value graphed is the ratio obtained when the *XmnI-DpnI* band quantity is divided by the *XmnI* band quantity. See materials and methods 2.2.2.6 for an explanation of the band quantitation procedure.

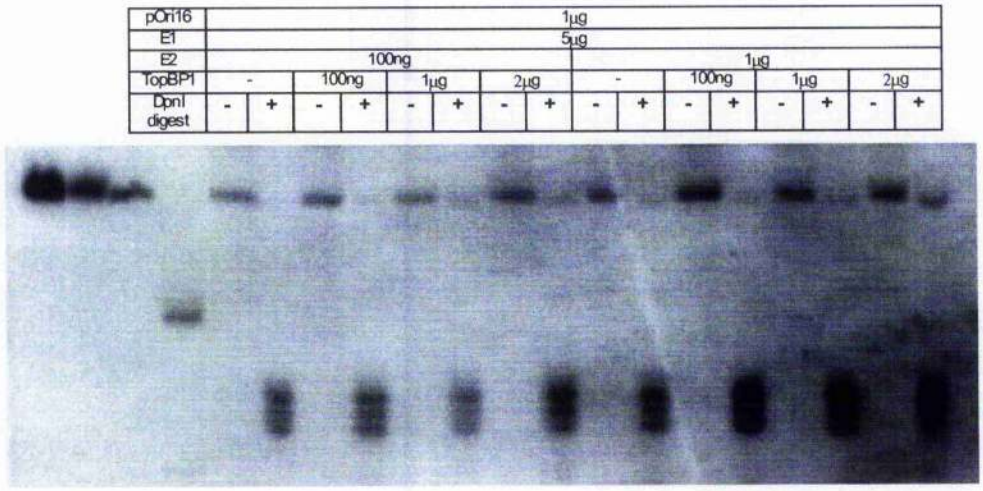
Figure 3.1.5

Effect of TopBP1 expression on replication function in C33a and U2OS cells

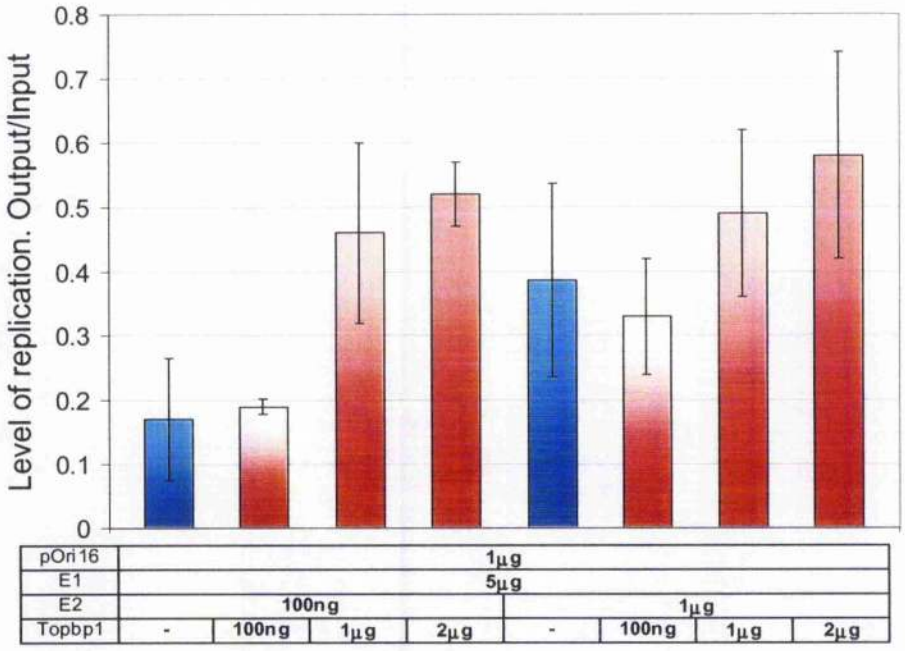
(a) Summary of replication in C33a cells



(b) Southern blot of replication in U2OS cells



(c) Summary of replication in U2OS cells



(a) The effect of TopBP1 overexpression on the level of replication in C33a cells. Graphed results from three experiments, with the pOri16 replication template, and the E1, E2 and TopBP1 expression plasmids transfected as indicated. The value graphed is the ratio obtained when the *XmnI-DpnI* band quantity is divided by the *XmnI* band quantity. Quantitation was done using a phosphorimager.

(b) The effect of TopBP1 overexpression on DNA replication in U2OS cells. The replication template, pOri16M, and the E1 and E2 expression vectors were transfected as indicated. Samples were first linearised by XmnI digestion, then digested with DpnI as indicated to reveal the replicated band at 3kb.

(c) Effect of TopBP1 overexpression on DNA replication in U2OS cells. Graphed results from three experiments, with the pOri16 replication template, and the E1, E2 and TopBP1 expression plasmids transfected as indicated. The value graphed is the ratio obtained when the *XmnI-DpnI* band quantity is divided by the *XmnI* band quantity. Quantitation was done using a phosphor-imager.

3.1.5

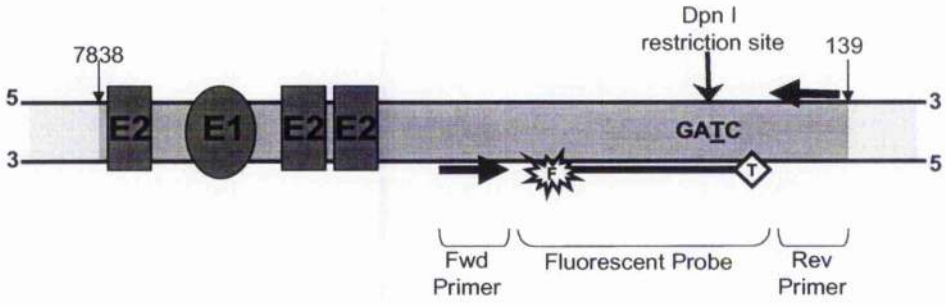
Design of a real-time PCR based HPV transient replication detection protocol

The traditional method of detection of E1/E2 mediated replicated molecules by Southern blot is not ideal for several reasons; the lower threshold for the detection of DNA is 1pg, the quantitation is not ideal due to its semi-quantitative nature as highlighted by the background in figure 3.1.4 b, the protocol is labour intensive resulting in limitations on experimental size and design, and Southern blotting is hazardous due to the use of radiolabeled probe. Real-time PCR is an established technique for the quantitative detection of specific DNA molecules. I therefore designed and set-up a real-time PCR based protocol for the detection of E1/E2 mediated replicated pOri16 molecules.

To detect and quantitate the target DNA molecules real-time PCR uses a specific primer set and a dual labelled DNA probe. In this case they were designed to detect the HPV16 replication origin (see figure 3.1.6, design protocol see section 2.2.2.19). To distinguish between transfected and replicated molecules a point mutation was introduced into a non-essential region of the origin within the real-time PCR amplicon, creating a *DpnI* site (see figure 3.1.6). Therefore when a sample is digested with *DpnI* all transfected pOri16M molecules will no longer support PCR amplification, but the *DpnI* resistant replicated pOri16M will be detected by real-time PCR. To enhance the distinction between replicated and transfected molecules the sample is subsequently digested with endonuclease III, which indiscriminately digests DNA from free ends. Replicated pOri16M DNA is resistant to exonuclease III digestion due to the molecules still being circular. This digestion reduces the background signal resulting from incomplete digestion of transfected pOri16M.

Figure 3.1.6

Design of the real-time PCR replication assay



Design of real-time PCR based replication assay. Outline of HPV16 origin sequence cloned into pOri16M. Dark grey shaded area is the minimal origin of replication from HPV16 (nt 7838 – 139) cloned into pSK II(-). This contains one E1 binding site and three E2 binding sites. The real-time PCR primers and probe were designed against nt 49 – 139 and their positions are shown. Within the probe binding site the sequence GATC, a *DpnI* restriction site, is highlighted. This was created by point mutation of nt 115 from C to T, the mutated T is underlined. "F" is the reporter FAM fluorochrome and "T" is the quencher fluorochrome.

3.1.6

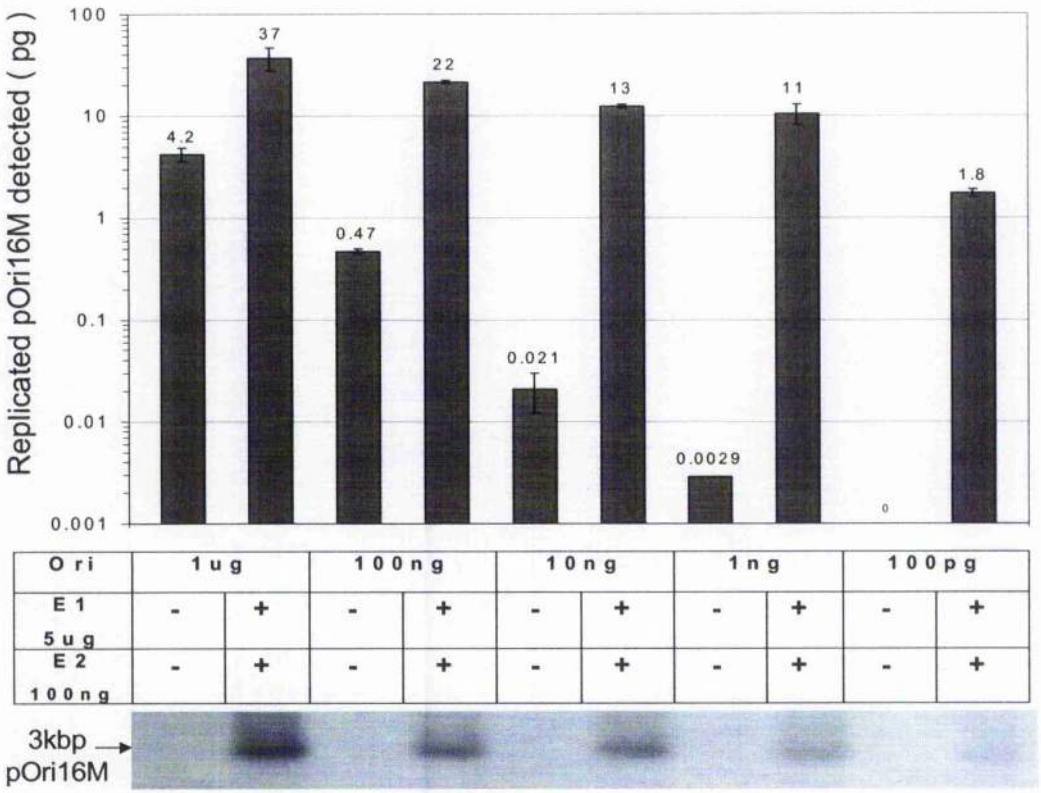
Titration of pOri16M

Initial experiments with the real-time PCR protocol used identical conditions to those used for the previous Southern blot experiments, with 1µg pOri16M, with 5µg pE2 and 100ng pE2 transfected into C33a cells to activate replication. These experiments were disappointing, as there was a very high background signal when there was no E1/E2 present. However there was a small and repeatable four to ten fold increase in the signal when E1 and E2 were transfected (data not shown). A possible explanation for this is that there is a small fraction of the transfected molecules that are resistant to *DpnI* or Endonuclease III digestion, and these molecules are heterogeneous in size and are therefore undetectable on a Southern blot. To test this hypothesis I titrated pOri16M into the system while keeping E1 and E2 expression constant.

At 1µg of input pOri16M there is a ~10 fold difference between non-replicating background samples and replicating samples (figure 3.1.7). As the amount of pOri16M is reduced by 10 fold the amount of background is reduced until it reaches 0 at 100pg pOri16M. Significantly as pOri16M is reduced the amount of replication as detected in this assay falls by a relatively small amount (~ 5 fold from 1µg pOri16M to 1ng pOri16M) whereas the background is reduced by ~400 fold. At 100pg pOri16M transfected the level of pOri16M significantly limits the amount of replication able to occur. The representative Southern blot for this experiment highlights that the signal detected when E1/E2 is present mirrors the bands on the blot, however the background signal observed with real-time PCR is not detected at all concentrations of pOri16M on the blot. Additionally at 1ng pOri16M the efficiency of viral replication is good, and the background is approximately 4000 fold less than the signal when there is replication activated by E1 and E2.

Figure 3.1.7

Titration of pOri16M



A titration of the amount of pOri16M transfected with either no E1 and E2, or with E1 and E2 co-transfected. Samples were analysed with both real-time PCR and Southern blot. Real-time PCR results are plotted on a bar chart and the Southern blot is below. Real-time PCR samples were digested with *DpnI* and Southern blot samples were digested with *DpnI* and *XmnI* to reveal linear replicated pOri16M at 3kb.

3.1.7

Evaluating the sensitivity of real-time PCR protocol

To evaluate the sensitivity of the real-time PCR protocol a titration of E2 expression plasmid was transfected into the system with 1ng pOri16M (Figure 3.1.8). Replication peaked with 100ng E2 transfected, at 1ng and 10ng E2 the replication is sub-optimal and at 1µg E2 there is a decrease in replication probably due to "squelching". Also there is no detectable replication with just E1 or E2 expressed individually confirming that co-operation between E1 and E2 is required for replication of pOri16M.

Comparing the results for the real-time PCR with the Southern blot reveals that the real-time PCR detection method is more sensitive at detecting replication. The Southern blot could only detect replication at 100ng and 1µg E2 with 10ng pOri16M and 100ng E2 with 1ng pOri16M. With both 1ng and 10ng pOri16M real-time PCR is able to detect viral DNA replication at levels at least 100 fold lower than the Southern blot can detect. This set of experiments highlights the increased sensitivity of the real-time PCR protocol.

3.1.8

Selective detection of transfected pOri16M by MboI digestion

To internally control a replication assay the quantitation of the amount of input pOri16M is an effective method for controlling against variation in transfection and DNA harvest efficiency. To use this method with real-time PCR, I digested the samples with *MboI*. The restriction site for *MboI* is the same as *DpnI* (GATC) except *MboI* can only digest DNA that is *dam* negative, it is blocked by the methylation on *dam* positive DNA. This results in the digestion of freshly replicated pOri16M in the sample and *dam* positive input DNA is detected using real-time PCR. An experiment

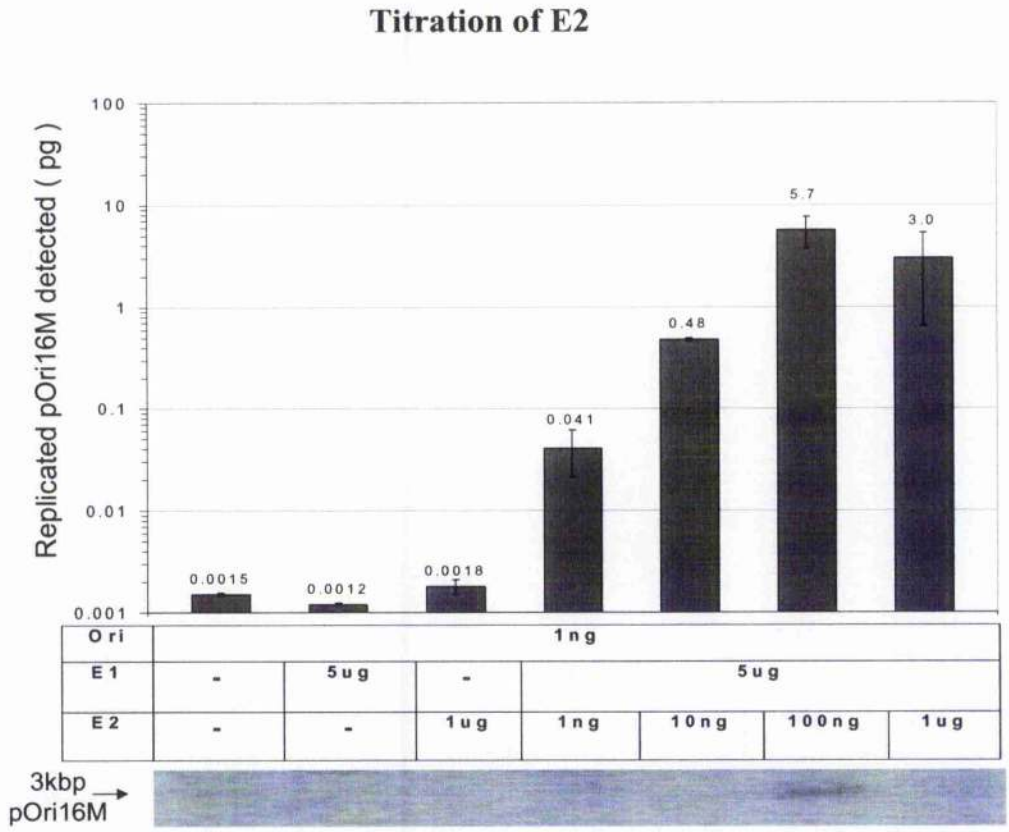
using *MboI* digested samples from Figure 3.1.8 was done using real-time PCR (see figure 3.1.9). This demonstrates the clear detection of the transfected input pOri16M and the consistency between samples. This information can be used in future experiments to internally control for variations in transfection efficiency and DNA harvest efficiency.

3.1.9

Enhancement of HPV replication by TopBP1 overexpression in C33a cells

I previously demonstrated that overexpression of TopBP1 enhances E1/E2 mediated replication in U2OS cells using Southern blotting. Similar experiments in C33a cells showed no reproducible difference in the level of replication observed with TopBP1 using Southern blotting (Boner *et al* 2002 and figure 3.1.5a). It is possible however that no difference was observed as the replication system was not fully optimised in C33a cells, and therefore the subtle effects that the overexpression of an E2 interacting protein may have on replication were undetectable. To highlight the benefits of the increased sensitivity the real-time PCR protocol offers, I overexpressed TopBP1 with sub-maximal levels of E2 (1ng and 10ng) with 1ng pOri16M in C33a cells. Overexpression of TopBP1 significantly enhances the E1/E2 dependent replication with a 5 fold increase using 1ng E2 and a 2.5 fold increase using 10ng E2. Therefore there is a significant effect on E1/E2 mediated DNA replication following TopBP1 overexpression that was undetectable using Southern blotting in C33a cells. This demonstrates that the real-time protocol described here has enhanced sensitivity and quantitation for assaying E1/E2 mediated DNA replication.

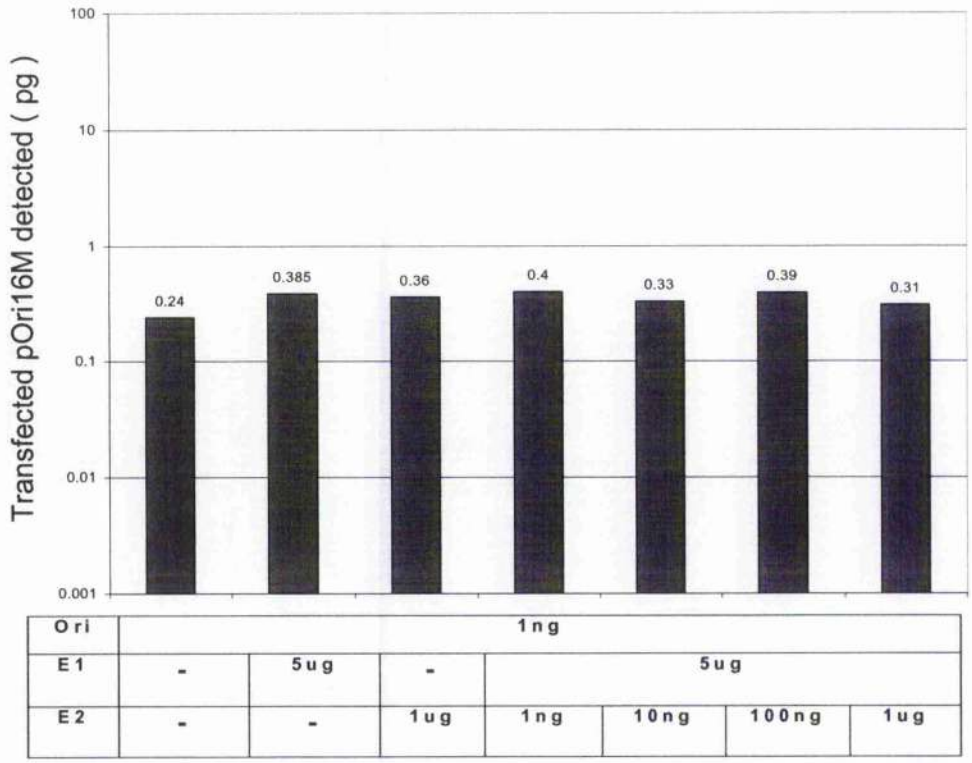
Figure 3.1.8



Titration of E2 with 1ng pOri16M transfected. Samples were analysed with both real-time PCR and Southern blot. Real-time PCR samples were digested with *DpnI* and Southern blot samples were digested with *DpnI* and *XmnI* to reveal linear replicated pOri16M at 3kb.

Figure 3.1.9

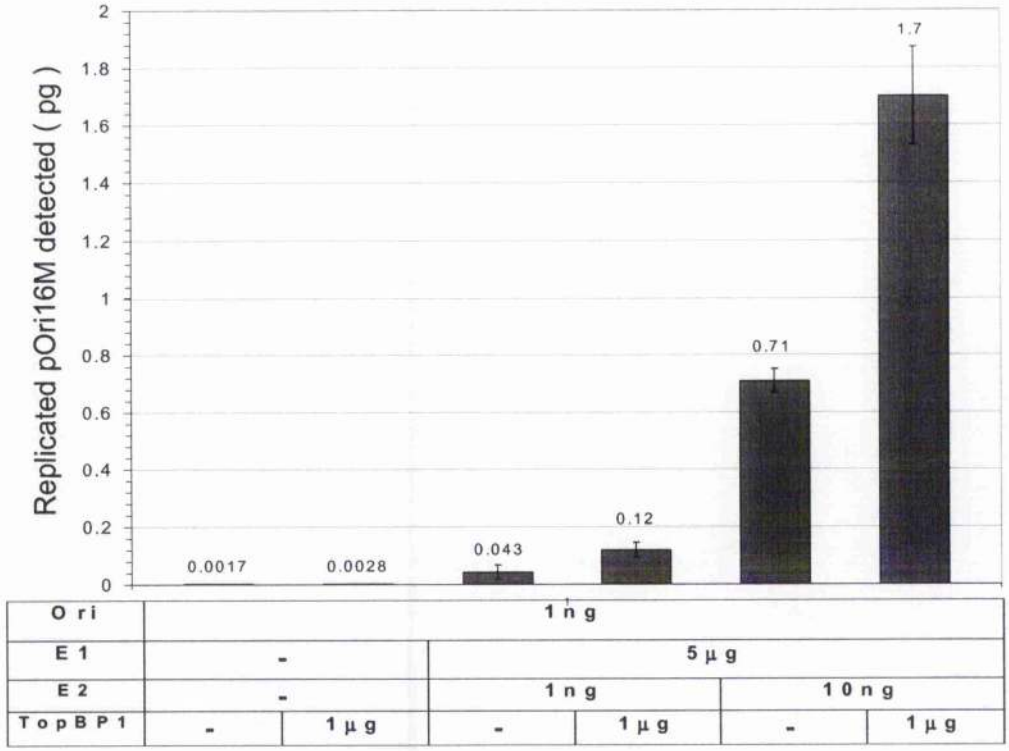
Detection of input pOri16M for use as an internal control



Detection of input pOri16M for use as an internal control. *MboI* digests the replicated *dam* negative DNA leaving the *dam* positive DNA intact and therefore detectable by real-time PCR. Samples from figure 3.1.8 were *MboI* treated and analysed using real-time PCR.

Figure 3.1.10

Increased replication with overexpression of TopBP1



Increased replication with overexpression of TopBP1 in C33a cells. Internally controlled detection of replication using either 1ng or 10ng E2, with or without 1µg TopBP1. Data shown is the *DpnI* digest of each sample detected with the real-time PCR protocol.

3.1.10

ΔTopBP1 does not activate E2 mediated transcription/replication

The enhancement of E2 mediated transcription and replication by the overexpression of TopBP1 suggests that TopBP1 is a functional partner for E2. TopBP1 has multiple domains potentially responsible for the enhancement of the transactivation function of E2 and E1/E2 dependent replication. The E2 binding region of TopBP1 that was identified in the yeast 2 hybrid screen for E2 interacting proteins was the C-terminal half of TopBP1. In yeast the two BRCT domains at the amino terminus of TopBP1 have the ability to activate transcription therefore a similar function may be present in mammalian cells (Makiniem *et al* 2001). Additionally the amino terminal half of TopBP1 has close structural homology to Cut5/Dpb11, while the C-terminal E2 binding domain has limited homology to the C-terminus of BRCA1. A deletion mutant of TopBP1 that binds to E2 yet lacks the amino-terminal 5 BRCT domains was used in transcription and replication assays for two reasons. Firstly, to test if the full length TopBP1 protein is required for enhancement of E2 function. Secondly, to potentially demonstrate a dominant negative effect from a deleted TopBP1 on E2 function.

To begin to understand what region of TopBP1 is responsible for the enhancement of E2 function, and if an amino terminal deleted TopBP1 can act as a dominant negative molecule for E2 function, transactivation and replication assays were repeated using a deletion mutant of TopBP1 (amino acids 776-1435, see figure 3.1.11). Over expression of ΔTopBP1 did not result in a reduction of E2 transactivation potential, and it did not enhance the transactivation function of E2 to the levels that the full length TopBP1 does in C33a cells (see figure 3.1.12). A similar experiment was

carried out in U2OS cells demonstrating that ΔTopBP1 does not significantly alter E2 mediated transactivation function (Boner *et al* 2002).

The effect of over expression of ΔTopBP1 on replication function was tested and a representative Southern blot is shown in figure 3.1.13. No significant effect on the levels of sub-maximal replication in U2OS cells was observed with ΔTopBP1 in contrast to the enhancement of replication with full length TopBP1 (see figure 3.1.13).

Figure 3.1.11

Diagram of TopBP1 deletion mutant

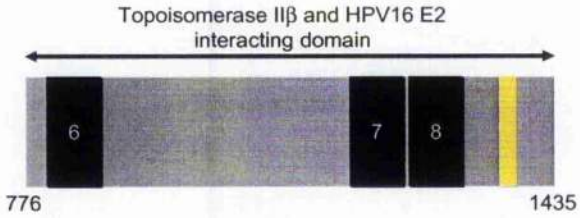
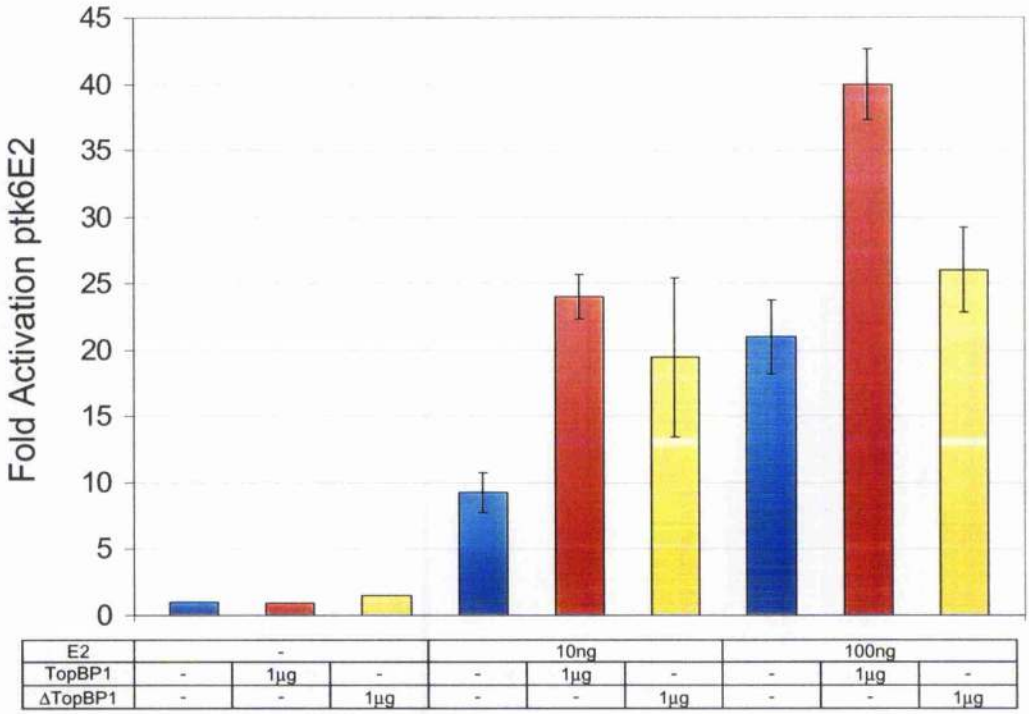


Diagram of the TopBP1 deletion mutant used in transcription and replication assays. Amino acid numbers are displayed below, the black numbered boxes are the BRCT domains and the yellow box is the nuclear localisation signal.

Figure 3.1.12

Effect of ΔTopBP1 overexpression on E2 dependent transcription

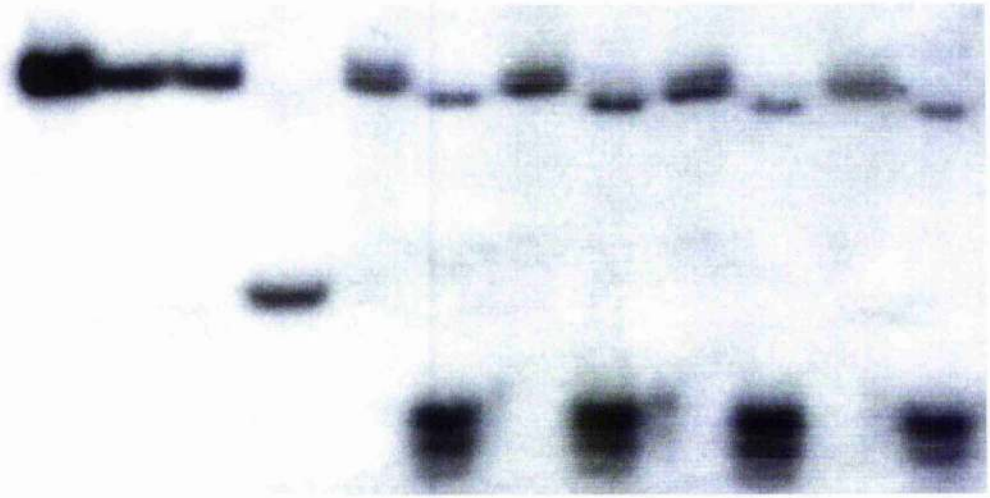


Effect of ΔTopBP1 overexpression on E2 dependent transcription. U2OS cells were transfected with 100ng ptk6E2 with the indicated amounts of HPV16 E2 expression plasmid with or without 1µg TopBP1 or ΔTopBP1 expression plasmid. Results are graphed as the fold activation of transcription relative to the basal level of transcription. Graphed results from three experiments.

Figure 3.1.13

Effect of expression of ΔTopBP1 on the level of E1/E2 mediated replication

pOri16	1µg							
E1	5µg							
E2	100ng							
TopBP1	-		1µg		-			
ΔTopBP1	-				1µg		5µg	
DpnI digest	-	+	-	+	-	+	-	+



Effect of expression of ΔTopBP1 on the level of E1/E2 mediated replication. The replication template, pOri16M, and the E1 and E2 expression vectors were transfected as indicated into U2OS cells. Samples were first linearised by *XmnI* digestion, then digested with *DpnI* as indicated to reveal the replicated band at 3kb.

3.1.11 Point mutation analysis of the amino-terminal of E2

ΔTopBP1 is unable to alter the transactivation function of E2 or E1/E2 mediated replication, suggesting that TopBP1 is not essential for either of these functions of E2. However endogenous TopBP1 may be able to functionally compete and maintain E2 function in the presence of the ΔTopBP1, or ΔTopBP1 may still have the domains responsible for E2 function and may only lack the enhancement effect of the amino terminus. The inability of ΔTopBP1 to repress the transactivation function of E2 or E1/E2 mediated replication also suggests that TopBP1 neither disrupts the hypothesised amino terminal homodimerisation of E2 necessary for transactivation function, or the interaction between E2 and E1 on the amino terminus of E2. A point mutant of HPV16 E2 that does not bind TopBP1 would be a useful tool in understanding if TopBP1 is an essential partner for E2 function. *In vitro* pull down data indicate that TopBP1 requires the first 25-110 amino acids of E2 for efficient interaction (I.Morgan, Boner *et al* 2002). This region contains many conserved amino acids essential for either transactivation or replication function and these residues are localised to discrete domains on the surface of E2 (Saki *et al* 1996, Antson *et al* 2000). There are also many conserved residues that have had no previous role in transcription or replication identified. These latter residues are either hydrophobic internal structural amino acids, or are external amino acids that potentially interact with E2 partner proteins. I therefore made targeted mutants of E2 with the aim of making a functional E2 protein that is unable to bind TopBP1.

The amino terminal crystal structure has been resolved and is therefore a useful tool in the prediction of possible surfaces for TopBP1 interaction with E2. The residues picked for point mutation were chosen due to their high conservation and their exposure on the surface of E2. Additionally, to prevent the disruption of the E2

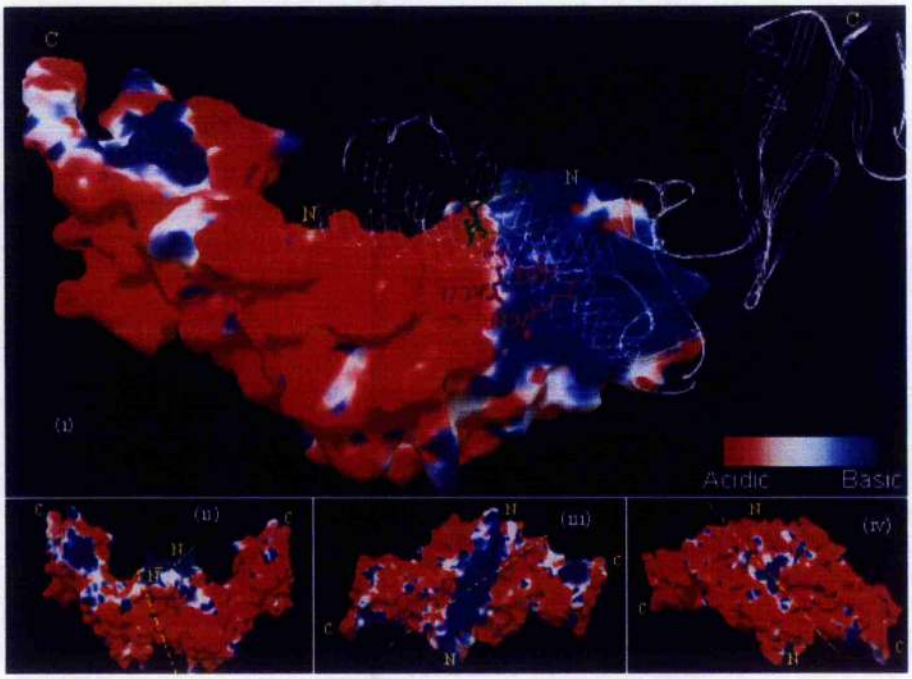
homodimerisation or the E1-E2 interaction the residues chosen were preferentially away from these two interaction surfaces. To aid this choice I used the amino terminal crystal structure and computer modelled the homodimerisation of two E2 amino terminal domains (see figure 3.1.14a); residues essential for transcription (R37, I73 and Q76) and replication (Q12 and E39) are highlighted (Saki *et al* 1996). The model produced for the homodimerisation of the amino terminal of HPV16 E2 was very favourable for electrostatic interactions, and was further refined by scoring for the involvement of three essential residues of transactivation (R7, R37 and I73). The model generated is similar to that produced by Antson *et al* 2000. The identification of the E2-E2 and the E1-E2 interaction surfaces aided the choice of four residues for point mutation distant from these interaction surfaces. The four residues chosen were Y44, R47, K68 and Y102. Two of these residues, R47 and K68, have previously been characterised as having no significant effect on E2 function (Saki *et al* 1996) and two residues have not been previously characterised (Y44 and Y102). Figure 3.1.14b shows the location of the four residues mutated, and it also shows the location of two residues essential for transactivation or replication function (R37 and E39 respectively).

Initial characterisation of the E2 point mutants by sequencing and western blotting ensured that the plasmids created expressed the correct proteins, and that these proteins were expressed at a similar level to the wild type E2 protein. Figure 3.1.15 is a western blot of the wild type and each point mutant E2 protein transiently expressed in C33a cells from 2μg of expression vector. No signal is visible when no E2 is transfected, however for the wild type and all the point mutants of E2 a similar strong level of expression of E2 was detected using the anti-E2 antibody.

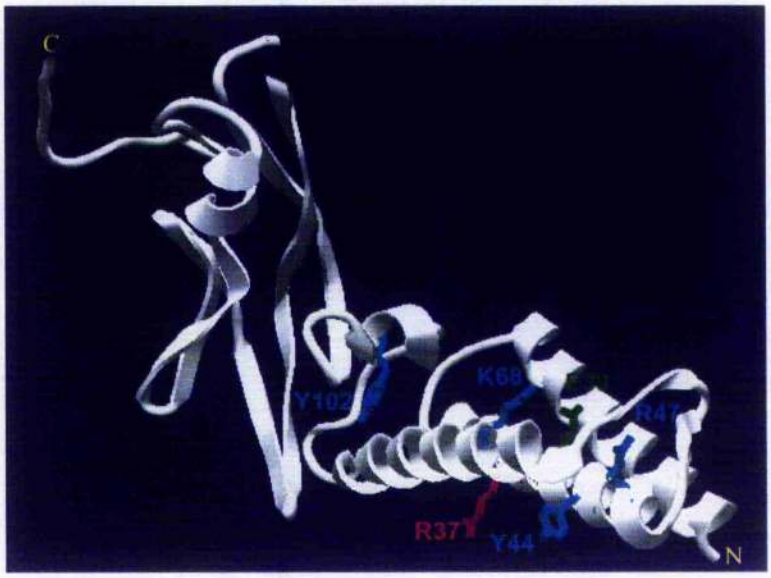
Figure 3.1.14

Design of point mutations in the amino-terminal domain of HPV16 E2

(a) Computer generated model of a dimeric amino terminal



(b) Diagram of the residues chosen for point mutation



(a) Computer generated model of a dimeric amino terminal of HPV16 E2. Using the crystal strucutre of HPV16 E2 (1dto.pdb) and the Chemera/Bigger protein docking prediction program a model of the dimeric configuration of HPV16 E2 amino terminal was created. The model was generated using the Bigger protein docking algorithm and the Chemera molecular graphics and modelling software. The Bigger algorithm searches every possible surface contact and orientation that two molecules can have with each other searching through a set of 1 A angular rotational steps. Each possible contact generated is then scored on surface contact, electrostatic interactions, hydrophobic interactions and side chain interactions. Further refinement of the model was done using the Chemera program to highlight those interactions where the residues R7, R37 and I73 are important for E2 homodimerisation.

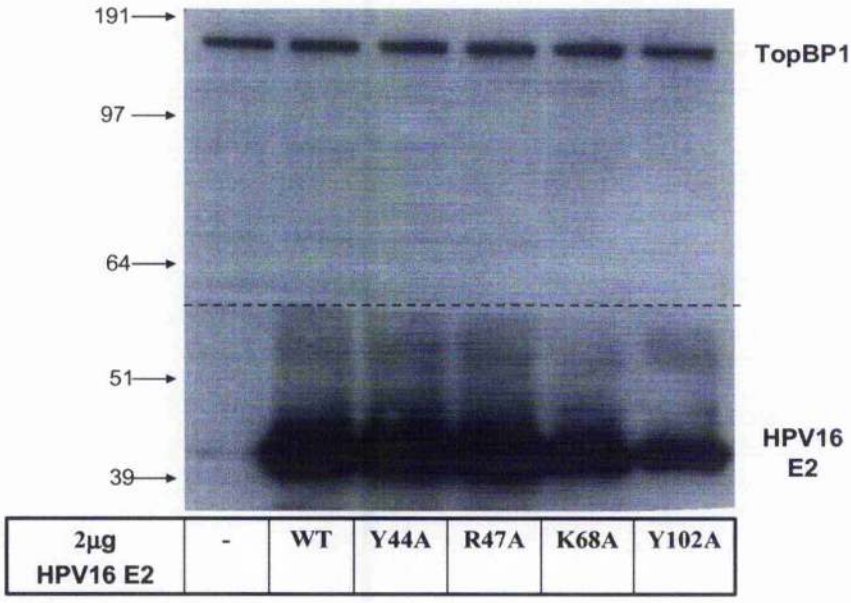
(i) Two HPV16 E2 amino terminal domains are illustrated. One subunit has its molecular surface shown with the molecules surface electrostatic potential shown. The second subunit is drawn as a cartoon, important residues (see text section 3.1.10) are highlighted and are drawn as sticks.

(ii), (iii) and (iv) all show the structure of a dimer of the amino-terminal domian of HPV16 E2 from various angles. The yellow dashed line highlights the contact surface between the two subunits. The amino and carboxy terminals of each subunit is highlighted as N and C respectively.

(b) Cartoon diagram of the residues chosen for point mutation. Cartoon of the crystal structure of HPV16 E2 amino terminal domain. The four residues chosen for point mutation were Y44, R47, K68 and Y102. They are drawn as sticks in blue. Two residues essential for transactivation (R37) and replication (E39) are drawn as sticks in purple and green respectively.

Figure 3.1.15

Western blot of E2 mutants



Expression of HPV16 E2 mutants. U2OS cells were transfected with 2µg of wild type and mutant E2 as indicated. Cellular protein extracts were prepared and were subjected to SDS-page electrophoresis and western blotted. The upper portion of the blot was probed with an α-TopBP1 antibody and the lower was probed using the TVG261 α-E2 antibody. The positions of the molecular markers are indicated on the left in kD.

3.1.12

Ability of E2 point mutant proteins to activate transcription and the effect of TopBP1 over expression

The transactivation function of the E2 point mutants was next analysed with the ability of over expression of TopBP1 to enhance this function also tested (see figure 3.1.16). In the absence of E2 the overexpression of TopBP1 leads to a repression of the tk6E2 promoter in ptk6E2, a result similar to that observed with the tk promoter in ptk in figure 3.1.2d. For wild type E2 there is a three-fold enhancement of the transactivation of tk6E2 promoter by the overexpression of TopBP1. With the Y44A mutant E2 there is no significant transactivation of the tk6E2 promoter by E2, and overexpression of TopBP1 leads to a repression of the tk6E2 promoter in a similar manner to that observed when no E2 is expressed. The R47A mutant of E2 behaves in a similar manner to wild type E2, with transactivation of the tk6E2 promoter by E2 and this is enhanced by the overexpression of TopBP1. The K68A and Y102A mutant E2 proteins share similar transactivation characteristics, both alone do not transactivate the tk6E2 promoter significantly, however with both proteins transactivation is enhanced by the overexpression of TopBP1 by two fold. The enhancement of transactivation by E2 with the R47A, K68A and the Y102A mutants suggests that TopBP1 can bind to and enhance the transactivation function of the proteins. The lack of transactivation by the Y44A mutant protein and the repression of the tk6E2 promoter could indicate one of three possibilities. Firstly, the Y44A mutant protein is itself unable to bind the ptk6E2 plasmid, and therefore the tk6E2 promoter is repressed by TopBP1 due to no E2 binding the plasmid. Secondly, the Y44A mutant protein is unable to activate transcription, and TopBP1 overexpression only enhances transcription from the tk6E2 promoter when E2 initiates the conformational

changes in chromatin at the promoter associated with transcription. Thirdly, the Y44A protein does not bind TopBP1 and therefore the overexpression of TopBP1 leads to repression of the tk6E2 promoter in trans, similarly to when no E2 is present.

3.1.13

Ability of E2 point mutant proteins to activate DNA replication and the affect of TopBP1 over expression

Further characterisation of the E2 mutant proteins was done by analysing their ability to activate DNA replication, and the ability of TopBP1 overexpression to enhance replication with each mutant was tested (figure 3.1.17). Wild type E2 activated DNA replication efficiently and the overexpression of TopBP1 resulted in a two-fold enhancement of the replication level. Each point mutant of E2 was able to activate DNA replication to a similar level as the wild type E2, and with each E2 point mutant TopBP1 overexpression affected DNA replication to varying degrees. The replication with the Y44A and R47A mutant E2 proteins was unaffected by TopBP1 overexpression, and with the K68A and Y102 mutants the replication was enhanced by two to four fold respectively. These results suggest that the Y44A and R47A mutants are unable to interact with TopBP1.

3.1.14

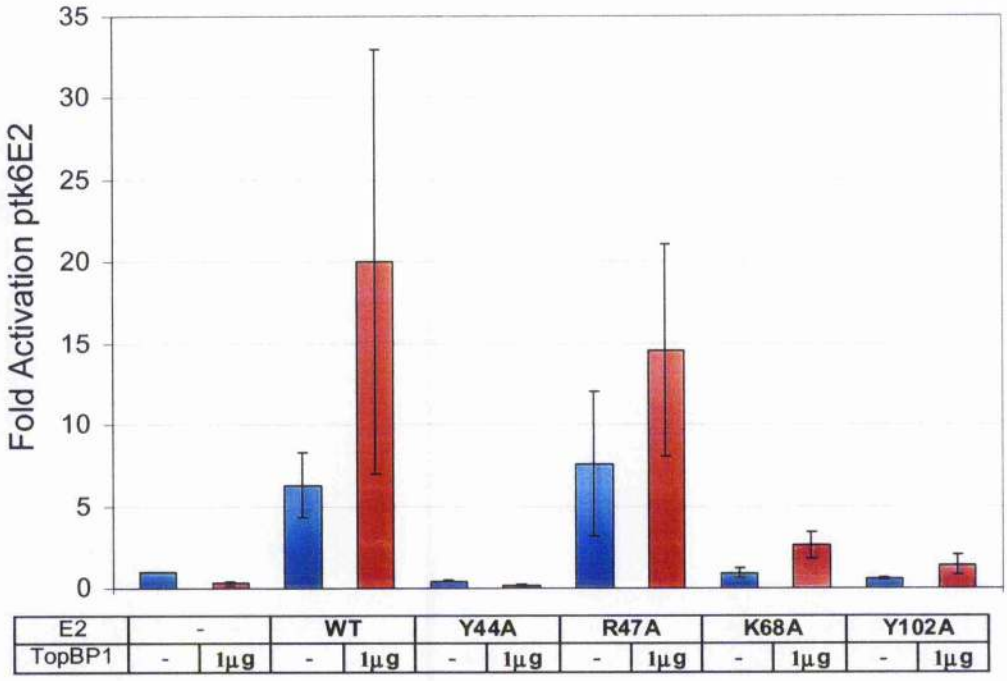
In vivo interaction between TopBP1 and the E2 point mutant proteins

To test if TopBP1 can bind to each of the E2 point mutants *in vivo* co-immunoprecipitation of TopBP1 with transfected E2 was performed to demonstrate the amount of E2 that can bind to TopBP1. Figure 3.1.18 is the western blot of the immunoprecipitated protein pulled down using a polyclonal TopBP1 antibody. The

upper half of the western blot is probed for TopBP1 and the lower half is for HPV16 E2. In lanes 1 and 2 when no E2 is expressed no signal is detected at ~43kD and TopBP1 is only visible when immunoprecipitated with the immune TopBP1 serum, not the pre-immune. In lanes 3 and 4 WT E2 is expressed and E2 is co-immunoprecipitated with TopBP1, no signal for E2 is visible when using the pre-immune serum. In lanes 5 to 9 the point mutants of E2 are expressed as indicated and co-immunoprecipitated using the immune TopBP1 serum. The amount of TopB1 pulled down is similar with each point mutant and with each point mutant of E2 there is a significant amount of E2 pulled down. While on the blot shown there is variation in the amount of E2 pulled down these differences were not repeatable. This experiment demonstrates that while the E2 point mutants have varying phenotypes in response to TopBP1 overexpression, these phenotypes cannot be explained solely due to an inability of the mutant E2 to bind TopBP1. On the contrary this result suggests that the phenotypes displayed by the E2 point mutants are due to alterations of E2 function that the point mutations cause.

Figure 3.1.16

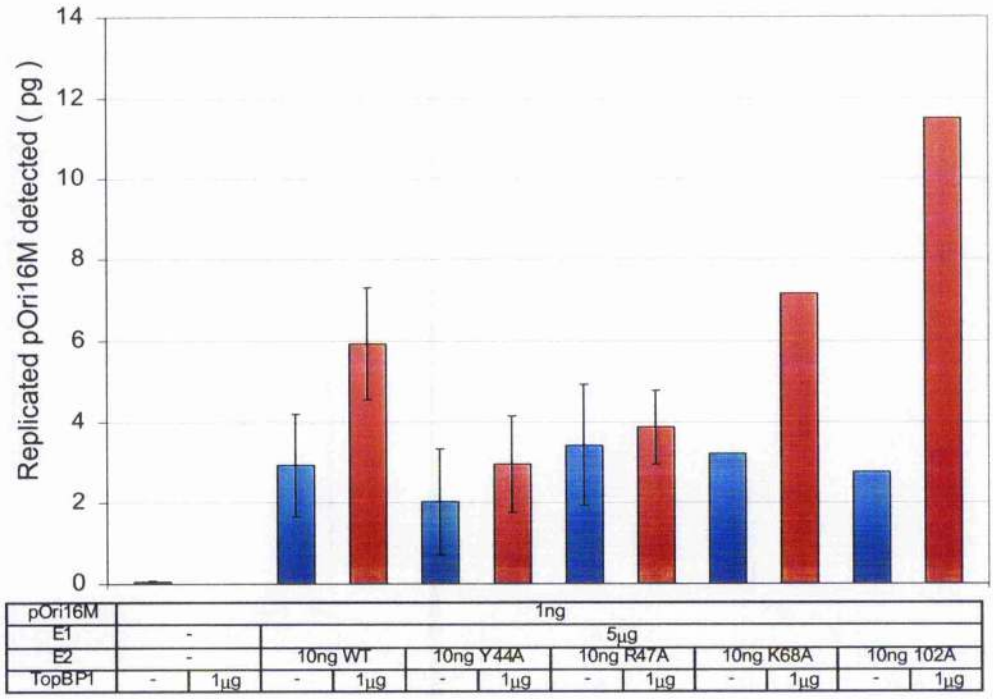
Effect of TopBP1 on mutant E2 proteins transactivation function



The effect of TopBP1 overexpression on the transactivation function of E2 mutants. U2OS cells were transfected with 1µg ptk6E2 with the indicated amounts of HPV16 E2 mutant expression plasmid with or without 1µg TopBP1 expression plasmid. Results are graphed as the fold activation of transcription relative to the basal level of transcription.

Figure 3.1.17

Effect of TopBP1 overexpression on E1/E2 replication function using E2 point mutants

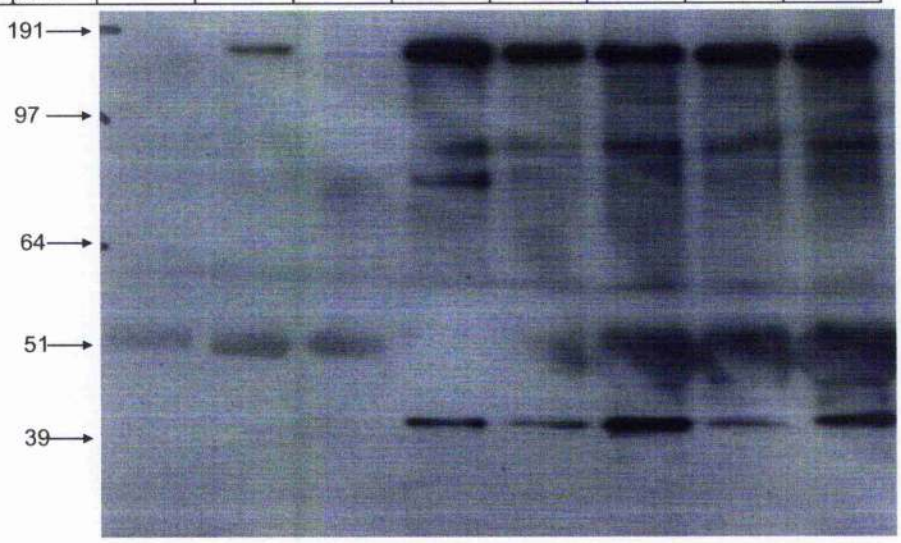


The effect of TopBP1 overexpression on the ability of mutant E2 proteins to activate E1/E2 mediated replication. C33a cells were transfected with E1, wild type or mutant E2 and TopBP1 as indicated. The amount of replicated DNA was determined by real-time PCR as described in section 3.1.8.

Figure 3.1.18

Co-immunoprecipitation of E2 mutants with TopBP1

E2	-	-	WT E2	WT E2	Y44A E2	R47A E2	K68A E2	Y102A E2
I.P. Pre-immune	+	-	+	-	-	-	-	-
I.P. Immune TopBP1	-	+	-	+	+	+	+	+



Ability of E2 mutant proteins to bind TopBP1. U2OS cells were transfected with 2μg of wild type and mutant E2 as indicated. Cellular protein extracts were prepared and immunoprecipitated with either pre-immune serum or α-TopBP1 serum as indicated. The immunoprecipitated material was subjected to SDS-page electrophoresis and western blotted. The upper portion of the blot was probed with an α-TopBP1 antibody and the lower was probed using the TVG261 α-E2 antibody. The positions of the molecular markers are indicated on the left in kD.

3.2

Response of HPV16 E2 protein to UVB irradiation

The HPV16 E2 protein has many cellular binding partners (see sections 1.6.2-1.6.4). Several of these proteins have central roles in signal transduction pathways that control the cellular response to DNA damage (p53, BRCA1, TopBP1, PARP, see sections 1.6.3-1.6.4). These proteins have altered levels, modifications and functions in response to genomic insult. These interactions suggest that E2 may itself respond to DNA damage stimuli thus the response of E2 to cellular UVB irradiation (and other DNA damage agents) was evaluated. UVB irradiation induces both direct and indirect damage to DNA (see Ravanat *et al* 2001 for review) and causes the activation of the cellular DNA damage checkpoint responses. U2OS cells were used for most assays in this study for two reasons. Firstly, U2OS cells are p53 wild type and on starting these experiments it was deemed desirable to have p53 present because p53 has important roles in the HPV life cycle, and is an E2 interacting protein. p53 suppresses E1/E2 mediated viral DNA replication (Lepik *et al* 1998), the E6 protein targets p53 for degradation, and HPV16 E2 induces apoptosis in HPV negative cells through a p53 dependent mechanism. Secondly, U2OS cells do not undergo apoptosis by HPV16 E2 expression even though they are p53 wild type, and this is highlighted by the creation of U2OS cells that stably express E2 (these cell lines were prepared by Dr Winifred Boner, see Taylor *et al* 2003 a). This point is important because a combination of E2 and UVB irradiation could be very toxic for many other cell lines. Unfortunately there are no known epithelial cell lines that are p53 wild type and resistant to E2 induced apoptosis.

3.2.1

Cellular UVB irradiation reduces the transactivation potential of E2

To test what effect UVB irradiation has on the transactivation function, cells transfected with ptk6E2 and E2 Were irradiated or mock irradiated with UVB irradiation 24 hours post transfection. 16 hours post irradiation cell extracts were harvested and luciferase counts were determined. With 10ng of E2 present there was a 25 fold activation of the tk6E2 promoter, UVB irradiation repressed the E2 dependent transactivation by 5 fold, see figure 3.2.1a and compare lane 4 with lanes 5 and 6. No significant effect on the activation of the tk6E2 promoter was observed with UVB irradiation in the absence of E2. To control for cell death and more general effects on transcription a parallel experiment was done using the ptk reporter plasmid (see figure 3.2.1b). When cells are irradiated there is no significant effect on transcription from the ptk plasmid. When E2 is expressed the transcription from ptk is repressed by 50% however this level of transcription is not affected by UVB irradiation. These results demonstrate that cellular UVB irradiation results in a significant reduction in the transactivation potential of E2 and this reduction is independent of cell death and general transcription inhibition.

HPV16 E2 is from a high-risk HPV subtype that infects mucosal epithelium, therefore to further characterise the effect of UVB irradiation on E2 the effect of UVB irradiation on HPV8 E2 transactivation function was tested. HPV8 is a virus that infects cutaneous epithelia (see table 1.1). With the ptk6E2 reporter HPV8 E2 activates the tk6E2 promoter twenty fold. With 300 J/m^2 UVB irradiation there is a four fold repression of this activation and at 400 J/m^2 UVB the activation by E2 is reduced to levels similar to the basal level of transcription (see figure 3.2.2a). When

the response of ptk was tested no significant effect on transcription was observed with UVB irradiation, either with or with-out E2 (see figure 3.2.2b). Also HPV8 E2 expression alone did not effect the level of transcription from the ptk reporter.

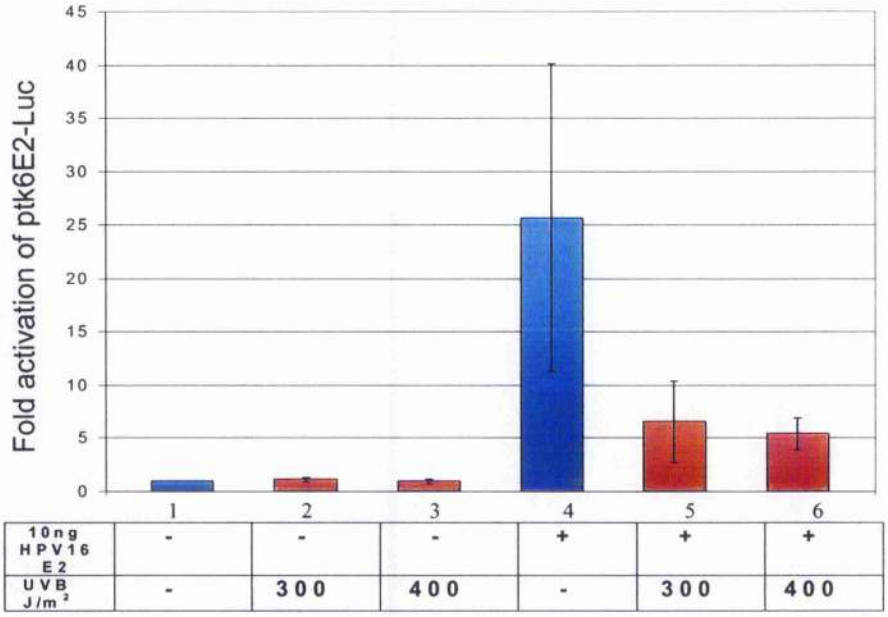
Additional characterisation of the response of E2 to UVB was done using a chimeric E2 molecule. VP16-E2 contains the hinge and DNA binding domain of BPV1 E2 and the herpes virus VP16 transactivation domain replaces the BPV1 E2 transactivation. With the ptk6E2 reporter VP16-E2 activates the tk6E2 promoter four hundred fold and with UVB irradiation there is a four fold repression of this activation. This result is of interest and will be discussed in detail later.

UVB irradiation stimulates multiple DNA damage signalling pathways therefore to test if the reduction of E2 transactivation function is due to a generic DNA damage signal cells were treated with the chemotheraputic agents hydroxyurea or etoposide (I.Morgan. Taylor *et al* 2003 a). Hydroxyurea is an inhibitor of ribonucleotide reductase resulting in the depletion of nucleotides available for DNA synthesis and causes the stalling of replication forks and a cell cycle arrest early in S-phase (Yarbro 1992). Etoposide is a DNA topoisomerase II inhibitor that results in double stranded DNA breaks during late S-phase and G2 that results in a G2 arrest (Chow & Ross 1987). Treatment of U2OS cells with both these agents does not result in a reduction in E2 transactivation potential even though a DNA damage repsonse is induced as demonstrated by the increase in p53 level (E.Dornan. Taylor *et al* 2003 a).

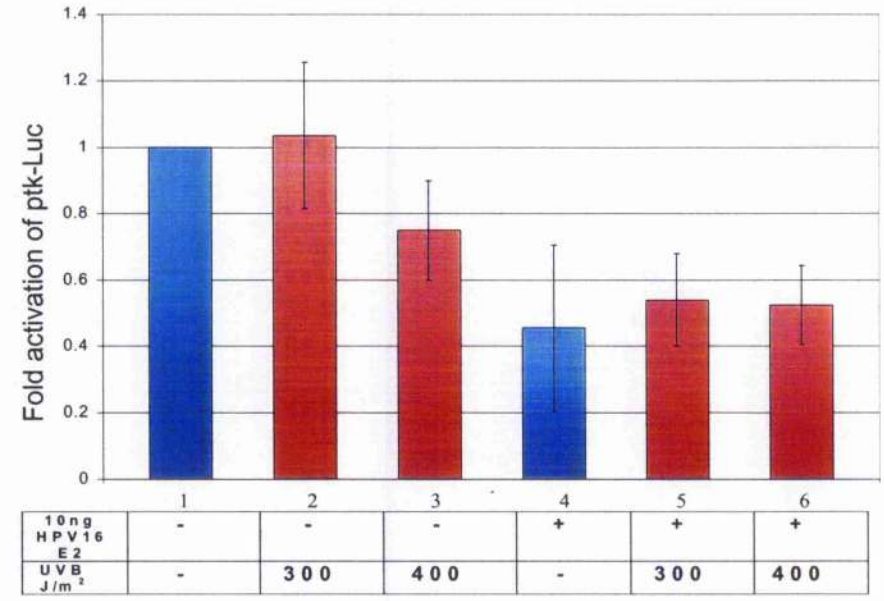
Figure 3.2.1

UVB mediated repression of HPV16 E2 transactivation function

(a)



(b)



UVB irradiation downregulates HPV16 E2 transactivation function

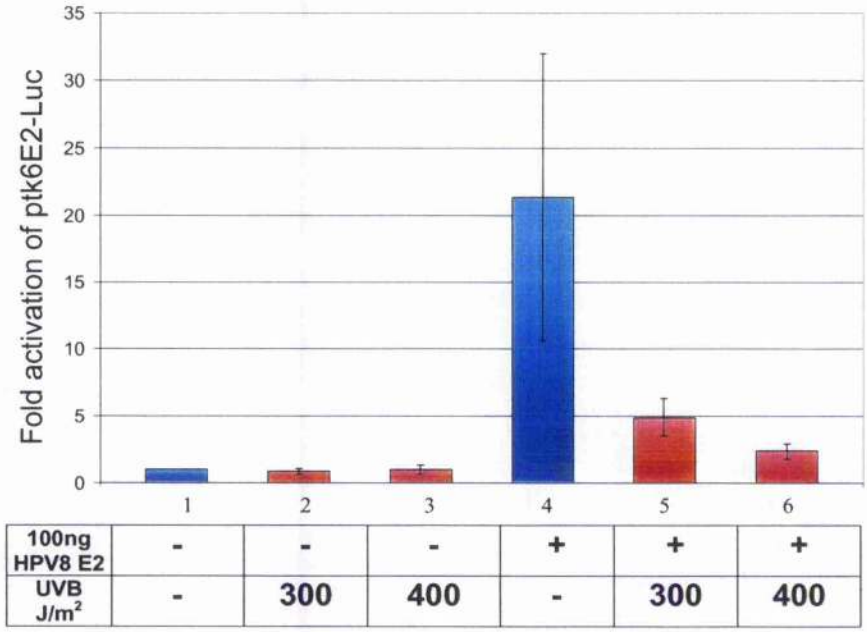
(a) U2OS cells were transfected with ptk6E2 (1μg), 10ng of E2 and UVB irradiated as indicated (red bars). Lanes 1-3 show the effect of UVB irradiation on the basal level of transcription from the tk6E2 promoter. Lanes 4-6 show the effect of UVB irradiation on E2 mediated transactivation.

(b) U2OS cells were transfected with ptk (1μg), 10ng of E2 and UVB irradiated as indicated (red bars). Lanes 1-3 show the effect of UVB irradiation on transcription from the tk promoter. Lanes 4-6 show the effect of UVB irradiation on transcription when E2 is present.

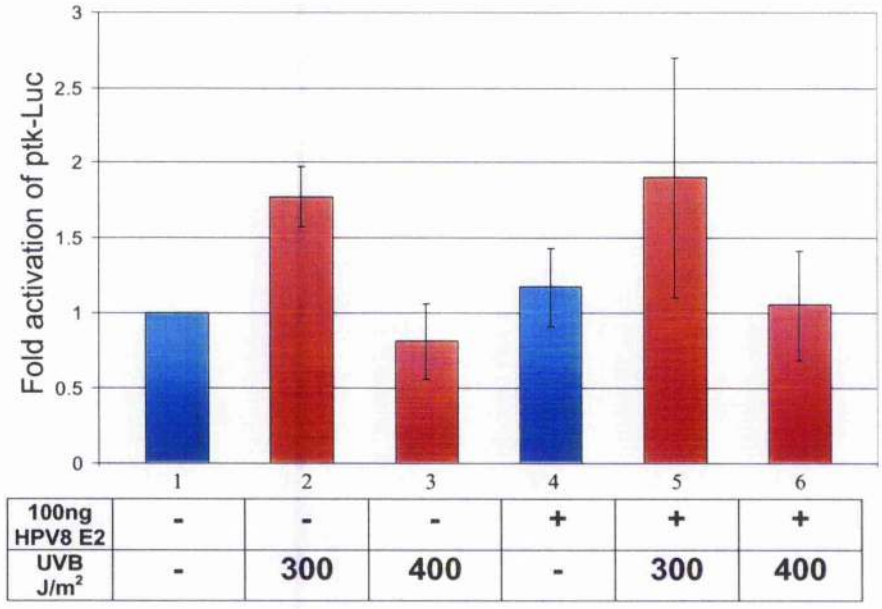
Figure 3.2.2

UVB mediated repression of HPV8 E2 transactivation function

(a)



(b)



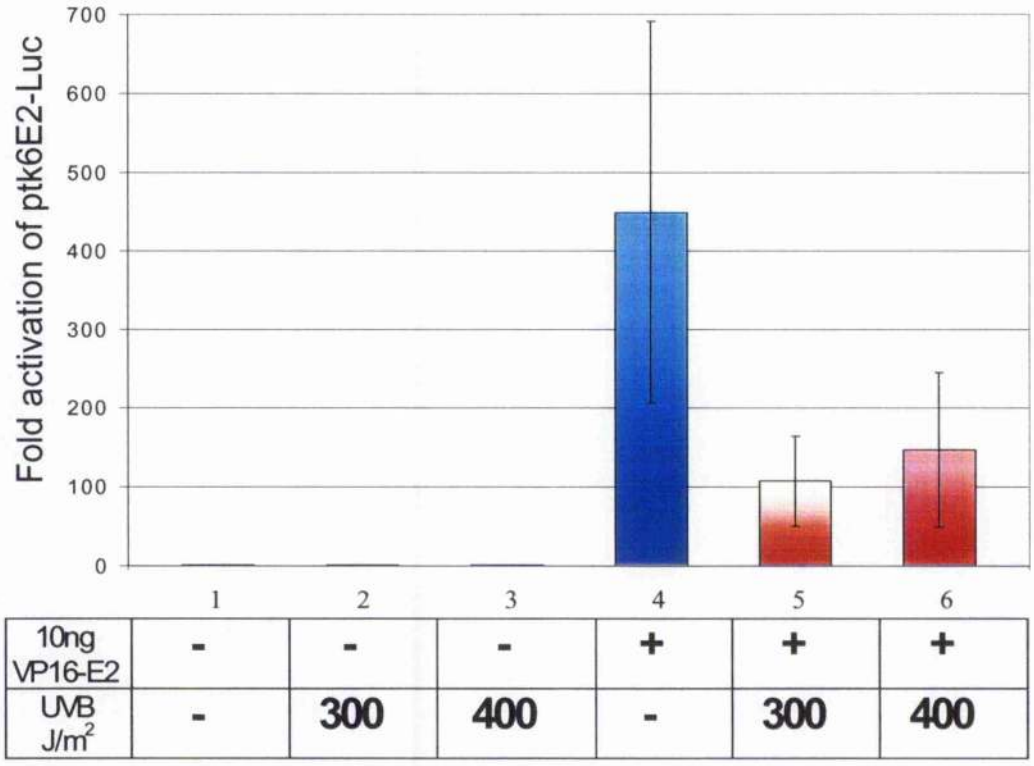
UVB irradiation downregulates HPV8 E2 transactivation function

(a) U2OS cells were transfected with ptk6E2 (1μg), 10ng of E2 and UVB irradiated as indicated (red bars). Lanes 1-3 show the effect of UVB irradiation on the basal level of transcription from the tk6E2 promoter. Lanes 4-6 show the effect of UVB irradiation on E2 mediated transactivation.

(b) U2OS cells were transfected with ptk (1μg), 10ng of E2 and UVB irradiated as indicated (red bars). Lanes 1-3 show the effect of UVB irradiation on transcription from the tk promoter. Lanes 4-6 show the effect of UVB irradiation on transcription when E2 is present.

Figure 3.2.3

UVB mediated downregulation of VP16-E2 transactivation function



UVB irradiation downregulates VP16-E2 transactivation function. U2OS cells were transfected with ptk6E2 (1μg), 10ng of VP16-E2 and UVB irradiated as indicated (red bars). Lanes 1-3 show the effect of UVB irradiation on the basal level of transcription from the tk6E2 promoter. Lanes 4-6 show the effect of UVB irradiation on VP16-E2 mediated transactivation.

3.2.2

Repression of E2 function is independent of p53 function

The p53 protein is functionally altered in response to DNA damage due to direct protein modifications, protein abundance and protein turnover (see review Wahl & Carr 2001). HPV16 E2 and p53 functionally interact therefore p53 is a good candidate cellular interacting protein for modifying E2 function. The HPV16 E6 protein binds and degrades p53 and can therefore be used as a tool to investigate the role of p53 on the response of E2 to UVB irradiation.

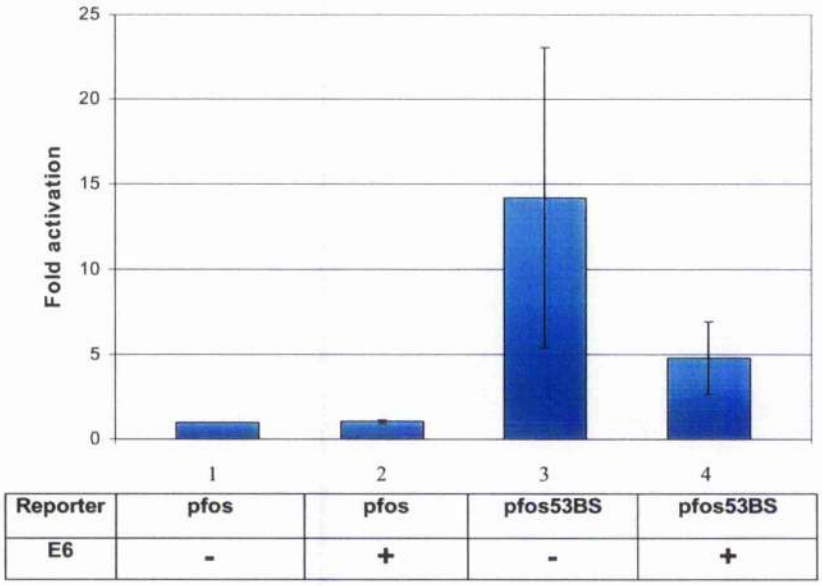
To demonstrate that transfection with E6 results in a reduction in p53 function reporter plasmids containing the fos promoter with or without upstream p53 binding sites (Midgley *et al* 2000) were transfected into U2OS cells. The fos promoter is activated fifteen fold when there is upstream p53 binding sites (see figure 3.2.4). Co-transfection with HPV16 E6 results in repression of this activation three fold, demonstrating that E6 is reducing p53 function in U2OS cells. Assaying the effect of E6 expression on the repression of E2 function by UVB irradiation was done using the ptk6E2 reporter (see figure 3.2.5a). The presence of E6 has no significant effect on the ability of HPV16 E2 to transactivate nor on the UVB mediated reduction of E2 transactivation of ptk6E2 (figure 3.2.5a compare lanes 5 and 6 with lanes 7 and 8). This indicates that p53 is not primarily responsible for the repression of E2 by UVB. This experiment was done in parallel with the ptk reporter to control for general effects on transcription and cell death, no significant differences were observed with E2, E6, UVB or with combinations of all three (see figure 3.2.5b).

To further highlight that the UVB mediated repression of E2 function is independent of p53 function, transcription assays similar to figure 3.2.5 were done by Eilidh MacKay in the human osteosarcoma cell line Saos2 that is p53 null. In Saos2 cells

HPV16 E2 activates transcription from ptk6E2 by 400 fold and UVB irradiation represses this activation by two fold (see Taylor *et al* 2003 a). This result confirms that p53 is not primarily responsible for the repression of the transactivation function of E2 by UVB irradiation.

Figure 3.2.4

Reduction of p53 function by HPV16 E6

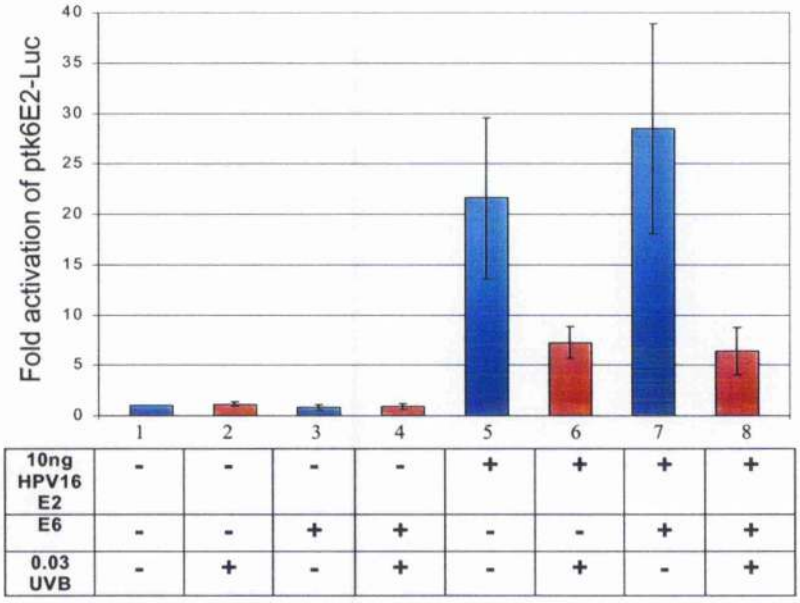


Reduction of p53 transactivation function by expression of HPV16 E6. pfos-Luc reporter (1μg) was co-transfected with or without 1μg of HPV16 E6 (lanes 1 and 2). p53BSfos-Luc reporter (1μg) was co-transfected with or without 1μg of HPV16 E6 (lanes 3 and 4). Lanes 1 and 2 show the effect of HPV16 E6 expression on the level of transcrioption from the fos promoter. Lanes 3 and 4 show the level of transcription from a fos promoter with consecutive p53 binding sites.

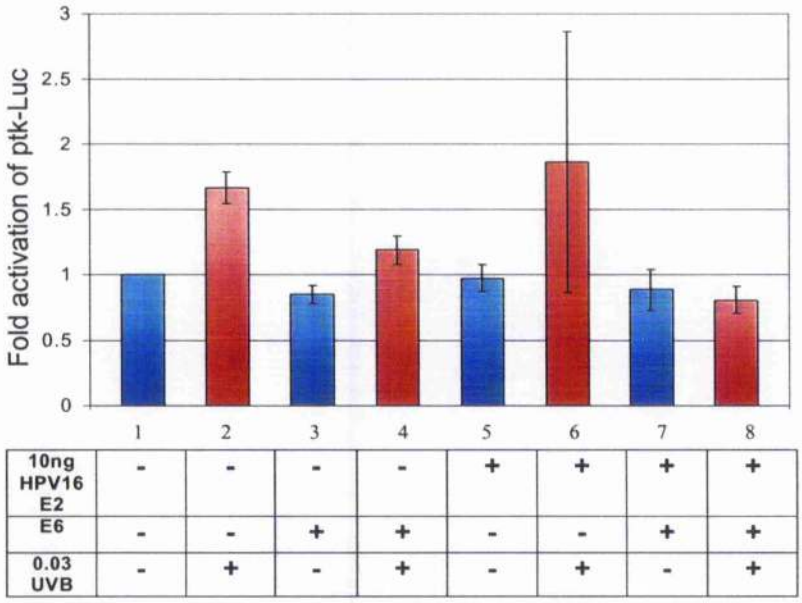
Figure 3.2.5

UVB reduces HPV16 E2 transactivation function in a p53 independent manner

(a)



(b)



Compromisation of p53 function by expression of HPV16 E6 has no effect on UVB downregulation of E2 transactivation function

(a) ptk6E2 (1μg) was co-transfected with 10ng E2 and 1μg E6 into U2OS cells as indicated. 300 J/m^2 UVB irradiation is as indicated (red bars). Lanes 1-4 show the effect of E6 and UVB irradiation of the basal level of transcription from the tk6E2 promoter. Lanes 5-8 show the effect of E6 and UVB irradiation on the transactivation of the tk promoter by E2.

(b) ptk (1μg) was co-transfected with 10ng E2 and 1μg E6 into U2OS cells as indicated. 300 J/m^2 UVB irradiation is as indicated (red bars). Lanes 1-4 show the effect of E6 and UVB irradiation of the level of transcription from the tk promoter. Lanes 5-8 show the effect of E6 and UVB irradiation of the level of transcription from the tk promoter when E2 is present.

3.2.3

E2 protein level is reduced after UVB irradiation

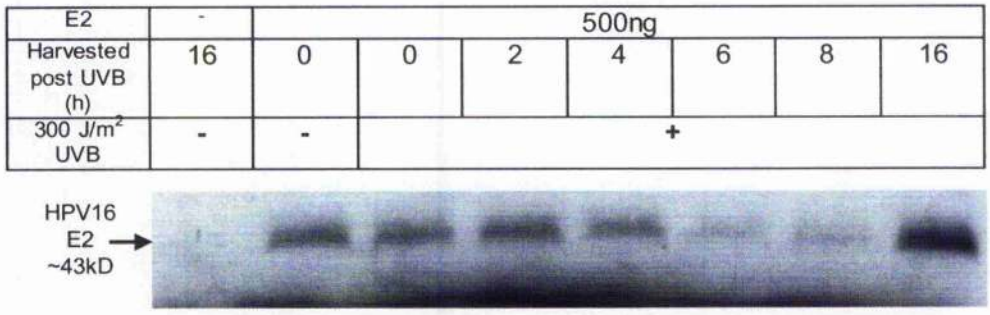
The reduction of the E2 transactivation function could be due to a reduction of E2 protein level. To test this U2OS cells were transfected with 500ng of pCMV-E2b then UVB irradiated. Western blot analysis of E2 protein levels was done at various time points after UVB irradiation as indicated in figure 3.2.6. E2 protein levels remain constant for the first two hours after irradiation after which E2 levels are significantly reduced with maximal effect eight hours after irradiation. Sixteen hours after irradiation E2 levels are restored. This experiment has been repeated by Dr Winifred Boner using the U2OS cells that stably express HPV16 E2 (see Taylor *et al* 2003 a). These experiments demonstrate a similar reduction in the level of HPV16 E2 post UVB irradiation. These western blots also show that post UVB irradiation p53 levels are increased significantly therefore demonstrating that there is activation of the DNA damage response post UVB irradiation. Additionally hydroxyurea treatment of these stable cell lines induces p53 however this does not lead to the degradation of HPV16 E2 (E.Dornan, Taylor *et al* 2003 a).

To ensure that the reduction of E2 protein level is not due to a reduction in E2 mRNA amount quantitative Taqman® RT-PCR was performed on mRNA extracts two and eight hours post irradiation. This was done using the U2OS cells that stably express HPV16 E2. The relative amount of mRNA present at these time points was compared to that prior to irradiation and the relative value plotted in figure 3.2.7, actin mRNA was used as an internal control. There was no significant reduction in E2 mRNA in response to UVB irradiation. This experiment indicates that the reduction in E2 level is done post transcriptionally.

Inhibition of proteasome function by MG132 resulted in stabilisation of E2 in response to UVB (W. Boner, Taylor *et al* 2003 a) indicating the involvement of ubiquitin mediated proteolysis as the mechanism for E2 degradation following UVB irradiation. Determination of E2 protein half-life demonstrated that the half-life of E2 is reduced two fold following UVB irradiation (E.Dornan, Taylor *et al* 2003 a).

Figure 3.2.6

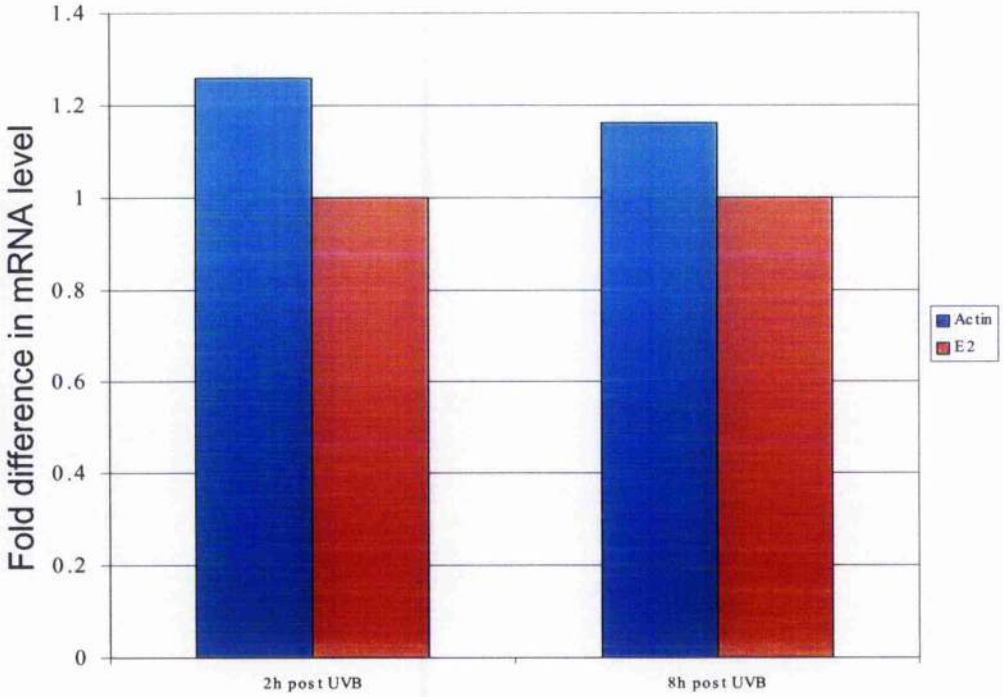
Reduction of HPV16 E2 protein level by UVB irradiation



Reduction in the level of HPV16 E2 in response to UVB irradiation. U2OS cells were transfected with 500ng E2 and subsequently UVB irradiated with 300 J/m^2 as indicated. Protein extracts were harvested at the time points post-irradiation as indicated. Protein concentrations were determined using the BCA protein assay (see 2.2.1.3) and an equal amount of protein were added to each well. Levels of HPV16 E2 were assessed by western blot.

Figure 3.2.7

Fold difference in E2 and actin mRNA level post UVB irradiation



Quantitation of HPV16 E2 and β actin mRNA levels post UVB-irradiation in U2OS cell lines that express HPV16 E2. Duplicate plates were set-up, with one irradiated and one left untreated. At the time points indicated post-irradiation mRNA was harvested and the amount of β actin (blue bars) and E2 (red bars) mRNA present was calculated using RT-PCR Taqman. Results are graphed as the fold difference in signal 2 and 8 hours post-irradiation, when comparing UVB irradiated samples to non irradiated.

3.2.4

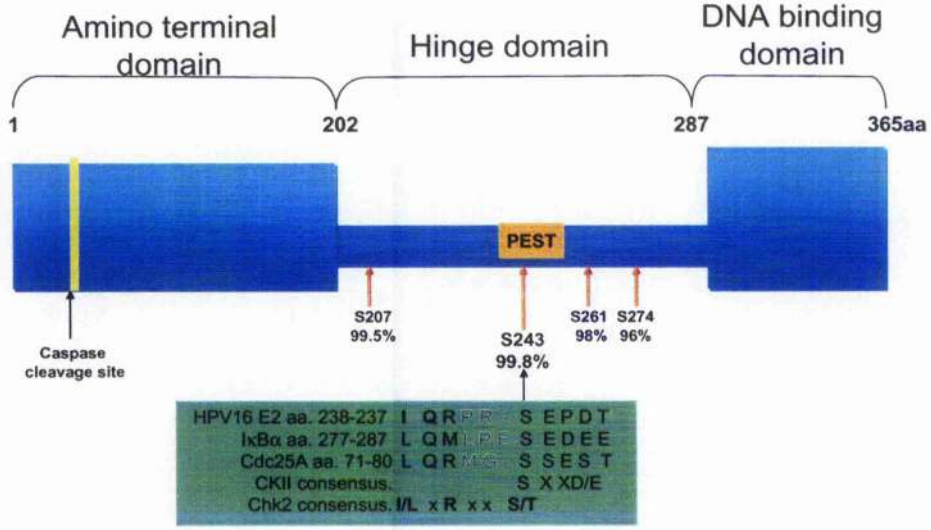
Bioinformatic search for future targets for understanding UVB mediated reduction of E2 protein level

Future study of the mechanism for the reduction of E2 protein half life by UVB irradiation should involve deletion mapping of HPV16 E2 to help identify what sequences are involved in the stability of the protein. Additionally HPV16 E2 is a phosphoprotein and phosphorylation protein modifications commonly control the stability and turnover of proteins. With BPV1 E2 protein it has been demonstrated that the two phosphorylation sites within the hinge region can influence protein half-life. Point mutation of serine 301 of BPV1 E2 to alanine increases the proteins half-life two fold and reduces the level of protein ubiquitination. This phosphorylation site is within a region of the hinge of BPV1 that contains a consensus PEST sequence. PEST sequences are a group of short divergent protein motifs that control protein turnover and are often controlled by protein phosphorylation. It is possible that the the half-life of HPV16 E2 is also controlled by similar phosphorylation events. Computer based bioinformatic analysis of protein sequences using motif prediction programs are useful for rapid assessment of suitable regions of a protein for deletion and point mutagenesis. Bioinformatic prediction of phosphorylation sites with the NetPhos2.0 program (see materials and methods 2.2.4.2, Blom *et al* 1999) on the HPV16 E2 protein suggests that like other E2 proteins the major phosphorylation sites are in the flexible hinge of the protein. Within this hinge there is a potential PEST sequence at amino acids 242-252 as determined using the PESTFIND program (materials and methods 2.2.4.3). Within this sequence there is the highest scoring predicted phosphorylation site (see figure 3.2.8). Further analysis of the sequence surrounding this serine reveals that this site is the consensus for both casein kinase II (CKII) and

the check point kinase Chk2. Serine 301 of BPV1 also has the consensus sequence for CKII suggesting that S243 of HPV16 E2 may have a similar function in protein stability. Both CKII and Chk2 are activated in response to DNA damage (gamma irradiation, UV irradiation) and phosphorylate multiple target proteins (e.g. p53, BRCA1). Also CKII has been demonstrated to control the turnover of IκBα in response to DNA damage by the phosphorylation of serine 283 (Shen *et al* 2001) and Chk2 controls the turnover of Cdc25a in response to UV irradiation by phosphorylation of serine 75 (Hassepass *et al* 2003). Both these residues are located within predicted PEST sequences. The sequence surrounding serine 243 of HPV16 E2 shows limited homology to those around S293 of IκBα and S75 of Cdc25a (see figure 3.2.8). Therefore serine 243 of HPV16 E2 is good target for investigation. Finally HPV18 E2 has a caspase cleavage consensus site within the amino terminus (Demeret *et al* 2003) and HPV16 E2 also contains an identical site between amino acids 19-23 compared to amino acids 23-28 of HPV18 E2. This is also worth investigation.

Figure 3.2.8

Bioinformatic search for possible motifs responsible for HPV16 E2 degradation



3.3

Analysis of the frequency of mutation during HPV16 E1/E2 mediated DNA replication

Replication of the viral genome is activated by the viral E1 and E2 proteins. E1 and E2 recruit cellular DNA polymerases to replicate the viral genome. Therefore maintenance of the genetic integrity of the viral genome through multiple generations is assumed to be dependent on cellular replication/repair processes. Cellular DNA replication has a low frequency of spontaneous mutation indicating that HPV DNA may be similar. However HPV DNA from naturally occurring lesions often contains HPV genomes with deletion/insertion rearrangements or parts of the HPV genome chromosomally integrated. A study using tonsillar cancer biopsies recently demonstrated that in three from eleven HPV16 episomal DNA positive samples examined there were episomal viral genomes containing deletions present, and two of these were coexistent with full length episomal HPV DNA (Mellin *et al* 2002). There have been cancerous lesions identified that contain only mutant episomal HPV genomes containing deletions or rearrangements in the viral LCR and capsid protein coding regions (Kasher & Roman 1988, Deau *et al* 1991). Additionally in the HPV16 model cell line, W12, episomal copies of the viral genome can be lost rapidly due to integration over the course of less than 10 passages (Alazawi *et al* 2002). Integration of the viral genome is an event closely linked to tumour progression. These observations indicate that HPV replication may be less stable and accurate compared to cellular DNA replication. Double-stranded DNA breaks created during replication/repair processes are efficient substrates for recombination events resulting in DNA rearrangement and integration. It is these events that will be responsible for

HPV genome rearrangement and integration. I therefore set out to examine the fidelity of HPV16 replication.

3.3.1 Determination of mutation frequency in C33a cells

Initial experiments were done in the C33a cell line as this cell line is the standard cell line used for HPV mediated DNA replication assays due to them being derived from a HPV negative cervical carcinoma. The system used is based on transiently replicating a HPV16 replication origin containing plasmid pOri16Lac (see figure 3.3.1), a plasmid that contains a phenotypic marker, LacZ. Subsequently the freshly replicated plasmids were rescued into DH10B *E.coli*. The α–complementation between the LacZ' gene on pOri16Lac and the deleted LacZ in DH10B means that DH10B carrying pOri16Lac with a wild type LacZ are blue, and those pOri16Lac with mutations in LacZ are light blue/white. Selected colonies were picked and grown overnight and restreaked on the same medium to confirm the phenotype. The frequency of mutation can then be calculated by scoring for LacZ-/LacZ+. Efficient replication was confirmed by Southern blotting (see figure 3.3.2a); this blot demonstrates that pOri16Lac is efficiently replicated by E1 and E2. To measure the fidelity of replication parallel experiments were carried out and the replicated molecules electroporated into DH10B and scored for the number of blue and white colonies. This experiment was carried out three times and the cumulative results from these experiments are shown in Table 3.3.1. The mutation frequency detected was rather high, being $4x10^{-3}$. To determine what types of mutations are responsible for the mutation frequency plasmid DNA was prepared from the transformed bacteria and digested with *BamHI*. This would be predicted to provide bands of 2.9 and 1.2 kb; however as shown in Figure 3.3.2b this is not what is seen in most cases. In many

colonies the pOri16Lac has undergone deletions insertions and more complex DNA rearrangements. These rearrangement mutants account for the majority of the plasmids and subsequent DNA sequence analysis demonstrated that many plasmids that showed a WT restriction map have smaller insertions or deletions of about 40bp (E.Dornan, J.Connolly & S.McNair, see Taylor *et al* 2003 b). These plasmids can only be created through inaccurate repair of double strand DNA breaks created during the replication process.

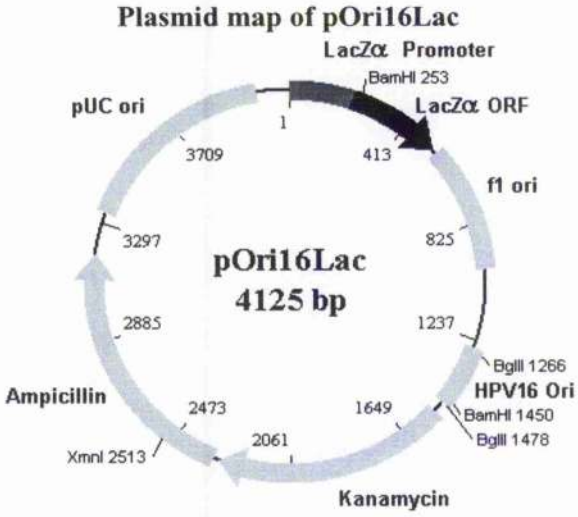
To gain further insight in to the reason for this high rate of spontaneous mutation I next tested to see if it is because of error prone replication by E1/E2 or is it due to the genetic background the plasmid is replicating in. C33a cells are a carcinoma derived cell line and have been shown to have a high rate of sister-chromatid exchange and microsatellite instability (Larson *et al* 1996).

3.3.2 Genetic defects that effect DNA replication fidelity

Xeroderma pigmentosum (XP) patients have increased sensitivity to UV irradiation due to genetic defects in proteins involved in nucleotide excision repair (NER) or trans-lesion synthesis (TLS). UV irradiation induces a range of DNA lesions (see section 4.2.2) the most common of which is the covalent bonding between two adjacent pyrimidine bases creating a cyclobutane pyrimidine dimer (CPD). Patients that lack NER functions have mutations in a group of proteins (XPA-XPG) that are involved in the excision of DNA lesions (Berneburg & Lehmann 2001). Patients with defective TLS (XP variant, XPV) lack the TLS DNA polymerase η (Cordonnier & Fuchs 1999). DNA polymerase η is a member of the Y family of polymerases that are involved in trans-lesion synthesis across DNA damage. Polη allows efficient by-pass

replication with relatively high fidelity. Defects in DNA polymerase η result in the error prone replication bypass or the collapse of the replication fork. Cell lines have been established from several XP patients by immortalizing primary fibroblasts using SV40 infection (Kannouche *et al* 2001). XPV cell lines, and those expressing restored polη, have been used in SV40 replication assays to confirm that polη is indeed involved in the efficient replication of CPD containing DNA (Stary *et al* 2003). To investigate if HPV mediated DNA replication can use cellular DNA TLS polymerases and cellular NER mechanisms the fidelity of replication was measured on both damaged and undamaged templates in a variety of XP cells with genetic defects in DNA repair.

Figure 3.3.1

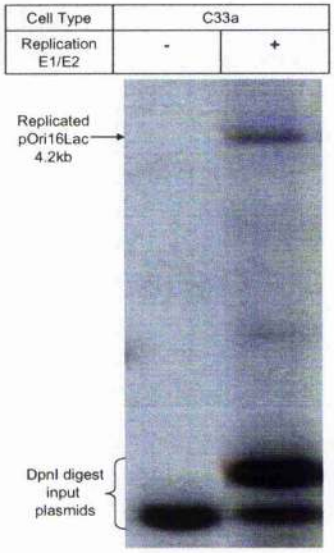


A map of the pOri16Lac replication vector. Darkened area highlights the LacZα promoter and ORF, the target for isolation of mutations arising during HPV16 E1/E2 mediated replication. The HPV16 origin sequence was cloned into the *BglII* site, and the kanamycin antibiotic resistance gene allows selective rescue of pOri16Lac plasmids.

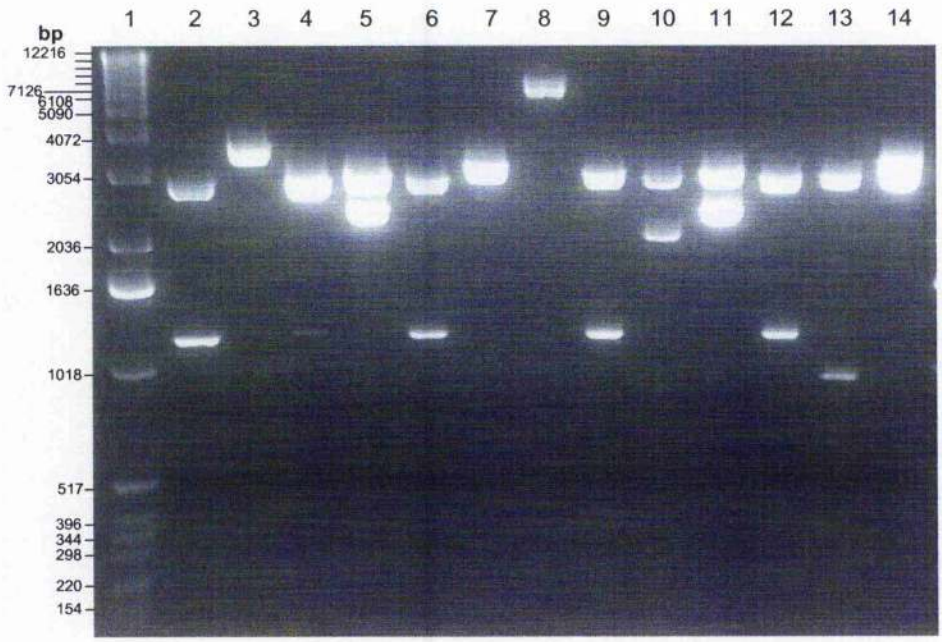
Figure 3.3.2

Replication and rescue of pOri16Lac in C33a cells

(a) Replication of pOri16Lac in C33a cells



(b) **Restriction digest analysis of rescued plasmids**



(a) Replication of pOri16Lac in C33a cells. C33a cells were transfected with plasmid DNA as indicated. Three days post-transfection low MW DNA was harvested. DNA was *XmnI/DpnI* digested to reveal a linear band of replicated pOri16Lac as indicated. Lane 1 has no E1/E2 present therefore there is no pOri16Lac replication. Lane 2 has E1/E2 present therefore pOri16Lac is replicated.

(b) Mutant pOri16Lac plasmids are commonly rearranged. 5µl of miniprep DNA from mutant pOriLac16 plasimd rescued was *BamHI* digested and resolved on an agarose gel. DNA was visualized using ethidium bromide staining. 1kb marker is loaded in lane 1 with the values in base pairs marked on the left of the gel. Wild type DNA is in lane 2, DNA from white colonies from E1/E2 mediated replication in C33a cells are in lanes 3-14.

Table 3.3.1

Mutation frequency of pOri16Lac by E1/E2 mediated replication in c33a cells

Cell Type	C33a
Total	9477
White	39
MF ($X10^{-5}$)[a]	**412**
Rearranged[b]	25
Rearranged MF ($X10^{-5}$)[c]	**263**

[a] MF; mutation frequency (calculated by white/total).

[b] As scored by *BamHI* digestion.

[c] Calculated by rearranged/total.

3.3.3 Determination of UVC mediated damage level on plasmid DNA

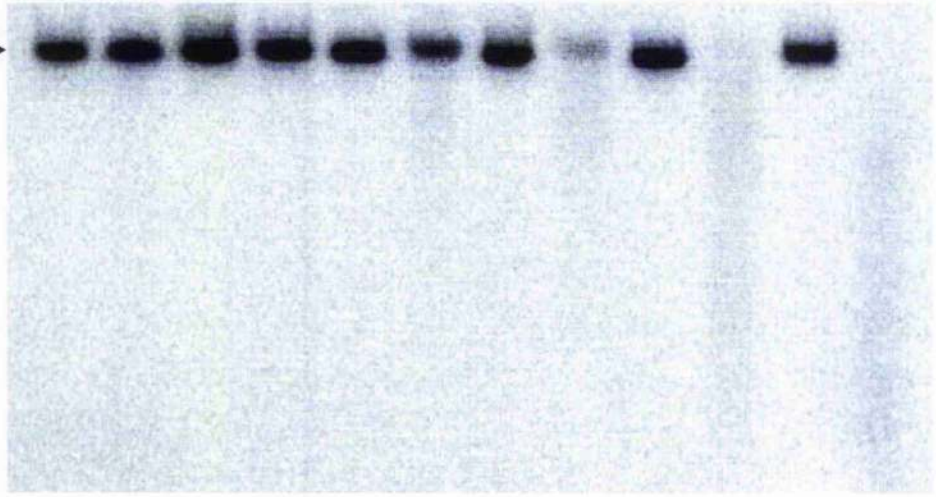
pOri16Lac was irradiated with UVC using a Stratagene cross-linker. To ensure the UVC irradiated DNA transfected contains multiple DNA lesions I tested UVC irradiated DNA *in vitro* for its sensitivity to T4 endonuclease V, an enzyme that nicks CPD lesions (see figure 3.3.3a). At lower levels of UVC irradiation (0-800 J/m^2) a significant band of full length DNA is visible at 4.2kb indicating that not all pOri16Lac DNA may be damaged. Optimal levels of UVC irradiation appear at 1600 J/m^2 UVC and greater with the clear demonstration that >99% of the DNA treated has multiple UVC lesions. 1600 J/m^2 UVC is the level of damage used for all future work. To ensure that the damaged pOri16Lac transfected is not completely repaired by nucleotide excision repair prior to replication, undamaged and damaged pOri16Lac DNA were harvested from C33a cells three days post-transfection. The DNA was linearised and digested with T4 endonuclease V. Figure 3.3.3b demonstrates that >90% of the DNA UVC irradiated prior to transfection still contains multiple CPD lesions 3 days post-transfection.

Figure 3.3.3

Detection of UV induced CPD DNA lesions *in vitro* and *in vivo*

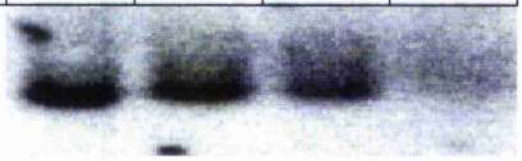
(a) Detection of UVC induced CPD lesions *in vitro*

UVC J/m²	0	0	100	100	400	400	800	800	1600	1600	5000	5000
T4 EndoV	-	+	-	+	-	+	-	+	-	+	-	+



(b) Detection of UVC induced CPD lesions in UVC damaged plasmid rescued 3 days post transfection

UVC J/m²	0	0	1600	1600
T4 Endo V	-	+	-	+



(a) Titration of *in vitro* UVC DNA damage to pOri16Lac. pOri16Lac was UVC irradiated as indicated, then mock digested or digested with T4 endonuclease V to remove CPD, all samples were then lincarised by *XmnI* digestion and 100pg of each sample was resolved on an agarose gel. DNA was visualized by Southern blot.

(b) UVC irradiated DNA is still DNA damaged three days post transfection. Mock or UVC irradiated DNA was transfected into c33a cells then three days post-transfection low MW DNA was harvested. DNA was treated and visualized as in figure 3.3.3a.

3.3.4 Genetic characterisation of the fidelity of E1/E2 to replicate damaged DNA.

HPV16 mediated E1/E2 replication assays were carried out in a range of cell lines; XP30 cells defective in Polη, XP30η cells are XP30 cells with stable expression of Polη, XP12 cells are defective in the XPA protein and lack NER, MRC5 cells are genetically wild type for DNA replication/repair proteins and C33a were used also and have been described previously. Replication assays were carried out in these cells using undamaged and UVC damaged pOri16Lac. These assays were carried out at least three times and Southern blots carried out to measure the amount of replication, a representative example of the Southern blots for each cell line is shown in figure 3.3.4. In both MRC5 and C33a there is little difference in the amount of replication that is detected with either the damaged or non-damaged template. However, in all XP cell lines there is a clear reduction in the amount of replication that is detected with the damaged template. This may be expected in the XP30 and XP12 cells due to the genetic lesions in these cells that hinder their response to DNA damage. However, restoration of polη, as demonstrated by XP30η, does not enhance the ability of this cell line to replicate damaged DNA.

To test the fidelity of E1/E2 mediated replication in each of these cells lines replicated pOri16Lac DNA was rescued into DH10B *E.coli* and the ratio of blue to white colonies was calculated. This was done at least three times and the total number for each cell line is shown in Table 3.3 To aid the study of these results figures 3.3.5 a-c are graphs summarising these results. A number of observations can be made about the fidelity of replication in the different genetic backgrounds.

Firstly, all of the cell lines have statistically insignificant differences in their background mutation frequency, apart from C33a cells where the number of mutants

is four fold significantly higher ($p<0.001$). This difference in mutation frequency may in part be due to the error phenotype of the cell. Secondly, damaging the template to be replicated with UVC significantly increased the numbers of mutants rescued in all cell types ($p<0.001$). These mutations are due to incorrect TLS or non-homologous recombination. Thirdly, restoration of polη to XP30 cells reduced the number of mutations observed around three-fold demonstrating once more the role of this polymerase in the replication of UV damaged DNA ($p<0.001$). Fourthly, upon restoration of polη the mutation frequency observed is no different from the MRC5 cell line. Fifthly, the number of mutations observed in MRC5 upon replication of the damaged template is significantly lower than that observed in XP30, XP12 and C33a cells ($p<0.001$). Sixthly, damaging the template to be replicated with UVC significantly increased the numbers of re-arrangement mutants rescued in all cell types ($p<0.001$). Seventhly, the number of rearranged recombinant plasmids detected following replication of the UVC damaged template are significantly increased in XP30, XP12 and C33a cells when compared with MRC5 ($p<0.001$). Finally, the large increase in mutations observed in the XP12 cells following replication of the UVC damaged template suggests that most of the mutagenic damage on the template is repaired using NER prior to replication of the template in S phase.

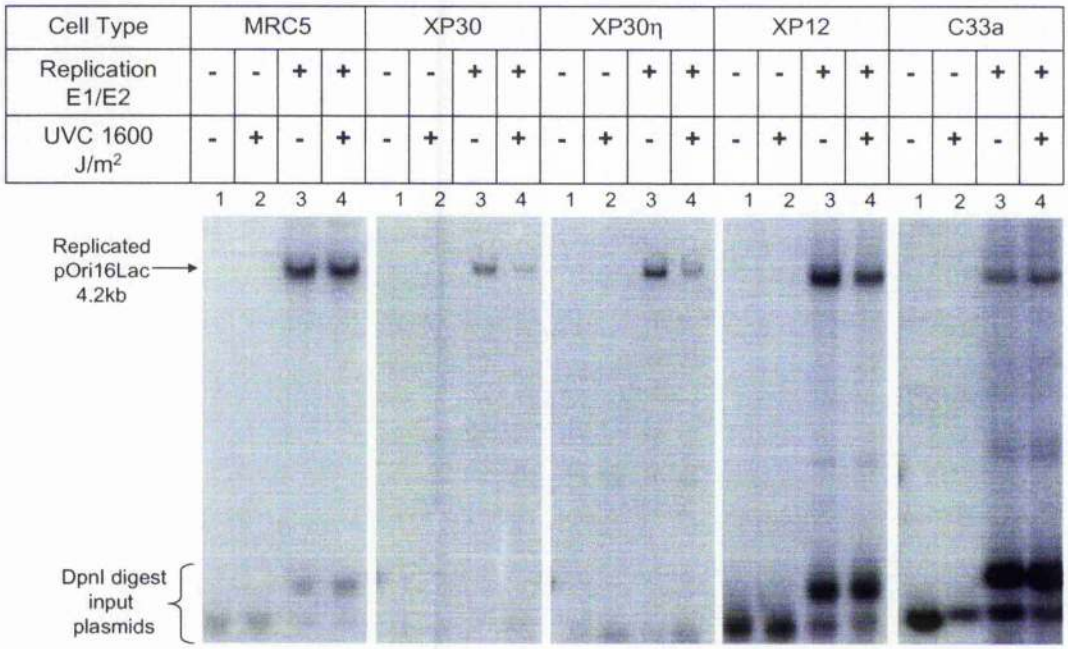
The higher mutation frequency with each XP cell line compared to MRC5 cells when the template is damaged is expected and highlights several points about the maintenance of the integrity of the viral genome. The cellular DNA replication complex that E1/E2 recruits is able to use cellular TLS mechanism to bypass DNA lesions during replication (XP30 compared to MRC5 and XP30η). Additionally the high mutation frequency (MF) in XP12 cells in response to DNA damage

demonstrates that the majority of the mutagenic DNA lesions caused by UVC irradiation are repaired via NER prior to the replication of the DNA.

These results give a unique insight into the role of cellular DNA replication/repair proteins during E1/E2 mediated DNA replication. With each cell line tested the MF observed using E1/E2 replication is reflective of the genetic background of the cell. This is highlighted in C33a cells by the error phenotype exhibited on both undamaged and damaged templates. The related polyoma virus SV40 large T antigen is thought to replicate DNA in a similar manner as HPV16 E1/E2. Therefore I investigated the MF of SV40 large T mediated replication in C33a cells on both undamaged and damaged templates to test if it is similar to that of HPV16 E1/E2 replication to confirm that the error prone phenotype is due to the cellular environment. Transient replication assays using a SV40 origin containing pCR2.1 derived vector pOriSV40Lac (1µg) was replicated by the expression of the SV40 Large T antigen protein (5µg). Southern blot analysis of replication demonstrates that the level of Large T mediated replication in C33a cells is not reduced when the template is UVC damaged (data not shown). Also in C33a cells SV40 replicated DNA with a similar fidelity to E1/E2. On an undamaged pOriSV40Lac the MF was $1042x10^{-5}$ and on a UVC damaged pOriSV40Lac the MF was $3531x10^{-5}$. All of the mutations on the undamaged template were due to DNA rearrangements. This experiment suggests that both HPV E1/E2 and SV40 large T replicate DNA with similar fidelity, and C33a cells have an error prone cellular environment.

Figure 3.3.4

Replication of UVC damaged pOri16Lac in various cell types



Response of E1/E2 mediated replication in response to UV damaged template in different DNA replication/repair deficient backgrounds. Plasmid DNA was transfected into cells as indicated, three days post transfection DNA was harvested and digested with both *XmnI* and *DpnI*. For each cell type lanes 1 and 2 have no E1/E2 present therefore there is no pOri16Lac replication. Lanes 3 and 4 have E1/E2 present therefore pOri16Lac is replicated. Lanes 1 and 3, no UVC irradiation of pOri16Lac, lanes 2 and 4 pOri16Lac was UVC irradiated prior to transfection.

Table 3.3.2

Mutation frequency of pOri16Lac by E1/E2 mediated replication in a variety of DNA repair/replication deficient cells

Cell Type	MRC5		XP30		XP30η		XP12		C33a	
pOri16Lac Treatment [a]	No UVC	1600 J/m^2 UVC	No UVC	1600 J/m^2 UVC	No UVC	1600 J/m^2 UVC	No UVC	1600 J/m^2 UVC	No UVC	1600 J/m^2 UVC
Total	10634	4104	8330	1502	6565	2262	10971	1157	9477	2850
White	10	50	5	46	5	20	3	83	39	104
MF ($X10^{-5}$)[b]	**94**	**1218**	**60**	**3062**	**76**	**884**	**27**	**7174**	**412**	**3649**
Rearranged[c]	4	14	5	11	3	9	2	14	25	27
Rearranged MF ($X10^{-5}$)[d]	**38**	**341**	**60**	**732**	**45**	**398**	**18**	**1210**	**263**	**947**

[a] UVC irradiation prior to transfection.

[b] MF; mutation frequency (calculated by white/total).

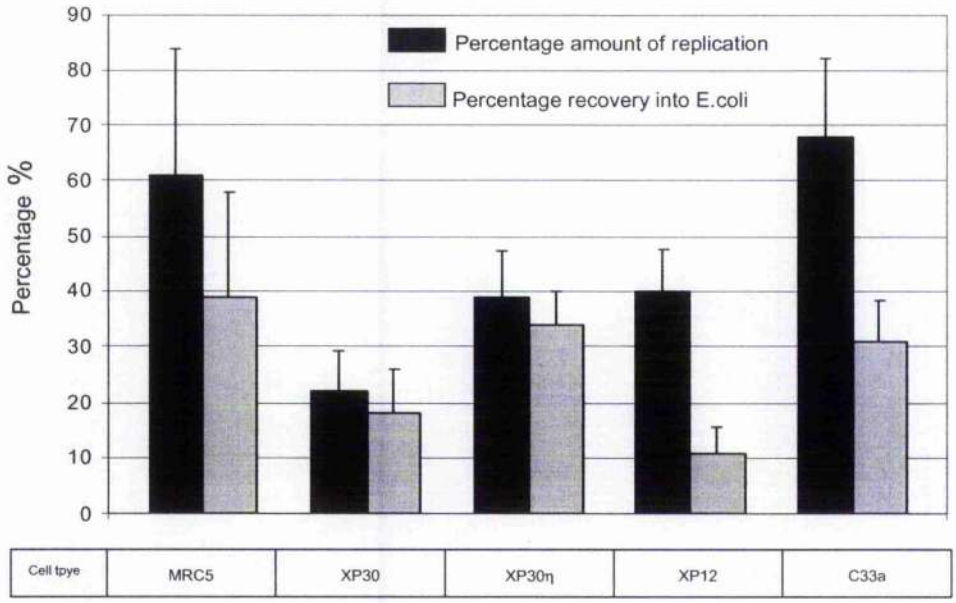
[c] As scored by *BamHI* digestion.

[d] Calculated by rearranged/total.

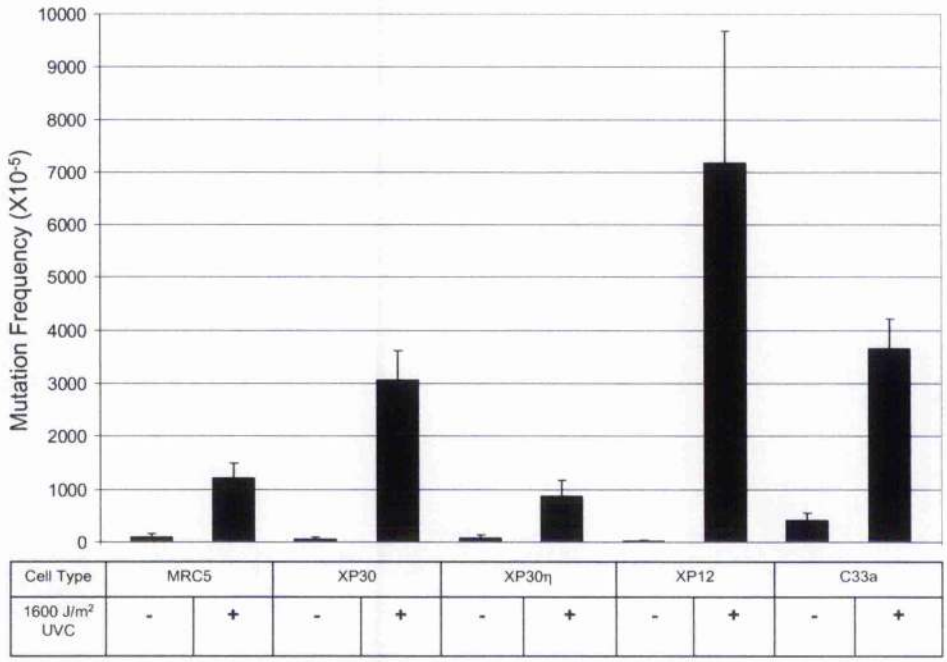
Figure 3.3.5

Summary of replication and mutation data from the replication of damaged pOri16Lac in various cell types

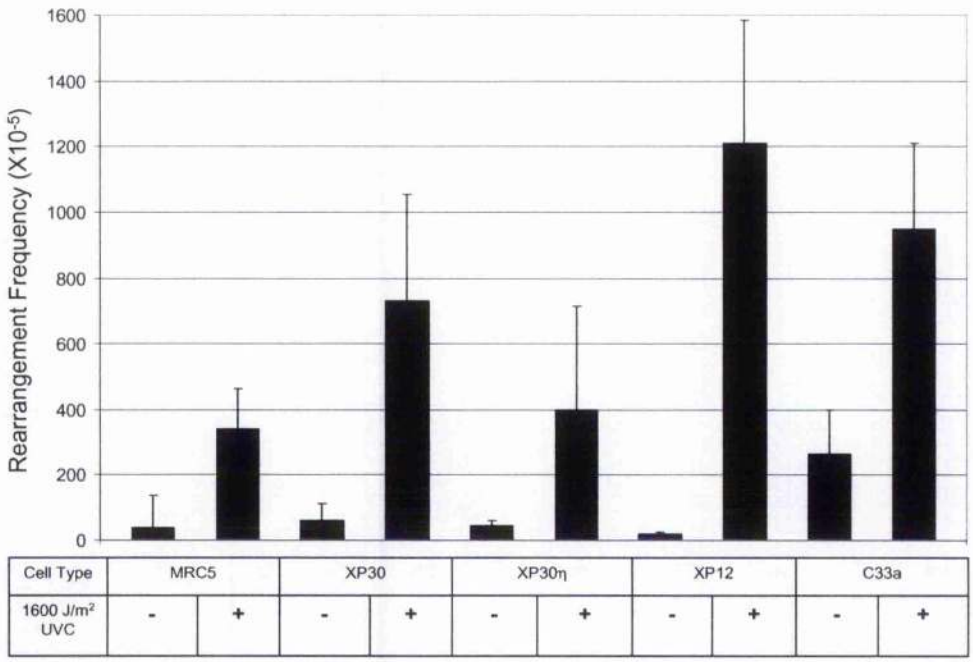
(a)



(b)



(c)



(a) Summary of the percentage of replication of pOri16Lac following UVC irradiation, and the percentage of bacterial colonies rescued. The results are shown as the percentage compared to when pOri16Lac is not UVC irradiated. Changes in the amount of replication following UVC irradiation were quantified from Southern blots.

(b) Summary of the frequency of mutations (MF) on the replicated pOri16Lac in a variety of cell types.

(c) Summary of the rearranged mutant frequency (rMF) on the replicated pOri16Lac in a variety of cell types. Data shown is the average of at least three independent experiments for each cell type in figure 3.3.5 a-c.

Discussion

This thesis is composed of three studies: the functional characterisation of the E2-TopBP1 interaction; the investigation of the effect on E2 function of DNA damage stimuli; and the investigation of HPV E1/E2 mediated DNA replication fidelity. The broad based approach to study the involvement of cellular DNA replication/repair processes in the modulation of E2 functions evolved from initial studies investigating the role of E2-TopBP1 interaction. The data presented in the three studies contained in this thesis highlight the functions of E2 and the possible roles of E2 during the HPV life cycle thus providing an extensive range of future targets to study. Also this thesis highlights the range of cellular processes that E2 can interact with. Therefore viral proteins like E2 have great future potential for use as molecular tools for the investigation of multiple cellular processes including transcription control, replication modulation and DNA repair.

4.1

Functional interaction between HPV16 E2 and the DNA damage response protein TopBP1

4.1.1 E2 and TopBP1

The major functions of HPV16 E2 protein are to regulate viral transcription, to regulate viral replication and to induce apoptosis. HPV E2 proteins can be structurally divided into three domains; an amino terminal transactivation domain, a central hinge domain and a carboxy-terminal DNA binding domain. The amino terminal transactivation domain of E2 is essential for the transcription, replication and apoptotic properties of E2 (see section 1.6). Using a yeast 2 hybrid screen a novel

interaction between the transactivation domain of HPV16 E2 and the carboxy terminal of the cellular DNA replication/repair protein TopBP1 was identified (Boner *et al* 2002, Boner & Morgan 2002). TopBP1 is a large nuclear protein that has 8 BRCT domains, the largest number in any known protein. BRCT domains are protein interaction domains therefore TopBP1 is a possible scaffold protein that may ensure the co-ordination of cellular processes. TopBP1 functions are essential for cellular DNA replication initiation, the sensing of DNA damage and DNA damage checkpoint responses (see introduction section 1.6.4). Therefore there could be multiple purposes for the E2-TopBP1 interaction. The function of the interaction between TopBP1 and HPV16 E2 was investigated, to assess the influence of TopBP1 on E2 mediated activation of transcription and replication.

The overexpression of TopBP1 results in an increase in E2 mediated transcription and replication. E2 interacts with several proteins that activate transcription and replication through the modification of chromatin structure. E2 interacting proteins p300/CBP and p/CAF possess intrinsic HAT activity. Another E2 interacting protein BRCA1 activates cellular promoters due to an interaction with p300/CBP (Pao *et al* 2000). This activation of cellular promoters and E2 transactivation is dependent on the carboxy-terminal BRCT domain of BRCA1 (Kim *et al* 2003). It is likely that TopBP1 is able to activate E2 transcription and replication function due to similar interactions by chromatin remodelling proteins with the BRCT domains of TopBP1. In contrast to full length TopBP1 the overexpression of a deletion mutant of TopBP1 that lacks the BRCT domains 1 to 5 is unable to activate E2 mediated transcription or replication. The BRCT domains 1 and 2 in TopBP1 are able to activate transcription in yeast therefore it is possible that they play a similar role in mammalian cells and are responsible for the enhancement of the transactivation function of E2 by TopBP1

(Makiniemi *et al* 2000). Further deletion mapping of TopBP1 will highlight which regions of TopBP1 are responsible for the enhancement of E2 mediated transactivation function. It is probable that TopBP1 enhances E2 mediated transcription function through the recruitment of chromatin modification proteins and/or through direct interaction with the transcription complex.

Overexpression of TopBP1 enhances the level of E1/E2 mediated DNA replication. Additionally overexpression of the amino terminal deletion mutant of TopBP1 has no significant effect on E1/E2 mediated DNA replication. However TopBP1 has several replication specific functions that E2 may use to facilitate replication. TopBP1 binds to DNA polymerase epsilon and this interaction is thought to be essential for DNA replication (Makiniemi *et al* 2000). The budding yeast TopBP1 homologue Dpb11 binds to both DNA polymerase alpha and epsilon. The localisation of Dpb11 to ARS (autonomously replicating sequence) DNA elements in yeast is essential for the association of DNA polymerase alpha and epsilon with ARS elements (Masumoto *et al* 2000). The region of TopBP1 that is homologous to Dpb11 encompases the BRCT domains 1, 2, 4 and 5, therefore this region may have similar functions in enhancing HPV DNA replication initiation. The Xenopus homologue of TopBP1, Xmus101/Xcut5, is essential for the initiation of DNA replication in egg extracts (Van Hatten *et al* 2002, Hashimoto & Takisawa 2003). Xmus101/Xcut5 is essential for the loading of cdc45 and DNA polymerase alpha and epsilon to the initiation complex. It is however not essential for the loading of the origin recognition complex (ORC) or Mcm 2-7 helicase proteins. Therefore TopBP1 has a hypothesised essential role in the formation of the DNA replication initiation complex which E2 may utilise. Additionally E2 could be viewed as the viral ORC complex as it is essential for

efficient recruitment of the viral E1 helicase hexamer and initiation of viral replication.

HPV DNA replication can be initiated several times per cell cycle, this is in contrast to cellular DNA replication where each replication origin can fire only once per cell cycle (Piirsoo *et al* 1996). Eukaryotic DNA replication initiation is tightly controlled to prevent the re-firing of cellular replication origins, thus ensuring the integrity of the genome (see Bell & Dutta 2002, Woo & Poon 2003, DePamphillis 2003 for reviews). Initiation of eukaryotic DNA replication involves the stepwise binding of initiation factors to form the pre-replication complex during G1. The ORC complex binds to eukaryotic replication origins during G1 and is essential for the loading of the Mcm2-7 helicase complex and additional factors (e.g. Cdt1, Cdc6p, Mcm10). Multiple events dependent on phosphorylation by CDKs (cyclin dependent kinases) and Cdc7 at the G1/S boundary result in the release of Cdt1 and Cdc6p, the loading of Cdc45, RPA and DDK and the phosphorylation of the Mcm 2-7 helicase proteins. This creates a licensed initiation complex that is able to load the DNA polymerases and initiate replication on entry into S-phase. The failure of any of these licensing steps (i.e. ORC, Mcm2-7, Cdc45 or DNA polymerase loading) prevents DNA replication. This control of DNA replication therefore prevents the re-replication of DNA during S-phase or G2. HPVs presumably achieve multiple firing of DNA replication origins by coding for their own ORC-like and DNA helicase proteins, E2 and E1 respectively. Unlike their cellular counterparts, it is presumed that E1 and E2 are able to form DNA replication initiation complexes multiple times during S-phase thus ensuring the exponential increase in papillomavirus genome copy number during few cell divisions (Ravnan *et al* 1992). Therefore there are two possible roles for the E2-TopBP1 interaction. Firstly, E2 may recruit TopBP1 to ensure the efficient recruitment of the

cellular DNA polymerases alpha and epsilon. This would facilitate the efficient initiation of DNA replication. E1 can also bind DNA polymerase α therefore E2, TopBP1 and E1 may exist as a stabilising multi-protein complex during HPV DNA replication initiation. Secondly, E2 may need to interact with TopBP1 so that it can efficiently license the E1/E2 replication initiation complex multiple times during S-phase thus ensuring replication of the HPV genome multiple times during S-phase. Amplification of the viral genome is essential for the establishment of viral infection and for the production of viral genomes suitable for package into new virions. TopBP1 therefore could be essential for the viral life cycle.

4.1.2 Point mutation analysis of E2-TopBP1 interaction

To further investigate the role of the TopBP1 interaction on E2 function an attempt to make an HPV16 E2 molecule that does not interact with TopBP1 was made. The point mutation substitution of four conserved amino acids in the E2 amino terminal to alanine were made. The four residues chosen (Y44, R47, K68 and Y102) were picked due to their conservation among different HPV subtypes, and because they were predicted to be distant from the E2 homodimerisation and E1 interaction domains on the surface of HPV16 E2. Each of the point mutants of E2 was tested for its ability to activate transcription and replication. Also the effect of TopBP1 overexpression on the transcription/replication functions of E2 and the ability of these mutant E2 molecules to bind TopBP1 *in vivo* were tested.

The wild-type and the R47A, K68A and Y102A mutant E2 molecules vary in their ability to activate transcription however the overexpression of TopBP1 results in an two to four fold enhancement of transcription from the tk6E2 promoter. In contrast Y44A is unable to activate the tk6E2 promoter and the overexpression of TopBP1

leads to a two fold repression of the tk6E2 promoter when the Y44A mutant E2 protein is co-expressed. This result has one of two implications. Firstly, TopBP1 may be essential for E2 mediated transactivation and the Y44A mutant is unable to bind TopBP1. Alternatively, the Y44A E2 mutant is fundamentally unable to activate transcription and the increased level of free TopBP1 therefore cannot enhance E2 mediated transcription. Each mutant E2 molecule tested can activate DNA replication with E1 to a similar degree however only with WT E2 and the K68A and Y102 mutant E2 proteins is E1/E2 mediated DNA replication enhanced by TopBP1. With the Y44A and R47A mutant E2 proteins there was no significant increase in the level of DNA replication. This result suggests that both Y44 and R47 mediate the interaction between TopBP1 and E2.

To investigate the ability of the mutant E2 proteins generated to bind TopBP1, co-immunoprecipitation experiments were done. E2 was pulled down by TopBP1 in each immunoprecipitation including with wild type E2 and each of the four point mutants E2 proteins tested. On the blot shown in figure 3.1.18 there is variation in the amount of E2 co-immunoprecipitated however these differences were unrepeatable. This experiment demonstrates that none of the E2 mutants proteins created totally abolishes the interaction between E2 and TopBP1. However the functional data in figures 3.1.16 and 3.1.17 suggests that the interaction may be disrupted *in vivo* for the Y44A and R47A mutant E2 proteins. It is possible that the conditions used for the immunoprecipitation experiment were not optimal for the detection of subtle effects on the interaction between the two molecules. Future optimisation of the protocol through titration of the level of TopBP1 antibody or the level of cell extract used may increase sensitivity of the technique.

4.1.3 Future work

While the attempt to find a TopBP1 non-interacting mutant of E2 was not a complete success in this study the principle of finding a non-interacting mutant is the most logical approach for future experiments. A mutant of E2 that fails to bind TopBP1 will provide a useful tool that will highlight the role of TopBP1 in the transactivation, replication and apoptosis functions of E2. Two experiments will demonstrate the precise role for the E2-TopBP1 interaction on the transactivation and replication functions of E2.

Firstly, the reverse yeast two-hybrid technique can be used to dissect the interaction between two proteins through the mutational screening of a protein region of interest (see Vidal & Legrain 1999 for review). Briefly, this methodology uses the principles of a convential yeast-two hybrid screen with the use of bait and prey chimeric proteins to activate transcription of a survival gene. The bait protein contains a DNA binding domain (e.g. LexA) and the protein sequence of interest. The prey protein contains a transcription activation domain (e.g. Gal4) and a library of coding sequence (e.g. cDNA library or point mutation library of target protein). A reverse yeast two-hybrid screen could be used to screen for a mutant of HPV16 E2 that can not bind to TopBP1. The carboxy-terminal of TopBP1 could be the interaction domain on the bait protein, and a degenerate library coding for the amino-terminal of E2 on the prey protein. Expression of bait and prey proteins that interact in yeast causes the expression of a protein that sensitises the yeast to cyclohexamide. Therefore this protocol will select E2 amino terminal protein sequences that are unable to interact with TopBP1. This more thorough approach to finding a non-interacting E2 mutant allows a more systematic approach to investigating the E2-TopBP1 interaction. Subsequent experiments with an E2 non-interacting mutant will test its ability to

activate transcription, replication, apoptosis and the ability of E2 to be tethered to mitotic chromosomes.

Secondly, the chromatin immunoprecipitation technique (ChIP) allows the investigation of the recruitment of proteins to DNA sequences (Kuo & Allis 1999). This technique has been used previously to study the loading of the ORC complex Mcm2-7 proteins and EBNA1 onto an EBV replication origin (oriP) during the passage of the cell cycle (Chaudhuri *et al* 2001, Ritzi *et al* 2003). A similar approach could be applied to investigate the recruitment of TopBP1 to activate transcription and replication. ChIP could be used to demonstrate recruitment of TopBP1 by E2 to E2 dependent promoters (e.g. on ptk6E2) and to the HPV replication origin (e.g. on pOri16). One important question to ask would be is a mutant of E2 that is unable to activate transcription but is wild type for replication function (e.g. R37A or Y44A) able to recruit TopBP1 to an E2 dependent promoter. In such a scenario TopBP1 may still enhance E1/E2 mediated replication, however it is of interest to find out if TopBP1 can still be recruited to E2 dependent promoters. Mutant E2 proteins like R37A and Y44A are likely to be still able to bind E2 binding sites however the failure to form a transcription initiation complex may prevent the recruitment of TopBP1. In contrast it is also of interest to see if a mutant of E2 that has very poor affinity for E1 and is defective for replication activation, yet wild type for transactivation (e.g. E39A) can recruit TopBP1 to the HPV replication origin as efficiently. This is of particular interest due to the ORC-like functions of E2 and the helicase function of E1, and this experiment would demonstrate if TopBP1 is preferentially recruited to an E1/E2 complex at the replication origin, or if E2 is enough. Additionally the effect of TopBP1 overexpression on the loading of E1 to replication origin by E2 is of interest. Finally if a mutant of E2 is found that is defective for TopBP1 interaction the ChIP

method described may demonstrate if any mutant E2 proteins fail to recruit TopBP1 to either transcription promoters or replication origins.

4.2

Reduction of E2 function in response to UVB irradiation

4.2.1 Reduction of E2 function in response to UVB irradiation

The HPV16 E2 protein interacts with a variety of DNA damage response proteins; p53, BRCA1, PARP and TopBP1 (see 1.6 and 3.1). Each of these proteins is functionally modified in response to DNA damage stimuli (see section 1.7). Therefore the ability of DNA damage stimuli to modulate E2 function was tested. The method of DNA damage first used was UVB irradiation, this was due the wide range of DNA lesions caused and the range of DNA damage pathways activated by UV damage. In response to UVB irradiation the transactivation function of HPV16 and HPV8 E2 were significantly reduced. This reduction was independent of effect to the tk promoter used and general effects on cell viability. UVB irradiation results in a rapid decrease in the level of E2 protein about four to eight hours post irradiation. This reduction is independent of mRNA levels and translation, and is due to a proteasome dependent reduction in the protein half-life. These observations suggest that it may be possible to down-regulate the function of HPV E2 proteins in HPV lesions. The reduction in E2 function is independent of p53 function, and other DNA damage treatments such as hydroxyurea and etoposide do not induce the degradation of E2 levels. Thus the identification of the pathway responsible for UVB mediated downregulation of E2 function will provide novel targets for HPV therapy.

4.2.2 Possible role in the viral life cycle

The reduction in both HPV16 and HPV8 E2 protein levels in response to UVB suggests a common response to UVB stimuli and could play a role in protecting the virus in response to UVB irradiation. Additionally the response of the HPV18 LCR to UVB irradiation was tested (W.Boner, Taylor *et al* 2003a) and UVB irradiation significantly repressed transcription from the LCR. Therefore there are several common results from UVB irradiation on the viral life cycle. Firstly, repression of the HPV18 LCR would result in a reduction of E6 and E7 levels, thereby elevating levels of p53 and pRb. These proteins are essential for mediating an appropriate cellular response to DNA-damaging agents such as UVB. Reactivation of p53 and pRb will therefore protect the cell from extensive damage and/or apoptosis thus protecting the infected cell. Secondly, the reduction in E2 level in response to UVB will reduce the level of viral replication allowing the viral genome to be repaired, and thereby maintain integrity. The HPV18 LCR has been demonstrated to contain elements that control the level of replication (Demeret *et al* 1995) and UVB irradiation may also influence HPV replication controlled by the LCR. Thirdly, the HPV16 E2 protein can itself induce apoptosis (Webster *et al.*, 2000) and therefore the presence of E2 with a DNA damage response could induce massive levels apoptosis in virus-infected cells. In summary, the inactivation of E6 and E7 proteins by transcriptional repression, and the reduction of the E2 protein levels, would provide an opportunity for repair of both the cellular and viral genome.

4.2.3 UVB mediated signal transduction pathways

The key to understanding why E2 responds to UVB irradiation is to understanding what UVB mediated signal(s) induce the degradation of E2. UVB irradiation induces

both direct and indirect damage to DNA (see Ravanat *et al* 2001 for review). The majority of DNA lesions caused by UV irradiation are due to the direct photochemical formation of intrastrand dimers between adjacent pyrimidine bases. The most common lesion created is the *cis-syn* cyclobutane pyrimidine dimer (CPD). The second most common is the 6-4 pyrimidine-pyrimidone (6-4pp) photoproduct. Additionally UV irradiation induces a significant amount of free radical formation and oxidative stress. This leads to the creation of a variety of lesions including 8-oxoguanine, pyrimidine hydrates and thymine glycol. Also oxidative stress induces single strand DNA breaks. All of these DNA lesions and the oxidative stress result in the activation of many cellular signalling pathways (Kulms & Schwarz 2002).

DNA damage activates an interlinking signalling network that is activated by the blockage of transcription and DNA replication processes, and DNA damage on transcription/replication inactive DNA (see Qin & Li 2003 for review). UV irradiation induces three checkpoints on the progression of the cell cycle, a G1 late arrest to repair DNA prior to S-phase, a lengthened S-phase, and a delay at the G2/M boundary. While the signalling cascade responsible for S-phase and G2/M checkpoints are relatively well characterised the G1 arrest is less clear. Unlike gamma irradiation UV induced G1 arrest is independent of p53/p21/pRb function. This has led to the suggestion that UV irradiation results in only a brief stall at the G1 boundary and results in an early and severe S-phase checkpoint (Al-Mohanna *et al* 2001, Loignon & Drobetsky 2002). However this delay in the initiation of DNA replication allows the activation of the NER and the removal of DNA lesions prior to DNA replication.

During S-phase the stalling of replication fork and induction of double stranded DNA breaks activates ATR/DNA-PK/ATM/Chk1/Chk2 dependent signalling to elicit a S-

phase checkpoint, the appropriate DNA repair and a subsequent G2/M arrest (Qin & Li 2003, Yang *et al* 2003). The stalling of the replication fork can occur through three mechanisms; through inhibition of the DNA polymerase e.g. aphidicolin, depletion of free dNTP pool e.g. hydroxyurea (Cobb *et al* 2003), and through blockage of DNA polymerase progression e.g. CPD lesion (Svoboda *et al* 1998). Replication dependent DNA breaks occur through two mechanisms; due to introduction of DNA nicks at the replication fork e.g. UV lesion or captothecitin (Furuta *et al* 2003) and through recombination mediated resolution of stalled replication forks and reversed "chicken-foot" replication forks e.g. HU or CPD (Limoli *et al* 2002, Sogo *et al* 2002, Sengupta *et al* 2003). Co-ordination of these events requires a wide range of phosphorylation events and protein-protein interactions that are currently being elucidated. At stalled and broken DNA replication forks the sensor DNA dependent kinases ATR and DNA-PK are recruited by protein complexes that bind single stranded DNA and double stranded breaks. Both these kinases are essential for the inhibition of replication in response to UV irradiation (Park *et al* 1999, Heffernan *et al* 2002). ATR is activated by both the stalling of replication forks and by replication dependent double strand DNA breaks (Tibbets *et al* 2000, Furuta *et al* 2003). Downstream targets of ATR include Chk1, Chk2, p53, and H2AX (Zhao & Piwnica-Worms 2001, Foray *et al* 2003, Ward & Chen 2001). DNA-PK is activated by DNA breaks throughout the cell-cycle. The downstream targets of DNA-PK are p53, RPA, JNK, and XRCC4 (Woo *et al* 2002, Allen *et al* 2002, Park *et al* 2001, Hsu *et al* 2002). Interestingly the SV40 large T antigen is though to be phosphorylated and functionally inhibited by DNA-PK (Chen *et al* 1991, Wang *et al* 1999). DNA-PK is part of a complex of proteins that are central to the repair of double stranded breaks. DNA-PK is essential for non-homologous end joining and is thought to have a role in

homologous. ATM is essential for multiple DNA repair and checkpoint functions in response to DNA breaks throughout the cell cycle (Abraham 2003). ATM is thought to have a role in the recognition of double stranded breaks induced during DNA replication. ATM hyperphosphorylates the RPA-32 subunit in response to UV irradiation, this phosphorylation is blocked by the inhibition of DNA replication by aphidicolin, therefore is dependent on the replication of damaged DNA (Oakley *et al* 2001). Therefore UV induced DNA lesions encountered during S-phase can cause both DNA breaks and stalled replication forks resulting in the activation of ATR, ATM and DNA-PK and their downstream signalling partners.

UVB irradiation also results in the activation of cellular MAP and stress induced kinase pathways (see Yang *et al* 2003 for review). This is thought to be both due to two stages of cellular stimuli. The early signal transduction is thought to be due to direct activation of cellular membrane receptors, this is due to UV induced oxidative stress (Rossette & Karin 1996, Marchese *et al* 2003). This early effect is characterised by the release of NFκB from its cytoplasmic inhibitor within the first hour of irradiation (Devary *et al* 1992). The activation of receptors like EGFR results in the activation of MEK and p38 dependent signal transduction processes. Late signal transduction is initiated about 6 hours post UV irradiation and is thought to be due to the persistence of lesions within the DNA (Kibitel *et al* 1998). The published literature on late UVB signal transduction events (e.g. TNFα induction) has no explanation of the trigger for late events, therefore I suggest that it is due to the activation of the replication checkpoint upon encountering UV induced DNA lesions in very early S-phase. Work on the elucidation of signalling responsible for the induction of TNFα has highlighted the rapamycin sensitive DNA-PK like kinase FRAP as a possible mediator of this effect (Yarosh *et al* 2000). In response to UVB

irradiation FRAP is thought to phosphorylate many cellular proteins, including BRCA1, p53, RNA polymerase II and $p70^{s6k}$ (Yarosh *et al* 2000, Canning *et al* 2003). Interestingly the DNA polymerase alpha inhibitor aphidicolin prevents the activation of $p70^{s6k}$ by UVB irradiation (Brenneisen *et al* 2000), therefore strengthening the idea that these late events are due to activation due to cellular replication machinery encountering the UV induced DNA lesions.

4.2.4 Future work

Future work should concentrate on elucidating the pathway(s) responsible for the UVB mediated degradation of E2. There are two logical methods to accomplishing this aim.

Firstly, the investigation of which region within HPV16 E2 is essential for the reduction of E2 function and protein level by UVB will aid the identification of the pathway responsible. Deletion and point mutation analysis will identify what amino acids are responsible (see section 3.2.4) and this may indicate the upstream protein that is essential for the degradation of E2.

Secondly, a broad approach to investigating the signalling of UVB irradiation and other DNA damage treatments will identify the UVB activated pathway responsible for the reduction of E2 function and protein level. Clearly the number of possible pathways responsible for the effect of UVB on E2 is very large. There are a wide range of DNA damage treatments that may be tried to mimic the effect of UVB on E2, however an important point to first address would be to establish if the UVB mediated signal to reduce E2 protein levels occurs during the replication of damaged DNA. The treatment of cells with aphidicolin prevents DNA replication and induces an early S-phase arrest, therefore aphidicolin pre-treatment before UVB irradiation

will demonstrate if the reduction in E2 level is caused by a signal induced during the replication of damaged DNA. Distinguishing if the UVB induced signal that leads to reduction of E2 function emanates during DNA replication will direct future targets for study. Additionally if the signal does occur during the replication of damaged DNA then this will highlight the differences in the DNA damage signals caused by hydroxyurea, etoposide and UVB irradiation. While both hydroxyurea and etoposide are known to activate similar DNA damage responses compared to UVB (e.g. ATR activity, p53 modification) it is possible that UVB irradiation stimulates a unique subset of DNA damage pathways. The understanding of the complexity of DNA damage signalling and the differences between different genotoxic insults is still in its infancy, therefore the reduction of E2 function by UVB may provide a unique insight to these processes.

If aphidicolin prevents the reduction of E2 function/level then both the interference with DNA damage signalling and mimicking UVB damage may highlight a pathway of interest. Various DNA damage pathways can be blocked through small molecules that inhibit the activity of DNA damage response proteins (e.g. Wortmannin inhibits ATM/ATR/DNA-PK (Sarkaria *et al* 1998), or 3-aminobenzamide inhibits PARP (Pivazyan *et al* 1992)). Use of these small molecule inhibitors or the use of cells that are genetically defective in any of the major signalling molecules may help the elucidation of the UVB mediated pathway involved. A similar approach can be used to investigate any possible roles of cellular MAP kinase pathways in the UVB mediated reduction of E2 function. There are many commercially available kinase specific inhibitors and genetically defective cell lines that will allow the investigation of the MAP kinase pathways.

Several different treatments can mimic the different types of DNA damage caused by UVB irradiation. 4NQO (4-nitroquinoline 1-oxide) induces bulky intrastrand DNA lesions that block DNA replication and transcription in a manner similar to UVB. Treatment of cells with the topoisomerase I inhibitor camptothecin results in a replication dependent double stranded DNA break at replication forks in a mechanism similar to UVB (Furuta *et al* 2003). Additionally, camptothecin induces a similar replication arrest to UVB irradiation. Induction of oxidative stress through use of H_2O_2 will mimic the activation of membrane receptors and the induction of DNA lesions by the oxidative stress caused by UVB irradiation. Alternatively oxidative stress could be limited by the pre-treatment of cells with an antioxidant like genistein. The experiments described above will provide initial direction for the understanding of the UVB mediated downregulation of HPV16 E2 and will provide targets for future study, and possibly future drug targets for treatment of HPV infection.

4.3

HPV DNA replication fidelity and the implications for HPV carcinogenesis

4.3.1 The significance of HPV genome disruption in HPV induced cancer

HPV infection has two recognised consequences; a complete infectious life cycle with efficient virion release and possible subsequent clearance of the virus, or an abortive (latent) life cycle that is linked with carcinogenesis and the development of HPV related tumours (Stubenrauch & Laimins 1999). The latter is thought to occur concurrently when portions of the viral genome become chromosomally integrated and results in the increased expression of E6 and E7 (Jeon & Lambert 1995, Jeon *et al*

1995). Also often found in HPV tumours are episomally maintained viral genomes that have significant DNA re-arrangements and often contain deletions within the "late" ORFs or the LCR (Kasher & Roman 1988, Deau *et al* 1991, Hall *et al* 1997, Kalantari *et al* 2001, Mellin *et al* 2002). Therefore progression to HPV induced carcinogenesis is linked to the genetic instability of the viral genome. The fidelity of E1/E2 mediated DNA replication was studied to investigate if HPV DNA replication is prone to events that may lead to viral integration/rearrangement.

4.3.2 Methods for double strand break creation

The substrates for viral DNA re-arrangement and integration are double stranded DNA breaks and DNA ends, these are subsequently repaired through recombination and strand invasion mediated repair (Van Gent *et al* 2001). Double stranded breaks occur by DNA replication independent and dependent mechanisms. Endogenous and carcinogen induced DNA damage (e.g reactive oxygen intermediates, gamma irradiation) can induce double stranded DNA breaks throughout the cell cycle, however these events are thought to be rare. During DNA replication there are several opportunities for the creation of double stranded DNA breaks. Inhibition of topoisomerase I by drugs like camptothecin or by DNA lesions like those created by UV irradiation can cause a nick in the template strand at the replication fork (Kuzminov *et al* 2001, Pourquier & Pommier 2001). This nick on the template in combination with the subsequent termination of the nascent DNA strand creates a double stranded break on the daughter DNA duplex. Intrastrand and interstrand DNA lesions (e.g. CPD and mitomycin C crosslink respectively) can also cause the reversal of the replication fork into a "chicken foot structure" that can be a substrate for recombination mediated repair and replication fork restart. In cells that have defects in

excision repair and translesion synthesis the creation of double stranded breaks at replication forks, as discussed above, is thought to be more frequent. This is highlighted in cells that are functionally null for the TLS DNA polymerase η, when these cells are UV irradiated there is ~14 fold more cells with the Mre11/Nbs1/Rad50 recombination foci (Limoli *et al* 2002, Cleaver *et al* 2002). Also the inhibition of replication fork elongation due to hydroxyurea results in the subsequent reactivation of the replication fork through recombination (Mirozeva & Petrini 2003).

4.3.3 Summary of results

E1/E2 mediated replication of undamaged template plasmid pOri16Lac results in a portion of daughter molecules that contain mutations within the phenotypic marker LacZ. The majority of these are due to DNA rearrangements resulting from >5bp DNA insertions and deletions. On UVC damaged pOri16Lac there is a significant increase in the number of DNA re-arrangements and a significant increase in the number of point mutations. These assays were repeated in a variety of different genetic backgrounds to dissect the role that cellular DNA replication/repair processes have on HPV replication fidelity.

The cells initially used for these assays were C33a cells, a non HPV related cervical carcinoma derived cell line commonly used for HPV replication assays. A number of SV40 immortalised fibroblast cell lines were used to investigate the role of cellular translesion synthesis (XP30, XP30η) and NER (XP12) functions on the frequency of mutagenesis. XP30 cells are functionally null for the major translesion synthesis DNA polymerase η. XP30η cells are derived from XP30 cells and express WT DNA polymerase η. XP12 cells are functionally null for the essential NER protein XPA.

Additionally the SV40 immortalised MRC5 fibroblast cell line was used as they are genetically wild type for DNA replication/repair functions.

On undamaged DNA templates replicated in C33a the rate of spontaneous mutation due to DNA re-arrangement is 4-8 fold higher than in all the fibroblast cell lines. C33a cells have an error prone phenotype and display a high rate of sister chromatid exchange and microsatellite instability. Therefore the rate of HPV mutation is reflective of the genetic background of the cell. On UVC damaged DNA templates the frequency of mutation due to re-arrangements is higher in C33a, XP30 and XP12 cells compared to both MRC5 and XP30η cells. The difference in MF between XP30 and MRC5 and XP30η demonstrates that HPV mediated DNA replication can use cellular TLS mechanisms to replicate UV induced DNA lesions. The significant increase in MF in XP12 cells demonstrates that a large proportion of the UV induced mutagenic lesions encountered during HPV DNA replication are repaired by NER prior to replication. The reason for the increase in the MF in C33a cells is likely due to the cellular error prone phenotype. Additionally when the replication template is irradiated there is a significant 3-4 fold reduction in the level of replication in XP30, XP30η and XP12 cells, however there is only a slight and insignificant decrease in C33a and MRC5 cells. All these results give a clear insight into the mechanisms involved in maintaining HPV genome integrity.

4.3.4 Role of cellular TLS and NER functions on viral mutagenesis

The increased MF in XP30 compared to MRC5 and XP30η demonstrates that the HPV replication fork is protected against point mutation and replication fork stalling in response to DNA lesions by the use of cellular TLS polymerases. The failure to replicate past DNA lesions often results in recombination mediated resolution of the

stalled replication fork i.e. due to double strand break or "chicken foot" (Cleaver *et al* 2002). The use of cellular TLS mechanisms by HPV16 E1/E2 mediated replication indicates that the error prone phenotype exhibited by HPV replication is not due to an inability to recruit TLS DNA polymerases to replicate past DNA lesions. The increase in the MF in XP12 cells compared to MRC5 shows that DNA lesions are repaired by cellular NER mechanisms probably prior to replication (Muotri *et al* 2002).

4.3.5 Viral replication error phenotypes

Replication of the viral genome is dependent on cellular DNA polymerases and the E1/E2 mediated DNA replication fork is able to recruit the cellular TLS DNA replication functions. The use of cellular DNA polymerases by HPV DNA replication thus ensures HPV viruses have a low frequency of point mutation when compared to RNA viruses like HIV (Munoz *et al* 1993). This is because RNA viruses replicate their genomes with a non-proofreading polymerase (e.g. reverse transcriptase) thus leading to a heterogeneous viral population due to frequent point mutation, often referred to as quasispecies. To RNA viruses this genetic heterogeneity enables antigenic variation and efficient evasion from the immune system. DNA viruses, including HPVs, are generally not thought to be found as heterogeneous populations due to their use of proofreading error free cellular DNA polymerases (Villarreal *et al* 2001). For HPV16 genomes detected in benign lesions few variants are found between unrelated infected individuals in the same continent. Instead HPV16 variation is limited to a few nucleotide changes between each of five different continental locations (Wheeler & Icenogle 1995). Consequently HPV16 genome variation has been dismissed as insignificant in the pathogenesis of DNA virus associated disease. However several clinical studies demonstrate that in HPV

associated cancerous lesions a heterogenous population of episomal viral genomes is found, and the mutations present in the HPV genome are thought to be responsible for the virally induced cancer (Kasher & Roman 1988, Deau *et al* 1991, Hall *et al* 1997, Kalantari *et al* 2001, Mellin *et al* 2002). For example the deletion of the transcription repressor site YY1 is thought to increase E6/E7 expression (Dong *et al* 1994, May *et al* 1994), and the deletion of the late ORFs L1/L2 may prevent completion of viral life cycle and possibly cellular differentiation (Deau *et al* 1991). However unlike the point mutations associated with RNA virus heterogeneity, in HPV lesions larger deletion or re-arrangement mutations have been predominantly detected. The above investigation into HPV replication fidelity demonstrates that HPV quasispecies can be recreated in cell culture, and this error prone phenotype is not due to a failure of HPV E1/E2 mediated replication to use cellular TLS and NER functions. An attractive future target to understand why HPV mediated DNA replication is prone to DNA deletion/re-arrangement is to investigate the differences between E1/E2 mediated replication control and cellular DNA replication control.

4.3.6 Role of replication initiation control on mutation frequency

Initiation and elongation of HPV replication requires the virally encoded DNA helicase E1 (Liu *et al* 1995). E1 is thought to functionally replace the cellular MCM 2-7 helicase complex and facilitates viral DNA replication initiation and elongation. Cellular MCM proteins are loaded onto cellular replication origins only once per S-phase in a process dependent on the loading of the origin recognition complex (ORC) to the origin (see Bell & Dutta 2002 for review, discussion 4.2). HPVs in contrast can load the E1 helicase multiple times onto the HPV origin, and this loading is enhanced by E2 (Piirsoo *et al* 1996). E2 could be viewed as the virally encoded ORC complex

that loads the helicase accessory factors to the viral origin to ensure efficient firing of DNA replication multiple times during S-phase. This would be highly beneficial to the virus due to limited number of S-phases that the one viral genome would encounter during the viral life cycle. The virus needs to rapidly expand the genome copy to first establish infection in the basal layers and in the upper layer to provide the genomes required for new virions. However replication origins that fire more than once per S-phase can cause the creation of secondary replication bubbles within active replication bubbles in a process called "onion skin" replication (Botchan *et al* 1979, Baran *et al* 1983, Mannik *et al* 2002). These structures occur when a replication origin initiates DNA replication before the previous replication bubble has terminated. If a second replication fork collides into the first replication bubble a complex DNA structure is created that requires recombinational repair mechanisms to resolve. Further if a DNA lesion blocks the first replication bubble then these collision events may occur more often. The collision of the first and second replication forks therefore could potentially produce double stranded DNA breaks or complex DNA structures thus providing substrates for homologous and non-homologous recombination, resulting in HPV genome re-arrangement or viral integration. In all lines tested when the pOri16Lac template is UVC irradiated there is a significant increase in the re-arrangement frequency. This is expected to be due to both recombination mediated replication past the UV lesion and due to recombination mediated repair of collided "onion skin" replication forks.

The potential role of "onion skin" type replication in viral mutation frequency is highlighted in a study that compares the fidelity of SV40 large T antigen mediated replication, and Epstein-Barr virus EBNA1 mediated DNA replication (James *et al* 1989). Large T antigen, like E1/E2, is not thought to be dependent on cellular ORC

related replication origin liscencing and can therefore initiate DNA replication multiple times per S-phase, thus creating "onion skin" type replication intermediates. In contrast EBNA1 mediated DNA replication is dependent on ORC related replication fork licensing (Yates & Guan 1991, Dhar *et al* 2001, Ritzi *et al* 2003) and is therefore unable to create "onion skin" type replication intermediates. Large T antigen mediated replication had a similar mutation frequency to that of HPV16 E1/E2 mediated replication (James *et al* 1989). Also a large proportion of the mutants plasmids recovered had DNA deletions/re-arrangements. In contrast the mutation frequency obtained from EBNA1 mediated DNA replication was 100 fold lower and over long term culture the EBNA1 maintained plasmids retained integrity and showed little sign of DNA deletion/re-arrangements. This study demonstrates that the ability to initiate viral DNA replication is a significant risk factor for the induction of genetic instability (James *et al* 1989). A recent study using BPV1 E1 and E2 demonstrates that BPV1 E1/E2 mediated replication in the presence of high levels of E1 creates a heterogeneous population of replication products (Mannik *et al* 2002). This is thought to be due to "onion skin" type DNA replication. Therefore it is possible that for HPV E1/E2 mediated DNA replication the ability to initiate DNA replication multiply is the primary reason for the error prone replication phenotype.

4.3.7 Possible HPV replication checkpoint

When the pOri16Lac template is UVC irradiated there is a significant reduction in the level of replication in all cells except C33a and MRC5. This observation poses several questions about why there is a reduction in the level of DNA replication. It may be due to a cell cycle delay in cells replicating UVC damaged pOri16Lac. The reduction in replication level could be due to fewer E1/E2 replication origins firing. It may be

due to a failure to re-licence E1/E2 origins for multiple firing during S-phase. It could be due to a significant inhibition of DNA elongation. Finally it could also be due to a complete failure and abortion of the replication fork upon encountering damage. The latter is the least likely because in C33a and MRC5 cells the replication level of UVC damaged template is similar to when undamaged. At high levels of cellular UV induced DNA damage one report demonstrates that there is a decrease in the rate of DNA replication elongation and a decrease in DNA initiation (Miao *et al* 2003). At lower levels of UV irradiation there is still an inhibition of DNA replication initiation however no inhibition of elongation. Therefore rate of HPV E1/E2 mediated DNA replication elongation needs to be tested.

The reduction of HPV replication levels by UVC damage on the template could be due to a replication checkpoint-like response that prevents replication initiation. The cellular replication checkpoint is activated in response to the blockage of replication fork progression by DNA lesions, nucleotide depletion or DNA polymerase inhibition (Heffernan *et al* 2002, Feijoo *et al* 2001). The subsequent responses mediated by ATR and Chk1/2 result in the stabilisation of the stalled replication fork and the delay in the firing of late replication forks. This is thought to be due to the modulation of the loading of TopBP1, cdc45, the MCM helicases and the DNA polymerases to the late origins, therefore preventing replication initiation. Replication at the HPV origin could also be modulated by the replication checkpoint. E1/E2 requires the recruitment of the cellular DNA polymerases to initiate replication. The recruitment of cellular DNA polymerases is dependent on the loading of the helicase complex (i.e. E1), the ORC complex (i.e. E2) and multiple DNA initiation factors (e.g. TopBP1, cdc45, cdt1, cdc6 and cdc7). It is unknown if E1/E2 can initiate DNA replication when cellular proteins essential for replication initiation are inhibited by the replication

checkpoint. TopBP1 is central to the co-ordination of DNA replication initiation and the transition from a pre-replication complex to active DNA replication. In Xenopus extract the TopBP1 homologue Xmus101/Xcut5 recruits the essential initiation factor Cdc45 to the replication origin. Further cdc45 and TopBP1 together are essential for the co-ordination of the initiation process. Therefore HPV DNA replication initiation may be inhibited by cellular initiation proteins, and therefore result in the protection of the viral genome. Future work to address this point will require the dissection of what components of the cellular initiation complex is required by E1/E2. This could be done using *in vitro* replication assays and through the selective depletion of each protein from the nuclear extracts used.

The functions of E1 and E2 themselves may also be targeted by the replication checkpoint response. Firstly, BPV1 E1 is phosphorylated *in vivo* by the cdk2/cyclinE cell cycle dependent kinase (Cueille *et al* 1998). Cdk2/cyclinE is thought to activate the function of E1 and *in vitro* HPV replication is dependent on cdk2/cyclinE function (Lin *et al* 2000). In response to DNA damage (p53/p21) and the replication checkpoint (Chk1/2) cdk2/cyclinE function is repressed by p53/p21 and Chk1/2. Therefore the function of HPV16 E1 may also be reduced in response to a checkpoint signal eminating from the replication of the UVC damaged pOri16Lac template. Secondly, in section 3.2 the HPV16 E2 protein is demonstrated to be functionally repressed and degraded in response to cellular UVB irradiation. It is thought that UV irradiation induces a very early S-phase arrest and induces the replication checkpoint (see section 4.2.2). Therefore the reduction in E2 levels could be in direct response to the activation of the replication checkpoint, or due to a signalling event that is preferentially activated during the replication of UVC damaged DNA.

Several E2 partner proteins are functionally modified in response to DNA damage. TopBP1 is phosphorylated and its levels are altered (Honda *et al* 2002, Greer *et al* 2003), the possible consequence of which on replication initiation is discussed above. p53 represses DNA replication of damaged DNA templates *in vitro* (Zhou & Prives 2003) therefore there may be a similar role *in vivo*. BRCA1 in response to replication fork stalling is rapidly phosphorylated and is essential for activating Chk1. BRCA1 recently been demonstrated to be essential for many ATM/R phosphorylation events, possibly due to BRCA1 acting as a scaffold ensuring efficient signal transduction. Therefore a similar role may exist with E2 and BRCA1 may modulate a possible checkpoint signal to E2 that may result from the replication of damaged pOri16Lac.

4.3.8 Future experiments

While the numbers of different possible roles of viral and cellular proteins in the control of HPV replication initiation, elongation and DNA repair are numerous, a few simple experiments will provide a great deal of insight into these processes.

The first target would be to try and knockout the reduction in the level of viral replication by targeting cellular pathways responsible for reducing replication rate in response to damage. Treatment of cells with the ubiquitous PIKK inhibitor wortmannin, or the Chk1 inhibitor UNC-01 during a transient replication assay will identify if any of these damage responsive kinases have a role (Limoli *et al* 2002, Heffernan *et al* 2002). Additionally the co-transfection of cells with dominant negative versions of p53 or BRCA1 during a transient replication assay may identify a role for either of these proteins. Further for all the DNA damage responsive proteins mentioned in this chapter there are cell lines available that are functionally null for all

these proteins and therefore provide different genetic backgrounds to do transient replication assays in. However the ideal system to use would be to do all transient replication assay in primary untransformed MRC5 cells. To dissect what cellular processes are essential for the reduction in replication siRNA would be used to knock out each protein of interest (Brummelkamp *et al* 2002).

Elucidation of the possible roles that E1 and E2 may have in the reduction of replication level may be a more subtle problem to solve. Due to the transient nature of the replication assay the cells are asynchronous and not all cells will be transfected with the E1/E2 expression plasmids and the pOri16Lac template. Additionally not all cells will be in S-phase replicating the pOri16Lac at the time of harvest. Therefore it is likely to be hard to determine possible modifications or regulation of overexpressed E1/E2 proteins in this system. Further refinement of the replication assay by synchronising the cells and enhancing the transfection protocol by electroporation may overcome these limitations. Investigation of any differences of E1/E2 binding to the HPV origin through use of chromatin immunoprecipitation (ChIP) will also be of interest (Keller *et al* 2002, Cobb *et al* 2003, Rizi *et al* 2003).

In budding yeast the structural study of stalled replication forks is done through the use of 2D agarose gel electrophoresis (Lopez *et al* 2001). This technique allows the separation and visualisation of DNA replication structures including both short and long replication bubbles and Y-shaped replication forks. When budding yeast with a mutation in the regulatory FHA domain of the Chk2 homologue Rad53 are incubated with hydroxyurea a distinct signal is observed on the 2D gel that is representative of the formation of X-shaped structures resulting from the collapse of replication forks (Lopez *et al* 2001). No similar work has been done yet in mammalian cells. Therefore using either E1/E2 or EBNA1 to initiate replication similar experiments to those done

by the lab of Foiani (Lopez *et al* 2001) can be done in human cells. 2D gel electrophoresis has been previously done using BPV1 replication templates before (Mannik *et al* 2002) therefore the setting up of an assay of this description is achievable. Due to the transient nature of these assays the stalling of the replication fork on the DNA template may be achieved by direct DNA damage (UV, mitomycin C) to the DNA replication template prior to transfection or the replication fork could be stalled by treatment of the cells with hydroxyurea. Subsequent 1D and 2D agarose gel electrophoresis will demonstrate the amount of replication completed and the structure of the DNA intermediates created (Lopez *et al* 2001, Mannik *et al* 2002). Additionally the replicated plasmids could be rescued into E.coli and screened for mutations that arise due to genetic rearrangement. Further the development of a vector to directly measure homologous recombination events will highlight the frequency that homologous templates are used to repair the replication fork. The potential power of this technology comes from its ability to detect DNA replication amount, DNA replication structures and the fidelity of DNA replication all from the same sample.

The work presented on E1/E2 mediated DNA replication fidelity demonstrates that while HPV DNA replication is error prone E1/E2 mediated DNA replication forks are able to use the error free TLS DNA polymerase η. The frequency of deletion and DNA rearrangements presents important questions about the reasons for the creation and the resolution of non-homologous recombination DNA repair events that will lead to these mutations. The future use of the HPV16 E1/E2 mediated replication system as outlined above will provide deep insight into both viral and cellular DNA replication initiation, elongation and repair mechanisms.

4.4 A novel technique with enhanced detection and quantitation of HPV-16 E1 and E2 mediated DNA replication.

The traditional method for the detection of HPV E1/E2 mediated replication is by Southern blot. This technique is hazardous, labour intensive and has limitation on its sensitivity. I have developed a real-time PCR based methodology for the detection of E1/E2 mediated replication. When an optimal level of pOri16M is used (1ng) the real-time PCR assay is a highly sensitive and quantitative method for detection of transient viral DNA replication. Importantly it allows for detection of replication at origin input levels where the replication mediated by E1/E2 is on a linear scale. This cannot be said for the Southern blotting technique. This advantage has resulted in the detection of enhanced replication by TopBP1 in C33a cells, something that was not possible using Southern blotting (Boner et al, 2002). The optimised conditions used in this assay and the enhanced sensitivity means that this method facilitates the detection of more subtle effects on DNA replication. This is ideal for looking at the way cellular partner proteins or HPV DNA elements and E1/E2 control viral DNA replication. Finally real-time PCR is a significantly faster way to generate results, the use of 96-well plates and a two-hour run time creates a high throughput assay that has potential for commercial application. Disruption of the E1/E2 interaction prevents viral DNA replication (Yasugi *et al* 1997, Kasukawa *et al* 1998) and is an attractive target for HPV drug development (Plumpton *et al* 1995, Clark *et al* 1998). The real-time PCR assay described here is an ideal template for the development of a screen for the discovery of HPV antiviral compounds. Several simple modifications of this assay will ensure success for commercial application. The current transfection protocol has a relatively low efficiency. Use of electroporation or lipid based transfection protocols

will allow the assay to be performed using less cells in multiwell plates. Also replacement of the DNA purification protocol with a mini-column based protocol may allow the technique to be performed by biological robots.

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