Investigating the role of Human Papillomavirus DNA replication in the development of cervical cancer

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Disclaimer:

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

Lauren E. King

December 2010

For the Kings

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This thesis is as much yours as it is mine.

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Abbreviations

˚ C	Degrees centigrade
µg	Microgram
µl	Microlitre
9-1-1	Rad9-Rad1-Hus1
A	Alanine
AAD	ATR activation domain
A/T	Adenine/Thymine
AAA+	ATPases Associated with diverse cellular Activities
ADP	Adenosine diphosphate
AIN	Anal intraepithelial neoplasia
AP1	Activator protein 1
APH	Aphidicolin
ASF	Alternative splicing factor
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	A-T Rad3-related protein
ATRIP	ATR interacting protein
BER	Base excision repair
bp	Base pair
BPV	Bovine papillomavirus
BRCA1	Breast cancer susceptibility Gene 1
BRCT	BRCA1 carboxyl-terminal
Brd4	Bromodomain-containing protein 4
Brg1	Brahma related gene 1
Brm	Brahma
BSA	Bovine serum albumin
C/EBPβ	CCAAT/enhancer-binding protein beta

CBP	CREB binding protein
cdc	Cell division cycle
Cdk	Cyclin dependent kinase
Chk	Checkpoint kinase
ChLR1	Chromosome loss-related protein 1
CHX	Cycloheximide
CIN	Cervical intraepithelial neoplasia
CMV	Cytomegalovirus
CPT	Camptothecin
DBD	DNA binding domain
ddH_2O	Double distilled water
DISC	Death inducing cellular complex
Dlg	Discs large
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNA-PK	DNA dependent protein kinase
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleic acid
DSB	Double strand breaks
dsDNA	Double stranded DNA
E. Coli	Escherichia Coli
E1BS	E1 binding site
E2BS	E2 binding site
E2-TA	E2 transactivator
ECL	Enhanced chemiluminescence
EGFR	Epidermal growth factor receptor
ETO	Etoposide
EV	Epidermodysplasia verruciformis
EVER1/2	Epidermodysplasia verruciformis 1/2
ExoIII	Exonuclease III
FADD	Fas-associated death domain

FCS	Foetal calf serum
FEN	Flap endonuclease
g	Gram
GINS	Go Ichi Ni San (5,1,2,3)
HA	Hemagglutinin
HEK293T	Human Embryonic Kidney Cells
HPV	Human papillomavirus
HR	Homologous recombination
HR-HPV	High risk human papillomavirus
HSP	Heat shock protein
HU	Hydroxyurea
IP	Immunoprecipitation
IR	Ionizing radiation
Kb	Kilobase pairs
kDa	Kilodaltons
L	Litre
LCR	Long control region
LR-HPV	Low risk human papillomavirus
LT	Large T antigen
LTAg	Large T antigen
M	Molar
MCM	Mini chromosome maintenance
MDC1	Mediator of DNA damage checkpoint protein 1
MDM	Murine double minute
mg	Milligram
MHC	Major histocompatibility complex
ml	Millilitre
mM	Millimolar
MMR	Mismatch repair
MRN	Mre11, Rad50, Nbs1
mRNA	Messenger ribonucleic acid
NBS	Nijmegen breakage syndrome

NER	Nucleotide excision repair
NES	Nuclear export signal
NF1	Nuclear factor 1
NHEJ	Non-homologous end joining
nm	Nanometres
OD	Optical Density
ORC	Origin replication complex
ORF	Open reading frame
ori	Origin of replication
PAGE	Polyacrylamide gel electrophoresis
pA_L	Polyadenylation late
pA_L	Polyadenylation late
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PIKK	Phosphatidylinositol 3-kinase-related kinase
PIN	Penile intraepithelial neoplasia
PML	Promyleocytic leukemia
pmol	Picomole
Pol	Polymerase
pre-RC	Pre replication complex
Q	Glutamine
Rb	Retinoblasta
RFC	Replication factor C
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
RPA	Replication protein A
S/Ser	Serine
SDM	Site Directed Mutagenesis
SDS	Sodium dodecyl sulphate
SEM	Standard Error of the Mean

SF2	Splicing factor 2
siRNA	Small interfering RNA
SNF	Sucrose non-fermentable
Sp1	Specificity protein 1
ssDNA	Single stranded DNA
SV40	Simian Virus 40
SWI	Switching deficient
T/Thr	Threonine
TBP	TATA binding protein 1
TCR	Transcription-coupled repair
TERT	Transcription of telomerase reverse transcriptase
TFIID	Transcription factor II domain
tk	Thymidine kinase
TMC	Transmembrane channel-like
TNF	Tumour necrosis factor
TopBP1	Topoisomerase binding protein 1
TopoI	Topoisomerase I
TRAIL	TNF-related apoptosis-inducing ligand
URR	Upstream Regulatory Region
UV	Ultraviolet
v/v	Volume per unit volume
VIN	Vulva intraepithelial neoplasia
w/v	Weight per unit volume
wt	Wild type

Abstract

Human papillomavirus type 16 (HPV16) is a causative agent of cervical cancer. In many HPV induced cancers the HPV genome is present integrated into the cellular chromosomes. Integration of HPV DNA into that of the host promotes genomic instability and progression to cancer. Factors that promote integration remain to be fully identified. HPV replication during DNA damage is a factor that may promote viral DNA integration. HPV origin replication is investigated in the presence of DNA damaging agents. HPV replication produces double strand DNA breaks in the viral genome and DNA damaging agents such as etoposide will result in double strand breaks in genomic DNA therefore creating substrates for viral integration. HPV origin replication during DNA damage is not inhibited *in vivo* and *in vitro.* In contradiction to these results, replication initiated by the SV40 helicase, Large T antigen, is arrested in response to DNA damage and ATR is the candidate kinase for mediating the arrest. In order to carry out replication of the viral origin, the HPV genome encodes for the E1 protein. E1 forms a di-hexameric helicase complex that replicates the viral genome. The failure of E1 to be targeted by ATR/ATM allows HPV replication in the presence of DNA damaging agents. Such replication will result in double strand breaks in the viral genome ultimately promoting viral integration and progression of cervical cancer.

Upon activation of the DNA damage response cellular DNA replication is stalled. The targets of the DNA damage pathways at the replication fork that result in the inhibition of DNA replication are not clearly defined. Evidence has shown that the MCM2-7 cellular helicase complex is targeted for phosphorylation by the ATR/ATM kinases and therefore LTAg helicase may also be targeted in a similar manner in response to DNA damage. The results presented in this thesis support the hypothesis that the replicative helicase is a direct target for phosphorylation by the DNA damaging signalling kinases. Using SV40 replication as a model for eukaryotic DNA replication the results in this thesis show that

LTAg is targeted for phosphorylation by the DNA damage signalling kinases ATR/ATM in response to DNA damage. Large T Antigen protein levels decrease after prolonged treatment with etoposide suggesting that the degradation may inhibit the replication functions of this viral helicase.

To carry out viral replication HPV encodes two proteins, E1 and E2, which interact with cellular factors to replicate the viral genome. E2 forms homodimers and binds to 12bp palindromic sequences adjacent to the viral origin and recruits the viral helicase, E1, to the origin. The regulatory consequences of the E1.E2 interaction have been controversial. The data in this thesis demonstrates the ability of E1 to stabilise E2 increasing E2 half life. Analysis using a mutant E2 that binds weakly to E1 demonstrates that the stabilisation is a consequence of a direct E1.E2 interaction. Furthermore this thesis also demonstrates that the presence of E1 results in the redistribution of HPV16 E2 by enhancing its affinity for chromatin. The E2 protein tethers the viral genome onto the host chromatin during cellular mitosis thereby ensuring the distribution of viral episomes into both daughter cells. The redistribution of E2 onto chromatin suggests a role for E1 in enhancing HPV genome segregation functions by stabilising the association of E2 with mitotic chromosomes. E2 also regulates transcription from the viral genome**. T**he data in this thesis shows that the E1 protein enhances E2 transcription function in a manner that suggests the E1 protein itself can contribute to transcriptional regulation not simply by E2 stabilisation but by a direct stimulation of E2-mediated transcription. E1 regulation of E2 function is again dependent on a direct protein-protein interaction. Taken together these results suggest that co-expression of E1 with E2 can increase E2 stability, enhance its affinity with chromatin and enhance E2-mediated transcription. These consequences are discussed with relation to the virus life cycle.

Chapter 1 – Introduction

1.1 Human papillomavirus

Papillomaviridae are an ancient and diverse family of non-enveloped DNA viruses, collectively known as *papillomaviruses*. Papillomaviruses infect a range of species including cattle, horses, birds, rabbit and humans causing persistent infections that are difficult to treat (Campo, 2002). The taxonomy of 120 papillomavirus has been described using sequence comparisons of the L1 open reading frame (de Villiers et al., 2002). There are sixteen genotypes of papillomavirus categorised. The alpha and beta genotypes contain the largest number of associated papillomavirus. All human papillomavirus (HPV) have a special affinity for epithelial cells and infection persists in the dividing basal cells of the cutaneous or mucosal epithelium. The life cycle is closely linked to the differentiation status of epithelial cells. The small circular double-stranded DNA virus naturally exists episomally and has the ability to cause a wide range of lesions in humans (Table 1.1).

1.2 Diseases associated with HPV

There are over 120 types of papillomavirus that have been identified that can infect humans (de Villiers et al., 2004). Symptoms vary or may be absent. Some types result in benign wart like growths on the skin typical of HPV types 1 and 2 (Table 1.1). Flat warts caused by HPV5 are most commonly found on the arms, face or forehead. Abnormal immune function is associated with the development of a cancer called epidermodysplasia verruciformis (reviewed by Dubina & Goldenberg, 2009). Epidermodysplasia verruciformis (EV) is a rare chronic inherited disease believed to be associated with HPV and in particular types 5 and 8 (Majewski et al., 2002; Lane et al., 2003). Mutations in two related human genes, EVER1/TMC6 and EVER2/TMC8, are believed to result in a primary deficiency of intrinsic immunity against HPV types that

would normally carry little oncogenic risk (Ramoz et al., 2002). HPV infections of the cutaneous epithelium are rarely fatal and carry a low oncogenic risk.

Over forty types of HPV infect the mucosal epithelial lining of the anogenital tract and other mucosal areas of the body. Mucosal epithelial infection is associated with a range of disease from benign anogenital warts to malignant carcinomas of the genital, oral or conjunctival mucosa. Of the types that infect the anogenital tract, two groups can be established based on the virus ability to cause malignancy. All of these types belong to the alpha genus (de Villiers et al., 2002). 'High-risk' (HR) sexually transmitted HPVs include types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68 (Muñoz et al., 2003).They may lead to the development of cervical intraepithelial neoplasia (CIN), vulva intraepithelial neoplasia (VIN), penile intraepithelial neoplasia (PIN), and/or anal intraepithelial neoplasia (AIN). The association of HPV and cervical cancer was first suggested by zur Hausen in 1975 (zur Hausen et al., 1975). It is now widely accepted that HPV16 and 18 are the causative agent of around 70% cervical cancer cases (Muñoz et al., 2004).

The 'low-risk' (LR) HPV's include types 6, 11, 40, 42, 43, 44, 53, 54, 61, 72 and 73. Types 6 and 11 are the most common and are associated with 90% of genital warts and laryngeal papillomas (Gale et al., 1994; Brown et al., 1999; Gale, 2005; Gale & Zidar, 2006; Potocnik et al., 2007). Most HPV infections are cleared rapidly by the immune system and do not progress to cancer. Papillomas can form weeks to years after contact with HPV infected individual. Hosts that are asymptomatic may become unknown reservoirs of infection.

Table 1.1

HPV strain	Cell-type	Disease	Oncogenic Risk
HPV1	Cutaneous epithelial	• Common wart • Plantar Wart	Low Low
HPV3	Cutaneous epithelial	• Flat wart	Low
HPV6/11	Mucosal epithelial	• Benign genital wart • Recurrent respiratory papillomatosis	Low High
HPV16	Mucosal epithelial	• Cervical intraepithelial neoplasia • Cervical carcinoma • Head and neck cancers	High High High
HPV18	Mucosal epithelial	• Cervical intraepithelial neoplasia • Cervical carcinoma • Head and neck cancers	High High High
HPV13/32	Mucosal epithelial	• Focal epithelial hyperplasia (Heck disease)	Low
HPV5	Cutaneous epithelial	• Epidermodysplasia verruciformis	High in immunocompromised host

Table 1.1 HPV type and associated diseases. Specific HPV types have been selected in table 1.1 to highlight the range of diseases the virus can cause in the human host.

1.3 Life cycle

HPV gains access to host cells through microabrasions in the epithelium. The target cell is the basal cells of the epithelium (Egawa, 2003; Schmitt et al., 1996). The basal layer consists of basal epithelial cells including the stem cells of the epithelium. To initiate infection the viral capsid proteins associate with the primary receptor, membrane associated heparan sulphate (Giroglou et al., 2001; Joyce et al., 1999). The nature of the precise membrane-associated secondary receptor is controversial but there is evidence supporting a role of α6 integrin (Joyce et al., 1999; Yoon et al. 2001). Following attachment via L1 and L2, endocytosis has been shown to take many hours. The virus

usually enters via clathrin coated pits or caveolar endocytosis depending on the type of papillomavirus (Smith et al., 2007; Hindmarsh et al., 2007).

After exit from the endosome, L2 protein transports viral DNA into the nucleus where a complex is formed with promyelocytic leukaemia protein (PML) bodies (Day et al., 1998). PML bodies enhance early transcription of the papillomavirus genes (Day et al., 2004). Also located within the nucleus are the host cell transcription/replication factors. The papillomavirus genome is compact and therefore the virus utilizes its host enzymes for its own transcription and replication requirements. The life cycle requires three different modes of viral DNA replication. The different modes are termed ‘‘establishment’’, ‘‘maintenance’’ and ‘‘amplification’’ (Reviewed in McBride, 2004). Expression of the E1 and E2 proteins results in viral replication so that the infected cell contains around 20-50 copies of the episome. After the initial amplification step, the viral genome must be maintained in the dividing basal cells to sustain a persistent infection. The virus exists episomally and replicates in synchrony with host cellular DNA (Evans et al., 2003).

The life cycle of HPV is strictly linked to the differentiation program of the host keratinocyte. Infection within the basal layer generates a pool of infected cells. As basal cells divide, a daughter cell is produced that harbours episomal viral DNA. This is a consequence of the E2 protein tethering the viral episome to chromatin during mitosis (Skiadopoulos & McBride, 1998; Oliveira et al., 2006). Post mitosis, one infected cell remains in the basal layer and the other migrates up through the suprabasal layers. The migrating cell begins a process of terminal differentiation. In an uninfected epithelium, the migrating cells exit the cell cycle. However papillomavirus infected cells remain mitotically active and they can undergo some differentiation. Amplificational (or vegetative) viral DNA replication permits viral genomes to be replicated to a high copy number destined to be packaged into the capsid to yield progeny virions (McBride, 2004). Some studies have reported that there is a switch in replication strategies of HPV in differentiated cells and that this partially explains the high copy plasmid number detected. The switch in replication mode is from bidirectional theta replication encountered in basal cells to a rolling circle mode in differentiating cells (Flores & Lambert, 1997; Rector et al., 2004).

Differentiated cells are withdrawn from the cell cycle therefore the virus relies on the proteins it encodes to maintain or induce a pseudo-S phase-like state in order to gain access to the cellular replication machinery. The E6 and E7 proteins modulate the cell cycle through associations with the tumour suppressors p53 and retinoblastoma (Rb) proteins respectively (reviewed by Howley, 2006). The E1^E4 and E5 proteins are also translated in a differentiation dependent manner. It has been shown that E1^E4 inhibits G_2 to M phase transition and that this arrest may play a role in creating an environment optimal for viral DNA replication (Davy et al., 2002; Davy et al., 2005). It has also been suggested that E5 contributes to cellular proliferation by cooperating with E7. E5 enhances E7-induced mitogenic response and this contributes to viral DNA synthesis (Bouvard et al., 1994).

The L1 and L2 capsid proteins undergo expression within the granular layer of the epithelium (Barksdale & Baker, 1993). Restricting the synthesis of the L1 and L2 antigens to the upper layers of the epithelium may aid viral evasion from the immune response. The capsid proteins assemble into the icosahedral capsid via assistance from chaperone proteins such as heat shock protein 70 (Hsp70) (Chromy et al., 2003; Buck et al., 2005; Chromy et al., 2006). Karyopherins may play a role in papillomavirus capsid assembly (Bird et al., 2008). It is unknown whether encapsidation of the viral episome takes place during or after capsid formation. The E1^E4 protein then interacts with cellular keratin networks to assist viral egression. The nature of the epithelium allows the virion to spread through the shedding of outer most cell layers (Figure 1.1).

Figure 1.1

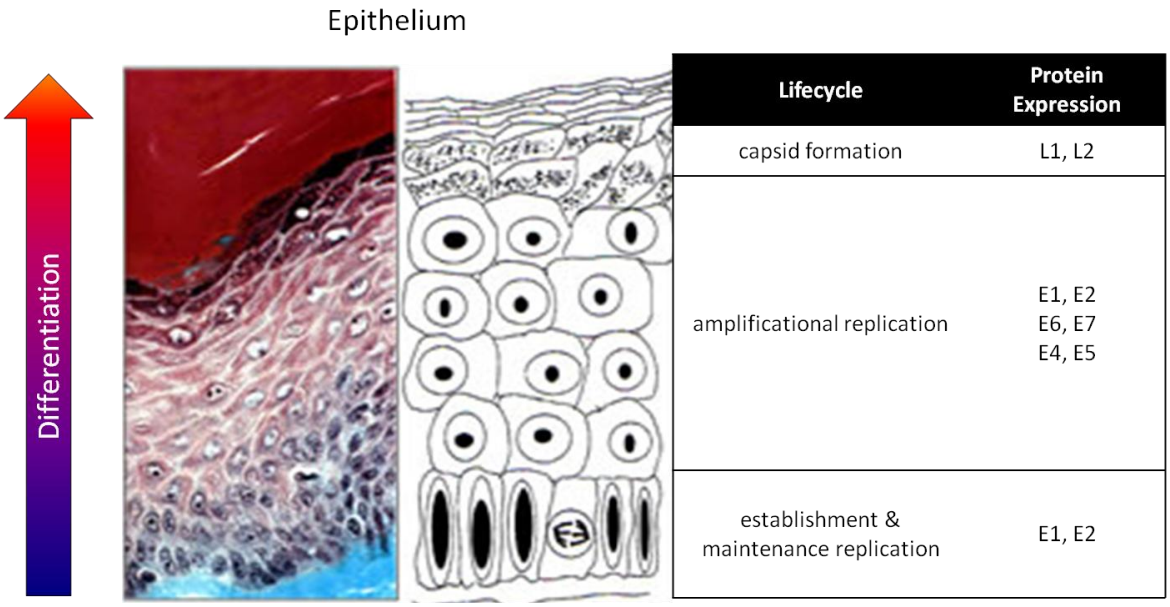


Figure 1.1 HPV life cycle within the epithelium. The different replication modes of the virus and where they take place within the layers of the epithelium are highlighted. The E1 and E2 proteins are expressed in the establishment and maintenance stages of the virus infection within the basal epithelial cells. Within the upper layers of the epithelium amplificational replication takes place which requires increased levels of E1 and E2 proteins. The E5, E6 and E7 oncoproteins and E1^E4 proteins are also expressed. Differentiated epithelial cells exit the cell cycle however these viral proteins create an environment that supports amplificational replication of HPV DNA. Within the granular layers the L1 and L2 proteins are expressed and form the viral capsid. Papillomavirus virions are released through natural sloughing of the epidermis.

1.4 HPV16 Genome

Off all the HPV types, HPV16 has the most defined association with malignancy. HPV16 DNA is not only detected in cancer of the cervix, but also in samples taken from oral and oropharyngeal cancer (reviewed in Marur et al., 2010). HPV16 has a 7.9kb circular double stranded DNA genome (Figure 1.2). The genome can be split into the three functional

regions. The first region is a non-coding regulatory region which is known as the Long Control Region (LCR) or Upstream Regulatory Region (URR). The LCR contains the sequences that are responsible for the regulation of HPV replication and transcription such as the p97 promoter (Desaintes & Demeret, 1996). The p97 promoter lies upstream of the HPV early oncogenes and is only functionally active in human keratinocytes (Cripe et al., 1990). The LCR also contains enhancer and silencer sequences as well as binding sites for cellular transcription factors and HPV proteins (Figure 1.2). Examples of the cellular transcription factors that can interact with the LCR include nuclear factor 1 (NF1), activator protein 1 (AP1), specificity protein 1 (Sp1) and transcription factor II domain (TFIID) (Chong et al., 1990; Chan et al., 1990; Gloss & Bernard, 1990; Chong et al., 1991; Tan et al., 2003). The viral origin of replication is also contained within this region. Off all HPV ORFs, the LCR contains the least homology between different sub-types of HPV.

The second region contains the ORF's for the 'Early' proteins E6, E7, E1, E2, E4 and E5 and occupies over 50% of the virus genome (Zheng & Baker, 2006). The proteins expressed from these genes are responsible for viral replication, transcription, evasion of immune detection, control of cell cycle and oncogenesis. The third region contains the 'Late' ORF's and codes for the L1 and L2 proteins. This region is responsible for the proteins that form the capsid of the virus. Directly after each coding region are the polyadenylation sites: early pA (pA_E) and late pA (pA_L). The pA_E is situated around nucleotide 4215 (Somberg & Swatch, 2010). There are three possible pA_L sites within the HPV16 genome situated at positions 7285, 7343 and 7680 (Kennedy et al., 1990) but only two are used (Milligan et al, 2007).

Figure 1.2

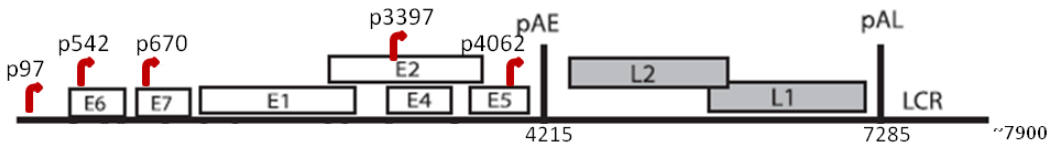


Figure 1.2 Map of the HPV16 genome. This diagram represents the main features of the HPV16 genomic map. The known viral promoters are highlighted including p97, p542, p670, p3397 and p4062. The HPV16 ORFs are represented by open boxes. The pA_E and pA_L and 850 base pair LCR/URR are also indicated. This figure has been modified from Somberg & Schwartz, 2010.

1.5 Transcription

Transcription involves the use of multiple promoters to create polycistronic RNA transcripts that are capable of encoding the viral proteins at the correct time and place throughout the differentiation-dependent life cycle (Hebner & Laimins, 2006). Multiple viral mRNA species are created from one DNA strand through alternative splicing and alternative polyadenylation (Oberg et al., 2005; Rush et al., 2005). In the initial stages of the HPV life cycle lower down in the epithelium layers, the early viral promoter is activated to express the viral E1, E2, E6 and E7 genes [Hummel et al., 1992; Ozbun et al., 1997). The multi-functional E2 protein interacts with four E2BSs in the long control region (LCR) of the HPV genome. E2 interaction results in either the activation (at low levels of E2) or repression (at high concentrations of E2) of E6 and E7 transcription from the early promoter (Bouvard et al., 1994; Steger& Corbach, 1997). E2 may repress the E6/E7 promoter by competing with cellular transcription factors or by preventing the assembly of a transcription complex on the viral chromatin (Demeret et al., 1994; Dong et al., 1994; Tan et al., 1994; Wu & Chiang, 2007).

Various promoters have been identified within the second region of HPV16 genome. A study has described the presence of a promoter in the HPV16 E6 ORF at nucleotide position 542 which controls the monocistronic expression of E7 (Glahder et al., 2003). As HPV-positive cells differentiate, the late promoters are activated in the upper layers of the epithelium leading to the expression of the late genes (Hebner & Laimins, 2006). The transcripts initiated at and around p670 have a coding potential for the late genes, E1^E4 protein and the E5 protein (Grassmann et al., 1996; Milligan et al., 2007). A promoter located within the E4 ORF has been identified (Doorbar et al., 1990; Milligan et al., 2007). This promoter in HPV31 has been shown to regulate late gene expression (Ozbun & Meyers, 1997). There is a promoter within the 3' end of the E5 ORF that has been suggested to have a role in the differentiation-dependent regulation of capsid protein expression (Geisen & Kahn, 1996; Milligane t al., 2007).

The change in viral transcription also involves host cell transcription factors. The cellular bromodomain protein (Brd4) interaction with E2 has been shown to be required for both E2 transcriptional activation and repression of the viral E6/E7 promoter (Schweiger et al., 2006; Nishimura et al., 2000; Goodwin et al., 1998). The CCAAT/enhancer-binding protein beta (C/EBPβ) is a key transcription factor that provokes chromatin opening and the terminal differentiation of keratinocytes. Within the differentiating epithelium it has been shown to inhibit transcription from the early p97 promoter and stimulate the p670 driven transcription of the HPV16 late genes (Kukimoto et al., 2006).

1.6 HPV16 proteins

The process of the transcription and translation of HPV proteins is a complicated and a not yet fully understood process. Activation of multiple promoters and alternative splicing are vital in order to guarantee the correct translation of viral proteins required during different stages on the virus life cycle. The proteins that are expressed from the eight HPV ORF's can be grouped by the roles that they engage in the viral life cycle.

1.6.1 The Replication Proteins

The episomal HPV DNA is replicated to different levels depending on the differentiation status of the epithelial cell. To support PV DNA replication, the virus encodes two proteins; the replicative helicase E1 and the dimeric origin-binding protein E2. All other replication proteins and enzymes required for HPV replication are borrowed from the host cell replication machinery.

1.6.1.1 E1

The 68kDa E1 protein is an essential papillomavirus replication factor without which viral replication cannot take place (Sarver et al., 1984; Lusky & Botchan, 1985; Yang et al., 1991; Ustav & Stenlund, 1991). The E1 ORF retains the most conservation in sequence amongst the other papillomavirus ORF (Danos et al., 1983; Clertant & Seif 1984; Lusky & Fontaine, 1991; Campione-Piccardo et al., 1991). It is the viral helicase and belongs to the family of ATPases associated with diverse cellular activities (AAA+) helicases. The E1 helicase shares sequence homology and similar functional motifs with other AAA+ helicases including the Large T Antigen (LT) of SV40 virus (Park et al., 1994; Mansky et al., 1997). The E1 protein can be split into four domains; an N terminal domain, a DNA binding domain, an oligomerization domain and a helicase domain at the C terminal.

The DNA binding, oligomerization and helicase domains of E1 are essential to carry out HPV replication (Hughes & Romanos, 1993; Mendoza et al., 1995; Chen & Stenlund, 1998; Amin et al., 1999; Enemark et al., 2002). E1 alone possesses minimal DNA binding activity however in the presence of E2 the formation of E1-DNA complexes increases (Lu et al., 1993; Frattini & Laimins, 1994; Sun et al., 1996; Dixon et al., 2000). E2 is the viral helicase loading factor. The N terminal domain of E2 binds to the helicase domain of E1 and this facilitates E1 interaction with the E1BS within the viral origin of replication (Sarafi and McBride, 1995; Yasugi et al. 1997; Sedman et al., 1997) (Figure 1.3 (b)). The E1BS consist of an 18bp palindromic sequence that contains multiple overlapping ATTGTT recognition

sequences for the E1 protein (Holt et al., 1994; Chen and Stenlund, 2001; Titolo et al., 2003; Auster and Joshua-Tor, 2004). E1 binds to DNA as a dimer (Auster & Joshua-Tor, 2004). Three closely spaced E2BS that are in close proximity to the E1BS facilitate the interaction of the E2 dimers with the E1 dimer (Figure 1.3(a)). Together, they have been shown to induce a loop in the supercoiled viral DNA. The extra twist in the DNA creates torsional stress that is relieved by denaturing of the AT-rich sequence in the origin (Sim et al., 2008). The denatured viral DNA alpha helicase may aid the loading of the E1 proteins into a functional hexameric complex. In BPV1 it has been shown that once E1 is recruited to the origin the E2 dimers displace from E2BS that are proximal to the E1BS (Sanders & Stenlund, 1998). Distal E2BS may continue to act as a reservoir for E1 proteins until the formation of the dihexameric complex is complete.

E1 alone possesses a weak affinity for the viral origin. Studies in the Broker and Chow lab reveal an interaction between E1 and the chaperone proteins Heat Shock Protein 40 and 70 (HSP40/70) (Liu et al., 1998; Lin et al., 2002). Using electrophoretic mobility shift assays this group identified that HSP40 and HSP70 independently and additively enhanced E1 binding to the origin of replication. The HSP70 and E1 interaction resulted in E1 hexamer formation whereas HSP40 bound to E1 resulted in the formation of a dihexameric E1 complex. *In vitro,* HPV11 replication assays where HSP40/70 was also expressed show an increase in replicated origin plasmid DNA compared to extracts without the chaperones (Liu et al., 1998). Further investigations into the role of chaperone proteins on the formation of papillomavirus pre-replication complex found that E2 partially inhibits E1-mediated DNA origin unwinding. E2 is displaced from an E1-origin complex by the addition of HSP70 and results in an increase in DNA unwinding. These studies suggest that the HSPs play a role in the assembly and activation of the unwinding activity of the E1 replicative helicase.

Figure 1.3

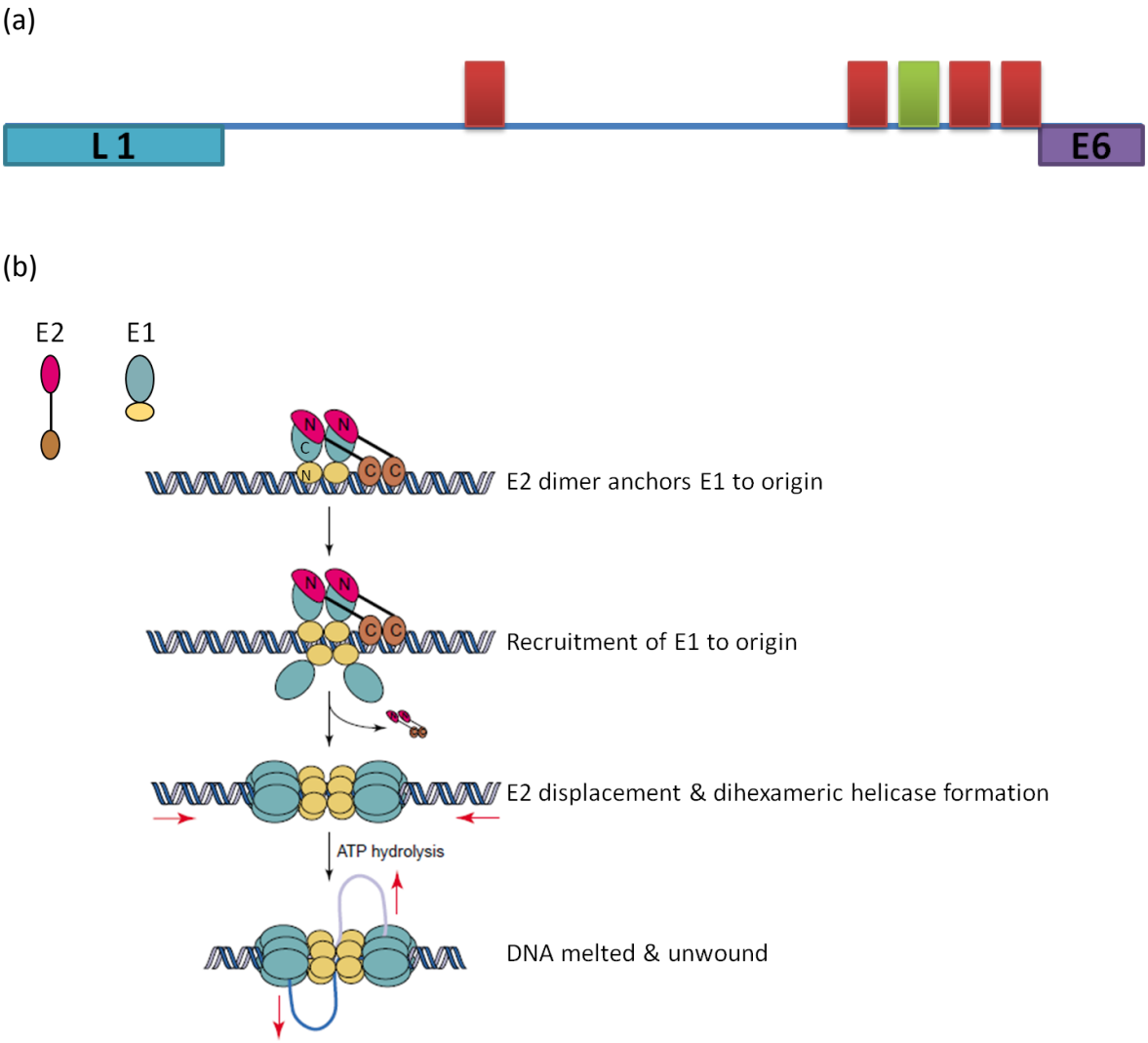


Figure 1.3 Initiation of E1-E2 mediated replication. (a) Map of E1BS (green box) and E2BS (red boxes) within the HPV16 LCR. Part of the L1 and E6 ORF are also indicated on this figure. The distances are approximate and not to scale. (b) The E2 dimer binds to E2BSs within the viral origin. The N terminal domain of E2 then interacts with the C terminal of an E1 dimer. This interaction recruits E1 to E1BS. E2 is displaced, and the hexameric helicase is assembled. As a result the DNA melts, unwinds and viral replication can commence (Modified from Hickman & Dyda, 2005).

The viral helicase melts and unwinds the alpha helix structure of the viral DNA. In order to do this E1 has further activity as an ATPase. E1 hydrolyses adenosine triphosphate (ATP) into adenosine diphosphate (ADP) and a free phosphate ion. The dephosphorylation releases energy which then drives the function of the helicase (Enemark & Joshua-Tor, 2006). The E1 complex can function on a variety of DNA substrates such as linear/circular single/double stranded DNA (Yang et al., 1993; Seo et al., 1993) but has a preference for 'tailed' DNA strands, like those seen at replication forks. Each helicase subunit contains a beta-hairpin that is projected into a central channel formed when the protein oligomerizes (Enemark & Joshua-Tor, 2006) (Figure 1.4). This structure is also conserved in Large T Antigen helicase complexes (Reviewed by Hickman & Dyda, 2005). The hairpin prises apart the DNA strands and makes contact with adjacent nucleotides in a sequential matter. Upon ATP hydrolysis each hexamer then translocates bidirectionally by feeding the DNA through the central channel with 3' to 5' polarity. This results in DNA unwinding and gives the cellular replication/transcription machinery access to the viral DNA.

Figure 1.4

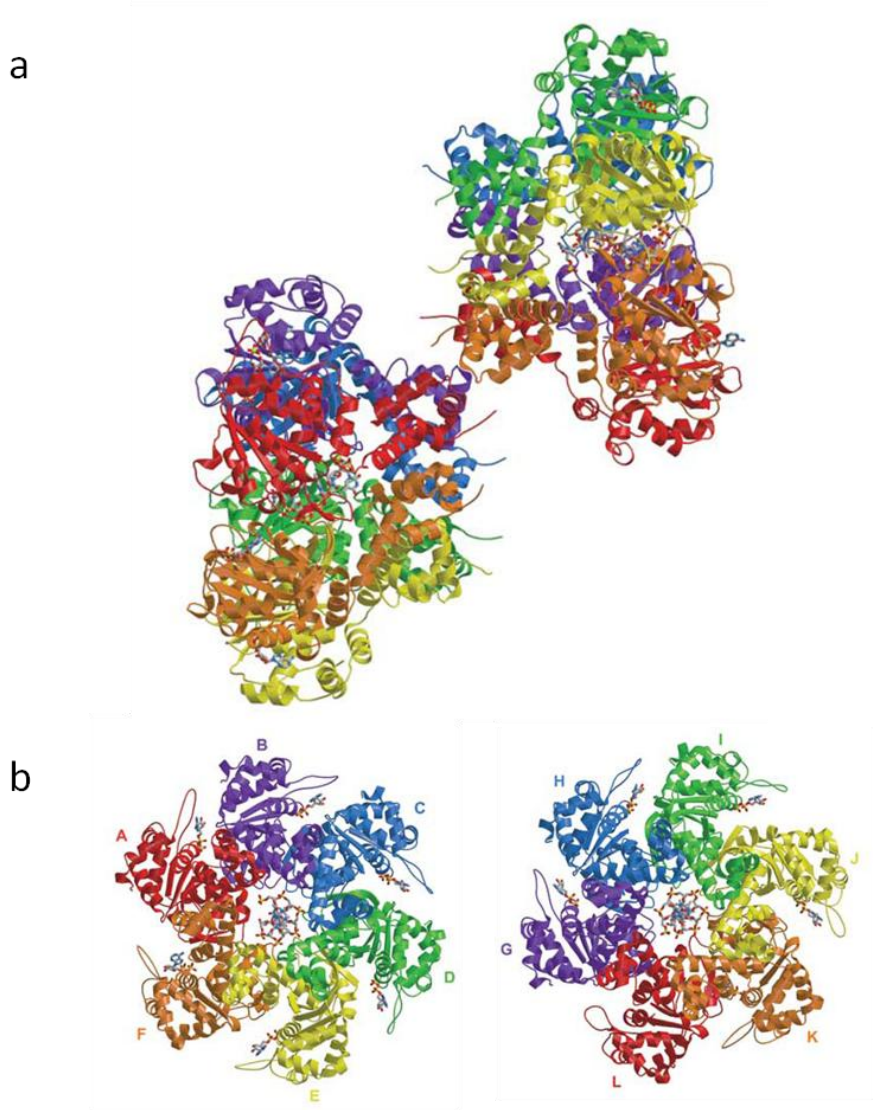


Figure 1.4 Structure of the E1 hexameric helicase in complex with DNA and ADP. Ribbon representations of two E1 helicase hexamers viewed perpendicular (a) and parallel (b) to the channels. (a) The oligomerization domains form a rigid collar, located between the AAA+ domains in (a) and projected towards the reader in (b) The DNA in the central channel and the ADP molecules at subunit interfaces are depicted in stick representation. Each individual E1 oligomerization domain has been lettered from A-L. Images were prepared with Bobscript34 and Raster3D (Enemark & Joshua-Tor, 2006)

The N terminal domain of E1 spans approximately the first 200 amino acids (Abbate et al., 2004). Multiple mutagenesis studies using transient replication assays to study the HPV11 and BPV1 E1 proteins reveal that replication is greatly reduced when the N terminal domain is missing (Ferran & McBride, 1998; Sun et al., 1998; Amin et al., 2000). Therefore the N terminal domain enhances papillomavirus replication. Further studies have suggested that the N terminal domain is involved in the regulation of papillomavirus replication. E1 interacts with multiple cellular cyclin/cdks and this interaction requires an RXL motif. An RXL motif is a docking site on a protein that recruits cyclin-cdk complexes to facilitate the phosphorylation of close proximity Ser/Thr phosphorylation sites (Chen et al., 1996). The RXL motif is located within the N terminal domain of the E1 protein (Ma et al., 1999). There are four cdk phosphorylation sites within E1 and three of which lie in the N terminal domain. Studies on HPV11 E1 have shown that E1 is targeted by cyclins E/cdk at cdk phosphorylation sites and that mutations at the sites greatly reduce the efficiency of E1 and E2 mediated replication (Deng et al., 2004; Ma et al., 1998).

The N terminal domain contains a conserved nuclear export signal (NES) (Deng et al., 2004; Rosas-Acosta & Wilson 2008). The exact role cytoplasmic E1 plays in the viral life cycle is still unclear. Work from the Archambault laboratory investigated the phosphorylation of NES sites in regard to E1 cellular location. They used mutagenesis studies to show that two cyclin E/A-cdk 2 phosphorylation sites located in and close to the NES of HPV31 E1 are phosphorylated and this inhibits nuclear export of E1 (Fradet-Turcotte et al., 2010). Cyclin E/A-cdk2 kinases are required for S phase entry and cell cycle progress (Lees et al., 1992; Dulic et al., 1992). Therefore nuclear import and export of E1 may restrict HPV replication to that of S phase of the cell cycle.

E1 has also been shown to interact with chromatin remodelling complexes. This potentially alters nucleosome positioning and would allow for efficient procession of the replication fork or transcription of viral RNA transcripts. The N terminal of E1 can to bind to histone 1 (H1). H1 is a linker histone that binds to regions of DNA between nucleosomes therefore condensing the chromatin into a tightly ordered structure (Thoma et al., 1979). H1 bound to viral DNA was displaced by the E1 protein (Swindle & Engler, 1998). The removal of H1 suggests that E1 alters the structure of nucleosome at the viral

origin and this may be critical for recognition by the E1-E2 complex in order to initiate replication. E1 has also been associated with alterations of chromatin in other studies. The switching deficient/sucrose non-fermentable (SWI/SNF) protein complex alters the structure of chromatin in order to transcriptionally activate genes. SWI/SNF disrupts chromatin structure at promoters to allow increased access to the transcription machinery (Peterson & Herskowitz, 1992; Travers, 1992). HPV18 E1 has been shown to interact with SNF proteins (Inil/hSNF5) and a consequence of this is enhanced viral replication seen in transient replication assays (Lee et al., 1999). Therefore E1 may play a role in chromatin modifications by recruiting proteins that can alter chromatin structure potentially enhancing viral replication or transcription of the viral ORF's.

The HPV genome is small and therefore limited in its capacity to synthesis proteins capable of carrying out essential functions. Therefore HPV has evolved to hijacks the cellular replication machinery in order to mediate it own replication. In order to do this E1 interacts with a variety of host cellular replication proteins that are involved in eukaryotic DNA replication. These include DNA polymerase α primase (polα-primase), replication protein A (RPA), topoisomerase 1 (topo 1) proliferating cell nuclear antigen (PCNA), replication factor C (RFC) and polymerase δ (polδ). (Park et al., 1994; Conger et al., 1999; Loo & Melendy., 2004; Clower et al., 2006; Melendy et al., 1995; Podust et al., 1995). Polymerase α primase has been shown to interact with E1 protein from BPV1 and HPV6 and this interaction is essential for viral replication (Park et al., 1994). The amino terminal of E1 forms a complex with the p180 polymerase subunit and recruits the protein to the viral origin allowing viral replication during *in vitro* studies. Later work with HPV16 also shows an interaction between the N terminal of E1 with polα-primase. The p68 subunit of polα-primase binds to amino acids 438-623 of HPV16 E1 and is competitive with E2 interaction (Masterson et al., 1998). These data support evidence of sequential interaction during the initiation of papillomavirus replication. The E1 protein of HPV11 interacts with the p180 and p70 subunits of polα-primase and is also essential for HPV replication. The p70 subunit competes with E2 for E1 interaction (Conger et al., 1999). Another protein that E1 recruits to the viral origin is the RPA. Work from Loo and Melendy has shown that BPV1 and HPV11 E1 interact with 70kDa subunit of RPA (Han et al., 1999; Loo & Melendy, 2004). Further studies from this group also identified the

interaction between BPV1 and HPV11 E1 and the topo 1 protein (Clower et al., 2006). An interaction within the N terminal DNA binding domain and C terminal helicase domain stimulates the activity of topoI. The replication factors RPA, RFC, PCNA, polα-primase and polδ are essential for BPV1 E1 and E2 mediated replication during *in vitro* replication assays (Melendy et al., 1994). Further to this polymerase δ and RPA can efficiently cooperate with BPV1 E1 in virus replication studies (Podust et al., 1995). These studies highlight the dependence of papillomavirus for cellular replication factors in order to carry out viral replication.

1.6.2 E2

The E2 proteins of papillomavirus function as dimers within the virus life cycle. The structure of E2 is prototypic of a transcription factor as it is divided into 3 functional domains (reviewed in McBride et al., 1991) (Figure 1.5). The N terminal transactivation domain is well conserved and it is within this domain that the multi-functional E2 protein can regulate viral transcription and replication (McBride and Myers, 1997). The central hinge region varies in sequence size and is not well conserved across varying HPV types. Little is known about the functions of this region but it has been shown as having a role in E2 protein turnover and nuclear localisation (Lai et al., 1999; Penrose & McBride, 2000; Zou et al., 2000). The C terminal domain is important for dimerization of the E2 protein, DNA binding at 12bp palindromic sites, interaction with the viral helicase E1 and interaction with the transcriptional protein TBP (reviewed in McBride. 2008).

Figure 1.5

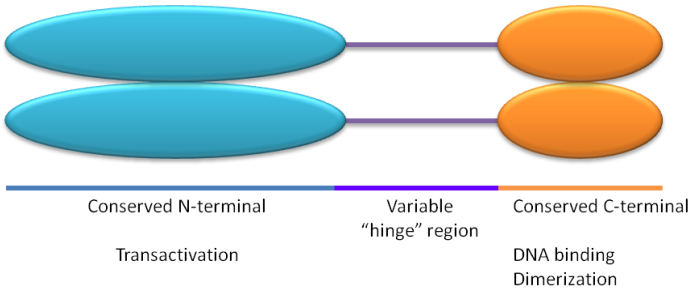


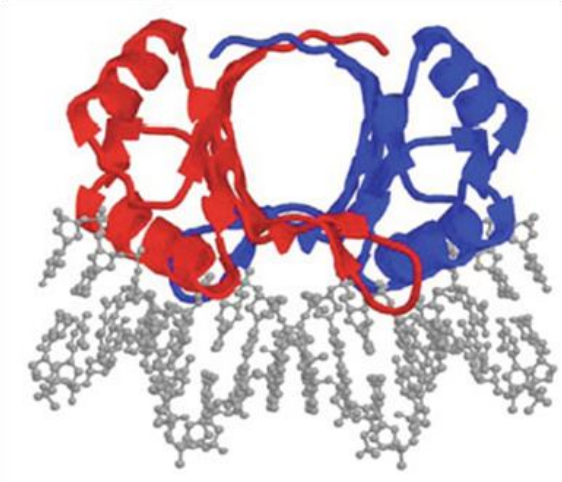
Figure 1.5 Structure of the E2 dimer. The three domains of E2 are highlighted. The N terminal domain is associated with the transactivation functions of the E2 dimer, and the C terminal domain is associated with DNA binding and E2 dimerization.

E2 is involved in the regulation of viral DNA replication through its association with E1. E2 interacts with DNA as a functional dimer at E2 binding sites within the viral LCR (Figure 1.6 (a)) (Mohr et al., 1990; Ustav & Stenlund, 1991; Ustav et al., 1991). The DNA binding domain of the E2 proteins forms a dimeric structure that positions two alpha helices to recognize and contact the E2 binding site (Hedge et al., 1992). The HPV16 E2 dimer interacts with 12bp palindromic sequences and has a high affinity for the DNA sequence: $ACCGN_4CGGT$ (Bedrosian & Bastia, 1990). There are four E2BS within the HPV16 LCR that are used in functions such as viral replication, transcriptional regulation and viral genome partitioning. As mentioned in Chapter 1.6.1.1, the amino terminal domain of E2 recruits the ATP dependent viral helicase E1, via its C terminus, to the viral origin therefore initiating E1/E2-mediated HPV DNA replication (Masterson et al, 1998) (Figure 1.6 (b)). E2 interacts with E1 at arginine 454 in the helicase domain of E1 (Abbate et al., 2004). A mutation of the glutamic acid residue at position 39 of HPV16 E2 to alanine (E39A) disrupts its E1 interaction activity and its replication function without affecting its transcriptional function (Sakai et al., 1996; Kasukawa et al., 1998). E2 bound to E2BS loads the E1 helicase onto the E1BS and increases the specificity of E1 DNA binding. The

E1.E2 interaction reveals specific DNA binding activity intrinsic to the E1 DNA binding domain (Stenlund, 2003). After E1 recognition of the origin E2 is displaced in an ATP dependent manner to facilitate the formation of the E1 dihexameric complex, therefore generating larger complexes that can modify the origin structure (Lusky et al., 1994; Hughes & Ramanos, 1993; Sedman & Stenlund, 1998). E2 also recruits cellular replication factors to the viral origin. The E2 protein from BPV1 interacts with RPA and this facilitates opening of closed DNA structures (Li & Botchan, 1993).

Figure 1.6

(a)



(b)

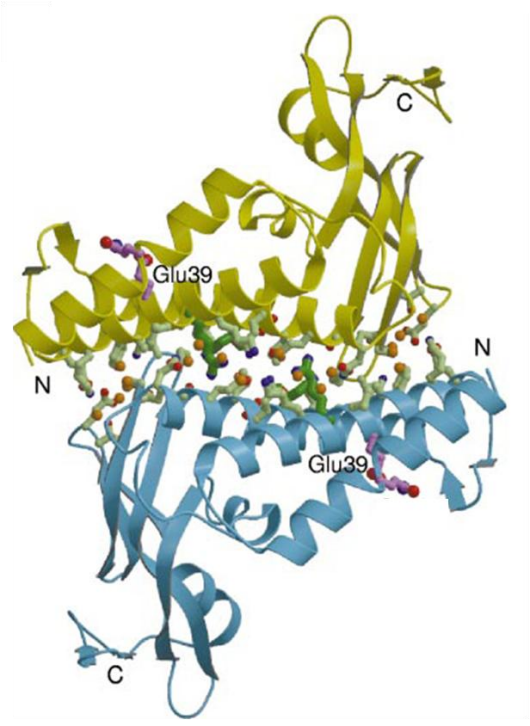


Figure 1.6 Functional interaction domains of the E2 dimer. (a)E2-DNA complex viewed from pdb file 2BOP. The BPV1 E2 (326-410) dimer (red/blue) is bound to its DNA target (grey) (Hedge et al., 1992). (b) Ribbon structure of two independent HPV16 E2 monomers are shown in blue and green. Side chains of glutamine 39 are highlighted in purple. This amino acid is essential for E2 interaction with E1 (modified from Anston et al., 2000).

The E2 protein can regulate viral gene transcription. The E2 gene can code for three E2 proteins that are the products of multiple transcription initiation sites and alternative splicing (Lambert et al., 1987; Vaillancourt et al., 1990; Sherman & Alloul, 1992). The full length E2 protein is called E2-Transactivator (E2-TA). Within the LCR the E2 dimer binds to the E2 specific enhancers (12bp palindromic sequence of ACCGNNNNCGGT). Several E2 dimers interact with the palindromic sequences in cooperation with cellular transcription factors such as p300, AP1 and NFK/1 factor (Gauthier et al., 1991; Kruppel et al., 2008) This allows E2 to regulate viral transcription. There is evidence of a complex interplay between E2 and cellular transcription factors within the LCR that indicates dose-dependent regulation, i.e. at lower concentrations of E2, E6 and E7 viral transcription is stimulated from the p97 promoter and at higher levels of E2, transcription is repressed (Bouvard et al., 1994; Steger& Corbach, 1997). It is possible that at high concentrations, E2 sterically hinders the cellular transcription factors interaction at the viral promoter elements such as Sp1 binding sites and the TATA box (Cripe et al., 1987; Thierry and Yaniv, 1987). The areas within the transactivation domain required for transcriptional regulation are different from those required for replication. The E2 residues arginine 37 and isoleucine 73 within the N terminal section of the transactivation domain are important for transcriptional regulation (Sakai et al., 1995; Antson et al., 2000) (Figure 1.7). Mutations at these residues severely repress E2 transcriptional activation from E2 reporter plasmids. E2 has also been shown to regulate a number of cellular promoters. This allows the virus to express/downregulate cellular proteins that may be required during different stages of the virus life cycle. HPV18 E2 protein downregulates the cellular human telomerase reverse transcriptase (hTERT) by interacting with Sp1 (Lee et al., 2002). E2 interaction with Sp1 has been shown to transactivate p21 expression (Steger et al., 2002). HPV16 E2 transactivation domain can transactivate the alternative splicing factor/splicing factor 2 (ASF/ SF2) promoter (Mole at al., 2009).

Figure 1.7

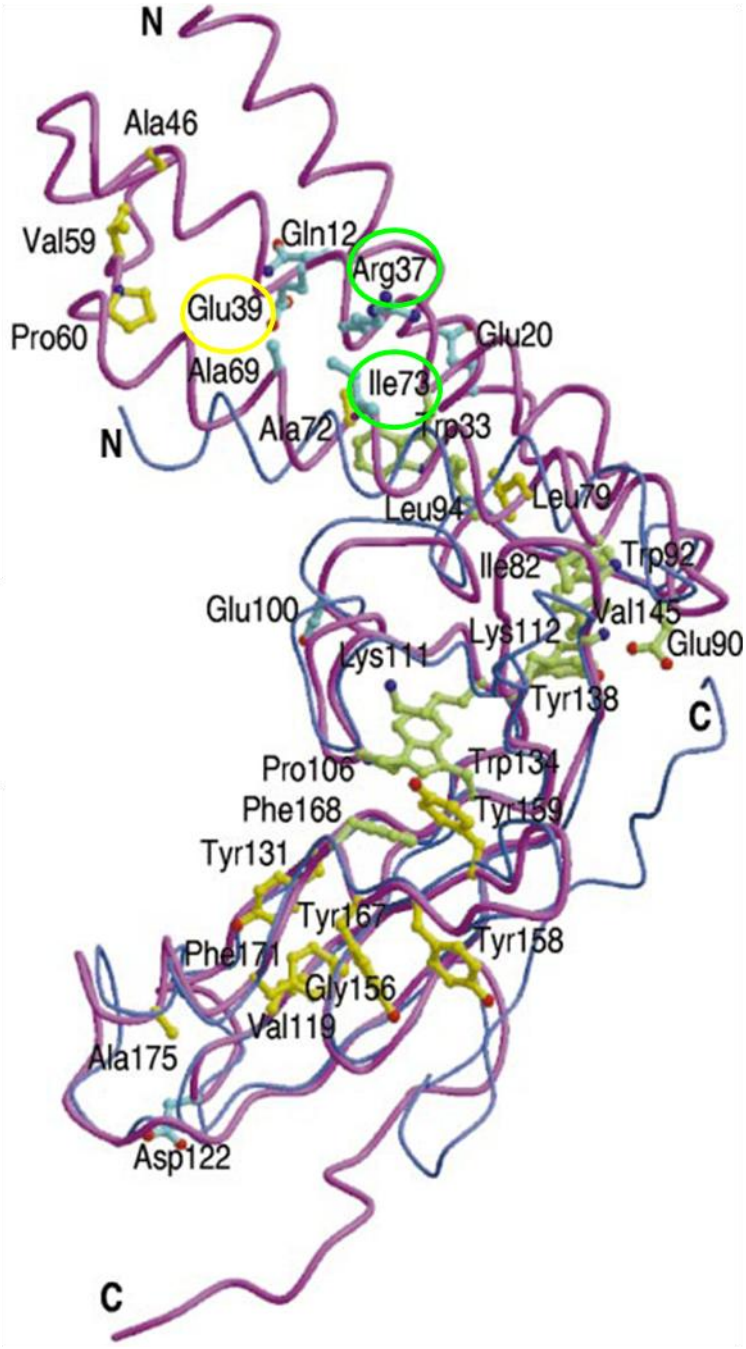


Figure 1.7 Structure of the transactivation domain of E2. Stereo diagram showing the distribution of conserved residues within the HPV18 E2 monomer. Side chains of internal structural residues are shown in yellow, those in the interface between the two domains are shown in green and those exposed on the surface with are shown in aquamarine. The structure is overlaid with that of the backbone HPV18 E2 (blue). Highlighted are the residues essential for transcriptional activation (green circle) and E1 interaction (yellow circle). Image generated with BOBSCRIPT27 and Raster3D (modified from Anton, 2000).

At the basal layer of the epithelium, cell division results in one daughter cell remaining in the basal layer and one daughter cell migrating up to the suprabasal layers to begin the differentiating process. E2 ensures that viral episomes are distributed into both new daughter cells and therefore maintains a source of HPV DNA for persistent infection. To achieve this, E2 either interacts directly with the chromatin or indirectly through cellular factors that tether the viral genome to the cellular chromatin (Skiadopoulos and McBride, 1998; Zheng et al., 2005). E2 can bind to mitotic chromosomes through a direct protein-protein interaction. The DNA binding domain binds to the E2BS within the viral LCR and tethers the viral genome to the condensed chromosomes via its transactivation domain (Bastien and McBride, 2000; Ilves et al., 1999).

The factors used by E2 to tether the viral chromatin during mitosis vary between papillomavirus type. For example HPV11 E2 has been shown to bind HPV origin plasmids directly to the mitotic spindle (Van Tine et al., 2004) (Dai et al, 2006). HPV8 E2 targets the short arms of acrocentric mitotic chromosomes, in particular the repeated ribosomal DNA genes (Poddar, 2009). Brd4 is a member of the BET family of double bromodomain proteins which interacts histones H3 and H4 and can bind to chromatin through mitosis (Florence and Faller, 2001; Dey et al., 2003). BPV1 E2 and HPV16 E2 have been shown to utilize Brd4 as an accessory factor in viral genome tethering and the complex on mitotic chromosomes (You et al 2004; Baxter et al 2005; Schweiger et al., 2006). Two highly conserved residues arginine 37 and isoleucine 73 in the N terminal section of the transactivation domain are critical for Brd4 binding (Baxter et al., 2005; McPhillips et al., 2006; Senechal et al., 2007). These residues are also essential for E2 transactivation function. The N terminal domains of the E2 dimer interact with two Brd4 peptides within the Brd4 C terminal (Abbate et al., 2006).

ChLR1, a DNA helicase that plays a role in sister chromatid cohesion, has been shown to interact with BPV E2 at early mitosis as a possible loading factor (Parish et al, 2006). E2 interacts with and co-localises with ChLR1, but only at early stages of mitosis. E2 protein that does not interact with ChLR1 cannot tether viral DNA to cellular chromatin. The viral genome is lost when ChLR1 expression is inhibited. Work carried out in this laboratory suggests that Topoisomerase binding protein 1 (TopBP1) could be the mitotic chromatin

receptor for HPV16 E2 in late mitosis (Donaldson et al 2007). E2 was shown to associate with the mitotic spindle via co-localisation with TopBP1 suggesting that this protein could be the mitotic chromatin receptor for HPV16 E2. The E2 protein of HPV8 has been shown to interact with the pericentromeric region of mitotic chromosomes. The precise target is the ribosomal RNA gene loci that are situated on the short arms of acrocentric chromosomes (Oliveira et al., 2006; Poddar et al., 2008).

Asides from its roles in viral replication, viral genome partitioning at cell mitosis and regulation of viral gene expression, E2 also has a role in the regulation of cell proliferation and apoptosis. A characteristic of HPV-induced cervical cancer is the loss of episomal HPV DNA and integration of the HPV genome into that of the host cell (Cooper et al., 1991; Kristiansen et al., 1994; Alazawi et al., 2002; Peitsaro et al., 2002; Arias-Pulido et al., 2006; Pett et al., 2006). This integration often results in a loss of the E2 ORF leading to derepression of viral oncoprotein expression resulting in a growth advantage in the cells (Romanczuk et al., 1990; Romanczuk & Howley, 1992; Jeon et al., 1995). The E2 protein was first described as having apoptotic properties by an investigation of BPV1 E2 introduction into a range of cervical cancer cell lines. The presence of E2 was shown to inhibit cellular proliferation in HeLa (human epithelial carcinoma cell line containing HPV18 integrated DNA), C-4I (squamous cell carcinoma cell line of the cervix containing integrated HPV18 DNA), MS751 (squamous cell cervical carcinoma cell line containing HPV18 DNA) and HT-3 (HPV-negative p53 mutated cervical carcinoma cell line) (Hwang et al., 1992). Due to the use of HT-3 cells, this study also suggests that the E2 protein appears to exert a growth-inhibitory effect that is independent of its effects on HPV gene expression. A separate study has shown that cell lines containing episomal HPV31 DNA have a higher rate of apoptosis after long term interferon (IFN-β) treatment compared to HPV-negative cell lines. Induced E2 expression resulted in a significant increase in apoptotic cells compared to non-induced cells, therefore supporting evidence of E2 pro-apoptotic effects (Chang et al., 2002).

Other studies have been carried out investigating the effects of HR-HPV E2 on cell proliferation. Multiple studies by the Gaston group have focused on the role of HPV16 E2 and its pro-apoptotic properties (Sanchez-Perez et al., 1997; Webster et al., 2000; Parish

et al., 2006). Their work, focusing on p53 mediated apoptosis, shows that E2 induces apoptosis in both HPV-transformed and non HPV-transformed cell lines via two independent pathways. In non HPV-transformed cells, mutations in E2 that abrogate its interaction with p53 inhibit E2-induced apoptosis (Webster et al., 2000). In contrast, E2 mutants that do not interact with p53 do not inhibit E2 mediated cell apoptosis in HPV transformed cell lines (Parish et al., 2006). The results from HPV-transformed cell lines suggest that E2 regulates cell proliferation by interfering with the expression of the anti-apoptotic viral proteins E6 and E7. The E6 and E7 proteins alter the cellular proliferation by negatively interfering with two regulatory proteins of the cell cycle, p53 and pRb (see Chapter 1.6.2.1 – 1.6.2.2). Investigations carried out in the Thierry lab on HPV18 show that E2 is able to cause apoptosis in Hela cells and has a negative effect of E6 and E7 transcription (Thierry & Howley, 1991; Desaintes el al., 1997; Desaintes et al., 1999). However, Saos-2 cells that are deficient in p53 function and are non HPV-transformed show that E2-mediated apoptosis is activated independently of viral functions, suggesting the use of the extrinsic apoptosis pathway (Demeret et al., 2003). Therefore the pathways with which E2 is able to activate apoptosis are still highly debatable and differences in results may be accountable to the type of papillomavirus used. Loss of E2 expression in cell lines containing integrated DNA most certainly contributes to cell immortality.

1.6.2 Oncoproteins

The association between HPV infection and cervical cancer was first reported three decades ago. It is widely accepted nowadays that HPVs are the causative agent of cervical cancer. Over 99% of cervical lesions and 40% of oral cancers are found to contain HPV DNA from ‘high risk’ strains of the virus (Walboomers et al., 1999; Parkin & Bray, 2006). The viral oncoproteins are well documented for their contribution in the development of cervical cancer. The E7 and E6 proteins support each other’s function and have transforming capabilities. More recent work has also indicated a role for E5 protein in contributing towards cancer development.

1.6.2.1 E7

The 17kDa HPV16 E7 protein is located primarily in the nucleus. Within the amino terminal of E7, there is a conserved LXCXE motif. The motif is the site where E7 interacts with the Rb tumour suppressor protein and related family members p107 and p130 and targets them for degradation (Dyson et al., 1989; Munger et al., 1989). 'High risk' E7 proteins have been shown to bind with the Rb family members with much higher affinity than proteins from 'low risk' HPV's and this may contribute to their oncogenicity (Gage et al., 1990).

The Rb family of proteins control G_1-S phase transition of the cell cycle by regulating the activity of the E2F family of transcription factors (reviewed by Dyson, 1998). The eight members of the E2F family are involved in the regulation of transcription of genes involved in cell cycle progression, differentiation, mitosis and apoptosis. Therefore E2F binding sites are found in the promoters of genes involved in these functions. In non HPV infected cells, Rb proteins inhibit transcription of E2F dependent promoters by directly interacting with the E2F transactivation domain (reviewed by Harbour & Dean, 2000). In HPV infected cells however, the E7 protein disrupts Rb-E2F interaction by binding to Rb protein. This interaction results in the expression of E2F responsive genes such as cyclin A and cyclin E and consequently promotes premature S phase progression and DNA synthesis (Chellappan et al., 1992). The E7 and E2F-6 interaction therefore maintains an S phase environment that is required for amplificational replication in differentiating cells (McLaughlin-Drubin et al., 2008). The HR-HPV E7 also target Rb family members for degradation through ubiquitination (Boyer et al., 1996).

The expression of E7 has also been associated with abnormal chromosome numbers (Duensing & Münger, 2003a; Duensing & Münger, 2003b). E7 rapidly induces centrosome amplification by the formation of multiple immature centrioles from a single maternal centriole (Duensing et al., 2001; Duensing et al., 2007). Centrosome abnormalities have the potential to lead to genomic instability. Therefore E7 proteins from HR-HPVs, contribute to the development of cervical cancer through targeting members of the Rb

family for degradation, activating E2F transcription factors that drive the expression of S phase genes and inducing centrosome abnormalities in HPV infected cells.

1.6.2.2 E6

The previously described interaction between E7 and Rb family of proteins can lead to inhibited cell growth and apoptosis through the activation of p53 dependent pathways (Jones et al., 1997; Eichten et al., 2004). To counter this, HPV expresses the E6 protein. E6 interferes with p53 functions by multiple mechanisms. E6 recruits the E3-ubiquitin ligase E6 associated protein (E6AP) and results in ubiquitylation and consequential proteasomal degradation of p53 (Scheffner et al., 1993; Lechner & Laimins, 1994). E6 can also alter p53 function by decreasing its stability. The histone acetylases p300 and CREB binding protein (CBP) have been shown to increase p53 stability through acetylation. HPV16 E6 binds to p300 and CBP therefore inhibiting their ability to stabilise p53 (Patel et al., 1999; Zimmermann et al., 1999).

E6 contributes to cell immortalisation through the activation of telomerase. Telomerase enzyme is often activated in many cancers and is important for replicating the DNA sequences at the end of chromosomes. HPV16 E6 can activate the transcription of telomerase reverse transcriptase (TERT) and increases its protein levels by directly associating with the nuclear transcription factor X-123 (Katzenellenbogen et al., 2007).

E6 interferes with the effects of a number of growth inhibitory cytokines in order to regulate cellular proliferation. When the virus first enters the cell a potent inhibitor of keratinocyte proliferation, the tumour necrosis factor alpha (TNF-α), is synthesised (Basile et al., 2001). Inflammatory cytokines such as TNF-α activate the extrinsic apoptotic pathways such as those belonging to the TNF receptor family. Members of this family include TNFR-1, FAS and TNF-related apoptosis-inducing ligand (TRAIL). HPV16 E6 inhibits TNF-α induced apoptosis by interacting with TNFR1 (Fillippova et al., 2002). This interaction inhibits the formation of the Death Inducing Cellular complex (DISC) resulting

in inhibition of apoptosis. HPV16 E6 also interacts with the Fas-associated death domain (FADD) and caspase-8, therefore protecting cells from Fas associated apoptosis (Fillippova et al., 2004; Garnett et al., 2006).

The human homologue of the *Drosophila* discs large tumour suppressor gene (hDlg) protein is a PDZ domain containing protein that is involved in the regulation of cell polarity and negative proliferation control. The HPV16 E6 protein has been shown to target hDlg for proteasomal degradation therefore enhancing morphological transformation of the cell (Gardiol et al. 1999; Watson et al., 2002). Prior to degradation mediated by E6, hDlg is phosphorylated by either Cdk1 or Cdk2 and a result of this is accumulation of hDlg in the cell nucleus. It is within the nucleus where hDlg encounters E6-induced degradation (Massimi et al., 2006; Narayan et al., 2009). E6 contributes to cell transformation through a number of mechanisms including targeting p53 and hDlg for degradation, activation of telomerase and preventing HPV induced cellular apoptosis.

1.6.2.3 E5

HPVs are known to cause long term persistent infections. Viral persistence is caused by a number of factors. The papillomavirus life cycle takes place exclusively in epithelial cells and therefore can go undetected by the immune cells of the dermis. Papillomavirus do not initiate an inflammatory response because they do not cause lysis of the host cell. Viral proteins inhibit activation of the host cell immune response. The hydrophobic membrane bound HPV E5 protein contributes to viral persistence by down-regulating the host cell immune defence. The Major Histocompatibility Complex class I (MHC class I) expression increases presentation of viral antigens to cytotoxic T cells. E5 protein down regulates MHC class I in HPV infected cells (Ashrafi et al., 2005; Campo et al., 2010). This is achieved by recruiting the MHC complex to the golgi apparatus as opposed to the cell surface where it would be recognised by CD8+ T cells (Ashrafi et al., 2006; Cortese et al., 2010). The accumulation of the MHC in the golgi is a consequence of E5 induced alkalinisation of the golgi membrane (Schapiro et al., 2000).

Malignant transformation by HPV can also be attributed to the E5 protein. The role of HPV16 E5 has been suggested mostly in early stage of cervical carcinogenesis. Evaluation of E5 expression levels by immunohistochemistry has shown that E5 is detectable in 80% of HPV infected low grade squamous intraepithelial lesions and that this percentage drops to around 60% in cervical carcinoma tissue (Chang et al., 2001). Further evidence supporting the role of E5 as a transforming protein is by its interaction with the epidermal growth factor receptor (EGFR) signalling pathway (Straight et al., 1993; Genther Williams et al., 2005; DiMaio & Mattoon, 2001). The downstream effects of EGFR are an increase in DNA synthesis and cell proliferation. Over expression of EGFR signalling has been correlated with a large number of cancers (reviewed in Zhang et al., 2007). E5 increases EGFR signalling by interfering with its degradation and by increasing the number of receptors expressed on the cell surface.

HPV16 E5 is capable of enhancing cell proliferation by interfering with tumour suppressor proteins p21 and p27. E5 proteins of HPV11 and HPV16 have been shown to repress p21 gene expression and this might be one of the mechanisms by which E5 stimulates cell proliferation (Tsao et al., 1996). The half life of p27 (Kip1) decreases in the presence of E5 favouring an S phase environment (Pedroza-Saavedra et al., 2010).

Resistance to apoptosis is a hallmark of many cancers. HPV16 E5 is reported to impair apoptotic pathways in HaCaT cells. E5 weakens FasL and TRAIL mediated apoptotic pathways by down regulating Fas expression involved in FasL signalling and altering the formation of the death-inducing signalling complex involved in TRAIL signalling (Kabsch & Alonso, 2002). Therefore, apart from its role in avoidance of the host immune surveillance, E5 is a potential oncogene through interaction with the EGFR, interfering with tumour suppressor protein expression and by inhibiting HPV activated apoptotic pathways.

1.6.3 Proteins expressed in the upper epithelial layers

1.6.3.1 E1^E4

The E4 protein is expressed from a spliced mRNA species as E1^E4. Spliced transcripts are formed between the N terminal of the E1 ORF and an almost complete E4 ORF (Nasseri et al., 1987). E1^E4 accumulates to high levels in the mid layers of the epithelium. The E1^E4 roles in the viral life cycle are a little unclear, however when this protein is lost it results in severe and adverse effects on viral genome amplification of HPV types 16, 18 and 31 (Nakahara et al., 2005; Wilson et al., 2005; Wilson et al., 2007). The cottontail rabbit papillomavirus, E1^E4 is essential for completion of the vegetative stage of the virus life cycle (Peh et al., 2004). Therefore one of the key roles for the E1^E4 protein in the upper epithelial layers appears to support vegetative replication.

E1^E4 may be involved in vegetative replication by inhibiting mitotic progression and inducing DNA re-replication (Knight et al., 2004). Inhibiting cellular replication could allow for the recruitment of the cell replication factors to the viral origin of replication. In the presence of the replication licensing factor cdc6 the HPV1 E4 protein inhibits the initiation of cellular DNA replication. E4 blocks cellular replication by preventing the loading of Mcm2 and Mcm7 onto cellular chromatin (Roberts et al., 2008).

E1^E4 has also been associated with having a fundamental role in viral egress. E1^E4 diminishes the integrity of the keratinocyte by disrupting the keratin cytoskeleton and cornified envelope formation (Doorbar et al., 1991; Roberts et al., 1993; Bryan & Brown, 2000; McIntosh et al., 2010). E1^E4 induces apoptosis through alteration of mitochondrial function (Raj et al., 2004). Therefore through breaking down the cell architecture, E1^E4 expression in the upper layers of the epithelium is able to assist HPV capsid release.

1.6.3.2 The capsid proteins: L1 & L2

The 55nm diameter HPV capsid is non-enveloped, has T=7 icosahedral symmetry and is formed from two HPV structural proteins, L1 and L2. Within the capsid is the 7.9kb circular genome that is associated with cellular histones to form chromatin like structures (Larsen et al., 1987; reviewed in Doorbar, 2005). There are 72 pentamers of L1 that make up the viral coat and they are associated with L2. The stoichiometry of L1:L2 in purified L1+L2 complexes is 5:1 indicating that a single molecule of L2 interacts with an L1 pentamer (Finnen et al., 2003) .The capsid proteins are essential for viral entry, and are only expressed in terminally differentiated keratinocytes demonstrating their role in capsid formation and egress (Barksdale & Baker, 1993).

The L1 protein is evolutionary conserved. Interaction between the viral capsid and the cell surface receptor relies primarily on L1. There has been speculation regarding what cell surface receptor L1 interacts with to fulfil capsid attachment to the cell. Glycosaminoglycans (GAG's) are the suggested initial attachment receptors, in particular heparin sulphate (Joyce et al. 1999; Giroglou et al., 2001; Combita et al., 2001; Drobni et al., 2003). Another potential candidate for L1 attachment is syndecan1. Heparin sulphate proteoglycans (HSPG) are frequently located on cell surfaces and in the extracellular matrix. Syndecans are the predominant HSPG in epithelial cells. Syndecan1 may therefore serve as the primary attachment receptor due to its high expression level in epithelial cells (Sapp & Day, 2009; Shafti-Keramat et al., 2003).

L1 expressed alone is sufficient to produce synthetic papillomavirus particles. Formation of non-infectious viral like particles is highly immunogenic. This is why L1 is used as a preventive vaccination against HPV types. Two HPV vaccines are currently on the market: Gardasil and Cervarix (reviewed by Harper, 2009). Both vaccines protect against two HR-HPV types, 16 and 18. Gardasil also protects against the two LR-HPV types, 6 and 11.

HPV binding and entry into the host cell is complex and involves virus attachment to multiple receptors. The multifunctional L2 minor capsid protein is internally located within the capsid. Initial low specificity binding of the L1 major capsid with HSPG results in

a conformational change in the viral capsid and subsequent exposure of the L2 N terminal (Roden et al., 2001; Yang et al., 2003; Sapp & Day, 2009). L2 interacts with the C terminal domain of L1. Proteolytic cleavage of L2 N terminal domain by furin is essential for successful HPV infection (Day et al., 2008). The cleavage site is conserved amongst all papillomaviruses and it is otherwise inaccessible at the surface of the virion (Richards et al., 2006). The location of this essential cleavage site helps prevents an immune response against L2 (Sapp & Day, 2009). Furin cleavage of L2 results in an additional conformational change that exposes a site on L2 for a yet undetermined secondary receptor. Interaction with the secondary receptor facilitates the release of a L2/genome complex into the host cytosol via endocytosis.

Aside from its role in virion attachment and endocytosis, L2 is responsible for the nuclear import of the viral genome. L2 interacts with syntaxin 18 facilitating the movement of the viral genome to a perinuclear complex (Bossis et al., 2005; Richards et al., 2006). The amino terminal of L2 contains a nuclear localisation site (Fay et al., 2004; Bordeaux et al., 2006). L2 may therefore translocate the viral genome into the nucleus, exposing it to the cellular transcription and replication machinery. L2 also plays a role in viral DNA encapsidation using the HPV E2 proteins (Heino et al., 2000; Gu et al., 2004; Holmgren et al., 2005).

1.7 HPV episomal integration

Failure of the immune system to detect and clear high-risk HPV infections can result in the development of cancerous cells, a process that may take several decades. Episomal viral DNA can be detected in precancerous lesions. However DNA from the HR-HPV types 16 and 18 are frequently found integrated into host cell DNA in high grade squamous intraepithelial lesions (SIL) and squamous cell carcinomas (Cooper et al., 1991; Kristiansen et al., 1994; Alazawi et al., 2002; Peitsaro et al., 2002; Arias-Pulido et al., 2006; Pett et al., 2006). HPV integration often occurs in a naturally occurring region of genomic instability. Integration is an accidental but critical event in the development of carcinomas. In

cervical malignancies, over half of HPV16-positive and nearly all of HPV18-positive malignancies contain integrated HPV genomes (reviewed in Pett & Coleman, 2007). There is also evidence of HPV16 integration in HPV-positive head and neck cancers. A study has found that 61% of head and neck cancers are HPV DNA positive. HPV16 was accountable for 84% of the malignancies and was found integrated in 50% of the samples (Koskinen et al., 2003). This supports evidence from other studies that have also detected integrated HPV DNA in HPV-positive head and neck cancer cells (Hafkamp et al., 2003). Other factors that must be taken into consideration are alcohol intake and whether the individual is a smoker, however this evidence suggests that head and neck cancers may also have viral integration aetiology.

In a HPV episomal infection, the E2 protein regulates the expression of the E6 and E7 oncoproteins. Viral integration normally results in loss of E2 expression and deregulated expression of the viral oncoproteins (Figure 1.8). Deregulated expression of E6 and E7 can contribute to cellular proliferation and transformation. Integration of HR-HPV DNA also correlates with a selective growth advantage of cells for clonal expansion (Jeon et al., 1995; Romanczuk & Howley, 1992; Romanczuk et al., 1990).

Figure 1.8

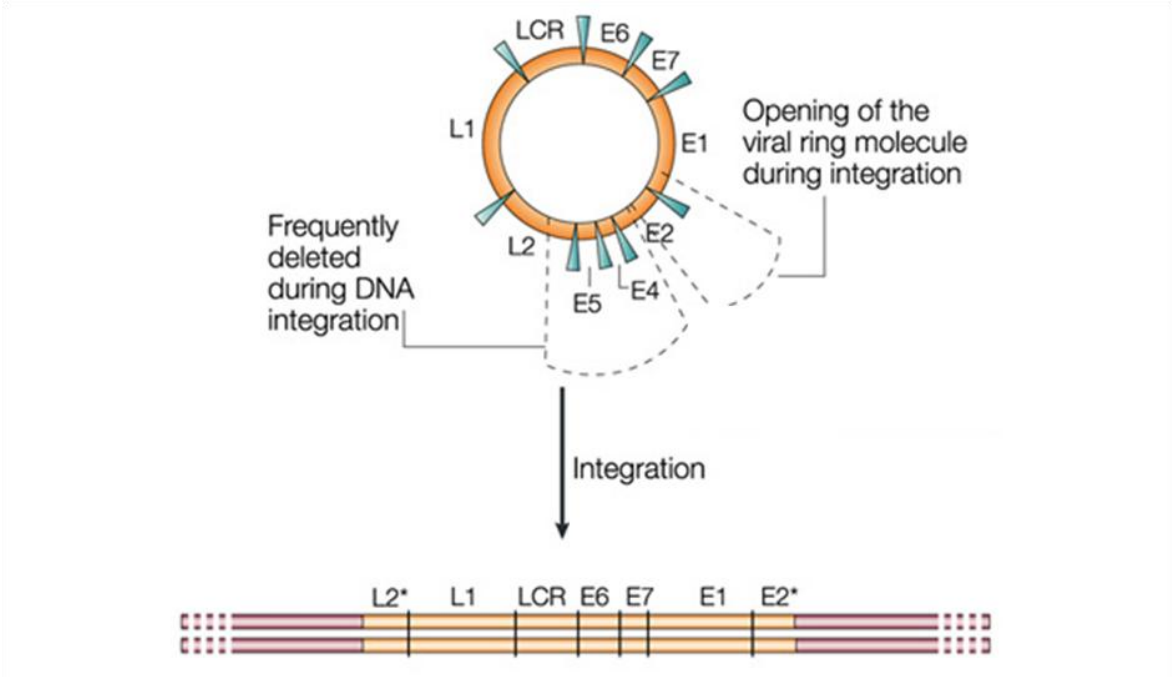


Figure 1.8 HPV episomal integration. In the course of cancer development the HPV episome frequently becomes integrated into host cell DNA. The episome is most often opened within the E2 ORF therefore the E2 gene is lost. E4, E5 and part of L2 ORF are often deleted after integration (partial genes are denoted by an asterisk). The E6 and E7 ORF are present and transcription is enhanced by flanking host-cell promoters (modified from zur Hausen, 2002).

Episomal HPV DNA is lost over time in HPV-integrated cells. However early on in malignant progression, episomal and integrated viral DNA exists within the host cell. It has been suggested that episomal E1 and E2 are able to stimulate viral replication from an integrated origin of replication. This would result in amplification of the integrated HPV origin and flanking sequences (Kadaja et al., 2009). A consequence of this replication may be local rearrangements within the DNA backbone (Figure 1.9). The theory of an 'onion skin' method of replication during S phase of the cell cycle would result in chromosomal abnormalities that could drive malignant progression. Replication of integrated HPV activates the DNA damage checkpoints which can cause chromosomal abnormalities through non homologous end joining (NHEJ) repair.

Figure 1.9

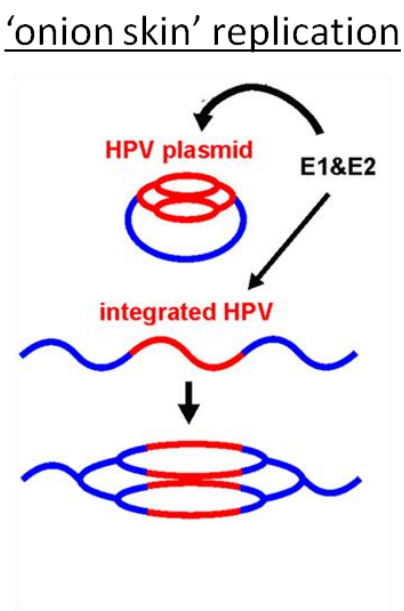


Figure 1.9 E1 and E2 expressed from episomal DNA can cause replication of integrated DNA. If the HPV episome is present in cells harbouring integrated HPV, DNA re-replication from the integrated HPV origin is initiated. The chromosomal abnormalities encountered may be further exasperated by 'onion skin' methods of replication (modified from Kadaja et al., 2009).

1.8 DNA replication and the cell cycle

The fundamental features and components of DNA replication are well conserved throughout evolution from viruses to mammals. Every cycling cell is required to duplicate its genome in a coordinated manner before it leaves the cell cycle and enters cytokinesis.

1.8.1 G_1

There are four stages of the cell cycle prior to cytokinesis. The G_1 phase is marked by synthesis of various enzymes that are required in S phase, mainly those needed for DNA replication. It is within this phase that the pre-replication complexes are formed (Figure 1.10). The DNA double helix must be opened to allow access of the components required to copy each strand. DNA is opened up at the origins of replication. The assembly of the prereplication complex (pre-RC) takes place at the origins (Kelly & Brown, 2000; Bell & Dutta, 2002). In eukaryotic cells the primary factor that initiates the formation of the pre-RC is the origin recognition complex (ORC) (reviewed in Méchali, 2010). The ORC is a heterohexamer with DNA dependent ATPase activity. The ORC then recruits cdc6 and CDT1. These proteins load the 'inactive' mini chromosome maintenance (MCM) complex onto the DNA strands (Cook et al., 2003). The MCM2-7 complex is a group of proteins that belong to the AAA+ ATPase family of proteins. Other members of this family include the HPV E1 protein and the SV40 Large T Antigen protein. The heterohexamer complex has DNA helicase activity and forms a ring around the origin. Once the helicase is bound to the DNA, the origin is deemed licensed for activation.

In order to activate origin firing, cellular factors including cdc45 protein, the GINS complex, Cdc7-DBf4, geminin and Cdk2 are recruited (Mailand & Diffley, 2005; Moyer et al., 2006; De Marco et al., 2009; Ilves et al., 2010; Balestrini et al., 2010) . The cdc6 and ORC become degraded. CDT1 is negatively regulated by Geminin in order to restrict origin firing to once per cell cycle. In the presence of geminin, CDT1 interaction with DNA is inhibited leading to the initiation of DNA replication.

Figure 1.10

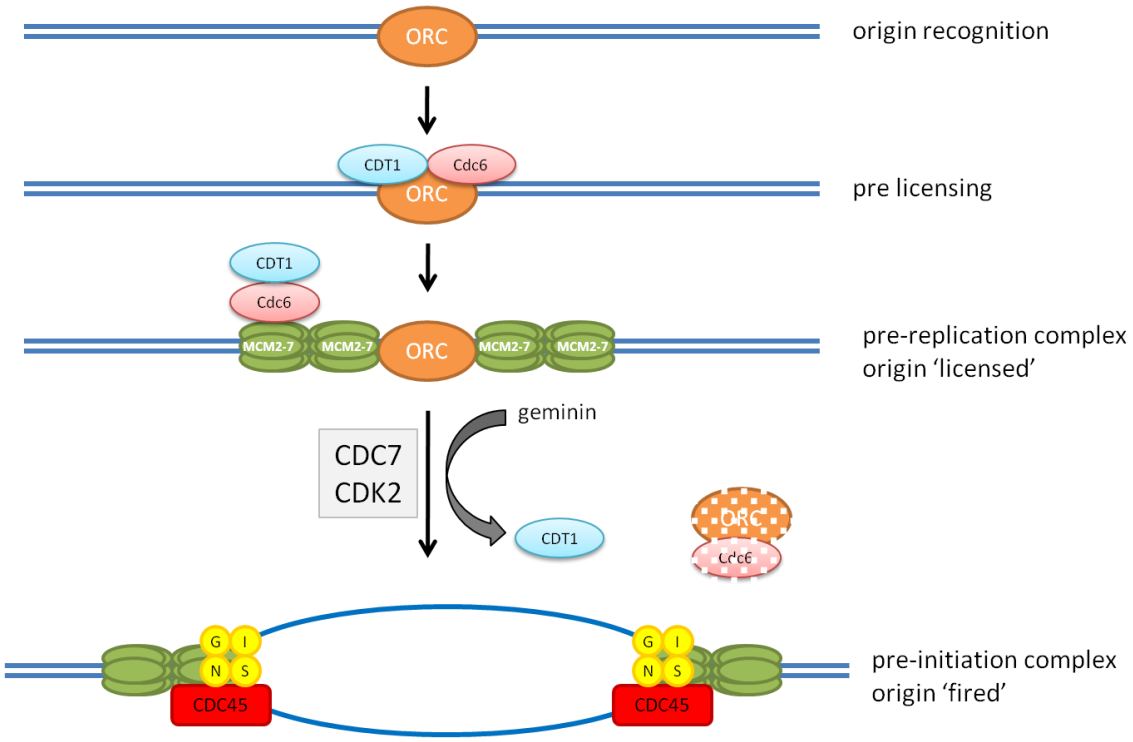


Figure 1.10 Assembly of the pre-replication complex at DNA replication origins In eukaryotes, ORC directly recognizes an origin of replication. Cdc6 and CDT1 are then recruited to load the MCM2–7 complex onto the replication origin. Once the helicase is bound to the DNA, the origins are 'licensed' marking the end of pre-replication complex (preRC) assembly. CDT1 is a major regulator of this reaction, as it is negatively regulated by Geminin to restrict licensing to only once per cell cycle. The preRC is further activated by Cdc45, GINS complex, CDC7–DBF4 and Cdk2. As cells enter S phase, CDT1 is inactivated by both its release from the origin by Geminin and by its degradation. These events enable the association of the DNA polymerase machinery and MCM2–7 to travel ahead of the replication fork to open the double-stranded DNA and allow the synthesis of the complementary strand.

1.8.2 S phase

The S phase of the cell cycle starts with the onset of DNA replication and continues until all chromosomes have been replicated. DNA replication takes place at an activated origin of replication. Replication of genomic DNA requires a number of factors (Figure 1.11). Briefly, the MCM2-7 complex unwinds the DNA double helix, the topoisomerase enzyme is recruited to remove DNA supercoiling ahead of the MCM2-7 helicase, RPA coats the single strands to prevent the DNA from re-annealing to itself and DNA primase synthesizes the short RNA primers for the initiation of complimentary DNA synthesis (Maniar et al., 1997; Iftode et al., 1999). Preventing the rebinding of the MCM complex to DNA is key to avoiding re-replication (Blow & Dutta, 2005). DNA polymerase delta and alpha (for the leading and lagging strand respectively) copy the DNA strands starting at the 3' end of the RNA primer and RNAse H degrades the RNA on the lagging strand. Okazaki fragments on the lagging are joined together by DNA ligase. At the core of the replication complex is the sliding clamp, PCNA (Sporbert et al., 2002; Sporbert et al., 2005). The clamp is loaded onto the separated DNA strands by the clamp loading complex, RFC. The sliding clamp helps hold this DNA polymerase onto the DNA as the DNA moves through the replication machinery

Figure 1.11

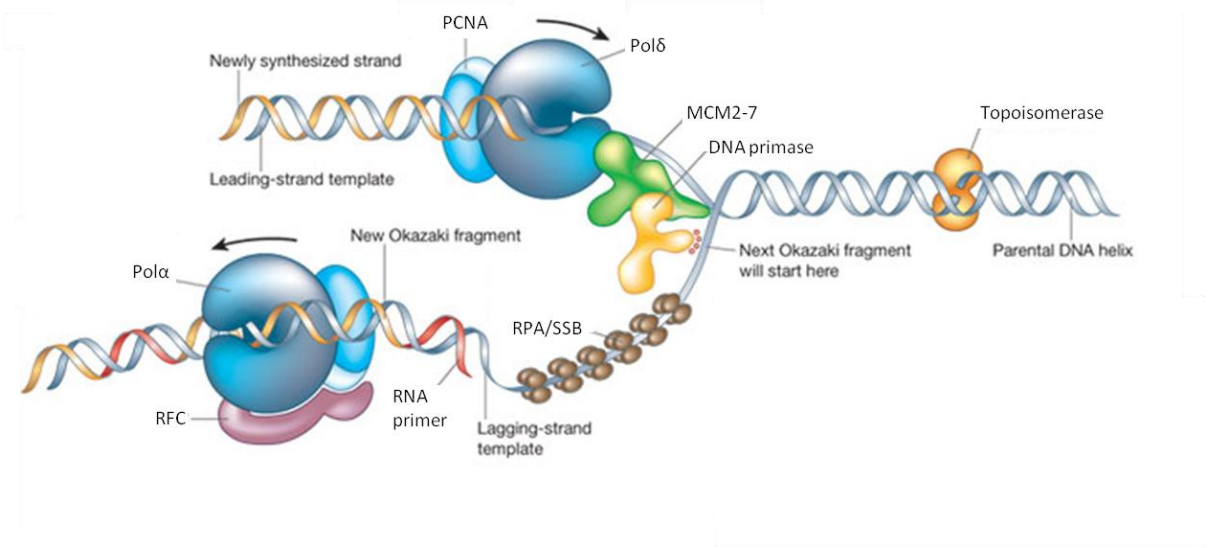


Figure 1.11 DNA replication fork. Two DNA polymerase molecules (delta and alpha) are active at the fork. Pol. δ produces the new DNA molecule on the leading strand, whereas Pol. α produces a series of short 'Okazaki DNA fragments' on the lagging strand. Both polymerases are anchored to their template by PCNA and RFC. MCM2-7 opens the DNA helix ahead of the replication fork. DNA topoisomerase aids DNA helix unwinding. Pol. α requires the action of a DNA primase. Single-stranded regions of DNA at the fork are coated with RPA that holds the DNA template strands open with their bases exposed (modified from Alberts, 2003).

1.8.3 G_2

G_2 phase follows successful completion of chromosomal replication and is the last stage in interphase prior to cell entry into mitosis. Within this phase the cell undergoes a period of rapid growth and produces new proteins in preparation for mitosis. The G_2 phase can be considered as a safety gap during which the cell can ensure that the correct duplication of its DNA prior to entering mitosis.

1.8.4 M phase

The completion of interphase allows the cell cycle to enter mitosis. M phase permits the equal segregation of the genome into two daughter cells. Mitosis occurs in different stages: prophase, prometaphase, metaphase, anaphase and telophase. As discussed previously, the E2 protein tethers the viral genome to the cell chromatin during mitosis.

1.8.5 Cytokinesis

The cytoplasm of a eukaryotic cell is divided to form two daughter cells. A contractile ring made up from myosin II and actin filaments forms in the middle of the cell at the cell cortex. Using the free energy released from ATP hydrolysis, myosin moves along the actin filaments (reviewed in Pollard & Wu, 2010). This causes the cell to pucker, a process called furrowing. The cleavage furrow begins to move inwards. Ingression continues until the process of abscission physically cleaves the cell into two and the process of cellular division is complete.

1.9 Regulation of the cell cycle

DNA checkpoint mechanisms have evolved that monitor the completion of cell cycle events. If DNA is damaged or DNA replication is blocked a signal transduction pathway is activated to maintain genome integrity. There are three regulatory checkpoints within interphase of the cell cycle: the G_1/S, intra-S and G_2/M (Abraham, 2001; Bartek & Lukas, 2003; Harper & Elledge, 2007). The G_1/S checkpoint determines whether the cell should divide, delay division, or enter a resting stage (G_0). The intra-S checkpoint will stall ongoing replication and initiation of late firing replication origins if DNA damage is detected during S phase. The G_2/M checkpoint ensures that DNA has been correctly duplicated prior to entry into mitosis.

Signal transduction pathways consisting of many factors that are involved in checkpoint regulation (Harper & Elledge, 2007). These proteins include DNA damage sensors, amplifying mediators, transducers and effectors. Their ultimate goal is to arrest the cell cycle, activate transcription of the DNA repair machinery and repair the damaged DNA or activate apoptosis if damage is too severe (Table 1.2). In parallel to cell cycle arrest, checkpoint signalling mediates the recruitment of DNA repair proteins.

There are two major branches of the kinase signalling cascade that are vital to chromosome integrity. The ataxia telangiectasia mutated (ATM) and ATM and Rad3-related (ATR) protein kinases are positioned at the apex of the DNA damage signal transduction pathway (Abraham, 2001; Harper & Elledge, 2007; Kastan & Bartek, 2004; Shiloh, 2003). Both of these proteins belong to a structurally unique family of serine-threonine kinases characterized by a C terminal catalytic motif containing a phosphatidylinositol 3-kinase domain called PIKK proteins (phosphatidylinositol 3-kinase-related kinase). The other member of this family is DNA-dependent protein kinase catalytic subunit (DNA-PK). Although ATM and ATR appear to phosphorylate many of the same cellular substrates, they are generally activated by distinct types of DNA damage.

Table 1.2

DNA Damage Response	Signal Transduction Protein
Sensors	RFC
	RPA
	PCNA
	9-1-1
	Pol alpha
	TopBP1
Amplifying mediator	Rad9
	Claspin
Transducers	
Primary:	ATM
	ATR
Secondary:	Chk2
	Chk1
Effector	P53
	Cdc25
Targets	Cdks/cyclin complex

Table 1.2 Functional role of cellular factors involved in DNA damage signal transduction pathways.

1.9.1 ATM activation

The 370kDa ATM protein is activated in response to double strand DNA breaks. Activation requires the recruitment of the MRN (Mre11, Rad50, Nbs1) mediator complex and phosphorylated H2AX histone (Fernandez-Capetillo et al., 2004; Celeste et al., 2003). Together they act as a signal amplifier that can recruit other factors to the dsDNA lesion. Nbs1 of the MRN complex is responsible for ATM activation. Under normal cellular conditions ATM exists as a multimer. Upon DNA damage, the protein dissociates into

active monomers (Bakkenist et al., 2003). ATM activation is regulated by post translational modifications. ATM has been shown to be phosphorylated at S1981 in response to damage sourced from ionizing radiation (IR). Separate studies have also found that ATM is acetylated at lysine 3016 in response to IR and this is essential for its activation (Sun et al., 2005; Sun et al., 2007). Activated ATM phosphorylates the variant histone H2AX at S139 to synthesise γH2AX (Harper & Elledge, 2007; Fernandez-Capetillo et al., 2004). This phosphorylation facilitates γH2AX interaction with the BRCT motif of the mediator of DNA damage checkpoint 1 protein (MDC1). In return, ATM phosphorylates MDC1, promoting tighter binding of the MRN complex and ATM at the site of the double strand break.

1.9.2 ATR activation

The 317kDa ATR protein is a serine/threonine-specific kinase that is involved in sensing DNA damage and activating the DNA damage checkpoint resulting in a cell cycle arrest. ATR is activated in response to persistent single-stranded DNA. Single strand DNA breaks are commonly formed during DNA damage detection and repair. Should the replication fork stall during DNA synthesis, the MCM helicase continues to unwind the DNA template upstream of the replication fork. This exposes single stranded DNA (ssDNA) coated with RPA. RPA acts as a sensor to DNA damage (Byun et al., 2005). It recruits ATR to stalled replication forks through a direct interaction with ATR Interacting Protein (ATRIP) (Zou & Elledge, 2003). ATRIP regulates the localization of ATR to stretches of RPA coated ssDNA at stalled replication forks. ATRIP is also essential for ATR signalling (Cortez et al., 2001; Zou & Elledge, 2003; Ball & Cortez, 2005).

The precise method for ATR activation however involves not only ATR-ATRIP complex but also a DNA associated complex involving Rad9-Rad1-Hus1 (9-1-1) complex and TopBP1 (Kumangai et al., 2006; Majka et al., 2006; Mordes et al., 2008; Lee & Dunphy 2010). The 9-1-1 complex serves as a heterotrimeric clamp that encircles DNA in a similar manner to PCNA. The complex is loaded onto DNA by a clamp loader consisting of Rad17 and four subunits of RFC. TopBP1 is then recruited in a phosphorylation dependent manner

(Parrilla-Castellar et al., 2004). As a result, the 9-1-1 complex facilitates the interaction between TopBP1 and the ATR-ATRIP complex. TopBP1 belongs to a family of evolutionary conserved proteins containing multiple BRCT domains which interact with phospho-proteins (Manke et al., 2003; Yu et al., 2003). TopBP1 contains a region between the sixth and seventh BRCT domains called the ATR Activation Domain (AAD). When TopBP1 interacts with ATR-ATRIP, it activates ATR kinase activity (Kumagai et al., 2006; Mordes et al., 2008).

The polymerase alpha protein also has a role in sensing stalled replication forks and activation of ATR (Muzi-Falconi et al., 2003; Michael et al., 2000). The DNA structure that the Rad17-RFC clamp recognises is an RPA coated 5'DNA single strand. Polymerase alpha can synthesis primers that produce the 5'DNA substrates and therefore may have a direct role in the recruitment of the 9-1-1 complex to stalled replication forks. Polymerase alpha has also been shown to accumulate on DNA at stalled replication forks to levels in excess over what is observed during normal DNA replication. TopBP1 depletion is shown to inhibit the hyper loading of polymerase alpha at these sites on the chromatin (Yan et al., 2009). Therefore TopBP1 may have a role in recruiting polymerase alpha to stalled replication forks.

ATM, ATR kinases belong to a series of primary transducers that can activate secondary kinases or directly target effector proteins. Two well known secondary signal transducer include Chk1 and Chk2.

1.9.3 Chk2

The checkpoint kinase 2 protein (Chk2) is a common target of ATM and DNA-PK. Chk2 exists as inactive monomers in unperturbed cells. Oligomerization of Chk2 during DNA damage is believed to increase the protein kinase activity (Ahn et al., 2004). In the presence of DNA damage, Chk2 undergoes multiple intermolecular phosphorylations. ATM phosphorylates Chk2 in a serine/threonine cluster at threonine 68 in response to IR treatment. Active Chk2 phosphorylates p53 at Serine20 and blocks p53-MDM2

interaction consequently stabilizing p53. Similarly, BRCA1 phosphorylates p53 efficiently at serine 15 after activation by ATM (Schwarz et al., 2003; Melchionna et al., 2000; Ahn et al., 2000).

1.9.4 Chk1

The Chk1 kinase is one of the best studied ATR substrates. Its activation by ATR-mediated phosphorylation requires the presence of the mediator protein claspin (Kumagai A, Dunphy, 2000; Kumagai et al., 2004; Liu, 2006). Phosphorylation of Chk1 by ATR takes place at serine residues 317 and 345 and results in a marked increase in kinase activity (Zhao & Piwnica-Worms, 2001).

1.9.5 Direct targets of ATR/ATM

The effector kinases are so named due to the effect they produce on the cell. These effects include DNA replication inhibition, recruitment of the DNA repair machinery or apoptosis. Active ATM phosphorylates downstream targets including p53, MDM2, BRCA1, and 53BP1. The p53 protein is stabilized and activated after being phosphorylated on multiple serine residues. Phosphorylation at serine 9 and serine 15 inhibits binding to MDM2. Phosphorylation at serine 20 and 46 has important consequences for apoptotic activity. ATM-mediated phosphorylation of MDM2, a negative regulator of the p53 tumour suppressor, increases MDM2 auto-ubiquitination. ATR has been implicated in the late phosphorylation of p53 at serine 15 after treatment with IR (Shiloh, 2001).

1.10 Cdc25 Phosphatases

The ATR/Chk1 and ATM/Chk2 pathways converge to inactivate members of the Cdc25 family of phosphatases (Donzelli & Draetta, 2003). The ability of cells to divide is mainly attributed to the presence of two classes of molecules, a family of serine/threonine kinases called the cyclin-dependent kinases (Cdks) and their binding partners the cyclins.

Cdks are highly conserved and become activated once associated with their regulatory cyclin subunits (Nurse, 2000). Cdk-cyclin complexes vary and are activated at different stages within the cell cycle (Table 1.3)

Table 1.3

Cdk	Cyclin Partner	Activation Phase
Cdk3	cyclin C	G_0/G1
Cdk4 Cdk2	cyclin D cyclin E	mid-late G1
Cdk2	cyclin A	S phase
Cdk1 Cdk2	cyclin A cyclin B	late G2-mitosis

Table 1.3 Regulatory partners of Cdk kinases (Sherr, 2000; Sage, 2004).

Cdk activity during the cell cycle is tightly controlled in order to ensure the integrity of DNA sequence and coordination of mitotic events. The regulation of Cdks is, in part, controlled by a family of proteins called the cell-division cycle 25 (Cdc25). Cdc25 phosphatases are conserved amongst all eukaryotic cells. In mammalian cells there are three Cdc25 genes (Cdc25A/B/C) (Galaktionov & Beach, 1991; Sadhu et al., 1990; Nagata et al., 1991). In unperturbed cells, Cdc25B activates the Cdk1/cyclin B complex at the centrosome therefore initiating mitosis (Jackman et al., 2003). The G_1/S phase transition is predominantly controlled by Cdc25A. Cdc25A is activated prior to S phase initiation and a result of this is activation of the Cdk2/cyclin E complex (Jinno et al., 1994; Blomberg & Hoffmann, 1999). During S phase, Cdc25A also activates the Cdk2/cyclin A complex driving the synthesis of DNA replication.

In response to damaged DNA, Chk1 and Chk2 delay cell cycle progression by targeting and inhibiting the Cdc25 proteins (Donzelli M, Draetta, 2003). Cdc25A is a critical substrate for activated Chk1 and Chk2. Chk1 phosphorylates Cdc25A at serine 76,124,178,279,293 and threonine 507. Chk2 protein also phosphorylates Cdc25A at serine 124, 178,279 and 293

(reviewed in Kiyokawa & Ray, 2008). Chk1Phosphorylation of Cdc25A at serine 76 serves as an initiation event to promote subsequent Cdc25A phosphorylation at serines 82 and 88. This subsequent phosphorylation facilitates the recognition of βTrCP ubiquitin ligase complex (Busino et al., 2003). Polyubiquitnated Cdc25A is then recruited to the proteosome for degradation thus arresting the cell cycle (Jin et al., 2003).

Cdc25B and C also undergo similar phosphorylation by Chk1 and Chk2. During interphase of the cell cycle, Chk1 negatively regulates Cdc25B and therefore prevents mitotic entry (Kramer et al., 2004; Loffler et al., 2006). Chk2 phosphorylates Cdc25C at serine 216 and assists binding with the 14-3-3 complex. Interaction with the 14-3-3 complex consequently sequesters Cdc25C to the cytoplasm therefore hindering cell cycle progression (Kiyokawa & Ray, 2008).

1.11 DNA repair - double stranded breaks

Double-strand breaks are hazardous to the cell. If left unrepaired they can lead to a loss of heterozygosity, mutations, deletions, genomic rearrangements and chromosome loss. These factors contribute to cancer and genetic disease. Mechanisms exist to repair double-strand breaks (DSBs) including non-homologous end joining (NHEJ), and homologous recombination (HR).

1.11.1 Non homologous end joining

Non homologous end joining is referred to as "non-homologous" because the break ends are directly ligated without the need for a homologous template. In contrast, homologous recombination requires a homologous sequence to guide repair. A DSB within the DNA is recognized by the Ku dimer (Ku70–Ku80). The two DNA ends are brought together by DNA-PKcs. DNA-PKcs/MRN and Artemis are phosphorylated, and the DNA ends are processed by a protein complex consisting of XLF, XRCC4 and DNA ligase IV (reviewed in Lieber et al., 2010). The DNA ends are ligated by DNA ligase IV, and the DNA-repair factors dissociate (Figure 1.12). Major themes of NHEJ include flexibility in handling diverse DNA end configurations by the nuclease, polymerase, and ligase activities and also repetitive processing of each DNA end (Lieber et al., 2010).

HPV DNA integration into that of the host genome is believed to occur through HNEJ. Studies into the pathogenic role of genomic integration of HPV16, HPV18 and HPV31 genomes have analysed integration sites in anogenital precancerous and cancerous lesions. At many integration sites, a short overlap between HPV and genomic sequences is observed. These data suggests that the integration of HPV genomes is mediated by HNEJ (Ziegert et al., 2003).

Figure 1.12

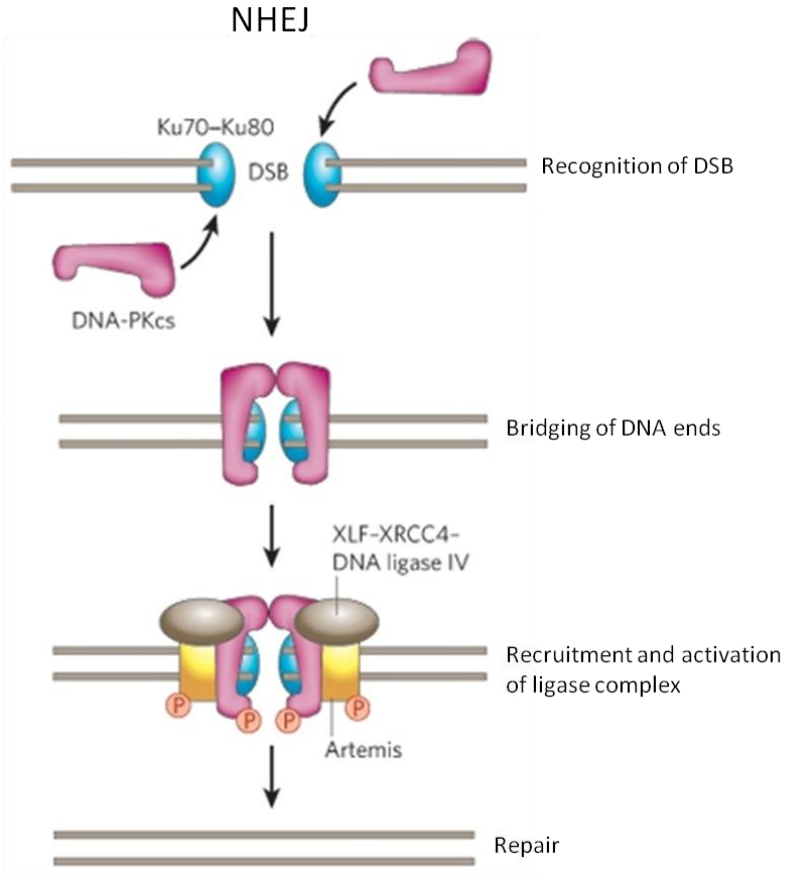


Figure 1.12 DNA damage repair: NHEJ. A DSB is recognized by the Ku dimer (Ku70–Ku80) and DNA-PKcs. The two DNA ends are then bridged together. DNA-PKcs and Artemis become phosphorylated and this results in the formation of the ligase complex (XRCC4-like complex, XRCC4, DNA ligase IV and Artemis). Upon activation, the DNA ends are ligated by DNA ligase IV, and the DNA-repair factors dissociate (modified from Downs et al., 2007).

1.11.2 Homologous recombination

Homologous recombination (HR) repairs DNA breaks without loss of genetic information (reviewed in van den Bosch et al., 2002). Homologous recombination uses a homologous DNA template and is therefore highly accurate (whereas NHEJ rejoins the broken ends without using a template and is often accompanied by loss of some nucleotides).

1.12 DNA repair – single stranded breaks

When only one of the two strands of a double helix encounters a DNA break, the undamaged strand can be used as a template to guide the correction of the damaged strand. A number of excision repair mechanisms that remove the damaged nucleotide and replace it with an undamaged nucleotide complementary to that found in the undamaged DNA strand exist.

1.12.1 Mismatch repair

The main role of mismatch repair (MMR) is to remove base mismatches and small insertion/deletion loops (IDLs) that can sometimes occur during replication. Most knowledge of MMR stems from research carried out in Escherichia coli (*E.coli*) (reviewed in Kolodner, 1996). The main proteins involved in *E.coli* MMR are MutS, MutL and MutH (Modrich, 1991).

1.12.2 Nucleotide excision repair

Nucleotide excision repair (NER) is the most flexible of the DNA repair pathways due to the diversity of DNA lesions it acts upon. These include pyrimidine dimers, bulky chemical adducts, DNA intrastrand crosslinks and forms of oxidative damage. The NER process requires the action of damage recognition, opening of the DNA helicase around the lesion, dual incision of the damaged strand, gap repair synthesis, and strand ligation. There two distinct forms of NER. Global genomic NER (GG-NER) repairs damage at transcriptionaly silent parts of the genome and transcription coupled NER (TC-NER), repairs damage at transcriptionaly active genes (reviewed in Schuck et al., 2008).

1 .12.3 Base excision repair

The base excision repair (BER) is the main cellular defence pathway against endogenous DNA damage. The rate of endogenous DNA damage is estimated to be at least 20,000 lesions per cell per day (Lindahl, 1993; Sung & Demple, 2006). The BER pathway is activated in response to damage including abasic sites, oxidative lesions, alkylated and alternative bases. There are two major BER repair pathways: short and long patch repair (Klungland & Lindahl, 1997; McCullough et al., 1999; Matsumotoet al., 1999).

1.13 Research Aims

DNA from the high risk HPVs 16/18 is frequently found integrated into host cell DNA in high grade squamous intraepithelial lesions (SIL) and squamous cell carcinomas (Cooper et al., 1991; Kristiansen et al., 1994; Alazawi et al., 2002; Peitsaro et al., 2002; Arias-Pulido et al., 2006; Pett et al., 2006). In cervical malignancies, over half of HPV16-positive and nearly all of HPV18-positive malignancies contain integrated HPV genomes (reviewed in Pett & Coleman, 2007). Factors that may promote integration include viral replication during DNA damage. Replication of the HPV episome will create substrates for NHEJ and this may promote HPV integration.

E2 is a multifunctional protein and it is involved in viral replication, transcription regulation and long term maintenance of the viral episome. Factors that promote E2's stability will affect the virus life cycle and may reveal antiviral therapeutic targets. The rate at which HPV DNA is replicated varies as part of the viral life cycle. Vegetative replication, where viral genomes are amplified to a high copy number, is triggered in differentiated epithelial cells. This may be due to an increase in the levels of the viral replication proteins. The levels of BPV1 E2 protein is greatly increased in BPV1 cells that undergo spontaneous amplification of the viral genome suggesting that increased E2 protein levels may account for amplificational replication (Burnett et al., 1990; Penrose and McBride, 2000). The E1 and E2 proteins are known to interact during the viral life cycle however the consequences of this interaction on E2 stability and function remains controversial.

The activation of DNA damage pathways is well described in the literature however the downstream target proteins at the replication fork are not as well defined. There is some evidence to suggest that the MCM2-7 cellular helicase is phosphorylated by the DNA damage response (Cortez et al., 2004; Ishimi et al., 2003a). SV40 DNA replication control mimics that of cellular DNA replication (Miao et al., 2003). The LTAg protein forms a dihexameric complex that is able to replicate the SV40 origin in a bidirectional manner

similar to the MCM2-7 and E1 helicase complexes. Therefore SV40 DNA replication is often used as a model for eukaryotic DNA replication.

Based on this knowledge, the following hypotheses were made:

- A factor that may promote HPV integration is the ability of HPV E1 and E2 mediated DNA replication to go unregulated by the DNA damage response pathways

- The SV40 LTAg viral helicase is targeted by an activated DNA damage response

- The interaction between the HPV E2 and E1 proteins will alter E2 protein stability and function.

This thesis has three main aims:

(i) To assess HPV E1/E2 mediated replication in the presence of DNA damage and an activated cellular DNA damage response.

This was investigated using transient *in vivo* replication assays. E1 and E2 expressed from transfected plasmids were used to study the replication of HPV origin plasmids in the presence and absence of DNA damaging agents.

(ii) To identify the downstream targets of the DNA damage response pathways.

This aim was investigated using the SV40 Large T Antigen viral helicase as a model for eukaryotic DNA replication. The phosphorylation of the viral helicase by ATR/ATM during DNA damage was studied using co-immunoprecipitation techniques. An antibody that detects phosphorylated targets of ATR and ATM was used to identify LTAg pulldown during an activated DNA damage response indicating that this protein is being targeted by the DNA damage response pathways.

(iii) To assess the regulatory and functional consequences of the E1.E2 interaction.

This was examined using a plasmid that expressed wild type HPV16 E2 and a plasmid that expressed an E2 mutant that did not interact with E1. The stability of the E2 protein was measured by calculating protein half life in the presence and absence of an E1 expressing plasmid. The transcriptional function of E2 was also assessed in the presence and absence of an E1 expressing plasmid using an E2 responsive reporter plasmid containing six E2 binding sites. For all experiments the E2 wild type protein was compared to E2 mutant that did not interact with E1.

Chapter 2 – Materials and Methods

2.1 Materials

2.1.1 Antibodies

Abcam plc. (Cambridge, United Kingdom)

Rabbit polyclonal to ATR

Catalogue Number: ab10312

Rabbit monoclonal to Chk1

Catalogue Number: ab40866

Covance Inc. (Princeton, USA)

HA.11 Clone 16B12 Monoclonal Antibody

Catalogue Number: MMS-101R

Dr. Merilyn Hibma (University of Otago, New Zealand)

The TVG 261, Human Papilloma Virus-16 early protein 2 (HPV16-E2) Antibody was a kind gift from Dr. Merilyn Hibma (University of Otago)

Dr. Thomas Melendy (University of Buffalo, USA)

pAb101, SV40 Large T Antigen Monoclonal Antibody was a kind gift from Dr. Thomas Melendy (University of Buffalo). The pAb101 recognizes a C terminal epitope within the last 190 amino acids of LTAg.

pAb419, SV40 Large T Antigen Monoclonal Antibody was a kind gift from Dr. Thomas Melendy (University of Buffalo). The epitope is contained between amino acids 1 and 82 of LTAg.

New England Biolabs Ltd. (Herts, United Kingdom)

Phospho-Chk1/2 Antibody Sampler Kit

Catalogue Number: 9931

Phospho-(Ser/Thr) ATM/ATR Substrate Antibody)

Catalogue Number: 2851

Sigma-Aldrich Company Ltd. (Dorset, United Kingdom)

Anti-Mouse IgG (whole molecule) - peroxidase antibody produced in goat

Catalogue Number: A4416

Anti-Rabbit IgG (whole molecule) peroxidase developed in goat

Catalogue Number: A6154

GTU-88, Monoclonal Anti-γ-Tubulin antibody produced in mouse

Catalogue Number: T6557

TopBP1 Antibody

TopBP1 polyclonal antibody R1180 against amino acids 861-1287 was raised in a rabbit, method of preparation of the antibody and the preimmune have been described previously (Boner et al 2002).

2.1.2 Bacteriology

Bibby Sterlin Ltd (Staffordshire, United Kingdom)

90mm bacteriology petri dishes - Catalogue Number: 502014

Invitrogen Ltd. (Paisley, United Kingdom)

S.O.C. medium - Catalogue Number: 15544-034

Subcloning efficiency DH5α competent cells - Catalogue Number: 18265-017

Sigma-Aldrich Company Ltd. (Dorset, United Kingdom)

Ampicillin - Catalogue Number: 10047

LB-Agar- Catalogue Number: L2897

LB-Broth -Catalogue Number: L3022

2.1.3 Cell lines

C33A cells are derived from a HPV negative cervical carcinoma and are defective for both p53 and Rb function.

Human Embryonic Kidney 293 (293) are a specific cell line originally derived from human embryonic kidney cells grown in tissue culture

Human Embryonic Kidney 293T (HEK293T) derived from the 293 cell line in which the SV40 T-antigen was inserted.

2.1.4 Chemicals

Amersham International PLC (Buckinghamshire, United Kingdom)

Enhanced chemiluminescence (ECLplus) detection substrate - Catalogue Number: PRPN2132

Hyperfilm X-ray film - Catalogue Number: 93184

Applied Biosystems (Warrington, United Kingdom)

Hi-Di™ Formamide - Catalogue Number: 4336697

Bio-Rad Laboratories Ltd. (Hertfordshire, United Kingdom)

Bio-Rad protein assay dye (Bradford) reagent concentrate (5X) – Catalogue Number: 500-0006

Merck Chemicals Ltd. (Nottingham, United Kingdom)

Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG-132) – Catalogue Number: 474791

3,8-Diamino-5-[3-(diethylmethylammonio)propyl]-6-phenylphenanthridiniumdiiodide (Propidium Iodide) – Catalogue Number: JA1654

Roche Biosystems (Basel, Switzerland)

Complete protease inhibitor cocktail tablets - Catalogue Number: 04 693 159 001

Sigma-Aldrich Company Ltd. (Dorset, United Kingdom)

3,8-Diamino-5-[3-(diethylmethylammonio)propyl]-6-phenylphenanthridinium diiodide (Propidium Iodide) – Catalogue Number: 81845

3-Hydroxy-4-(2-sulfo-4-[4-sulfophenylazo] phenylazo)-2, 7-naphthalenedisulfonic acid sodium salt (Ponceau S) - Catalogue Number: P7170

4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) - Catalogue Number: H4034

4'-Demethylepipodophyllotoxin 9-(4,6-O-ethylidene-β-D-glucopyranoside) (Etoposide) – Catalogue Number: E1383

Acetic acid sodium salt (Sodium acetate) - Catalogue Number: S2889

Aphidicolin – Catalogue Number: A0781

Bovine serum albumin protein standard - Catalogue Number: P0834

Caffeine 99% tissue culture grade - Catalogue Number: C8960

Calcium chloride solution, 1M - Catalogue Number: 21115

Camptothecin – Catalogue Number: C9911

Cycloheximide - Catalogue Number: C4859

D-(+)-Glucose (Dextrose) - Catalogue Number: G8270

Magnesium chloride (MgCl2) anhydrous beads - Catalogue Number: 449172

Nonident P-40 (NP40) - Catalogue Number: I8896

Phenol: chloroform: isoamyl alcohol (25:24:1) - Catalogue Number: P3803

Phosphate buffered saline - Catalogue Number: P4417

Potassium chloride - Catalogue Number: P3911

Propan-2-ol (Isopropanol) - Catalogue Number: 24137

Ribonuclease 3'- pyrimidinooligonucleotidohydrolase (Ribonuclease A) – Catalogue Number: R4642

Sodium chloride - Catalogue Number: S7653

Sodium dodecyl sulphate (SDS) AnalaR® - Catalogue Number: L4509

Tris base (2-Amino-2-(hydroxymethyl)-1, 3-propanediol) - Catalogue Number: T6066

Tween 20 (polyoxethylene sorbitan nonolaurate) - Catalogue Number: P1379

University of Glasgow (University of Glasgow Stores)

Crude Ethanol

Crude Methanol

VWR International Ltd. (Leicestershire, UK)

Absolute 99.7-100% AnalaR® ethanol - Catalogue Number: 10107EP

Acetic acid AnalaR® - Catalogue Number: 10001CU

Crystal Violet - Catalogue Number: 340245L

Disodium hydrogen phosphate (Na_2HPO_4) AnalaR® - Catalogue Number: 102494C

Dimethyl sulfoxide (DMSO) - Catalogue Number: 8029122500

Ethylenediaminetetraacetic acid (EDTA) AnalaR® - Catalogue Number: 100938B

Hydrochloric acid - Catalogue Number: 101254H

Methanol AnalaR® - Catalogue Number: 10158FK

Sodium Hydroxide Pellets AnalaR - Catalogue Number: 102527R

2.1.5 Enzymes

New England Biolabs Ltd. (Herts, United Kingdom)

DpnI – Catalogue Number: R0176L

Exonuclease III (*E.coli*) - Catalogue Number: M0206L

2.1.6 Kits

Applied Biosystems (Warrington, United Kingdom)

ABI Prism® BigDye®Terminator v3.1 Cycle Sequencing Kits – Catalogue Number: 4337436

DNA-*free*™ - Catalogue Number: AM1906

RNaseZap - Catalogue Number: AM9780

Invitrogen Ltd. (Paisley, United Kingdom)

NuPAGE® 4-12% Bis-Tris 10 well 1.0 mm gels - Catalogue Number: NP0321

NuPAGE® 4-12% Bis-Tris 12 well 1.0 mm gels - Catalogue Number: NP0322

NuPAGE® Antioxidant - Catalogue Number: NP0005

NuPAGE® *LDS* Sample Buffer – Catalogue Number: NP0007

NuPAGE® MES SDS Running Buffer (20X) - Catalogue Number: NP0002

NuPAGE® Sample Reducing Agent (10X) - Catalogue Number: NP0009

NuPAGE® Transfer Buffer (20X) - Catalogue Number: NP0006-1

Purelink® plasmid maxiprep kit - Catalogue Number: K2100-07

SeeBlue Plus2 prestained standard - Catalogue Number: LC5925

SuperScript® III first-strand synthesis system for RT-PCR - Catalogue Number: 18080-051

Agilent Technologies UK Limited. (Cheshire, United Kingdom)

AffinityScript QPCR cDNA synthesis kit – Catalogue Number: 600559

Brilliant® QPCR master mix – Catalogue Number: 600549

© Merck KGaA (Darmstadt, Germany)

KOD Hot Start DNA polymerase – Catalogue Number: 71086

Promega UK Ltd. (Southampton, United Kingdom)

Luciferase assay substrate – Catalogue Number: E151A

Reporter lysis buffer 5X – Catalogue Number: E397A

Qiagen Ltd. (West Sussex, United Kingdom)

RNeasy mini kit – Catalogue Number: 74104

QIAshredder – Catalogue Number: 79654

Sigma-Aldrich Company Ltd. (Dorset, United Kingdom)

Protein A-Sepharose® 4B, fast flow from *Staphylococcus aureus* – Catalogue Number: P9424

Protein G Sepharose®, fast flow recombinant expressed in *Escherichia coli* – Catalogue Number: P3296

2.1.7 Cell culture

Corning Incorporated (Leicestershire, United Kingdom)

100mm x 20mm non-pyrogenic cell culture dishes - Catalogue Number: 430167

60mm x 15mm non-pyrogenic cell culture dishes - Catalogue Number: 430166

25ml disposable serological polystyrene pipettes - Catalogue Number: 4251

10ml disposable serological polystyrene pipettes - Catalogue Number: 4101

5ml disposable serological polystyrene pipettes - Catalogue Number: 4051

Cell scraper - Catalogue Number: 3010

Invitrogen Ltd. (Paisley, United Kingdom)

0.05% Trypsin-EDTA 1X – Catalogue Number: 25300

DMEM + GlutaMAX™ Dulbeccos Modified Eagle Medium – Catalogue Number: 31966

Fetal calf serum – Catalogue Number: 16101

Lipofectamine™ 2000 Transfection Reagent – Catalogue Number: 11668027

Opti-MEM® reduced serum media – Catalogue Number: 31985

Penicillin/streptomycin solution – Catalogue Number: 15140

Greiner Bio-One Ltd. (Gloucestershire)

15ml polypropylene centrifuge tubes - Catalogue Number: 188261

50ml polypropylene centrifuge tubes - Catalogue Number: 210201

Tissue culture flasks 175cm^2 – Catalogue Number: 660160

Tissue culture flasks 75cm^2 – Catalogue Number: 658170

© Merck KGaA (Darmstadt, Germany)

GeneJuice® Transfection Reagent – Catalogue Number: 70967

2.1.8 Miscellaneous

BD Biosciences (Oxford, United Kingdom)

5ml falcon tube - Catalogue Number: 352052

Elkay International (Basingstoke, United Kingdom)

1.5ml microcentrifuge tubes with attached cap - Catalogue Number: MICR050

0.5ml microcentrifuge tubes with attached cap - Catalogue Number: MICR051

Plastic pasteur pipettes - Catalogue Number: P511

VWR International Ltd. (Leicestershire, United Kingdom)

Cuvet – Catalogue Number: 634-2501

STARLAB (UK), Ltd. (Milton Keynes, United Kingdom)

TipONE 1-20µL Bevelled Filter Tips - Catalogue Number: S1120-1810

TipONE 1-100µL Graduated Filter Tips - Catalogue Number: S1120-8810

TipONE 101-1000µL Extended Length Filter Tips - Catalogue Number: S1122-1834

Thermo Fisher Scientific Inc. (Hertfordshire, United Kingdom)

0.5ml thermo PCR tube - Catalogue Number: AB-0350

Thermo fast 96 well PCR plate - Catalogue Number: AB-0600

Optical 8-cap strip - Catalogue Number: 4323032

Thermo Labsytems Corporation (Warwickshire, United Kingdom)

Luminoskan Acsent Luminometer and Ascent software (version 2.4.2)

Global Medical Instrumentation, Inc.

MJ Research PTC-200 Thermo Cycler

Leica Microsystems (UK) Ltd. (Milton Keynes, UK)

Illumination Leica DM IL LED Microscope

Invitrogen Ltd. (Paisley, United Kingdom)

iBlot® Dry Blotting System

Wm Morrison Supermarkets plc (Anniesland, UK)

Marvel dried skimmed milk

Kimberley Clark (Brighton, United Kingdom)

Safeskin Disposable Purple Nitrile Exam Gloves - Catalogue Number: 014602

2.1.9 Plasmids

pGL3 control contains SV40 promoter and enhancer sequences driving the expression of firefly luciferase (Promega Ltd Southampton, United Kingdom).

pGL3 basic lacks SV40 promoter and enhancer sequences but contains the firefly luciferase (Promega Ltd Southampton, United Kingdom).

ptk6E2luc contains the thymidine kinase promoter from HSV-1 cloned into the pGL3 luciferase vector upstream of 6 HPV16 E2 binding sites (Vance *et al.* 1999).

pCMV-E2 contains the entire HPV16 E2 open reading frame. cloned into *Xba*I-*Sma*I sites of the cytomegalovirus immediate-early promoter/enhancer-based expression vector pCMV4 (Sakai et al, 1995).

pCMV-E39A E39A contains HPV16 E2 ORF that had been mutated at amino acid position 39 by replacing a glutamic acid with alanine. The mutation was synthesised in the pCMV E2 vector.

pCMV-E1 (E1-Rep) was constructed by inserting the HPV16 DNA fragment (nt 865 to 2813), into *Xba*I-*Sma*I sites of pCMV4 (Sakai et al, 1995)

pMH1-16E1HA was constructed by inserting E1 ORFs from HPV -16 (83–2814 nt) into pMH1. The major splice donor site (AGGT) at the beginning of E1 ORFs was disrupted by inserting influenza hemagglutinin epitope tag (HA) in-frame into the E1 coding sequence. The inserted HA tag had no effect on the E1 protein activities (Kadaja et al 2007). This plasmid was a kind gift from Mart Ustav, University of Tartu.

pMH1-11E1HA was constructed as described above. Additional point mutation was introduced into the splicing acceptor site of HPV11 E1 ORF (2622 nt), (ACA → ACC), and this did not change the coding capacity. This plasmid was a kind from Mart Ustav, University of Tartu.

pOri16M is a modified version of pOri16 described (Sakai et al., 1996). The HPV16 Ori (nt 7838–139) was PCR amplified from pOri16 with a primer containing a point mutation at nt 115 (C to T) of the HPV16 genome creating a *Dpn*I site (GATC). The PCR fragment was cloned as an *Eco*RI/*Bam*HI fragment into pSKII (−)

pLT contains the entire SV40 LTAg open reading frame.

pLTAg-S120A contains SV40 LTAg ORF that had been mutated at amino acid position 120 by replacing a serine amino acid with alanine. The mutation was synthesised in the pLT vector.

pLTAg-T517A contains SV40 LTAg ORF that had been mutated at amino acid position 517 by replacing a threonine amino acid with alanine. The mutation was synthesised in the pLT vector.

pLTAg-S639A contains SV40 LTAg ORF that had been mutated at amino acid position 639 by replacing a serine amino acid with alanine. The mutation was synthesised in the pLT vector.

pLTAg-S664,666A contains SV40 LTAg ORF that had been mutated at amino acid positions 664 and 666 by replacing a serine amino acid with alanine. The mutations were synthesised in the pLT vector.

pLTAg-S676A contains SV40 LTAg ORF that had been mutated at amino acid position 676 by replacing a serine amino acid with alanine. The mutation was synthesised in the pLT vector.

pcDNA 3.1 contains CMV promoter and was used as a carrier plasmid, Invitrogen Ltd. (Paisley, United Kingdom)

pCMV E2 (b) contains HPV16 E2 ORF and was a kind gift from Peter Howley.

pCMV R37A contains the HPV16 E2 ORF with mutation at amino acid position 37 by replacing an arginine for an alanine. This plasmid was a kind gift from Peter Howley.

pTM1-E1 contains the full length HPV11 E1 ORF inserted into pTM1. This plasmid was a kind gift from Jacques Archambault.

pCR3-E2 contains the full length HPV11 E2 ORF inserted into pCR. This plasmid was a kind gift from Jacques Archambault.

2.1.10 Oligonucleotides

2.1.10.1 Real-Time PCR analysis

Eurogentec Ltd. (Southampton, United Kingdom)

Table 2.1 Oligonucleotide for RT-PCR analysis

Oligonucleotide	Sequence Written 5' to 3'
Beta actin probe	ATTTCCCGCTCGGCCGTGGT
Beta actin FWD	AGCGCGGCTACAGCTTCA
Beta actin REV	CGTAGCACAGCTTCTCCTTAATGTC
HPV16 E1 probe	ATGATAATGATTATTTAACACAGGCAGAAACAGAGACAGC
HPV16 E1 FWD	CGAGAACGAAAATGAACAGTGATACA
HPV16 E1 REV	TTTTGGTTCCTGTGCAGTAAACA
HPV16 E2 probe	CAACCACCCCGCCGCGA
HPV16 E2 FWD	CCTGAAATTATTAGGCAGCACTTG
HPV16 E2 REV	GCGACGGCTTTGGTATGG
HPV ori probe	ACCAAAAGAGAACTGCAATGTTTCAGGATCC
HPV ori FWD	ATCGGTTGAACCGAAACCG
HPV ori REV	TAACTTCTGGGTCGCTCCTG
Large T Antigen probe	AAGCAACTCCAGCCATCCATTCTTCTATGTC
Large T Antigen FWD	TTTGGGCAACAAACAGTGTAGC
Large T Antigen REV	AATGTTTGGTTCTACAGGCTCTGC
Luciferase probe	CACTGATCATGAACTCCTCTGGATCTACTGGTC
Luciferase FWD	TCCTTCGATAGGGACAAGACAATT
Luciferase REV	GGCAGAGCGACACCTTTAGG

2.1.10.2 Site directed mutagenesis

VH Bio Ltd. (Gateshead, United Kingdom)

Table 2.2 Oligonucleotides for site directed mutagenesis

Oligonucleotide	Sequence 5' to 3'
LTAg: S120A	
FWD	GATGAGGCTACTGCTGACGCTCAACATTCTACTCCTCCAAAAAAG
REV	CTTTTTTGGAGGAGTAGAATGTTGAGCGTCAGCAGTAGCCTCATC
LTAg: T517A	
FWD	AAACACCTAAATAAAAGAGCTCAAATATTTCCCCCTGGAATAG
REV	CTATTCCAGGGGGAAATATTTGAGCTCTTTTATTTAGGTGTTT
LTAg: S639A	
FWD	GATGATGATGATGAAGACGCCCAGGAAAATGCTGATAAAAATGAAG
REV	CTTCATTTTTATCAGCATTTTCCTGGGCGTCTTCATCATCATCATC
LTAg: S664,666A	
FWD	GAAACAGGCATTGATGCACAGGCCCAAGGCTCATTTCAGGCC
REV	GGCCTGAAATGAGCCTTGGGCCTGTGCATCAATGCCTGTTTC
LTAg: S676A	
FWD	TTTCAGGCCCCTCAGTCCGCACAGTCTGTTCATGATCATAATC
REV	GATTATGATCATGAACAGACTGTGCGGACTGAGGGGCCTGAAA
E2: E39A	
FWD	TATTGGAAACACATGCGCCTAGCATGTGCTATTTATTACAAGGCC
REV	GGCCTTGTAATAAATAGCACATGCTAGGCGCATGTGTTTCCAATA

2.1.10.3 siRNA knockdown

Dharmacon Inc. (Leicestershire, United Kingdom)

Table 2.3 Oligonucleotides for siRNA knockdown

Oligonucleotide	Target Sequence
siATR	CCCGCGUUGGCGUGGUUGAdTdT
siLuciferase	CGUACGCGGAAUACUUCGAdTdT
siTopBP1	GUGGUUGUAACAGCGCAUCdTdT

2.1.10.4 Sequencing

VH Bio Ltd. (Gateshead, United Kingdom)

Table 2.4 Oligonucleotides for DNA sequencing

Oligonucleotide	Sequence 5' to 3'
LTAg S120A	GGAACTGATGAATGGGAG
LTAg T517A	GATTTGCCTTCAGGTCAG
LTAg S639A	GAGAGATTGGACAAAGAG
LTAg S664,666A	GAGAGATTGGACAAAGAG
LTAg S676A	GAGAGATTGGACAAAGAG
E2 E39A	CGTGGATCCGAGACTCTTTGC

2.2 Methods

The protocols used in this thesis are described in the following section. The manufacturer and distributor details for the materials used are given in the previous section.

2.2.1 Cell culture methodology

2.2.1.1 General growth

All cell culture experiments were carried out in a tissue culture laboratory and under strict aseptic conditions. All *cell culture* procedures were carried out in a Class II laminar flow hood (*BioMAT 2*, *MAT*, Derby, U.K.). The 293T, 293 and C33A cell lines were grown in DMEM+GlutaMAX with 10% (v/v) foetal calf serum and 1% (v/v) penicillin/streptomycin mixture (Invitrogen) at 37 °C in a 5% CO2/95% air atmosphere (*LEEC* GA2000 Cell Culture *Research* Incubator, LEEC Ltd.). Cell lines were passaged at least twice a week. The cells were harvested by trypsonisation

2.2.1.2 Calcium phosphate transient transfection

Cells were transiently transfected using the calcium phosphate precipitation technique. Cells were counted and were plated out onto sterile plates with 5mls of media for 60mm^2 plates or 10ml of media for 100mm^2 plates and left over night in the incubator to adhere. The following day, the plasmid DNA of interest was mixed with distilled water to a final volume of 375µl. 125µl of 1M $CaCl_2$ was then added to the mix. This 500µl solution was then added drop wise to 2X HEPES buffered saline (50mM HEPES, 280mM NaCl, and 1.5mM $Na_2HPO_4.2H_2O$ to pH 7.0). The solution was left at room temperature until the formation of an opaque precipitate could be seen. The precipitate was then added to each monolayer with gentle rocking of the medium and left to incubate for approximately

16 hours. The cell monolayer was then washed twice with PBS and fresh medium was added before an additional 24 hour incubation.

2.2.1.3 GeneJuice transient transfection

293 cells were transfected with pLTAg mutated using the GeneJuice® method of transfection (Novagene). Briefly $1x10^6$ 293 cells were plated out in normal growth media onto 100mm dishes (so as to achieve around 80% confluence at harvest) and incubated at 37°C (5% CO_2) overnight. For each dish to be transfected, 800µl of Opti-MEM® was placed into a sterile tube. GeneJuice® was then added drop wise to the serum free media and incubated at room temperature for 5 minutes. The volume of GeneJuice® used was 2µl per µg of plasmid DNA being transfected. (i.e. 2µg of $pLTAg^{mutated}$ with 4µl of GeneJuice®). After incubating, the plasmid DNA was added to the mix and gently mixed by pippeting. The GeneJuice/DNA mixture was incubated at room temperature for 15 minutes. The entire volume of GeneJuice/DNA mixture was added dropwise to the dish whilst gently rocking motion to ensure equal distribution. Cells were incubated for 24-48 hours and harvested for protein extraction.

2.2.1.4 Lipofectamine 2000™ transfection

For siRNA knockdown experiments, siRNA oligonucleotides against ATR, TopBP1 and luciferase were designed (Dharmacon). Two transfections solutions were prepared; firstly 2.5µl of a 75µM stock of the desired oligonucleotide was added to 497.5µl Opti-MEM® (Invitrogen), and secondly 15µl Lipofectamine 2000™ was added to 485µl Opti-MEM®. These two separate cocktails were incubated at room temperature for 5 minutes before being mixed and incubated for a further 20 minutes at room temperature. Following the incubation 0.5 x 10^6 293T cells were added to the Lipofectamine/oligo solution and plated out onto a 60mm tissue culture plate incubated at 37°C (5% CO_2) (v/v). The

following day, media was replaced with fresh media and cells were harvested 24 hours later.

2.2.1.5 Cell count assay

$2x10^6$ 293T cells were plated onto 100mm plates. The following day the cells were transfected with oligonucleotides for siATR/siTopBP1/siLuciferase using the Lipofectamine 2000 protocol (2.2.1.4). Forty eight hours post transfection, cell were harvested as normal with trypsin, washed and counted. $2x10^5$ cells were plated onto 60mm plates (in triplicate). The remaining unused cells were checked for successful knockdown using the western blot technique. Every 2-3 days, the cells were harvested and recounted and $2x10^5$ cells were replated onto 60mm plates (in triplicate). A running cell total is calculated for data analysis.

2.2.1.6 Colony survival assay

3×10^5 293T cells per 100mm culture dish were plated out in 7ml DMEM (containing 10% foetal calf serum and penicillin), and siRNA treated with siATR/TopBP1/Luciferase as described (2.2.1.4). 48 hours later the cells were trypsinised, counted and 2000 cells per 100mm culture dish were plated out in 7ml DMEM (containing 10% foetal calf serum and penicillin). 12 days later the medium was removed and the cells washed twice with 5ml PBS. The surviving colonies were visualised by staining with 2mls 0.5% crystal violet solution (25% methanol, ddH_2O) for 20 minutes at room temperature with gentle shaking. The plates were washed with tap water until the solution ran clear, dried overnight at room temperature before the colonies were counted.

2.2.1.7 Luciferase assay

$2.5x10^5$ cells were plated onto $60mm^2$ plates and transfected using the calcium phosphate precipitation protocol as described (2.2.1.2). The total volume of the calcium phosphate ($CaPO_4$) mixture was 1ml but it contained plasmid DNA for two reactions. At transfection therefore, 500µl of $CaPO_4$ and DNA mixture was transfected onto each plate. Twenty four hours later the plates were removed from incubation and the medium removed. The tissue culture dishes were gently washed twice with 4ml of PBS. At the final wash all excess PBS was removed by pipetting. 300µl of 1x Reporter Lysis Buffer (Promega) was added to each plate and left to incubate for 15 minutes at room temperature. The cell lysate was scraped using a sterile plate scraper into a clean microcentrifuge tube. The samples were centrifuged at 14000g for 10 minutes at 4˚C. To measure luciferase activity, 80µl of each sample was added into a 96 well plate. The luminescence was measured using the Luciferase Assay System (Promega) at a 1 in 3 dilution and a Luminoskan Ascent plate reader (Thermo Labsystems).

2.2.1.8 Drug treatments

Prior to drug treatment the media was removed from the plates and any excess was removed by a pipette. A master mix of drug and medium was made up separately so that each plate received the same concentration of drug. The following concentration of each drug was used, unless otherwise stated; etoposide (50µM), aphidicolin (2.5µg/ml) MG132 (30µM), cycloheximide (100µM) hydroxyurea (3mM) and camptothecin (10µM). Treatment times are indicated in the results section.

2.2.1.9 Preparation of protein extract

Cells were grown and transfected so as to achieve around 80% confluence at harvest. The medium was removed and the cells were washed once with PBS. 1ml trypsin was added

to each plate and incubated at 37°C until the cells detached from the plate. Medium was then added to the mix (at least 3mls). The cells were then transferred to a 15ml centrifuge tube and centrifuged at 1000g for 5 minutes. The resultant cell pellet was washed in 5ml PBS and centrifuged for a second time. After the PBS was removed by pippeting, the cell pellet was lysed in NP40 lysis buffer (0.5% NP40, 150mM NaCl, 50mM Tris pH 8.0 (containing 1 in 10 volume protease inhibitor cocktail tablet), and incubated on ice for 30 minutes. The lysate was then centrifuged at 14000g for 30 minutes at 4°C. The protein extract was stored at -80°C until required.

2.2.2 Molecular methods

2.2.2.1 DNA purification using phenol chloroform

DNA samples were purified using phenol:chloroform to remove all residual contaminants. Sample volumes were typically completed to 500µl prior to extraction. An equal volume of phenol: chloroform: isoamyl alcohol (24:25:1 v/v/v) was added to each sample. After a thorough vortex the samples were centrifuged at 14000g for 10 minutes at room temperature. The top aqueous layer was gently removed and put into fresh microcentrifuge tube. The procedure was repeated for a second time.

2.2.2.2 Ethanol precipitation

Following purification of DNA samples using phenol:chloroform (2.2.2.1) DNA was precipitated using ethanol precipitation. 1/10th of the sample volume of 3M sodium acetate, pH 5.2 and 2x the sample volume 100% ethanol, was added to the sample. The samples were vortexed and left to incubate at –20˚C for 1-24 hours. The samples were centrifuged at 14000g at 4˚C for 30 minutes. The ethanol was removed gently. 1x sample volume of 70% ethanol was used to wash the resulting DNA pellet. The samples were centrifuged at 14,000g for 15 minutes at 4˚C. The ethanol was carefully removed with a pipette. The pellet was dried either by the use of a heating block or at room temperature until no ethanol remained. The precipitated DNA was then resuspended in distilled H_20.

2.2.2.3 Oligonucleotide synthesis

Oligonucleotides used as primers in RT PCR reactions were synthesised by Eurogentec Inc. Each oligonucleotide probe was dual labelled (5'FAM, 3'TAMRA) and purified using

Reversed Phase High-performance liquid chromatography (HPLC-RP). The primers were purified using SePOP (Selective Precipitation Optimized Process). SePOP desalting is based on a differential precipitation process. It increases the purity level of the deprotected and desalted Oligonucleotides from 15 to 80 bases. All purification was done by Eurogentec. The oligonucleotides arrived lyophilised and were re-suspended in ddH_2O to a concentration of 100µM and stored at -20°C until required.

Oligonucleotides used as primers for site directed mutagenesis and sequencing protocols were synthesised by VH Bio Ltd. The primers were purified by RP-Column at VH Bio and arrived lyophilised. The oligos were resuspended in water so as to achieve a stock concentration of 100µM and stored at -20°C until required.

RNA oligonucleotides used during siRNA experiments were synthesised by Dharmacon. The siRNA duplex is provided in the 2'-deprotected and desalted form. Upon arrival, the lyophilised form was resuspended in siRNA Buffer (20mM KCl, 6mM HEPES-KOH pH 7.5, 0.2mM $MgCl_2$) to make a stock of 75µM and stored at -20°C until required.

2.2.2.4 Real-Time PCR detection for transient DNA replication assay

A description of each probe and primer set is described in Table 2.1. For HPV E1 and E2 mediated replication, the primer set chosen amplifies a 99bp region of the HPV16 origin which has been cloned into pOriM. For SV40 Large T antigen mediated replication, the primer set chosen amplifies the luciferase gene found in pGL3 plasmids. To differentiate between replicated and input plasmid DNA, 25µl of each sample was digested with Dpn1 overnight followed by 1 hour digest with Exonuclease III (ExoIII). ExoIII was heat inactivated by heating each sample for 30 minutes at 70°C.

5µl of each sample was analysed in triplicate using real time PCR. Quantification was performed using a standard curve from pOri16M (HPV replication) or pGL3-Control/Basic (SV40 replication) dilutions of 100pg to 10^{-5}pg. Standard curves were prepared in

duplicate. A non DNA control (ddH2O) was prepared in quadruplicate. Five microlitres of each sample was prepared in a solution containing 12.5µl of Mastermix (Stratagene), 900nM of each primer, 100nM of probe, 0.5µl of reference dye (supplied with Mastermix) and 6µl of ddH2O so that the final volume was 25µl. Triplicates were prepared in a single tube to eliminate a source of variability. 25µl was pipetted into each well in a 96-well real-time PCR plate. To measure the quantity of pOri16M or pGL3-Control, DNA was detected by assaying the real-time PCR plate on an ABI Prism 7500 using the universal real-time PCR conditions (95°C 10 minutes 'hot-start', 95°C for 30 seconds and 60°C for 1 minute in 40 cycles). Data was analysed using the 7500 System Software. This calculates the standard curve and the quantity of DNA for each individual sample well. The average signal for each sample was calculated from the sample repeats. The undigested pOri16M/pGL3-C represents the freshly replicated molecules.

2.2.2.5 *In vitro* DNA replication assay

Viral *in vitro* DNA replication assays were performed by John Fisk at the University of Buffalo. Thirty nanograms of template DNA containing the SV40 or HPV origins of replication (pSV011 or p7974-99) was incubated with 200ng of SV40 TAg or 40ng of HPV11 E1 and 20 ng of HPV11 E2, replication buffer (30mM HEPES (pH 7.5), 40mM creatine phosphate, 7mM MgCl2, 0.5mM DTT, 4mM ATP, 200µM CTP, 200µM UTP, 200µM GTP, 100µM dCTP, 100µM dGTP, 100µM dTTP, 25µM dATP, 0.1mg/ml acetylated BSA, 0.625µg creatine phosphokinase (Sigma), 1µCi α^{32}P dATP, and 30–50ng of HEK 293 cell extract in 10µl reactions. Reactions were carried out for one hour at 37 °C and were terminated by addition of stop buffer (20mM Tris (pH 7.5), 10mM EDTA (pH 8.0), 0.1%SDS, 1 µg/µl proteinase K) for twenty minutes at 37 °C. Replication was evaluated by resolution of replication products on 0.8% agarose gels followed by phosphorimager autoradiography.

2.2.2.6 *In vitro* checkpoint kinase assay

In vitro checkpoint kinase assays were carried out by John Fisk at the University of Buffalo. To determine the effect of the kinase inhibitors wortmannin, NU7026, and KU55933, either dimethylsulfoxide (DMSO) or the inhibitor dissolved in DMSO was first incubated with cellular extracts for 5 minutes on ice prior to assembly of the replication reaction. Non-template plasmid added to the reactions (pUC19) was isolated using standard protocols, and then either added directly or first digested with the restriction enzyme RsaI to completion, and then phenol:chloroform extracted and precipitated prior to addition. Several restrictions enzymes, with varying termini (3′ overhand, 5′ overhang, and blunt ended) were also used and all types of ends produced very similar results (data not shown). Quantification of DNA replication activity was by phosphorimager analysis; incorporation of isotope from the top of the replication intermediates all the way through Form I DNA was quantified, background from an unused lane was subtracted, and the results were compared to the DMSO only positive control lane (set to 100%).

2.2.2.7 Bradford assay

Prior to western blotting and immunoprecipitation, protein extracts were analysed for protein concentration so as to ensure that equal amounts of protein were used in each experiment. Bovine serum albumin (BSA) protein standard solutions were made up at the following concentrations from a stock of 2mg/ml (Invitrogen); 2, 1, 0.4, 0.2, 0.1mg/ml. 5µl of each protein extract was added to 995µl of Bio-Rad Protein Assay Dye (Bradford) Reagent Concentrate (5X) in a cuvet. The Bradford reagent was diluted to a concentration of 1x with distilled water. Samples were shaken to mix and absorbance was measured on a spectrophotometer (O.D 595). The absorbance readings for the BSA standards were used to derive a standard curve, from which the sample concentrations were calculated.

2.2.2.8 Immunoprecipitation

In preparation for immunoprecipitation (IP) experiments, protein A/G sepharose beads were washed 5 times in lysis buffer (0.5% NP40, 150mM NaCl, 50mM Tris pH 8.0)and were resuspended after the final wash at a bead:slurry ratio 1:1 (v/v).The concentration of protein lysate used for coimmunprecipitation was 300µg.

For IPs involving the phospho - (Ser/Thr) ATM/ATR substrate antibody, protein lysate and antibody (1/20) were incubated in a 1.5ml eppendorf tube on a spinning rotor overnight at 4 °C. The following morning 20µl of protein G sepharose beads were added to the lysate and incubated for 1 h at 4 °C on the rotor.

For IPs involving the HA antibody, protein lysate and antibody (1/200) were incubated in a 1.5ml eppendorf tube on a spinning rotor overnight at 4 °C. The following day 20µl of protein A sepharose beads was added to each sample in a post-clearing step. The incubation was for 1-2 hours on a spinning rotor at 4°C. The eppendorf tube was centrifuged briefly at 3000g and the lysate was transferred to a fresh eppendorf tube. 20µl of protein A sepharose was added to the samples for a second overnight incubation.

For IPs involving the LTAg antibody; pAb419, protein lysate and antibody (1/100) were incubated in a 1.5ml eppendorf tube whilst spinning at 4°C for 1-2hours. Protein A sepharose beads (20µl) were added to the mixture for 1 hour under the same conditions. The complexes containing the antibody-protein A/G sepharose-cell lysate were pelleted by centrifugation and washed five times with 500µl of lysis buffer. The beads were boiled in 15µl of LDS buffer (Invitrogen) and 5µl of sample reducing agent (Invitrogen) for 10minutes at 70°C. Protein inputs (10%) were mixed with 5 µl of LDS buffer and 2 µl of sample reducing agent and boiled under the same conditions. Both the beads and supernatant were electrophoresed using SDS-PAGE system and subjected to western blot.

2.2.2.9 Western blotting

Protein concentrations were determined using a Bradford assay as described in chapter 2.2.2.7. Equal amounts of protein (20-30ng) were added to 2µl NuPage® sample reducing agent (10X) and 5µl NuPAGE sample buffer (4x) (Invitrogen) and heated to 70°C for 10 minutes. The pre-cast gels (either 10/12 well) were placed into the gel tank filled with 1x MES running buffer. 500µl 10X antioxidant was added to the running buffer before loading the sample. 10µl See-Blue® kDa marker (Invitrogen) was also loaded as marker using fine pipette tips. SDS-Page gels were electrophoresed for 1 hour at 200V. The buffer was discarded and the gel cast was carefully cracked open and the top plate peeled off to leave the gel in place. The gel was soaked in transfer buffer NuPAGE® (1X) for 10 minutes prior to transfer. The wells were removed and the gel was transferred to a nitrocellulose membrane using the dry Invitrogen i-blot transfer system following the manufacturer's instructions. Following transfer the nitrocellulose membrane was incubated with 5% milk PBS-T (1 x PBS 0.1% Tween) on a mechanical shaker for either 1 hour at room temperature or overnight at 4°C. Following blocking, the membrane was incubated with the primary antibody in 5% milk PBS-T using the following concentrations: ATR (1:1000), Chk1 (1:1000), HA.11 (1:2000), TVG 261 (1:250), pAb101 (1:1000), pAb419 (1:1000), phospho-Chk1/2 antibodies (1:1000). For 1-2 hour incubations the membranes were gently shaken at room temperature. For overnight incubations, the membranes were gently shaken in a 4°C cold room. The antibody was then removed and the membrane was washed in PBS-T for 3 x 5 minutes at room temperature with gentle agitation. The secondary antibody was then added diluted in 5% milk PBS-T and incubated at room temperature for 1 hour with gentle agitation. The secondary antibodies were used at the following concentrations: anti-rabbit IgG (1:10000), anti-mouse IgG (1:10000) and GTU-88 (1:10000). The antibody was then removed and the wash steps repeated. The proteins were detected using ECLplus (GE Healthcare) according to the manufacturer's instructions. Briefly 1ml detection reagent A and 25µl detection reagent B was mixed and added to the membrane and incubated at room temperature for 5 minutes. The excess was removed and the membrane placed into a cassette and developed with X-ray Hyperfilm (Amersham).

2.2.2.10 Stripping of nitrocellulose membrane

Membranes were washed in PBS-T for 3x 5 minutes whilst gently shaking therefore removing any excess ECL reagent. Membranes were incubated on the shaker in 0.1M NaOH for 10-15 minutes. The NaOH was removed and membrane washing steps were performed (3 x 5 minutes in PBS-T whilst gently shaking). The membranes were then blocked with 5% milk PBS-T for 1 hour at room temperature in preparation for incubation with the primary antibody.

2.2.2.11 Site directed mutagenesis

The site directed point mutagenesis was carried out using KOD Hot Start Polymerase kit according to the manufacturer's instructions. The primers used to generate point mutations in SV40 large T antigen and HPV16 E2 are described in Table 2.2. Primers were designed to be around 25-45 nucleotides long and have a melting temperature (Tm) value >65°C. The Tm of each primer was calculated using the formula: Tm (°C) = $2(N_A + N_T) + 4(N_G + N_C)$. For each mutation generated 1ul pLTAg/E2 (100ng/µl) was mixed with 5ul 10x KOD buffer, 15µl of 2mM dNTPs, 4µl of 25mM MgCl2, 1µl DMSO, 1µl of 10µM Primer 1, 1µl of 10µM Primer 2, 1µl KOD polymerase and 31µl ddH2O. PCR amplification was carried out using an MJ Research PTC-200 Thermo Cycler. Samples were heated to 94°C for 5 minutes (polymerase activation), 94°C for 15 seconds (denature), lowest primer Tm for 30 seconds (annealing) and 72°C for 20 seconds/kb (extension). The cycle of denaturing, annealing and extension was repeated another 17 times. To finish, the samples remained at 72°C for 5 minutes then were cooled to 4°C. Non mutated template DNA was then digested using 1µl of DpnI for 1 hour at 37°C. The mutated plasmids were cleaned up using phenol chloroform extraction and ethanol precipitation techniques. The mutated plasmid DNA was transformed into DH5α cells and grown up for DNA miniprep. The DNA was then sequenced to check for mutations.

2.2.2.12 DNA sequencing

To check plasmids for mutation they were sequenced using an Applied Biosystems 3100 automated sequencer. The region of interest was sequenced using a primer complementary to the appropriate region (Table 2.4). Reactions were carried out in 0.5ml PCR tubes and contained around 250ng/µl of plasmid DNA, 2µl of 5x Big Dye buffer, 3.2pmol primer, 4µl of Big Dye Terminator Reaction mix made up to 20µl with ddH2O. Samples were heated to 96˚C for 10 seconds, 50˚C for 5 seconds, and 60˚C for 4 minutes. This cycle was repeated 25 times and the sequencing reaction held at 4˚C till required. PCR reactions were purified using phenol:chloroform ethanol precipitation (as described in 2.2.2.1). Following purification the reactions were resuspended in 25µl Hi-Di formamide and analysed using an ABI Prism 300 automatic sequencer.

2.2.2.13 DNA/RNA concentration determination

The concentration of plasmid DNA was measured using an absorbance measurement at 260nm (Biotech spectrophotometer -model UV1101). An optical density reading (OD) of 1 is equivalent to 50µg/ml of double stranded DNA. RNA concentration was determined similarly and OD of 1 is equivalent to 40µg/ml RNA. A 260nm/280nm absorbance ratio was measured for all plasmid preparations, a reading of between 1.8 and 2.1 being indicative of a pure sample.

2.2.2.14 Transformation of chemically competent bacterial cells

Transformation of DH5α competent bacterial cells took placed prior to large scale preparation of DNA. 10ng of plasmid DNA was added to 50µl DH5α cells and incubated on ice for 30 minutes. Cells were immediately heat shocked at 42˚C for 45 seconds and placed back on ice for 2 minutes. Each sample was gently mixed with 100µl SOC medium

(25ml bactotryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$ and 20mM glucose). The samples were incubated at 37˚C whilst shaking (225rpm) for 1 hour. The samples were aseptically plated out on to Ampicillin containing agar plates. The plates were then left inverted and incubated overnight at 37˚C. Colonies were picked the following day and grown in 5ml L-broth, 50mg/ml Ampicillin, overnight at 37˚C with shaking (225rpm) prior to both small and large scale preparations of plasmid DNA.

2.2.2.15 RNA extraction

RNA was extracted from cells using the Qiagen RNeasy kit. Cells were washed twice with PBS and trypsinised and pelleted into a 1.5ml Eppendorf tube. Pellets were lysed in 750µl of RTL buffer. The lysate was transferred into a QIAshredder spin column then centrifuged to homogenise the sample. An equal volume of 70% ethanol was added to the lysate and the sample was transferred to an RNeasy mini column by centrifugation. The flow through was discarded and the column was washed with 700µl RW1 once, and twice with 500 µl of RPE buffer by centrifugation. Purified RNA was eluted from the column by 100µl of RNase-free H_2O. RNA concentration was measured as described (2.2.2.12).

2.2.2.16 DNase treatment

Following RNA extraction, 0.1 volume 10X DNase I buffer and 1µL rDNase I (Applied Biosystems) were added to the RNA, and mixed gently. The sample was incubated at 37°C for 30 minutes. DNase Inactivation Reagent (typically 0.1 volume) was added to each reaction and mixed well. The reaction was incubated for 2 minutes at room temperature mixing occasionally. The samples were then centrifuged at 10000g for 1.5 minutes and the RNA was transferred to a fresh tube for cDNA synthesis.

2.2.2.17 Synthesis of complementary DNA

RNA was converted to cDNA using the SuperScript III Reverse Transcriptase kit from Invitrogen. In sterile 0.2µl PCR tubes, 1-500ng of RNA, 50–250ng of random primers, 1µl of 10mM dNTP Mix (10mM each dATP, dGTP, dCTP and dTTP at neutral pH) and sterile ddH_2O (to 13 µl) were mixed, incubated at 65°C for 5 minutes and then were placed on ice for 1 minute. Prior to PCR, 4 µl of First Strand Buffer (5X), 1 µl of 0.1M DTT and 1 µl of the SuperScript III enzyme were added to the reaction. First strand cDNA synthesis was carried out using a MJ Research PTC-200 gradient cycler. Samples were incubated at 25°C for 5 minutes, heated to 50°C for 1 hour and terminated at 70°C for 5 minutes. The samples were stored at -20°C until required.

2.2.2.18 Large scale preparation of plasmid DNA (maxiprep)

1ml of a 5ml overnight bacterial culture was used to inoculate 500ml of L-broth containing antibiotic. The culture was incubated overnight in a 2L conical flask with vigorous shaking. The following day, the culture was poured into 50ml tubes and cells were pelleted by centrifugation at 3500g for 10 minutes at 4°C. The supernatant was discarded. The bacterial pellet was resuspended in 10ml resuspension buffer (50mM Tris-Cl pH 8.0, 10mM EDTA, 100µg/ml RNase A) by vortexing and pipetting. 10ml of lysis buffer (200mM NaOH, 1% SDS) was added by gentle inversion 5 times. The lysed pellets were incubated for 5 minutes at room temperature. 10ml of precipitation buffer (3.0M potassium acetate pH 5.0) was then added and mixed gently to homogenize the sample. The precipitated sample was centrifuged at 3500g for 10 minutes at 4°C. To prepare the extraction columns for the sample, 15ml of equilibration buffer (750mM NaCl, 50mM MOPS, pH 7.0, 15% isopropanol (v/v) and 0.15% Triton r X-100 (v/v)) was added directly into the top. The buffer was allowed to drain through.

Following centrifugation the supernatant was filtered to remove the precipitate and the clear lysate was added to the extraction column. The lysate was left to drain by gravity

flow. 60ml of wash buffer (1.0M NaCl, 50mM MOPS pH 7.0, 15% isopropanol) was added and left to drain by gravity flow. The DNA was eluted by adding 15ml elution buffer (1.25M NaCl, 50mM Tris-Cl (pH 8.5) and 15% isopropanol), and collected into 50ml centrifuge tubes. 10.5ml of isopropanol was then added to the eluted DNA. The sample was centrifuged at 3500g at 4˚C for 1 hour. The resulting supernatant was removed and the DNA pellet was washed in 10ml 70% ethanol before centrifugation at 3500g for 15 minutes at 4˚C. The pellets were allowed to air dry before being resuspended in 300µl of TE Buffer, and stored at –20˚C until required.

2.2.2.19 Propidium iodide staining and flow cytometry analysis

Cells were plated out in duplicate onto 100mm plates so as to achieve a confluence of 80% at harvest. Forty eight hours post transfection, cells were harvested, and washed twice with ice cold PBS (centrifuged at 1000 rpm, for 10 minutes at 4°C between each wash). After the final wash, the pellet was resuspended in 0.5ml of residual PBS. Samples were kept cool on ice. On a vortex at a low speed, 4.5ml of ice cold 70% ethanol was added dropwise to each sample. If the samples were being used straight away, they were left on ice for 30 minutes. If samples were being stored to use on a separate day, they were stored at -20°C. Each sample was washed twice in ice cold PBS following the same conditions prior to fixing. Each pellet was resuspended in 0.5ml of fresh PBS, 10µg/ml of Propidium Iodide and 1µl of ribonuclease A. The staining was carried out for at least 3h at 4 °C. The samples were analysed using the EXPO32ADCXL4 Colour program on a Beckman Coulter Epics XL-MCL machine.

2.2.2.20 Salt extraction

Cells were lysed in CSK buffer (10nM PIPES pH6.8, 100mM NaCl, 300mM sucrose, 3mM $MgCl_2$, 1mM EGTA) containing 0.5% Triton X-100, and the insoluble material was pelleted by centrifugation. The pellet was then serially extracted with increasing concentrations of

NaCl in CSK (0.15/0.2/0.4/0.6/0.8/1.0M salt). The first extraction (0.15M) was incubated on ice for 15 minutes then centrifuged at 14000g for 30 minutes at 4 °C. For the following extractions (0.2-1.0M), the pellet was incubated on ice for 20 minutes before being centrifuged at 14000g for 30 minutes at 4 °C. Lysates were then prepared for western blot analysis.

2.2.2.21 DNA plasmid miniprep using alkaline lysis

Three to five mls of *E.coli* culture containing the plasmid were grown overnight. The culture was centrifuged in a 1.5ml Eppendorf tube at 14000g. The supernatant was removed and the pellet was resuspended in 300µl of buffer 1 (50mM Tris-HCl pH 8.0, 10mM EDTA, 100µg/ml RNase A). Buffer 1 is stored at 4°C. To lyse the pellet, 300µl of buffer 2 (200mM NaOH 1% SDS) was added and the eppendorf tube was inverted 5 times and incubated for 5 minutes at room temperature. 300µl of ice cold buffer 3 (3.0 M potassium acetate pH 5.5) was added and the tube inverted 5 times. The sample was then incubated on ice for at least 5 minutes and then centrifuged at 14000g for 10 minutes. The supernatant was transferred to a new tube, phenol:chloroform extracted and centrifuged at 14000g for 5 minutes. The aqueous phase was transferred to a fresh eppendorf tube. 0.7 volumes of Isopropanol was added, mixed by inversion and centrifuged at 14000g for 15 minutes at 4˚C. The pellet was washed by ethanol precipitation and resuspended in TE buffer.

2.2.2.22 Harvesting low molecular weight DNA

Low molecular weight DNA was harvested for real time PCR analysis as part of the *in vivo* transient replication assays. 3 x 10^5 293T cells were counted, plated onto 100mm^2 cell culture dishes and incubated for 24 hours at 37°C in a 5% CO2/95% air atmosphere. Calcium phosphate transfections of HPV replication plasmids (pCMV-E2, pCMV-E1 and pOri16M / pTM1-E1, pCR3-E2 and pOri16M) or SV40 replication plasmids (pGL3 control /

pGL3 basic) took place the following day. Sixteen hours post transfection the calcium phosphate and medium was removed, the cells were washed twice with PBS and incubated in fresh medium for a further 48 hour. The cells were then washed once with PBS and excess PBS was removed by a pasteur pipette. Cell were lysed in 800µl of Hirt solution (10mM EDTA pH???, 0.5% SDS) and scraped into a 1.5 ml Eppendorf tube. 200µl of 5M NaCl was added and the samples were left at 4°C overnight. Samples were centrifuged at 5000g for 30 minutes at 4°C, extracted twice with phenol: chloroform: isoamyl alcohol (2.2.2.1) and precipitated with ethanol (2.2.2.2). The DNA pellet was dried and resuspended in 100µl of ddH2O and the samples were analysed by real time PCR (2.2.2.4).

2.2.3 Statistics

2.2.3.1 Mean and standard error of the mean

The mean ± the standard error of the mean (SEM) are shown in several experiments as a control for experimental reproducibility. The standard error of the mean (SEM) gives a measure of the precision of the mean giving a representation of the spread of the data, indicating how far the mean of a sample is from the true mean. The SEM is calculated by dividing the standard deviation by the square root of the total number of samples used. This is calculated in Microsoft Excel using = STDEV/SQRT (COUNT(X,-Y)).

Where STDEV (σ) =

$$\sigma = \sqrt{\frac{\sum (x - \bar{x})^2}{n}}$$

σ = standard deviation

$\sum$ = sum of

x = each value in the data set

$\bar{x}$ = mean of all values in the data set

n = number of value in the data set

$$\text{SEM} = \frac{\text{Standard Deviation}}{\sqrt{[\text{Sample number (n)}]}}$$

2.2.3.2 Statistical Significance

Statistical significance for the data presented in this thesis was typically calculated using a Students-T-test to give a significance value (p). This was carried out using Microsoft Excel (T-TEST function). The cut off for all experiments was taken as 0.05, therefore if two sets of data return a p value of <0.05 there is a less than 5% chance that the result observed is due to chance, and the two groups are therefore statistically significantly different.

Chapter 3 – Results

3.1 HPV E1-E2 mediated DNA replication is not arrested by DNA damage.

The DNA from the high risk HPVs is often found integrated into the host cell DNA in high grade squamous cell carcinomas specimens (Cooper et al., 1991; Kristiansen et al., 1994; Alazawi et al., 2002; Peitsaro et al., 2002; Arias-Pulido et al., 2006; Pett et al., 2006.) Viral integration has been shown to be a factor in cell transformation from studies carried out in HPV positive cervical cancer specimens and *in vitro* model systems (Wentzensen et al., 2004; Dall et al.,2008.). Integration results in the unregulated expression of the oncogenes E6 and E7 which can lead to the development of cancer. Factors that may promote viral integration include replication of the viral genome during cellular DNA damage. This would promote double stranded breaks in the viral and cellular DNA and increase the chance of integration through a DNA repair mechanism such as NHEJ (Ziegert et al., 2003). It has been suggested that replication of integrated viral DNA by E1 and E2 may also cause genomic damage and this could lead to further integration events (Kadaja et al., 2007; Kadaja et al., 2009). Another dsDNA virus that has controversial findings regarding its replication status during an activated DNA damage response is the SV40 virus. Some reports suggest that SV480 infection requires an activated DNA damage response for a productive life cycle (Shi et al., 2005). Whereas there is evidence to suggest that an activated DNA damage response inhibits SV40 replication (Miao et al., 2003).

The regulation of DNA replication is well described in the literature however the precise targets at the replication fork that respond to DNA damage pathways have not been clearly defined. There is evidence that the components of the cellular MCM2-7 helicase is targeted for phosphorylation by ATR/ATM in response to DNA damage (Cortez et al., 2004; Ishimi et al., 2003a; Ishimi et al., 2003b). Therefore targeting of the helicase by DNA

damage pathways may inhibit DNA replication in the presence of DNA damage. E1, LT and MCM2-7 are all ATPases Associated with a variety of cellular Activities (AAA+) domain containing helicases. The 200-250 amino acid AAA+ domain contains conserved ATPase sequences that use the energy released from ATP hydrolysis to alter the structure of DNA during replication (White & Lauring, 2007).

The large T antigen (LT) of SV40 initiates viral replication in a similar manner to HPV E1. Both proteins share structural homology in both the DNA binding and helicase domains and functional similarities (Luo et al., 1996; Enemark et al., 2000; Li et al., 2003; Meinke et al., 2006; Enemark & Joshua-Tor, 2006). LT is a viral origin binding protein that forms double hexameric rings encircling the viral DNA. Similarly to E1 it distorts DNA and initiates replication bidirectionally (Valle et al., 2000). The major mechanistic difference between E1 and LT is that LT does not require a viral loading factor to bind to the viral origin.

In this study the replication of the papillomavirus origin replication was compared to SV40 origin replication. The hypothesis is that HPV is able to replicate in the presence of DNA damage. To determine this hypothesis, the replication of HPV and SV40 origin containing plasmids by E1:E2 or LT is first examined using *in vivo* and *in vitro* replication assays. Both viruses contain double stranded circular DNA and replicate in a similar manner using host cell replication proteins to undertake their own replication. Therefore SV40 origin replication was used as a control to identify any differences between HPV and SV40 replication that may help understand factors that can lead to HPV integration. Low risk and high risk (HPV11 and HPV16 respectively) replication was also compared. The low risk HPV11 can cause genital warts whereas the high risk HPV16 can cause cervical cancer. The high risk types can cause oncogenesis when the viral DNA becomes integrated and the E6 and E7 protein expression becomes unregulated. Comparing replication of HPV11 and HPV16 may help to understand why some strains of papillomavirus can cause cancer and why some strains do not.

3.1.1 HPV11 E1-E2 mediated replication *in vivo* is not arrested by DNA damaging agents.

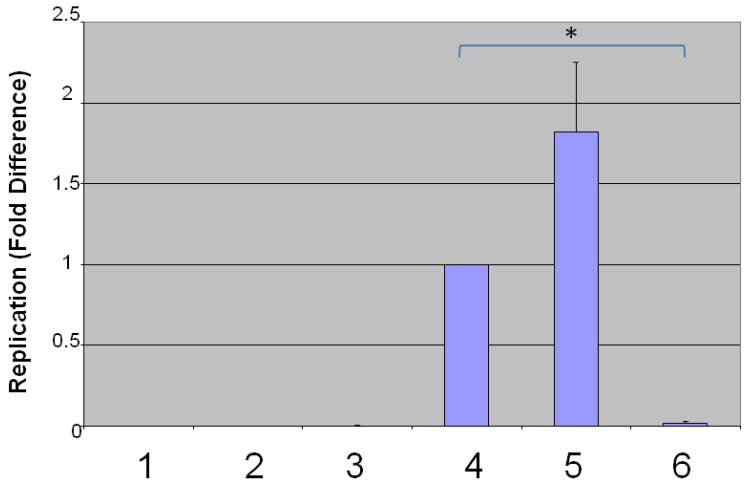
To investigate whether HPV11 E1–E2 mediated DNA replication is arrested following DNA damage, transient DNA replication assays were carried out in 293T cells using the real-time PCR protocol developed in previously in our laboratory (Taylor and Morgan, 2003; Morgan and Taylor, 2005). Initially this protocol was used with C33A and U2OS cells however the combination of calcium phosphate transfection and drug treatment resulted in high amounts of cell death. Although 293T cells are not a cervical epithelial cell line they were chosen due to their high transfectability of the viral protein expressing plasmids and ability to withstand subsequent drug treatment. Briefly, plasmids containing HPV11 E1, HPV11 E2 and the HPV16 origin of replication were transfected into 293T cells using the calcium phosphate method of transfection (2.2.1.2). In these experiments, the pOri-16M (designed for HPV16 replication) can be used to investigate HPV11 E1-E2-mediated replication because the origin sequences are well conserved in mucosal HPVs. Cells were then treated with a DNA damaging agent or aphidicolin. In separate experiments etoposide and camptothecin were used as DNA damaging agents. Etoposide inhibits topoisomerase IIa, resulting in double strand DNA (dsDNA) breaks during the S phase of the cell cycle. To create DNA damage a relatively high concentration of etoposide was used (50μM). This concentration has been shown to produce dsDNA breaks in 293T cells (Abramson et al., 2010). Camptothecin inhibits the DNA enzyme topoisomerase I and creates dsDNA breaks. It was used at 10μM as this concentration has previously been shown to cause dsDNA breaks and is tolerated by 293T (Reinhardt et al., 2007; Fu et al., 2007). Cells were also treated with Aphidicolin (2.5μg/ml) as a control. Aphidicolin inhibits the replicative DNA polymerase (alpha/delta/epsilon) inhibitor (Ikegami et al, 1978). Therefore aphidicolin treatment should arrest both cellular and viral (HPV and SV40) DNA replication. A previous publication had used aphidicolin at a concentration of 5μg/ml to arrest U2OS cells that stabley express HPV16 E2 (Johansson et al., 2009). Concentrations slightly above and below this value were tested and it was found that 2.5μg/ml could arrest HPV replication therefore this was the concentration used in these assays. Forty eight hours later the cells were lysed and DNA extracted using HIRT extraction (2.2.2.22). DNA was purified by phenol.chloroform extraction (2.2.2.1) and ethanol precipitation (2.2.2.2). Digesting DNA with DpnI and ExoIII resulted in freshly

replicated origin plasmid DNA. The *E.coli* strains used for propagation of viral plasmid DNA cause methylation of the plasmid DNA using DAM methyltransferase. The Dpn1 digests DAM-methylated (non-replicated) DNA and ExoIII digests single stranded DNA overhangs. The amount of freshly replicated DNA which was then measured using Real Time PCR (2.2.2.4).

The results obtained with HPV11 E1 and E2 replication *in vivo* are shown in Figure 3.3.1. In Figure 3.1.1.(a), origin plasmid replication is not detected in the non-transfected cells (lane 1), HPV origin plasmid transfected alone (lane 2) and HPV origin plasmid with E1 expression plasmid (lane 3). Replication of the origin plasmid is detected when the E1 and E2 expression plasmid are co-transfected (lane 4). After treatment with the DNA polymerase inhibitor aphidicolin, this replication signal is greatly reduced. The small level of replication detected may be a result of origin plasmid replication prior to drug treatment (lane 6). There is a statistical difference between untreated and aphidicolin treated cells. When etoposide is added to the HPV plasmid transfected cells, there is an apparent increase in origin plasmid DNA replication (lane 5). However, there is no significant difference between HPV11 ori replication with and without etoposide treatment. In Figure 3.1.1 (b) again no DNA replication signal is detected in non-transfected cells or when a plasmid containing the HPV origin is transfected alone or with an E1 expression plasmid (lanes 1–3). DNA replication is detected when the E2 expression plasmid is also co transfected (lane 4). When the cells are treated with aphidicolin following transfection there is a minimal level of replicated origin plasmid (lane 6). As $p<0.05$, there is a statistical difference between untreated HPV11 E1-E2-ori transfected and aphidicolin treated cells When the transfected cells are treated with camptothecin HPV11 E1-E2 mediated replication of the origin is not inhibited (lane 5). As $p>0.05$, there is no statistical difference between HPV11 E1-E2-ori transfected untreated and camptothecin treated cells. This result may be ascribed to one of the repeats showing an exceptionally large increase in origin replication when treated with camptothecin.

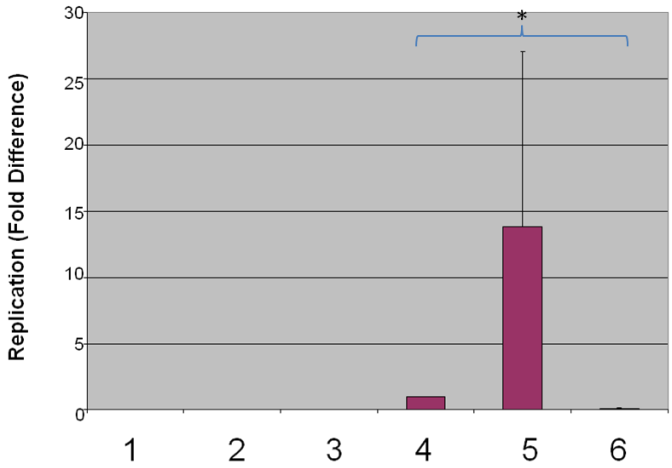
Figure 3.1.1 HPV11 E1-E2 mediated replication *in vivo* is not arrested by DNA damaging agents.

(a) HPV11 origin replication assay with etoposide treatment



pOri-16M	-	100pg	100pg	100pg	100pg	100pg
HPV11 E1	-	-	5µg	5µg	5µg	5µg
HPV11 E2	-	-	-	2µg	2µg	2µg
Drug	-	-	-	-	ETO	APH
F.D	-	-	0.001	1	1.8	0.02

(b) HPV11 origin replication assay with camptothecin treatment



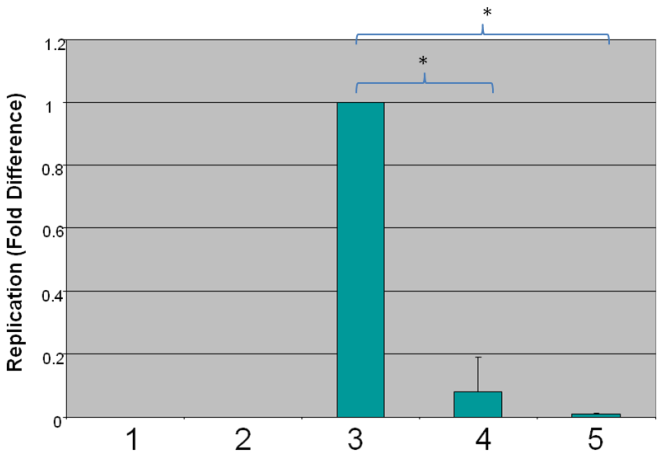
pOri-16M	-	100pg	100pg	100pg	100pg	100pg
HPV11 E1	-	-	5µg	5µg	5µg	5µg
HPV11 E2	-	-	-	2µg	2µg	2µg
Drug	-	-	-	-	CPT	APH
F.D	-	-	-	1	13.8	0.08

Figure3.1.1 (a) HPV11 E1-E2 mediated replication is not arrested by etoposide *in vivo*. 293T cells were transfected with 100pg of pOriM (lanes 2–6), 5µg of pCMV11-E1 (lanes 3–6) and 2µg of pCMV11-E2 (lanes 4–6). 16 hours after transfection, cells were left untreated (lanes 1–4) or treated with 50µM of etoposide (lane 5) or 2.5 µg/ml of aphidicolin (lane 6). Forty-eight hours later low molecular weight DNA was harvested from the cells for transient replication assays. The results shown represent the summary of three experiments with mean and standard error of the mean shown. Results are shown as a fold difference (F.D) to lane 4 (ori, E1 &E2 with no drug treatment). Statistical significance by Student's *t*-test is indicated as * ($p < 0.05$). **(b) HPV11 E1-E2 mediated replication is not arrested by camptothecin *in vivo*.** The replication assay was carried out as described in figure 3.1.1(a). 16 hours after transfection cells were treated with 10 µM of camptothecin (lane 5) instead of etoposide. The results shown represent the summary of three experiments with mean and standard error of the mean shown. Lane 4 is standardised to 1 and the results shown are a fold difference of this value. Statistical significance by Student's *t*-test is indicated as * ($p < 0.05$).

3.1.2 SV40 virus LT mediated replication *in vivo* is arrested by etoposide.

It was feasible that HPV replication during treatment with DNA damaging agents might be explained by a lack of DNA damage response in the 293T cells. Therefore another DNA virus was used to confirm that HPV replication during DNA damage was simply not a result of the 293T cells failing to sense and activate a DNA damage response pathway. The LT-mediated SV40 origin DNA replication was used to assess this *in vivo* (Figure 3.1.2). 293T cells stably express SV40 LT therefore only the SV40 origin containing plasmid was required to be transfected into the cells. In 293T cells only, there is no replication signal detected (lane 1). A control plasmid that contains the luciferase gene but does not contain the sequence for the SV40 origin (p-SV40 ori) does not produce a replication signal (lane 2). Transfection of a plasmid containing the SV40 origin sequence (p+SV40 ori) produces a replication signal in lane 3. In lane 5 the 293T cells have been transfected with p+SV40 ori and aphidicolin. In a similar trend to HPV E1 and E2 mediated replication, aphidicolin arrests LT mediated replication. The p value is calculated to be $5x10^{-7}$. As $p<0.05$ there is a significant difference between p+SV40 ori replication with and without aphidicolin treatment. In a direct contrast to HPV E1-E2 mediated replication, etoposide treatment of p+SV40 transfected cells results in an arrest in LT mediated SV40 origin plasmid replication (lane 4). The p value is calculated to be 0.005. As $p<0.05$ then there is a significant difference between p+SV40 ori with and without etoposide treatment.

Figure 3.1.2 SV40 virus LT mediated replication *in vivo* is arrested by etoposide.



p –SV40 ori	-	1ng	-	-	-
p +SV40 ori	-	-	1ng	1ng	1ng
Drug	-	-	-	ETO	APH
F.D	-	-	1	0.08	0.01

Figure3.1.2 SV40 virus LT mediated replication *in vivo* is arrested by etoposide. 293T cells were transfected with 1ng of luciferase gene containing plasmid DNA (lanes 2–5). In lane 2, the plasmid contained no SV40 origin of replication in lanes 3–5 the plasmid contained an SV40 origin of replication. One day post transfection, drug treatments were added in lane 4 (50µM of etoposide) and lane 5 (2.5 µg/ml of aphidicolin). The other lanes are all untreated (lanes 1-3). Forty-eight hours later low molecular weight DNA was harvested. The results shown represent three independent experiments and are standardised to LT mediated p+SV40 replication without any drug treatment (lane 3). Statistical significance by Student's *t*-test is indicated as * ($p < 0.05$).

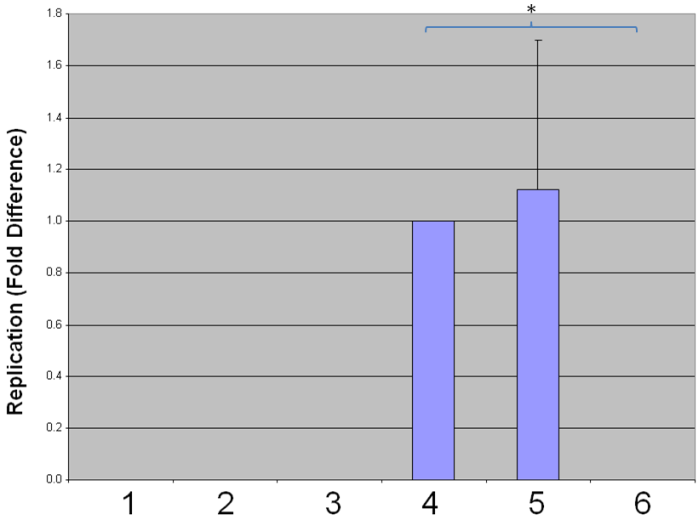
3.1.3 HPV16 E1-E2 mediated replication *in vivo* is not arrested by etoposide.

The ability of a HR-HPV to replicate during DNA damage was investigated. Replication during DNA damage of a HR-HPV could be a factor promoting viral integration that is often detected with cancer causing HPVs. Integration of the HPV16 genome into the host cell chromosome is a key event in the pathway to cervical cancer as it results in unregulated expression of the viral oncoproteins E6 and E7 (Jeon et al., 1996; Romanczuk & Howley, 1992; Romanczuk et al., 1990). The low risk types do not cause cancer because they do not express oncoproteins. To investigate whether HPV16 E1–E2 mediated replication of the ori plasmid is arrested by DNA damage caused by etoposide transient DNA replication assays in 293T cells using the real-time PCR protocol as described in 3.1.1 were carried out. Fig. 3.1.3 represents the results obtained with HPV16 E1 and E2 mediated replication *in vivo*. In lane 1 no DNA replication is detected in the untransfected cells. No replication is detected when a plasmid containing the HPV origin (pOri-16M) is transfected with or without an E1 expression plasmid (lanes 2–3). As anticipated, DNA replication is detectable when the E2 expression plasmid is co-transfected with pOri-16M and E1 (lane 4). When the cells are treated with aphidicolin following transfection E1-E2 replication of the origin plasmid is inhibited (lane 6). The p value is calculated to be $4x10^{-7}$. As $p<0.05$ there is a significant difference between E1-E2 mediated replication of pOri-16M with and without aphidicolin treatment. HPV16 E1 and E2 replicate the origin plasmid in the presence of etoposide treatment (lane 5). The p value is calculated to be 0.85. As $p>0.05$ there is no significant difference between E1-E2 mediated replication of pOri-16M with and without etoposide treatment.

These data suggest that the HR-HPV16 E1-E2 can replicate the origin plasmid during etoposide treatment. However to definitively prove whether replication is a factor that may contribute to viral integration, the type of DNA damage caused by etoposide treatment should be assessed. A comet electrophoresis assay could be used to visualize and quantify double strand DNA breaks (Singh et al., 1988). Prior to electrophoresis the DNA is situated within the comet 'head'. During electrophoresis undamaged DNA is too large to migrate and does not leave the "head". However damaged DNA migrates towards the anode creating a 'tail' which can be clearly seen using a fluorescent

microscope. Image analysis of the fluorescence intensity can be compared between the 'head' and 'tail' to detect dsDNA breaks. To further validate the presence of dsDNA breaks during etoposide treatment, H2AX phosphorylation could be examined in these samples. H2AX is phosphorylated on serine 139 in response to DNA double stranded breaks (Fernandez-Capetillo et al., 2004; Harper & Elledge, 2007). A simple western blot using a phospho-H2AX Ser 139 antibody may detect differences in DNA damage/repair in etoposide-untreated and treated cell lines.

Figure 3.1.3 HPV16 E1-E2 mediated replication *in vivo* is not arrested by etoposide.



pOri-16M	-	100pg	100pg	100pg	100pg	100pg
HPV16 E1	-	-	5µg	5µg	5µg	5µg
HPV16 E2	-	-	-	2µg	2µg	2µg
Drug	-	-	-	-	ETO	APH
F.D	-	-	-	1	1.2	-

Figure 3.1.3 HPV16 E1-E2 mediated replication *in vivo* is not arrested by etoposide. 293T cells were transfected with 100pg of pOri-16M (lanes 2–6), 5µg of pCMV16-E1 (lanes 3–6) and 2µg of pCMV16-E2 (lanes 4–6). The day after transfection, cells were left untreated (lanes 1–4) or treated with 50µM of etoposide (lane 5) or 2.5 µg/ml of aphidicolin (lane 6). Forty-eight hours later low molecular weight DNA was harvested from the cells for replication assays. The results shown represent the summary of three experiments and are standardised to E1+E2 without treatment (lane 4) equalling 1. Statistical significance by Student's *t*-test is indicated as * ($p < 0.05$).

3.1.4 *In vitro* results show that LT replication arrest is inhibited via a checkpoint response, while E1–E2 replication is not

In Figures 3.1.1-3, LT-mediated replication of the SV40 origin plasmid is inhibited in response to etoposide treatment whereas HPV11 and HPV16 E1 and E2-mediated replication of the HPV16 origin replication plasmid is not. To address whether these differences are a result of an activated DNA damage checkpoint response, the role of the DNA checkpoint kinases (ATR/ATM/DNA-PK) in replication control was investigated. Previous studies have shown that MCM2-7 helicase is targeted for phosphorylation by ATR/ATM in response to DNA damage (Cortez et al., 2004). It was therefore feasible that a checkpoint kinase may specifically target SV40 LT for phosphorylation and this may explain why replication of SV40 origin is inhibited during etoposide treatment. *in vivo* experiments such as those in Figures 3.1.1-3, using either knockdown of DNA checkpoint kinases in 293T cells or the use of checkpoint kinases inhibitors were attempted. However the combination of siRNA oligonucleotides/checkpoint inhibitors with etoposide treatment was highly toxic to the cells meaning that this approach was not feasible..

To address this problem, *in vitro* viral DNA replication assays were utilized and carried out by John Fisk at the University of Buffalo. The protocol for studying *in vitro* HPV and LT replication has been previously established for HPV11, BPV1 and SV40 origin replication in 293 cell extracts (Melendy et al., 1995; Narahari et al., 2006). This protocol was adapted in order to study HPV/LT origin replication during an activated checkpoint response (2.2.2.5). The pUC19 plasmid DNA was digested with RsaI enzyme. This produced linear DNA strands which mimic dsDNA breaks. The undigested or digested plasmid was added to the 293 cell extract to determine differences in HPV and SV40 origin replication in the presence of undamaged or damaged DNA. Checkpoint kinase inhibitors were used to establish whether an activated DNA damage response can regulate LT or E1 mediated replication *in vitro* (2.2.2.6). As a control, the same amount of undigested plasmid DNA was added. This DNA did not contain blunt DNA ends and therefore did not mimic DNA damage. The addition of undigested circular DNA did not modify the DNA replication capabilities of LT or E1. Figure 3.1.4 (a) represents LT replication *in vitro*. When 50ng of undigested supercoiled pUC19 is added to the cell extract, it has no effect on LT origin replication. Replication intermediates or Cairns structures are formed during the

bidirectional replication of circular DNA molecules (Tapper & DePamphilis, 1977). There is no change in replication intermediate products between lanes 1 and 2. When 50ng of linearised DNA (mimics dsDNA breaks) is added in lane 4, there is a significant reduction in the production of LT replication intermediates. Form I and II DNA are the replicated progeny of replication intermediates. Form I DNA exists as supercoiled circular DNA and form II exists as circular DNA which has a nick in one of the strands. There appears to be a reduction in the quantity of form I and II DNA in lane 4 compared to the lanes that contain supercoiled uncut pUC19 DNA (lanes 2 and 3). DNA ends can therefore induce an arrest of LT mediated replication *in vitro*.

Wortmannin inhibits the phosphoinositide 3-kinase related kinase (PIKK) family of proteins. ATR, ATM and DNA-PK are checkpoint kinases that belong to this family of proteins. To determine whether the arrest in LT mediated SV40 origin replication was a result of an activated checkpoint response, wortmannin was included in the assay. Wortmannin in the presence of super coiled DNA does not affect LT mediated replication (compare lane 3 to lane 2). On the contrary, wortmannin in the presence of DNA ends does effect LT mediated replication. In lane 4, linearised pUC19 DNA inhibits LT mediated replication. LT mediated replication was evident in the presence of linearised DNA and wortmannin (lane 5). Therefore wortmannin can relieve arrest in LT-mediated replication in the presence DNA ends suggesting that a PIKK protein is responsible for regulating LT mediated replication during the presence of DNA ends.

To determine whether HPV replication was inhibited by the addition of DNA ends, identical experiments were carried out comparing the SV40 LT with HPV11 E1 *in vitro* (Figure 3.1.4 (b)). Similarly to the previous experiment, the addition of 50 and 100ng of digested pUC19 reduces the quantity of replication intermediates and replicated DNA suggesting that LT mediated replication is inhibited by DNA ends (lanes 2 and 3). However, the addition of linearised DNA does not inhibit HPV11 E1 mediated DNA replication as can be seen by presence of replication intermediates after 100ng of digested pUC19 was added (lane 5). These *in vitro* results imitate the *in vivo* DNA damage replication assay results. Therefore LT mediated replication is sensitive to checkpoint kinase signalling via a PIKK pathway whereas E1 mediated replication is not.

By using PIKK inhibitors that prevent either ATM or DNA-PK activation, it was possible to investigate which of the kinases was responsible for the inhibition of LT induced DNA replication *in vitro*. As there is no specific ATR inhibitor, a process of elimination using the ATM and DNA-PK inhibitors could determine whether ATR was responsible (Figure 3.1.4 (c)). As was seen previously in Figures 3.1.4 (a) and (b), the addition of RsaI digested pUC19 DNA resulted in a decrease in LT replication (down to around 38% compared to the control DMSO treatment) in lane 2. The addition of wortmannin in lane 3 results in LT mediated replication in the presence of DNA ends. When NU7026, a DNA-PK checkpoint inhibitor, or KU55933, an ATM specific checkpoint inhibitor, are added individually or together, replication remains low at levels similar to that seen with the addition of digested pUC19 DNA (lanes 4,5,6 respectively). Therefore, through a process of elimination, it can be hypothesised that ATR checkpoint kinase is responsible for regulating LT mediated replication in the presence of DNA ends *in vitro* and this may serve as a model for LT mediated replication *in vivo*. In addition, the inhibitors for DNA-PK and ATM were tested in order to confirm their functional activity in these experiments. The Promega DNA-PK assay kit (DNA-PK inhibition) and NBS1 hyper-phosphorylation detection (ATM inhibition) were used to verify inhibition (data with Melendy laboratory, University of Buffalo).

Figure 3.1.4 *In vitro* results show that LT replication arrest is inhibited via a checkpoint response, while E1–E2 replication is not.

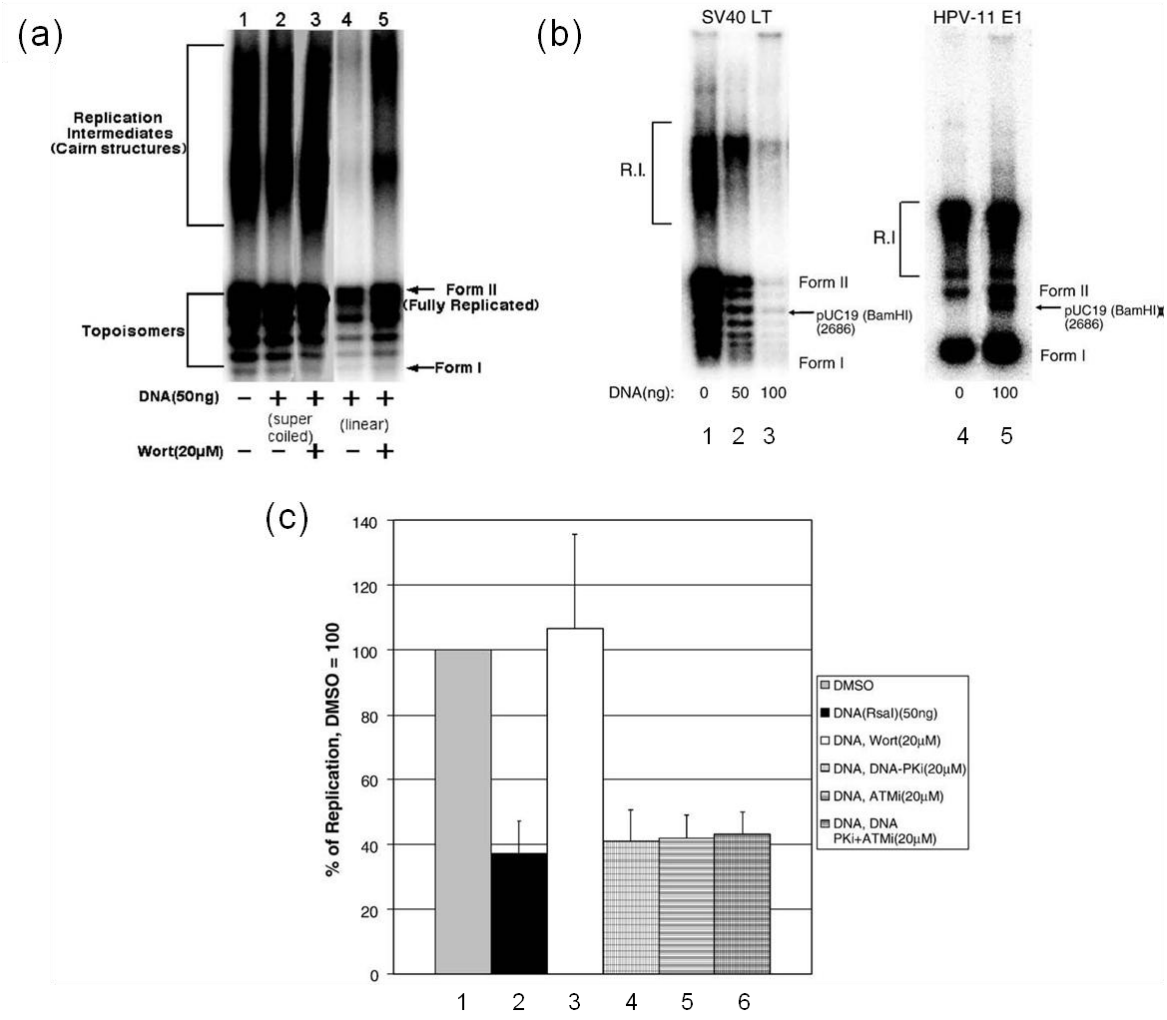


Figure 3.1.4 *In vitro* results show that LT replication arrest is inhibited via a checkpoint response, while E1–E2 replication is not. a) *In vitro* SV40 DNA replication as described in 2.2.2.5 was carried out in the presence of 0.1% DMSO (lane 1). Undigested pUC19 DNA (50ng) was added to the 293 cell extract prior to the assembly of replication reaction, either in the absence (lane 2) or presence of 20μM wortmannin (lane 3). Restriction-enzyme digested pUC19 (50ng) was added to the 293 cell extract prior to the assembly of SV40 replication reaction either in the absence (lane 4) or presence of 20μM wortmannin (lane 5). b) Left panel: SV40 replication reactions were carried out in a similar method to (a). 0, 50 or 100ng of restriction enzyme digested pUC19 DNA was added as indicated. In the right panel: HPV11 DNA replication reactions were carried out as described in 2.2.2.5

using the same extract and reaction buffer as in the left panel. The only variant is the replication template DNA and the use of HPV11 E1 in place of SV40 LT. Zero or 100ng of restriction enzyme digested pUC19 DNA was added as indicated. c) The PIKK inhibitors (DNA-PKi=NU7026; ATMi=KU55933) were added to the 293 extracts on ice, 5 minute prior to addition of template and replication reaction components (concentrations of the inhibitors given are final concentration in the reaction). LT replication levels were quantified as described in chapter 2.2.2.6. Results shown represent four independent experiments; error bars indicate the standard deviation.

3.1.5 LT is a substrate for DNA checkpoint kinases *in vivo*.

During an activated DNA damage response, DNA replication is inhibited to prevent replication of a mutated genome. It has been suggested that the cellular MCM2-7 helicase is targeted by checkpoint kinases in response to DNA damage and this may result in the inhibition of DNA replication (Cortez et al., 2004; Ishimi et al., 2003a; Ishimi et al., 2003b). The results in Figure 3.1.6 also suggest that ATR is the checkpoint kinase that regulates LT mediated replication *in vitro*. It was therefore possible that the checkpoint kinases were responsible for the inhibition of LT-mediated replication of the origin plasmid by targeting the SV40 LT helicase during DNA damage. Similarly to HPV replication, SV40 replication requires many of the same cellular replication proteins to initiate replication from the SV40 origin such as DNA pol α/δ and RFA (Replication factor A) (Muller et al., 1994; Melendy et al., 1995). Therefore an obvious difference between SV40 and HPV replication is the viral helicase. LT is the only viral protein that is required for SV40 replication whereas E1 in combination with E2 are the viral proteins required to carry out HPV origin replication. A previous study has shown that LT is phosphorylated at Ser120 by ATM in response to ionizing radiation treatment (Shi et al., 2005). Therefore, similar to phosphorylation of the MCM-2-7 helicase complex, the checkpoint kinases may target LT (and not E1) for phosphorylation and this may explain the differences in viral replication during DNA damage.

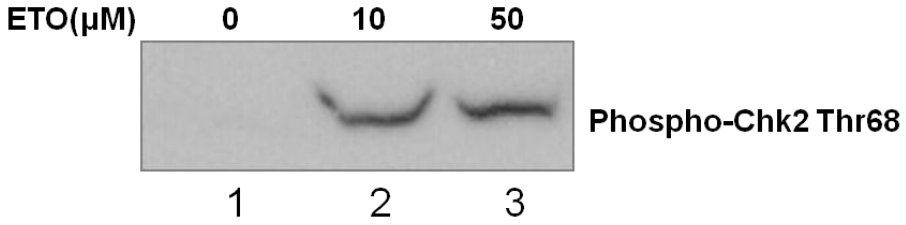
To investigate whether the DNA damaging agent etoposide is able to activate a checkpoint kinase pathway the phosphorylation of a downstream target protein, Chk2 was investigated in 293T cells. The phosphorylation of Chk2 protein is an indication of an activated DNA damage response (Matsuoka et al., 1998; Chaturvedi et al., 1999). In Figure 3.1.5(a) 293T cells were treated with a range of concentrations of etoposide for two hours. The phosphorylation of Chk2 is evident at 10 and 50μM of etoposide (lanes 2 and 3) but not in untreated 293T cells (lane 1)). Therefore phosphorylation of Chk2 protein demonstrates the activation of a checkpoint kinase pathway.

To investigate whether LT is phosphorylated in a similar manner to MCM2-7 in response to checkpoint kinase activation, LT protein co-immunoprecipitation pull down with the protein kinases ATR and ATM in response to etoposide treatment was investigated

(2.2.2.8). 293T cells that stably express the LT protein were untreated or treated with 50µM of etoposide. Cells were harvested and protein extracts were immunoprecipitated with a phospho-(Ser/Thr)-glutamine ATR/ATM substrate antibody (pS/Q antibody). This antibody pulls down proteins that have been phosphorylated on serine/threonine residues by the protein kinases ATR/ATM. The samples were subjected to immunoblotting for LT (Figure 3.1.5 (b)). In the input lanes, 3 and 4, it is evident that the LT protein is not degraded by 30 minutes of etoposide treatment therefore ruling out the possibility of protein degradation. The pS/Q antibody co-precipitates a significant amount of LT protein after etoposide treatment (lane 2) whereas LT shows no pull down with the pS/Q antibody in untreated 293T cells (lane 1). This result suggests that, similar to MCM2-7, the LT helicase is phosphorylated by either ATR or ATM in response to an activated DNA damage response *in vivo*.

Figure 3.1.5 LT is a substrate For DNA checkpoint kinases *in vivo*.

(a)



(b)

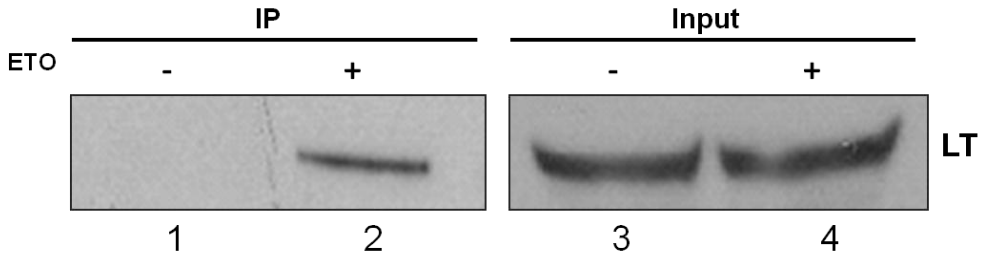


Figure 3.1.5 LT is a substrate For DNA checkpoint kinases *in vivo*. a) 293T cells were treated with 0/10/50µM of etoposide for 2 hours. Cells were harvested and protein

extracts were prepared for western blot analysis using a phospho-Chk2 Threonine-68 antibody to confirm ATR/ATM checkpoint activation. (b) Cells were left untreated (-) or treated with 50μM of etoposide (+) for 30 minutes and protein extracts were prepared. A fraction of the protein extract was then immunoprecipitated with an S/Q antibody (lanes 1 and 2). The precipitate was probed with LT antibody (pAb101).

3.1.6 E1 is not a substrate for DNA checkpoint kinases *in vivo*.

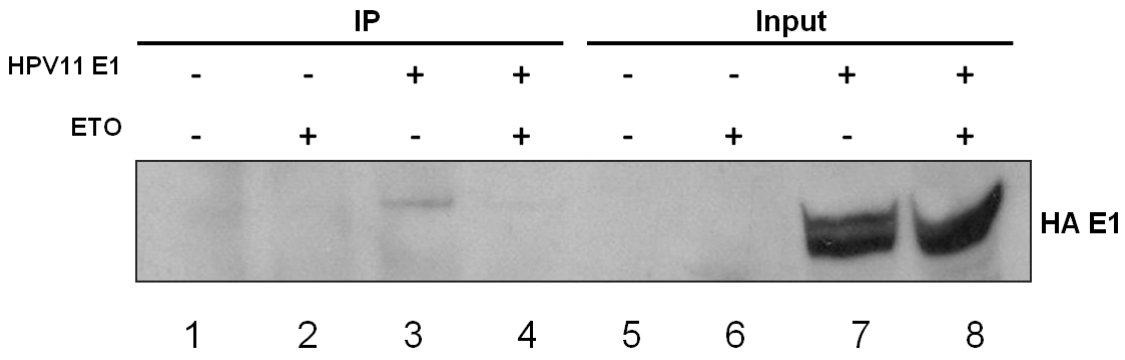
Having established ATR activation with etoposide treatment and shown that LT is a substrate for ATR phosphorylation *in vivo*, it was hypothesised that E1 may not be targeted for phosphorylation by ATR/ATM *in vivo* in the presence of an activated DNA damage response. To investigate this HPV11 and HPV16 E1 protein was used in a similar experiment to Figure 3.1.5. 293T cells were transfected with an HA-tagged E1 plasmid (pMH1-11E1HA/pMH1-16E1HA), expressing either HPV11 or HPV16 E1 respectively and were then treated with 50μM of etoposide. Protein extracts were immunoprecipitated with the phospho-(Ser/Thr)-glutamine ATR/ATM substrate antibody (pS/Q antibody). These extracts were then subjected to immunoblotting for E1 protein pull down. In Figure 3.1.6 (a), cells were calcium phosphate transfected with 1μg of HPV11 HA-tagged E1 or an empty control plasmid of the same concentration. Forty eight hours post transfection, samples were left untreated or treated with 50μM of etoposide for 30 minutes prior to harvest and protein preparation. A fraction of the protein extract was incubated with the pS/Q antibody and extracts were then prepared for western blotting. The membranes were probed against E1 (HA). In lanes 1, 2, 5 and 6, there is no E1 present in these samples therefore they act as negative controls. There appears to be phosphorylation of E1 in the absence of DNA damage (lane 3). However, the pS/Q antibody fails to pull down E1 in the presence of DNA damage (lane 4). In lanes 7 and 8, the HA-tagged E1 plasmid is detectable in the input lanes. In lane 8, E1 is not degraded as a consequence of etoposide treatment and this therefore rules out any suggestions that E1 is simply not co-immunoprecipitated with the pS/Q antibody because it has been degraded. Studies on BPV E1 protein suggest that E1 is phosphorylated and this may regulate BPV replication (Lentz et al., 1993; Zanardi et al., 1997; Lentz et al., 2002). Therefore the phosphorylation of HPV11 E1 in undamaged cells may be a consequence of E1 regulation. A recent paper has also suggested that BPV1 E1 may contain an ATR phosphorylation site (Lentz et al., 2006). Further evidence is required to confirm this hypothesis however the result in Figure 3.1.6 (a) suggests that either ATR or ATM phosphorylates HPV11 E1 in untreated cells, suggesting a regulatory role for the protein kinases.

Figure 3.1.6 (b) shows the results obtained with HA-tagged HPV16 E1. This experiment was carried out in an identical manner to the experiment in 3.1.6 (a) with the exception

of transfection of a HPV16 HA-tagged E1 instead of HPV11. Lanes 1 and 3 serve as negative controls as they have not been transfected with E1. Unlike HPV11 E1 the pS/Q antibody appears not to pull down E1 in the absence of etoposide treatment (lanes 2). Similarly to HPV11, HPV16 E1 does not appear to be phosphorylated by ATR/ATM in response to etoposide treatment (lane 4). E1 is detected in the input lanes (5 and 6). Therefore the results from our checkpoint activated co-immunoprecipitation assays demonstrate that the E1 protein of HPV11 and HPV16 is not targeted for phosphorylation by the ATR/ATM checkpoint kinases in response to DNA damage.

Figure 3.1.6 E1 is not a substrate for DNA checkpoint kinases *in vivo*.

(a)



(b)

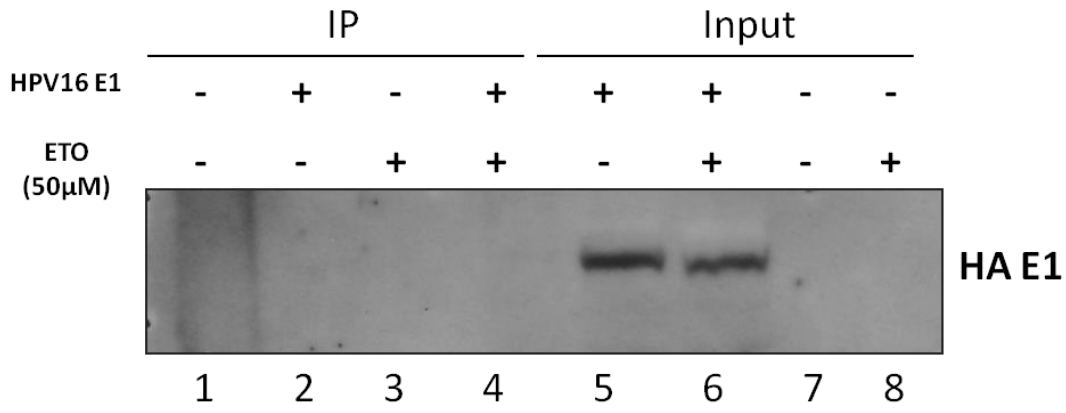


Figure 3.1.6 E1 is not a substrate for DNA checkpoint kinases *in vivo*. (a) Cells were transfected with 1µg of HPV11 HA-E1 (lanes 3, 4 and 7, 8) or 1µg of empty control vector (lanes 1, 2 and 5, 6). Twenty-four hours later the cells were treated with 50µM of etoposide for 30 minutes (+) or left untreated (−) as indicated and protein extracts prepared. A fraction of the protein extract was then immunoprecipitated with an S/Q antibody (lanes 1–4) and the resultant precipitate probed with an HA antibody to detect any immunoprecipitated E1 b) 293T cells were transfected with 1µg of HPV16 HA-E1 (lanes 2, 4 and 5, 6) or 1µg of empty control vector (lanes 1, 3). Twenty-four hours later the cells were treated with 50µM of etoposide for 30 minutes (+) or left untreated (−) as indicated and protein extracts prepared. A fraction of the protein extract was then

immunoprecipitated with an S/Q antibody (lanes 1–4) and the resultant precipitate probed with an HA antibody.

3.1.7 LT protein levels are reduced following prolonged etoposide treatment

The DNA replication assay results in Figures 3.1.1-3.1.3 are extended over a 48 hour period following etoposide treatment. To investigate the levels of the LT protein following extended etoposide treatment, a time course of etoposide treatment followed by LT western blotting was carried out and the results are shown in Figure 3.1.7 (a). The level of LT protein begins to drastically decrease after 8 hours of etoposide exposure. After 24 hours there is a significant reduction in the LT proteins levels as it is barely detectable by western blotting. To confirm that etoposide had activated an ATR DNA damage response, a phospho-Chk1 antibody was probed against these extracts. Etoposide treatment clearly phosphorylates Chk1. Chk1 is a substrate for activated ATR/ATM (Zhao and Piwnica-Worms, 2001). Chk1 phosphorylation persists over the 24 hour period of the experiment. Gamma-tubulin levels are shown as a loading control.

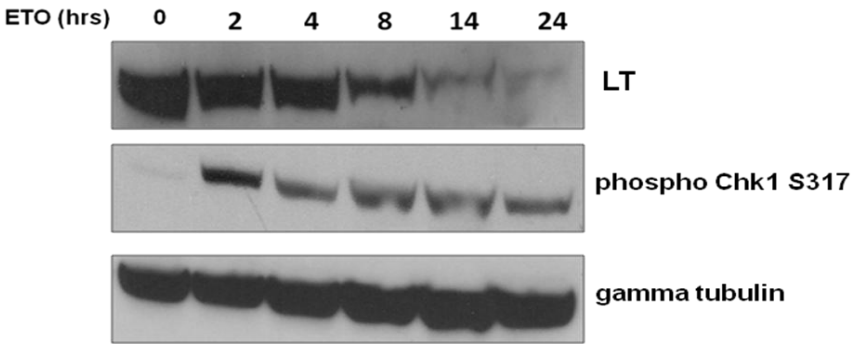
LT RNA levels were also analysed to investigate whether LT protein reduction after prolonged etoposide treatment was a post translational event, and not simply due to a reduction in LT mRNA levels (Figure 3.1.7 (b)). Although LT mRNA levels decreased by 24 hours to around 36% of RNA levels without treatment (100%) this was not representative of the protein levels detected by western blot (2.2.1.15). Therefore LT mRNA reduction is not fully responsible for the reduction of protein levels after prolonged etoposide treatment. In addition, the *in vitro* experiments in Figure 3.1.4 demonstrate that the regulation of LT mediated DNA replication by DNA damaging agents is due to a direct regulation of the LT protein. Therefore although there is a reduction in LT RNA levels following etoposide treatment, this is unlikely to fully account for the inhibition of DNA replication function. When aphidicolin is used, an immediate arrest of LT replication occurs (Figure 3.1.2). The replication signals after etoposide and aphidicolin treatment are very similar in Figure 3.1.2, suggesting an immediate arrest of LT mediated DNA replication by etoposide.

To determine whether part of the reduction of LT protein is due to proteosome mediated degradation, the proteasomal inhibitor MG132 was used in the LT-etoposide experiments. The result of this experiment is shown in Figure 3.1.7 (c). In lane 1, LT

protein is detected in untreated 293T cells. Following treatment with etoposide, protein levels decrease (lane 2). LT protein levels appear to decrease slightly in response to MG132 treatment (lane 3). The LT protein can be easily detected by western blot when treated in combination with etoposide and MG132. The results from three identical experiments were quantified using Image J software and graphed in Figure 3.1.7 (d). The addition of etoposide to 293T cells drastically reduces LT protein levels to around 5% of that detected in untreated 293T cells. Treatment with MG132 also reduces LT levels to around 30% less than the untreated cells. MG132 is dissolved in DMSO. DMSO has been reported to affect protein stability therefore this may explain the reduction in LT protein levels (Tjernberg et al., 2006). 293T cells treated with etoposide and MG132 have around 60% less LT protein than untreated cells. These results suggest that LT protein levels are reduced by long term etoposide treatment because of a decrease in LT RNA levels, possibly due the health of the cells beginning to deteriorate, and by degradation of LT via the proteasome.

3.1.7 LT protein levels are reduced following prolonged etoposide treatment

(a)



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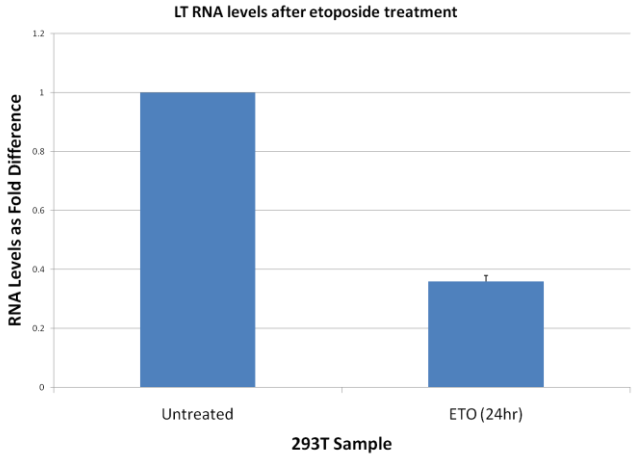
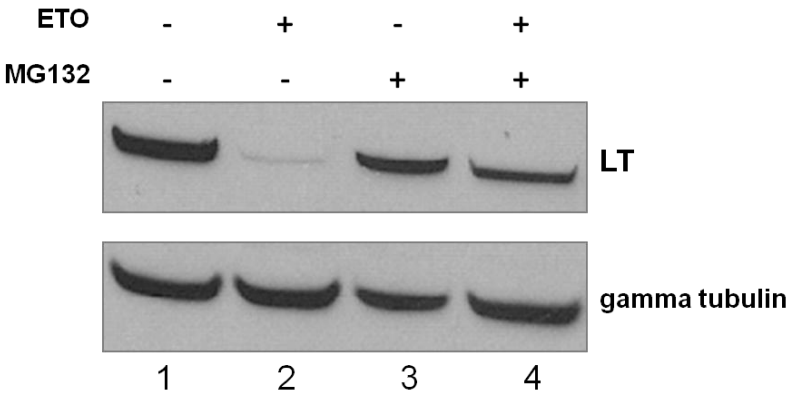


(c)



(d) LT protein levels following etoposide and MG132 treatment.

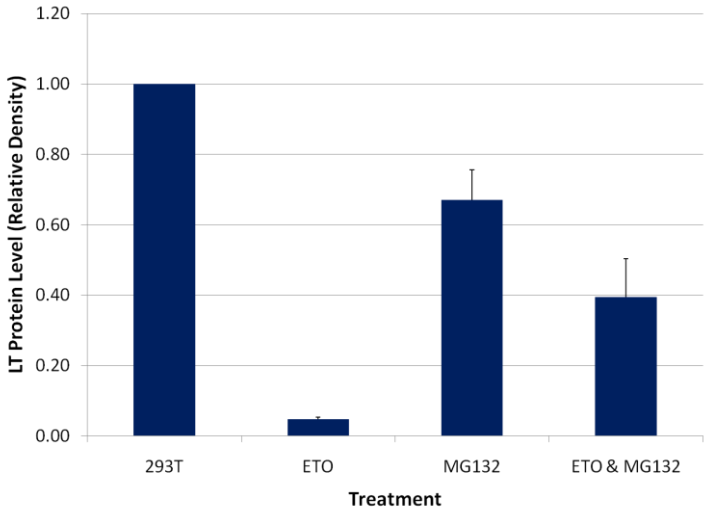


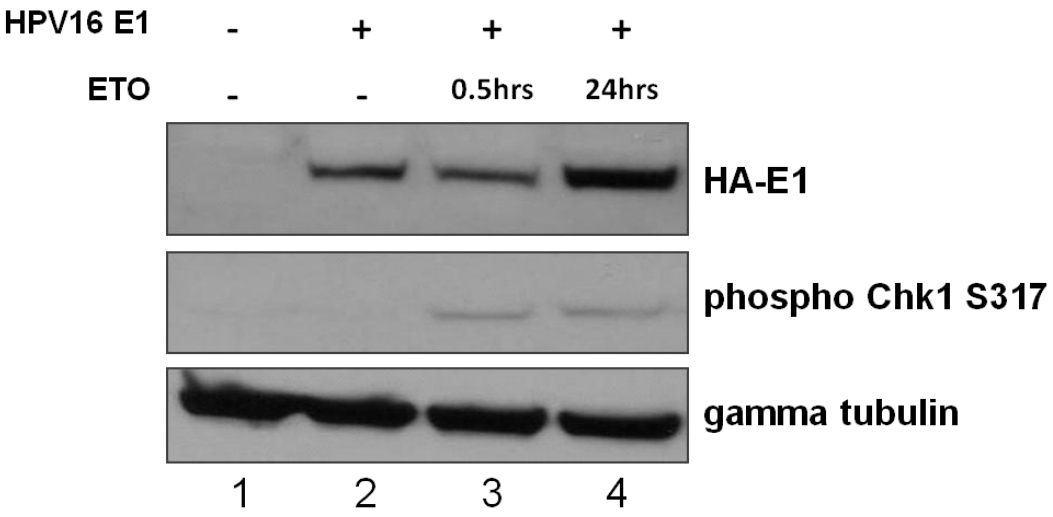
Figure 3.1.7 LT protein levels are reduced following prolonged etoposide treatment. a) 293T cells were treated with 50µM of etoposide for the time periods shown and then protein extracts prepared. These extracts were then western blotted for the proteins indicated. Chk1 S317 serves as a control to confirm activation of the DNA damage response and gamma-tubulin as a loading control. b) 293T cells were treated with 50µM of etoposide for 24 hours. RNA extracted from the cells was converted to cDNA and levels of LT were detected using real-time PCR. The graph represents 3 experiments with results normalised to β-actin. c) LT protein levels are reduced via the proteosome following etoposide treatment. 293T cells were treated with 50µM etoposide (lanes 2 and 4) and/or 100µM MG132 as indicated and protein extracts prepared 24 hours later. Western blotting was then carried out for LT and gamma-tubulin as a loading control. (d) Three replicates from 3.1.7 (c) were quantified using Image J software and gamma tubulin as a loading control. The results displayed show the mean and standard error of the mean.

3.1.8 HPV16 E1 protein levels are not reduced following prolonged etoposide treatment.

Previously it was shown that ATR targets LT for phosphorylation as a result of DNA damage, and a consequence of long term exposure to DNA damage is a large reduction in LT protein levels (Figures 3.1.4 and 3.1.7 respectively). Therefore it was hypothesised that the ability of HPV E1 protein to avoid phosphorylation by the ATR checkpoint kinase would result in unchanged protein levels after long term etoposide exposure. The protein levels of the HPV16 E1 helicase were investigated following long term exposure to etoposide. In Figure 3.1.8 (a), 293T cells were calcium phosphate transfected with 1µg of HPV16 HA-tagged E1 plasmid. The following day cells were treated with etoposide for the time points indicated, and harvested for western blot analysis against E1 (HA), phospho-Chk1 S317 (to show DNA damage checkpoint activation) and gamma tubulin (loading control). Lane 1 does not contain any transfected E1 plasmid and serves as a negative control. E1 is detected when transfected in lane 2. Figure 3.1.8 also demonstrates that calcium phosphate transfection does not activate a DNA damage response because no phosphorylated Chk1 is detected by western blot. Short term exposure (0.5 hours) with etoposide does not alter E1 protein levels during the activation of a DNA damage response (lane 3). In contrast to the LT helicase, E1 protein levels do not appear to decrease after 24 hours of etoposide treatment. Results from three experiments were quantified using Image J software and gamma tubulin as a loading control (Figure 3.1.8 (b)). Treatment with etoposide results in a doubling of E1 protein levels after 24 hours. E1 protein levels appear to remain high after long term etoposide treatment. Treatment with etoposide may arrest the 293T cells in the S phase of the cell cycle (Chow & Ross, 1987). E2 protein has been shown to be stabilised in S phase of the cell cycle and there may be a similar mechanism for E1 stabilisation (Johansson et al., 2009). E1 associates with cyclin E/Cdk2 and prevents E1 degradation via the proteosome (Cueille et al., 1998; Ma et al., 1999). Cyclin E/Cdk2 is a key regulator that initiates progression into the S phase of the cell cycle and therefore it is feasible that E1 may also be stabilised during s phase.

3.1.8 E1 protein levels are not reduced following prolonged etoposide treatment.

(a)



(b)

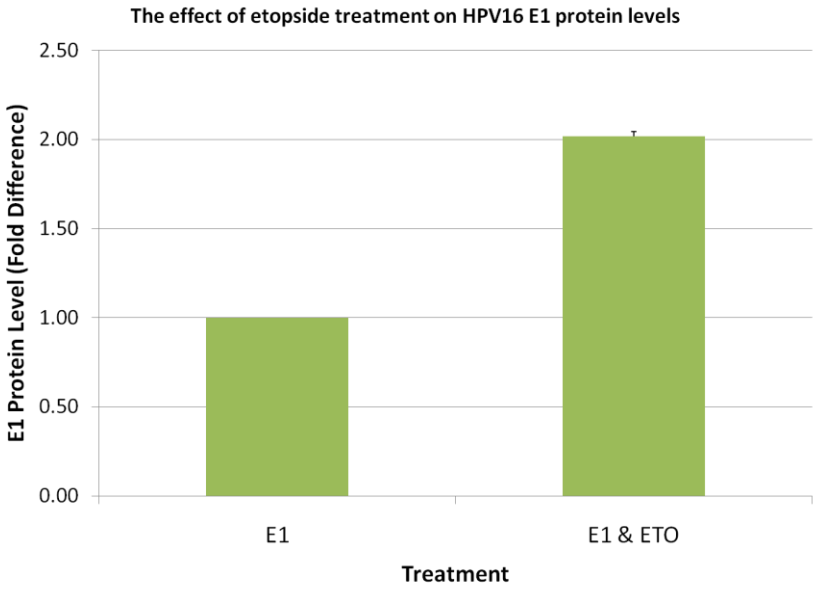


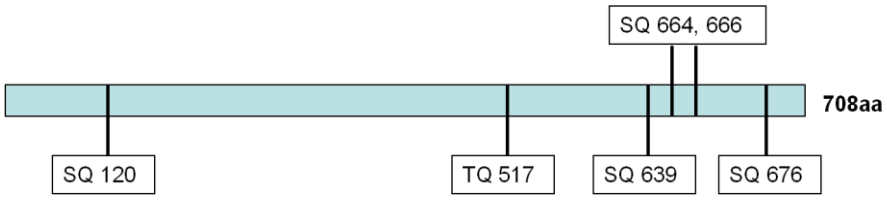
Figure 3.1.8 E1 protein levels are not reduced following prolonged etoposide treatment. (a) 293T cells were transfected with 1µg of HPV16 HA-E1 plasmid and the following day cells left untreated (−) or treated with 50µM of etoposide (+) for 0.5 hours (lane 3) and 24 hours (lane 4). Protein extracts were then prepared and western blotted for the proteins

indicated. Phospho-Chk1 S317 serves as a control to confirm activation of the DNA damage response and gamma-tubulin is used as a loading control. (b) Three similar experiments were quantified after 24 hours of etoposide treatment using Image J software and gamma tubulin as a loading control. The results show the mean and standard error of the mean from three independent experiments.

3.1.9 Site directed mutagenesis of LT phosphorylation sites.

LT is phosphorylated on serine and threonine sites by ATR in response to DNA damage (Figure 3.1.5). ATR is known to target serine/threonine sites that are followed by a glutamine (SQ/TQ sites) (Traven & Heierhorst 2005). Activities of LT are differentially regulated by phosphorylation of serine and threonine residues (Shi et al., 2005). Site directed mutagenesis of LT at known S/Q or T/Q sites was carried out. The aim was to find the specific S/Q site that is phosphorylated by ATR in response to DNA damage. Mutations were made in the LT plasmid using the site directed mutagenesis protocol (2.2.2.11 & 2.2.2.12). The serine or threonine were mutated and replaced with an alanine (Figure 3.1.9).

Figure 3.1.9 Site directed mutagenesis of LT phosphorylation sites.



Amino Acid Sequence SV40 LTAg

MDKVLNREES LQLMDLLGLE RSAWGNIPLM RKAYLKKCKE FHPDKGGDEE KMKKMNTLYK
KMEDGVKYAH QPDFGGFWDA TEIPTYGTDE WEQWWNAFNE ENLFCSEEMP SSDDEATADS
QHSTPPKKKR KVEDPKDFPS ELLSFLSHAV FSNRTLACFA IYTTKEKAAL LYKKIMEKYS
VTFISRHNSY NHNILFFLTP HRHRVSAINN YAQKLCTFSF LICKGVNKEY LMYSALTRDP
FSVIEESLPG GLKEHDFNPE EAEETKQVSW KLVTEYAMET KCDDVLLLLG MYLEFQYSFE
MCLKCIKKEQ PSHYKYHEKH YANAAIFADS KNQKTICQQA VDTVLAKKRV DSLQLTREQM
LTNRFNDLLD RMDIMFGSTG SADIEEWMAG VAWLHCLLPK MDSVVYDFLK CMVYNIPKKR
YWLFKGPIDS GKTTLAAALL ELCGGKALNV NLPLDRLNFE LGVAIDQFLV VFEDVKGTGG
ESRDLPSGQG INNLDNLRDY LDGSVKVNLE KKHLNKRTQI FPPGIVTMNE FSVPKTLQAR
FVKQIDFRAK DYLKHCLERS EFLLEKRIIQ SGIALLLMLI WYRPVAEFAQ SIQSRIVEWK
ERLDKEFSLS VYQKMKFNVA MGIGVLDWLR NSDDDDEDSQ ENADKNEDGG EKNMEDSGHE
TGIDSQSQGS FQAPQSSQSV HDHNQPYHIC RGFTCFKKPP TPPPEPET

Figure 3.1.9 Site Directed Mutagenesis of known LT phosphorylation sites. Image showing the known S/Q and T/Q sites on LT. The sites highlighted were chosen to be mutated in wild type pLT using site directed mutagenesis.

3.1.10 LT mutated plasmids are recognised by LT antibodies.

To ensure that the LT epitope recognition site for the LT antibodies, pAb101 and pAb419 was retained following mutation of the LT plasmid a simple co-immunoprecipitation was carried out in 293 cells. Using the GeneJuice method of transfection, either wild type LT plasmid DNA, or one of the mutant plasmid (pLTAg S120A, Q517A, S639A, S664/666A, S676A) were transfected into cells. Two days post transfection, cells were harvested and a fraction of the extract was immunoprecipitated with the LT antibody, pAb419. Samples were then prepared for western blot analysis and probed against the LT antibody pAb101 (Figure 3.1.10). Unlike 293T cells, 293 cells do not stably express the wild type LT protein (lane 1). Wild-type (wt) LT was detected in the pull down (Lane 2). All but one of the mutants (pLTAg S664/666A) were pulled down with the wild type LT antibody. This mutant contained a double mutation due to the close proximity of the S/Q sites. It was therefore possible that this mutation was detrimental to the correct LT protein folding as the antibody could not recognise the epitope of the mutated plasmid. The mutants that were capable of pull down with wild type LT antibody demonstrate that the epitope recognition site for the antibodies pAb101/419 on the mutated LT protein is maintained despite site directed mutagenesis.

Figure 3.1.10 LT mutated plasmids are recognised by LT antibodies.

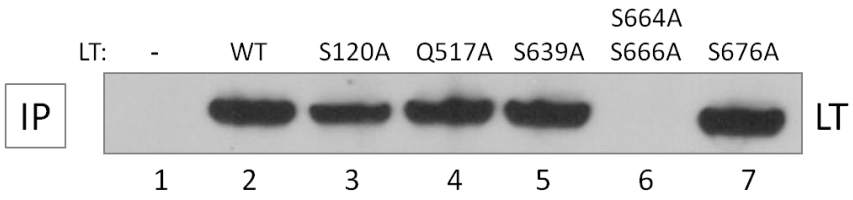


Figure 3.1.10 LT mutated plasmids are recognised by LT antibodies 293 cells were transfected with 2µg of pLT or with 2µg of one of the mutants; pLTAg S120A, Q517A, S639A, S664/666A, S676A. Forty eight hours post transfection, cells were harvested for immunoprecipitation. Protein extracts were immunoprecipitated with pAb419 (LT) and samples were prepared for western blot analysis probing against pAb101 (LT).

3.1.11 ATR targets LT SQ/TQ mutants for phosphorylation after etoposide treatment.

To determine if ATR/ATM phosphorylates a specific LT SQ/TQ site during an activated DNA damage response, co-immunoprecipitation was carried out using the LT mutated plasmids. The mutant and wild type LT plasmids were transfected into 293 cells using the GeneJuice method of transfection (2.2.1.3). Two days later, cells were treated with 50μM etoposide for 6 hours. Cells were treated for 6 hours because previous results indicated that LT protein levels do not substantially decrease after this time and phosphorylation of Chk1, indicative of an activated checkpoint response is still detected (Figure 3.1.7). Cells were then harvested and protein extracts were made. A fraction of the extract was then immunoprecipitated with the phospho-S/Q antibody that had been used previously (Figure 3.1.5 (b)). The extracts were then prepared for western blot analysis and the membranes were probed with the LT antibody (pAb101). If a LT mutant that had previously shown pull down with the wild type LT but failed to co-immunoprecipitate with the pS/Q antibody, it would reveal a specific site on LT that is phosphorylated by ATR/ATM in response to DNA damage. In Figure 3.1.11 wild type LT is phosphorylated by ATR/ATM in response to etoposide treatment (lane 2). The pLTAg S664/666A mutant was not detected by co-immunoprecipitation (lane 6). This mutant previously failed to co-immunoprecipitate with wild type LT and it was not detected in the input lanes therefore this mutation has resulted in loss of confirmation of the antibody recognition site. The remaining LT mutants all pull down with the pS/Q antibody. Therefore none of the potential ATR/ATM target residues are individually responsible for phosphorylation of LT following DNA damage. At this point it was decided that proteomic studies would be more effective at determining the phosphorylation of LT following DNA damage. Proteomic studies would allow for the identification of post translational modification of phosphorylation sites on the LT protein after etoposide treatment.

Figure 3.1.11 ATR targets LT SQ/TQ mutants for phosphorylation after etoposide treatment.

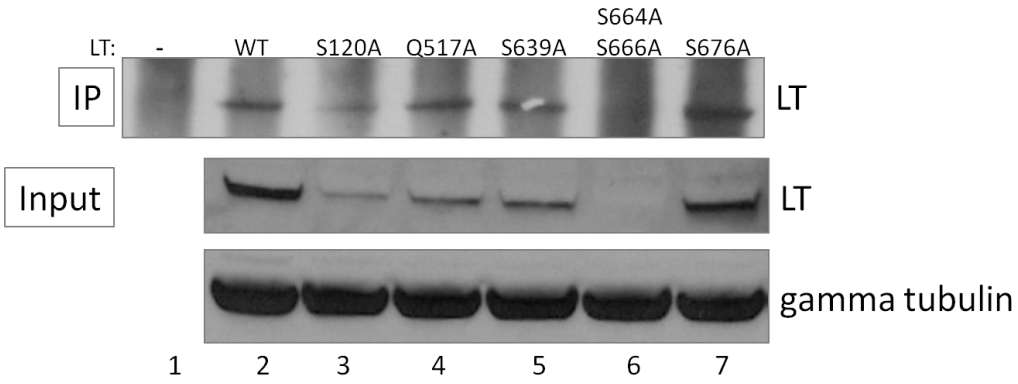


Figure 3.1.11 ATR targets SQ/TQ LT mutants for phosphorylation after etoposide treatment. 293 cells were transfected with 2µg of pLT or a pLTAg mutant. Two days post transfection cells were treated with 50µM of etoposide for 6 hours. Cells were harvested and protein extracts prepared. A fraction of the extract was immunoprecipitated with the phospho-S/Q antibody and prepared for western blot analysis against pAb101. Input extracts are also shown and gamma tubulin represents a loading control.

3.2 *In vitro* cell extract preparation of ATR and TopBP1 deficient cell extracts

In Chapter 3.1 replication assays showed that replication of the SV4O origin by the SV40 helicase LT is inhibited *in vivo* after treatment with etoposide and *in vitro* by linear digested DNA plasmid. Further to this, *in vitro* data suggested that the ATR kinases are responsible for the activation of the checkpoint pathway that regulates replication by LT. Co-immunoprecipitation using an antibody that binds to proteins that have been phosphorylated by ATR/ATM suggested that LT is targeted by these proteins in response to etoposide treatment *in vivo*. Chapter 3.2 focuses on determining whether the ATR kinase is indeed responsible for the regulation of LT-mediated SV40 origin plasmid replication.

3.2.1 ATR and TopBP1 are essential for activation of Chk1 protein kinase.

The *in vitro* results in Figure 3.1.4 suggest that ATR is responsible for regulating LT replication. This result was extended in the *in vivo* co-immunoprecipitation assay where LT was shown to be targeted for phosphorylation by ATR/ATM in response to etoposide treatment (Figure 3.1.5). Attempts were made to generate protein extracts lacking in ATR activity. The ATR^{-}deficient extract could then be used during *in vitro* replication studies to investigate LT-mediated replication during DNA damage without ATR activation. This would validate the hypothesis that ATR is the protein kinase that regulates LT mediated replication. Two proteins that were targeted for knockdown were ATR and TopBP1. TopBP1 plays the role of a sensor protein in the DNA damage response that is essential for ATR activation (Kumagai et al., 2006). TopBP1 coordinates activation of the DNA damage checkpoint response by coupling the interaction of the 9-1-1 checkpoint clamp at sites of ssDNA. In return this activates the ATR-ATRIP checkpoint kinase complex (Chapter 1.9.2). The activation of ATR triggers a cascade of phosphorylation events which ultimately leads to cell cycle arrest. Owing to the fact that previous studies have demonstrated the essential nature of ATR (making it unlikely to generate sufficient amounts of ATR depleted cell extract for replication assays) the dual approach of TopBP1

and ATR knockdown offered a greater chance of success. The phenotype of TopBP1 depleted cells is well defined.

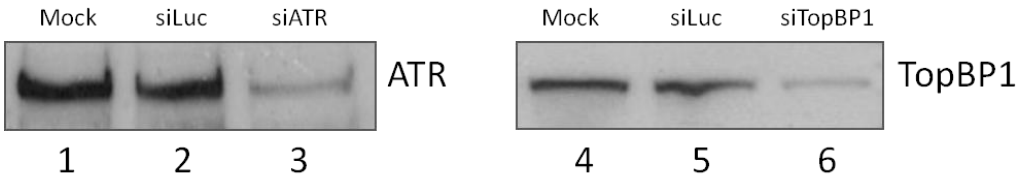
Oligonucleotides were designed against ATR and TopBP1 (siATR and siTopBP1). The siRNA oligonucleotides were transfected using the Lipofectamine 2000 protocol (2.2.1.4) into 293T cells that were harvested forty eight hours later for western blot analysis (Figure 3.2.1 (a)). Levels of knockdown were measured by probing the membranes for ATR and TopBP1. In lane 1 and 4, 293T cells were mock transfected. A control oligonucleotide (siLuciferase) was transfected into the cells in order to control for any protein knockdown due to the presence of an oligonucleotide targeting a non essential gene (lanes 2 and 3). In lane 3, ATR protein levels have greatly reduced confirming the knockdown by the siATR oligonucleotide. TopBP1 protein levels are also significantly reduced after transfection with siTopBP1 (lane 6). Transfecting siRNA oligonucleotides into cells can cause off target effects such as the silencing of non specific target genes and reduced protein levels (Jackson et al., 2003; Snove & Holen, 2004; Jackson & Linsley, 2004). Studies have demonstrated that the knock down of TopBP1 reduces the activation of ATR (Kumagai et al., 2006; Pedram et al., 2009). However due to the off target effects or siRNA oligonucleotides, ATR and TopBP1 protein levels should have been determined after knockdown of TopBP1 and ATR (respectively). This would have been useful to determine whether knock down of TopBP1 could reduce DNA damage signalling independently from ATR.

The effect of ATR and TopBP1 knockdown on the activation of the DNA damage response was investigated by examining Chk1 phosphorylation. TopBP1 was knocked down as described above. Twenty four hours later cells were treated with hydroxyurea (HU) (Figure 3.2.1 (b)). Hydroxyurea has previously been shown to activate ATR DNA damage pathways (Hammond et al., 2003). After HU treatment, the cells were harvested for western blot analysis. In lanes 1 and 2, cells were mock treated. In lane 2 Chk1 is phosphorylated at S317 in response to HU treatment, indicating an activated checkpoint response. In lanes 3 and 4, cells were transfected with the control oligonucleotide, siLuciferase. In lane 4, Chk1 is phosphorylated when cells are treated with HU. In lanes 5 and 6 the cells were transfected with siTopBP1. Levels of phosphorylated Chk1 protein are activated upon HU treatment in lane 6, however these levels are much lower than

those seen in lanes 2 and 4. This may be a result of a partial knockdown of TopBP1. A pan-Chk1 antibody that detects the total amount of Chk1 in each protein extraction was used as a control for Chk1 levels, and gamma tubulin antibody was used as a loading control. These results indicate that TopBP1 knock down in 293T cells results in reduced Chk1 phosphorylation. ATR was knocked down and cells treated with hydroxyurea in a similar way to TopBP1 (Figure 3.2.1 (c)). In lane 1 and 2, cells were mock treated. In lane 2 Chk1 is phosphorylated at S317 in response to HU treatment. In lanes 3 and 4, cells were transfected with the control oligo, siLuciferase. In lane 4, Chk1 is phosphorylated in response to HU treatment. In lanes 5 and 6 the cells were transfected with siATR. In lane 6, siATR transfected-HU treated cells do not appear to contain any phosphorylated Chk1 protein. A pan-Chk1 antibody that detects the total amount of Chk1 is each extraction was used as a control for Chk1 levels, and gamma tubulin antibody was used as a loading control. These results indicate that when ATR or TopBP1 are knocked down, DNA damage signalling through Chk1 kinase is significantly inhibited indicating a reduction in DNA damage signalling.

Figure 3.2.1 ATR and TopBP1 are essential for activation of Chk1 protein kinase.

(a)



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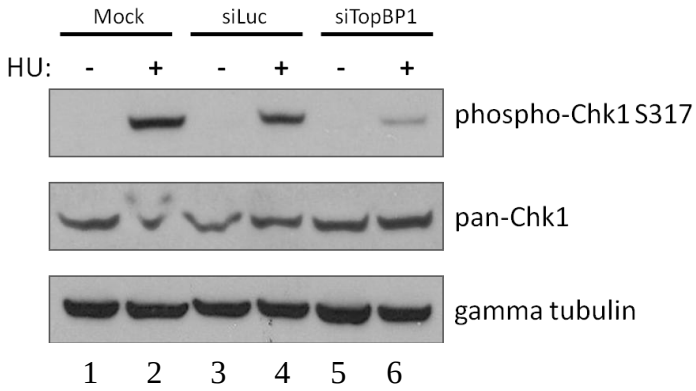


(c)

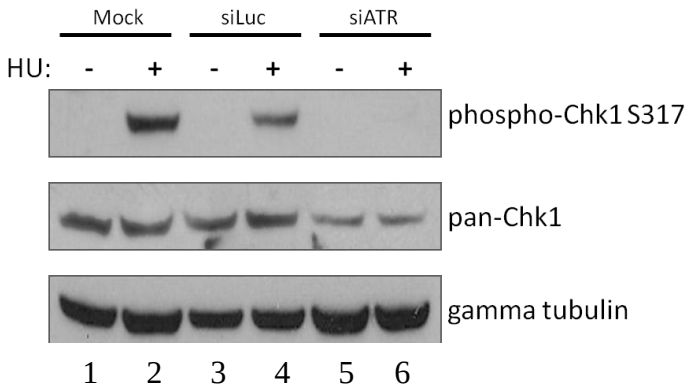


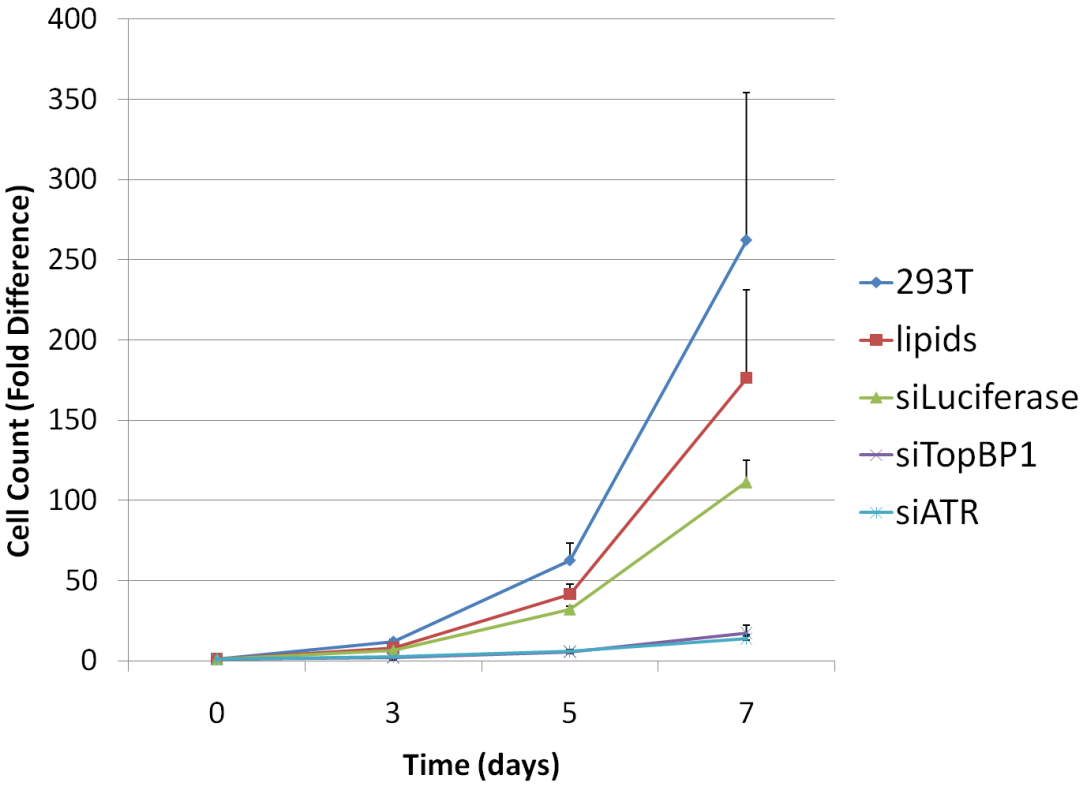
Figure 3.2.1 ATR and TopBP1 are essential for activation of Chk1 protein kinase. (a) 293T cells were mock transfected or transfected with oligonucleotides against Luciferase/ATR/TopBP1 genes. The oligonucleotide efficiency for ATR/TopBP1 knockdown was analysed by western blotting using antibodies against ATR and TopBP1. (b) 293T cells were mock transfected (lanes 1 and 2), transfected with siLuciferase (lanes 3 and 4) or

siATR (lanes 5 and 6). Lanes 2, 4, and 6 are samples that were treated with 3mM of HU for 4 hours. Cells were then harvested and analysed for western blotting for antibodies against phospho-Chk1 S317, pan-Chk1 and gamma tubulin. (c) cells were mock transfected (lanes 1 and 2), transfected with siLuciferase (lanes 3 and 4) or siTopBP1 (lanes 5 and 6). Lanes 2, 4, and 6 are samples that were treated with 3mM of HU for 4 hours. Cells were then harvested and analysed by western blotting for antibodies against phospho-Chk1 S317, pan-Chk1 and gamma tubulin.

3.2.2 Cell survival is diminished when ATR and TopBP1 are knocked down

Having established that siATR and siTopBP1 treatment of 293T cells inhibited DNA damage response signalling, the next step was to investigate the effects of ATR and TopBP1 knockdown on cell growth. This would determine whether it was possible to generate enough TopBP1/ATR knockdown extract for use in the *in vitro* DNA replication assays. TopBP1 knockdown in 293T cells has previously been shown to result in no aberrant changes in cell growth (Wright, 2007). This result was validated using the siTopBP1 oligonucleotide and cell growth during ATR knockdown was also investigated. A simple cell count assay was completed to investigate whether cells could replicate and grow in the absence of ATR/TopBP1 siRNA. HEK 293T cells were transfected with lipids, siLuciferase, siATR or siTopBP1 using the Lipofectamine2000 protocol. Two days after transfection cells were harvested, counted and 2 x 10^5 cells were replated in triplicate for each transfection. Every 2-3 days, cells were harvested, counted and replated (Figure 3.2.2). These experiments were carried out in triplicate, with the standard error bars shown. In contrast to the findings from Wright, 2007 TopBP1 appears to be essential for normal 293T cell growth because transfected cells were unable to replicate efficiently. ATR is also essential for cellular replication. Since TopBP1 and ATR appear to be essential for normal cell growth *in vivo*, it would be very difficult to generate cell extracts depleted of ATR activity for *in vitro* replication assays.

Figure 3.2.2 Cell survival is diminished when ATR and TopBP1 are knocked down



3.2.2 Cell survival is diminished when ATR and TopBP1 are knocked down. 293T cells were either untreated, or treated with siLuciferase/siTopBP1/siATR/mock. A cell count assay was performed after knockdown. Day 0 is representative of 2 days post transfection. Cells were harvested and counted at the time points indicated. The experiments were carried out three times in triplicate and this data shows the standard error of the three experiments. Values are shown as a fold difference where day zero is standardised to 1.

3.2.3 Long term cell survival is abolished when ATR and TopBP1 are knocked down

To validate previous findings that TopBP1 and ATR are essential to cell survival crystal violet colony survival assays were carried out over fourteen days (2.2.1.6). In these assays TopBP1 or ATR were knocked down using TopBP1/ATR targeted siRNA and similarly to the previous cell count assays; siLuciferase and mock (lipofectamine only) controls were also used. The results for the colony survival assay are shown in Figure 3.2.3. This experiment was carried out three times in triplicate; this figure is representative of the triplicate plates from the same repeat experiment. It is visually very clear that knocking down TopBP1 or ATR expression has a profound effect on 293T cell growth over long periods of time. The results are clear that TopBP1 and ATR deficient 293T cells are unable to grow, ruling out the possibility of generating enough knocked down lysate for *in vitro* replication assays.

3.2.3 Long term cell survival is abolished when ATR and TopBP1 are knocked down

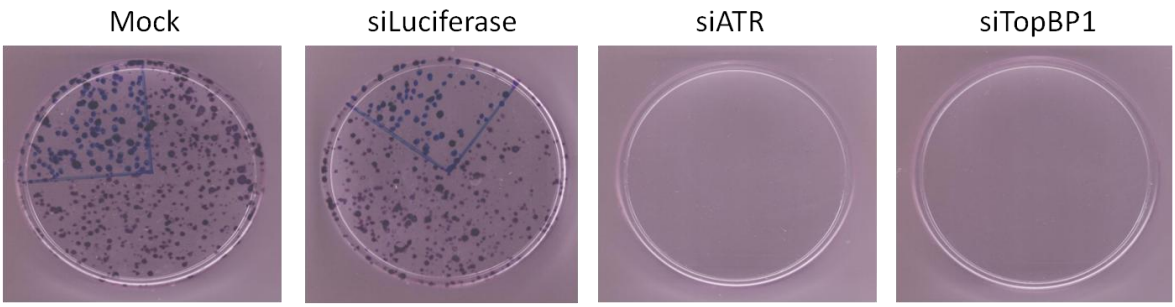


Figure 3.2.3 Long term cell survival is abolished when ATR and TopBP1 are knocked down. Photographs from 293T colony survival assay. These experiments were carried out three times in triplicate. The plates shown here are representative of those obtained for the three experiments. 293T cells were plated out and mock transfected, siLuciferase transfected, siATR transfected or siTopBP1 transfected. All colonies were visualized fourteen days following siRNA treatment using a 0.5% crystal violet solution.

3.3 HPV16 E2 is stabilised by E1 and etoposide

In Figure 3.1.8, HPV16 E1 protein levels appear to increase in the presence of etoposide although the mechanism used to explain this observation was not investigated. The E1 and E2 proteins interact during the virus life cycle at the viral origin therefore it was feasible that E2 protein levels may also increase during etoposide treatment. E2 is a multifunctional protein and any changes in protein levels would affect many stages of the virus life cycle such replication and regulation of transcription. Changes in E2 protein levels will affect levels of virus replication therefore the levels of E2 protein in the presence of E1 was also investigated.

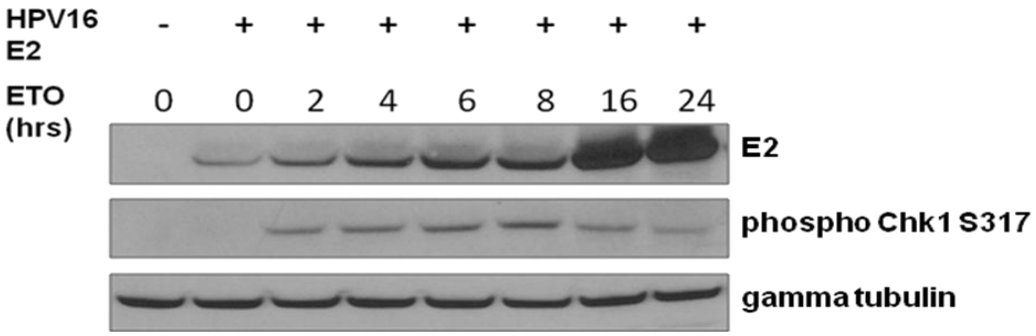
3.3.1. HPV16 E2 is stabilised by prolonged etoposide treatment

In Figure 3.1.2, the HPV viral helicase E1 avoids phosphorylation by ATR during an activated DNA damage response. This result may explain why E1 protein levels were shown to increase after long term exposure to etoposide (Figure 3.1.8). The E2 protein is also required to initiate HPV replication therefore the consequences of prolonged etoposide treatment on E2 protein levels was also investigated. 293T cells were calcium phosphate transfected with HPV16 E2. Two days post transfection cells were untreated or treated with etoposide for 2/4/6/8/16 and 24 hours. Cells were then harvested and prepared for western blot analysis. In a similar trend to E1, there appears to be an increase in E2 protein levels following long term etoposide exposure (Figure 3.3.1(a)). This result suggests a stabilisation of E2 following etoposide treatment. To determine whether the increase in E2 protein levels is a post translational event or simply due to increased E2 RNA levels, E2 RNA was measured after 24 hours of etoposide treatment. E2 transfected 293T cells were untreated or treated with 50μM of etoposide. Cells were harvested and RNA was extracted using Qiagen RNeasy kit and contaminating DNA removed using the DNA-free kit (2.2.2.15 & 2.2.2.16). Complimentary DNA was synthesised using SuperScript III reverse transcriptase kit (2.2.2.17). E2 levels were detected using real time PCR and standardised with a beta actin control (Figure 3.4.2. (b)). In the presence of etoposide, E2 RNA levels increase to approximately 5 fold more, than untreated E2 transfected cells (p

value >0.05). Therefore although there is an increase increase in E2 mRNA levels after prolonged etoposide treatment it is not a significant difference.

Figure 3.3.1 HPV16 E2 is stabilised by prolonged etoposide treatment

(a)



(b)

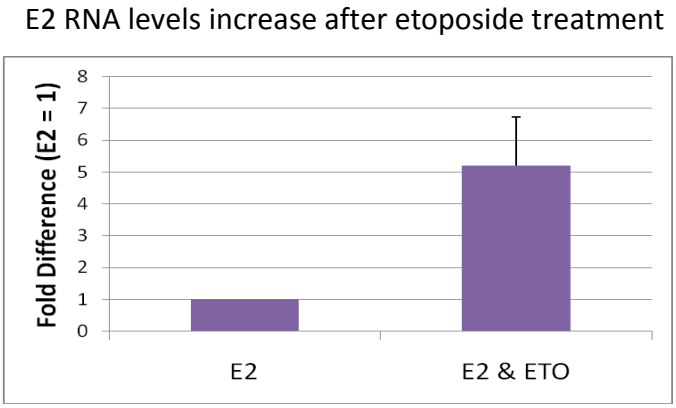


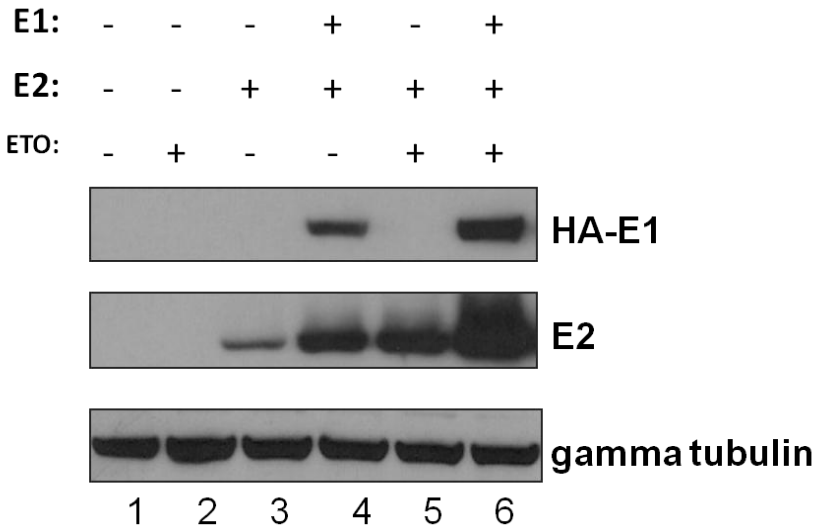
Figure 3.3.1 E2 is stabilised by prolonged etoposide treatment (a) 293T cells were transfected with 2µg of HPV16 E2 plasmid. 48 hours later cells left untreated (−) or treated with 50µM of etoposide (+) for the times indicated. Cells were harvested and prepared for western blot analysis and probed for E2 (TVG261) and gamma tubulin. Phosphorylated Chk1 S317 serves as a control to confirm activation of the DNA damage response. (b) 293T cells were transfected with 2µg of HPV16 E2 plasmid and untreated or treated with 50µM of etoposide for 16 hours. RNA extracted from the cells was converted to cDNA and levels of E2 were detected using real-time PCR. The graph represents 3 experiments with results normalised to beta actin. The values shown are representative of fold difference where E2 is equal to 1.

3.3.2 HPV16 E2 is stabilised by E1 through post translational modifications.

The consequence of E1 and etoposide on E2 protein levels was next investigated. 293T cells were transfected with HPV16 E2, and either the HPV16 HA tagged E1 or an empty vector plasmid. Two days post transfection the cells were treated with 50μM etoposide for 24 hours or were left untreated. The treated cells were then harvested and prepared for western blot analysis probing with antibodies against E2 (TVG261), E1 (HA) and gamma tubulin (Figure 3.3.2 (a)). E2 protein is detectable when transfected by itself (lane 3). When co-transfected with the HA tagged E1 plasmid, E2 protein levels increase (lane 4). In the presence of etoposide, E2 protein levels again increase. There appears to be an additive effect on E2 protein levels when both E1 and etoposide treatment are present (lane 6). The increase seen in E2 protein levels in etoposide treated cells is attributed to a rise in E2 mRNA (Figure 3.3.1). To investigate whether the changes in E2 protein levels in the presence of E1 is a result of additional E2 mRNA or due to post translational modifications, E2 RNA levels were analysed (Figure 3.3.2 (b)). There is an increase in E2 RNA in the presence of E1 by around two fold however this is not significantly different as the p value is >0.05.

Figure 3.3.2 HPV16 E2 is stabilised by E1 through post translational modifications.

(a)



(b)

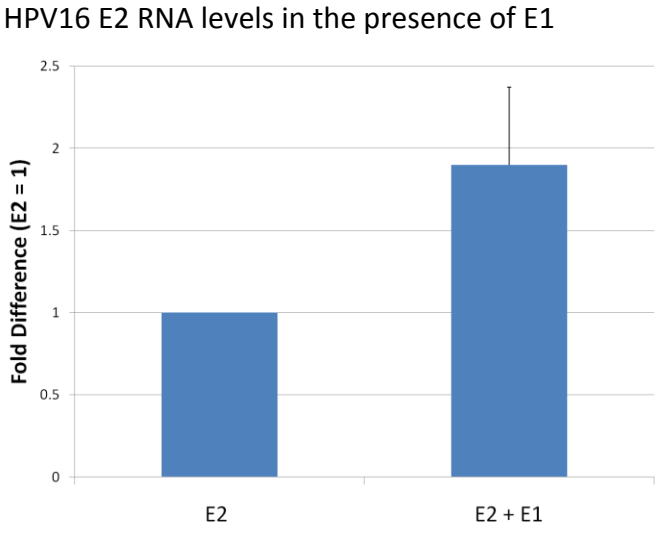


Figure 3.3.2 HPV16 E2 is stabilised by E1 through post translational modifications. (a) Cells were transfected with 2µg of HPV16 E2 (+), 100ng HPV16 E1 HA tagged (+) and left untreated (-) or treated with 50µM etoposide (+) for 16 hours. Cells were then harvested and prepared for western blot analysis by probing for E2 (TVG261), E1 (HA) and gamma tubulin as the loading control. (b) 293T cells were transfected with 2µg of HPV16 E2 plasmid with or without 1µg of HPV16 E1 HA tagged. RNA extracted from the cells was converted to cDNA and levels of E2 were detected using real-time PCR. The graph represents 3 experiments with results normalised to beta actin.

3.3.3 Etoposide arrests HPV16 E1 and E2 transfected 293T cells at the G_2/M checkpoint

Etoposide inhibits the cell cycle by creating double stranded DNA breaks. It has been shown that HPV16 E2 is stabilised in the S phase of the cell cycle in E2 stably expressing U2OS cells (Johansson et al., 2009). It was possible that the stabilisation of E2 observed with long term etoposide exposure and E1 co-transfection could be a consequence of an S phase arrest. Flow cytometry analysis was carried out to determine the cell cycle profile of E2 and E1 transfected 293T cells that were either untreated or treated with etoposide. Briefly, 293T cells were mock calcium phosphate transfected or transfected with HPV16 E2 (with and without HA tagged E1). Two days post transfection the cells were treated with etoposide for 16 hours and prepared for flow cytometry analysis. The profiles in the top row are untreated and the bottom row is the profiles of cells treated with etoposide (Figure 3.3.3). Mock transfected 293T cells treated with etoposide arrest the cells at the G_2/M checkpoint (bottom row, left panel). Cells transfected with E2 have a normal cycle profile (top row, middle panel). When treated with etoposide these cells also arrest at the G_2/M checkpoint (bottom row, middle panel). E2 and E1 co-transfected cells have a normal profile (top row, right panel). After etoposide treatment the cells arrest at the In G_2 phase (bottom row, right panel). Etoposide arrests E1 and E2 transfected 293T cells predominantly at the G_2 phase of the cell cycle. Etoposide and E1 do not arrest cells in S phase and therefore E1 does not increase E2 protein levels by arresting the cell cycle in S phase. Therefore the mechanisms by which E1 increases E2 protein levels were investigated further.

Figure 3.3.3 Etopsoide arrests HPV16 E1 and E2 transfected 293T cells at the G_2/M checkpoint

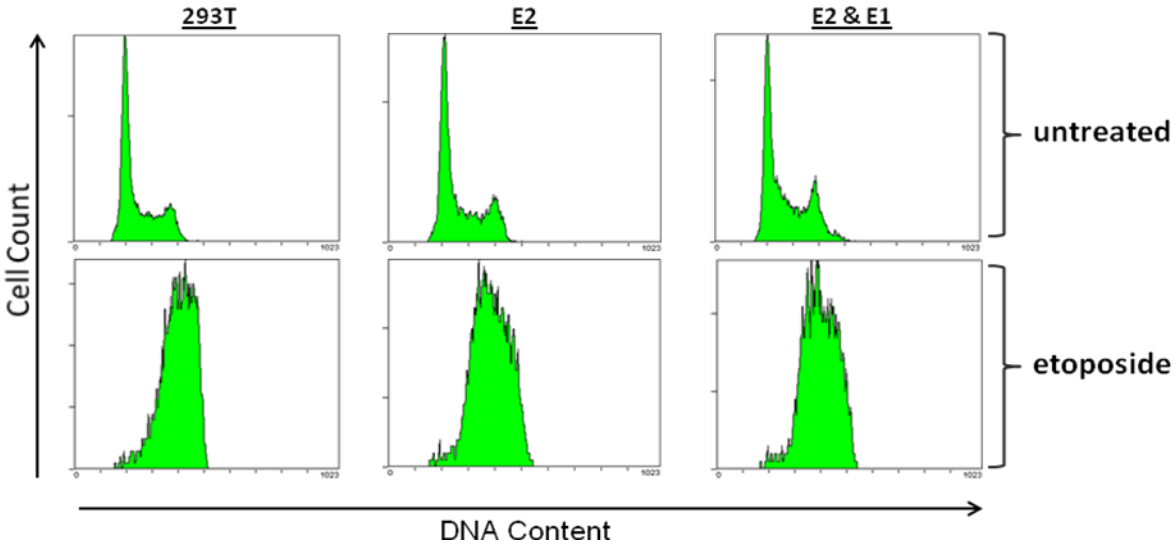


Figure 3.3.3 Etopsoide arrests HPV16 E1 and E2 transfected 293T cells at the G_2/M checkpoint. 293T cells were either mock calcium phosphate transfected, transfected with 2µg of HPV16 E2 or co transfected with E2 and 1µg of HA-tagged HPV16 E1. Forty eight hours later cells were left untreated or treated to 50µM of etoposide for 16 hours. Cells were then harvested and prepared by propidium iodide staining for flow cytometry analysis.

3.4 HPV16 E2 has increased stability and function due to a direct interaction with the viral helicase E1.

The results in Chapter 3.3 indicated that HPV16 E1 increases E2 protein levels through post translational mechanisms. E2 RNA analysis showed that there was an increase in RNA by E1 to around 2 fold higher, however this was not significantly different (Figure 3.3.2 (b)). Cell cycle analysis revealed that E1 transfected cells were not arrested in S phase ruling out this mechanism to explain E2 stabilisation (Figure 3.3.3). This chapter investigates the E1.E2 interaction, the effects it has on E2 protein stability and possible mechanisms that E1 uses to alter the levels of E2 protein within the cell. E2 is a multifunctional protein within the virus life cycle and therefore the functional consequences of this interaction were also investigated.

3.4.1 HPV16 E1 increases E2 protein levels.

It became obvious that E2 protein levels were higher in the presence of E1 whilst investigating the effect that etoposide had on both proteins stabilisation (Figure 3.3.2(a)). In the western blot, E2 protein levels increased in the presence of the HA-tagged E1 expression vector. As further evidence of stronger E2 expression in the presence of E1 a different E1 expressing vector was used in Figure 3.4.1. HPV16 E2 (pCMV-E2) and the alternative HPV16 E1 plasmid (pCMV-E1) were calcium phosphate transfected into 293T cells. Forty eight hours post transfection, cells were harvested, and protein was extracted and prepared for western blot analysis. There is no antibody for HPV16 E1 however the E1 protein expressed from this plasmid is functional as it is able to replicate HPV16 origin plasmid in the presence of an E2 expressing plasmid (King et al., 2010). E2 protein bands appear to be stronger in the presence of the alternative E1 plasmid. This result supports previous evidence that E1 can increase E2 protein levels.

Figure 3.4.1 HPV16 E1 increases E2 protein levels.

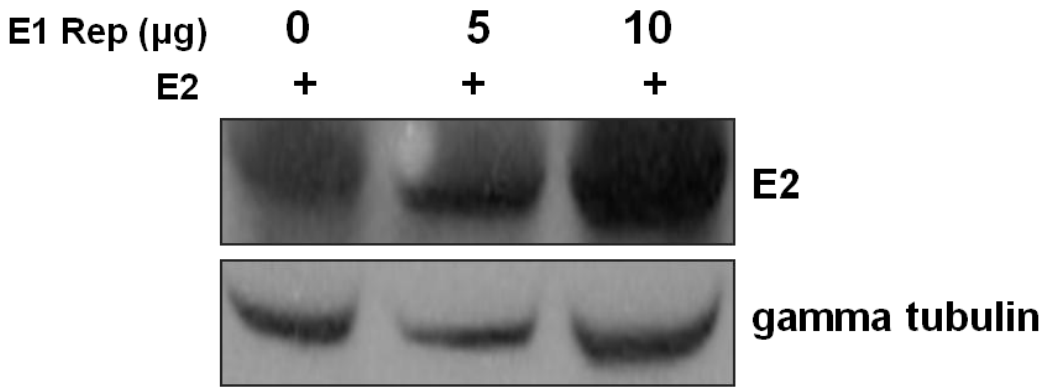


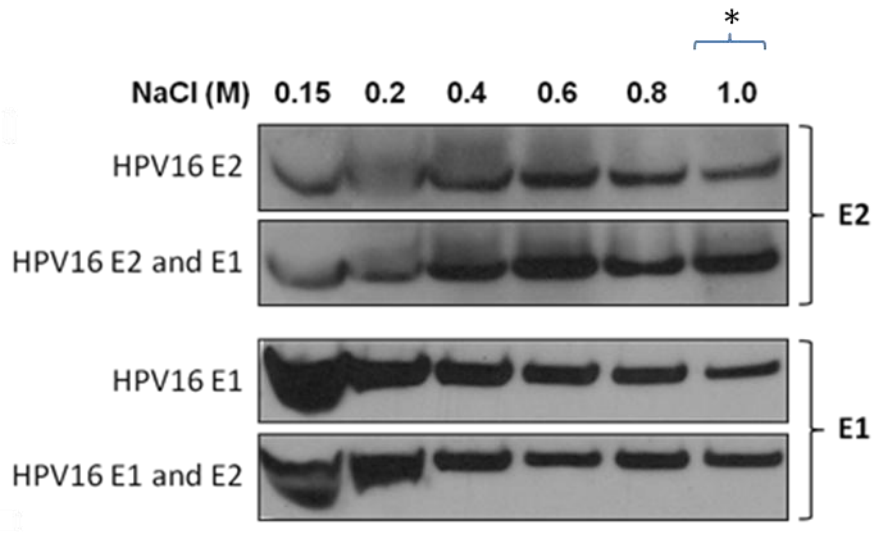
Figure 3.4.1 HPV16 E1 increases E2 protein levels. 293T cells were transfected with 2µg of E2 and either 0/5/10µg of the alternative E1 plasmid. Forty eight hours later the cells were harvested and prepared for western blot analysis to probe for E2 and gamma tubulin. There is no available antibody available for the alternative HPV16 E1 plasmid.

3.4.2 HPV16 E1 can alter E2 chromatin affinity

HPV16 E2 that interacts with chromatin is more stabilised than E2 that does not have a high affinity for chromatin (Donaldson et al., 2007). During salt extraction assays, E2 is shown to be detectable in soluble cell extracts with a high salt concentration more than low salt concentration extracts. From this previous work it was hypothesised that E1 may increase E2 protein levels by shifting E2 onto chromatin. Salt extractions were carried out in 293T cells to investigate this hypothesis. HPV16 E2 and HPV16 E1 tagged with HA were co-transfected into the cells and forty eight hours post transfection the cell lysate was serially extracted in increasing concentrations of NaCl in CSK (0.15/0.2/0.4/0.6/0.8/1.0M salt) (2.2.2.20). The extractions were prepared for western blot analysis and probed with antibodies against E1 (HA) and E2 (TVG261) (Figure 3.4.2(a)). The results from Figure 3.4.2 (a) are representative of three independent experiments. Visually there appears to be a slight shift in E2 towards the 1.0M salt extraction whilst in the presence of E1. To confirm this by densitometry the blots were quantified using Image J software and graphed showing a percentage of the total amount of E2 from all the extractions (Figure 3.4.2 (b)). There is a significant difference between E2 at 1M in the absence or presence of E1. The remaining extracts (0.15-0.8) did not show a significant difference between E2 levels with and without E1 ($p>0.05$).

Figure 3.4.2 HPV16 E1 can alter E2 chromatin affinity

(a)



(b)

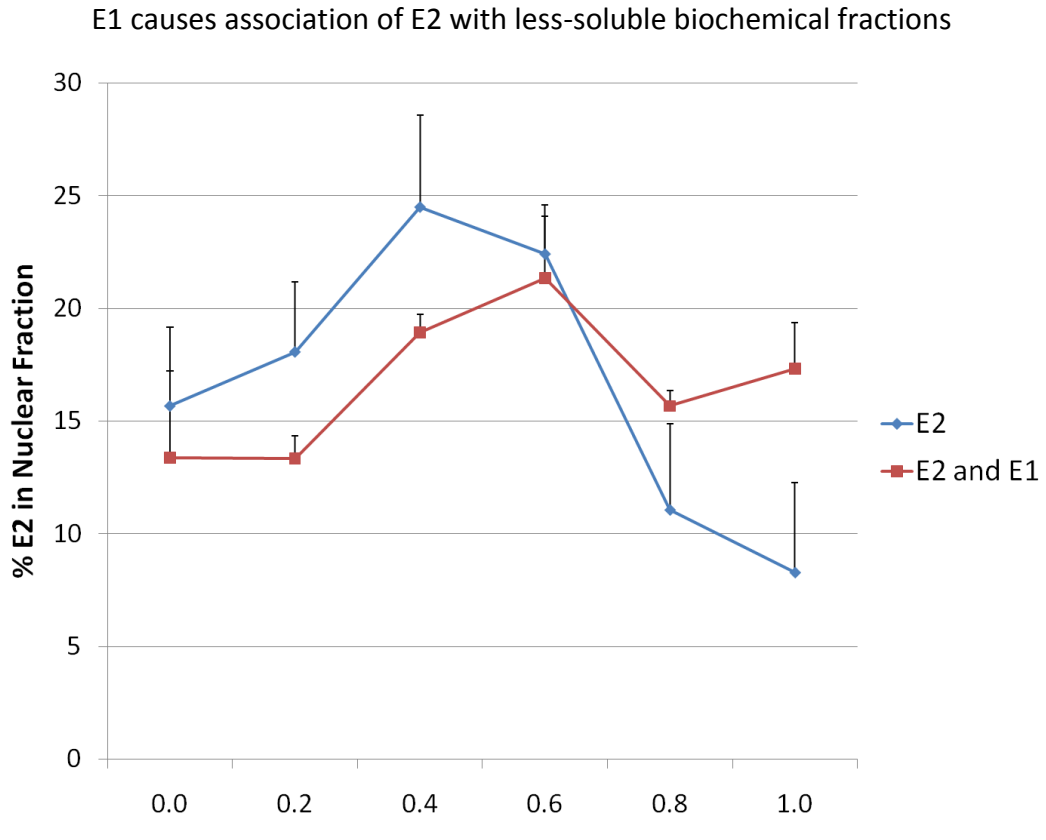


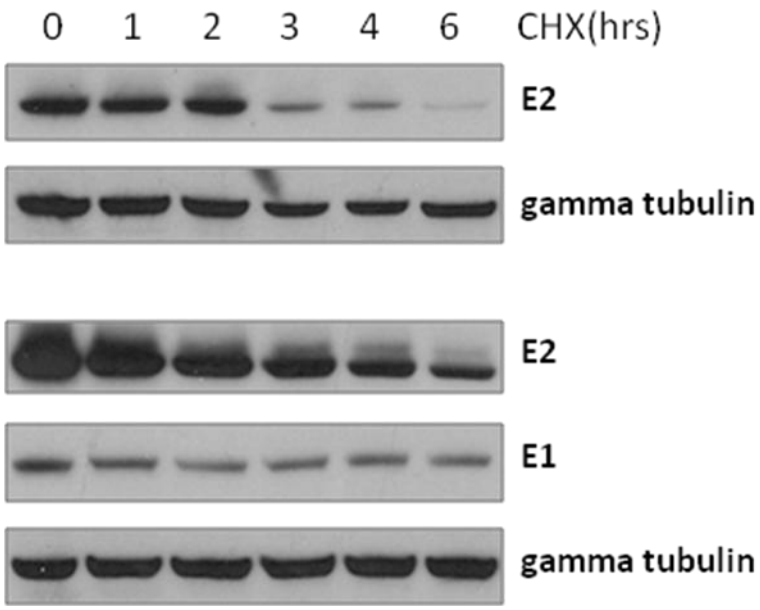
Figure 3.4.2 HPV16 E1 can alter E2 chromatin affinity. 2µg of HPV16 E2 with or without 1µg of HPV16 E1HA were transfected into 293T cells. Proteins were serially extracted using Triton-X lysis buffer with salt concentration ranging from 0.15 to 1M. The E2 and E1 proteins were detected using western blot analysis using the TVG261 antibody for E2 detection and HA antibody for E1 detection. Statistical significance by Students *t*-test is indicated as * ($p<0.05$). (b) Three repeats were quantified using Image J software. The graph displays the mean and the standard error of the mean for each extraction. The value for E2 was calculated as a percentage of the total amount of E2 for all the extractions (0.15-1.0M).

3.4.3. HPV16 E2 is stabilised by E1.

E2 protein levels increase in the presence of E1. E1 may therefore increase the half-life of E2 by increasing the stability of the protein. Cycloheximide (CHX) drug treatment was used to investigate whether E1 extends E2 protein stability as this drug is known to inhibit protein biosynthesis. HPV16 E2 was transfected in 293T cells with and without HA tagged E1 expression plasmid. The transfected cells were treated with 100µM of the protein synthesis CHX for either 0/1/2/3/4 or 6 hours. The cells were then harvested and protein was extracted for western blot analysis using antibodies against E1 (HA) and E2 (TVG-261). In Figure 3.4.3(a) E2 protein levels are reduced as a result of CHX treatment. This reduction is less obvious when E1 is co-transfected with E2. Three repeats were quantified using Image J software using gamma tubulin as a loading control (Figure 3.4.3 (b)). The graph shows the mean and the standard error of the mean from three independent experiments. E2 protein half life was calculated using the Microsoft Excel programme. This programme predicted the best fit line for each data set and the equation of this line was used to calculate the half life at 50% of E2 protein level without CHX treatment. The half life of E2 alone was calculated to be 2.6 hours and in the presence of E1 it increases to 3.5 hours.

Figure 3.4.3 HPV16 E2 is stabilised by E1.

(a)



(b)

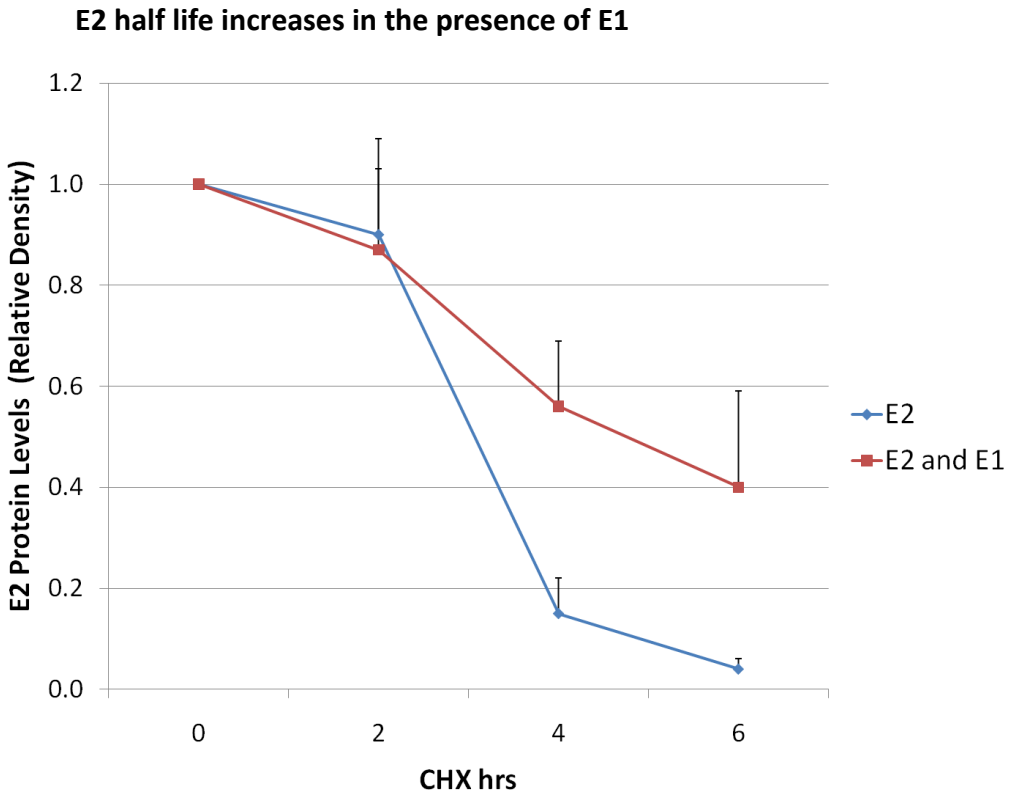
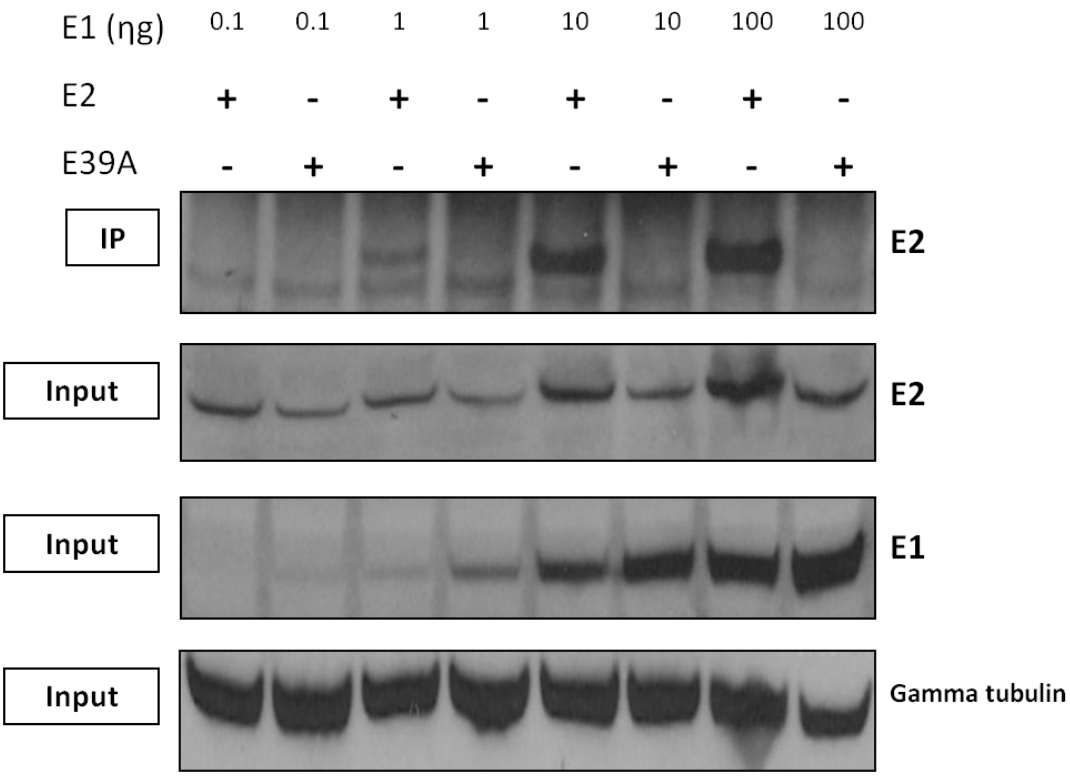


Figure 3.4.3 HPV16 E2 is stabilised by E1. (a) 293T cells were transfected with 50ng of E1-HA and 1 μg of E2. Forty eight hours post transfection the cells were treated with 100μM CHX for the time points indicated and cells were then harvested for western blot analysis for E2 (TVG261) and E1 (HA). (b) Protein bands were quantified using E2 levels relative to pre-CHX treatment and the gamma tubulin signal was used for normalisation of each band. The graph represents the mean and standard error of the mean for three independent experiments. Image J software was used for quantification of the protein bands and therefore made it possible to estimate the half life of E2.

3.4.4 HPV16 E2 E39A mutant does not interact with HA-tagged E1

E1 and E2 have been shown to interact at glutamic acid 39 on HPV16 E2. A mutant E2 expression plasmid harbouring a glutamic acid to alanine substitution at amino acid position 39 is unable to support HPV replication but does not loose its transactivation function (Sakai et al 1996). This mutation was synthesised into the HPV16 E2 expression plasmid used throughout this thesis by Mary Donaldson using site directed mutagenesis (2.2.2.11 & 2.2.2.12).. The mutated E2 expressing plasmid was then utilized to investigate whether E1 stabilisation of E2 is a result of a direct interaction between both proteins. To test whether E2 expressed from the mutated plasmid had lost its ability to interact with E1 protein, co-immunoprecipitation experiments were carried out in 293T cells. The E39A E2 mutant or E2 wild type expressing plasmids were co-transfected with 0/0.1/1/10/100ng of E1-HA expression plasmid. Two days post transfection the cells were harvested and proteins were extracted. Protein lysates were incubated with an antibody against HA to detect E1 (Figure 3.4.4). E2 wild type and E39A mutant pull down was determined using the E2 antibody, TVG261. The western blot shows that the E2 wild type co-immunoprecipitates with E1. No pull down was detected with the E39A mutant. Input lanes also show that wild type E2 protein bands appear to increase in intensity with increasing amounts of transfected E1. On the contrary E39A mutant show no change. This result suggests the E39A mutant may not be stabilised by E1. Future work was focused on testing this theory.

3.4.4 HPV16 E2 E39A mutant does not interact with HA-tagged E1



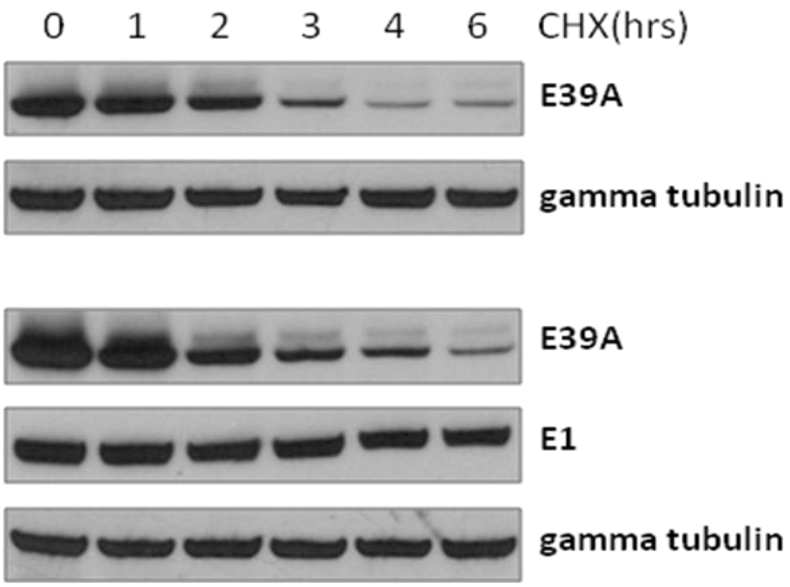
3.4.4 HPV16 E2 E39A mutant does not interact with HA-tagged E1. 293T cells were transfected with a concentration range of E1-HA plasmid (0.1/1/10/100ng) and either 2µg of E2 wild type or 2µg of E39A E2 mutant. Cell lysates were harvested forty eight hours later and protein extracted. To detect E2/E39A interaction with E1, lysates were immunoprecipitated with the anti-HA antibody. Samples were then prepared for western blot analysis and immunoblotted with antibodies against E2 (TVG 261), E1 (HA) and gamma tubulin as a loading control. The image shown is representative of three independent experiments.

3.4.5 HPV16 E2 stabilisation by E1 is a result of a direct E1.E2 interaction

E1 and E2 interact during the virus life cycle to mediate replication from the viral origin. Therefore E1 may stabilise E2 to enhance viral replication during the viral life cycle. This may be important during viral vegetative replication in the differentiating epithelial cells. E1 may increase the stability of E2 as a result of the proteins interacting directly. Therefore the ability of E1 to stabilise the E2 E39A mutant was assessed next (Figure. 3.4.5 (a)). This figure is representative of three independent experiments. E2 E39A and E1 transfected 293T cells were treated with 100µM of the protein synthesis inhibitor cycloheximide (CHX) for the indicated time points. The cells were then harvested and prepared for western blot analysis and probed for E2 (TVG 261) and E1 (HA). The E2 E39A protein bands appear to lose intensity after 2 hours of CHX treatment. In the presence of E1, E39A protein levels show only a slight increase in intensity compared to E39A alone at 2 hours. The actual E39A half life was calculated from three independent experiments using ImageJ software (Figure 3.4.5 (b)). This figure represents the quantified results with associated mean and standard errors of the mean. The half life of E39A was calculated as 2.8 hours. In the presence of E1, the half life was marginally increased to 2.9 hours. This result suggests that the E1.E2 interaction is essential for the increase in E2 stability by the E1 protein.

Figure 3.4.5 HPV16 E2 stabilisation by E1 is a result of a direct E1.E2 interaction

(a)



(b) The E39A E2 mutant does not show increased stabilisation by E1

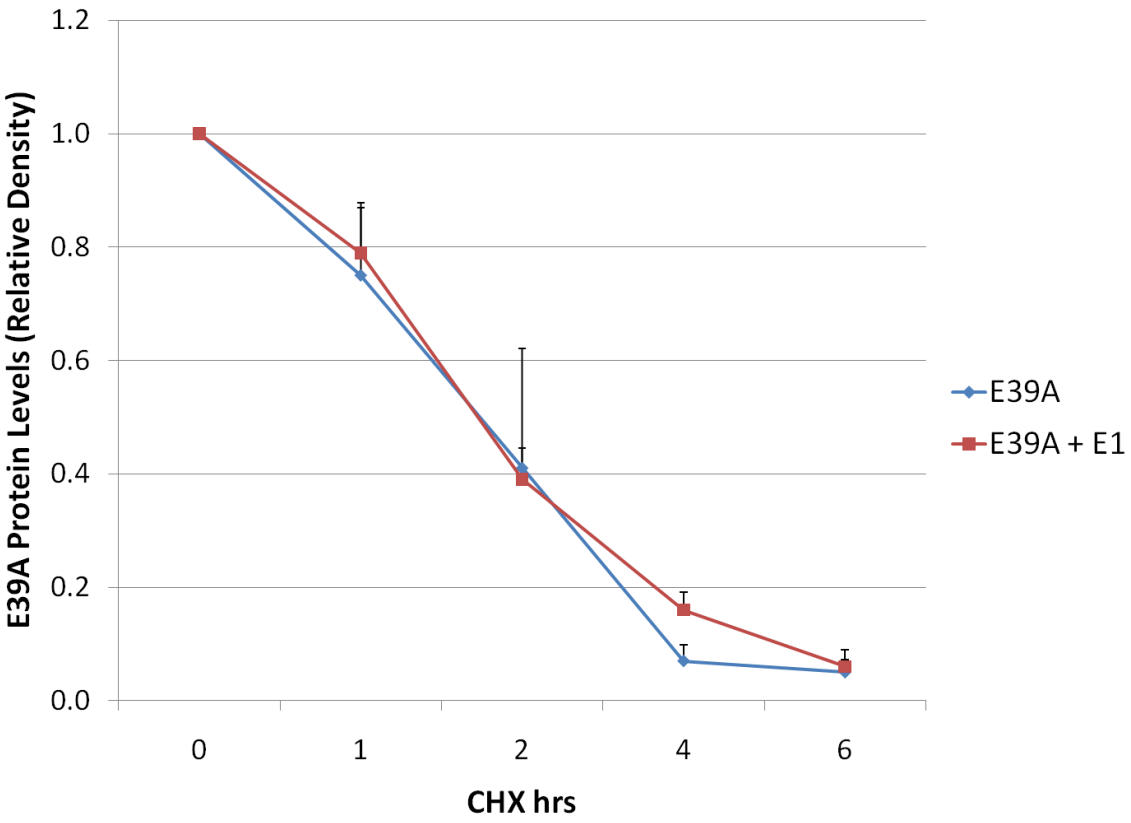


Figure 3.4.5 HPV16 E2 stabilisation by E1 is a result of a direct E1.E2 interaction a) 293T cells were calcium phosphate transfected with 2µg of E39A plasmid and 50ng of either E1-HA or an empty vector plasmid DNA. Two days post transfection, cells were treated with CHX (100µM) for the time points indicated and harvested for western blot analysis. E39A was detected using the TVG261 antibody. E1 was detected using the HA antibody. (b) E39A half life in the absence and presence of E1 was calculated using densitometry. Protein bands were quantified using E39A levels relative to pre-CHX treatment and the gamma tubulin signal was used for normalisation of each band. The graph represents the mean and standard error of the mean from three independent experiments. Image J software was used for quantification of the protein bands and therefore made it possible to estimate the half life of E39A.

3.4.6 HPV16 E1 enhances E2 functional activity as a result of the E1.E2 interaction.

Studies have demonstrated that chromatin adaptor protein, Brd4, can alter E2 protein stability *and* functional activity therefore it was hypothesised that a consequence of the direct interaction between E1 and E2 would be a change in E2 transcriptional activity (Schweiger et al., 2006; Lee & Chiang, 2009; Gagnon et al., 2009; Zheng et al., 2009). To investigate any changes that E1 may influence on E2 transcriptional activity, transcriptional assays were undertaken that use the tk6E2 plasmid containing six HPV16 E2 binding sites, a downstream thymidine kinase promoter and a luciferase reporter gene (Vance, 1999). The HPV16 E2 expression plasmid (1/10/100ng) and tk6E2 plasmid was transfected into 293T cells with and without HPV16 E1-HA (Figure 3.4.6 (a)). Luciferase activity was normalised using the tk6E2 plasmid only (normalised to 1) and the values shown are represented as fold differences (*n*-fold). At all concentrations of E2, luciferase expression increased with increasing amounts of E2 and there was no trans-repression at the highest amount of E2 used (blue bars). The effect of an E1-E2 interaction was investigated by co-transfection of the HA-tagged E1 with HPV16 E2 plasmids (red bars). There is an approximate 8 fold increase in transcriptional activation in the presence of E1 compared to E2 alone when 1ng of E2 plasmid is co-transfected with HA-tagged E1 (p value < 0.05). There is an approximate 9 fold increase when 10ng of E2 plasmid is transfected with E1 expression plasmid (p value < 0.05). E1 results in an approximate 4 fold increase with 100ng of E2 plasmid. However because the p value is 0.007, there is no significant difference between these data sets. Compared to 1ng and 10ng of E2, E1 enhancement of E2 transcriptional activation at 100ng of E2 is reduced. This may be attributed to E2 binding site saturation.

The increase in E2 transcription by the presence of E1 may be attributed to a direct interaction between these proteins. To test this hypothesis, the E2 mutant plasmid that has reduced interaction with E1 was used in a similar experiment to Figure 3.4.6 (a). The only deviation between the experiments was the use of E2 E39A mutant in place of E2 wild type expression plasmid, all plasmid concentrations were kept the same (Figure 3.4.6 (b)). At the concentrations 1/10/100ng, luciferase expression of E2 E39A mutant is higher than with the reporter plasmid alone, demonstrating that this mutant retains

transcriptional functions comparable to wild type E2. The transcriptional activity of wild type E2 and E2 E39A expression plasmids are very similar. For all concentrations of E39A (1/10/100ng) there is only a small rise detected in E2 transcriptional activation when E1 is present (red bars). The 2 fold increase in E2 transcription and small increase in E2 E39A protein half life seen previously in the presence of E1 (Figure 3.3.3) may be a result of a weak E1 and E2 interaction that was not detected by western blot (Figure 3.4.4). In Figure 3.3.2 (b) E2 RNA is increased by almost two fold when co-transfected with HA-E1 plasmid. The E2 and E39A ORFs are cloned into the same plasmid so contain the same promoter and enhancer sequences. Therefore any increase in transcription or half life may reflect the increase in RNA levels seen previously. The data presented shows evidence that a direct interaction between E1 and E2 is accountable for an increase in E2 functional activity.

Figure 3.4.6 HPV16 E1 enhances E2 functional activity as a result of the E1.E2 interaction.

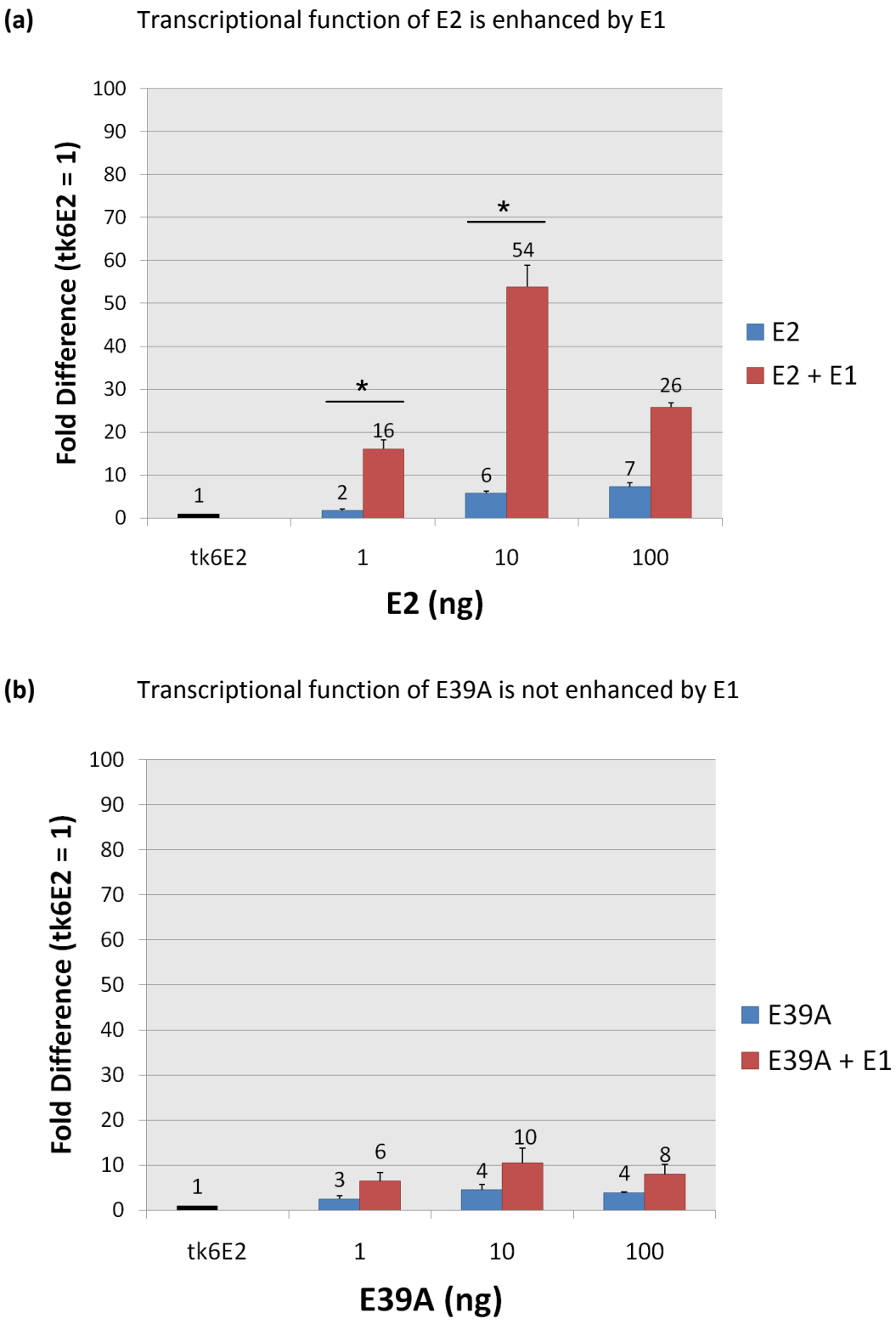


Figure 3.4.6 HPV16 E1 enhances E2 functional activity as a result of the E1.E2 interaction. (a) One microgram of the tk6E2 reporter plasmid was co-transfected with

increasing amounts of HPV16 E2 in the absence (blue bars) or presence (red bars) of 50ng HPV16 E1HA. Cells were harvested 48 hours post transfection and assayed for luciferase activity (Promega). Luciferase activity for the lysate containing tk6E2 only (black bar) is normalized to 1 and other activities are calculated as a fold difference (*n* fold). The results shown are an average of three independent experiments with mean and standard error of the mean bars displayed. Statistical significance by Students *t*-test is indicated as * ($p<0.05$). (b) One microgram of the tk6E2 reporter plasmid was co-transfected with increasing amounts of HPV16 E39A mutant in the absence (blue bars) or presence (red bars) of 50ng of HPV16 E1. Cells were harvested 48 hours post transfection and assayed for luciferase activity. Luciferase activity for the lysate containing tk6E2 only (black bar) is normalized to 1 and other activities are calculated as a difference (*n* fold). The results shown are an average of three independent experiments showing the mean. Standard error of the mean are also displayed.

3.4.7 Functional activity of the transcriptionally defective HPV16 R37A E2 mutant in the presence of E1

Previous work has shown that a mutated E2 (R37A) was able to bind to E1, retained reasonable replication function but had greatly reduced transactivation function (Sakai et al., 1996). It was later discovered by the same group that this mutation severely impeded E2 and Brd4 interaction (Schweiger et al 2006). The Brd4-E2 interaction was therefore deemed essential for mediating E2 transcriptional function. It was considered whether E1 could stimulate E2 R37A mutant transcriptional activity and therefore whether E1 contributes directly to transcriptional activation. 293T cells were transfected with the tk6E2 reporter plasmid, with increasing amount of E2/R37A in the presence or absence of HA tagged E1. The R37A mutant was constructed in the E2 plasmid that is used in this particular experiment, by Peter Howley's group (Harvard, Boston). Both wild type and mutant plasmids are identical with the exception of the arginine to alanine mutation at amino acid position 37.

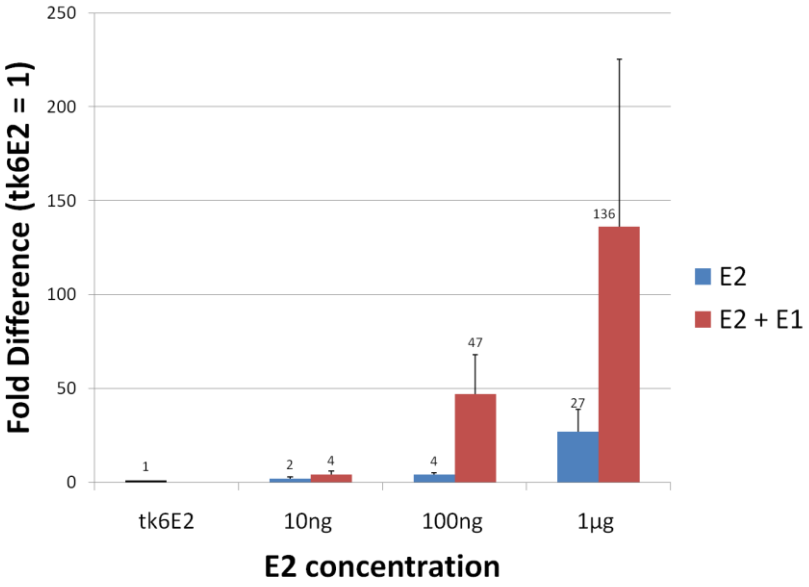
Forty eight hours post transfection duplicate samples were harvested and prepared for a transcription assay. Luciferase activity for the tk6E2 plasmid was normalised to 1, and activities from other samples were calculated as a fold difference (*n*-fold). The values shown represent an average of three independent experiments. As can be seen in Figure 3.4.7 (a), the new E2 plasmid mimicked our previous results in shown in Figure 3.4.6. As E2 concentration is increased, the luciferase signal from tk6E2 activation increases (blue bars). This demonstrates that the E2 expression plasmid shows expression of increasing E2. When E1 is present, again an enhancement of E2 function is observed (red bars). At 10ng of E2, E1 increases activation by approximately 2 fold, at 100ng of E2 this enhancement increases to around 10 fold and at 1µg of E2, E2 activity increases by approximately 4 fold. These results are similar to the previous observations of E1 stimulated E2 functional activity however the p values for each concentration where >0.05 so it cannot be said that there is a significant difference.

The ability of E1 to increase the R37A mutant transcriptional activity was investigated in Figure 3.4.7 (b). Preparation of the transcription assay was carried out in a similar fashion to Figure 3.4.7(a) with the only variation being the transfection of R37A and not wild type

E2. Previous work with R37A mutant has demonstrated severely diminished transcriptional functions of this plasmid compared to wild type E2. Our results are not reflective of this observation. R37A appears to be transcriptionaly active with the tk6E2 reporter plasmid. At 10ng of R37A, the signal is 3 fold higher compared to tk6E2 alone, at 100ng this signal is 5 fold higher and at 1µg of R37A this signal is 10 times higher. Therefore the R37A mutant is functionally active with the tk6E2 reporter plasmid. The disparities in the results may be a consequence of differences in reporter plasmids. The previous study used chloramphenicol acetyltransferase (CAT) reporter plasmids and this study used luciferase reporter plasmids.

Figure 3.4.7 Functional activity of the transcriptionaly defective HPV16 R37A E2 mutant in the presence of E1

(a) HPV16 E2 transcription in the presence of E1



(b) HPV16 E2 R37A mutant transcription in the presence of E1

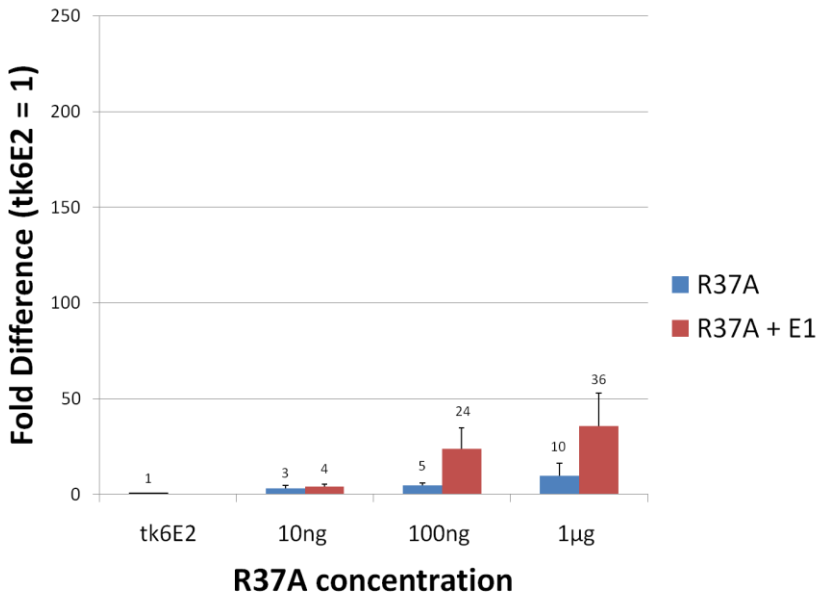


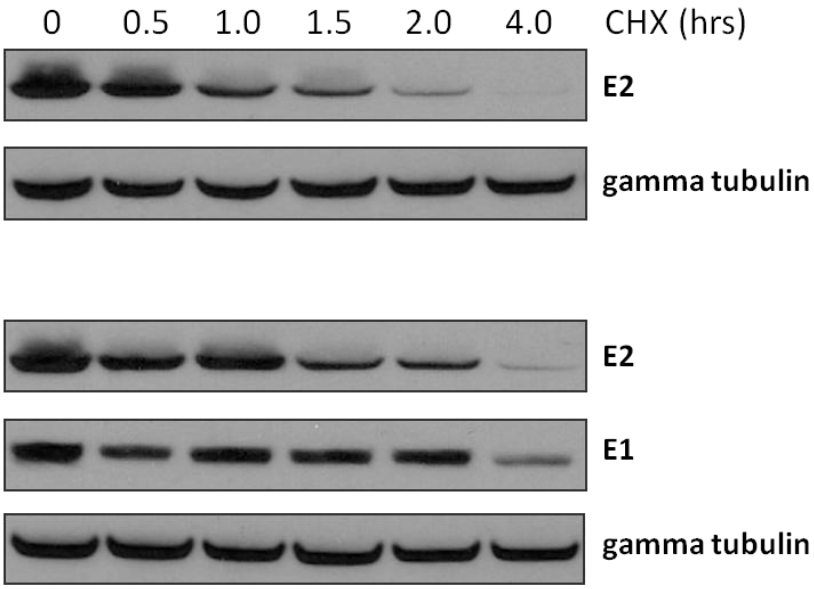
Figure 3.4.7 Functional activity of the transcriptionaly defective R37A E2 mutant in the presence of E1 (a) One microgram of the tk6E2 reporter plasmid was co-transfected with increasing amounts of HPV16 E2 in the absence (blue bars) or presence (red bars) of 50ng of HPV16 E1HA. Cells were harvested 48 hours post transfection and assayed for luciferase activity (Promega). Luciferase activity of lysate containing tk6E2 only (black bar) is normalized to 1 and other activities are calculated as a difference (*n* fold). The results shown are an average of three independent experiments with standard error of the mean displayed. (b) One microgram of the tk6E2 reporter plasmid was co-transfected with increasing amounts of HPV16 R37A mutant in the absence (blue bars) or presence (red bars) of 50ng of HPV16 E1HA. Cells were harvested 48 hours post transfection and assayed for luciferase activity. Luciferase activity of lysates containing tk6E2 only (black bar) is normalized to 1 and other activities are calculated as a difference (*n* fold). The results shown are an average of three independent experiments with standard error of the mean displayed.

3.4.8 HPV16 E1 increases E2 stability in a cervical cancer cell line.

The data shown in Chapter 3.4 has been carried out in the HEK 293T cell line. The biggest threat to human health caused by HPV16 is its ability to cause cancer in cervical epithelial cells. Similar experiments were therefore carried out in a cell line that mimicked the oncogenic environment that HPV may encounter during tumourigenesis in the cervix. The C33A cell line is a HPV-negative, human cervical carcinoma cell line. This cell line was used to investigate the consequences of the E1 and E2 interaction. Protein half life experiments were completed using cycloheximide as an inhibitor of protein synthesis to investigate whether E1 can increase E2 stability in a cervical cancer cell line. HPV16 E2 was transfected with or without HPV16 E1 HA tagged plasmid. The cells were treated with CHX two days after transfection for the time points indicated (Figure 3.4.8 (a)). C33A cells were then harvested for western blot analysis to detect E2 (TVG261), E1 (HA) and gamma tubulin (loading control). The image is representative of three independent experiments. At 1 hour of CHX treatment, the intensity of the E2 protein band appears significantly less with E2 alone, compared to E2 in the presence of E1. Densitometry is used in Figure 3.4.8 (b) to estimate the half life of E2 in C33A cells. The quantitated results show the average of three independent experiments with associated means and standard error of the mean. Using the Microsoft Excel programme to calculate the equation of the best fit line for each data set the half life was calculated. E2 has a calculated half life of 3.6 hours in C33A cells. The half life increases substantially to 5.7 hours in the presence of E1. Therefore in a cervical carcinoma cell line, E1 is able to increase E2 stability by increasing the protein's half life.

Figure 3.4.8 HPV16 E1 increases E2 stability in a cervical cancer cell line.

(a)



(b) HPV16 E1 increases E2 stability in C33A cells

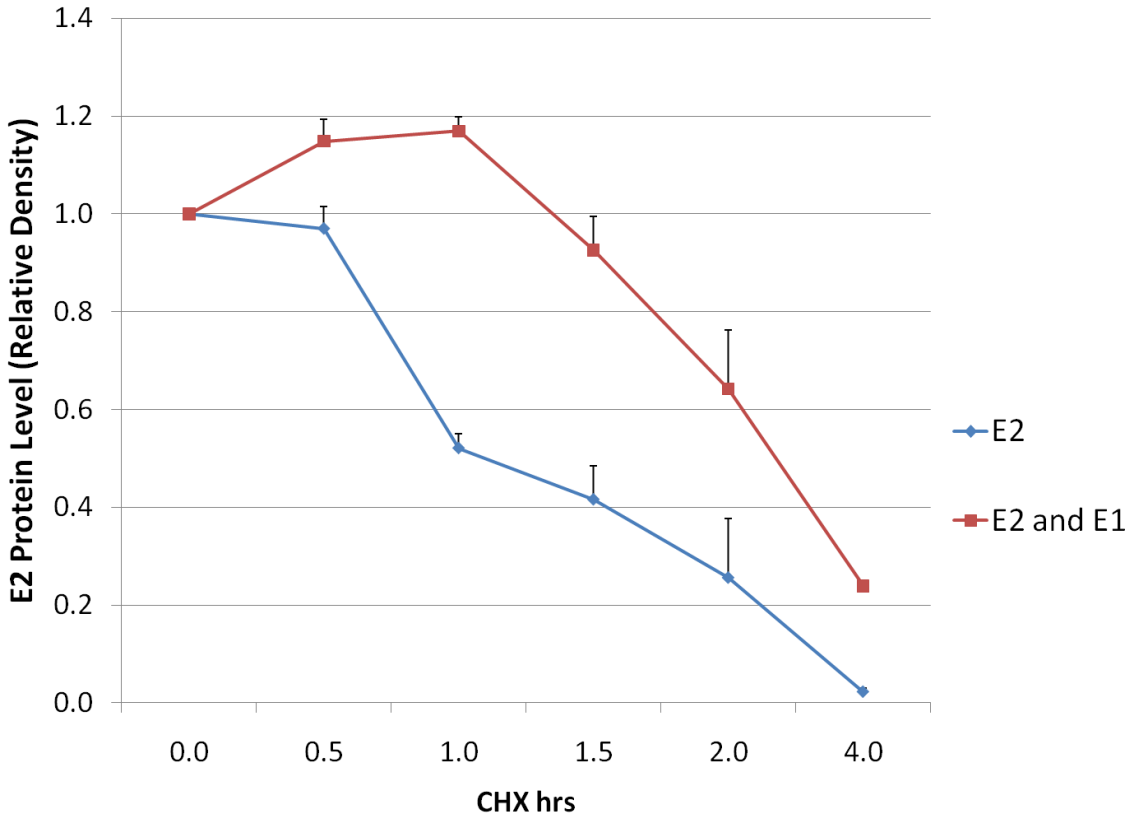


Figure 3.4.8 HPV16 E1 increases E2 stability in a cervical cancer cell line. (a) C33A cells were transfected with 2µg HPV16 E2 with or without 2µg E1-HA. Two days post transfection, cells were treated with 100µM of CHX for the time points indicated and harvested for western blot analysis using TVG261 (E2) HA antibody (E1) and gamma tubulin as a loading control. (b) E2 half life in the absence and presence of E1 was calculated using densitometry. Protein bands were quantified using E2 levels relative to pre-CHX treatment and the gamma tubulin signal was used for normalisation of each band. The graph represents the average percentage of protein with standard error of the mean of three independent experiments. Image J software was used for quantification of the protein bands and therefore made it possible to estimate the half life of E2 in C33A cells.

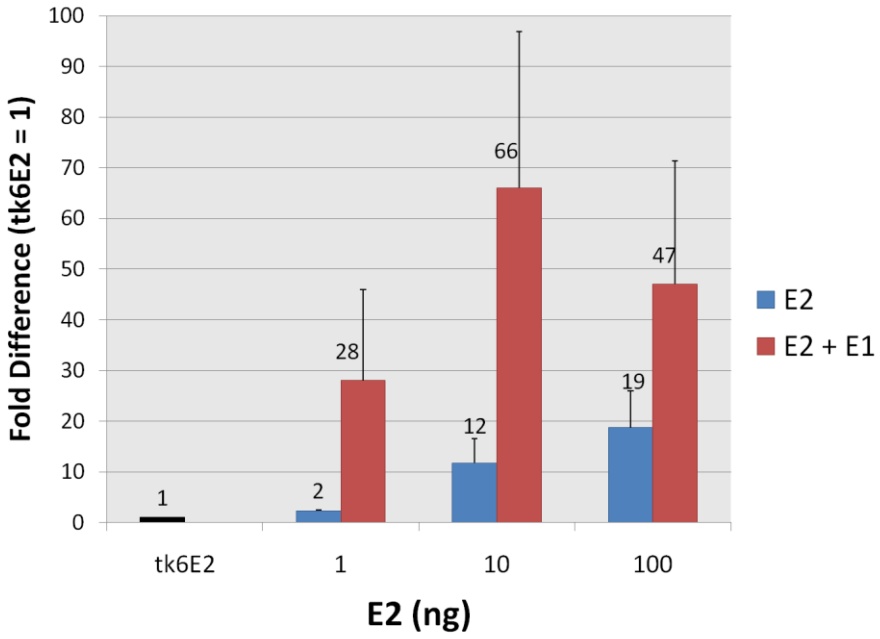
3.4.9 E2 transcriptional activity in a cervical cancer cell line.

A previous observation in the 293T cell line demonstrated that E2 stability is improved by a direct interaction with E1 and a functional consequence of this interaction is increased E2 transcriptional activity (Figure 3.4.6). Therefore the effect E1 has on E2 transcriptional activity was also investigated using the C33A cell line. Figure 3.4.9 (a) shows the results of the E2 transcription assay in C33A cells. Luciferase expression from the tk6E2 reporter plasmid increases with increasing amounts of transfected E2 (blue bars). There was no signal repression at the highest amount of E2 used. All signals were stronger than the tk6E2 plasmid alone (black bar) and this demonstrates that E2 is capable of activating the tk promoter in C33A cells. The effect of E1-E2 interaction was investigated by co-transfection of 50ng of E1 and 10/100/1000ng of E2 (red bars). At 1ng E2, there is an approximate 12 fold increase in transcriptional activation in the presence of E1 compared to E2 alone. At 10ng E2 there is an approximate 6 fold increase and at 100ng E2, there is an approximate 3.5 fold increase. However these increases are not significantly different because the p value was greater than 0.05 at each concentration of E2 used.

To determine whether the increase in E2 transcription function is a consequence of an E1-E2 interaction the E39A mutant was used in place of wild type E2 (Figure 3.4.9 (b)). The values and standard errors shown are an average of three independent experiments. At all concentrations of E39A (blue bars) used, luciferase expression was higher than the tk6E2 reporter plasmid (black bar), demonstrating that this mutant retained normal E2 transcriptional functions in C33A cells. Luciferase expression increases from 1ng to 100ng E39A (blue bars). The red bars represent samples where E39A and E1 have been co transfected. At 1ng E39A, there is an approximate 3 fold increase in transcriptional activation in the presence of E1 compared to E39A alone. At 10ng E39A there is an approximate 2 fold increase and at 100ng E39A, there is an approximate 1.2 fold increase in the presence of E1. The p values was greater than 0.05 at each concentration of E39A therefore there is no significant difference between E39A mutant with and without E1. This result may reflect some residual binding between E1 and the E39A mutant or an increase in E39A RNA levels as mentioned previously. The enhancement of E39A mediated transcription in the presence of E1 is much smaller compared to E2 wild type.

Figure 3.4.9 E2 transcriptional activity in a cervical cancer cell line.

(a) HPV16 E2 transcriptional activity in the presence of E1



(b) HPV16 E2 E39A mutant transcriptional activity in the presence of E1

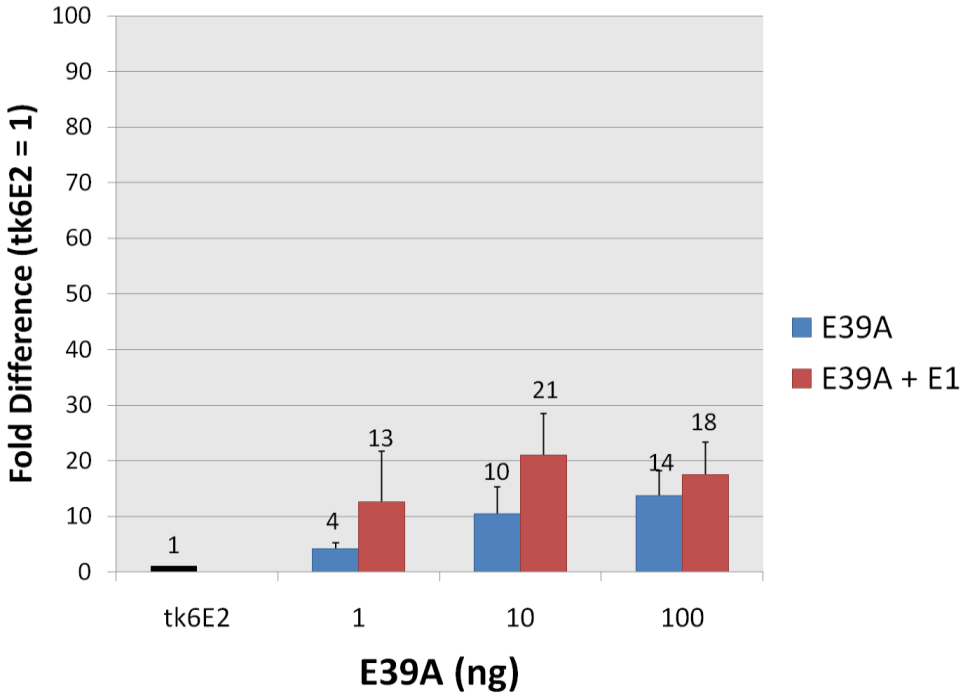


Figure 3.4.9 E2 transcriptional activity in a cervical cancer cell line. (a) One microgram of the tk6E2 reporter plasmid was transfected alone (black bar) or with increasing amounts of HPV16 E2 in the absence (blue bars) or presence of 50ng of E1HA (red bars). C33A were

harvested 48 hours post transfection and assayed for luciferase activity. Luciferase activity of lysates containing tk6E2 only is normalized to 1 and other activities ware calculated as a fold difference (*n* fold). The values and standard errors of the mean are shown and are an average of three independent experiments. (d) One microgram of the tk6E2 reporter plasmid was transfected alone (black bar) or with increasing amounts (1/10/100ng) of the E39A mutant in the absence (blue bars) or presence of 50ng E1HA (red bars). C33A were harvested 48 hours post transfection and assayed for luciferase activity. Luciferase activity of lysate containing tk6E2 plasmid only is normalized to 1 and other activities are calculated as a fold difference (*n* fold). The values and standard error of the mean are shown and are an average of three independent experiments.

3.5 Summary of Chapter 3

The data presented in this chapter shows the ability of E1 and E2 to replicate the HPV origin in the presence of DNA damage despite the activation of checkpoint kinases. In contrast, SV40 LT mediated replication is arrested in response to treatment with DNA damaging agents. Evidence from *in vitro* replication assays suggest that it is ATR that targets LT and prevents replication during DNA damage. Further evidence supporting this hypothesis was hampered because ATR/TopBP1 knock down cells were unable to grow.

In vivo, ATR/ATM phosphorylates LT in response to DNA damage and a consequence is LT degradation by the proteosome. Mutational work of predicted ATR/ATM targeted sites in LT failed to indicate an individual site responsible for phosphorylation. In comparison activated ATR/ATM does not target E1 for phosphorylation *in vivo* and this may explain the differences between HPV E1-E2 and SV40 LT mediated replication.

Analysis of the E1.E2 interaction in 293T and C33A has shown that E1 stabilises E2 through post translational modifications. E1 does not alter the cell cycle but does alter E2 affinity for chromatin. E2 shows a higher affinity for chromatin in the presence of E1. Functional analysis using a reporter plasmid that contains six E2 binding sites has demonstrated the ability of E1 to significantly enhance E2 transcriptional functions in the 293T cell line. These results will be discussed in detail in Chapter 4.

Chapter 4- Discussion

4.1 E1-E2 mediated HPV origin replication is not inhibited in response to DNA damage

HPV is the causative agent of cervical cancer and the integration of viral DNA into the host genome can enhance malignant transformation (Pett & Coleman, 2007). Deletions in both E1 and E2 genes are often observed during integration with the maximum number of losses observed within the E2 gene region (zur Hausen, 2002). Loss of E2 expression results in unregulated transcription of the E6 and E7 oncogenes, predisposing the cell to genomic instability.

Factors promoting HPV integration may include the ability of the virus to replicate at the same time as DNA breaks in the cell genome. Figures 3.1.1 & 3.1.3 demonstrate the ability of the replication proteins E1 and E2 to replicate the HPV origin of replication under conditions of DNA double strand breaks therefore increasing the chances of viral integration. Etoposide and camptothecin cause DNA damage however other factors that may promote naturally occurring DNA breaks are endogenous factors such as the female hormone estrogen. Estrogen is an important issue in cervical cancer research because many women take the estrogen-containing contraceptive pill. Some studies have shown that woman who use estrogen as a contraceptive pill have higher rates of cervical dysplasia than those who do not (Moulten & Le, 1991; Moreno et al., 2002; Smith et al., 2003; McFarlane-Anderson et al., 2008; Vanakankovit & Taneepanichskul, 2008). Data from one multicentre study showed that use of the oral contraceptive increased the risk of developing cervical cancer in a duration-dependent manner compared to women who did not use the pill. The risk increased by 2.72 fold for women using the contraceptive for 5-9 years. Women that used the contraceptive pill for ten or more years had a 4.48 fold increase in cervical cancer rates (Moreno et al., 2002). A separate study of 12531 cases of cervical cancer also showed an increase in cervical cancer rates with increasing

contraceptive pill duration (reviewed in Smith et al., 2003). Women who tested positive for HPV DNA and used the contraceptive pill for 5-9 years had increased rates by 1.3 fold and for 10 years or more of use showed increased rates of 2.5 fold compared to woman who did not take contraceptive pill. The same study also suggests that the incidence rate of cervical cancer decreased after the oral contraceptive stopped being used. Later studies have also linked long term use of the oral contraceptive with increased rates of cervical cancer (McFarlane-Anderson et al., 2008; Vanakankovit & Taneepanichskul, 2008). Estrogen has also been shown to contribute to the onset, persistence and malignant progression of cervical cancer in HPV-transgenic mouse models) and cause resistance to apoptosis in cervical cancer cells (Arbeit et al., 1996; Brake and Lambert, 2004; Chen et al., 2004).

Further to this, several estrogen metabolites can cause DNA damage through redox cycling processes that produce reactive radical species (Yager & Liehr, 1996). Redox cycling of estrogen quinone in cells generates free radicals, which induce DNA single strand breaks in estrogen-sensitive breast cancer cells (Nutter et al., 1991; Nutter et al., 1994). ATR is the predominant Ser/Thr kinase that is activated in response to single strand breaks. 17-βestradiol substantially blocks ATR by reducing the enhanced association of ATR and TopBP1 proteins that follows after DNA damage (Pedram et al., 2009). This study showed that 17-βestradiol inhibits ATR activation through rapid PI3K/AKT signalling in breast cancer cells. AKT phosphorylates TopBP1 thereby preventing the enhanced interaction of ATR with TopBP1 after DNA damage. 17-βestradiol also inhibited claspin and Chk1 protein association therefore preventing Chk1 signalling to the G_2/M checkpoint (Pedram et al., 2009). Inactivation of ATR signalling could result in HPV replication in the presence of DNA breaks therefore increasing the risk of viral integration.

Taken together with our findings a hypothetical model can be built. HPV replicates in estrogen-sensitive cervical cells. The presence of estrogen potentially results in DNA

damage. As HPV replication is not inhibited by DNA damage pathways, the virus replicates in the presence of DNA breaks and this increases the chances of viral integration into the host genome. Recurrence rates after treatment for CIN I are estimated to be around 10% (Flannelly et al., 1997). Part of this recurrence may be attributed to estrogen. Future work should focus on studying integration rates of HPV DNA in the presence and absence of estrogen and to consider the effects of anti-estrogen therapy in combination with current treatments to reduce HPV recurrence ultimately to reduce cervical cancer rates.

4.2 SV40 LT is targeted for phosphorylation by activated ATR

HPV E1-E2 mediated replication is not inhibited despite the activation of a DNA damage response, whereas SV40 LT mediated origin replication was (compare Figures 3.1.1 and 3.1.3 with 3.1.2). The findings in this thesis suggest that ATR is the protein kinase responsible for this inhibition *in vitro* and after etoposide treatment ATR/ATM was also shown to target LT for phosphorylation *in vivo* (Figure 3.1.4 & 3.1.5 respectively). Previous studies on SV40 infection have demonstrated that the DNA damage response is activated in the infected cells and that the replication functions of LT are regulated by consequent phosphorylation. In particular, phosphorylation of serine 120 results in a stimulation of LT mediated replication (Shi et al., 2005). SV40 infected cells alter the host DNA damage response by targeting the MRN complex for degradation via the proteosome (Digweed et al., 2002; Zhao et al., 2008). Large T Antigen interacts with the Nbs1 component of the MRN complex to disrupt DNA replication control and therefore enhancing SV40 replication (Wu et al., 2004). Therefore the host cell DNA damage response is re-programmed by SV40 infection to promote viral DNA replication. 293T cells used in this study retain the ability to signal via the DNA damage signalling kinases despite stably expressing LT (Shirata et al., 2005). In contrast, DNA damage signals can suppress SV40 DNA replication in mammalian cells via activation of the DNA damage signalling kinases (Miao et al., 2003). The replication functions of LT are regulated by phosphorylation, where low levels of phosphorylation appear to promote LT origin

binding and replication, and high levels of phosphorylation inhibit replication (Wang et al., 1993).

The results described in this thesis agree with the previous studies demonstrating arrest of LT-mediated DNA replication following DNA damage (Miao et al., 2003). It appears that treatment with DNA damaging agents (which activate damage signalling pathways but suppress replication) may be related to differential modification of LT in different circumstances. Phosphorylation of serine 120 results in stimulation of LT replication function following viral infection and it is possible that following exposure to DNA damaging agents LT is differentially modified resulting in replication repression (Shi et al., 2005). The LT mutation studies of hypothesised ATR phosphorylation target sites did not reveal a single SQ/TQ site that was responsible for ATR phosphorylation (Figure 3.1.11). Future work should focus on proteomic studies as it is possible that the ATR kinase targets multiple sites on LT for phosphorylation in response to exogenous DNA damage. A suggestion as to how the proteomic studies could be carried out is to use the pS/Q antibody to enrich for LT sequences that have been phosphorylated by ATM/ATR after etoposide treatment. Alterations in the LT amino acid sequence indicative of post translational modification could be identified using sequencing of the known LT SQ/TQ sites and by mass spectrometry.

Upon activation of the DNA damage response DNA replication is stalled (Abraham, 2001; Bartek & Lukas, 2003; Harper & Elledge, 2007). Protein kinase activation and signal transduction pathways have been clearly defined, however what is not clear is which targets at the replication fork result in the inhibition of DNA replication. Previous work has demonstrated that following DNA damage SV40 DNA replication control mimics that of cellular DNA replication (Miao et al., 2003). The results presented in this thesis support the hypothesis that the replicative helicase is a direct target for phosphorylation by the DNA damaging signalling kinases. The cellular replicative helicase complex consisting of the MCM2-7 proteins becomes phosphorylated in response to DNA damage (Cortez et al., 2004; Ishimi et al., 2003a, b). Phosphorylation of the helicase proteins appears

fundamental in the arrest of DNA replication following DNA damage. Future work should focus on determining what sites of LT are targeted for phosphorylation by ATR upon activation of DNA damage kinases as this will be a useful model for studying eukaryotic DNA replication. LT plasmids could be synthesised harbouring mutations in the known ATR target sites. The mutated plasmids could then been utilized in checkpoint kinase transient replication assays. The results will support evidence that activated ATR directly targets the DNA helicase for phosphorylation therefore inhibiting replication during DNA damage.

4.3 E1 is not a substrate for ATR kinase in response to DNA damage.

An important result to consider is that neither HPV11 nor HPV16 E1 proteins are substrates for DNA damage kinases upon activation of ATR (Figure 3.1.6). Similar to SV40, recent work has demonstrated that HPV can activate DNA damage signalling kinases during their life cycle (Moody and Laimins., 2009). However in contrast to SV40, HPV E1-E2 mediated replication does not arrest in response to DNA damage (King *et al*., 2010). The findings from this thesis suggest for the first time that in the presence of DNA damage the E1 helicase is not targeted for phosphorylation despite the activation of ATR, whereas the LT helicase (model for MCM complex) is. Therefore the difference in the DNA replication response of LT and E1–E2 is likely related to either the differential phosphorylation of LT and E1 following DNA damage or to differential interactions of the viral helicases with cellular factors phosphorylated by DNA damage response pathways.

The consequences of this in relation to the virus life cycle should be taken into consideration. Regulation of cellular replication limits genomic duplication to once per cell cycle. However in the differentiating layers of the epithelium the papillomavirus is able to replicate despite the fact that differentiating cells exit the cell cycle (amplificational replication). Work carried out in the Laimins laboratory has shown that HPV activates components of the ATM/ATR pathways and this is required for viral

genome amplification in differentiating cells (Moody & Laimins, 2009). The findings described in this work also imply that HPV uses checkpoint kinase activation in order to carry out its replication. These observations suggest that the ATR/ATM kinase pathway may be a potential therapeutic target to inhibit HPV replication. ATR is essential for cell survival (Figure 3.2.2 & 3.3.3) therefore the inhibition of ATR signalling using downstream components of this pathway may be a better antiviral target than ATR itself (e.g Chk1 kinase).

Long term exposure to the DNA damaging agent etoposide results in the degradation of LT by the proteosome (Figure 3.1.7). This degradation is unlikely to explain the inhibition of LT DNA replication reported in Figure 3.1.2. Treatment of cells with aphidicolin results in an immediate arrest of DNA replication because it inhibits the DNA polymerase α and δ. LT mediated replication shows similar trends in replication of the SV40 origin plasmid for aphidicolin and etoposide arrested replication, suggesting an immediate arrest of LT replication following etoposide treatment. In contrast, HPV E1 protein is not degraded after long term etoposide exposure. This evidence suggests that by avoiding downstream targeting by DNA damage kinases HPV is able to replicate in the presence of DNA damage.

4.4 TopBP1 is essential for DNA replication and cell survival

In Chapter 3.2, attempts to create ATR-deficient cell lysate in order to carry out *in vitro* SV40 checkpoint kinase replication assays were unsuccessful. The task of generating enough ATR deficient lysate was always going to be a challenge because ATR is essential for completion of the cell cycle. ATR deficiency in mice is found to be lethal to embryonic development (Brown & Baltimore, 2000; de Klein et al., 2000). Seckel syndrome, ATR deficiency in humans, is characterized by growth retardation, microcephaly, mental retardation and craniofacial abnormalities (O'Driscoll et al., 2004). The lack of ATR-deficient cells is an obstacle to functional studies of this protein. Therefore it was deemed feasible to knock down TopBP1 because this protein is involved in ATR activation. Further

to this, previous reports have suggested that TopBP1-deficient 293T cells can grow for a short period of time (Wright, 2007). TopBP1 KD cells prevent ATR inactivation (Figure 3.2.1) however cell growth was severely diminished in cells transfected with TopBP1 or ATR siRNA oligonucleotides (Figure 3.2.2 and 3.3.3). Therefore TopBP1 deficient 293T cells were unable to grow ruling out the possibility of ATR knock down *in vitro* replication assays.

4.5 A consequence of the E1-E2 interaction is increased E2 stability

E2 is a multifunctional protein which has numerous roles in the papillomavirus lifecycle. It is involved in the regulation of viral transcription, viral genome segregation during mitosis and it anchors E1 to the viral origin of replication to initiate viral replication (McBride, 2008). Therefore the regulation of E2 function is important to the virus lifecycle because E2 has the potential to disrupt the viral life cycle at several steps. The activities of E2 within a cell are likely to be affected by the amount of available E2 protein and one way of controlling this is to regulate the turnover of this protein. In Chapter 3.3, HPV16 E2 protein levels appear to increase when treated with etoposide and when co-transfected alongside E1 (Figure 3.3.2). Previous work reports that E2 protein levels increase within the S phase of the cell cycle (Johansson et al., 2009). Therefore it was considered whether the DNA damage induced by etoposide was causing the E2 transfected cells to arrest in S phase and this would explain E2 stabilisation. Flow cytometry analysis shows that E2 transfected cells were arrested in G_2/M phase after etoposide treatment and not S phase therefore this did not explain the rise in E2 protein levels after etoposide treatment (Figure 3.3.3). E2 RNA levels were examined in the presence of etoposide because it was possible that the increase in E2 protein levels was a result of increased CMV promoter activity from the E2 plasmid. Real-Time PCR measuring HPV16 E2 mRNA confirmed that etoposide increased the CMV promoter activity in the HPV16 E2 plasmid. Therefore E2 stabilisation by etoposide is not a post translational event.

Worthy of great consideration from Chapter 3 is the ability of HPV16 E1 to stabilise E2 in response to CHX treatment (Figure 3.3.2). Further analysis of E2 RNA levels in the presence of E1 revealed that the stabilisation is a post translational event. Further evidence using an alternative E1 plasmid to increase E2 protein levels is demonstrated in Figure 3.4.1. One hypothesis that may explain the increase in E2 stability in the presence of E1 protein is the ability of E1 to alter the cell cycle. E1 may alter the cell cycle to favour an S phase environment which is suitable for HPV replication. A previous study in this laboratory highlighted HPV16 E2 stabilisation in the S phase of the cell cycle (Johansson et al., 2009). However E1 and E2 transfected 293T cells were shown to have a normal cycling profile and therefore this does not explain the stabilisation of the E2 protein in the presence of E1 protein (Figure 3.3.3).

Another post translational modification that may explain the mechanism behind E2 increased stability by E1 is the ability of E1 to shift E2 onto chromatin. Previously, in TopBP1 depleted cells, E2 was shown to have a higher affinity for chromatin and this subsequently resulted in higher levels of E2 protein compared to TopBP1 containing cells (Donaldson et al., 2007). It was therefore possible that E1 interacts with E2 encouraging its affinity for chromatin. The results in Figure 3.4.2 suggest that E1 results in E2 redistributing into the chromatin pellet. In the viral life cycle the virus genome associates with the host chromatin. E2 ensures accurate segregation of the replicated viral episomes to the daughter cells during host cell division by interacting with host mitotic chromosomes (Skiadopoulos and McBride, 1998; Florence and Faller, 2001; Dey et al., 2003; Van Tine et al., 2004; You et al 2004; Zheng et al., 2005; Baxter et al 2005; Dai et al, 2006; Schweiger et al., 2006; Parish et al, 2006; Oliveira et al., 2006; Donaldson et al 2007; Poddar et al., 2008; Poddar, 2009). The data presented in this thesis suggest that the E1 protein enhances E2 interaction with chromatin and therefore E1 may have a role in viral genome segregation. E1 may stabilise E2 and enhance its tethering function with the chromatin. This finding is in disagreement with a previous study. This study carried out in the Botchan laboratory suggested that wild type BPV1 E1 protein could relocate E2

from mitotic chromosomes therefore interfering with its tethering functions (Voitenleitner & Botchan, 2002). However these studies were carried out in BPV1 and not HPV16. The proteins that papillomavirus E2 uses in chromatin tethering and genome partitioning vary between different papillomavirus types (McPhillips et al., 2006). Therefore differences between the results presented here and those reported previously may be explained by the use of different papillomavirus types. Future work should consider the E1.E2 interaction at varying points during the cell cycle using different types of papillomavirus. This could be achieved using fluorescence microscopy of both E1 and E2 proteins and examining the interaction of E2 with chromatin during mitosis and cytokinesis. E2 protein half life in the presence of E1 protein should also be examined in chromatin (high salt) pellets. This would confirm whether E1 stabilises E2 that is in close proximity to cellular chromatin.

Previous studies on E2 regulation have shown that the E2 proteins of HPV16, HPV18 and BPV1 are ubiquitinated at the amino terminal and subsequently targeted by the proteosome for degradation (Bellanger et al., 2001; Penrose and McBride, 2000; Taylor et al., 2003). E2 has a relatively short half life and measurements from this laboratory approximate it to be 45 minutes (Taylor et al., 2003). However in a natural HPV infection, E2 does not exist as an isolated viral protein, but it is expressed with other HPV proteins during the virus lifecycle. Therefore it is important to consider E2 turnover in the presence of other HPV proteins. Viral proteins have been previously reported to alter E2 turnover and function. Studies have demonstrated that E4 can influence the stability and function of the E2 protein (Davy et al., 2009). The N terminal domain of E2 directly binds the HPV16 E1^E4 protein altering E2 solubility and decreasing turnover. In the presence of E1^E4 E2 protein becomes less soluble. Therefore the E1^E4 protein may influence E2 activity by altering its cellular location. Through a direct interaction E2 and E6 proteins are both capable of regulating each other's activity resulting in changes in E6 targeting of PDZ-containing substrates and the functional activities of E2. In the presence of E6, transcriptional activity of E2 is elevated whilst viral DNA replication is inhibited (Grm et

al., 2005). The L2 capsid protein may regulate the transcriptional functions of E2 without affecting its ability to interact with the E1 protein during replication (Okoye et al., 2005).

In this thesis the results have demonstrated that via a direct interaction, HPV16 E1 can regulate the stability of E2 and is also capable of enhancing E2 transcription functions. Previous work from this laboratory has shown that HPV16 E2 is degraded via the proteosome following ubiquitinylation (Taylor et al., 2003). It was hypothesised that E1 stabilises E2 by preventing its turnover via the proteosome. Brd4, a major binding protein for E2, binds the N terminal transactivation domain of E2 and co-localizes with E2 on mitotic chromosomes. Besides tethering the viral episome to mitotic chromosomes the E2-Brd4 interaction also plays important roles in E2 turnover. Recent studies demonstrate that expression of the Brd4 C terminal domain blocks the interaction between E2 and the cullin-3 complex of the proteosome therefore preventing E2 degradation (Zheng et al., 2009). Tax1BP1 has been shown to interact with E2 from HPV16, HPV18 and BPV1. In BPV1 the C terminal region of Tax1BP1 interacts with the N terminal transactivation domain of E2. This protein functions as an essential component of an A20 ubiquitin-editing complex (Valck et al., 1999; Shembade et al., 2007; Iha et al., 2008) and has been shown to plays a role in the regulation of E2 by regulating its proteasomal degradation (Wang et al., 2009)

The work carried out in this thesis investigated some of mechanisms that may explain the increase in E2 stability by E1 and found it was not simply due to an increase in E2 RNA levels or modulation of the cell cycle (Figures 3.3.2 & 3.3.3). It is therefore possible that E1 acts in a similar manner as Brd4 and Tax1BP1 by blocking E2 degradation. Alternatively, additional modifications of E2 may occur following interaction with E1 that contribute to protein stabilisation and function. Sumoylation counteracts ubiquitinylation and subsequent proteasomal degradation via competition with the same lysine residue of substrates (Hoege et al., 2002; Bergink & Jentsch, 2009). E2 interacts with components of

the SUMO family of proteins resulting in increased E2 stability (Wu et al., 2008; Wu et al., 2009). The SUMO conjugating protein, Ubc9 and SUMO1 have been shown to interact directly with E1 from BPV1, HPV11 and HPV16 although the functional consequences are not yet completely defined (Rangasamy and Wilson, 2000; Fradet-Turcotte et al., 2009). Mutational analysis of the E1 protein at serine 330 was shown to be defective for Ubc9 interaction and this lead to a loss of replication capabilities (Yasugi et al., 1997). It is therefore possible that the sumoylation of the E2 and E1 proteins is involved in the regulation of E2 protein turnover. E1 may recruit Ubc9 to an E1.E2 complex, therefore preventing E2 degradation through SUMOylation.

The life cycle of HPV is closely linked with the differentiation of epithelial cells. As cells migrate and begin to differentiate, maintenance replication switches to amplificational replication, and the factors that trigger this switch are unknown. It has been shown that the level of E2 increases during the epithelial differentiation process (Burns et al., 2010; Xue et al., 2010). E2 is essential for the regulation of HPV replication therefore the factors that regulate E2 may ultimately be contributing to the 'switch' in viral replication modes. This report demonstrates that the regulation of E2 protein turnover should be analysed in the presence of E1 because these proteins are often co-expressed in the HPV life cycle. It will be interesting to study the mechanisms involved in E1 stabilisation of E2 as this will provide targets and strategies for viral life cycle intervention.

4.6 E1 enhances E2 transcriptional function

The work in this thesis also demonstrates that, via a direct interaction, E1 is capable of enhancing E2 transcriptional functions in 293T cells (Figure 3.4.6). There have been many studies involving the regulation of E2 transcription function. The E1 protein of BPV1 has been shown to activate and repress E2 transcriptional function (Ferran and McBride, 1998; Le Moal et al., 1994; Parker et al., 2000; Sandler et al., 1993). Variation in results may be a consequence of the reporter plasmid/cell line used in these studies. The work in

this thesis supports evidence that E1 enhances E2 transcriptional function and this is in concordance with previous HPV studies (Piccini et al., 1995; Demeret et al., 1998). E1 is shown to enhance E2 transcriptional function in HPV16 and HPV18 chloramphenicol acetyltransferase (CAT) transcription assays (Piccini et al., 1995; Demeret et al., 1998). Similar to transcription assays carried out in this thesis, the reporter plasmids in the HPV studies all contained a thymidine kinase promoter.

In Figure 3.4.6 E2-mediated transcription activation is greatly stimulated by E1 to levels that suggest the involvement of additional factors. Activation of thymidine kinase requires the modification of chromatin. HPV18 E1 protein has been shown to activate transcription when targeted to DNA by fusion of the full length protein with the BPV1 E2 C terminal dimerization/DNA binding domain (Demeret et al, 1997). This suggests that E1 is a transcriptional activator and that it can recruit chromatin modification complexes to promoters. These studies highlight the close association between the mechanisms used in HPV replication and gene transcription.

E1 may also aid E2-recruitment of cellular transcription factors. Studies involving BPV1 E1 mediated increased E2 transactivation suggest that when E1 interacts with the E2 activation domain it acts as an allosteric activator and that the E1.E2 interaction may stabilise a particular structure of E2, increasing its transcriptional functions (Parker et al, 2000). E1 modification of E2 structure may mediate the interaction of E2 with transcriptional co-factors such as Brd4 (Lee and Chiang, 2009; You et al., 2004), BRCA1 (Kim et al., 2003), Tax1BP1 (Wang et al., 2009), TopBP1 (Boner et al., 2002) and p300 (Peng et al., 2000). The virus uses the host chromatin remodelling proteins to promote viral transcription (Kumar et al., 2007). E2-activated transcription is enhanced by its interaction with the Brahma (Brm) containing SWI/SNF chromatin remodelling complex (Kumar et al., 2007). It would be interesting to investigate whether E1 enhances this interaction.

Papillomavirus transcription is controlled by E2 binding sites and multiple regulatory elements within the virus LCR. E1 mediated enhancement of E2 transcriptional function may be attributed to a combination of increased E2 stability, improved E2 interaction with transcriptional co-factors and enhanced recruitment of chromatin modification complexes. The exact molecular mechanisms by which HPV16 E1 influences the transcriptional activity of E2 are still to be identified. Future studies should aim to identify the substrates of the E2/E1/DNA complex. E2 interaction with transcriptional co-factors and chromatin modifying complexes should be investigated in the presence and absence of E1 using co-immunoprecipitation techniques. The E2 mutant that does not interact with E1 (E2 E39A) would be a useful control in determining whether E1 enhances E2 interaction with the cellular transcription proteins.

4.7 Conclusions

This thesis had three main aims identified in Chapter 1. Efforts have been made to draw possible conclusions to each aim and are as follows:

(i) To assess HPV E1/E2 mediated replication in the presence of DNA damage and an activated cellular DNA damage response.

HPV origin replication in the presence of DNA damage suggests that this may be factor that will contribute to viral integration. This thesis shows the ability of HPV origin plasmid to replicate during DNA damage and an activated DNA damage response. Future work should investigate HPV integration rates in the absence and presence of DNA damaging agents. It would also be useful to look at the entire HPV genome replication in the presence of DNA damage. The W12 cell line is derived from human cervical keratinocytes grown from a CIN I lesion that expresses episomal HPV16 (Stanley et al., 1989). The use of these cells would allow for investigations into papillomavirus replication in response to DNA damage whilst in the presence of all viral proteins. The cellular factors that can cause DNA damage will contribute to viral integration during a HPV infection. Anti-estrogen therapy has been discussed as a potential cofactor for cervical cancer treatment.

(ii) To identify the downstream targets of DNA damage response pathways.

The activation of DNA damage pathways is well described in the literature however the downstream targets at the replication fork are less well defined. Our findings suggest that the SV40 LT helicase is a substrate for ATR during DNA damage. This result supports evidence that the MCM2-7 cellular helicase is a target for ATR/ATM in response to checkpoint activation. Future work should aim to generate LT plasmids that are mutated at known ATR/ATM target sites. *In vivo* checkpoint replication assays using the mutated LT plasmids will identify whether activated ATR/ATM inhibits LT replication. This will be a useful model for studying cellular replication.

(iii) To assess the regulatory and functional consequences of the E1.E2 interaction.

Papillomaviruses encode the E1 and E2 proteins to carry out replication at the viral origin of replication. The regulatory and functional consequences of the E1.E2 interaction are controversial. The work in this thesis supports evidence that E2 stability and transcriptional activation is enhanced by E1 via a direct protein-protein interaction. This will have consequences for E2 functions in the viral life cycle as both these proteins may be co-expressed. Future work should aim to determine what factors E1 uses to enhance E2 stability and functions as it will lead to the identification of novel therapeutic targets. E1 is a functional interacting partner for HPV16 E2 in DNA replication. Evidence from this thesis suggests that E1 regulates the association of E2 with chromatin. Therefore E1 may have a role in enhancing the genome segregation functions by stabilising the association of E2 with mitotic chromosomes.

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