https://theses.gla.ac.uk/

Theses Digitisation:

https://www.gla.ac.uk/myglasgow/research/enlighten/theses/digitisation/

This is a digitised version of the original print thesis.

Copyright and moral rights for this work are retained by the author

A copy can be downloaded for personal non-commercial research or study, without prior permission or charge

This work cannot be reproduced or quoted extensively from without first obtaining permission in writing from the author

The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the author

When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given

Enlighten: Theses
https://theses.gla.ac.uk/
research-enlighten@glasgow.ac.uk

Regulation of splicing related SR proteins during the life cycle of human papillomavirus type 16

By

Sarah Mole

A thesis presented for the degree of Doctor of Philosophy

In

The Faculty of Biomedical & Life Sciences

At the University of Glasgow

Division of Infection and Immunity

Glasgow Biomedical Research Centre

120 University Place

Glasgow

G12 8TA

ProQuest Number: 10390575

All rights reserved

INFORMATION TO ALL USERS
The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.

ProQuest 10390575

Published by ProQuest LLC (2017). Copyright of the Dissertation is held by the Author.

All rights reserved.
This work is protected against unauthorized copying under Title 17, United States Code
Microform Edition © ProQuest LLC.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

GLASGOW
UNIVERSITY
LIBRARY:

Acknowledgements

Firstly, I would like to thank my supervisor, Dr. Sheila Graham, for her patience, guidance and support during the course of my PhD, and also critical reading of this thesis. In addition, I would like to thank members of the SVG lab, past and present for their advice, discussion and help. In particular, my thanks go to Steve Milligan and Sarah Cumming for their excellent teaching and encouragement, especially during my first year. Furthermore, I would like to thank Jim Scott for his knowledge of all things lab related; I think we would have all been lost without you following the move to the new building.

My thanks also go to staff and students within the MRC virology unit. Not only have they made my time enjoyable but they have also helped me with many techniques and problems that have been encountered on the way. Special thanks go to the PhD students, Jon Hubb, Louise Yule, David Dalrymple, Tanya Chaudry, Amanda Sykes, James Towler and Alijan Tabarraei, who have endured and enjoyed the past three years with me. We finally made it!! I would also like to give particular thanks to my assessors, Prof. Darren Monckton and Prof. Barklie Clements, for objective discussion of the project and general support.

Very special thanks go to my family, who have supported me every step of the way. My parents and sister for having believed in my abilities and given encouragement, even when work wasn't going so well, and my grandparents for their support and the occasional £10. In addition, I would like to thank all my friends, a number of whom deserve special mentions: Jon Hubb (again), for everything, Adele McLean, for your belief in me, Ali Saville, for getting me drunk when necessary and Dan Jones, for making the last year bearable. I would also like to thank Joy Kean, Amanda Bradley, Lesley Heaney and Bobbie Heaney for the gigs – need I say more!

The author received funding from the Wellcome Trust. Unless otherwise stated, all the work presented in this thesis is by the author's own efforts.

Abstract

HPV-16 is a causative agent of cervical cancer. Gene expression from the virus is tightly controlled depending on the differentiation status of the epithelial cells it infects. Whilst early proteins are produced in undifferentiated cells and are thought to be continuously expressed throughout differentiation, late proteins are expressed only in differentiated cells. In particular, expression of the highly immunogenic capsid proteins, L1 and L2, is confined to the most differentiated cells, where immune surveillance is low. However, L1 and L2 transcripts have been detected in less differentiated cells, suggesting mechanisms exist to prevent their expression in these cells. A number of *cis*-acting RNA regulatory elements have been identified within the HPV-16 late region, which may be involved in regulating late gene expression during differentiation. Of particular relevance is the 79 nucleotide late regulatory element (LRE), which spans the final L1 exon and ends in the late 3'UTR. This element has been shown to confer negative regulatory activity upon reporter genes in undifferentiated epithelial cells. In addition, several cellular proteins with roles during RNA metabolism are known to associate with the element. One such protein, splicing related SR protein, SF2/ASF, associates with the LRE indirectly in both undifferentiated and differentiated HPV-16 infected epithelial cells, potentially via a splicing-like complex. This protein is also upregulated during differentiation of HPV-16 infected epithelial cells, and for these reasons, it is the main focus of this project.

The aim of this project was to investigate the role of SF2/ASF in the life cycle of HPV-16, and to determine how the virus regulates this protein, as well as other SR proteins. As SF2/ASF is upregulated in cells expressing HPV-16 E2, experiments were undertaken to determine if SF2/ASF expression is transactivated by E2. Promoter transactivation studies were performed using the CAT reporter gene fused to the SF2/ASF promoter. Results demonstrate that the promoter is transactivated in cells expressing E2. Furthermore, to determine a direct role for E2 in transactivation, chromatin immunoprecipitation assays were used to show presence of E2 at the endogenous SF2/ASF promoter. However, a direct interaction could not be observed *in vitro*, using electrophoretic mobility shift assays. Whilst direct interaction is possible *in vivo*, it is likely that one or more cellular proteins aid the interaction. As the SF2/ASF promoter is as yet undefined, nothing is known about its cellular regulation. Therefore, further EMSAs were

performed to determine if cellular partners of E2 could be found to associate with this promoter. In the case of Sp1, potential association of the protein with the SF2/ASF promoter was observed *in vitro*, using supershift assays. However, association of this protein with the promoter was not determined *in vivo*. This suggests Sp1 may be involved in E2-mediated regulation of SF2/ASF; however, further experiments are required to confirm this role.

The next aim was to discover if HPV-16 regulates SR proteins, other than SF2/ASF, during the life cycle. This was achieved using western blot and immunofluorescence techniques, comparing undifferentiated and differentiated HPV-16 infected epithelial cells and cell extracts. Whilst elevated levels of a sub-set of SR proteins, including SF2/ASF, SRp20, SRp55, SRp40 and SC35 were observed upon differentiation, this was not the case for all SR proteins analysed. This suggests the SR proteins which are regulated during HPV-16 infection may be important for processing of late transcripts in differentiated cells. Furthermore, SF2/ASF and SRp20 were observed to redistribute from nuclear speckles to diffuse cytoplasmic localisation upon differentiation. However, redistribution was observed inconsistently, the reason for which is unclear. To determine if viral proteins E2 and E1^E4 were involved in regulating potential SR protein shuttling, they were expressed in undifferentiated and differentiated epithelial cells. However, this had no effect on SR protein distribution, and little or no co-localisation was observed. This suggests that either SF2/ASF and SRp20 do not shuttle during differentiation of HPV-16 infected epithelial cells, or that shuttling is reliant on other viral proteins or processes. Furthermore, levels of a major protein kinase for SR proteins, SRPK1, was also shown to increase upon differentiation of HPV-16 infected epithelial cells, and cellular distribution of this protein was changed upon differentiation.

The final aim of this PhD was to determine a role for SF2/ASF in LRE mediated regulation of late gene expression. SF2/ASF is shown to interact indirectly with the LRE in both undifferentiated and differentiated cells. However, protein levels are elevated and hyperphosphorylated within differentiated cells. This change in abundancy and/or phosphorylation may be involved in activation of the LRE in differentiated cells. To study this, undifferentiated epithelial cells were transfected with reporter constructs containing the late 3'UTR from HPV-16, either including or lacking the LRE. Undifferentiated epithelial cells were co-transfected with these constructs and vectors expressing SF2/ASF. Potential changes in reporter gene

expression were then assayed. Results showed no observable change in reporter protein activity upon overexpression of SF2/ASF. In contrast, changes were observed at the RNA level. Polyadenylated RNAs seemed to increase upon expression of SF2/ASF in the presence of the LRE. The data also suggests the LRE may act as an instability element, and may regulate transport and/or translation independently of SF2/ASF.

Table of Contents

Figures and Tables

Abbreviations

APS	ammonium persulfate
bp	base pair
BPV	bovine papillomavirus
BS	binding site
CAM	chloramphenicol
CAT	chloramphenicol acetyl transferase
CBP	cAMP response element-binding protein-binding protein
CF	cleavage factor
ChIP	chromatin immunoprecipitation
CIN	cervical intraepithelial neoplasia
cpm	counts per minute
CMV	cytomegalovirus
CPSF	cleavage and polyadenylation specificity factor
CSt-F	cleavage stimulatory factor
CTD	carboxy terminal domain
DAPI	4', 6'-diamino-2-phenylindole hydrochloride
DBD	DNA binding domain
DCS	donor calf serum
DEPC	diethyl pyrocarbonate
DMEM	Dulbecco's modified eagles medium
DMSO	dimethyl pyrocarbonate
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetre-acetic acid
EGF	epidermal growth factor
EMSA	electrophoretic mobility shift assay
ESE	exonic splicing enhancer
ESS	exonic splicing silencer
FCS	foetal calf serum
HEPES	N-2-hydroxyethyl piperazine-N'-2-ethane sulphonic acid
hnRNP	heterogenous nuclear riboprotein
HSV	herpes simplex virus
HPV	human papillomavirus
HuR	human antigen R
LCR	long control region
LRE	late regulatory element
mRNA	messenger RNA
ORF	open reading frame
PAB	poly(A) binding protein
pAE	early polyadenylation signal
PAGE	polyacrylamide gel electrophoresis
pAL	late polyadenylation signal
PAP	poly (A) polymerase
PBS	phosphate buffered saline
PBS-T	PBS-Tween
p/CAF	p300/CREB-binding protein-associated factor
PCR	polymerase chain reaction
PIC	pre-initiation complex
PV	papillomavirus
qRT-PCR	quantitative reverse transcriptase PCR
RNA	ribonucleic acid
rpm	revolutions per minute

RRM	RNA recognition motif
SDS	sodium dodecyl sulphate
SF2/ASF	splicing factor 2/alternative splicing factor
snRNA	small nuclear RNA
snRNP	small nuclear ribonucleoparticle
SR	serine and arginine rich
SRPK	SR protein kinase
SV40	simian virus 40
TA	transactivation
TAF	TBP-associated factor
TBE	Tris-borate EDTA buffer
TBP	TATA-box binding protein
TE	Tris-EDTA buffer
TF	transcription factor
µ	micro
U	units
U2AF	U2 snRNP auxiliary factor
UTR	untranslated region
v/v	volume per volume
w/v	weight per volume

1 Introduction

1.1 Papillomaviruses

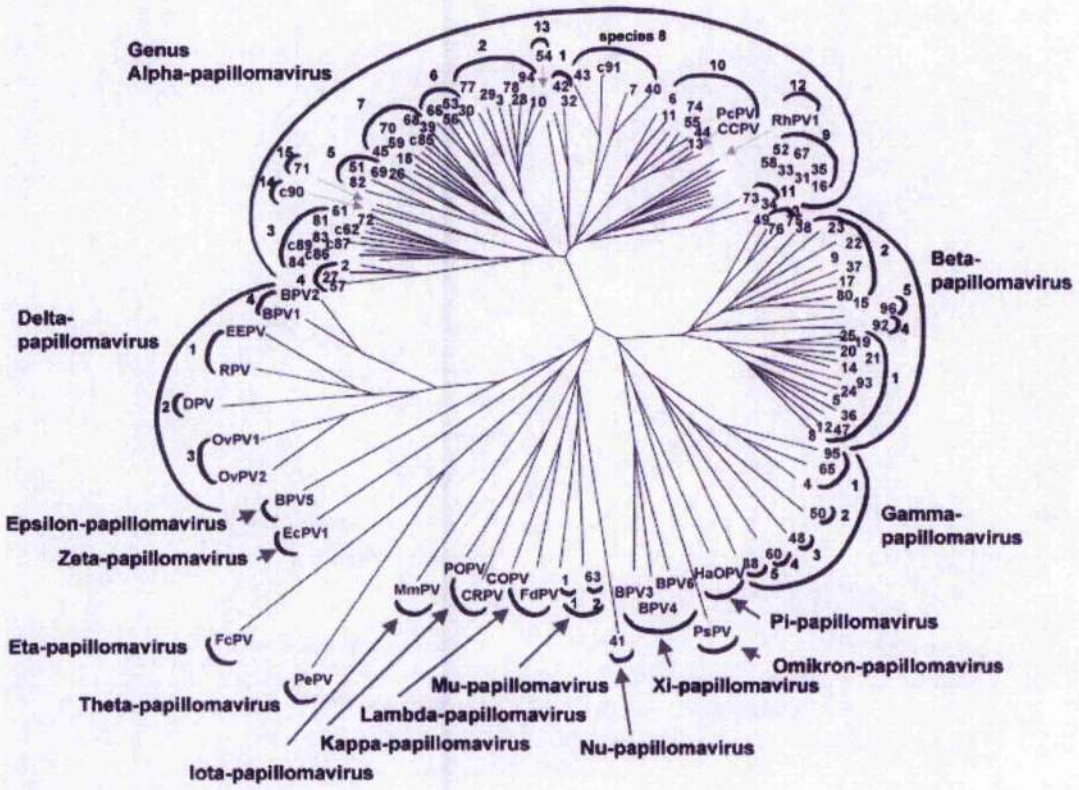
Papillomaviruses (PVs) are small, circular, double-stranded (ds) DNA viruses with genomes of ~8.0kb. They infect epithelial tissue and are the causative agent in many benign skin lesions, such as warts and papillomas. Furthermore, papillomavirus are associated with cervical carcinoma and other tumours in infected individuals (Walboomers et al. 1999). The PV family is divided into different types based on host specificity and genome homology (reviewed by zur Hausen and de Villiers 1994). Many types have been found infecting a wide variety of species, including humans, cattle, dogs and rabbits. In humans, over 100 types have been identified which infect a variety of epithelial tissues throughout the body (reviewed by Bernard 2005). Each type is specific for the epithelial tissue and in most cases, the anatomical site it infects. For example, human papillomavirus type 16 (HPV-16) is known to infect the mucosal epithelium of the anogenital tract, whilst HPV-2 infects cutaneous tissue of the hands. Furthermore, host specificity is absolute, although homology is greater between mucosal types compared with cutaneous types, irrespective of species (reviewed by zur Hausen 1996).

1.1.1 Classification

PVs were classified as members of the *Papovaviridae* family, which also includes polyomaviruses, in the mid-1950s to 1960s. These are non-enveloped DNA viruses, with circular genomes, which have icosohedral capsids of 45-55nm in diameter. However, similarities between the two virus types are superficial, with little genetic homology being observed (Danos et al. 1982). Therefore, PVs were recently characterised as a separate family (reviewed by de Villiers et al. 2004). PV classification is based on either serotype or genotype identification. Although serotype is useful for some HPV types, most phylogenetic research is based on genetic analysis of PV genomes. Initially, typing was done using Southern blot hybridisation and restriction patterns, however, more recent methods utilise PCR. Most commonly a region of the L1 gene is analysed as it is thought to be the most conserved region of the PV genome. A new HPV type is established where L1 sequences differ by at least 10% compared with that of any other HPV type.

Furthermore, differences of 2-10% define sub-types and less than 2%, a variant. The designation, e.g. HPV-(number), is given following isolation and complete genome characterisation.

Following the classification of PVs as a family of viruses, alterations were made in terminology relating to the phylogenetic tree. This led to the introduction of the term genus to replace supergroups, and species to replace groups. Whilst virus types within a genus are not always associated with similar lesions, each species is composed of highly related viruses with similar pathogenicity. A phylogenetic tree based on L1 ORF sequences and showing 118 PV types is depicted in figure 1.1 (reviewed by de Villiers et al. 2004). Here, viruses are classified into 16 genera, alpha-PV being the largest containing 15 species and 61 types. Alpha-PVs, previously termed supergroup A, are clinically most important, as HPV types associated with mucosal and genital lesions are found within this genus. Species 7 and 9 contain the majority of the 'high-risk' HPV types, so named due to their association with cervical carcinogenesis. For example, species 7 contains HPV-18 and species 9 contains HPV-16 and -31. The second largest genera are the beta-PVs, which contains epidermodysplasia verruciformis (EV) associated HPVs. EV is a rare cutaneous disorder characterised by persistent HPV infection. Beta-PVs are composed of 25 HPV types, divided into 5 species. They mainly cause flat-to-papillomatous, wartlike benign lesions of cutaneous tissue, however viral DNA has previously been detected in mucosal epithelial tissue. Species 1 is the largest group containing 10 virus types including HPV-5 and -8, which are commonly detected in EV-associated squamous cell carcinoma. Furthermore, virus types from other species within the genus are also associated with malignant cutaneous lesions. There are a further three genera which contain HPV types, all of which are less well defined than types from alpha and beta genera. The remaining genera contain types found in various mammals and birds.



Taken from de Villiers et al. 2004

Figure 1.1: **A phylogenetic tree of papillomavirus types**

The tree shows 118 PV types based on L1 ORF sequences. Genera are given by the outermost semicircles, whilst numbers show species in the inner semicircles. There are 16 genera, and high-risk mucosal types are found with the alpha genus.

1.1.2 Pathogenicity

HPVs infect epithelial cells at three target sites: the skin, the respiratory tract and the anogenital tract. Each type has a particular tissue tropism, either cutaneous or mucosal, often for a particular site. Infection occurs through micro-lesion in the epithelium and the viral genome enters the basal layer cells (see section 1.1.5: The life cycle). Following infection, the viral genome can be maintained as an extrachromosomal episome, and replication is linked with the host chromosome, within basal layer cells. Furthermore, the genome can be amplified during the productive phase, allowing for completion of the virus life cycle. In these instances the virus causes benign lesions, such as warts and papillomas. Warts from a number of HPV types, such as 1, 2 and 7 generally regress spontaneously. However, the viral genome can also integrate into the host chromosome, which in some cases results in progression of a lesion towards malignancy (Figure 1.2). The tumourigenic property of papillomaviruses was first elucidated from studies of cottontail rabbit papillomavirus (CRPV) (reviewed by Howley 1996). This was the first example of a mammalian tumour virus. It was found to cause papillomas in cottontail rabbits, extracts from which resulted in disease transmission if inoculated onto scarified cottontail or domestic rabbit skin (Shope 1933). Malignant transformation was observed within 6 months in 25% of animals, with the majority of these progressing to metastatic tumours (reviewed by Breitburd et al. 1997). Many properties of virus-induced cancer biology were elucidated from CRPV studies. For example, it was found that productive infection no longer occurs with malignant progression (Kidd and Rous 1940) and that application of certain chemicals accelerates the progression rate (Rous and Kidd 1936).

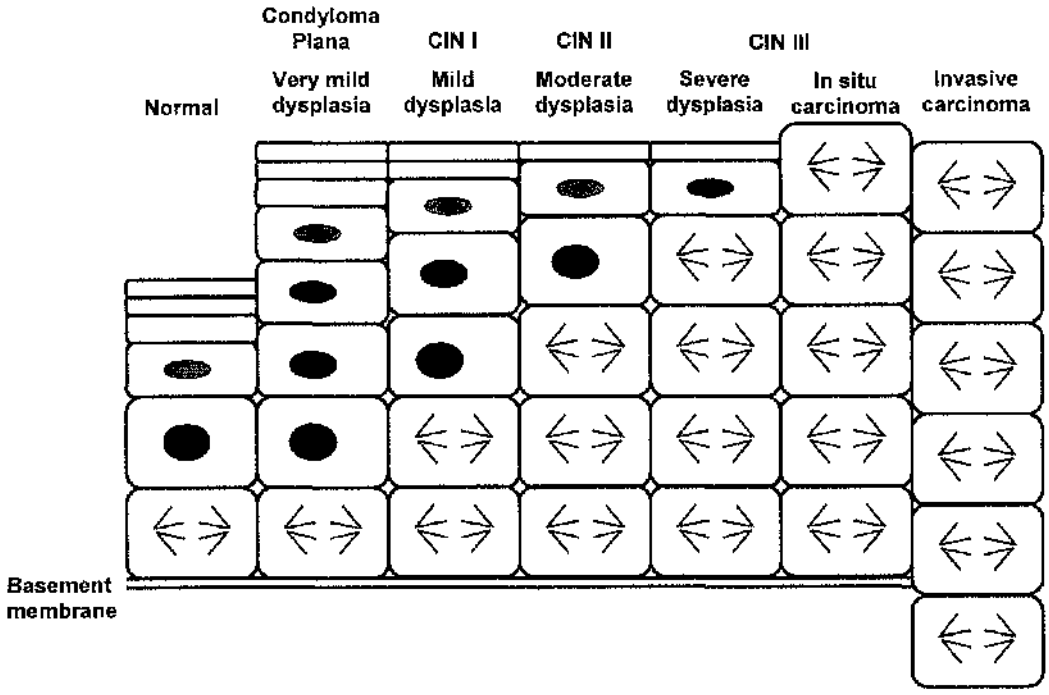


Figure 1.2: **Progression towards malignancy**

A Schematic diagram of epithelium, from normal tissue to invasive carcinoma. Note the increase in thickness from normal tissue to very mild dysplasia (episomal papillomavirus infection), with increasing basal:suprabasal cells ratio upon progression of CIN severity (Grade I to III) and in situ carcinoma, indicative of integrated genomes and mutagenesis of cellular genes. Eventual result is invasion of cells through the basal membrane.

HPV types can be divided into 'high' and 'low' risk categories depending on their capacity to cause HPV-related tumours. For example, cervical cancer is the second most common cancer of women in the developed world and the major causative agent is HPV-16 (Walboomers et al. 1999). Other HPV types are known to infect the anogenital tract. However, only the 'high-risk' types, such as HPV-16, -18, -31, -33 and –45 are frequently found in anogenital malignancies (reviewed by Shah and Howley 1996). HPV-16 is associated with 50-60% of cervical cancers (Walboomers et al. 1999), whilst the next most common, HPV-18, is detected in 10-20% of cases (Koutsky 1997; Sasieni and Adams 2001). Cancer develops from precursor lesions, known as cervical intraepithelial neoplasias (CIN) (Figure 1.2). These are graded I-II, depending on severity, with CIN I resembling production infection and CIN II and III showing progressive increase in the proliferative phase of the life cycle and increased expression of viral oncoproteins (reviewed by Doorbar 2005). Time taken to clear infection is thought to be involved in progression to malignancy, and indeed HPV-16 tends to persist for longer periods than other high-risk types (Giuliano et al. 2002). High-risk HPVs are also associated with carcinoma at a number of lower genital sites; however, cervical cancer is the most common (reviewed by Canavan and Doshi 2000). This is suggested to be due to the high susceptibility of the transformation zone, where cells are rapidly turned over, to carcinogens (reviewed by Shah and Howley 1996). The 'low-risk' types, HPV-6 and –11, cause genital warts (Condylomata acuminate), which generally regress spontaneously or with treatment and are very rarely associated with tumours. Studies have shown that whilst HPV-11 is detected most often in immunosuppressed individuals, control patients are more often infected with HPV-6 (Brown et al. 1999). Furthermore, these low-risk HPVs are commonly found in Recurrent Respiratory Papillomatosis (RRP), where infection of the upper respiratory tract results in growth of papillomas, which may cause airway obstruction. HPV-6 and -11 cause the vast majority of RRP cases, with HPV-11 causing more severe disease (Maloney et al. 2006). Virus transmission is believed to be vertical during birth; however, cases have been identified in individuals born by caesarean section (reviewed by Shah and Howley 1996). Therefore, in utero transmission is also suggested to be the route of transmission in rare cases. There is a similar relationship between HPV types and other malignancies such as EV-associated squamous cell carcinoma (SCC) (Harwood et al. 2004; reviewed by Sterling 2005). EV is rare condition in which sufferers develop warty and scaly areas of skin, especially on sun-exposed sites.

Generally lesions progress to SCC before the age of 40. In this case, HPV-5 and -8, are highly correlated with tumour formation, and are found in about 90% of cases. However, integration of the viral genome into the host chromosome is not required for progression to malignancy (Harwood et al. 2004).

1.1.3 Risk factors

HPV infection is associated with a number of risk factors. For example, immunosupression is known to result in development of HPV-associated lesions more frequently than in normal patients (Brown et al. 1999) and sexual activity (Kjaer et al. 2001), as well as age of first intercourse (Parazzini et al. 1992), are also known to play a role in acquisition of HPV. Furthermore, certain types of sexual behaviour, such as multiple partners results in increased HPV incidence (Clavel et al. 1998; Clavel et al. 1999). In general, HPV infection is cleared by the immune system, with only a relative few high-risk HPV infected women developing high-grade cervical lesions (Frisch and Goodman 2000; reviewed by Goodman 2000). In addition, incidence of high-grade lesions increases in women using oral contraceptives, suggesting this affects disease progression, rather than HPV infection (Negrini et al. 1990). Although, there are general risks factor, such as smoking, infection with other STI's, diet, multiple pregancies and family history, studies have shown that social class, religion and economic opportunities also play a role, with incidence of cervical cancer showing regional and cultural differences (Drain et al. 2002). Furthermore, infection with more than one HPV type is also associated with higher-grade lesions (Fife et al. 2001).

1.1.4 Detection and treatment

Detection of genital HPV infection is currently not performed on a population basis. Instead techniques using clinical diagnosis of potential pre-cancerous cells are employed. Techniques, each with different strengths, are Papanicolaou (Pap) smears and colposcopic morphology. During a Pap smear exfoliated genital cells are examined for increased nuclear/cytoplasmic ratio with darker and more irregular nuclei (dyskaryosis). Dyskaryosis can be used as a marker for severity of the CIN lesion, with very severe dyskaryosis indicating invasive cancer (reviewed by Hudson 1990). Whilst this technique is very specific (~90%), it has poor sensitivity for HPV infection (reviewed by Trofatter 1997). Upon persistence of abnormal pap smears, a patient is referred for colposcopy (Gamzu et al. 2002). In

this case cells of the cervix are swabbed with acetic acid and a colposcope is used to view the cells, with dysplasia showing as white areas. A biopsy is taken, followed by removal of abnormal cells. Colposcopy is very sensitive but, like pap smears can only detect clinically apparent infection (reviewed by Trofatter 1997).

Whilst population based Pap smear testing has reduced the incidence of cervical cancer, it is costly, time consuming and prone to human error. Furthermore, this type of screening is not performed in developing countries. Consequently, research is ongoing to produce reliable methods to detect infection with high-risk HPVs. Two such methods are PCR-ELISA and Hybrid Capture II (HC-II). PCR-ELISA involves labelled probe detection of a PCR product, generally amplified from the E7 or L1 ORFs (Venturoli et al. 1998; Zerbini et al. 2001), whilst direct interaction of an RNA probe with viral DNA, followed by antibody recognition and chemiluminescent detection is the basis of HC-II (Ferris et al. 1998; Poljak et al. 1999). Both techniques show high correlation for HPV detection, and can be used to determine viral types, however HC-II is less complex (Venturoli et al. 2002). One problem with HC-II is its inability to detect integrated genomes (Clavel et al. 1998), suggesting a combination of approaches may increase sensitivity and specificity of HPV detection.

Treatment against HPV infection generally involves physical removal of lesions. For example, periodic laser ablation of RRP lesions is currently the most effective treatment for the disease. However, this is costly and can be distressing for patients. Therefore, research is ongoing into production of vaccines targeting primarily cervical cancer associated HPV types, but also those associated with RRP. In June 2006 the American Food and Drug Administration (FDA) licensed a vaccine for prevention of cervical cancer and associated diseases (Gardasil, produced by Merck). This vaccine composes prophylactic quadrivalent virus like particles, comprising L1 protein from HPV-16, -18, -6 and –11. Furthermore, GlaxoSmithKline (GSK), have developed a bivalent vaccine containing VLPs for HPV-16 and –18. Phase III trails for Gardasil and phase II for the GSK vaccine show no side effects and 99.7% of those vaccinated develop an antibody response (Koutsky et al. 2002; Harper et al. 2004; Villa et al. 2005). There is some debate as to when HPV vaccines should be administered. Studies suggest immunisation of pre-adolescent girls is most effective, however this is somewhat controversial, as these HPVs are sexually transmitted.

1.1.5 The life cycle

Epithelial cells become more differentiated and less metabolically active the closer they move towards the surface of the epidermis, and the only layer that is mitotically active is the basal layer (Figure 1.3). Upon division, one daughter cell becomes committed to differentiation and moves into the spinous layer (reviewed by Fuchs and Byrne 1994). Spinous layer cells are metabolically active and are mainly involved in production of keratins, K1 and K10 (reviewed by Fuchs 1993). Lysine-rich envelope proteins, such as involucrin, begin to be produced in the upper spinous and granular layer (reviewed by Fuchs 1990). In the upper granular layer cells stop producing keratins and begin to express components of the cornified envelope and keratin filament matrix proteins. Intracellular degradation begins as cells reach the cornified layer; cells become flattened and are sloughed from the surface. Major morphological changes associated with terminal differentiation of normal epithelial tissue are disintegration of the nucleus, extensive keratinisation (cross-linking of keratin intermediate filaments), formation of cornified envelopes and lipid secretion (reviewed by Madison 2003). These changes are required to form a physical barrier against the environment.

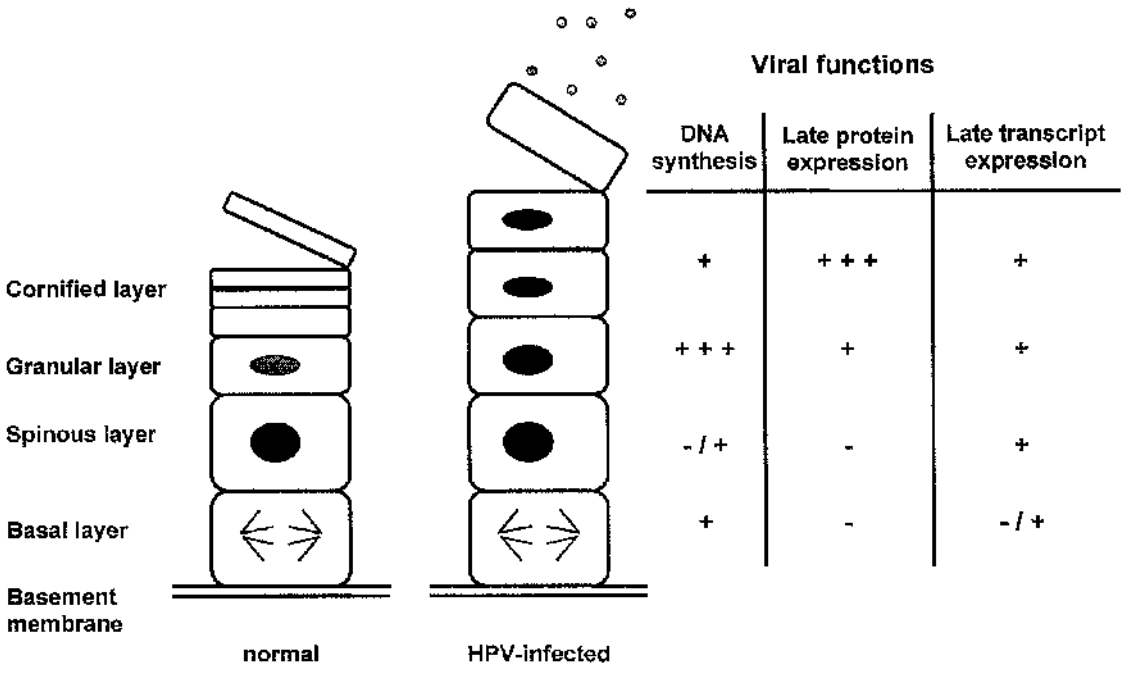


Figure 1.3: **Uninfected and HPV infected epithelial tissue**

A Schematic diagram of epithelial tissue, both uninfected and HPV infected. Epithelial tissue is composed of basal layer cells, capable of division and a number of suprabasal layers, cells of which become more differentiated and less metabolically active the closer to the surface they become. Upon HPV infection, the nucleus is retained to allow viral replication and gene expression. Whilst not depicted here, extensive keratinisation is required to allow virus exit from the outer layers of the epithelium. Expression of capsid proteins, L1 and L2 is restricted to only the most differentiated cells; however, transcripts are observed in less differentiated cells.

PVs gain entry into basal epithelial cells through micro-lesions, via a receptor on the surface of the cell. There is some controversy as to the identity of the receptor with both heparan sulphate (Giroglou et al. 2001) and α_6 integrin (Evander et al. 1997) being implicated. Giroglou et al. have shown that infection using HPV-16 and 33 pseudovirions can be blocked by heparin, a heparan sulphate ligand (Giroglou et al. 2001). Furthermore, HPV-16 VLPs interact with heparan sulphate, and binding can be inhibited by increasing heparin concentration (Shafti-Keramat et al. 2003). However, other studies indicate heparan sulphate is not required for HPV-31b infection of human keratinocytes, although it is necessary for infection of some other cells (Patterson et al. 2005). Furthermore, HPV-6b VLP interaction with cells can be blocked by anti-α_6 integrin antibodies (Evander et al. 1997), and expression of α_6 integrin in negative cells lines results in VLP binding (McMillan et al. 1999). HPV-16 L1 VLP binding to cells also correlates with α_6 integrin expression (Yoon et al. 2001). In addition, HPV-11 virions and VLPs interact with laminin 5 (LN5), a ligand of α_6 integrin (Culp et al. 2006). Expression of α_6 integrin is shown to be essential for infection of LN5 bound particles. In contrast, BPV-4 infects α_6 integrin positive and negative cells with similar efficiency (Sibbet et al. 2000). It has been proposed that binding to a secondary receptor is required for infection (Kawana et al. 2001; Yang et al. 2003). HPV-16 L2 interacts with an unknown cell surface protein and enhances infection, a process which is thought to be partly involved in epithelial tropism (Kawana et al. 2001). In addition, Yang et al. showed that regions of HPV-16 and BPV-1 L2 are required for infection but are dispensible for binding to the cell surface (Yang et al. 2003), and Embers et al. showed neutralisation of HPV-16, -6 and 11 infection using anti-L2 antibodies (Embers et al. 2004). It has been suggested more recently that internalization of virions is relatively slow and that endocytosis of clathrin coated vesicles is required (Day et al. 2003; Culp and Christensen 2004; Embers et al. 2004). BPV-1 virions co-localise with AP-2, a clathrin adaptor molecule, and HPV-16 VLPs co-localise with BPV-1 virions (Day et al. 2003). HPV-16 L1 VLPs endocytosis is also inhibited by nystatin, an inhibitor of clathrin mediated endocytosis (Bousarghin et al. 2002). However, the same study indicates that infection with HPV-31 L1 and L1+L2 VLPs is not inhibited by nystatin and instead endocytosis is mediated by another pathway. Translocation to the nucleus and nuclear entry are thought to be mediated primarily by L1 and L2, and are discussed further in section 1.1.6.5: L1 and L2. Once in the nucleus, the genome is established as an episome at ~10-200 copies per cell. It is thought that a productive phase follows initial infection,

resulting in an increase in the number of basal cells containing the viral episome (reviewed by Doorbar 2005). PV-infection of basal layer cells allows persistence of the viral genome, as it is maintained and segregated equally into daughter cells at each cell division. This is achieved via association with the host chromosome and the genome replicates via theta structures in a bi-directional fashion.

Upon initiation of differentiation, virus infected cells of the spinous layer are induced to divide once more. Blockage of the cell cycle at G_2 is then thought to create an environment for massive amplification of the virus genome via vegetative replication (Davy et al. 2002). Rolling circle replication of viral DNA is thought to occur here as multiple copies of the genome are generated from one initiation event (reviewed by Lambert 1991; Flores and Lambert 1997). In accordance, the nucleus is retained not only to allow replication but also to enable expression and processing of late gene transcripts (Figure 1.3). Following genome amplification, capsid proteins are expressed and virions are produced. This is thought to occur at PML bodies within the nucleus, as these may be the sites of viral DNA replication (Day et al. 1998; Swindle et al. 1999). The virus is thought to escape desquamated cornified cells (DCCs), which are continuously shed from the stratum corneum. DCCs are shown to have fragile envelops and are suggested to act as a vehicle for delivery of virions to fresh sites of infection (Bryan and Brown 2001).

1.1.6 Gene expression

Although there are many differences between HPV types, and PVs infecting other organisms, the genome structure for all is largely similar (reviewed by Shah and Howley 1996). PV gene expression is divided into early (E) and late (L) phases. E and L genes are encoded on one strand of the dsDNA genome and are produced depending upon the differentiation state of the cell (Figure 1.4) (reviewed by Laimins 1996). Replication and transcription are controlled by *cis*-acting sequences within a region of the PV genome called the Long Control region (LCR) (reviewed by zur Hausen 1996). Production of viral proteins as well as commencement of genome amplification occurs at differing stages of differentiation depending on the PV type. For example, genome amplification is observed in the mid or upper layers for alpha-PV types, such as HPV-16, where as HPV-1, a Mu-PV type, previously found in supergroup E, begins genome amplification as soon as cells leave the basal layer (Middleton et al. 2003). Gene

expression is highly complex and is controlled at both transcriptional and post-transcriptional levels. A number of polycistronic RNAs are expressed using various promoters and polyadenylation signals. Furthermore, *cis*-acting RNA elements are involved in directing the appropriate splice patterns depending on cell differentiation. Transcripts produced by HPV-16 in infected undifferentiated and differentiated epithelial cells have been mapped using RT-PCR and primer extension (Figure 1.5) (Milligan et al., 2006; Wildridge and Graham, unpublished). Extensive splicing is observed and a number of splice sites and promoters were found to be used. Transcripts with the potential to express novel, as well as the well-established proteins described below, were found. There are thought to be two major promoters, P_{97}, immediately upstream of the E6 ORF (reviewed by Smotkin and Wettstein 1986; Grassmann et al. 1996), and P_{670}, the differentiation specific promoter in the E7 ORF (Grassmann et al. 1996). Promoters are found at similar positions within the genome of other HPV types, such as HPV-18 and –31 (Schneider-Gadicke and Schwarz 1986; Hummel et al. 1992; Ozbun and Meyers 1998). It is suggested that switching occurs between the two promoters several times during the virus life cycle. Further promoters have been proposed for HPV-16; P_{542} and P_{482}, in front of the E7 ORF (Braunstein et al. 1999); P_{430}, P_{441}, P_{446}, P_{483}, and P_{489}, within E6 (Rosenstierne et al. 2003); P_{3392}, within E4 and $P_{4062/64}$, within in E5 (Milligan et al., 2006), each of which is thought to contribute to overall protein expression.

In terms of polyadenylation signals, HPV-16 seems to be complex. There are 3 polyadenylation signals: the early signal (pAE) at nt 4213, upstream of the L2 ORF, and two late signals (pAL), at nts 7286 and 7343 (Kennedy et al. 1990). Polyadenylation is regulated in both undifferentiated and differentiated cells by *cis*-acting RNA elements, particularly in the early and late 3'UTRs. For example, a region of the early HPV-16 3'UTR which is U rich and interacts with components of the polyadenylation and cleavage machinery, enhances polyadenylation from the pAE in HeLa cells (Zhao et al. 2005). In addition, the pAL signals differ in strength, with the more downstream containing a good consensus GU-rich region, which would bind polyadenylation factor CstF with more efficiency (see section 1.2.3: Cleavage and Polyadenylation); however, both are used *in vivo*, with the major site at nt 7343 being used only in differentiated HPV-16 infected epithelial cells (Milligan et al., 2006).

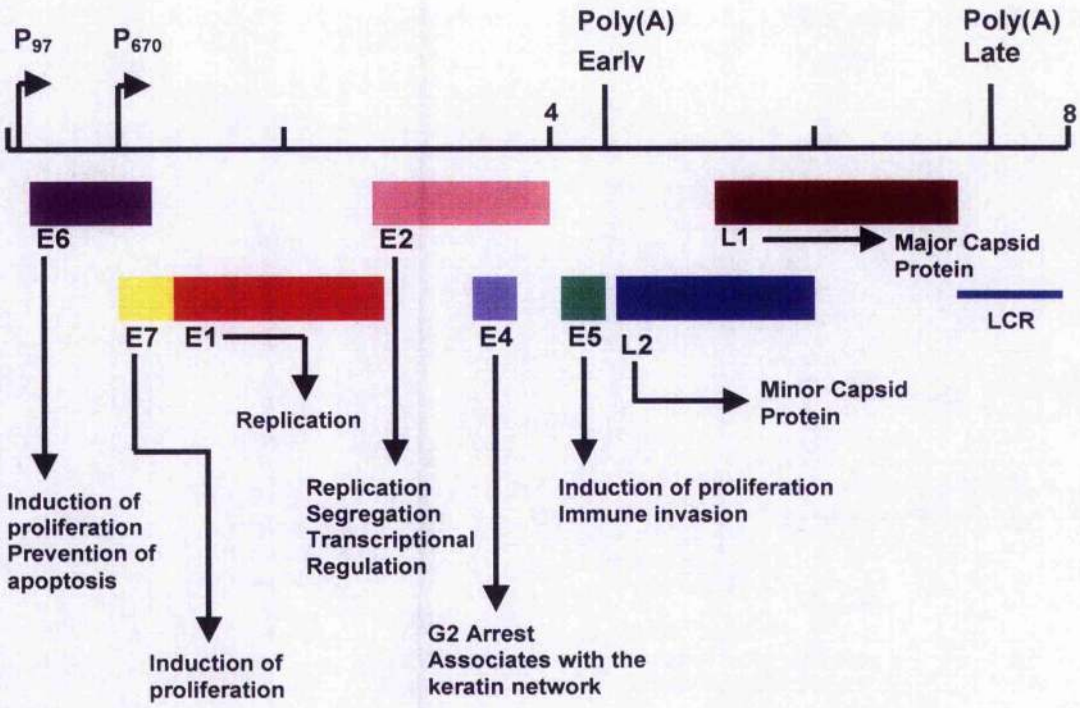


Figure 1.4: **Papillomavirus genome structure**

A Schematic diagram of a linearised version of the HPV-16 genome (not to scale). The major promoters, P_{97} and P_{670}, as well as the early and late polyadenylation signal are shown. ORFs are depicted as coloured rectangles, with a summary of major gene functions given below each. The LCR is located after the L1 ORF.

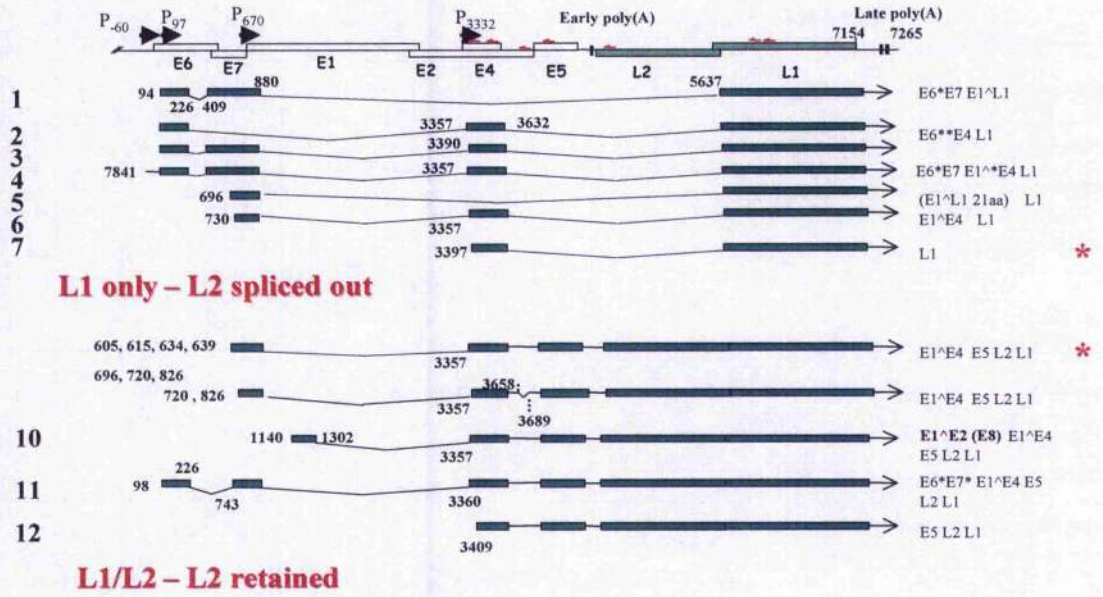


Figure 1.5: **Transcripts produced by HPV-16 in differentiated W12E cells**

A Schematic diagram of transcripts terminating at poly(A) Late produced by HPV-16 in differentiated infected epithelial cells (not to scale). A linearised version of the genome is shown at the top with transcripts depicted below. ORFs are shown as open rectangles with the late genes, L1 and L2, in green. Arrowheads represent promoters, whilst thick bars indicate polyadenylation signals. Within the transcripts, diagonal thin lines represent regions that are removed by splicing and coding potential of each transcript is given to the right. Arrows indicate inferred but not tested termination at the late polyadenylation signal. Red stars indicate the most abundant transcripts. Transcripts containing E1 and E2 ORFs have been detected in differentiated cells, but terminate at poly(A) Early, and therefore are not shown on this figure.

1.1.6.1 E6 and E7

Two early HPV proteins are E6 and E7, which are involved in inhibition of apoptosis and promotion of the cell cycle in basal layer cells, and are the major transforming proteins of the virus (reviewed by Mantovani and Banks 2001; reviewed by Munger et al. 2001). Both proteins are expressed at low levels in basal layer cells (Stanley et al. 1989) and continue to be expressed as the cell differentiates. E6 and E7 protein from both high and low-risk HPVs are thought to be involved in episomal maintenance, extending their roles within the virus life cycle (Thomas et al. 1999; Oh et al. 2004). Mutant E6 or E7 HPV-31 genomes were able to replicate in transiently transfected cells; however, only genomes with functional E6 and E7 were maintained in high copy number (Thomas et al. 1999). Similary, HPV-11 genomes lacking E6 or E7 expression were also unable to maintain the episomal genome in transfected cells (Oh et al. 2004). It is suggested that E6 and E7, modify the cellular environment to prevent removal of the extrachromosomal viral genomes.

E6 is ~150 amino acids in length and contains two zinc finger-like motifs (Barbosa et al. 1989). It interacts with a number of cellular proteins involved in tumour suppression, apoptosis and transcription, amongst other things. The most extensively studied interaction with E6 is that of tumour suppressor p53. p53 is activated in response to DNA damage and halts the cell cycle at specific checkpoints, at which point damage is repaired or the cell is committed to the apoptotic pathway (Lowe et al. 1994; Wu and Levine 1994). It is phosphorylated by cellular kinases ATM and ATR, and the phosphorylated form acts to transcriptional activate expression of genes involved in growth arrest and apoptosis (reviewed by Mercer et al. 2007). HPV-16 and –18 E6 are shown to interact with p53 *in vitro* (Werness et al. 1990). Furthermore, Lechner & Laimins showed binding affinities varied between p53 and E6 from different HPV types (Lechner and Laimins 1994). HPV-16 E6 bound most strongly, followed by –31, -18 and lastly –11, suggesting affinity may contribute to oncogenic potential. The HPV-16 E6-p53 interaction is important *in vivo* to stimulate ubiquitin mediated p53 proteasome degradation (Scheffner et al. 1990). Furthemore, the half-life of p53 is significantly reduced in cells expressing HPV-18 E6 (Lechner et al. 1992). This is thought to be involved in inhibition of p53 mediated growth suppression and allows the G1/S checkpoint, which normally serves as a control for DNA-damaged cells, to be bypassed. For example, expression of HPV-16 E6 leads to reduction in p53,

which correlates with it's ability to block actinomycin D induced growth arrest (Foster et al. 1994). In addition, inhibition of DNA synthesis in the epidermis following treatment with ionising radiation is abrogated in transgenic mice expression HPV-16 E6 (Song et al. 1998). Degradation of p53 is mediated via interaction with cellular protein, E6-associated protein (E6AP). E6AP is an E3 ubiquintin ligase and via interaction with HPV-16 and –18 E6, it mediates p53 ubiquitination (Huibregtse et al. 1991; Huibregtse et al. 1993; Scheffner et al. 1993). The role of E6AP in p53 degradation is also observed in HeLa cells, where overexpression results in decreased levels of p53 (Talis et al. 1998). Furthermore, a catalytically inactive E6AP can act as a dominant negative upon p53 regulation. No such effects were observed using an HPV negative cell line, suggesting E6AP mediates p53 degradation only in the presence of E6 (Talis et al. 1998). High-risk E6 also associates with other pro-apoptotic proteins, such as Bak (Thomas and Banks 1998). Bak is a Bcl-2 family member which is involved in reinforcing upstream apoptotic signals to push cells through apoptosis and is highly expressed in differentiating keratinocytes (Krajewski et al. 1996). HPV-18 E6 is shown to induce degradation of Bak, again via association with E6-AP, a process that inhibits apoptosis (Thomas and Banks 1998).

E6 is also implicated in the degradation of PDZ-domain containing proteins, human discs large (hDlg), MAGI-1, -2 & -3, MUPP1 and human scribble (hScrib). These proteins are thought to act as tumour suppressors, and hDlg, MAGI-1 and MUPP1 are strong inhibitors of cellular transformation (Massimi et al. 2004). Furthermore, HPV-18 E6 can overcome the growth suppression effects of hDlg. Kiyono et al. showed interaction between hDlg and both HPV-16 and –18 E6 via a PDZ-binding domain in the C-terminal region of E6 (Kiyono et al. 1997). This interaction was not observed with HPV-11 and –5 E6, suggesting it is a unique function of high-risk cervical HPVs. In addition, expression of HPV-16 but not HPV-11 E6 results in downregulation of hDlg via proteasome mediated degradation (Gardiol et al. 1999). A similar situation has been observed for MAGI-1, -2 & -3, MUPP1 and hScrib, where an interaction is necessary for proteasome mediated degradation (Glaunsinger et al. 2000; Lee et al. 2000; Nakagawa and Huibregtse 2000; Thomas et al. 2002). In addition to degrading hScrib, HPV-16 E6 expression is shown to result in loss of integrity of tight junctions, as shown by the relocalisation of tight junction component ZO-1 (Nakagawa and Huibregtse 2000). However, there is some debate as to the mechanisms involved in degradation. In the case of hScrib, HPV-39 E6 acts via E6AP, in a similar manner

as described above for p53 (Nakagawa and Huibregtse 2000). HPV-16 E6 interaction with E6-AP is also implicated in hDlg and hScrib degradation (Matsumoto et al. 2006). However, Pim et al. showed that mutant HPV-16 and –18 E6 proteins which are unable to bind E6AP and do not degrade p53, are still able to target hDlg for degradation (Pim et al. 2000). In addition, immunodepletion of E6AP from rabbit reticulocyte lysate inhibits HPV-16 E6 induced p53 but not MAGI-1 degradation (Sterlinko Grm et al. 2004). This suggests that mechanisms involved in PDZ-domain containing protein degradation may differ for each protein and possible HPV types. Regardless of the mode of degradation, the C-terminal PDZ-binding domain of HPV-16 and –18 E6 is necessary for cell transformation (Kiyono et al. 1997). In addition, transfections using PDZ-binding E6 mutant HPV-31 genomes, showed retarded growth and reduced viral copy number when compared to wild type (Lee and Laimins 2004), and mutant HPV-16 E6 was unable to induce epithelial hyperplasia in transgenic mice, despite retaining the ability to inactivate p53 (Nguyen et al. 2003). This indicates that PDZ-domain containing protein binding by high-risk E6 is an important function of E6.

Apart from its function in degradation of a number of cellular proteins, E6 is involved in transcriptional regulation. In particular, interaction of HPV-16, -18 and BPV-1 E6 with, and subsequent inhibition of transcriptional activity of co-activators CBP/p300, is required for optimal transforming activity (Patel et al. 1999; Zimmermann et al. 1999; Zimmermann et al. 2000). CBP/p300 play roles in activation of a number of genes involved in cell cycle regulation, differentiation and immune responses. HPV-16 E6 is shown to downregulate p53-mediated transcriptional regulation via interaction with CBP/p300 (Zimmermann et al. 1999). This is achieved in an E6AP independent manner, and E6 can displace the CBP/p300-p53 interaction, which is required for p53 to transactivate cellular promoters. Therefore, HPV-16 E6 not only causes degradation of p53, but also inhibts its activity. Furthermore, expression of HPV-16 E6 decreases the ability of CBP/p300 to activate p53 and NF-kappaB responsive promoters (Patel et al. 1999). NF-kappaB can regulate genes such as interleukins (ILs), and IL-8 expression, which is involved in the local immune response, requires NF-kappaB, CBP/p300 and related co-activator p/CAF for activation (Huang and McCance 2002). HPV-16 E6 represses IL-8 expression by competing with NF-kappaB for CBP/p300 binding. Furthermore, HPV-16 and –18 E6 also interact with other co-activators, such as hADA3, the yeast homologue of which bridges transcription factors with histone acetylation and the basal transcription machinery (Kumar et al.

2002). E6 is shown to cause hADA3 degradation *in vivo* and also abrogates its co-activation function with p53.

E7 is a small protein of ~100 amino acids and contains an LXCXE motif and two zinc finger motifs (reviewed by Hebner and Laimins 2006). An important function of high-risk E7 oncoproteins is interaction with and degradation of the Rb family of proteins. Rb is a downstream target of p53 and is involved in cell-cycle control at the G_1/S phase checkpoint, via differential phosphorylation. Hypophosphorylated Rb interacts with E2F, preventing it from activating transcription of genes involved in S phase. Expression of HPV-16 E7 is shown to cause failure of G1 arrest following gamma-irradiation; however, whilst decreased hypophosphorylated Rb is detected, p53 levels are elevated (Demers et al. 1994; Slebos et al. 1994). In Caski cells, which express HPV-16 E7, both Rb and E7 are regulated by ubiquitin mediated proteasome degradation (Wang et al. 2001). E7 is shown to promote Rb ubiquitination, and inhibition of proteasomal degradation leads to an increase in hypophosphorylated Rb; however, Rb is not observed to complex with E2F, suggesting that E7 acts to block this interaction. Furthermore, HPV-18 E7 is shown to disrupt the Rb-E2F interaction in HeLa cells, without affecting E2F-cyclin A association, a complex that promotes E2F mediated transactivation and proliferation (Pagano et al. 1992). Although this is thought to be important to E7 function, blocking the Rb-E2F interaction is not sufficient for HPV-16 E7 to inhibit anti-proliferative signals (Helt and Galloway 2001). Furthermore, a number of studies have revealed that inactivation of Rb by E7 is not the only factor involved in E7-induced transformation. For example, Balsitis et al. showed that Rb inactivation in transgenic mouse models does not account for the entire phenotype observed in mice expressing HPV-16 E7 in undifferentiated epithelial cells, and expression of E7 in Rb null mice was shown to increase hyperplasia and dysplasia (Balsitis et al. 2003; Balsitis et al. 2006). Furthermore, when HPV-16 E7 transgenic mice were crossed with Rb mutant mice, in which Rb was unable to bind E7, a modest delay in terminal differentiation was observed (Balsitis et al. 2005). Indeed, HPV-16 E7 complexed with E2F-cyclin A/cdk2 in S phase (Arroyo et al. 1993). HPV-16 E7 interacts directly with E2F and synergism is seen between the two proteins upon activation of an E2F responsive promoter for cyclin E (Hwang et al. 2002). This is also observed in Rb negative cells, showing that this function of E7 is Rb-independent. Interaction of E7 with other Rb family members has also been observed. Whilst HPV-16 E7 disrupts association of E2F with Rb, and family members p107 and p130, HPV-6 E7 only affects p107 and

p130 interactions with E2F (Armstrong and Roman 1997). This possibly reflects the transforming potential of the two E7 proteins.

E7 proteins are also shown to interact with cyclins and cyclin dependent kinases (cdk's), as well as cyclin kinase inhibitiors (CKIs). HPV-16 E7 is thought to interact with cdk2, via cyclin A, an interaction which does not require Rb (Tommasino et al. 1993). In addition, HPV-18 E7 associates with cyclin E *in vitro* and *in vivo* (McIntyre et al. 1996). Interaction is shown to be via p107 and the complex phosphorylates p107, a function which is dependent upon cyclin E. HPV-16 E7 is also able to repress the CKI, $p21^{CIP1}$ inhibitory activity upon cyclin E/cdk2 (Funk et al. 1997). This is achieved via direct interaction between the two proteins and is thought to allow uncoupling of proliferation and differentiation in HPV-16 E7 expressing keratinocytes (Jones et al. 1997). HPV-16 E7 also interacts with CKI $p27^{KIP1}$ (Zerfass-Thome et al. 1996). This represses $p27^{KIP1}$ inhibition of cyclin E and also its suppression of transcription of cyclin A. In addition, expression of HPV-16 E6 or E7 is suggested to cause genomic instability, which is likely involved in malignant progression. For E6 this is thought to be achieved via inactivation of p53 (White et al. 1994); whereas, E7 is shown to cause abnormal centromere duplication when expressed in human keratinocytes (Duensing et al. 2000). Expression of mutant HPV-16 E7 that cannot inactivate CKI $p21^{CIP1}$, does not induce centromere abnormalities. Furthermore, inhibition of cdk2, which is involved in linking centromere duplication with the G1/S transition, also abrogates E7s ability to induce centrosomal abnormalities (Duensing et al. 2000). In addition, HPV-16 E7 induces mis-segregation and aneuploidy, requiring the region spanning the Rb binding domain, but in a pRb independent manner (Duensing and Munger 2003). Expression of HPV-16 E6 or E7 in human keratinocytes also results in un- or misaligned chromosomes and an increase of genetic material is observed at metaphase (Duensing and Munger 2002). The anaphase checkpoint is also by-passed and a number of anaphase bridges, which reflect structural chromosomal abnormalities, are observed. However, expression of HPV-6 E6 and E7 have no effect on centromere or chromosomal stability, suggesting this is a function specific to the high-risk oncoproteins (Duensing and Munger 2002). This function for HPV-16 E6 and E7 is also evident in basal epithelial cells transfected with HPV-16 genomes (Duensing et al. 2001). In addition to the role in malignant progression by mutation of cellular genes, it is suggested that genome instability may also stimulate integration of viral genomes (Melsheimer et al. 2004). Melsheimer et al. showed that a greater proportion of HPV-16 associated cervical

lesions with integrated genomes show aneuploidy, when compared with the lower percentage of aneuploid cell lines without integrated HPV-16 genomes.

1.1.6.2 E5

The E5 protein also has a role in cell transformation in some PV types, such as BPV-1 (DiMaio and Mattoon 2001); however, E5 is not detected in HPV associated tumour cells (DiMaio and Mattoon 2001). This does not rule out a proliferation stimulating activity of E5, which may function in benign lesions or in initiation of the carcinogenic process. E5 is predominantly expressed upon differentiation, although low levels are thought to be present in undifferentiated cells. Although there seems to be functional homology between E5 from different papillomavirus types, there is little sequence homology. In agreement, E5 from a number of papillomavirus types are known to down-regulate the major histocompatablilty complex (MHC) class I (Ashrafi et al. 2002; Ashrafi et al. 2005). MHC class I (or HLA I in humans) is involved in antigen presentation, followed by lysis by cytotoxic T lymphocytes of infected cells. The molecule is a ternary complex composed of heavy chain, β2 microglobulin and peptide (Solheim 1999). Down-regulation is achieved by several mechanisms including inhibition of heavy chain gene transcription or protein stability, and repression of complex assembly and/or transport (Ashrafi et al. 2006). HPV-16 E5 is known to cause retention of HLA I in the Golgi apparatus, hence preventing its transport to the cell surface (Ashrafi et al. 2005). This is achieved via interaction with the heavy chain component of the complex (Ashrafi et al. 2006). HPV-16 E5 also interacts with the 16kD subunit of the vaculolar H^+-ATPase and leads to increased presentation of the epidermal growth factor receptor (EGFR) on cells expressing E5 (Straight et al. 1993; Straight et al. 1995). Furthermore, HPV-16 E5 interacts with ErbB-4, a growth factor receptor which is related to EGFR (reviewed by Carpenter 2003). This interaction inhibits ErbB-4 induced c-jun expression and phosphorylation, resulting in increased cell proliferation (Chen et al. 2007). These data suggest HPV-16 E5 may have a proliferative stimulatory function, which may function within benign lesions.

1.1.6.3 E1 and E2

E1 and E2, the viral replication and maintenance proteins are expressed at early stages in differentiated cells to massively increase the number of viral genomes by

vegetative replication (Mohr et al. 1990; Winokur and McBride 1992). Although E1 and E2 expression is observed throughout the PV life cycle, the highest levels are observed upon differentiation when vegetative viral genome replication occurs (Maitland et al. 1998). Interaction of E2 with the viral LCR is thought to facilitate interaction of E1 with the proximal AT-rich, E1 binding site, via direct E1-E2 interaction, whilst E1 ATP-dependent helicase activity is then involved in DNA unwinding and recruitment of cellular replication factors. Kuo et al. showed that both E1 and E2 are essential for HPV-11 DNA replication in a cell-free system complemented with cell extracts (Kuo et al. 1994). Furthermore, E2 is shown to inhibit E1 driven origin-independent replication. HPV-11 E1 DNA binding is largely non-specific and an E1 binding site is not essential for high affinity E1/E2 complex formation on the origin (Dixon et al. 2000). HPV-16 E1 is also unable to associate with the origin in the absence of E2, and origin interaction is shown to require direct binding between E1 and E2 (Masterson et al. 1998). Similarly HPV-31b E1-origin association requires E2 binding sites and is enhanced by E2 (Frattini and Laimins 1994). In contrast, BPV-1 replication using a cell-free system complemented with mouse or HeLa cell extracts, show that E2 is not an absolute requirement for replication (Muller et al. 1994). However, at low levels of E1, E2 stimulates E1 origin binding and origin-specific unwinding, without affecting helicase activity (Seo et al. 1993). In addition, like HPV-11 E2, BPV-1 E2 suppresses non-origin dependent replication by E1 (Bonne-Andrea et al. 1997). HPV-1a E1 is also sufficient for replication of HPV-1a and BPV-1 origin containing plasmids *in vivo*; however, both HPV-1a E1 and E2 are required for replication from HPV-6b and HPV-18 origins (Gopalakrishnan and Khan 1994). This suggests the intrinsic strength of the E1 binding site, rather than the E1 protein itself, may determine whether E2 is required or not. However, in either case, E2 seems to ensure replication from the viral origin and not any other site.

In addition to E1 and E2, papillomavirus replication is shown to require a number of cellular proteins. For example, HPV-11 cell-free DNA replication requires the HPV-11 origin sequence, DNA polymerase α and δ, replication protein A and topoisomerase I and II (Kuo et al. 1994). DNA polymerase α/primase is the key enzyme involved in initiation of DNA synthesis and is composed of four subunits: p180, the polymerase, p58 and p49, with primase activity and p70, a cell cycle dependent phospho-protein. HPV-16 and HPV-11 E1 interact with the p70 subunit (Masterson et al. 1998; Conger et al. 1999). In addition, HPV-11 and BPV-1 E1 associate with the p180 subunit (Park et al. 1994; Conger et al. 1999). Interaction

of HPV-11 E2 with both p70 and p180 subunits was shown to be essential for viral DNA replication (Conger et al. 1999). In addition, antibody's recognising p180 inhibited BPV-1 viral replication in cell-free systems (Park et al. 1994). This suggests that recruitment of DNA polymerase α/primase to the viral origin is essential to replication. Furthermore, BPV-1 E1 interacts with the replication protein A (RPA) complex (Han et al. 1999). The RPA complex, which is composed of three subunits, associates with single stranded DNA (ssDNA) and has roles during DNA replication, repair and recombination. Loo and Melendy showed that BPV-1 E1-RPA interaction can be inhibited by increasing concentrations of ssDNA (Loo and Melendy 2004). It is suggested that disruption of the E1-RPA complex results in continuous recruitment of RPA to the replication fork, due to the proximity of ssDNA from the lagging strand throughout replication. More recently, HPV-11 and BPV-1 E1 have been found to interact with topoisomerase I (topo I), a protein involved in decreasing torsional stress of supercoiled DNA (Clower et al. 2006). Interaction was shown to stimulate topo I relaxation activity upon the PV origin (Clower et al. 2006) and viral DNA unwinding mediated by HPV-11 E1 requires topo I, as well as RPA, to proceed (Lin et al. 2002). In addition, recruitment of BPV-1 E1 to the viral origin was stimulated by topo I, but not RPA or DNA polymerase α/primase, *in vitro*. HPV-11 E2 also interacts with topo I (Clower et al. 2006). This interaction stimulates DNA relaxation activity in an origin independent manner, but does not affect E2 DNA binding.

Other cellular proteins involved in PV replication include chaperones. HPV-11 E1 associates with molecular chaperons hsp70 and hsp40 (Liu et al. 1998). These interactions independently enhance E1-origin binding and replication in a cell-free system, however no synergism was shown between the two proteins. Whilst E1 predominantly binds DNA as a hexamer in the absence of chaperons, hsp 40 promotes di-hexamer formation (Liu et al. 1998). As E1 functions as a hexamer in DNA unwinding, formation of di-hexamers would provide enough E1 for bi-directional unwinding of DNA. Phosphorylation of E1 is also important, and BPV-1 and HPV-11 E1 interact with cyclin E/cdk complexes, via cyclin E, a regulator of S phase during the cell cycle (Cueille et al. 1998; Ma et al. 1999). Phosphorylation of HPV-11 E1 is important for replication (Ma et al. 1999) and is shown to be achieved using one or more cyclin/cdk complexes *in vivo* (Cueille et al. 1998). Deng et al. showed that non-phosphorylated E1 has cytoplasmic localisation due to a nuclear export signal (NES) (Deng et al. 2004). This was inactivated by cdk

phosphorylation, resulting in E1 nuclear retention, where it is available to regulate viral DNA replication. In contrast, cyclin A/cdk2 phosphorylation of BPV-1 E1 on Serine 283 is not required for viral replication (Hsu et al. 2006). Instead this promotes nucleo-cytoplasmic shuttling in S phase, when cyclin A/cdk2 is active, a process thought to prevent viral replication.

E2 is a well-studied protein of approximately 42 kDa, and has many roles in both undifferentiated and differentiated cells. The E2 protein has been functionally divided into three regions: (1) the N-terminal, transactivation domain, (2) the C-terminal, DNA binding and dimerization domain and (3) the hinge region joining the two (Hegde and Androphy 1998; Antson et al. 2000). In BPV-1, splice variation results in expression of three forms of the protein: E2-TA (transactivation), containing all three domains, and two truncated versions, E2-TR and E8/E2-TR (trans-repression) (Choe et al. 1989). E2-TR contains the hinge and C-terminal domain, whilst the E8 ORF, which is located in the E1 ORF spliced to the E2 mRNA, also containing the C-terminal region, encodes E8/E2-TR. It is thought that E2-TR and E8/E2-TR act as dominant negatives of E2-TA, as they retain DNA binding and dimerization activity but lack the ability to control transcriptional regulation (Lambert et al. 1987). The transactivation and DNA binding domains are largely conserved among different PV types; however, the hinge is variable in both length and sequence (McBride et al. 1989; Gauthier et al. 1991).

In basal layer cells E2 is known to tether virus genomes to the cellular chromosome to ensure equal partitioning to daughter cells during cell division. For example, BPV-1 genomes, as well BPV-1 E2 are shown to associate with mitotic chromosomes, in a DNA binding independent manner, via the N terminal region, throughout mitosis (Skiadopoulos and McBride 1998; Bastien and McBride 2000). In contrast, HPV-11, -16 and –18 E2 co-localise with mitotic chromosome during prophase and metaphase and migrate to the central spindle microtubules at anaphase, requiring the C-terminal region (Dao et al. 2006). This is again achieved via interactions with cellular proteins and recently interaction with mitotic chromosome binding protein, Brd4 was observed between the E2 protein of some PV types (You et al. 2004; Baxter et al. 2005). You et al. have shown that BPV-1 E2 interacts with Brd4 via the Brd4 C-terminal domain (Brd4-CTD) and that expression of Brd4-CTD inhibited viral episome association with mitotic chromosomes and BPV-1 mediated cell transformation, in a dominant negative

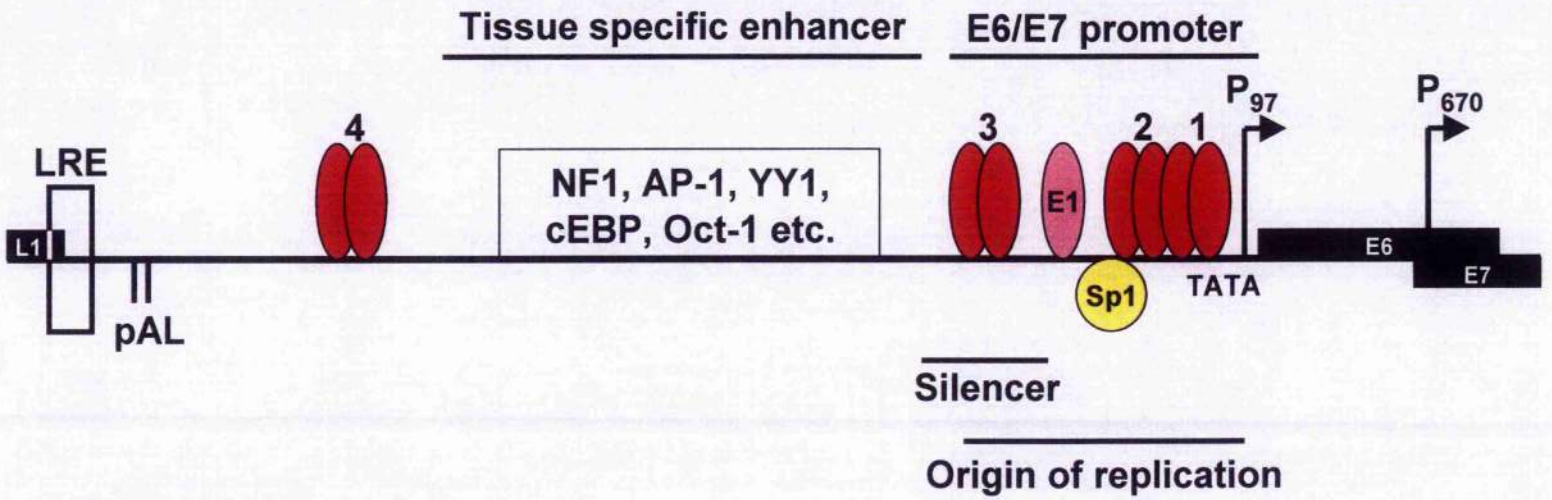
fashion (You et al. 2004). Brd4 was seen to relocate from diffuse coating of condensed chromatin, to random punctuate dots in BPV-1 E2 expressing cells (McPhillips et al. 2005). Furthermore, studies using yeast plasmids containing an autonomous replication site (ARS) and E2 binding sites to replace the centromeric (CEN) maintenance element, have shown that Brd4 is required for BPV-1 E2 induced maintenance of the plasmid (Brannon et al. 2005). Stable expression of Brd4-CTD in BPV-1 transformed cell lines also results in morphological reversion and complete elimination of viral DNA (You et al. 2005). HPV-16 E2 was also shown to interacts with Brd4 and abolishment of binding eliminates chromosome association (Baxter et al. 2005). However, further analysis has revealed that this interaction is not required for viral replication and instead Brd4 acts to regulate HPV-16 E2-mediated transcription (Schweiger et al. 2006). In support of this, co-localisation of HPV-16, as well as –11, -31 and –57 E2 with mitotic chromosome does not require Brd4 interaction (McPhillips et al. 2006) and no interaction is observed between HPV-11, -16 and –18 E2 with Brd4 during mitosis (Dao et al. 2006). Therefore, Brd4 interaction with α-PV E2 proteins is suggested to regulate transcription rather than replication or maintenance of viral genomes. It is also thought that binding of HPV-11 E2 to E1BS proximal sites within the LCR may play a role in preventing binding of negative regulators, such as CCAAT displacement protein (CDP) to the origin of replication (Narahari et al. 2006). CDP association with the origin is decreased in E2 expressing cells. Furthermore, E2 is shown to alleviate CDP mediated inhibition of replication (Narahari et al. 2006).

Another cellular process E2 is known to be involved in is apoptosis. HPV-18 and BPV-1 E2 have been shown to repress endogenous E6/E7 expression in HeLa cells (Desaintes et al. 1997). An increase in p53 transcription activity is observed, which is required for E2-induced G1 arrest but not apoptosis. Furthermore, HPV-18 and BPV-1 E2 do not activate the main p53 responsive gene involved in promotion of apoptosis, Bax, even though p53 levels are increased (Desaintes et al. 1999). p53 accumulates after E2-induced apoptosis has begun, suggesting it is not involved in this process. In contrast $p21^{WAF1/CIP1}$, a downstream target of p53 is transcriptionally activated by E2 expression, leading to G1 arrest (Desaintes et al. 1999). HPV-16 E2 has also been shown to interact directly with p53 via the C-terminal domain (Massimi et al. 1999). This interaction correlates with the ability of p53 to inhibit HPV DNA replication. Furthermore, HPV-16 E2 expression in HPV transformed cells results in decreased growth and cell death (Sanchez-Perez et al. 1997; Webster et al. 2000). HPV-16 E2 also promotes apoptosis in non-HPV

transformed cells, which correlates strongly with p53 binding (Parish et al. 2006). However, HPV-18 E2 can induce apoptosis in p53 negative cells (Demeret et al. 2003). This is found to be achieved via activation of Caspase 8 by autocleavage (Demeret et al. 2003; Blachon et al. 2005). In addition, p53 binding is dispensable for E2-induced apoptosis in HPV-transformed cells, in agreement with previous data (Parish et al. 2006). In the same study, HPV-6 and –11 E2 failed to induce apoptosis and did not interact with p53. Blanchon et al. show that induction of apoptosis by E2 proteins from different PV types may be a consequence of cellular localisation (Blachon et al. 2005). HPV-16 and –18 E2 are found to shuttle between the nucleus and the cytoplasm, whereas HPV-6 and –11 E2 are predominantly nuclear. Cytoplasmic location is shown to correlate with apoptosis (Blachon et al. 2005).

E2 proteins are known to transcriptionally activate/repress the viral LCR, via a number of E2 binding sites (E2BSs). In the case of HPV-16 and other mucosal PV types, such as HPV-18 and BPV-4, 4 E2BSs are present within the LCR, each differing in E2 binding affinity (Figure 1.6) (reviewed by Kalantari and Bernard 2006). Differential binding of E2 to these sites, which is at least partly due to E2 levels, is known to affect the repression/activation of E6 and E7 (or E5 and E7 in the case of BPV-4), as well as viral genome replication, via the early promoter (Steger and Corbach 1997; Morgan et al. 1998). For example, at low to intermediate concentrations BPV-4 E2 activates E5/E7 transcription (Morgan et al. 1998). Conversely, as E2 levels rise, E5/E7 expression is inhibited and mutation of E2BS1, which is 3bp upstream of the TATA-box, results in abrogation of trans-repression by E2. A similar situation is observed for HPV-18, where low E2 expression allows transactivation at the early promoter, whereas high E2 causes repression (Steger and Corbach 1997). HPV-18 E2 has high affinity for BS4, binding of which is thought to be involved in transactivation of the early promoter. Furthermore, affinity for E2BS1 is low; suggesting binding of this site at high concentrations of E2 is involved in repression. In agreement, BPV-1 E2 binds E2BS1 from the HPV-18 LCR with high affinity and is able to repress the promoter more efficiently than HPV-18 E2 (Steger and Corbach 1997). The proximity of E2BS1 with the TATA-box is suggested to inhibit recruitment of TBP to the promoter, and hence to prevent Pre-initiation complex formation, as in the case of BPV-1 (Dostatni et al. 1991). Indeed, mutation of E2BS1 in the BPV-4 LCR inhibits repression of the early promoter at high levels of E2 (Morgan et al. 1998). For HPV-18, E2BSs 1-3 are required for full repression of the early promoter in

epithelial cells, and displacement of Sp1 when E2 occupies BS2 is thought to contribute to this (Demeret et al. 1997). HPV-16 E2 is also shown to displace Sp1 from the overlapping Sp1 BS when bound to BS2, and E2BS1 & 2 are again shown to be necessary for repression (Tan et al. 1994). Sp1 is elevated in epithelial cells, such as HaCaT cells, when compared with fibroblasts, and it is suggested to be a major cellular factor controlling expression from the HPV-16 early promoter (Apt et al. 1996). In addition, Sp1 can transactivate the HPV-18 LCR via the promoter proximal binding site overlapping E2BS2 (Hoppe-Seyler and Butz 1992). This suggests displacement of Sp1 from the E2BS2 proximal Sp1 binding site within mucosal PV LCRs is important to repression of the early promoter by E2. However, binding of HPV-16 E2 to BS1 & 2 results in increased repression, compared with binding to either site alone, indicating that displacement of both Sp1 and TBP are required for efficient repression (Tan et al. 1992; Tan et al. 1994). In contrast, Bechtold et al. reported that high expression of HPV-16 E2 represses E6 and E7 from integrated HPV-16 genomes; however, no such repression was observed in cells containing episomal copies of HPV-16 (Bechtold et al. 2003). This is suggested to be due to the closed conformation of the HPV-16 p97 promoter in episome containing cells. However, it is not clear at what stage of differentiation cells were assayed for E6/E7 repression and it is possible that the chromatin conformation of the promoter may change throughout differentiation to help control E6/E7 expression. Therefore, HPV-16 E2 may be able to repress the p97 promoter in episomally infected cells only at certain stages of differentiation.



Adapted from Kalantari and Bernard, 2006

Figure 1.6: **HPV-16 Long Control Region (LCR)**

A schematic diagram of the HPV-16 LCR (not to scale). E2 is shown as red circles, binding as dimers to binding sites 1-4, and pink and yellow circles indicate E1 and Sp1, respectively, located at their relevant binding sites. ORFs are depicted as black rectangles, promoters as arrows, polyadenylation signals as short lines and the LRE as an open rectangle. Positions of the tissue specific enhancer, E6/E7 promoter and origin of replication are indicated.

In addition to E2 responsive regions and Sp1 binding sites, a number of other cellular transcription factor (TF) binding sites are found within the LCR, and interaction of E2 with cellular proteins is known to affect transcriptional activity. A region between E2BS 3 and 4, the tissue specific enhancer, is shown to cause transactivation in only keratin expressing cells via synergism of a number of cellular TFs (Figure 1.6). For example, expression from the HPV-18 LCR is specifically enhanced in epithelial cells (Bernard et al. 1989). Similarly, sequences in the HPV-16 LCR respond to cellular factors in keratinocytes and cervical cancer cells, but not fibroblasts (Cripe et al. 1987). The BPV-4 LCR, and specifically a region between E2BS3 & 4, is also shown to confer expression of reporter constructs in keratinocytes, but not fibroblasts (Morgan et al. 1999). In addition, BPV-4 E2 shows similar tissue specificity with respect to BPV-4 early promoter regulation (Morgan et al. 1998) This was found to be a promoter specific affect, as E2 was able to transactivate the BPV-4 LCR, when it was fused to an SV40 promoter, in both epithelial cells and fibroblasts. HPV-16 E2 is also shown to activate the BPV-4 early promoter in epithelial cells (Vance et al. 1999). In this study, a thymidine kinase (tk) promoter was fused to the 5' region of the LCR containing the early promoter and E2BS1 & 2, with 6 adjacent consensus E2BSs upstream. HPV-16 E2 activated this promoter with 10-fold more efficiency in epithelial cells, compared with fibroblasts. Therefore, epithelial specificity of E2 is not reliant on the enhancer region (Vance et al. 1999). As well as E2, cellular proteins are suggested to be involved in tissue specificity via the LCR. As discussed, Sp1 levels are elevated in epithelial cells, suggesting this may be involved (Apt et al. 1996). Furthermore, cellular factors, such as C/EBP and AP-1 are shown to associate with the HPV-31 LCR enhancer region *in vitro* (Sen et al. 2004). Similarly, binding sites for several TFs, including C/EBP, AP-1, NF1, and Oct-1 are found within the HPV-16 LCR (Sibbet and Campo 1990; Chong et al. 1991). Using DNAse I protection a number of footprints are observed within the HPV-16 LCR in the presence of HeLa extracts in the epithelial enhancer region between E2BS3 & 4 (Gloss et al. 1989). In addition to E2 responsive elements and the epithelial specific enhancer, there are also repressor sequences within the LCR. A region of the BPV-4 LCR, upstream of E2BS2 and overlapping the origin of replication, confers repression in the context of the LCR, as well as the SV40 promoter (Vance et al. 2001). This element binds a 50Kda cellular protein in epithelial cells and fibroblast, suggesting its repressive activity is ubiquitous. A similar element is found within the HPV-16 LCR, which contains two similar

sequences, found to bind the same complex *in vitro* (O'Connor et al. 1998). This element also represses in the context of the SV40 enhancer and tk promoter, and is found to be orientation-independent but position-dependent. A silencer, which is acted upon by YY1, is also located within the HPV-16 LCR (O'Connor et al. 1996). Repression is achieved by inhibition of AP-1 transactivation via interactions with a binding site within the silencer region (O'Connor et al. 1996). In addition, siRNA mediated knock-down of Brd4 is shown to reduce repression of the E6 promoter in HeLa cells (Wu et al. 2006). E2 association with the LCR is enhanced in increasing Brd4 concentrations, whilst there is a reduction in TFIID and RNA polymerase II recruitment. This suggests the LCR of mucosal PV types is complex and is acted on by a number of cellular factors to ensure correct transcriptional regulation during the viral life cycle.

E2 is known to regulate cellular genes, for which cellular factors are again necessary. For example, BPV-1 E2 interacts with Sp1, a cellular transcription factor, an interaction which can target E2 to promoters lacking E2 binding sites, resulting in regulation of transcription (Li et al. 1991). Sp1-E2 interaction also facilitates downregulation of telomerase via Sp1 binding sites within the hTERT promoter, a function common to both high and low-risk types, HPV-6, -11, -16 and -18 (Lee et al. 2002). In addition, HPV-8 E2 has been shown to interact with Sp1 and activate $p21^{WAF1/CIP1}$ expression via a region proximal to the transcription start site containing a number of Sp1 binding sites (Steger et al. 2002). In this case, the HPV-8 E2 hinge region is shown to be necessary for synergism with Sp1, whereas HPV-18 E2 transactivation domain mutants are unable to support $p21^{WAF1/CIP1}$ transactivation. Other cellular proteins HPV-8, -16, -18 and BPV-1 E2 are known to interact with include C/EBPα and β, which are involved in regulation of keratinocyte differentiation in the epithelium (Hadaschik et al. 2003). The interaction is mediated by the C-terminal region of HPV-8 E2 and synergises transcriptional activation from both a synthetic promoter containing C/EBPβ and the proximal involucrin promoter containing C/EBPα sites. Transactivation is independent of E2 binding sites within the DNA, but is dependent upon interaction of E2 with C/EBP, as mutant forms of E2 that do not interact with C/EBP, do not synergise transactivation. This suggests E2 may promote keratinocyte differentiation. In addition, HPV-8 and –18 E2 downregulate β4-integrin expression, which is involved in detachment of keratinocytes from the basement membrane (Oldak et al. 2004). Whilst HPV-18 E2 is also shown to induce

apoptosis, HPV-8 does not and can bind the β4-integrin promoter directly, which results in displacement of one or more cellular factor.

Like many viral transcriptional regulating proteins, E2 from several PV types is known to interact with a number of components of the basal transcription machinery. Interaction with these components can affect formation of the pre-initiation complex (PIC), composed of a number of TF complexes and RNA polymerase II itself, and can therefore affect the frequency of transcription initiation. For example, interaction has been observed between BPV-1 E2, and TATA-box binding protein (TBP), via the C-terminal region and TFIIB, via the transactivation domain (Rank and Lambert 1995; Benson et al. 1997). TBP is a component of TFIID that binds DNA directly via the TATA-box, whilst TFIIB is recruited to the complex following TFIIA and TFIID, but prior to RNA polymerase II. HPV-8 E2 also associates with TBP, requiring its C-terminal region (Enzenauer et al. 1998), whilst HPV-16 E2 interacts with TFIIB (Benson et al. 1997). In the case of BPV-1, co-operative association of TBP and E2 to a minimal promoter containing only E2 binding sites (E2BSs) and a TATA element is involved in E2 mediated transcriptional control, depending on the distance between the elements (Dostatni et al. 1991; Steger et al. 1995). When E2BSs are placed 3nt from the TATA-box, TBP binding is inhibited. In the case of HPV-11, repression of transcription is not alleviated by pre-formation of TBP-TATA complex, suggesting E2 does not simply cause steric hindrance but represses PIC formation at a stage later than this (Hou et al. 2000). However, for BPV-1 E2, with 8nts separating E2BSs and the TATA-box the two proteins interact with the promoter co-operatively, requiring the E2 hinge region (Ham et al. 1994; Steger et al. 1995). In this case E2 is shown to activate transcription not by increasing recruitment of TBP, but by decreasing its dissociation rate with the TATA element, thereby stabilising formation of the PIC (Ham et al. 1994). HPV-8, -18 and BPV-1 E2 proteins are also known to interact with other components of TFIID, known as TBP-associated factors (TAFs) (Enzenauer et al. 1998; Carrillo et al. 2004). HPV-8 E2 interacts with TAFII55 *in vitro* via the hinge and C-terminal region (Enzenauer et al. 1998), whilst HPV-18 and BPV-1 associate with TAFII80 and TAFII250 (Carrillo et al. 2004). Interaction with TAFII250, which inhibits binding of TBP to the TATA-box (Liu et al. 1998), was found to differ in strength between the two BPV-1 and HPV-18 E2 proteins (Carrillo et al. 2004). As TAFII250-E2 interaction has the potential to affect TBP-promoter association, this difference may reflect

the fact that BPV-1 E2 is a stronger viral transactivator than HPV-18 E2. However, both E2 proteins interact with TAFII80 with similar strength, suggesting this interaction may be important to the transcriptional activity of E2 proteins in general.

Further to interactions observed with cellular transcription factors, E2 also interacts with co-activators, which affect transcription via indirect association with DNA. For example, HPV-18 E2 interacts with cellular co-activator, cAMP response element-binding protein-binding protein (CBP) and p300, via the N-terminal transactivation domain (Lee et al. 2000). CBP/p300 has a role in preventing the G_0/G_1 transition during the cell cycle and its transactivation function is necessary for E2-mediated growth arrest in HeLa cells. This interaction was found to be necessary for HPV-18 E2 dependent transcription as sequestration of CBP/p300 by Adenovirus E1A, a protein known to interact with it, resulted in inhibition of E2 activated transcription from constructs containing E2 binding sites within the promoter (Lee et al. 2000). Furthermore, CBP/p300 contains Histone Acetyl Transferase (HAT) activity, which when lacking from the protein synergises with HPV-18 E2 only modestly, suggesting this may be necessary for E2 mediated transcription. HPV-8 E2 also binds to p300, an interaction which again synergises transcription (Muller et al. 2002). p300 is shown to be upregulated during epithelial differentiation in this report, suggesting it may be involved in differentiation specific transcriptional regulation by E2. Furthermore, a similar co-activator, which is known to interact with CBP/p300, p300/CREB-binding protein-associated factor (p/CAF), also contains HAT activity and can synergise HPV-18 E2 transcription in a similar fashion to CBP/p300 (Lee et al. 2002). Interaction was observed between p/CAF and HPV-18 E2 *in vivo* and with HPV-6b, -11 and –16 E2 *in vitro*, requiring the transactivation domain. Indeed, overexpression of both CBP/p300 and p/CAF increases HPV-18 E2 mediated transcription to greater extent than either protein alone, suggesting possible co-operative recruitment of the two proteins by E2. Furthermore, as for CBP/p300, the HAT function of p/CAF is essential as HAT mutants are shown to inhibit transcription (Lee et al. 2002). HPV-16 E2 also interacts with topoisomerase II β-binding protein 1 (TopBP1) (Boner et al. 2002). TopBP1 has 8 BRCA1 domains, which are involved in the response to DNA damage, transcription and replication, in its C-terminal region. Interaction is mediated via the N-terminal domain of E2 and is shown to enhance transcription and possibly replication. E2 mutants within the N-terminal domain do not support

TopBP1 mediate transactivation; however this domain does not act as a dominant negative (Boner et al. 2002), suggesting TopBP1 may not be an essential cellular partner for E2-mediated transcription.

E2 has also been found to interact with other papillomavirus proteins. As discussed, E2 binds to E1 to facilitate viral genome replication (Mohr et al. 1990; Berg and Stenlund 1997). Furthermore, E2 is known to associate with both viral oncoproteins E6 and E7 (Grm et al. 2005; Gammoh et al. 2006). HPV-16 E2 is shown to cause redistribution of HPV-18 E6 from diffuse staining throughout the cells to predominantly nuclear localisation (Grm et al. 2005). Direct interaction with HPV-16 E6 is shown to be mediated by HPV-16 E2 C-terminal sequences. This interaction results in increased E2 transcriptional activity but inhibits E2 mediated replication. The ability of HPV-16 and –18 E6 to degrade MAGI-1 and MAGI-3 is also disrupted in cells expressing HPV-16 E2 (Grm et al. 2005). Furthermore, binding of HPV-16 E2, via the hinge region, to HPV-16 E7 is thought to inhibit cellular transformation by E7 (Gammoh et al. 2006). As expression of E2 results in stabilisation of E7, it is suggested that E2 inhibits transformation by sequestering E7 to mitotic chromosomes. For HPV-16, interaction of E2 with the minor capsid protein L2 is also known to inhibit E2 mediated transactivation; however, replication was unaffected (Okoye et al. 2005). An L2-E2 interaction is not essential for inhibition. E2 levels were seen to decrease in HaCaT but not C33A cells, upon co-transfection with L2. This suggests L2 may not act by decreasing E2 levels, as a similar degree of transactivation inhibition was observed in each cell line. Although it is not known whether E2 can simultaneously interact with factors controlling replication and transcription, it is possible that the choice of interaction may influence the replication and transcription states of the virus (Rank and Lambert 1995). Furthermore, the choice of interaction, with both viral and cellular proteins, may also regulate which genes are up and down-regulated by E2 in a particular cell.

1.1.6.4 E1^E4

As the cell differentiates, late proteins begin to be expressed. E4, which is expressed as a chimera with five amino acids from the N-terminus of E1 and is known as E1^E4, was named an early protein due to its position within the genome. However, although it is expressed throughout the life cycle, it is most abundant at later times during productive infection (Doorbar et al. 1997). E1^E4 is

a small protein which accumulates in the cytoplasm and there is little sequence homology between different HPV types (Doorbar et al. 1989; Roberts et al. 1997). HPV-1 E1^E4 is progressively cleaved from the N-terminus to produce 4 isoforms: 17K, 16K, 11K and 10K. The protein is also known to oligomerise and little is thought to be in monomeric form within infected cells (Doorbar et al. 1996; Ashmole et al. 1998). It is a multifunctional protein and there is some debate as to the functions of E1^E4 from different viral types. For example, interaction with keratin intermediate filaments is observed for both HPV-1 and -16 E1^E4 (Doorbar et al. 1991; Roberts et al. 1994). This is achieved via a LLXLL motif in the N-terminal region (Roberts et al. 1994). HPV-16 E1^E4 binds keratin 18 directly via this motif and can homo-oligomerise via the C-terminus, providing a mechanism whereby E1^E4 can cause filament cross-linking (Wang et al. 2004). Whilst the cytokeratin network is seen to collapse in cells overexpressing HPV-16 E1^E4, this is not the case in HPV-1 E1^E4 expressing cells (Doorbar et al. 1991; Roberts et al. 1993). In addition, mutations in the C-terminus of HPV-16 E1^E4 are shown to inhibit cytokeratin collapse, indicating that homo-oligomerisation of the protein may be important to this function (Roberts et al. 1997). Following network collapse HPV-16 E1^E4 associates with mitochondria, causing their detachment from microtubules (Raj et al. 2004). This results in a reduction in the membrane potential and induction of apoptosis, which is suggested to make cells more fragile ready for virion egress.

HPV-16 E1^E4 has been shown to block cells in G2 of the cell cycle possibly creating an environment for vegetative replication of the genome to occur (Davy et al. 2002). A region within the N terminus containing a putative NLS, cyclin-binding site and cdk phosphorylation site is shown to be required. HPV-11 and -18 E1^E4 can also induce G2/M arrest (Davy et al. 2002; Nakahara et al. 2002). In contrast HPV-1 E1^E4 is shown in one study to cause arrest (Knight et al. 2004); however in another no such phenonmenon is observed in E1^E4 expressing cells (Davy et al. 2002). The discrepancy is probably due to expression of different E1^E4 isoforms, as the latter study focused on the full length 17K form, whilst Knight et al. used the N terminal truncated 16K isoform. In all instances studied so far however, G2/M arrest is achieved by retaining cdk1/cyclin B1, the cyclin kinase complex required for progression into mitosis, in the cytokeratin networks of infected cells (Nakahara et al. 2002; Knight et al. 2004; Davy et al. 2005). For HPV-16, E1^E4 mutants which are unable to bind cdk1 are unable to induce G2 arrest (Davy et al. 2005). Furthermore, HPV-16 E1^E4 associates with cyclin A2,

which is involved in the G2/M transition (Davy et al. 2006). This interaction is observed in G2 cells and results in redistribution of cyclin A2 to the cytoplasm. The requirement for E1^E4 for vegetative viral genome replication is also somewhat controversial. Studies investigating this use keratinocytes transfected with E1^E4 null mutant genomes in raft culture. Whilst HPV-11 E1^E4 is shown to be dispensable for viral genome amplification (Fang et al. 2006), both HPV-16 and –31 E1^E4 seem to be necessary (Nakahara et al. 2005; Wilson et al. 2005). Furthermore, HPV-16 E1^E4 is shown to contribute to episome replication in undifferentiated cells, and low levels of expression are observed in these cells (Nakahara et al. 2005); however, HPV-31 E1^E4 does not affect episome maintenance or early gene expression (Wilson et al. 2005). In addition, HPV-16, but not HPV-1 or –6, E1^E4 associates with E4-DEAD box protein (E4-DBP), via the C-terminal region (Doorbar et al. 2000). E4-DBP is a shuttling protein with RNA helicase activity. Interaction between the two proteins results in cytoplasmic relocalisation of E4-DBP, and loss of E1^E4 keratin binding activity causes both proteins to become localised to the nucleoli. E1^E4 is also shown to partially inhibit the RNA helicase activity of E4-DBP. Furthermore, E4-DBP is known to interact with HPV-16 late RNAs, suggesting HPV-16 E1^E4 may have functions during post-transcriptional processing of late transcripts (Doorbar et al. 2000).

1.1.6.5 L1 and L2

L1 and L2 encode the major and minor capsid proteins, respectively, and are expressed following genome amplification in the upper layers of the epithelium. Each HPV-16 capsid contains 256 copies of L1 and 72 copies of L2, forming a capsomere icosohedral shell (Modis et al. 2002). Virus-like particles are known to form in the absence of L2, however L2 is thought to enhance packaging and infectivity. During infection the capsid proteins are involved in cell contact, endocytosis (see section 1.1.5: The life cycle), translocation across the cytoplasm and nuclear entry. HPV-16 L2 binds β-actin, which is thought to facilitate transport across the cytoplasm during infection (Yang et al. 2003). In support of this, BPV-1 L1 co-immunoprecipitates with tubulin, and virions co-localise with micro-tubules in the cytoplasm by electron microscopy (Liu et al. 2001). Furthermore, HPV-16 L2 is suggested to be required for endosome escape as pseudovirions containing L1 only are retained in the vesicular compartment (Kamper et al. 2006). Nuclear entry is facilitated by L2. L2 protein from a number of PV types interacts with Karyopherin (Kap) subunits, which are nuclear import adaptor/receptor proteins

also known as importins (Darshan et al. 2004; Fay et al. 2004; Bordeaux et al. 2006; Klucevsek et al. 2006). Kap's form heterodimers of one α and one β subunit, and L2 from different PV types is shown to interact with Kap α_2, β_1, β_2, and β_3 via nuclear localisation signals in the L2 N and C-terminal regions. L2 is also involved in viral DNA binding and it is suggested that it is responsible for translocation of the genome to the nucleus (Day et al. 2004; Fay et al. 2004; Bordeaux et al. 2006; Klucevsek et al. 2006).

L2 also has functions during production of mature virions. HPV-33 L2 contains an ND10 localisation domain, which is necessary for L2 induced recruitment of Daxx and loss of Sp100 from ND10 domains (Becker et al. 2003). This is thought to be important as ND10 domains are suggested to be the sites of viral DNA replication. Furthermore, involvement of PML, a major component of ND10 domains, is somewhat controversial. Whilst authentic BPV-1 infection is elevated in PML positive cells, as compared to PML negative cells (Day et al. 2004), assembly of HPV-33 into VLPs requires L2 to be in the nucleus, but neither ND10 localisation or PML are necessary (Becker et al. 2004). Formation of L1 capsomeres occurs in the cytoplasm, followed by translocation to the nucleus via the Kap α2β1 heterodimer (Merle et al. 1999; Nelson et al. 2002). HPV-16 and –45 L1 interacts with Kap α2 to facilitate import and this requires RanGDP and GTP (Nelson et al. 2002). HPV-11 L1 also enters the nucleus using the same adapter heterodimer via interaction with Kap α2 (Merle et al. 1999). In addition, interaction of L1 with Kap β_2 in the case of HPV-16 and –45, and Kap β_3 for HPV-11 is shown to cause inhibition of import of cellular proteins containing an M9 NLS, such as hnRNP A1 (Nelson et al. 2002; Nelson et al. 2003). It is suggested that this import is inhibited during the productive phase of the life cycle.

1.1.6.6 Tumour Progression

In tumour cells, E6 and E7 of high-risk HPVs, are expressed at high levels, which drives proliferation (Durst et al. 1991). The high levels of expression are commonly caused by integration of the viral genome into cellular DNA resulting in loss of expression of other HPV genes. Specifically, E2 is lost and the break points of integration are often within the E2 ORF. E2 is thought to transcriptionally down-regulate E6 and E7 in episomally infected cells, during differentiation (see section 1.1.6.3: E1 and E2) (Dowanhick et al. 1995; Goodwin et al. 2000). Loss of

this down-regulation may result in uncontrolled E6 and E7 expression. In addition, endogenous expression of Sp1 which is thought to be a major transactivator of the HPV-16 E6/E7 promoter, is elevated in transformed cells (Apt et al. 1996). Furthermore, steady-state E6/E7 mRNA levels are increased upon integration of the HPV-16 genome in HPV-16 infected epithelial cells (Jeon and Lambert 1995). However, this is not only due to elevated transcriptional activation but is also a product of increased RNA stability (Jeon and Lambert 1995). As the E6/E7 transcripts that are produced in cells with integrated genomes are not polyadenylated using the HPV-16 early 3'UTR, it was suggested this region might contain an instability element. Indeed, when fused to the β-globin gene, the HPV-16 early 3'UTR was able to substantially reduce stability of β-globin transcripts in fibroblasts (Jeon and Lambert 1995). In addition, deletion of the HPV-16 early 3'UTR from a truncated version of HPV-16 genome containing only the early region, driven by a CMV promoter, increases mRNA steady-state levels in HeLa cells (Zhao et al. 2005). This suggests that increased mRNA stability, as well as more efficient transactivation of the early promoter, results in the high levels of E6/E7 expression observed in transformed cells. However, this has not been shown to be sufficient to cause tumour progression and mutagenesis of cellular genes plays a role. This is backed up by cell culture observation, in which integration of the genome results in a growth advantage over episome-containing cells and loss of differentiation but cells are not tumourigenic (Jeon et al. 1995). However, expression of BPV-1 E2 in HeLa cells to repress HPV-18 E6 and E7 expression results in inhibition of cellular DNA synthesis, and p53 and pRb induction (Goodwin et al. 2000). Furthermore, expression of HPV-16 E6 and E7 expression alleviates E2-mediated growth inhibition; suggesting continued E6 and E7 expression is necessary for optimal proliferation of transformed cells. In transgenic mice expressing HPV-16 E6 or E7 in undifferentiated epithelial cells, treatment with carcinogens known to act at different stages of tumour progression, reveals differing roles for E6 and E7 at different stages of tumourigenesis (Song et al. 2000). E7 primarily affects the promotion stage, whilst E6 acts more strongly in malignant conversion. *In vivo*, the eventual result is loss of differentiation capabilities, uncontrolled growth and progression to invasive carcinoma.

1.1.7 Tissue culture

Due to the tight association of the PV life cycle to epithelial differentiation, tissue culture analysis has been problematic. Many cell lines have been derived from cervical carcinomas in which the HPV genome is integrated. Although the transforming mechanisms of the E6 and E7 proteins can be studied in these systems, they are not indicative of the natural life cycle of HPV. W12 is a cell line derived from an HPV-16 infected CIN I lesion, in which the genome is episomal and is maintaining the natural pattern of HPV infection (Stanley et al. 1989). A sub-clone of W12 cells, W12E has been derived which whilst maintaining the features of W12 cells, can also be maintained in culture for more passages (up to 18) before genome integration occurs (Jeon et al. 1995). These cells also exhibit typical epithelial cell morphology consistent with CIN I lesions and can be differentiated *in vitro*, allowing for the comparison of gene expression and the effects thereof, between undifferentiated and differentiated cells. For example, in monolayer culture the density of W12E cells influences the state of differentiation. At low density cells are undifferentiated, whereas higher density results in differentiation (McPhillips et al. 2004). Cells can be harvested at different time points after seeding to obtain undifferentiated and differentiated samples. Furthermore, cells can be grown in organotypic-raft cultures to allow the formation of epithelial tissue *in vitro*. This tissue can be used in staining experiments, to detect when and where within a three-dimensional structure, certain RNAs and proteins are expressed.

1.2 RNA processing

To understand fully the relevance of the protein complexes binding papillomavirus *cis*-acting RNA elements, it is necessary to outline the mechanisms of RNA processing. In eukaryotic cells, there are a number of proteins that direct the appropriate processing, transport and stability of pre-mRNAs. These processes, as well as transcription itself, are intertwined, creating a complex machine from which mature mRNA transcripts are produced (reviewed by Minvielle-Sebastia and Keller 1999). RNA processing factors are known to associate with the RNA polymerase II C-terminal domain, and are thereby in close proximity to the nascent transcript as soon as it is produced. Each process will be discussed in isolation,

but links will be drawn where relevant, particularly with respect to interacting proteins involved in each process.

1.2.1 Capping

The first process to occur during RNA processing is capping of the 5' end of the pre-mRNA (reviewed by Shatkin and Manley 2000). This occurs in nucleus after synthesis of ~20-25 nts, when a 7-methylguanosine triphosphate 5' cap is added (reviewed by Bentley 2002). Methylation of the cap stabilizes mRNAs against 5' exonuclease activity and is involved in inhibiting decapping (reviewed by Furuichi and Shatkin 2000). Capping is achieved via three enzymes: RNA triphosphatase, guanylyltransferase (GT) and 7-methyltransferase (MT). GT and MT associate with the Serine 5 phosphorylated CTD of RNA polymerase II (reviewed by Bentley 2002). Whilst RNA triphosphatase and GT are released from the mRNA following capping, MT can be found associated with the CTD at the 3' end of the gene. Furthermore, MT is shown to associate with importin α, a molecule which along with importin β is involved in nuclear import of proteins containing nuclear localisation signals (reviewed by Nakielny and Dreyfuss 1999). This interaction promotes targeting of MT to its substrate and also increases cap methylation. A complex composed of two subunits, 20 and 80KDa, called the cap-binding complex (CBC) associates with the mRNA cap (reviewed by Izaurralde and Adam 1998). Capping and polyadenylation are linked during mRNA processing. CBC may be involved in cleavage at the 3' end of an mRNA as its depletion from HeLa nuclear extracts, results in decreased levels of cleavage (Flaherty et al. 1997). It is thought that interaction between CBC and 3' end processing complexes, stabilises 3' end complex formation. In addition, translation of uncapped RNA is reduced in *in vitro* translation assays (Both et al. 1975; Muthukrishnan et al. 1975). The cap associates with eukaryotic translation initiation factor 4F (eIF4) family and this promotes ribosome attachment which in turn results in increased initiation of translation (Gingras et al. 1999). Circularisation of the mRNA via cap and poly(A) tail complex interactions also increases translation initiation (reviewed by Sachs et al. 1997). Therefore, the cap is essential to the stability, processing and translation of an mRNA molecule.

1.2.2 Splicing

In eukaryotic cells splicing occurs via formation of a number of complexes, E, A, B and C (Figure 1.7). The E splice complex is formed via RNA-RNA, protein-RNA and protein-protein interactions. This involves recognition of the 5' and 3' splice sites. The 5' splice site is defined by one sequence (GURAGU, where R represents a purine), whilst the 3' splice site composes three parts; a branch point site (BPS) (YNYURAY, where Y is a pyrimidine and N is any nt), a poly-pyrimidine tract and the splice site itself (YAG/N) (reviewed by Graveley 2000). Splice sites within eukaryotic genomes can be highly degenerate and recognition often depends on nearby *cis*-acting elements, which either repress or enhance splicing from a particular site via the action of a number of RNA-binding proteins (see section 1.4: SR proteins). Association occurs between the U1 small nuclear ribonucleoprotein (U1 snRNP) complex and a 5' splice site (Wassarman and Steitz 1992), whilst the U2 snRNP auxiliary factor (U2AF) complex, containing $U2AF^{35}$ and $U2AF^{65}$, forms by interaction with the 3' splice site and polypyrimidine tract, respectively (Figure 1.7E). One or more SR proteins, so called because of the arginine/serine rich (RS) domain contained in the C-terminal region, bridge the gap between U1 snRNP and the U2AF complex, via protein-protein interactions, creating a splice site recognition complex necessary for the latter stages of splicing (reviewed by Smith and Valcarcel 2000). In subsequent stages many more components join the complex, such as snRNPs, SR proteins and splicing factor-associated proteins (SAPs), resulting in production of two molecules: an intronless mRNA and a lariat intron (Figure 1.7A-C) (reviewed by Reed and Palandjian 1997). Briefly, in formation of the A complex, U2 snRNP associates with the BPS, followed by displacement of U1 snRNP by a U6/U4.U5 tri-snRNP particle to form the B complex, in the pre-spliceosome to spliceosome transition. Loss of U2AF then results in the C complex and catalysis is completed, producing the intronless mRNA and the lariat intron, associated with U2, U5 and U6 snRNPs.

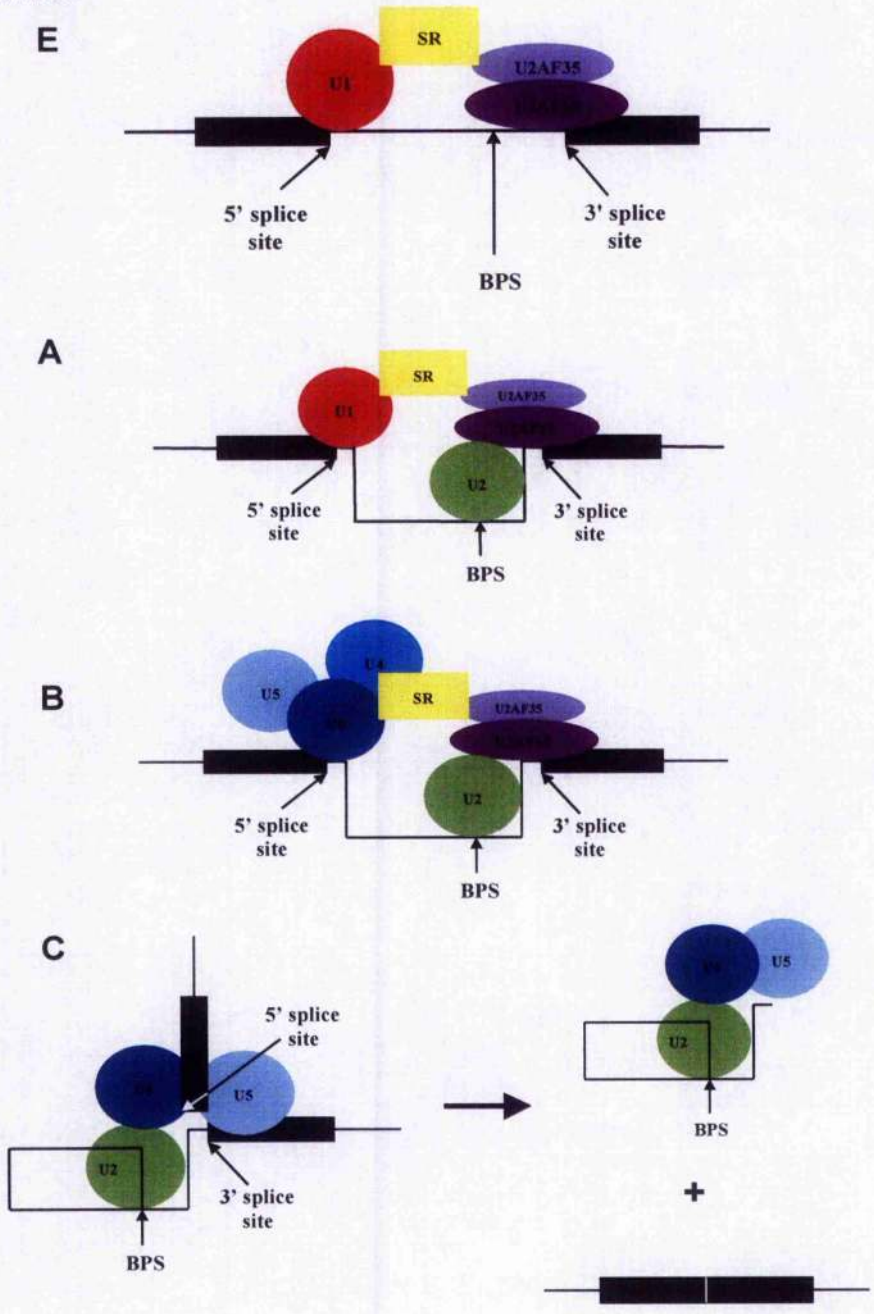


Figure 1.7: **Splicing**

E) shows the early (E) complex with U1 snRNP (red) bound at the 5' splice site, U2AF (purple) bound at the 3' splice site and one or more SR proteins (yellow) bridging the gap to adjoin the two. A) Shortly after E complex formation, U2 snRNP (green) binds the branch point site (BPS) to form the A complex. B) U1 snRNP is displaced by a U6/U4.U5 tri-snRNP particle (blue). C) U2AF is lost from the complex and catalysis is completed to produce the lariat intron, associated with U2, U5 and U6 snRNPs and the intronless mRNA.

1.2.3 Cleavage and Polyadenylation

In processing of the 3' end of most mRNAs, the transcript is cleaved and polyadenylated, two processes which are tightly coupled. There are a number of factors involved in these processes, and, as with splicing, RNA sequences are required. The core polyadenylation signal is almost invariable, consisting 5'-A(A/U)UAAA-3', located approximately 10 to 30 nucleotide upstream of the cleavage and polyadenylation site, and a more variable GU or U rich element is often found 10 to 50 nucleotides downstream (Figure 1.8A) (reviewed by Proudfoot 1991). The downstream element may be involved in modulating efficiency of polyadenylation from a specific site. A third sequence, UGUA, can be found in one or more copies at variable distance upstream of the cleavage site (Hu et al. 2005). Protein complexes involved in cleavage and polyadenylation include, poly (A) polymerase (PAP), cleavage and polyadenylation specificity factor (CPSF), cleavage stimulatory factor (CStF), cleavage factors (CFI/CFII) and poly(A) binding protein II (PABII) (Figure 1.8). The first event is thought to be interaction of the CPSF complex, composed of 4 subunits of 30, 70, 100 and 160KDa, with the A(A/U)UAAA sequence within the core element (Jenny et al. 1994; Murthy and Manley 1995). CstF, composed of CstF-77, CstF-64 and CstF-50, associates with the GU rich region via CstF-64 and stabilises CPSF-RNA interactions by direct protein interaction (Weiss et al. 1991; MacDonald et al. 1994). PAP then binds to the complex, followed by cleavage. It is suggested that requirement for PAP within the complex prior to cleavage may ensure tight coupling between cleavage and polyadenylation (reviewed by Keller 1995). CFI and CFII are also required for cleavage. Although their exact functions are as yet undefined, CFI interacts with the UGUA sequence and can allow polyadenylation in the absence of an A(A/U)UAAA motif via recruitment of CPSF and PAP (Brown and Gilmartin 2003; Venkataraman et al. 2005). Polyadenylation then proceeds rapidly requiring PAP, CPSF and PABII. PABII joins the complex shortly after polyadenylation starts to regulate efficiency and tail length (Wahle 1991; Bienroth et al. 1993). It stimulates the reaction after addition of approximately 10 nucleotides, and then slows polymerisation when the tail reaches 200-250 nucleotides (reviewed by Wahle and Keller 1996). As length of the poly(A) tail is consistent amongst different transcripts, it is thought that A's are counted by continued binding of PABII (Figure 1.8E) (Wahle 1995).

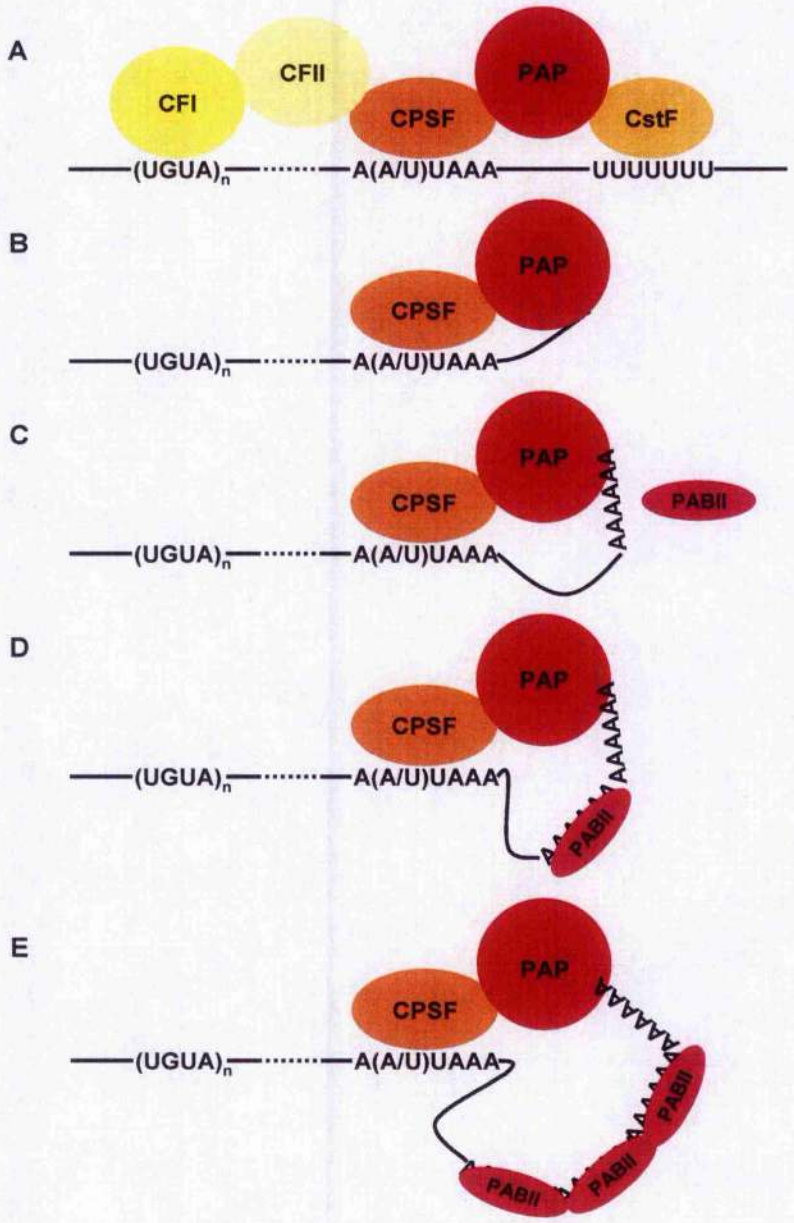


Figure 1.8: **Cleavage and Polyadenylation**

A) CPSF interacts with the A(A/U)UAAA sequences and CstF associates with the GU or U rich region cooperatively. CFI interacts with a further sequence, UGUA at variable distance upstream of the cleavage site. Although the RNA is shown linearised here, it is likely that the complex is formed by bending of the molecule. CFII is also required; however it's binding properties are not known. PAP then enters the complex via interactions with CPSF. B) Following cleavage, CstF and CFI and II are lost from the complex. C) Approximately 10 A's are added to the cleaved RNA before PABII associates with the complex. D) PABII binds the poly(A) tail and induces productive polyadenylation. E) Continuous PABII binding is thought to regulate tail length.

1.2.4 Exon definition

In many lower eukaryotes, splicing complexes are formed across the relatively small introns. This results in each splice junction contributing to the splicing of only one intron (Figure 1.9A). However, in mammalian cells where introns can be thousands of base pairs in length and exons small in comparison, the complex often forms across the exon in a process called 'exon definition' (Figure 1.9B) (reviewed by Berget 1995). Interaction then occurs between complexes formed at adjacent introns to facilitate splicing. Here each splicing complex contributes to the splicing of two introns, on each side of the exon in question. For internal exons the process of exon definition is adequate; however, problems arise when splicing the terminal introns. An E complex cannot form over the 5' and 3' most exons due to the lack of a 3' splice site at the start of the first exon and 5' splice site and the end of the last. In this situation it is thought that the splicing machinery interacts with end processing machinery to facilitate splicing (Figure 1.9C). For example, interaction of the CBC associated with the 5' cap interacts with U1 snRNP which enhances splicing of the first intron (reviewed by Izaurralde and Adam 1998). Similarly, splicing machinery bound at the 3' splice site of the 3' most exon interacts with cleavage and polyadenylation factors, thus creating a complex capable of splicing the final intron. For example, U1A, a component of the U1 snRNP complex, interacts with cleavage and polyadenylation specificity factor (CPSF) (Lutz et al. 1996). This interaction affects both polyadenylation rate and tail length by stabilising A(A/U)UAAA-CPSF interactions. Furthermore, U1A, whilst complexed in U1 snRNP, binds the upstream efficiency element (USE) of the SV40 late polyadenylation signal (Lutz and Alwine 1994). U1A also is known to inhibit polyadenylation of its own mRNA (Gunderson et al. 1994), whilst U1-70K is involved in inhibition of BPV-1 late gene transcript polyadenylation (Gunderson et al. 1998). Another component of U1 snRNP, U1 snRNA also interacts with the SV40 late and adenovirus L3 polyadenylation signals (Wassarman and Steitz 1993). For SV40, addition of a 3' splice site upstream enhances binding and polyadenylation (Niwa et al. 1990). In addition, mutation of the polyadenylation signal inhibits splicing of the 3' most intron *in vitro* (Niwa and Berget 1991). Furthermore, presence of a 5' splice site within the 3' most exon has been shown to repress polyadenylation *in vitro* and *in vivo* (Niwa et al. 1992). Interaction between $U2AF^{65}$ and poly (A) polymerase (PAP) has also been observed, which is believed to facilitate splicing (Vagner et al. 2000). Furthermore, mRNAs which

have been spliced but not polyadenylated have never been observed *in vivo*. Therefore, the relationship between splicing and polyadenylation is of great important to correct post-transcriptional processing in mammalian cells.

1.2.5 mRNA transport

All communication between the nucleus and the cytoplasm is mediated via the central channel of nuclear pore complexes (NPCs) (reviewed by Taura et al. 2005). For many molecules and complexes this is using Ran-dependent transport via importins. In contrast, export of bulk cellular mRNA is mediated by Ran-independent mechanisms. The majority of mRNA is thought to be exported from the nucleus via nucleo-cytoplasmic shuttling, export receptor, TAP (reviewed by Cullen 2003; Taura et al. 2005). TAP forms a heterodimer with p15/NXT1, which then associates with both polyadenylated mRNA and NPCs. Interaction between TAP and p15/NXT1 is essential to the shuttling activity of TAP and also stimulates NPC association (Katahira et al. 2002; Wiegand et al. 2002). However, TAP is not a good RNA binding protein and is thought to associate with mRNAs via components of the mRNP complex. For example, Aly/REF1, which forms a complex with a number of other proteins ~20-24 nts upstream of the exon-junction, termed the exon-junction complex (EJC), interacts with TAP (Le Hir et al. 2000; Zhou et al. 2000; Rodrigues et al. 2001). Aly is also a shuttling protein, which is shown to enhance mRNA export *in vivo* (Zhou et al. 2000). In addition, TAP is known to associate with splicing associated proteins such as $U2AF^{35}$ (Zolotukhin et al. 2002) and SR proteins, SF2/ASF, SRp20 and 9G8 (Huang et al. 2003). However, although splicing may seem to be important for export of mRNAs, those lacking introns are still efficiently exported from the nucleus to the cytoplasm in transfected cells. Furthermore, RNA interference mediated knock-down of Aly/REF1 shows this protein is not essential for bulk mRNA export, suggesting the EJC and splicing may not be essential for export of mRNAs (Gatfield and Izaurralde 2002). In addition, mRNA export is tightly linked to cleavage and polyadenylation. It is thought that this is achieved via protein-protein interaction between cleavage and polyadenylation factors and export proteins. In support of this, yeast strains defective for certain mRNA 3' end processing proteins, such as PAP, also show defective mRNA export (Hammell et al. 2002). However, at present little is known about how export is coupled with cleavage and polyadenylation.

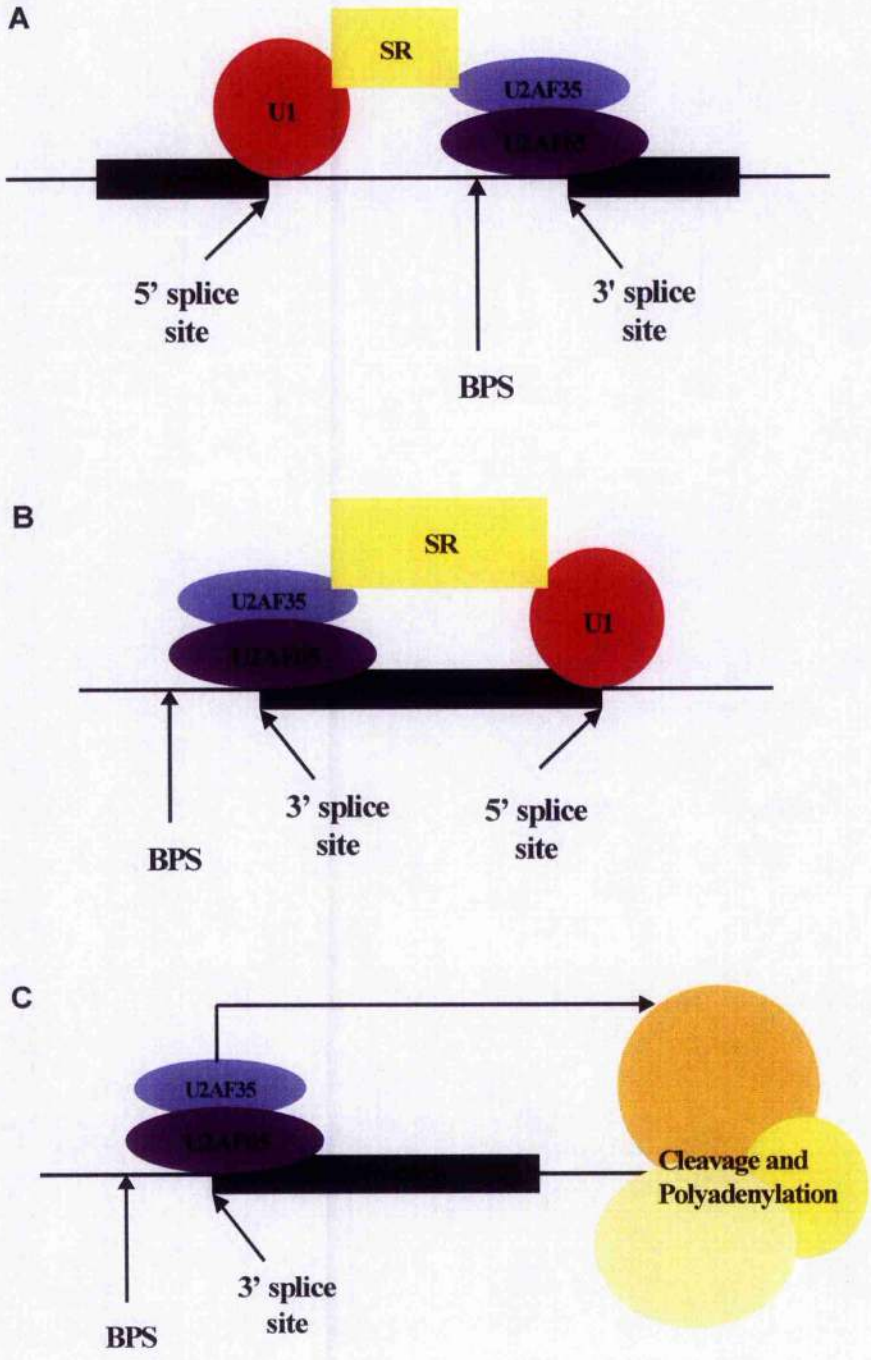


Figure 1.9: **E complex formation in eukaryotic splicing**

A) A U1 snRNP complex forms on the 5' splice site, whilst U2AF65 and U2AF35 bind the 3' splice site. The complex is bridged by SR proteins. B) In mammalian cells where introns are much larger than exons the E complex forms over exons. However, this becomes a problem when splicing terminal exons, which is overcome by interaction of the splicing machinery with end processing factors. C) depicts the possible interaction at the 3'end.

1.2.6 mRNA stability

Another important mechanism for controlling gene expression post-transcriptionally is by regulating RNA stability. Half-lives of different mRNA molecules can differ markedly from several minutes, in the case of human cytokine mRNAs (reviewed by Ross 1995), to many hours, as for globin family members (Weiss and Liebhaber 1995). Whilst *cis*-acting RNA elements regulating stability can be found in coding regions, they are most commonly found within the 3'UTR. In particular, AU-rich elements (AREs) are often found to regulate stability of an mRNA in a *cis*-acting manner. AREs act via interaction with cellular proteins known as ARE-binding proteins, which includes the Hu family. The Hu family contains 4 members; HuC and HuD, which are expressed only in neurons, HuB, which is expressed in neurons and gonads, and HuR, which is ubiquitously expressed in proliferating cells. As HuR is known to associate with *cis*-acting RNA elements within the late region of HPV-1 and –16, we will focus of this member of the family. HuR is shown to interact with AREs within the 3'UTR of some mRNAs, and act as a nuclear export adapter. For example, HuR is shown to interact with pp32 and APRIL, which associate with nuclear export factor CRM1 (Brennan et al. 2000). In addition, inhibition of CRM1 mediated export specifically represses export of mRNAs containing a *c-fos* ARE, to which HuR is known to bind, in the 3'UTR. Once in the cytoplasm, HuR is thought to regulate stability of the bound mRNA molecule. Overexpression of HuR in mouse cells, results in increased cytoplasmic stability of mRNAs containing a *c-fos* ARE (Peng et al. 1998). This correlates with HuR binding to the ARE, as confinement of HuR in the cytoplasm via inhibition of transcription, increases HuR-ARE interaction and also increases cytoplasmic mRNA stability.

1.3 Papillomavirus late gene expression

Papillomavirus capsid proteins are highly immunogenic and their expression is tightly controlled until productive genome replication is complete. For different PV types, expression is observed at different stages of differentiation. HPV-16 has a late productive phase compared with other mucosal HPVs, resulting in expression of capsid proteins in only the most differentiated cells. However, late unprocessed RNAs can be detected in less differentiated infected cells during the HPV-16 life cycle (Milligan et al., 2006). Therefore, regulatory mechanism must exist at the

post-transcriptional level to prevent late gene expression in undifferentiated cells. In accordance, many PV late transcripts have been the subject of studies investigating this and a number of elements have been found which result in down-regulation of late gene expression in undifferentiated epithelial cells, as well as conferring repression upon reporter gene constructs (Kennedy et al. 1990; Furth and Baker 1991; Kennedy et al. 1991; Yes: Schwartz 1998; Sokolowski et al. 1998; Cumming et al. 2002). These sequences can be found in both coding and non-coding regions of the RNA and seem to be divergent between papillomavirus types. However, for some there are similarities with respect to sequence and proteins binding, which may indicate a largely similar mechanism of late transcript down-regulation by different papillomavirus types (Sokolowski et al. 1999; Koffa et al. 2000; Collier et al. 2002; Cumming et al. 2003; McPhillips et al. 2004; McPhillips et al. Unpublished).

1.3.1 Late 3'UTR Regulatory Elements

Although down-regulation of late gene expression is observed in the presence of *cis*-acting elements found within PV coding regions, it has also been shown that stronger negative elements are often found partially or fully within non-coding regions (Sokolowski et al. 1999; Cumming et al. 2002). For example, a 53nt element immediately upstream of the poly (A) signal in the late 3'UTR of the BPV-1 genome is known to affect late gene expression (Furth and Baker 1991). The element inhibited reporter gene expression ~6-10 fold in undifferentiated cells, when cloned upstream, but not downstream of the polyadenylation signal. Furthermore, deletion of the 53nt element from L1 expression vectors containing the BPV-1 late 3'UTR resulted in increased levels of cytoplasmic L1 mRNA, but did not alter the stability of the molecule (Furth and Baker 1991). Therefore, it has been postulated that presence of this regulatory element reduces efficiency of RNA processing. In agreement with this, a consensus 5' splice site is contained within the 53nt element: a 9nt region which coincides with inhibitory activity (Furth et al. 1994). This study suggests that that U1 snRNA (small nuclear RNA), a component of U1 snRNP, which associates with 5' splice sites during splicing (see section 1.2.2: Splicing), interacts with the BPV-1 LRE, via the 5' splice site, and interaction is necessary for inhibition of gene expression. In addition, a U1 snRNP complex, containing U1 snRNA, U1A and U1-70K, is shown to form on the 5' splice site *in vitro* (Gunderson et al. 1998). Inhibition of gene expression is shown

to be caused via by U1-70K, via interaction with and subsequent inhibition of poly A polymerase (PAP) (see section 1.2.3: Polyadenylation).

A similar, yet more complex element has been found within the late region of the HPV-16 genome (described in more detail in Chapter 5). Termed the late regulatory element (LRE), this element resides at the 3' end of the L1 coding region and spans into the late 3'UTR (Kennedy et al. 1990; Kennedy et al. 1991). It is 79nt in length and contains 4 weak 5' splice sites within its 5' region and a 3' GU rich region, of which both regions have been shown to confer negative regulatory activity to some degree upon reporter gene expression in undifferentlated epithelial cells (Cumming et al. 2003). Within the 5' region, the 4 5' splice sites are shown to act in an additive manner, the second of which is the closest to 5' splice site consensus sequence and has the most inhibitory activity. Furthermore, many proteins have been shown to interact, either directly or indirectly with the LRE, via both the 5' and 3' regions, and of those identified, all have roles in RNA processing, transport or stability (Table 1.1) (Dietrich-Goetz et al. 1997; Koffa et al. 2000; McPhillips et al. 2004; McPhillips et al. Unpublished). For example, CstF-64 and HuR interact, requiring the 3' GU rich element of the LRE (Cumming et al. 2002). CstF-64 is sub-unit of the cleavage stimulating factor CstF, which is known to stabilise the formation of polyadenylation and cleavage complexes (see section 1.2.3: Cleavage and polyadenylation) (Wilusz et al. 1990) and HuR is involved in stabilisation of mRNA in the cytoplasm and transport of from the nucleus to the cytoplasm (see section 1.2.6: mRNA stability) (reviewed by Keene 1999). Heterogenous nuclear riboprotein (hnRNP) A1, a cellular splicing regulatory protein known to antagonise the action of SF2/ASF in terms of splice site selection, is also shown to associate with the HPV-16 LRE (Veerapraditsin et al. unpublished). hnRNP A1 associates with *cis*-acting RNA elements, known as exonic splicing silencers (ESSs), and directs splicing to more distal splice sites (Eperon et al. 2000). This is achieved by interfering with U1 snRNP binding to proximal 5' splice sites. In addition, the protein has roles during mRNA export and can affect mRNA stability in the cytoplasm (Izaurralde et al. 1997; Mili et al. 2001). A further protein complex thought to bind the LRE bears similarity to a splicing early (E) complex (see section 1.2.2: Splicing). It has been shown that a U1 snRNP like complex, containing Sm and U1A, interacts via U1 snRNA with the 5' region of the LRE (Cumming et al. 2003), whilst $U2AF^{65}$ binds the 3' GU rich region (Koffa et al. 2000). This is in accordance with their binding properties as observed in an E complex. Further studies have shown that a splicing related SR

protein, Splicing Factor 2/Alternative Splicing Factor (SF2/ASF) also interacts with the LRE indirectly when complexed with $U2AF^{65}$ (McPhillips et al. 2004). Therefore, there is the potential for a splicing-like complex, similar to that formed over an exon during exon definition (see section 1.2.4: Exon definition), to form on the LRE (Figure 1.10). This complex may facilitate and/or interfere with processing of late transcripts and in particular may exert effects on polyadenylation and splicing of the final intron (see Chapter 5).

Element	Associated cellular proteins	Publication
E4 ESE	Not Known	
L2 Polyadenylation enhancer	hnRNP H CstF-64	Oberg et al. 2005
L1 ESS	hnRNP A1	Zhao et al. 2004
LRE	U1 snRNP-like complex U2AF-65 SF2/ASF CstF-64 HuR hnRNP A1	Cumming et al. 2003 Dietrich-Goetz et al. 1997 McPhillips et al. 2004 Koffa et al. 2000 Koffa et al. 2000 Veerapraditsin and Graham, Unpublished

Table 1.1: Cellular RNA processing factors interacting with HPV-16 late cis-acting RNA elements

Cis-acting RNA elements are also found within the late 3'UTRs of HPV-1 and –31. For HPV-31, a 101nt region spanning the L1 coding region and late 3'UTR, was shown to have significant sequence similarity to the HPV-16 LRE (Cumming et al. 2002). This region contains 3 weak consensus 5' splice sites and a 3' GU rich region. When the HPV-31 late 3'UTR, encompassing this element was fused to a reporter gene, reporter activity was reduced to a similar extent as observed for comparable constructs containing the HPV-16 3'UTR. However, precise deletion of the 101nt element increased gene expression only ~2-3 fold (Cumming et al. 2002). Additional deletions of the 3'UTR, 3' of the proposed negative element, spanning a 130nt region, further increased reporter activity. Moreover, deletion of 110nt region downstream of the late polyadenylation signal also alleviated repression of gene expression to some degree. This suggests that the HPV-31 late 3'UTR contains two *cis*-acting RNA regulatory region, termed the major inhibitory element (MIE), containing the LRE-like element, and the minor element (SIE), downstream of the polyadenylation signal (Cumming et al. 2002). UV cross-linking and electrophoretic mobility shift assays (EMSAs) indicated that the MIE interacts with a number of proteins also known to associate with the HPV-16 LRE

(Cumming et al. 2002). For example, GST-tagged CstF-64 was able to cross-link with an MIE probe. In addition, HuR and U2AF^{65} could be affinity purified from HeLa nuclear extracts using MIE probes. An Inhibitory element has also been found in the HPV-1 late 3'UTR (Tan and Schwartz 1995; Sokolowski et al. 1997). A region spanning nt 6939-7184 was able to inhibit reporter gene activity and the amount of cytoplasmic polyadenylated mRNA produced from constructs containing the entire HPV-1 3'UTR was reduced, compared with those containing a deletion in this region (Tan and Schwartz 1995). A near consensus 5' splice site was found in this region; however, this was not found to be required for inhibitory activity and instead an AU-rich element, termed the h1ARE, located between nts 6938 and 7014, is associated with repression (Tan and Schwartz 1995; Sokolowski et al. 1997). This region contains two AUUUA and three UUUUU sequences, and U to C mutation within these sites abolishes inhibitory activity (Sokolowski et al. 1997). Co-immunoprecipitation of h1ARE UV cross-linked proteins revealed that hnRNP C1 and C2 interact with this element (Sokolowski et al. 1997). In addition, GST-HuR is able to bind the h1ARE, via the AUUUA and UUUUU sites, in EMSA and UV cross-linking experiments (Sokolowski et al. 1999). HuR binding affinity to the element is shown to correlate with its inhibitory activity (Sokolowski et al. 1999). In addition, levels of HuR are elevated in the cytoplasm of cells in which the h1ARE is less inhibitory (Carlsson and Schwartz 2000). It is suggested that HuR may regulate the h1ARE by increasing stability and/or promoting translation in the cytoplasm.

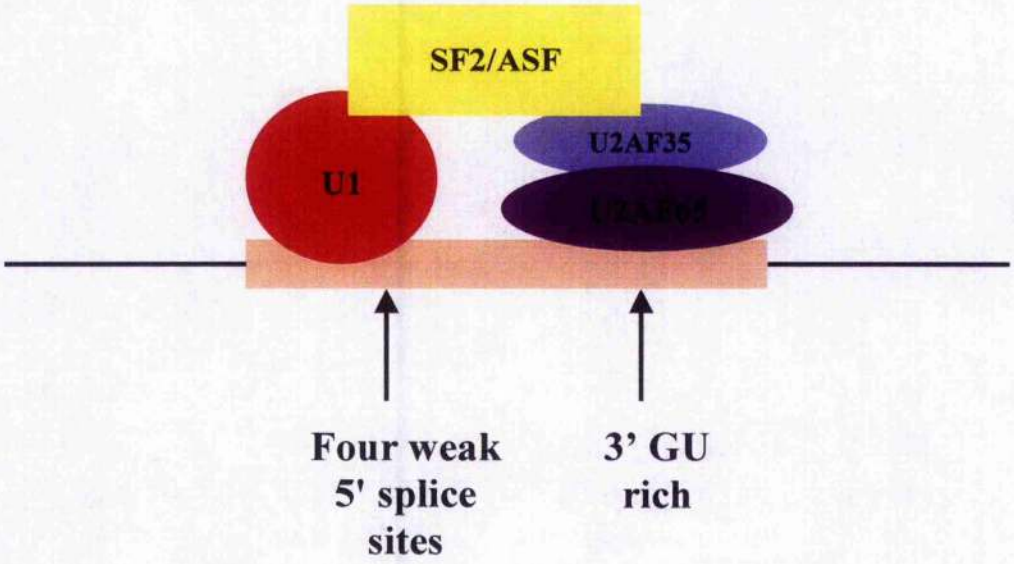


Figure 1.10: **An early splicing complex forms over the LRE**

A schematic diagram of a complex known to form on the LRE in both undifferentiated and differentiated HPV-16 infected cells. A U1 snRNP like complex interacts with the 5' region, whereas U2AF interacts with the 3' GU rich region. Furthermore, SF2/ASF is found to associate indirectly when complexed with U2AF, creating an early splicing like complex.

1.3.2 Cis-acting RNA elements

As well as elements within non-coding regions, studies of several HPV types have revealed regulatory elements within the coding regions. These elements have been shown to be involved in either regulation of alternative splicing, polyadenylation or RNA stability. With particular focus on HPV-16, a number of *cis*-acting RNA elements are shown to act via interaction with cellular RNA processing factors. For example, an exonic splicing enhancer (ESE), 65 nt in length has been observed within the E4 ORF of HPV-16 (Figure 1.11) (Rush et al. 2005). The ESE is AC-rich and bears similarity to AC-rich enhancers, which are known to regulate splicing of cellular and viral genes. This has been shown to direct splicing to the 3' splice site at position 3358, upstream of the ESE, hence inhibiting use of the differentiation specific 3' splice site at position 5639, upstream of the L2 ORF, within the late region of the genome (Rush et al. 2005). Optimization of the 3' splice site at 3358, negates the presence of the ESE, suggesting this element is necessary to direct splicing to a sub-optimal splice site. Presence of this ESE also results in polyadenylation from the early poly (A) site (pAE), which produces transcripts lacking L1 and L2 (Rush et al. 2005). Furthermore, an exonic splicing silencer (ESS) has been found downstream of the differentiation specific 3' splice site at 5639, which also acts to inhibit use of this splice site (Figure 1.11) (Zhao et al. 2004). This element is shown to associate with hnRNP A1 (Table 1.1) and directs splicing to more distal 3' splice sites. This highlights the importance of balance between cellular RNA processing factors in the control of late gene expression. Furthermore, deletion of these elements results in increased levels of late RNAs, suggesting they may also affect RNA stability (Zhao et al. 2004; Rush et al. 2005).

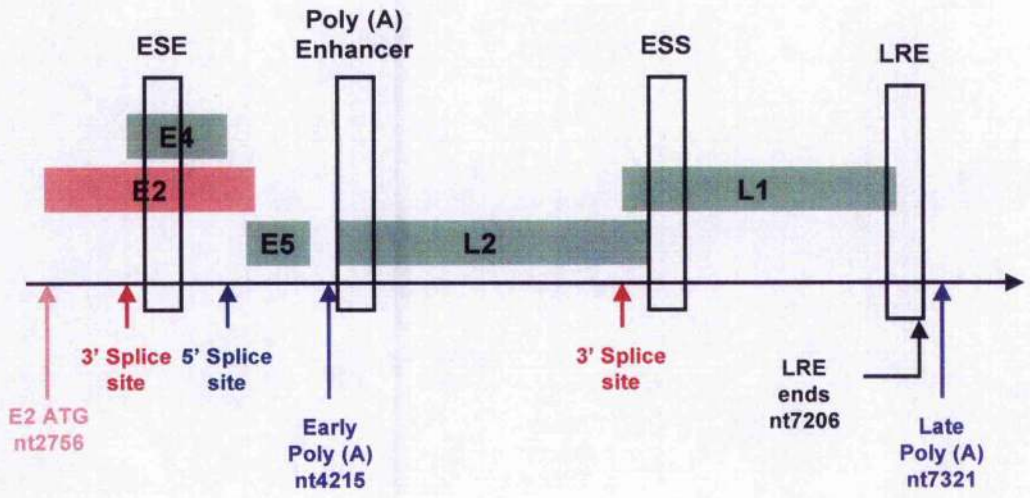


Figure 1.11: **Cis-acting RNA elements within the late region of HPV-16**

A schematic diagram of the 3' portion of the HPV-16 genome, showing the late region (not to scale). ORFs are sown as coloured rectangles, splice sites' and polyadenylation signals as vertical arrows and cis-acting RNA elements as open rectangles. Each element acts, via interactions with cellular RNA processing factors, to regulate splicing, stability, polyadenylation, export and potentially translation of late transcripts.

Further regulatory elements have been observed within the L2 ORF. These are generally less well defined but are shown to inhibit translation and result in instability of L2 containing mRNAs (Oberg et al. 2003). Proteins interacting with the L2 RNA elements include hnRNP K and poly(rC) binding protein 1 and 2 (PCBP-1 and 2), causing inhibition of translation (Collier et al. 1998). Furthermore, an element within the 5' region of L2 enhances polyadenylation from pAE (Figure 1.11) (Zhao et al. 2005). The region spanning ~170-400bp downstream of the pAE encodes multiple GGG motifs and can interact with polyadenylation factor, CstF-64 and, more strongly, with hnRNP H (Table 1.1). Consistent with a role for hnRNP H in enhancing polyadenylation from pAE, levels of the protein are seen to diminish upon differentiation of apparently normal cervical epithelium (Zhao et al. 2005). However, levels of certain RNA processing proteins are regulated during the HPV-16 life cycle, indicating that hnRNP H abundance could also respond to infection. There are also elements within the late region of HPV-31, which act to inhibit read-through at the early polyadenylation signal in undifferentiated cells (Terhune et al. 2001). In this case inhibitory activity is localised to the first 800 nts of the L2 ORF; however, little is known about how the region acts to enhance polyadenylation from the early signal.

1.4 SR proteins

As previously discussed, an important family of proteins essential to splicing are known as SR proteins. These contain one or two RNA recognition motifs (RRMs) which binds RNA within the N-terminal region and an RS domain, important for protein-protein interactions, in the C-terminal region (Wu and Maniatis 1993; reviewed by Caceres and Krainer 1997; reviewed by Graveley 2000). A glycine-rich hinge separates the two domains. RS domains are common to splicing factors, however the RRM is a conserved region of SR proteins. The family contains more than 12 members and is divided into two groups: those containing one RRM and those containing two (reviewed by Caceres and Krainer 1997). Of particular interest here is SF2/ASF, containing two RRMs, which is the key SR protein and is the only member of the family found to have essential function in *Caenorhabditis elegans* (Longman et al. 2000). As outlined previously, a complex is formed upon initiation of splicing which contains at least one SR protein (see section 1.2.2: Splicing). RS domains mediate interactions between SR proteins

and U1 snRNP and U2AF complex proteins (Cao and Garcia-Blanco 1998). SF2/ASF and SC35 are shown to associate with both U1-70K and $U2AF^{35}$ (Wu and Maniatis 1993). In addition, the SR protein RS domain and one of the RS domains of U1-70K is required for this interaction (Cao and Garcia-Blanco 1998). This is important as SR proteins are thought to bring together splicing components over splice junctions and therefore are important in defining splice patterns. Furthermore, the phosphorylation status of the RS domain has an impact on this. SR proteins can be phosphorylated by three types of kinase: SR protein kinases (SRPK1 and SRPK2) (Gui et al. 1994; Wang et al. 1998), the Clk/Sty family (Colwill et al. 1996) and DNA topoisomerase I (Rossi et al. 1996). It has been shown that phosphorylation mediates the transition between stages of splicing. For example, hyperphosphorylated SR proteins promote the pre-spliceosome to spliceosome transition (Mermoud et al. 1994; Roscigno and Garcia-Blanco 1995), whilst subsequent de-phosphorylation is thought to be required for the first transesterification to occur (Cao et al. 1997). *In vitro* phosphatase treatment of SR proteins prevents formation of the spliceosome and inhibits stable binding of U2 and U4/U6.U5 snRNPs to the pre-mRNA (Mermoud et al. 1994; Roscigno and Garcia-Blanco 1995). In contrast, whilst thiophosphorylated SF2/ASF, which is not readily de-phosphorylated, facilitates spliceosome formation in HeLa nuclear extracts, splicing is still inhibited, suggesting that SF2/ASF de-phosphorylation is required for the latter stages of splicing (Cao et al. 1997). In agreement with this, it has been shown that phosphorylation of the SF2/ASF RS domain affects protein-protein interactions (Xiao and Manley 1997; Xiao and Manley 1998). Phosphorylation of the RS domain enhances interaction with U1-70K *in vitro* (Xiao and Manley 1997). In contrast, hyperphosphorylation of SF2/ASF inhibits homodimerisation and binding to other SR proteins (Xiao and Manley 1998). Furthermore, SF2/ASF-$U2AF^{35}$ association is unaffected by phosphorylation. This indicates that SR protein phosphorylation, which results in changes in protein-protein interactions, mediates splicing.

Alternative splicing is controlled by hyperphosphorylated SR proteins via interaction with *cis*-acting elements, such as ESEs and ESSs (Kanopka et al. 1996). Some evidence suggests that ESEs promote 3' splice site usage via recruitment of $U2AF^{65}$ to adjacent 3' splice sites (Zuo and Maniatis 1996). However, this is somewhat controversial, and other studies have failed to detect a difference in U2AF binding in the presence or absence of an ESE (Ll and Blencowe 1999). More recently the RS domain from SR proteins has been shown

to support splicing when tethered to an ESE and subsequent interaction with the BPS is thought to promote pre-splicesome assembly (see section 1.3.1: Splicing) (Shen et al. 2004). Furthermore, RS domains from other splicing factors, such as $U2AF^{65}$ are able to associate with the BPS. It has been shown that the RS domain of $U2AF^{65}$ contacts the BPS first, in the E complex, whilst the ESE bound RS domain associates during pre-splicesome assembly (Shen and Green 2004). However, a further SR protein is required to complete spliceosome assembly and the RS domain of this is shown to interact with the 5' splice site (Shen and Green 2004). There is also evidence suggesting that ESE bound SR proteins may function not only during splice site selection but may also regulated the latter steps of the splicing reaction (Chew et al. 1999). Furthermore, ESEs have been implicated in 5' splice site selection, perhaps via SR protein interaction with U1-70K, a component of U1 snRNP (Caceres et al. 1994; Ryner et al. 1996). In contrast, interaction of hnRNP proteins with ESSs antagonises the action of ESE-bound SR proteins. For instance, expression of hnRNP A1 reduces occupancy of U1 snRNP with 5' splice sites, whereas SF2/ASF increases U1 snRNP-5' splice site interactions (Eperon et al. 2000). Furthermore, hnRNP A1 was shown to reduce interaction of U1 snRNP with 5' splice sites by direct competition for binding. However, this is thought to be achieved by indiscriminate binding of hnRNP A1 to the RNA molecule, which is displaced by SF2/ASF binding to ESEs. In contrast, hnRNP A1 association with an ESS is thought to inhibit splice site usage by mechanisms other than inhibition of U1 snRNP binding (Eperon et al. 2000).

A sub-set of SR proteins have roles in RNA metabolism other than splicing. For example, SF2/ASF, SRp20 and 9G8 are known to shuttle between the nucleus and the cytoplasm (Caceres et al. 1998). This was shown using HeLa cells in which transcription was inhibited, where shuttling proteins are restricted to the cytoplasm. In addition, SF2/ASF, SRp20 and 9G8 can be found in the mouse nucleus in interspecies heterokaryons. Whilst shuttling is dependent upon transcription in the case of SF2/ASF and SRp20, 9G8 is able to shuttle even when transcription is inhibited. Furthermore, SRp20 and 9G8 are able to facilitate export of intronless histone H2a mRNA by binding to a 22nt RNA element (Huang and Steitz 2001). Both proteins were shown to associate with polyadenylated RNA in the nucleus and the cytoplasm, and antibodies that inhibited this interaction, decreased the rate of mRNA export. For SF2/ASF, localisation and nucleo-cytoplasmic shuttling is regulated by the RS domain (Cazalla et al. 2002).

Shuttling is thought to be an intrinsic property of SR proteins; however, presence of a dominant nuclear retention signal in SC35, inhibits nuclear export. In addition, shuttling SR proteins also interact with export adaptor TAP (Huang et al. 2003). Truncated versions of 9G8, containing only the N-terminal region, were able to inhibit mRNA export in a dominant negative manner, and could also inhibit binding of TAP with SF2/ASF and REF family members. This suggests that all three associate with TAP via the same region, shown to be within the N-terminus of the protein (Huang et al. 2003). In addition, the RS domain of SF2/ASF and 9G8 is not required for TAP interaction *in vitro*. SF2/ASF is also known to regulate translation, as expression is seen to increase from transcripts to which SF2/ASF is tethered in the absence of SF2/ASF binding sites (Sanford et al. 2004). The shuttling activity of SF2/ASF is vital for this function, as mutant proteins that are unable to shuttle between the nucleus and the cytoplasm are unable to enhance translation. In addition, the phosphorylation status of the protein is important to each function. Whilst hyperphosphorylated forms are vital for alternative splicing, hypophosphorylation is necessary for shuttling and translational activity (Sanford et al. 2005). Hypophosphorylated SF2/ASF associates with cytoplasmic RNA with greater affinity than hyperphosphorylated forms, and hence is more active in regulating translation. In addition, phosphorylation of specific serine residues of the RS domain during certain cellular processes has not been determined. This suggests SF2/ASF has an integral role during post-transcriptional regulation of gene expression, and that phosphorylation of the protein, potentially at different serine residues, regulates protein function.

1.4.1 Regulation by SF2/ASF

SF2/ASF has been implicated in regulation of many targets in mammalian cells. Of most interest here is its influence on virus gene expression. Expression from integrated HIV genomes is as a single RNA which is alternately spliced into over 40 mRNAs. This is highly complex and relies on several alternate 5' and 3' splice sites. Furthermore, efficient replication is dependent upon the tightly controlled regulation of alternative splicing. Regulation is thought to be achieved using numerous *cis*-acting RNA elements and SR proteins are implicated in the process. Depletion of SF2/ASF has been shown to increase the level of HIV-1 tat pre-mRNA splicing (Wang et al. 1998), suggesting that SF2/ASF represses tat pre-mRNA splicing. SF2/ASF also interacts with the Rev response element (RRE), a

region of the HIV-1 genome involved in post-transcriptional regulation, in the presence but not absence of Rev (Powell et al. 1997). When bound to the RRE, Rev regulates the expression of structural, enzymatic and ancillary viral proteins. This is achieved partly via activation of Rev mediated splicing, a function which can be overcome by SF2/ASF overexpression (Powell et al. 1997). Furthermore, an ESE within exon 3 in shown to act in a bi-directional manner upon a downstream 3' splice site and an upstream 5' splice site (Caputi et al. 2004). This is achieved via interactions with SF2/ASF and SRp40, and involves stabilisation of U1 snRNP binding to the upstream 5' splice site.

Adenovirus temporal regulation of the L1 late unit is controlled via alternate 3' splice site usage. During early infection, use of the IIIa 3' splice site is minimal, whilst IIIa pre-mRNA splicing is activated to more than 200-fold in nuclear extracts prepared from late adenovirus-infected cells (Muhlemann et al. 2000). SR proteins are involved in repression of the IIIa splice pattern by interacting with an intronic repressor element and preventing recruitment of U2 snRNP to the BPS at this 3' splice site (Kanopka et al. 1996). SF2/ASF has been implicated in this regulation, as its overexpression prevents the early to late shift in mRNA expression (Molin and Akusjarvi 2000). This is vital to the viral life cycle as viral DNA replication is also blocked, resulting in lower virus yields. Furthermore, the virus-encoded protein E4-ORF4 has been shown to activate de-phosphorylation of SR proteins, via interaction with cellular protein phosphatase 2A (PP2A), inactivating them as alternative splicing regulators in late adenovirus-infected cells (Kanopka et al. 1998). Furthermore, E4-ORF4 can interact with hyperphosphorylated forms of SF2/ASF directly (Estmer Nilsson et al. 2001). E4-ORF4 mutants unable to interact with PP2A or SF2/ASF are unable to alleviate SR mediated repression of IIIa splicing. An additional protein found to inhibit the splicing enhancer and repressor activities of SF2/ASF is splicing factor-associated protein, p32 (Petersen-Mahrt et al. 1999). This function of p32 is established via inhibition of SF2/ASF RNA binding and phosphorylation, again highlighting the importance of SF2/ASF phosphorylation status to alternative splicing.

1.4.2 SF2/ASF and the HPV-16 life cycle

As mentioned previously SF2/ASF is found to interact, although not directly, with the HPV-16 LRE (McPhillips et al. 2004). Direct binding of a U1 snRNP-like complex and $U2AF^{65}$ to the 5' and 3' LRE regions respectively suggests that a

splicing like complex forms over the LRE. This complex has been implicated in regulation of late gene expression and it has the potential to affect splicing, polyadenylation, export and translation of LRE containing transcripts. Although a similar complex is thought to form in both undifferentiated and differentiated cells, SF2/ASF is found to be hyperphosphorylated and upregulated in differentiated cells (McPhillips et al. 2004). It has been suggested that this change in abundance and phosphorylation status affects the mode of regulation by the complex upon late viral transcripts (see Chapter 5 for more detail). Furthermore, there are a number of transcripts produced by the virus in differentiating cells, and as discussed *cis*-acting regulatory elements have been found within late RNAs. In addition, SF2/ASF is upregulated in cells stably expressing HPV-16 E2. Therefore, SF2/ASF may be regulated by HPV-16 to enhance late gene expression and hence ensure completion of the viral life cycle.

1.5 Aims

The aim of this PhD was to investigate the role of SF2/ASF in regulation of HPV-16 late gene expression by the LRE, and to determine how the virus regulates this protein, as well as other SR proteins. As reported previously, SF2/ASF is regulated in response to HPV-16 infection (McPhillips et al. 2004). As SF2/ASF is also upregulated in cells expressing HPV-16 E2, experiments were undertaken to determine if SF2/ASF expression is transactivated by E2. Promoter transactivation studies were performed using reporter constructs fused to the SF2/ASF promoter. To determine a direct role for E2 in transactivation, chromatin immunoprecipitation (ChIP) assays and electrophoretic mobility shift assays (EMSAs) were performed to show presence of E2 at the SF2/ASF promoter. As the SF2/ASF promoter is as yet undefined, nothing is known about its cellular regulation. Therefore, further EMSAs were performed to determine if cellular partners of E2 could be found to associate with this promoter. To establish if SF2/ASF levels were more likely to be regulated by transcription, rather than protein stability for example, levels of steady-state SF2/ASF mRNA were compared between undifferentiated and differentiated W12E cells. In addition, regulation of at least a sub-set of SR proteins may be expected due to the number of potential *cis*-acting RNA elements, which may act as ESEs, within the HPV-16 late region, and the extensive alternative splicing observed from the genome in HPV-16 infected differentiated cells (Milligan et al., 2006). To investigate the

possibility that SR proteins, other than SF2/ASF, were regulated during the HPV-16 life cycle, western blot and immunofluorescence techniques were used, comparing undifferentiated and differentiated W12E cells and cell extracts. Antibodies recognising a number of SR proteins and SRPK1, an SR protein kinase, were used to determine any changes in abundance or localisation upon differentiation. In addition, the role of HPV-16 protein E2 and E1^E4 in SR protein regulation was also determined. This was achieved using cells lines that stable express HPV-16 E2, or epithelial cells transiently transfected with HPV-16 E2 or E1^E4 expressing constructs. The final aim of this PhD was to determine a role for SF2/ASF in LRE mediated regulation of late gene expression. To study this, undifferentiated epithelial cells were transfected with reporter constructs containing the late 3'UTR from HPV-16, either including or lacking the LRE. Both HeLa and undifferentiated HaCaT cells (a non-tumourigenic spontaneously immortalised epithelial cell line) were co-transfected with these constructs and vectors expressing SF2/ASF. Undifferentiated W12E cells were not used in these experiments due to problems with transfections. Potential changes in reporter gene expression were then assayed. CAT assays were performed to determine any changes in protein abundance in the presence and absence of elevated SF2/ASF, whilst quantitative RT-PCR was used to analyse total and polyadenylated CAT RNA levels.

2 Materials and Methods

2.1 Materials

2.1.1 Enzymes

All enzymes were purchased from Roche Applied Science unless otherwise stated.

2.1.2 Primers

All primers used in PCR for cloning of inserts, Chromatin Immunoprecipitation, RT-PCR and sequencing were purchased from Sigma-Genosys. The name, sequence and application of these primers are given in table 2.1.

2.1.3 Plasmids/Vectors

pGEM®-T Easy (Promega): Used to clone PCR products generated by amplification using Taq DNA polymerase. The vector is supplied pre-cut with EcoRV, with 3' terminal thymidine residues added to both ends. This provides sticky ends which complement the 3' terminal adenosine residues added by Taq DNA polymerase to amplified products. Insertion of the PCR product into the ß-galactosidase enzyme gene causes inactivation of the enzyme, allowing detection of recombinant clones via blue/white selection on Xgal/IPTG plates.

pBluescript (BS) KS (Stratagene): Used to clone PCR products generated by amplification using Pfu DNA polymerase, and as an intermediate cloning vector. pBS contains T3 and T7 bacteriophage promoters at either end of the MCS. The MCS is contained with the ß-galactosidase enzyme gene, causing inactivation of the enzyme upon cloning of an insert.

pCI-neo (Promega): A mammalian expression vector containing a Cytomegalovirus (CMV) immediate-early enhancer/promoter and an SV40 late polyadenylation signal. A chimaeric intron, composed of the 5' splice site from the first intron of the human ß-globin gene and the branch and 3' splice site from the intron of an immunoglobulin gene heavy chain region, is found upstream of the

MCS. The plasmid also contains a neomycin phosphotransferase selectable marker for selection of stably transfected mammalian cells lines with G418.

pCI-E2: Nucleotides (nt) 2735-3852 of the HPV-16 genome containing the E2 ORF were cloned into the XbaI/SalI sites of pCI-neo, to produce a mammalian HPV-16 E2 expression vector.

pET15b:E2(16) (supplied by Julie Burns, University of York): A bacterial expression vector with a 20 amino acid histidine tag as an in-frame N-terminal fusion with HPV-16 E2.

pCG (supplied by Javier Cacares, MRC Human Genetics Unit, Edinburgh): A mammalian expression vector containing a CMV promoter and an SV40 late polyadenylation signal.

pCG-SF2/T7-SF2 (supplied by Javier Cacares, MRC Human Genetics Unit, Edinburgh): A mammalian expression vector for SF2/ASF, either containing or lacking a T7 tag. Derived from pCG.

pGCAT3-Basic(B) (adapted from pGL3-Basic, Promega): A Chloramphenicol Acetyltransferase (CAT) reporter vector containing a late SV40 polyadenylation signal.

pGCAT3-B-SF2 promoter: pGCAT3-Basic with a region spanning 1kb 5' of and including the SF2/ASF transcriptional start site, cloned into KpnI/SalI sites (Figure 3.1).

pLW2: A CAT reporter construct containing an HSV-1 immediate early 5 promoter and an SV40 late polyadenylation signal.

pLW1+LRE: A CAT reporter construct containing an HSV-1 immediate early 5 promoter and the late 3'UTR of HPV-16 from the PstI site at nt 7005 to the EcoRI site at nt 7456 (Figure 5.3).

pLW1-LRE: A CAT reporter construct containing an HSV-1 immediate early 5 promoter and the late 3'UTR of HPV-16 from the SspI site at nt 7226 to the EcoRI site at nt 7456 which lacks the LRE (Figure 5.3).

pLW2(intron)/pLW1(intron)+/-LRE: CAT reporters as described above containing the chimeric intron from pCI-neo in the BamHI site upstream of the CAT gene. pLW1(intron)-LRE differs in that the late 3'UTR of HPV-16 is cloned from the PstI to the EcoRI site, with the LRE from nt 7127-7206, precisely deleted (Figure 5.5).

Primer	Sequence (5' to 3')	Application
SF2pr –1024F	GTTACGGTTCTCACATCCATTTTGC	Cloning of SF2pr EMSA probe 1
SF2pr –829 R	TGACACTAGTGTGGGACTAGCGCAGA ATGC	EMSA probe 1
SF2pr –852 F	TGACACTAGTCTTCATTAAGTACCGTT CC	EMSA probe 2
SF2pr –650 R	CAGGCATTCTGCGCTAGTC	EMSA probe 2
SF2pr –684 F	TGACACTAGTGTTCTGACGAGAAGGC GGAAC	EMSA probe 3
SF2pr –482 R	GAAACCCGGGTATCTTCGTAG	EMSA probe 3
SF2pr –524 F	TGACACTAGTCAGCTCTGGATTAGAC GCAC	ChIPs EMSA probe 4
SF2pr –325 R	GCAATGAGGATCTTTGAAAGC	ChIPs EMSA probe 4
SF2pr –402 F	ATGGTGGGACAACGCTTTAG	Endogenous ChIPs
SF2pr –222 R	TTTGCGAACAGAGTGACCAG	Endogenous ChIPs
SF2pr –364 F	TGACACTAGTCTTTGAAGGCGCCGAG TTGC	EMSA probe 5
SF2pr –162 R	GACGTCACCCTCCCCACGAAG	EMSA probe 5
SF2pr –199 F	TGACACTAGTGGGACTTTTTTACCCCC TTC	EMSA probe 6
SF2pr +5R	CTCCCGCGGCCCCTCCAAAATG	Cloning of SF2pr EMSA probe 6
CAT5' F	AATCACTGGATATACCACCGTTGA	ChIPs
CAT5' R	TGAACGGTCTGGTTATAGGTAC	ChIPs
5S RNA F	GGCCATACCACCCTGAACGC	ChIPs
5S RNA R	CAGCACCCGGTATTCCCAGG	ChIPs
SF2 F	CTCATCATTCCCCAGAAACC	ChIPs RT-PCR
SF2 F	GGCAGGAATCCACTCCTATG	ChIPs RT-PCR
GAPDH F	TCCACCACCCTGTTGCTGTA	RT-PCR
GAPDH R	ACCACAGTCCATGCCATCAC	RT-PCR
CAT3' F	CTGGCCTATTTCCCTAAAGG	RT-PCR
CAT3' R	CAAACGGCATGATGAACCTG	RT-PCR

Table 2.1: Sequence and application of primers used.

2.1.4 Antibodies

The antibodies used in western blotting, imuunofluoresence and immunoprecipitation experiments are listed in table 2.2 together with their dilution and source.

Antibody	Western blotting	Immnuo-flourescence	ChIP/EMSA supershift	Source
HPV-16 E2	1/5000 with 1M NaCl		5µl – ChIP	rabbit, Lawrence Banks (ICGEB, Trieste, Italy)
			5µl - ChIP	SCT, rabbit, Julie Burns, (University of York)
		neat		TVG 261, mouse, Iain Morgan
TBP			1ml - ChIP	4C8/26, mouse, Robert White
Involucrin	1/1000	1/1000	5µl – ChIP	clone SY5, mouse, Sigma
Sp1			1µl - supershift	ab13405, rabbit, Abcam
C/EBP β			1µl - supershift	H-7, mouse, Santa Cruz
SF2/ASF	1/500			clone 96, mouse, Zymed Laboratories
		1/500		clone 103, mouse, Zymed Laboratories
SRp20	1/250	1/50		clone 7B4, mouse, Zymed Laboratories
9G8	1/10	1/25		clone 98, mouse, James Stevenin
Phosphorylated SR proteins	neat			Mb104, mouse, (Hanamura et al. 1998)
SC35		1/250		mouse, Sigma
SRPK1	1/100	1/100		clone G211-637, mouse, BD Pharmingen
GAPDH	1/2000			6CS, mouse, Biodesign International
HPV-16 E1^E4		1/100		B11, FITC conjugate, John Doorbar

Table 2.2: Dilution and source of all antibodies used.

2.1.5 Bacterial Culture

The DH5α *E. coli* strain was used to propagate and maintain all plasmid DNA. L-Broth (10g NaCl, 16g Bactopeptone, 5g yeast extract in 1 litre H_2O, pH 7.5) and agar plates made with 1.5% (w/v) agar in L-Broth were used to grow the bacteria. When necessary, L-Broth and agar plates were supplemented with 100μg/ml ampicillin.

2.1.6 Radiochemical

Radiochemicals were purchased from the following sources at the stated activity level:

α – [^{32}P] dCTP	10mCi/ml	Amersham (GE Healthcare)
[^{14}C] Chloramphenicol	50μCi/ml	Perkin Elmer

2.1.7 Cell lines

HeLa: An epithelial cell line derived from an aggressive cervical lesion containing integrated HPV-18.

J2 3T3: J2 clone of random-bred Swiss mouse 3T3 cells.

W12E: An epithelial cell line containing extrachromosomal copies of HPV-16, subcloned from the parental W12 cell line (Stanley et al. 1989; Jeon et al. 1995).

HaCaT: A spontaneously immortalised epithelial cell line (Boukamp et al. 1988).

U2OS: An osteosarcoma cell line.

All cell lines were grown at 37°C in the presence of 5% CO_2.

2.1.8 Common Reagents, chemicals and solutions

30% mix	Acrylamide/bisacrylamide	29% (w/v) acrylamide, 1% (w/v) N,N'-methylene bis-acrylamide

10 x DNA loading buffer	15% (v/v) Ficoll Type 400, 0.25% (w/v) bromophenol blue, 0.25% (w/v) Xylene cyanole
PBS	170mM NaCl, 3.4mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4, pH 7.2
PBS-Tween (PBS-T)	PBS, 0.1% (v/v) Tween 20
5x Protein loading buffer	62.5mM Tris-HCl, 2% (w/v) SDS, 715mM (v/v) β-mercaptoethanol, 0.3% (w/v) bromophenol blue, 20% (v/v) glycerol, pH 6.8
5x Tris-Glycine Buffer	1.25M Glycine, 125mM Tris, 0.5% (w/v) SDS
10x TBE	890mM Tris, 10mM EDTA, 890mM boric acid, pH 8.3
TE buffer	10mM Tris-HCl, 1mM EDTA, pH 8.0
Coomassie blue stain	0.02% (w/v) Coomassie Brilliant Blue, 50% (v/v) methanol, 7% (v/v) acetic acid
Coomassie blue destain	5% (v/v) methanol, 7% (v/v) acetic acid

2.2 Methods

2.2.1 DNA manipulation and cloning

2.2.1.1 Preparation and transformation of competent bacterial cells

1ml of a 5ml overnight culture of DH5α cells was inoculated into 100ml L-Broth and grown whilst shaking for approximately 2.5-3 hours until the OD_{600} was 0.6. The culture was then chilled on ice for 10 minutes followed by centrifugation at 2500rpm for 10 minutes at 4°C (Sorvall RT7 bench top centrifuge). After the supernatant was discarded, the pellet was resuspended in 20ml ice-cold sterile 0.1M $CaCl_2$ and incubated on ice for 30 minutes. Cells were pelleted by centrifugation at 2000rpm for 10 minutes at 4°C (Sorvall RT7 bench top centrifuge) and the supernatant was again discarded. The pellet was resuspended in 3.6ml ice-cold sterile 0.1M $CaCl_2$ and 10% glycerol was added. Competent cells were stored in aliquots of 200µl at 4°C for use within 48 hours or –70°C for prolonged storage.

Competent cells were thawed on ice prior to transformation. 1-50ng of plasmid DNA (to a volume of 10µl or less) was mixed with 100µl competent cells and incubated on ice for 30 minutes. Heat shocking at 42°C for 90 seconds was performed followed by transfer to ice for 1-2 minutes to allow cooling. 900µl L-Broth was added and cells were incubated for 30-90 minutes at 37°C whilst shaking. 100µl cells up to the entire solution following pelleting, were then transferred to agar plates containing 100µg/ml ampicillin and incubated at 37°C overnight.

2.2.1.2 Plasmid DNA purification

Small-scale purification of plasmid DNA

Plasmid DNA was isolated using a Wizard® Plus SV Miniprep kit (Promega) according to the manufacturer's protocol. Alternatively, cells were pelleted from 1ml of a 5ml overnight culture by centrifugation for 2 minutes at maximum speed in a microcentrifuge (Eppendorf 5415C). The supernatant was discarded and the pellet was resuspended in 100µl Solution I (50mM Glucose, 25mM Tris-HCl, 10mM EDTA, pH8.0). After addition of 150µl Solution II (0.2M NaOH, 1% SDS), the solution was mixed by inversion. 150µl Solution III (3M Potassium acetate,

11.5% (v/v) Acetic acid) was then added and the solution was again mixed by inversion. Following centrifugation at maximum speed for 2 minutes in a microcentrifuge (Eppendorf 5415C), the supernatant was transferred to a fresh eppendorf and extraction of nucleic acids using 150µl phenol:chloroform:isoamylalcohol (25:24:1) was performed. 720µl isopropanol and 40µl 3M NaOAc were added and the solution was mixed by inversion. Microcentrifugation was performed at maximum speed for 10 minutes (Eppendorf 5415C) and the supernatant was removed. 400µl 70% EtOH was added and microcentrifugation was repeated at maximum speed for 5 minutes (Eppendorf 5415C). Following removal of the supernatant and air drying, the pellet was resuspended in 20µl TE buffer.

Large scale Caesium Chloride purification of plasmid DNA

5ml overnight culture was inoculated into 200ml L-Broth plus 100µg/ml ampicillin and incubated at 37°C overnight, with shaking. Cells were pelleted at 8000rpm for 10 minutes at 4°C in a Beckman J2-21 centrifuge (JA-14 rotor). Following removal of the supernatant cells were resuspended in 10ml Solution I (50mM Glucose, 25mM Tris-HCl, 10mM EDTA, 2mg/ml lysozyme, pH8.0). 20ml freshly prepared Solution II (0.2M NaOH, 1% SDS) was added to lyse cells, followed by incubation at room temperature for 10 minutes. After addition of 20ml ice-cold Solution III (3M Potassium acetate, 11.5% (v/v) acetic acid), the solution was mixed by inversion and incubated on ice for 10 minutes. Cell debris was pelleted at 8000rpm for 10 minutes at 4°C in a Beckman J2-21 centrifuge (JA-14 rotor). The supernatant was filtered into to a fresh centrifuge bottle through 8ply gauze and plasmid DNA was precipitated from the filtrate by addition of 0.6 volumes isopropanol. Centrifugation was performed at 8000rpm for 15 minutes at room tempreature in a Beckman J2-21 centrifuge (JA-14 rotor). After air drying the DNA was resuspended in 5ml TE buffer, to which 5.4g CsCl and 400µl Ethidium Bromide (EtBr) (10mg/ml) was added. Following centrifugation at 10000rpm for 10 minutes at 18°C in a Beckman J2-21 centrifuge (JA-20 rotor), the supernatant was transferred to a 6ml ultracentrifuge tube and centrifuged at 48000rpm, overnight at 18°C in a Beckman L7-65 Ultracentrifuge (Ti70 rotor). The plasmid DNA band was extracted by puncturing the tube with a needle and syringe, removing the plasmid DNA band and transferring it to a 15ml Falcon tube. Repeated extraction (x5) with H_2O-saturated butan-2-ol was used to remove EtBr from plasmid DNA, then 3 volumes TE buffer was added followed by DNA precipitation with 2.5

volumes 100% ethanol. Following washing with 5ml 70% EtOH, the resulting DNA pellet was resuspended in 500µl TE buffer and analysed by restriction digestion.

2.2.1.3 Restriction endonuclease digestion and blunt-ending DNA

Digestion of DNA was performed for 1-3 hr at 37°C in the presence of approximately 2U of the restriction enzyme per µg DNA in 1x the appropriate buffer (supplied by the manufacturer). Over-hanging 5' DNA ends were filled-in using 2.5U Klenow DNA Polymerase I, Large Fragment, for 10-30 minutes at 25°C, in the presence of all four dNTPS at a final concentration of 33µM each.

2.2.1.4 Phenol:Chloroform extraction and ethanol precipitation

In general, an equal volume of phenol:chloroform (1:1) (phenol saturated in TE buffer, pH 7.9) was added to the DNA solution, mixed by vortexing and microcentrifuged at maximum speed for 5 minutes (Eppendorf 5415C). The upper aqueous phase was transferred to a fresh tube, an equal volume of chloroform was added and the solution was vortexed and microcentrifuged as before. Following transfer of the upper aqueous phase to another fresh tube, 1/10 volume 3M sodium acetate, pH 5.2, and 2.5 volumes 100% EtOH were added. DNA was precipitated at –20°C overnight, or on ice for 30 minutes. Pelleting of DNA was performed by microcentrifugation at maximum speed for 10 minutes (Eppendorf 5415C). The pellet was then washed with 70% ethanol, centrifuged for 5 minutes, air-dried and resuspended in dH_2O or TE buffer.

2.2.1.5 DNA ligation

Inserts were ligated into vectors with compatible ends following restriction digestion with the appropriate enzymes and gel extraction (Section 1.2.1.6). Reactions were carried out in 20µl volume with 1x ligase buffer and 40U T4 DNA ligase per reaction at differing molar ratios, typically 1:3, 1:1 and 3:1. Ligation was performed at room temperature for more than 4 hours, or at 4°C overnight. PCR products were ligated into pGEM T Easy (Promega) following the manufacturer's instructions.

2.2.1.6 Gel electrophoresis of DNA and recovery

Agarose gel electrophoresis

DNA was resolved in a horizontal slab gel of 1-2% (w/v) agarose (Roche) in 1xTBE or 1xTAE and electrophoresed in 1xTBE/TAE buffer at ~100V. 1x TBE was used to product more defined DNA bands, whilst 1xTAE was used when DNA was to be extracted. 100µg/µl 1kb DNA ladder (Invitrogen) was used as a standard size marker. Staining of gels in 0.5µg/ml EtBr for 15 minutes was performed following electrophoresis and DNA was visualised under UV light. Gel slices containing the appropriate DNA were excised from 1xTAE gels and the DNA was extracted using a QIAquick® Gel Extraction Kit (50) (Qiagen) following the manufacturer's instructions.

Acrylamide gel electrophoresis

Resolution of small DNA fragments, less than 1kb in size, was performed on a 6% polyacrylamide gel prepared as follows:

30% acrylamide/ bisacrylamide mix (BioRad)	3ml
10xTBE	1.5ml
dH_2O	10.5ml
10% APS	100µl
TEMED	12µl

Electrophoresis was performed at ~120V, followed by staining with 0.5µg/ml EtBr for 15 minutes and visualisation under UV. DNA was extracted from gel slices by incubation at 4°C overnight in 400µl 0.5M NaCl, 1mM EDTA and precipitation with 2.5 volumes 100% ethanol. Following pelleting and washing with 70% EtOH, the DNA was resuspended in dH_2O or TE buffer.

2.2.1.7 Quantification of nucleic acids

The concentration of nucleic acid solutions was calculated using a spectrophotometer (Eppendorf BioPhotometer). The A_{260}/A_{280} ratio was also used to determine the purity of the solution, a reading of ~1.8 indicating the sample was relatively pure.

2.2.2 Tissue Culture

2.2.2.1 Growth and maintenance of cell lines

HeLa, HaCaT and U2OS cells were passaged in Dulbecco's Modified Eagles Medium (DMEM), supplemented with 10% foetal calf serum, 2mM L-glutamine with or without 50U/ml Penicillin/ 50µg/ml streptomycin (all supplied by Life Technologies). 500µg/ml G418 was added if necessary.

J2 3T3 cells were passaged in DMEM, supplemented with 10% donor calf serum (Life Technologies) and 2mM L-glutamine.

W12E cells were grown on a feeder layer of J2 3T3 cells in DMEM, supplemented with 10% foetal calf serum, 2mM L-glutamine, 0.1nM Cholera Toxin and 0.4µg/ml Hydrocortisone. In preparation for W12E cell plating, J2 3T3 cells were treated with Mitomycin C at 4µg/ml for 2-5 hours. Cells were then washed twice with PBS, trypsinised and pelleted at 1000rpm at 4°C for 5 minutes (Sorvall RT7). Following resuspension, cells were counted using trypan blue exclusion, to identify live cells, and plated at 1.10^6 per 100mm dish. W12E cells were then plated at 2.10^5 cells per 100mm dish from liquid nitrogen stocks. After 24 hours, 0.5ng/ml epidermal growth factor was added. During growth, medium was changed every 2 days until harvesting, at 5 days for undifferentiated cells and 10 days for differentiated cells. To harvest W12E cells, J2 3T3 cells were first trypsinised and washed from plates. W12E cells remained adhered to the plate and were harvested either by trypsinisation or using one of the methods described in section 2.2.2.4.

2.2.2.2 Preparation of cell stocks

Cells were trypsinised then counted via trypan blue exclusion and resuspended in medium to 1.10^6 cells/ml. 10% (v/v) dimethylsulphoxide (DMSO) was added and aliquots of 1.10^6 cells were transferred to screw cap tubes, left at –70°C overnighted, followed by storage in liquid nitrogen.

2.2.2.3 Transfection with lipofectamine reagent

One day prior to transfection $2\text{-}8.10^4$ cells were plated in 10mm diameter wells in culture medium lacking Penicillin/Streptomycin. The following day the transfection

solution, of the appropriate amount of plasmid DNA, 1µl 2mg/ml Lipofectamine™ 2000 (Invitrogen) in 100µl DMEM per well, was incubated at room temperature for 15 minutes. The solution was then transferred to cells, which were incubated for 24-48 hours.

2.2.2.4 Preparation of cell extracts

Preparation of total cellular protein extracts

Following removal of media, cells were washed twice in PBS. Protein loading buffer was added and plates were incubated at 4°C for 15 minutes whilst shaking. To shear DNA, lysates were passed through a 22-guage needle 10 times and transferred to screw cap tubes. Storage was at –20°C. Prior to electrophoresis, lysates were incubated at 100°C for 5 minutes, before centrifugation at 12,000rpm for 2 minutes (Eppendorf 5415C). Alternatively, a freeze/thaw method was used. Cells were trypsinised and pelleted at 13,000rpm at 4°C for 5 minutes (Hettich zentrifugen EBA 12 R). Following washing with PBS, pellets were resuspended in 0.25M Tris-HCl, pH 7.5. Lysates were then frozen on 100% EtOH with dry ice and thawed at 37°C, three times. Following centrifugation at 13,000rpm at 4°C for 10 minutes (Hettich zentrifugen EBA 12 R), the supernatant was transferred to a fresh eppendorf and stored at –20°C.

Preparation of Nuclear Extract

Following removal of media and a single PBS wash, cells were scraped into 5ml PBS and pelleted at 4°C for 5 minutes at 4000rpm (Sorvall RT7). The pellet was then resuspended in 4ml Buffer A (10mM Tris-HCl, pH 7.9, 1.5mM $MgCl_2$, 10mM KCl, 0.5mM DTT, 0.5mM PMSF) and incubated on ice for 10 minutes. Following homogenisation (x10-15), nuclei were pelleted at 4°C for 5 minutes at 4000rpm (Sorvall RT7) and resuspended in 200µl Buffer C (20mM Tris-HCl, pH 7.9, 25% (v/v) Glycerol, 1.5mM $MgCl_2$, 0.2mM EDTA, pH 8.0, 0.3M NaCl, 0.5mM DTT, 0.5mM PMSF). Samples were incubated at 4°C for 30 minutes with rotation, then centrifuged at 4°C for 15 minutes at 10000rpm (Hettich zentrifugen EBA 12 R). The supernatant was transferred to fresh ice-cooled tubes and stored at –70°C.

Preparation of Total RNA

Cells were washed with PBS before addition of 1ml TRIzol® Reagent (Invitrogen) per 1.10^6 cells. Following transfer to 12ml snap-capped tubes (Greiner), samples were incubated at room temperature for 5 minutes. 1/5 volume of chloroform was

added and the sample was shaken vigorously for 15 seconds. After 2-3 minutes incubation at room temperature, centrifugation was performed at 10,000rpm for 15 minutes at 4°C in a Beckman J2-21 centrifuge (JA-20 rotor). The aqueous phase was transferred to a fresh tube and ½ volume isopropanol was added. Following incubation at room temperature for 10 minutes, samples were centrifuged at 10,000 rpm for 10 minutes at 4°C. The pellet was washed with 75% EtOH and allowed to air dry, followed by resuspension in DEPC-H_2O to ~1µg/µl. To remove DNA contaminants, samples were DNase treated using RQ1 RNase-Free DNase (Promega) as per the manufacturer's instructions.

2.2.3 Protein Analysis

2.2.3.1 Quantification of proteins

1-10µl neat or diluted sample was added to 200µl 1x Bradford Assay reagent (BioRad) in a 24-well plate. Standards were also set up in the same way using 10µl serial dilutions of BSA (0.1mg/ml, 0.2mg/ml, 0.4mg/ml, 0.6mg/ml, 0.8mg.ml and 1mg/ml), as well as a blank control. Concentrations were then determined against standards using an Anthos HT II labtech platereader.

2.2.3.2 SDS-PAGE

Fractionation of proteins, from lysates made in protein loading buffer, was performed on a 12% SDS-Polyacrylamide gel, with stacking gel, prepared as follows using a mini protein gel electrophoresis system:

	12% Separating gel	Stacking gel
30% acrylamide/ bisacrylamide mix	4ml	650µl
1.5M Tris-HCl, pH8.8	2.5ml	N/A
1M Tris-HCl, pH6.8	N/A	2.5ml
10% SDS	100µl	50µl
dH_2O	3.3ml	3.05ml
10% APS	100µl	50µl
TEMED	10µl	10µl

Electrophoresis was performed at 120V in 1x Tris-Glycine buffer, until the bromophenol blue reached the bottom of the gel. Proteins were visualised by

coomassie blue staining of the gel for 30 minutes at room temperature, followed by destaining overnight.

2.2.3.3 Western blotting

Following resolution by SDS-PAGE, proteins were transferred to nitrocellulose membrane (Amersham) using a Bio-Rad transblot system. Electroblotting was performed at 250mA for 2 hours or 100mA, overnight at 4°C. Blocking of the membrane was carried out in PBS-T with 5% dried skimmed milk (PBS-T, 5% milk) at room temperature for 1 hour, or 4°C overnight. The membrane was then incubated in the appropriate dilution of primary antibody in PBS-T, 5% milk for 1 hour at room temperature, or 4°C overnight. Following washing with PBS-T (1x30 seconds, 1x15 minutes and 2x5 minutes), the membrane was incubated in secondary antibody, either horseradish peroxidase conjugated anti-mouse IgG or protein A to detect monoclonal or polyclonal primary antibodies respectively, at a dilution of 1:1,000 in PBS-T, 5% milk, for 1 hour at room temperature. The membrane was then washed in PBS-T (1x30 seconds, 1x15 minutes and 2x5 minutes) and subjected to enhanced chemiluminescence (ECL) using the Amersham ECL system. Luminescence was visualised to Kodak X-OMAT S film and developed using a Konica SRX-101 A film processor. Stripping of membranes was performed as required in 50ml Stripping buffer (100mM β-mercaptoethanol, 2% SDS, 62.5mM Tris, pH 6.8) at 50°C for 30 minutes, followed by washing with large volumes of PBS-T (1x30 seconds, 1x15 minutes and 2x5 minutes).

2.2.3.4 Immunofluorescence

Cells were grown as described previously. Following trypsinisation, cells were transferred to 10mm coverslips and grown to ~70% confluence. The media was then removed and cells were washed three times in PBS. Fixing cells to coverslips was performed in 500µl Fix solution (58mM Sucrose, 5% formaldehyde, in PBS) for 10 minutes at room temperature. Following washing three times with PBS, coverslips were either stored at 4°C in PBS with 1% (v/v) Foetal calf Serum (PBS/FCS), or permeabilised in 500µl –20°C permea mix (70% acetone, 30% methanol) for 5 minutes at –20°C. Permea mix was then removed and coverslips were again washed three time with PBS. After this cells were incubated at room temperature for 1 hour with primary antibody at the desired dilution in PBS/FCS,

followed by washing six times with PBS. Secondary antibody, FITC or Texas Red-conjugated anti-mouse antibody was diluted 1:100 in PBS/FCS and incubated with cells for 30 minutes at room temperature. Following washing six times with PBS, coverslips were air dried and mounted with mounting fluid (Vector Laboratories) onto slides. Slides were stored at 4°C until required for confocal microscopy examination using a LSM 510 meta confocal microscope (Zeiss).

2.2.3.5 His Tagged protein purification

His tagged E2 protein was purified from BL21 bacterial cells. 5ml overnight culture was inoculated into 100ml L-Broth with 100µg/ml Ampicillin and incubated at 37°C for 3 hours, whilst shaking. Following addition of 1mM IPTG, the culture was incubated at 25°C for 4 hours, whilst shaking. Bacterial cells were recovered from culture via centrifugation at 6000rpm for 10 minutes at 4°C in a Beckman J2-21 centrifuge (JA-14 rotor) and the supernatant was discarded. Cells were then washed with 50mM NaCl, 2mM EDTA, 50mM Tris-HCl, pH 8.0 and centrifuged at 6000rpm for 10 minutes at 4°C in a Beckman J2-21 centrifuge (JA-14 rotor). The supernatant was again discarded and cells were resuspended in 10ml Binding buffer (5mM imidazole, 0.5M NaCl, 20mM Tris-HCl, pH 8.0). DNA was sheared by sonication (6x30seconds, with 1 minute intervals) and cell debris was pelleted at 13000rpm at 4°C for 10 minutes (Hettich zentrifugen EBA 12 R). The supernatant was then transferred to a fresh universal and applied to a Nickel resin column. The column was prepared with 1ml His•Bind® resin (Novagen), a 1:1 slurry in 20% EthOH, to a bed volume of 500µl, and allowed to drain. The column was then washed with 3 bed volumes dH_2O before application of 5 bed volumes 50mM $NiSO_4$. 3 bed volumes of binding buffer was then added, followed by addition of the lysate. At each stage fluid was allowed to drain through the column before addition of the next, to ensure maximum purity. To further remove impurities the column was subjected to 10x1 bed volume washes with binding buffer and 6x1 bed volume washes with wash buffer (60mM imidazole, 0.5M NaCl, 20mM Tris-HCl, pH 8.0). E2 protein was then eluted from the column via 6x1 bed volume washes with elution buffer (1.5M imidazole, 0.5M NaCl, 20mM Tris-HCl, pH 8.0). All washes and elutions were stored at –70°C and analysed by SDS-PAGE.

2.2.4 Analysis of reporter gene expression

2.2.4.1 Chloramphenicol Acetyltransferase (CAT) assays

Transfections were performed as described previously and protein lysates were made using the freeze/thaw method. 1-20µl lysate was added to a reaction mix of 625µM Acetyl Coenzyme A (Sigma), 0.05µCi [^{14}C] Chloramphenicol and 0.25M Tris-HCl, pH 7.5 to 40µl, and incubated at 37°C for 1 hour. 200µl ethyl acetate was added and the solution was vortexed for 10 seconds, before centrifugation at 13000rpm for 5 minutes at room temperature (Hettich zentrifugen EBA 12 R). The upper organic layer was then transferred to a fresh tube and lyophilised in an Eppendorf Concentrator 5301. Following resuspension in 25µl ethyl acetate, the solution was applied to a TLC plate (Merck) and samples were separated in 95% chloroform, 5% methanol. Plates were visualised to a phosphoimaging screen (Bio-Rad) and analysed using QuantityOne software.

2.2.5 DNA-Protein interaction assays

2.2.5.1 End-labelling of a dsDNA probe

DNA was digested with restriction enzymes leaving a 5' overhang and gel purified into 30µl dH_2O, as described above. α-[^{32}P] dCTP was then added to the DNA in a reaction mix consisting of 1x reaction buffer, 3.3µM dATP, dGTP and dTTP mix and 2.5U DNA Polymerase I, Large (Klenow) Fragment. The reaction was performed at room temperature for 10-30 minutes, then 3.3µM dCTP was added, followed by a further 5 minutes incubation at room temperature. The probe was then purified using a mini Quick Spin column (Roche). The column was first centrifuged at 6500rpm for 5 minutes at room temperature (MSE Micro Centrifuge). The probe was applied to column and centrifuged for a further 3 minutes at 6500rpm at room temperature (MSE Micro Centrifuge). Free α-[^{32}P] dCTP nucleotides were retained in the column. Specific activity of the probe was then determined using a Beckman liquid scintillation counter.

2.2.5.2 Electrophoretic Mobility Shift Assay (EMSA)

Reactions for DNA mobility shifts assays were prepared as follows:

α-[^{32}P] labelled DNA probe	2,000 cpm
1µg/µl poly dI-dC	2µl
10µg/µl BSA	0.45µl (to 300µg/ml)
3x reaction buffer	5µl
(10mM Hepes, pH 7.9, 100mM KCl, 5mM $MgCl_2$, 10% glycerol, 0.5mM DTT)	
E2	3µl
Nuclear Extract (~5µg/µl)	3.5µl
dH_2O	to 15µl

The reactions were incubated at room temperature for 15 minutes, and then resolved on a 4.5% non-denaturing acrylamide gel (9ml of 30% acrylamide/bis-acrylamide mix, 1xTBE, 44.5ml dH_2O, 500µl 10% APS, 50µl TEMED, pre-run for 45 minutes at 200V) for 2.5 hours at 200V in 1xTBE. The gel was then dried and potential DNA-protein complexes were visualised to Kodak X-OMAT film at -70°C.

2.2.5.3 Chromatin Immunoprecipitation (ChIP)

Following washing with PBS, cells were scrapped into 1ml PBS and pelleted at 1500rpm for 5 minutes at 4°C (Sorvall RT7). Cells were then washed in PBS and resuspended in 1ml PBS. The solution was added to 10ml pre-warmed PBS, 0.5% NP40 (PBS/NP40), 1% formaldehyde and incubated at 37°C for 10 minutes. Centrifugation was performed at 1500rpm for 5 minutes at 4°C (Sorvall RT7), followed by washing with PBS/NP40. Samples were kept on ice from this point. Cells were then resuspended in 40ml ice-cold High Salt buffer (PBS/NP40, 1M NaCl) and incubated on ice for 30 minutes. Following a second PBS/NP40 wash, cells were resuspended in 40ml ice-cold Low salt buffer (0.1% NP40, 0.1M NaCl, 10mM Tris-HCl, pH 8.0, 1mM EDTA) and incubated on ice for 30 minutes. Centrifugation was performed at 1500rpm for 5 minutes at 4°C (Sorvall RT7) and the pellet was resuspended in 1 ml Low Salt buffer, before being passed through a needle three times. 1.7ml Low salt buffer and 300µl 20% Sarkosyl were added and the solution was applied carefully to the top of 40ml Low salt buffer, 100mM Sucrose with cut blue tips, followed by centrifugation at 7000rpm for 10 minutes at 4°C in a Beckman J2-21 centrifuge (JA-20 rotor). Following resuspension of the pellet in 3ml TE buffer, the solution was again applied to 40ml Low salt buffer, 100mM Sucrose and centrifuged as before. The pellet was then resuspended in 2ml TE buffer and DNA was sheared by sonication (10x10 second pulses with 10 second intervals). Once transferred to two eppendorf (1ml each), 100µl 11x NET

buffer (5.5% NP40, 1.65M NaCl, 550mM Tris-HCl, pH 7.4, 5.5mM EDTA, 11mM MgCl2) was added, followed by centrifugation at 13000rpm for 5 minutes at 4°C (Hettich zentrifugen EBA 12 R). The supernatant was then transferred to fresh eppendorf tubes, one per immunoprecipitation, plus a 1/10 input control aliquot. Samples were then incubated in the presence of the appropriate concentration of antibody at 4°C, overnight with rotation. Following incubation with Protein-A-Sepharose beads at 4°C, for 2 hours with rotation, samples were applied to 5ml Polypropylene columns (Pierce) and washed with 20ml RIPA buffer (1% NP40, 150mM NaCl, 50mM Tris-HCl, pH 8.0, 0.1% SDS, 0.5% Deoxycholate), 20ml LiCl buffer (250mM LiCl, 0.5% NP40, 0.5% , 1mM EDTA, pH 8.0) and 20 ml TE buffer. DNA-protein complexes were then eluted from the column in 400µl TE buffer, 1% SDS. Proteinase K was added to 125µg/ml and samples were incubated at 42°C overnight before two phenol:cholorform:isoamylalcohol (25:24:1) and one chloroform extraction. Following ethanol precipitation, DNA was resuspended in 40µl TE buffer and analysed using PCR.

2.2.6 RNA

2.2.6.1 RT-PCR

Total RNA was purified as described previously. 1µl 0.5µg/µl Oligo dT or 1µl 1µg/µl random primer was incubated with 1µg RNA in 14.5µl DEPC-H_2O at 70°C for 10 minutes to denature RNA. Following cooling on ice, 2.5µl 10x $MgCl_2$ free PCR buffer, 2.5µl 25mM $MgCl_2$, 1µl 10mM dNTPs, 2.5µl 0.1M DTT and 1µl rRNasin® RNase inhibitor (Promega) were added and the sample was incubated at 42°C for 1 minute. 1µl Superscript II (Invitrogen) was then added followed by incubation for a further 50 minutes at 42°C. Reactions were stopped by incubation at 70°C for 15 minutes. RNA was then degraded by treatment with 1µl 2U/µl RNase H (Invitrogen) for 30 minutes at 37°C and RT-PCR was performed with 1/10 to 1/100 dilutions of each sample.

2.2.6.2 Quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) reactions were set up using total or poly(A)+ cDNA, as follows:

DNA	1µl
SYBR green mastermix (Applied Biosystems)	12.5µl
Forward primer (10µM)	0.75µl
Reverse primer (10µM)	0.75µl
dH_2O	10µl

Reaction were carried out on a Applied Biosystems GeneAmp® 5700 Sequence Detection System, using the following program:

50°C	2 minutes	
95°C	10 minutes	
95°C	15 seconds	40 cycles
60°C	1 minutes	

Each sample was compared to an arbitrary standard curve, calculated from reactions using dilutions of unknown concentration of the CAT PCR product. qRT-PCR was carried out in the same way using GAPDH primers. Samples were normalised by division by the GAPDH value.

3 HPV-16 E2 Transactivates Expression of Splicing Associated SR Protein, SF2/ASF

3.1 Introduction

W12E cells are a subcloned cell line from W12 cells, which were initiated from a low-grade cervical lesion, diagnosed as CIN I (Stanley et al. 1989). They contain ~100 episomal copies of the HPV-16 genome, which can be maintained through several passages and exhibit typical epithelial cell morphology consistent with cells from CIN I lesions. These cells can be induced to differentiate using a number of methods, perhaps the simplest being in monolayer culture depending on their density and calcium concentration in the growth medium. Cells are plated at low density in culture medium supplemented with 1.2mM calcium, with regular media changes to limit the onset of differentiation. McPhillips et al. showed that differentiation begins to occur at day 6 after plating, when cells start to form colonies of more than a few cells (McPhillips et al. 2004). Growth from this point results in increasing differentiation and markers for differentiation, such as involucrin, are found in abundance by day 7. Using this method of differentiation ~10-20% of the cells are positive for involucrin by immunofluorescence at day 5, whereas this increases to ~75-85% at day 10 (McPhillips et al. 2004). Therefore, samples are taken at day 5 and day 10 to compare expression between undifferentiated and differentiated cells. In western blotting experiments comparing undifferentiated and differentiated W12E protein lysates, abundance of splicing associated SR protein, SF2/ASF increased ~4-8 fold in differentiated samples (McPhillips et al. 2004). In contrast, SF2/ASF upregulation was not observed in uninfected differentiated epithelial cells, such as HaCaT cells: a spontaneously immortalised epithelial cell line, which can also be induced to differentiate in monolayer culture depending on cell density, in a similar manner to W12E cells (Boukamp et al. 1988). During HaCaT cell differentiation SF2/ASF levels diminish (McPhillips et al. 2004). This suggests that SF2/ASF is specifically upregulated in response to HPV-16 infection in infected differentiated epithelial cells. Therefore, this chapter was undertaken to determine what viral protein(s) were involved in SF2/ASF upregulation.

To look at which viral proteins may be involved in SF2/ASF regulation, a further cell line analysed for its expression during differentiation was W12G. W12G are

also subcloned from W12 cells, however the genome is integrated in these cells (Jeon et al. 1995). As such, expression of viral proteins other than E6 and E7 is lost and cells have a growth advantage over W12E cells, containing episomal HPV-16. However, they are non-tumourigenenic and can be induced to differentiated, albeit to a lesser extent, using the same procedures as described for W12E cells (Jeon et al. 1995; McPhillips et al. 2004). Therefore, using these cells, involvement of HPV-16 proteins other than E6 and E7, could be ruled out, should SF2/ASF expression still be shown to be elevated upon differentiation. However, comparing W12G undifferentiated and differentiated protein lysates, McPhillips et al. showed that integration of the HPV-16 genome abrogated SF2/ASF upregulation (McPhillips et al. 2004). Therefore, these data suggest a role for episomal HPV-16 in the regulation of SF2/ASF. The virus may achieve this by two possible routes. Firstly, one or more viral protein may act to stabilise SF2/ASF in differentiated cells. Candidates for this would include E6 and E7. As E6 and E7 are expressed in W12G cells where SF2/ASF is not regulated during differentiation, it is unlikely that they act to stabilise the protein. However, they may be involved in targeting SF2/ASF for degradation. E6 and E7 are known to target several cellular proteins for ubiquitin mediated proteasomal degradation, and may act in a similar fashion to regulate SF2/ASF abundance. However, E6 and E7 are expressed during W12E differentiation, where SF2/ASF is elevated, indicating they are unlikely to be involved in targeting it for degradation. Furthermore, SF2/ASF is downregulated during differentiation of uninfected keratinocytes, suggesting that HPV-16 specifically upregulates protein levels and does not simply lose repressive activity in differentiated cells. A further potential candidate for regulation of SF2/ASF stability was E1^E4. E1^E4 is not known to have roles during regulation of protein stability, however, it has many functions late in infection and expression is shown to increase in differentiated cells (Doorbar et al. 1997). In these cells it could have somehow been involved in stabilisation of SF2/ASF, resulting in the elevated levels observed during HPV-16 infection.

A second possible mechanism by which HPV-16 may regulate SF2/ASF abundance is via regulation of gene expression. SF2/ASF is a constitutively active gene in undifferentiated epithelial cells, where it is an essential protein. However, during differentiation of uninfected epithelium cells become less metabolically active and cellular functions begin to shut down. Expression of SF2/ASF is lost during differentiation of uninfected epithelial cells, as the protein is no longer required (McPhillips et al. 2004). Therefore, even if SF2/ASF stability was

increased in HPV-16 infected differentiated cells, it is possible that levels would still decrease, due to the lack of transcriptional activation. With this hypothesis in mind, it is likely that SF2/ASF is upregulated via transactivation in differentiated cells. E6 and E7 have roles during regulation of gene expression. HPV-16 E6 inhibits p53 expression via interactions with cellular co-activator, CBP/p300 (Zimmermann et al. 1999). In addition, HPV-16 E6 and E7 repress IL-8 transcription through associations with CBP/p300 and related co-factor, p/CAF, respectively (Huang and McCance 2002). E6 and E7 also target p53 and pRb for degradation. p53 is itself a transcription factor (TF), whilst pRb interacts with the TF, E2F. Therefore, these proteins have the potential to transcriptionally repress SF2/ASF expression in undifferentiated W12E cells. However, as discussed, both E6 and E7 are expressed during differentiation of W12E cells, and upregulation of SF2/ASF is unlikely to be caused by loss of repression of expression. Therefore, the most likely candidate for SF2/ASF regulation is the viral transcription factor, E2. HPV-16 E2 is known to control viral gene expression and can interact with cellular proteins, which is thought to be involved in transcriptional regulation. Furthermore, SF2/ASF is found to be upregulated in cells which stably express HPV-16 E2, suggesting a role for this viral protein in transcriptional regulation of SF2/ASF (McPhillips et al. 2004).

HPV-16 E2 is known to both transcriptionally activate and repress the viral Long Control Region (LCR) via four binding sites ($ACCN_6GGT$), which differ in E2 affinity (Steger and Corbach 1997; Morgan et al. 1998). Level of expression of E2 is at least partly responsible for activation/repression activity. Differential occupation of these sites, as well as interaction with various cellular proteins, also plays a role (Bouvard et al. 1994; Morgan et al. 1998). The LCR contains an enhancer and TF binding sites, and can be controlled by cellular factors such as Sp1, AP-1 and Oct-1 (Cripe et al. 1987; Sibbet and Campo 1990; Chong et al. 1991). Interaction of E2 with various cellular partners throughout epithelial differentiation is thought to regulate transactivation/repression activity of the viral LCR, at the appropriate stage of differentiation. Furthermore, E2 is known to regulate cellular genes, for which cellular factors are again necessary. For example, HPV-6, -8, -11, -16, -18 and BPV-1 E2 interact with Sp1 (Li et al. 1991; Lee et al. 2002). For HPV-18 E2 this interaction is involved in transcriptional downregulation of hTERT (Lee et al. 2002), whereas HPV-8 and –18 E2 transactivate p21 expression via Sp1 interactions (Steger et al. 2002). In addition,

HPV-8, -16, -18 and BPV-1 E2 interact with C/EBP α and β, which is shown to result in transactivation of involucrin (Hadaschik et al. 2003). Some PV E2 proteins also interact with components of the basal transcription machinery. For example, BPV-1 E2 interacts with TBP and TFIIB, as well as TBP-associated factors, TAFII80 and TAFII250 (Rank and Lambert 1995; Carrillo et al. 2004). In addition, HPV-11 E2 interacts with TBP (Hou et al. 2000), HPV-8 binds to TBP and TAFII55 (Enzenauer et al. 1998), and HPV-18 associates with TAFII80 and TAFII250 (Carrillo et al. 2004). These interactions are thought to be involved in regulation of pre-initiation complex formation. HPV-8 E2 has also been reported to downregulate β4-integrin expression by causing displacement of one or more important cellular factors from its promoter (Oldak et al. 2004). Furthermore, co-activators, which regulate gene expression via indirect DNA association, CBP/p300 and p/CAF, are also known to interact with HPV-6b, -8, -11, -16 and –18 E2 and, in the case of HPV-18 and -8, synergise transcription (Lee et al. 2000; Lee et al. 2002; Muller et al. 2002). The interaction with CBP/p300 and p/CAF is thought to be essential to E2-mediated transcription. In addition, HPV-16 E2 interacts with TopBP1, which also results in enhanced transcription (Boner et al. 2002). This suggests E2 is a multifunctional transcription factor that regulates transcription via complex associations with a number of proteins.

E2 from HPV-8 and –18 are known to affect not only viral transcriptional regulation, but are also involved in regulation of cellular genes, such as hTERT, p21 and β4-integrin (Lee et al. 2002; Steger et al. 2002; Oldak et al. 2004). In contrast, HPV-16 E2 has not previously been reported to transactivate expression of any cellular genes. To demonstrate a function for E2 in HPV-16 mediated SF2/ASF transcriptional regulation, a number of techniques were employed. Firstly, reporter assays were performed using SF2/ASF promoter region containing constructs. Association of E2 with the promoter was also determined both *in vitro* and *in vivo*, using electrophoretic mobility shift assays (EMSAs) and chromatin immunoprecipitation (ChIP). Furthermore, binding of cellular binding partners for E2, Sp1 and C/EBP, with the SF2/ASF promoter was analysed *in vitro*, using supershift EMSAs. Lastly, RT-PCR was used to establish steady-state SF2/ASF mRNA levels in undifferentiated and differentiated W12E cells.

3.2 HPV-16 E2 transactivates the SF2/ASF promoter region

To further investigate the effect of E2 upon SF2/ASF regulation, it was first necessary to confirm the transactivation function of E2. To achieve this, pGCAT3-B-SF2pr was produced (Figure 3.1). This plasmid contains a region spanning ~1kb upstream of and including the putative transcription start site of SF2/ASF, fused to the chloramphenicol acetyl transferase (CAT) gene. Due to the uncharacterised nature of the SF2/ASF promoter, this region was chosen as we expected it would likely contain most *cis*-acting sequences involved in E2 regulation. Furthermore, the placement of the putative transcription start site was chosen as this was where the longest sequenced cDNA clone began. Therefore, there is no direct evidence that this is the correct transcription start site and was used as the best guess when choosing the promoter region. Following PCR amplification from human genomic DNA using primers SF2pr -1024 F and +5 R, this region was cloned into pGCAT3-Basic, a CAT construct derived from pGL3-Basic (Promega), which contains the CAT gene and an SV40 polyadenylation signal, with no promoter. To ensure that results presented here are on a linear scale, assays using serial dilutions of CAT enzyme were performed and quantified by the same method as for subsequent experiments. Figure 3.2 shows a linear calibration curve of percentage chloramphenicol (CAM) convertion, as measured by optical density, with increasing CAT activity. pGCAT3-B and pGCAT3-B-SF2pr were then co-transfected into HeLa cells along with either an empty expression vector (pCI) or an HPV-16 E2 expression vector (pCI-E2) (Figure 3.3). As E2 causes cell growth arrest and apoptosis of HeLa cells, titration experiments were performed to ensure the level of E2 expression was optimal to observe transactivation at the SF2/ASF promoter (Figure 3.3A). No CAT activity was observed in cells transfected with pGCAT3-B either in the presence or absence of E2 (data not shown). However, a two-fold increase in CAT activity was observed from pGCAT3-B-SF2pr when co-transfected with 50ng pCI-E2, as compared to empty vector (Figure 3.3A). Transactivation decreased with increasing pCI-E2 concentration, suggesting E2 may not activate the SF2/ASF promoter at higher concentrations; however it does not trans-repress either. It is possible that higher E2 expression may be having an effect on cell proliferation, making cells less healthy, and in turn causing inhibition of normal cellular functions, such as SF2/ASF expression. The effects of E2 expression on cell viability were not

assayed; however cells counts, comparing live versus dead cells may have given an indication of whether E2 expression was causing apoptosis. In contrast, transcription of CAT constructs driven by the herpes simplex virus immediate early 5 promoter (HSV-IE5) was unaffected by E2 expression (Figure 3.3A). This suggests that low concentrations of E2 specifically transactivate the SF2/ASF promoter region as described above.

To verify this result, CAT activity was determined from pGCAT3-B-SF2pr transfected U2OS cell clones, A4 and B1, which stably express E2 to differing levels and have previously been shown to have upregulated SF2/ASF when compared to non-transfected U2OS cells (McPhillips et al. 2004). Therefore, non-transfected U2OS cells were used as a negative control. Similar to results obtained with HeLa cells, CAT activity from pGCAT3-B-SF2pr increased in the presence of E2 (Figure 3.4A), but no activity was observed in cells transfected with pGCAT3-B, either with or without E2 (data not shown). However the extent to which E2 transactivates the SF2/ASF promoter in this assay differs between cell clones, which is possibly due to the level of E2 expression (Figure 3.4B). Clone A4 expresses lower levels of E2 than clone B1. In agreement with the data obtained using HeLa cells, it is possible that higher E2 expression does not transactivate the promoter due to its affects on cell growth. However, as the effect of E2 expression on cell viability was not assayed, no firm conclusion can be drawn to account for the differences in SF2/ASF promoter transactivation between A4 and B1 clones. Furthermore, E2 failed to transactivate a β-galactosidase expression vector driven by the CMV promoter (Figure 3.4A). However, variation was observed in the level of reporter activity between cell clones, suggesting there are potentially differences between the cells clones with respect to general transcriptional activity, and/or transfection efficiency.

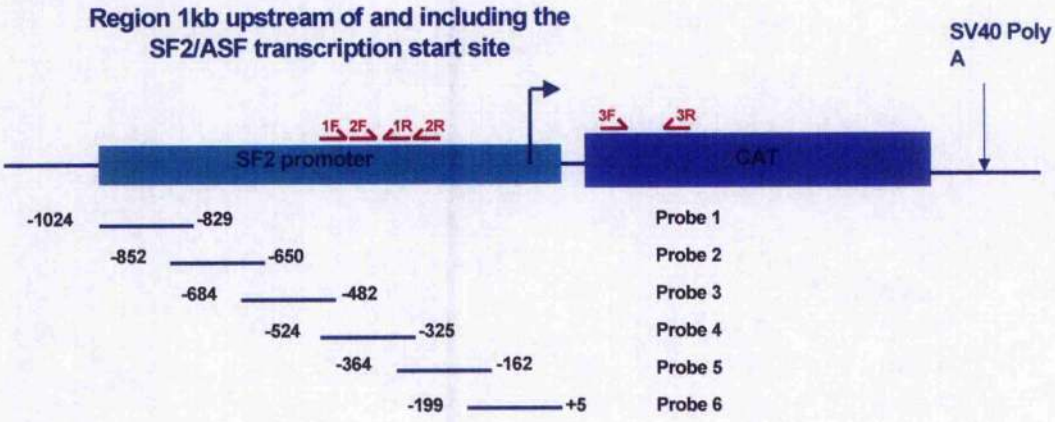


Figure 3.1: **pGCAT3-B-SF2pr**

Schematic diagram of the SF2/ASF promoter construct used in CAT assays. The SF2/ASF promoter (turquoise rectangle) was PCR amplified from nt –1024 to +5 with respect to the SF2/ASF transcription start site (arrow) and cloned into pGCAT3-B, a CAT vector with no promoter, to produce pGCAT3-B-SF2pr, and the insert was sequenced using vector specific primers. Probes used in EMSA experiments with positions within the promoter are depicted below. Horizontal lines above the SF2/ASF promoter region and CAT gene represent primers used in ChIP assay. 1F & R are SF2pr –524F & -325R; 2F & R are SF2pr –402F & -222R; 3F & R are CAT5'F & R.

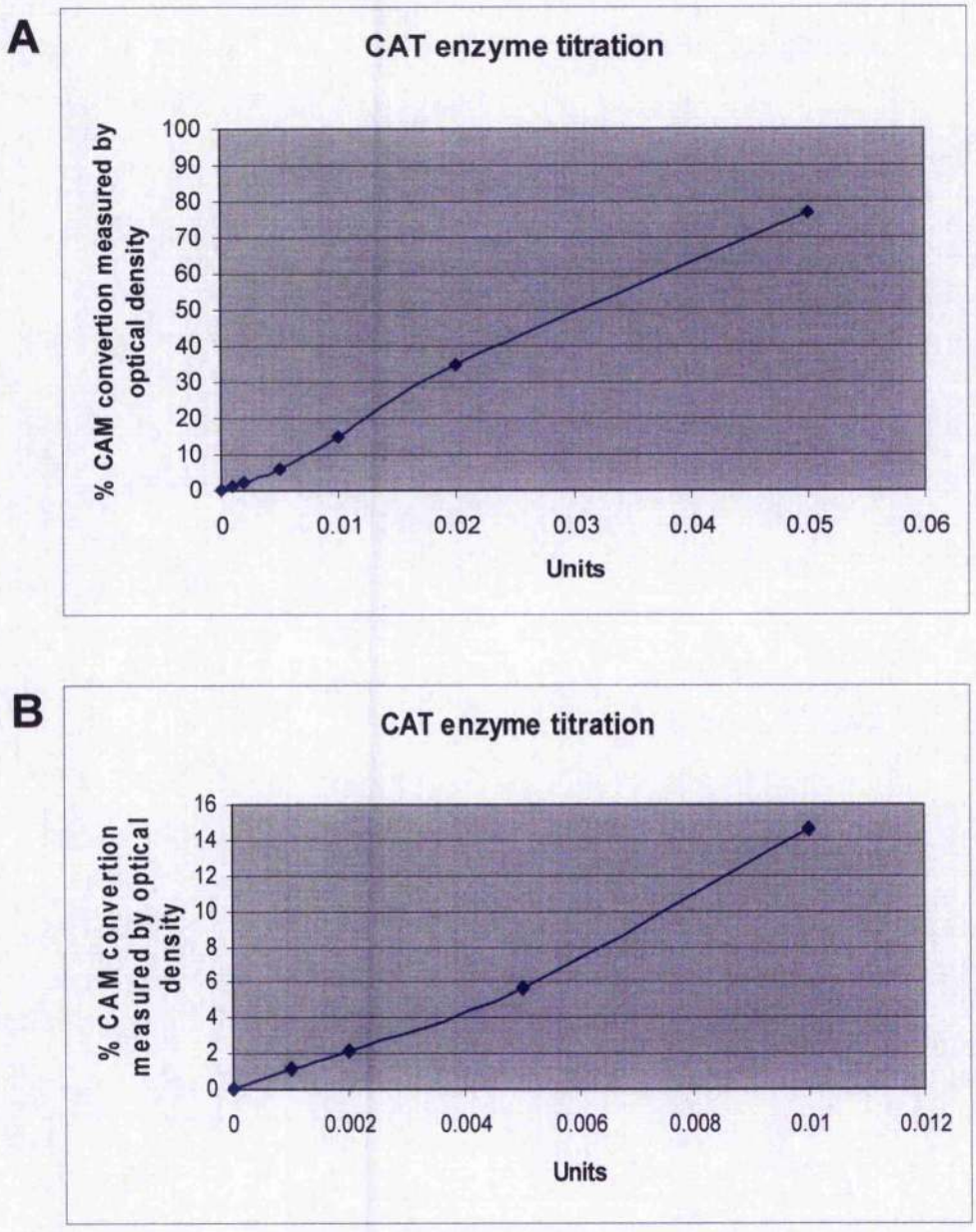


Figure 3.2: **CAT activity is linear in CAT assays using the TLC method**

Increasing units of CAT enzyme were added to CAT assays and percentage chloramphenicol (CAM) convertion was determined by comparing total and converted CAM. Phosphoimaging of TLC run samples, followed by measurement by optical density were used to measure total and converted CAM. (A) shows almost total convertion and (B) shows convertion of a lower range as this is where experimental values fall.

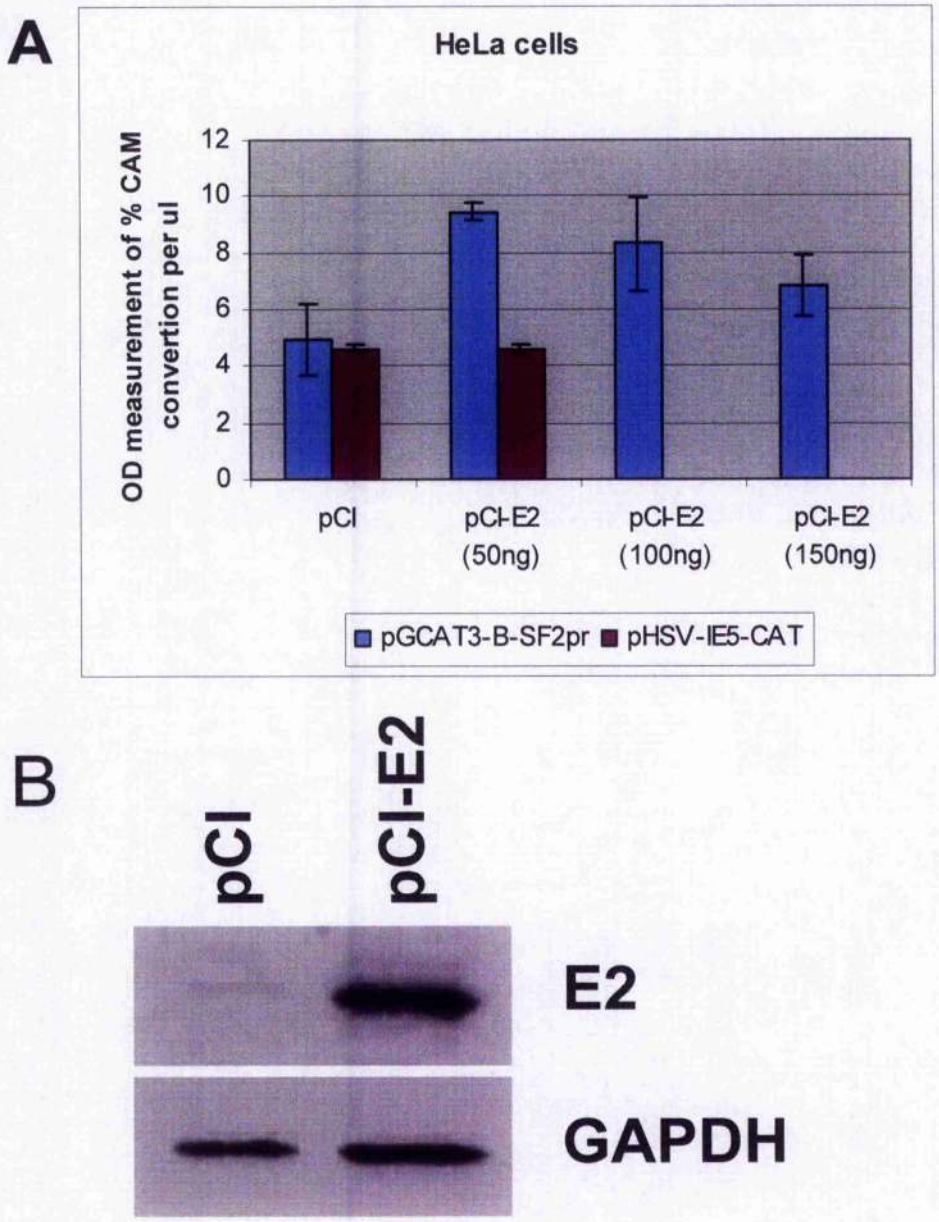


Figure 3.3: **Increased CAT activity from pGCAT3-B-SF2pr in the presence of E2**

A) Protein lysates were produced from HeLa cells co-transfected with pGCAT3-B-SF2pr and pCI or 50ng, 100ng or 150ng pCI-E2 and CAT assays were performed. Transfections were performed in triplicate and the graph represents mean and standard deviation about the mean from three separate experiments. B) Western blots using equal quantities of protein lysates from HeLa cells transfected with pCI or pCI-E2 probed using anti-E2 and anti-GAPDH antibodies. GAPDH acts as a loading control.

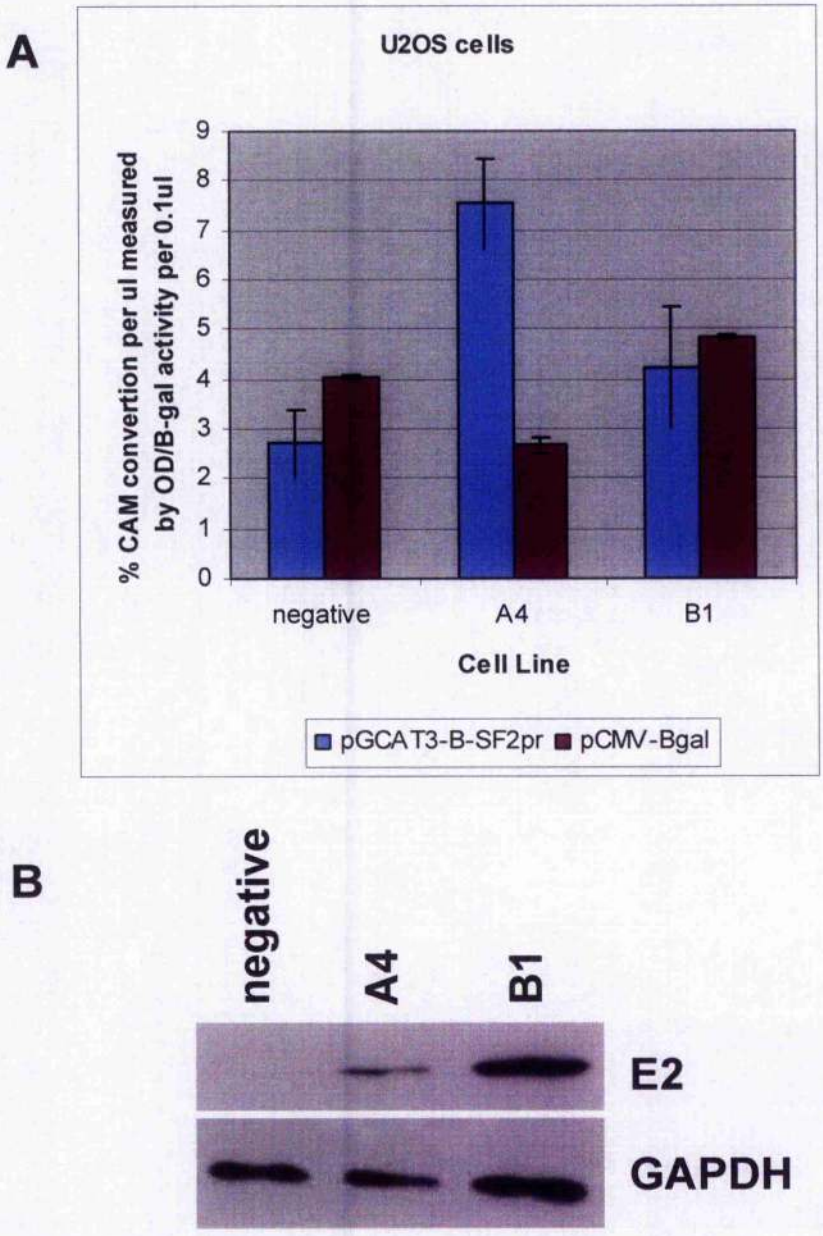


Figure 3.4: **Increased CAT activity from pGCAT3-B-SF2pr in cells stably expressing E2**

A) Protein lysates were produced from U2OS cells, A4 and B1, which stably express E2, as well as non-transfected U2OS cells (negative) transfected with pGCAT3-B-SF2pr and CAT assays were performed. Transfections were performed in triplicate and the graph represents mean and standard deviation about the mean from three separate experiments. B) Western blots using equal quantities of protein lysates from U2OS negative, A4 and B1 cells probed using anti-E2 and anti-GAPDH antibodies. GAPDH acts as a loading control.

3.3 E2 interacts with the SF2/ASF promoter in HeLa cells

As the SF2/ASF promoter is transactivated in cells expressing E2, it was necessary to confirm that this is achieved by E2 directly and not via regulation of other cellular proteins. To determine this, presence of E2 at the SF2/ASF promoter was assayed. E2 interacts with many cellular proteins, making it possible that it may not interact directly with the promoter, but may be tethered to it via involvement in a transcription complex. Therefore, chromatin immunoprecipitation (ChIP) experiments, which can detect indirect interaction of a protein with DNA, were performed. In these assays protein-DNA complexes are immunoprecipitated from cross-linked cellular extracts, purified, the protein is degraded and DNA is subjected to analysis by PCR. For this study, HeLa cells were co-transfected with pGCAT3-B-SF2pr and either empty vector (pCI) or an E2 expressing vector (pCI-E2). As both the anti-E2 antibodies used in these experiments were polyclonal and had the potential to recognise epitopes from other cellular proteins, it was important to make sure that they were not immunoprecipitating any other component involved in SF2/ASF transcriptional regulation. Two anti-E2 antibodies were used, both of which were polyclonal, one detecting only C-terminal epitopes (E2 CTD), and one raised against the entire protein (E2 poly). Not only would this ensure that it was E2 being detected, but it was also thought that it might give further information regarding the E2 domain involved in SF2/ASF promoter association. Should E2 poly but not E2 CTD detect E2 in promoter complexes, it may suggest that E2 associates with the promoter via its C-terminal region. If this were the case, it is possible that the C-terminal domain of E2 would not be accessible due to its association with a transcription complex on the SF2/ASF promoter. Furthermore, as positive controls, 1/10 volume of cell extract was removed prior to immunoprecipitation and an anti-TBP antibody was used during immunoprecipitation, as this is known to be necessary for transcription at all promoters. Antibodies against involucrin, an epithelial cell differentiation-specific protein with no reported functions during transcription, and beads alone with no antibody were used as negative controls. Following purification, immunoprecipitated DNA was assayed using CAT5' F & R primers, which amplify a ~200bp region within the 5' region of CAT gene, ~200bp downstream of the initiation site, SF2pr -524 F & -325 R, amplify a ~200bp region within the middle of the promoter, and 5S RNA F & R, which amplify a region

~200bp of the 5S RNA gene. The 5S RNA promoter is controlled by RNA polymerase III, which also requires TBP for its action. However, E2 is not reported to regulate RNA polymerase III transcription and should therefore not be detected at this promoter.

Figure 3.5 shows pGCAT3-B-SF2pr can be detected in the input control, using the primers within CAT5' and SF2 promoter regions, indicating successful transfection. Furthermore, TBP can be immunoprecipitated with the SF2/ASF promoter in cells transfected with both pCI-E2 and pCI, as observed using CAT5' F & R and SF2pr -524 F & -325 R primers. E2 interaction is detected only in cells transfected with pCI-E2 but not pCI, using CAT5' and SF2pr primers. This indicates that E2 is interacting, either directly or indirectly, with the SF2/ASF promoter in HeLa cells and that the E2 antibodies used are not cross reacting with any other proteins involved in SF2/ASF promoter complexes. In addition, whilst TBP is interacting with the 5S promoter region in cells transfected with pCI-E2, E2 cannot be detected. This indicates that E2 is interacting specifically with the SF2/ASF promoter. Finally, ChIP experiments were performed using cells transfected with pCI-E2 and pGCAT3-B, to ensure E2 is not associated with the CAT construct at a region other than the SF2/ASF promoter. Whilst CAT DNA can be detected from input samples, TBP and E2 cannot, suggesting E2, like TBP, associates with pGCAT3-B-SF2pr via the SF2/ASF promoter region.

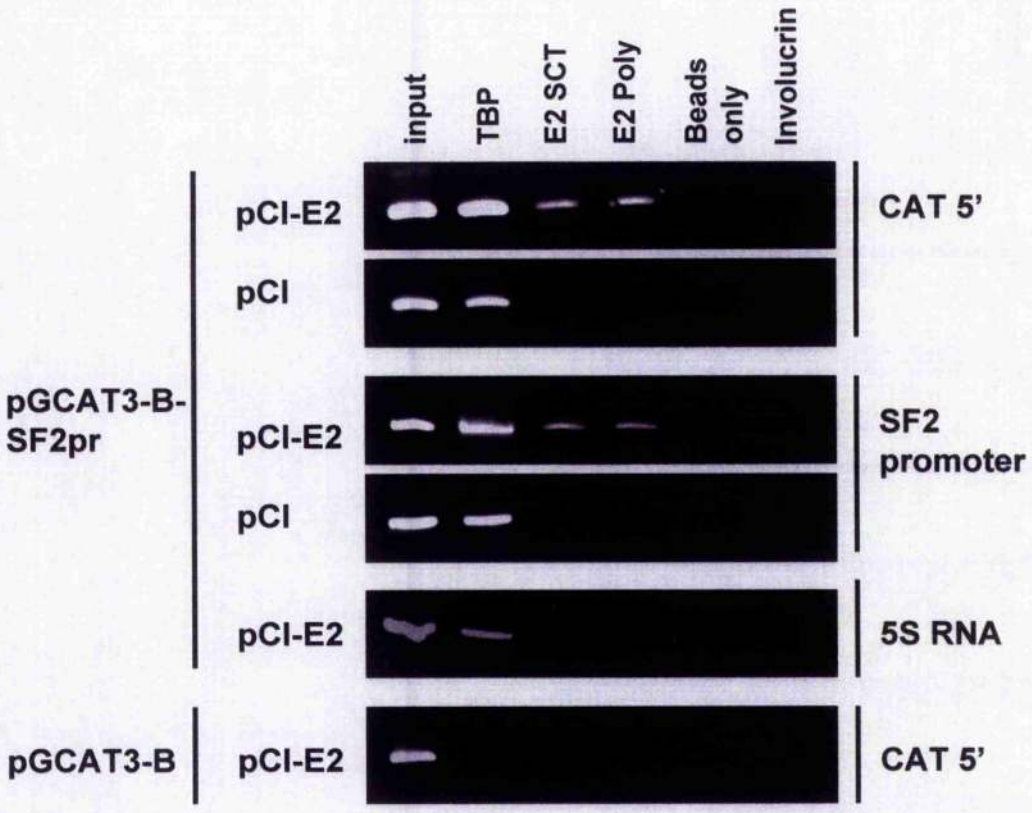


Figure 3.5: **E2 associates with the SF2/ASF promoter in HeLa cells**

HeLa cells were co-transfected with pCI or pCI-E2 and pGCAT3-B-SF2pr or pGCAT3-B (lacking the promoter). DNA-protein complexes from cell extracts were sonicated into fragments of 200bp-1kb and subjected to ChIP assays using anti-TBP, two polyclonal E2 antibodies (E2-SCT and E2 Poly), anti-involucrin and beads only. Primers were used to amplify a region of the CAT gene (CAT5' F & R) and SF2/ASF promoter (SF2pr –524F & -325R) to determine association with the SF2/ASF promoter (Figure 3.1), and 5S RNA (5S RNA F & R) as a negative control.

To build on this, association of E2 with the endogenous SF2/ASF promoter was also assayed. This time HeLa cells transfected with either pCI-E2 or pCI, but not pGCAT3-B-SF2pr, were subjected to ChIP experiments and primers, SF2pr -402 F & -222 R, amplifying a ~200bp region, within the SF2/ASF promoter were used to determine association. Furthermore, primers amplifying a ~200bp fragment within the 3' region of the SF2/ASF coding sequence ~2.5kb downstream from the promoter, SF2 F & R, were used as negative controls. Figure 3.6 indicates that E2 associates with the endogenous SF2/ASF promoter in the presence but not absence of E2. However, this is determined using only the E2 polyclonal antibody, as E2 CTD, does not immunoprecipitate with the endogenous promoter. As suggested, this may indicate differences in E2 binding to the endogenous and plasmid promoters. It is possible that E2 associates with the SF2/ASF promoter via its C-terminal region and due to the chromatin conformation of the endogenous promoter, E2 CTD is not able to access the complex. In contrast, SF2 F & R primers which amplify within the 3' region of SF2/ASF coding sequence, failed to detect DNA using anti-TBP or anti-E2 antibodies. This suggests E2 interacts specifically with the endogenous SF2/ASF promoter region in HeLa cells.

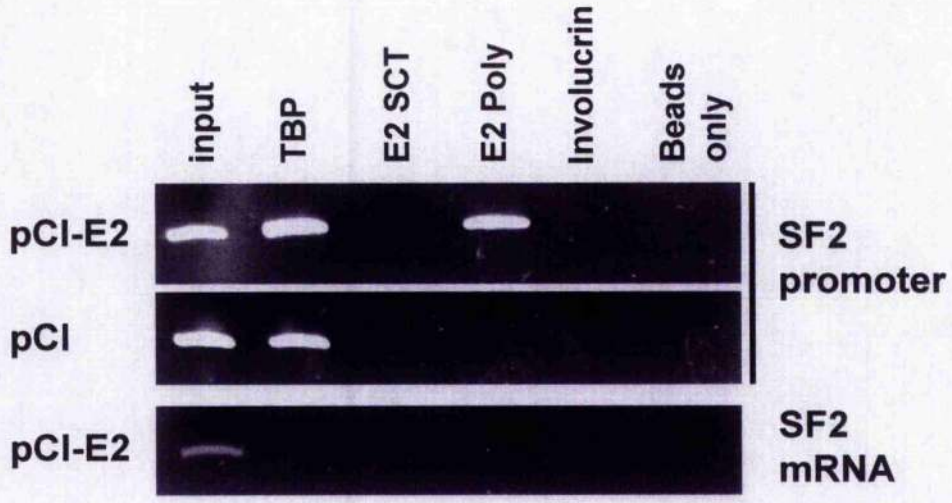


Figure 3.6: **E2 associates with the endogenous SF2/ASF promoter**

HeLa cells were transfected with pCI or pCI-E2. DNA-protein complexes from cell extracts were sonicated into fragments of 200bp-1kb and subjected to ChIP using anti-TBP, two polyclonal E2 antibodies (E2-SCT and E2 Poly), anti-involucrin and beads only. Primers were used to amplify a region of the SF2/ASF promoter (SF2pr –402F & -222R) to determine association with the endogenous SF2/ASF promoter (Figure 3.1), and a 3' region of the SF2/ASF mRNA (SF2 F & R) as a negative control.

3.4 E2 does not bind to a potential binding site within the distal region of the SF2/ASF promoter

As E2 is a DNA binding protein, analysis of the SF2/ASF promoter was performed to determine the presence of any potential E2 binding sites (E2BS). At nt -760 from the transcription start site, a near consensus E2BS was found with one substitution from C to T at nt 9, which may have the potential to bind E2 (Figure 3.7A). To investigate this possibility, electrophoretic mobility shift assays (EMSAs) were performed, in which protein-DNA complexes can be revealed by retardation of a DNA probe during electrophoresis. In this case an ~200bp probe spanning ~100nt either side of the potential E2BS was produced by Klenow end labelling of SpeI cut DNA. Furthermore, ~100bp probes were constructed in the same way using a region of the HPV-18 LCR which contains 2 E2BSs and the same region with mutated E2BSs to use as positive and negative controls, respectively. E2BSs were mutated by C to T substitutions at position 10 in BS1 and positions 2 & 3 in BS2, which has previously been shown to abolish E2 binding (Thierry and Howley 1991). His-tagged HPV-16 E2 was purified from BL21 bacterial cells, however a number of contaminates were observed (Figure 3.7B). To counteract this, wash 6 (lane 3) was used as a negative non-E2 containing lysate, for any non-specific binding, whilst elution 1 (lane 4) was used as the E2 containing fraction. EMSAs were then performed as described. Figure 3.7C shows that whilst E2 binds the positive control probe and occupation of one and two binding sites can be observed, E2 fails to interact with either the negative control probe or the SF2/ASF promoter region containing the potential E2BS. This indicates that E2 does not interact with the potential E2BS within the SF2/ASF promoter in this assay.

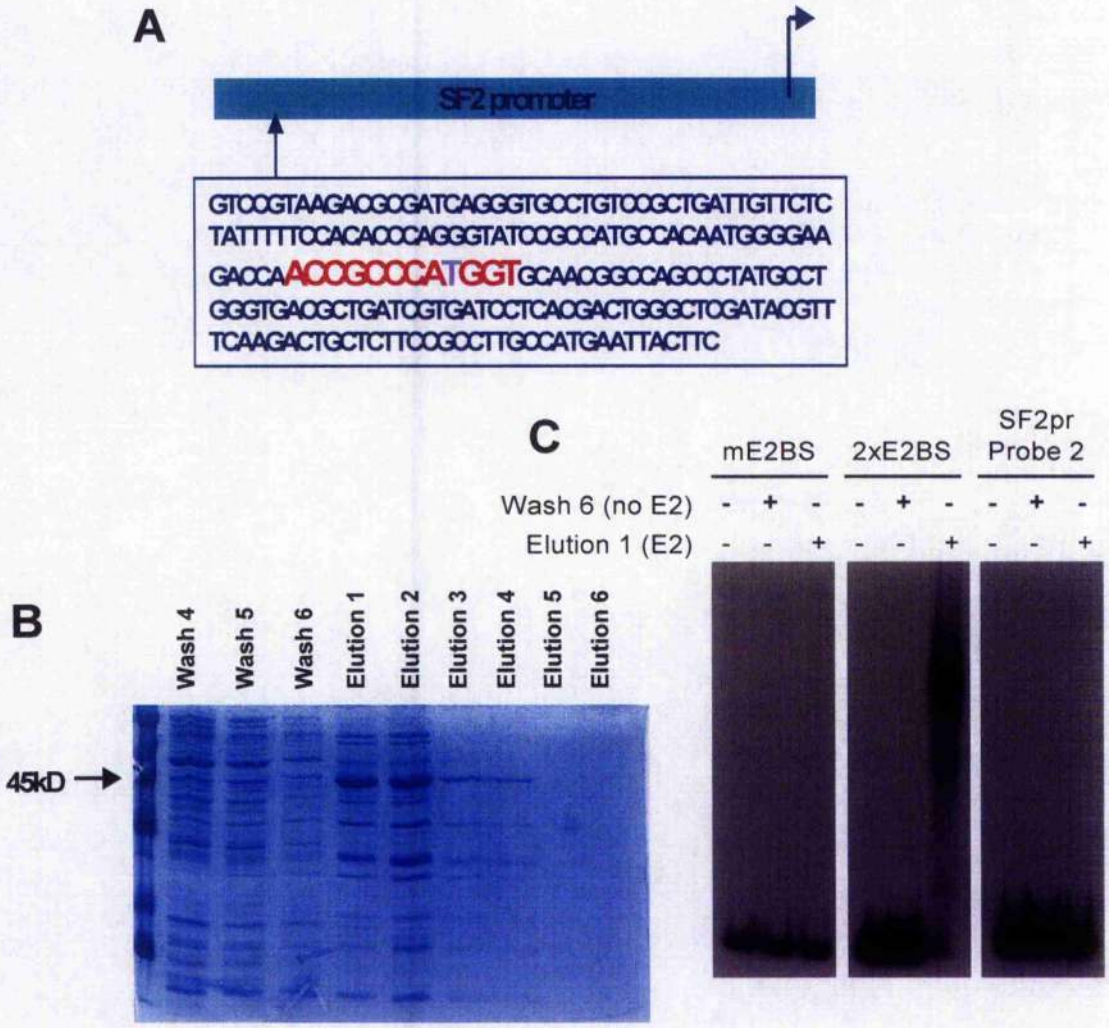


Figure 3.7: **E2 does not bind a near consensus-binding site within the distal SF2/ASF promoter**

A) Sequence of Probe 2 (Figure 3.1) within the distal SF2/ASF promoter. The potential E2BS is shown in red with the wobble position highlighted in pink. B) Coomassie stained SDS-PAGE gel of wash and elution samples from column purification of bacterial expressed His-tagged E2. Washes 4-6 (lanes 2-4) and elution's 1-6 (lanes 5-10) are shown, along with marker (lane 1). C) EMSAs using a region of the HPV-18 LCR containing two binding sites either mutated (mE2BS), or intact (2xE2BS), and the SF2/ASF promoter probe (Probe 2). EMSAs were performed using probe only, wash 6 (lacking E2) and elution 1 (containing E2).

3.5 E2 does not interact with the SF2/ASF promoter *in vitro*

Following results that E2 does not bind at a potential E2BS within the SF2/ASF promoter in EMSA experiments, it was necessary to determine the possibility of E2 binding to a non-consensus sequence. Therefore, six overlapping probes of ~200bp each, covering the entire promoter were synthesised by Klenow end labelling of SpeI cut DNA (Figure 3.1). To detect whether E2 bound DNA in complex with any cellular proteins, assays were also performed in the presence of HeLa nuclear extract, with and without E2. This would firstly show any complexes which form on the promoter in the absence of E2 and may also elucidate any changes brought about by E2. Figure 3.8, panels 1-2 show EMSAs using E2 and HeLa nuclear extract with probes 1 and 2 spanning the region nt -1024 to -650 from the SF2/ASF transcription start site. Probe 2 contains the potential E2BS described above. For both regions no complexes are formed either in the presence of E2 or nuclear extract. EMSAs using the three most proximal probes spanning nt -524 to +5 from the transcription start site are shown in Figure 4.8, panels 4-6. Whilst E2 does not interact with these probes, a number of complexes are observed upon addition of nuclear extract. This is not surprising as transcription is likely to be controlled from this region. However, addition of E2 has no effect on complex formation. In contrast, EMSAs using probe 3, nt -684 to -482 from the start site, a shift emerged upon addition of E2 and a comparable complex was not observed with wash 6 (Figure 3.8, panel 3). A shift was observed using wash 6 but it does not correspond to the complex observed using E2. Furthermore, addition of nuclear extract strengthens potential E2 binding (lane 6). Therefore, E2 may interact with the SF2/ASF promoter via a region nt -684 to -482 from the transcription start site.

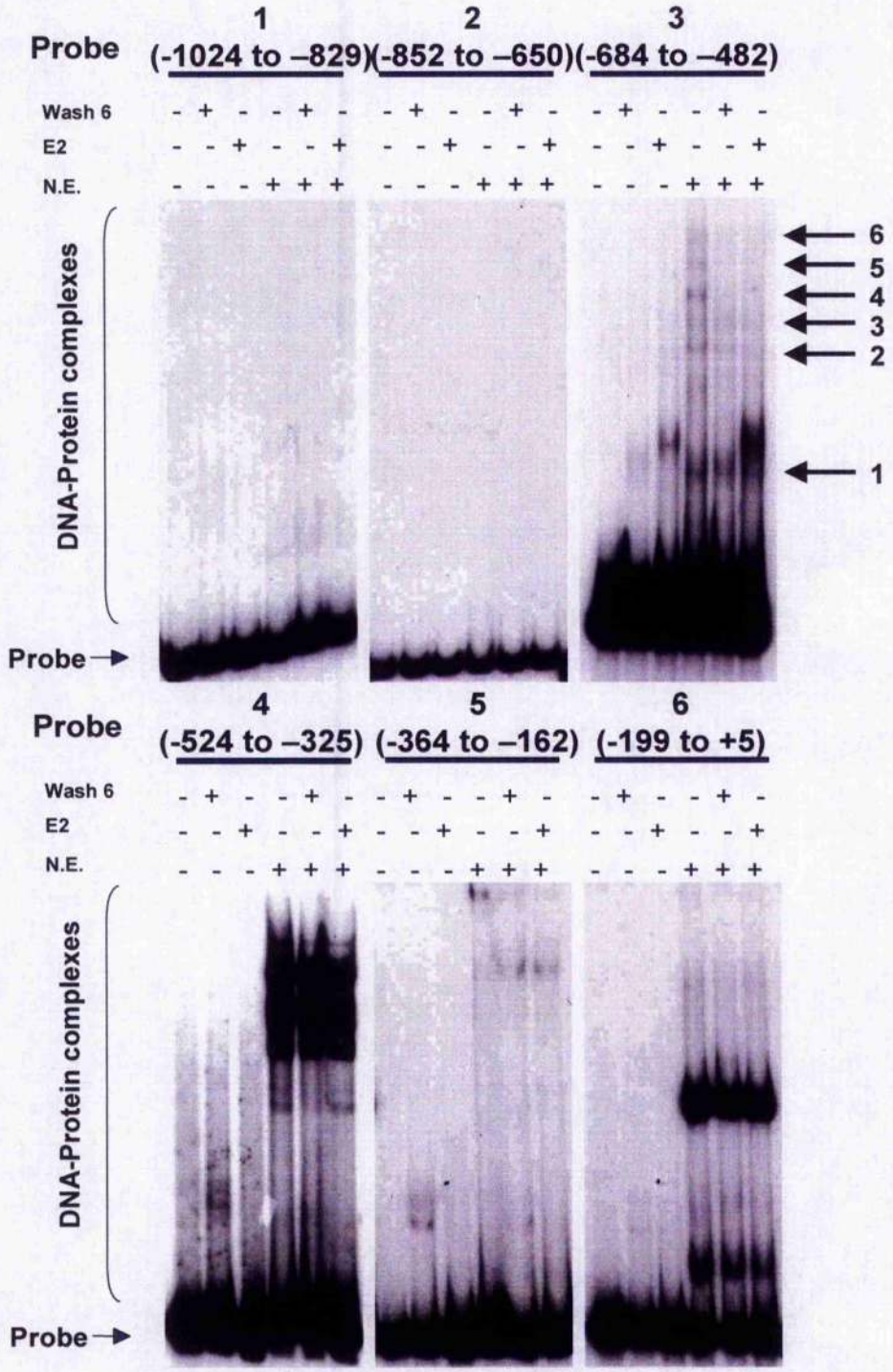


Figure 3.8: **E2 may bind a non-consensus site within the SF2/ASF promoter *in vitro***

EMSAs using probes 1-6 from the SF2/ASF promoter (Figure 3.1), with wash 6 (lacking E2), E2 (elution 1) (Figure 3.7B) and HeLa nuclear extract (N.E.). The most distal probes are shown in the top panel and the more proximal probes are shown in the bottom panel.

To investigate whether the protein causing a shift using probe 3 in the above experiments is indeed E2, this probe was used in further EMSAs in which non-radioactive labelled probe 3 was added to reactions in increasing concentrations to determine specificity of binding. Non-radiolabelled probe was added to reactions 15 minutes prior radiolabelled probe. Figure 3.9 shows a slight reduction in E2 binding upon addition of cold probe; however, binding does not diminish as greater concentrations of cold probe are added. This suggests that shifts obtained using both wash 6 and E2 may be non-specific, indicating that the shift observed using probe 3 might be either unspecific binding of E2 or one of the other proteins within the lysate. In contrast, it is possible that E2 associates with the probe in a dynamic fashion and that addition of increasing amounts of cold probe has little effect on this.

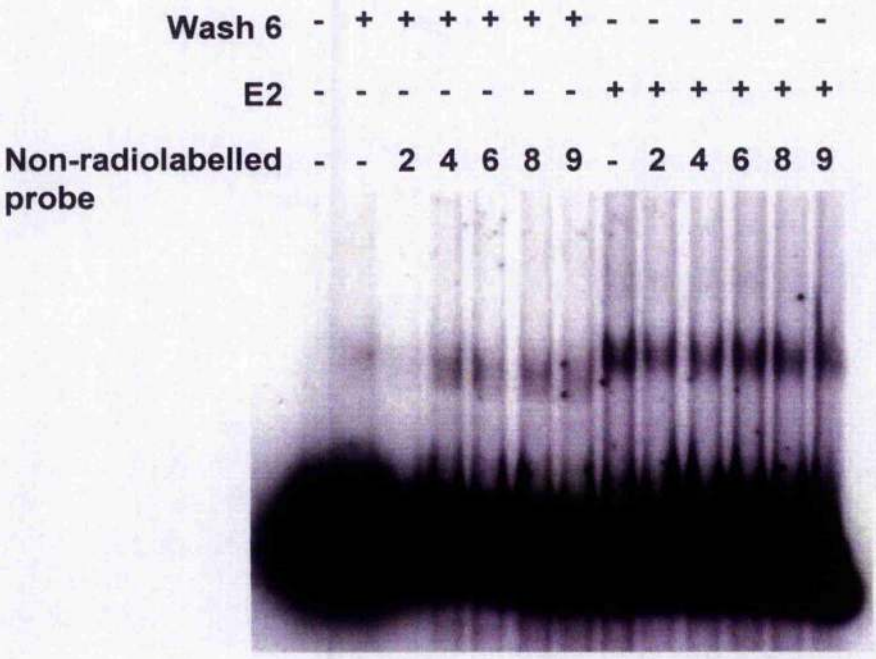


Figure 3.9: **Probe 3 binding by a component of the E2 lysate is not specific**

EMSA using probe 3 with wash 6 (lanes 2-7) and E2 (lanes 8-13), with increasing concentration of non-radiolabelled probe, at 2, 4, 6, 8 and 9-fold molar excess over radiolabelled probe. Non-radiolabelled probe was added prior to radiolabelled probe.

3.6 Sp1 may interact with the SF2/ASF promoter *in vitro*

Failure of E2 to interact directly with the SF2/ASF promoter suggests indirect association via a co-activator complex. As mentioned previously, E2 interacts and co-operates with a number of cellular partners, one or more of which may be involved in SF2/ASF transactivation. However, due to the uncharacterised nature of the SF2/ASF promoter, it is not known what cellular proteins are found to associate with it and regulate expression. Whilst direct E2 interaction with the SF2/ASF promoter could not be determined *in vitro*, there are a number of complexes forming in the presence of nuclear extract. Using TFSearch (http://www.cbrc.jp/research/db/TFSEARCH.html) a number of potential binding sites for cellular proteins were found within the sequence (Figure 3.10). Of particular interest, binding sites for Sp1 and C/EBP β, are found at –575 and –647, respectively, from the transcription start site. Both of these sites fall within probe 3, which produced 6 complexes when incubated with nuclear extract (Figure 3.11, lane 2). Therefore, to determine if any of the complexes formed contain Sp1 and/or C/EBP, supershift experiments were performed. In these assays, antibodies are added to the EMSA reaction, and changes in the complexes formed, due to antibody binding, are determined. Results indicate that whilst no change was observed in the presence of anti-C/EBP antibody, anti-Sp1 antibody was able to shift one of the complexes, when added either before (lane 3) or after nuclear extract (lane 4) (Figure 3.11). Shifting seems to result in lower levels of complex 4 suggesting this complex may contain Sp1. Therefore, this experiment indicates that Sp1 may interact with the SF2/ASF promoter *in vitro*.

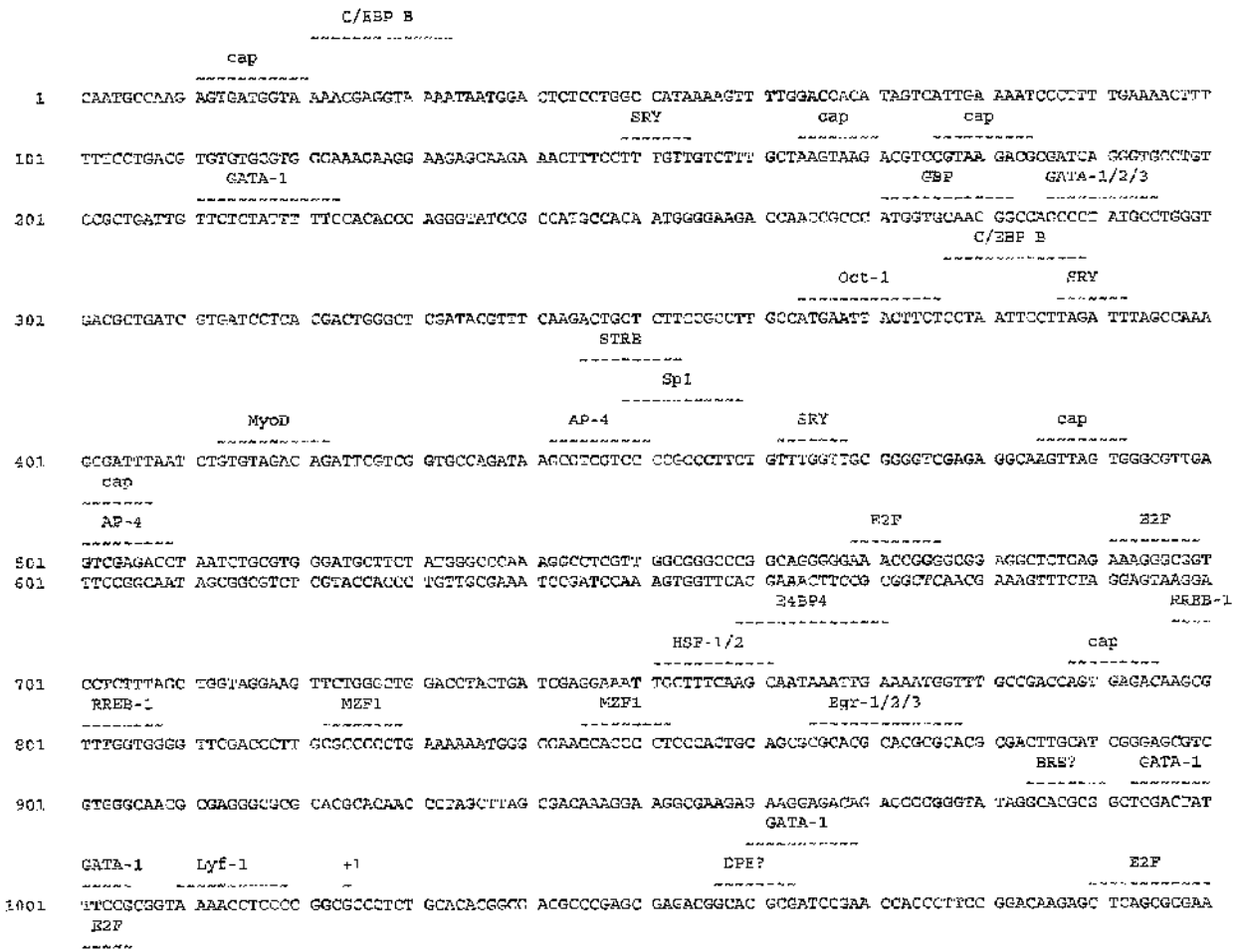


Figure 3.10: **Potential transcription factor binding sites within the SF2/ASF promoter**
The SF2/ASF promoter sequence, from –1024 to +89, is shown with potential cellular transcription factor binding sites indicated as lines above the sequence. The transcription start site is shown by at +1, and a potential BRE and DPE are shown with question marks.

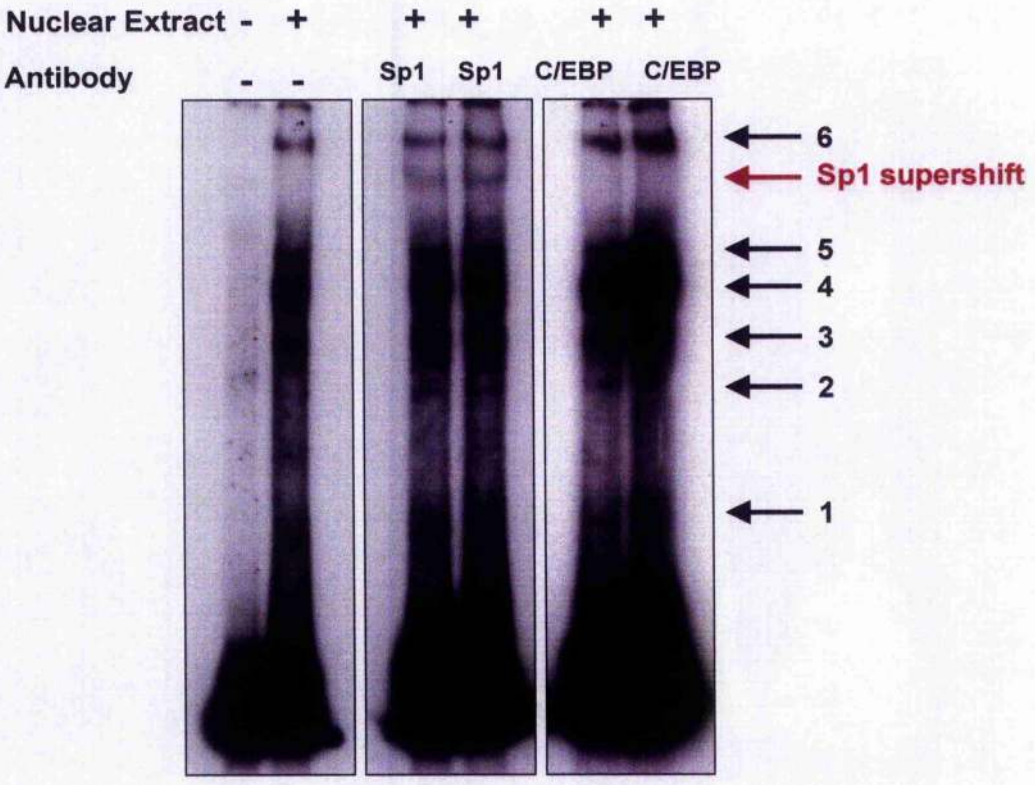


Figure 3.11: **Anti-Sp1 supershifts complexes form between HeLa nuclear extract and probe 3**

EMSAs using probe 3 (Figure 3.1) and HeLa nuclear extract (N.E). Anti-Sp1 and anti-C/EBP antibodies were added to binding reactions either 15 minutes prior to (lanes 3 and 5), or 15 minutes following (lanes 4 and 6) addition of nuclear extract. Horizontal arrows to the right indicate complexes formed.

3.7 SF2/ASF mRNA levels are elevated in HPV-16 infected differentiated cells

Whilst data presented here suggests a role for HPV-16 E2 in SF2/ASF transactivation, this role for the protein has not been demonstrated in the context of the HPV-16 life cycle. E2 function is known to be affected by other papillomavirus proteins, which may affect the transcription function described here. Furthermore, E2 expression levels as observed in cells stably or transiently transfected with E2 expression vectors, likely differ from those observed during the HPV-16 life cycle. To provide a link between the two, levels of SF2/ASF mRNA were determined within undifferentiated and differentiated W12E cells. An increase in SF2/ASF mRNA in differentiated cells would suggest that protein abundance might be regulated at the level of transcription and not via translation or protein stability, for example. Although this would not prove the transactivating role of E2 during the virus life cycle, it would indicate that SF2/ASF levels might be controlled transcriptionally, most likely by E2. To achieve this, total RNA was purified from both undifferentiated and differentiated W12E cells, cDNA was produced and RT-PCR was performed using primers within the SF2/ASF coding region (SF2 F & R), which produce an ~200bp fragment. As an internal control, primers amplifying an ~500bp region of the GAPDH (GAPDH F & R) gene were used in the same RT-PCR, as levels of this are shown to decrease during differentiation (Steele et al. 2002). Reverse transcription reactions were performed in the presence and absence of enzyme to control for possible effects of DNA contamination. Figure 3.12 shows upregulation of steady state SF2/ASF mRNA in differentiated W12E cells, when compared to undifferentiated cells, whereas GAPDH mRNA levels decrease. To control for the effects of differentiation, HaCaT cells were used, as they are an HPV-16 negative epithelial cell line, which can differentiate in culture. Results here again show no PCR products from reactions lacking RT. Although GAPDH mRNA does not decrease upon differentiation to the extent observed in W12E cells, SF2/ASF mRNA levels also decrease, in accordance with observations at the protein level. Therefore, steady state SF2/ASF mRNA levels are specifically upregulated during HPV-16 infection. This suggests SF2/ASF may be transactivated in HPV-16 infected differentiated epithelial cells, most probably by E2.

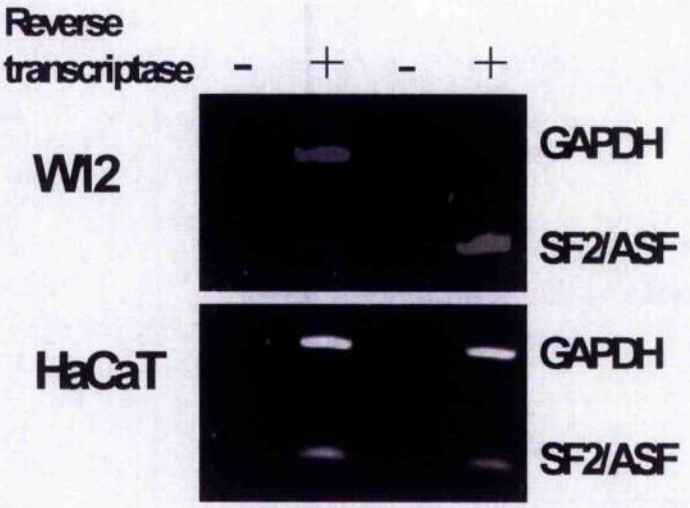


Figure 3.12: **Increased SF2/ASF mRNA in differentiated W12 cells**

Total RNA was produced from undifferentiated and differentiated W12E and HaCaT cells. RT-PCR reaction were performed in the absence (lanes 1 and 3) and presence (lanes 2 and 4) of reverse transcriptase. Primers amplifying a region of the GAPDH and SF2/ASF mRNAs were used in the same PCR reactions to determine steady state levels of each mRNA.

3.8 Discussion

Papillomavirus E2 proteins are known to be involved in regulation of cellular proteins, as well as acting upon the viral long control region, from which viral gene expression is controlled. Here we show regulation of splicing related SR protein, SF2/ASF, by HPV-16 E2. This is the first example of transactivation of a cellular promoter by HPV-16 E2. Furthermore, SF2/ASF is the first RNA regulatory protein found to be controlled by HPV-16. As discussed previously, epithelial cells become less metabolically active, the more differentiated they become. Therefore, many cellular proteins are no longer expressed, as they are no longer required for the cell to function. One such family are RNA processing proteins, such as SF2/ASF, as little gene expression, and hence RNA processing, is observed in differentiated epithelial cells. Therefore, in HPV-16 infected differentiated epithelial cells, the virus must regulate at least a subset of the factors controlling RNA processing to ensure completion of its life cycle. SF2/ASF is known to be elevated in HPV-16 infected differentiated epithelial cells, a phenomenon not observed in uninfected epithelial cells (McPhillips et al. 2004). Furthermore, endogenous SF2/ASF is upregulated in cells expressing HPV-16 E2. This was shown using U2OS cells, osteosarcoma cell clones, which stably express E2. Although E2 is known to induce apoptosis of many cell types, U2OS cells are less sensitive to this and therefore, higher levels of prolonged E2 expression are achievable in these cells. We show here that reporter gene expression driven by the SF2/ASF promoter region, is also increased in the presence of E2 and E2 is found to associate with the promoter in HeLa cells using ChIP assays. However, no direct interaction between E2 and the promoter could be proven *in vitro*, suggesting the association is indirect, possible via a co-activator complex. Although cellular partners for E2 have not been identified here, Sp1, a cellular transcription factor known to interact with E2, may associate with the promoter *in vitro*. We postulate that E2 is tethered to the promoter by one or more cellular proteins, such as Sp1, and then it is able to regulate SF2/ASF abundance at the level of transcription. To add weight to this role of E2 during the HPV-16 life cycle, levels of SF2/ASF mRNA were found to be elevated in infected differentiated epithelial cells, suggesting control of protein abundance may be at the level of transcription, rather than translation or protein stability.

Levels of reporter gene activity driven by the SF2/ASF promoter region as defined above were consistently elevated in cells expressing E2. However, the extent of upregulation differed depending on E2 abundance. With increasing E2 expression in HeLa cells, transactivation of the SF2/ASF promoter decreased. Furthermore, high E2 expression observed in U2OS cell clone B1, transactivated the promoter to a lesser extent than the lower levels observed in U2OS A4. It is therefore possible that high levels of E2 have an inhibitory effect on SF2/ASF transactivation. High levels of HPV-16 E2 are known to result in inhibition of cell growth and promote apoptosis (Sanchez-Perez et al. 1997; Webster et al. 2000; Parish et al. 2006). Therefore, with higher E2 levels, cells may be less healthy and therefore, expression of SF2/ASF controlled by cellular factors may be reduced. In this case, whilst E2 may still transactivate the promoter, it would not be observed in reporter assays due to a reduction in normal endogenous expression. In addition to variations in SF2/ASF promoter transactivation depending on E2 expression levels, there are also differences between HeLa cells and the U2OS cell clones. The greatest degree of transactivation observed in HeLa cells is two-fold, whereas reporter expression increases ~4-fold in U2OS clone A4, when compared with the control. Therefore, the two cells types may differ in levels of cellular proteins involved in E2 mediated transactivation. Alternatively, the difference may be due to the susceptibility of the cells to growth inhibition and apoptosis. HeLa cells may be more susceptible than U2OS cells and hence SF2/ASF expression may suffer to some degree at the levels of E2 expression assayed.

E2 associates with the promoter in HeLa cells, as determined using ChIP assays, suggesting it directly transactivates SF2/ASF. However, direct interaction between E2 and the promoter, or even changes in complexes formed in the presence of nuclear extract and E2 could not be determined *in vitro*. In particular E2 interaction with a near consensus binding site could not be determined. The E2BS is defined as ACCG(N_4)CGGT, with N representing any four bases. The site found within the SF2/ASF promoter differs from the consensus at position 9, where an T is found instead of a C. Furthermore, HPV-16 E2 binding to an E2BS is found to be susceptible to the spacer region sequence, with preferential binding to sites with AT-rich spacers (Hines et al. 1998). It is thought that this provides a pre-bent DNA molecule, to which E2 binds more easily (Hegde and Androphy 1998). However, the SF2/ASF site contains CCCA, within the spacer. In addition, HPV-16 E2 has more recently been shown to bind larger sites composed of

AACCG(N_4)CGGTT more efficiently (Dell et al. 2003). Although an A is found directly upstream of the near consensus SF2/ASF potential E2BS, the immediate downstream nucleotide is G, not T. Therefore, it is possible that whilst one of these discrepancies may be tolerated, all three within the same site prevent E2 from binding. Furthermore, whilst association of E2 with the region-spanning probe 3 of the promoter could not be conclusively competed for with increasing concentrations of cold probe, it is possible that E2 and/or probe concentration have affects on this. The amount of E2 used in EMSA experiments is difficult to compare with levels of expression in HeLa or U2OS cells. Furthermore, the amount of promoter sequence available within transfected cells is unlikely to correspond with the levels of probe used in EMSAs. Therefore, it is possible that E2 interacts with sequences within the probe 3 region in a dynamic manner, and that the concentration of E2 in *in vitro* experiments is high enough to allow transient interaction with both radiolabelled and non-radiolabelled probes, without affecting the observed shift obtained with radiolabelled probe. To definitively determine that E2 is associating with this probe, similar experiments using non-radiolabelled HPV-18 LCR probe, which contains E2BSs proven to associate strongly with E2 *in vitro*, should be performed.

DNA structure and/or cellular proteins may also be important for E2 to associate with the SF2/ASF promoter via the near-consensus or a non-consensus site. For example, methylation status and/or looped control regions may be important. However, methylation of E2BSs has been found to inhibit E2 binding (Thain et al. 1996). Furthermore, methylation of an E2 responsive promoter has been shown to cause inhibition of transactivation (Kim et al. 2003), suggesting the non-methylation status of the DNA in the EMSAs presented here, is unlikely to be the reason no interaction was observed. Additionally, if specific DNA looped control regions were required for E2 binding, observing an *in vitro* interaction would be difficult. Therefore, to further assay the E2 association with the promoter would require *in vivo* analysis. There is some support for a role of the DNA binding domain (DBD) in SF2/ASF transactivation in the data obtained from ChIP assays. Whilst a polyclonal E2 antibody recognising many regions throughout E2 was able to immunoprecipitate the endogenous SF2/ASF promoter from HeLa cells, an antibody recognising only the DBD was not. Therefore, it is possible that the DBD may be involved in protein-DNA complexes, which the antibody is unable to access. Analysing which domain(s) of E2 are involved in SF2/ASF transactivation, using transactivation (TA) and DBD mutants in transient transfections, may give

further indication as to how E2 associates with the promoter. If DBD mutants were able to transactivate the promoter, this suggests E2 is not binding DNA directly and is probably associating via one or more cellular proteins. Furthermore, the DBD of E2 is also involved in association with cellular proteins, such as C/EBP and TBP (Rank and Lambert 1995; Enzenauer et al. 1998; Hadaschik et al. 2003). Therefore, if these mutants were able to transactivate it would suggest that these proteins are not involved in E2-mediated transactivation of the SF2/ASF promoter. In addition, inability of E2-TA mutants to regulate SF2/ASF transactivation, would suggest that this region of the protein is vital to this function. The TA domain of E2 associates with CBP/p300 and p/CAF (Lee et al. 2000; Lee et al. 2002), which would implicate these proteins in bringing E2 to the SF2/ASF promoter. Conversely, should E2-TA mutants transactivate the SF2/ASF promoter, this would suggest that E2 functions to recruit cellular factors, which associate with the DBD, to enhance transcription. Therefore, these data would give considerably more information as to the mechanism by which E2 may associate with the SF2/ASF promoter.

The SF2/ASF promoter is likely to be a constitutively active promoter, due to the essential nature of SF2/ASF within cells. As such, it would be expected to harbour a number of binding sites for cellular transcription factors (TFs) to ensure to high level of expression. However, bioinformatic analysis of the promoter to determine potential binding sites for cellular TFs indicates that whilst there are sites for a number of TFs, no site is found in abundance (Figure 3.10). In addition, no TATA-box was observed, indicating that TBP is recruited to the promoter by other cellular proteins. A short T-rich stretch is observed at –25 from the start site; however, this does not look like any TATA element observed so far. In addition, the conical initiation signal (YYANWYY; where Y is C or T, N is any nt and W is A or T) is not observed at nt +1. Whilst TATA-less promoters often contain a number of Sp1 binding sites, this promoter region, has very few sites for TF binding within the transcription start site proximal region. This suggests that SF2/ASF expression is regulated by a number of different TFs, which may act at some distance from the transcription start site. In addition, a imperfect TFIIB recognition element (BRE) (SSRCGCC; where S is G or C and R is A or G), which is generally found immediately upstream of the TATA-box and directly binds TFIIB, is found at –38, and an imperfect downstream promoter element (DPE) (RGWCGTG), which interacts with TAFs, is located at +35 (Figure 4.10). Therefore, it is possible that these elements are involved in regulation of this promoter. The lack of TF binding

sites may suggest that the promoter is in open chromatin conformation, allowing easy recruitment of the TFs that have the potential to bind. Furthermore, in open conformation it would be more accessible to viral regulators, such as HPV-16 E2.

A known cellular binding partner of E2, Sp1 may interact with the promoter *in vitro*; however, this is not proven and association *in vivo* has not yet been established. Therefore, further EMSA and ChIP experiments are required to determine if Sp1 does indeed interact with the promoter. In contrast, TBP is shown in all ChIP assays to associate. As mentioned, BPV-1, HPV-8 and -18 E2 proteins are known to interact with TBP and/or a number of components of the basal transcription machinery (Rank and Lambert 1995; Enzenauer et al. 1998; Carrillo et al. 2004). Furthermore, HPV-16 E2 interacts with yeast TFIIB (Benson et al. 1997), providing a possible mechanism for E2-mediated transactivation of SF2/ASF. Furthermore, there are binding sites within the promoter, such as for Oct-1 and AP-1, which are also found within the HPV-16 LCR (Figure 3.10). Mutation of these sites, as well as the Sp1 binding site, may be useful in binding assays and transient transfections. Inability of E2 to subsequently associate with and transactivate the SF2/ASF promoter would indicate that this cellular protein was involved. A similar approach could be employed for other transcription factors known to interact with E2, such as C/EBP. C/EBP is not shown to interact with a potential binding site in *in vitro* assays here; however, a further potential C/EBP ß binding site is found ~1kb from the transcription start site (Figure 3.10). Potential interaction with this site has not been assayed. In contrast, mutation of binding sites would not be possible for co-activators, CBP/p300 and p/CAF, which do not bind DNA. Therefore, overexpression of the protein to induce further activation of reporter gene activity driven by the SF2/ASF promoter, or siRNA mediated silencing to abolish transactivation, could determine a role for that protein.

Although a role for E2 has been suggested in SF2/ASF transactivation, we must prove that this function is performed during virus infection. This is important, as E2 is known to associate with many proteins, including other papillomavirus proteins. As well as the well-characterised interaction with E1, which facilitates replication, the HPV-16 minor capsid protein, L2, inhibits E2 mediated transactivation (Okoye et al. 2005). Furthermore, interaction of HPV-16 E2 with HPV-16 E6 causes elevated transcriptional activity (Grm et al. 2005) and an HPV-16 E2-E7 interaction has been observed which is shown to inhibit E7 induced cellular transformation (Gammoh et al. 2006). However, the functional relevance

of E7 interaction with respect to E2 transcriptional activity, if any, is not yet known. Whilst initial experiments suggest that SF2/ASF protein abundance is possibly controlled at the level of transcription, further experiments are required to definitively determine a role for E2 in SF2/ASF transactivation in HPV-16 infected differentiated cells. For example, detection of E2 association with the endogenous SF2/ASF promoter in differentiated W12E cells using ChIP assays, would suggest a role for it in transactivation. This was not attempted initially due to problems amplifying the endogenous SF2/ASF promoter from HeLa cells in ChIP assays. In addition, levels of E2 are lower in differentiated W12E cells, when compared with transiently transfected HeLa cells, suggesting the antibodies used may be less efficient at immunoprecipitating E2-containing complexes. Furthermore, siRNA mediated silencing of E2 in infected cells could be employed. Loss of elevated SF2/ASF levels in siRNA treated cells would indicate that E2 is responsible for transactivation. However, loss of E2 may also impact viral gene expression, as well as other aspects of the viral life cycle, possibly making interpretation of this experiment difficult. Perhaps siRNA mediated knock-down of cellular factors thought to be involved would be useful, not only to determine if E2 is indeed acting via these partners, but also to establish whether SF2/ASF is transcriptionally activated by E2.

Whilst a role for HPV-16 E2 has been suggested in SF2/ASF transactivation and the protein can be found to associate with the promoter in HeLa cells, cellular partners have, as yet, not been identifled. Sp1, a cellular binding partner of E2, may associate with the SF2/ASF promoter *in vitro*. However, further experiments are required to prove this association, to determine whether it associates *in vivo* and to discover if it acts to enhance E2-mediated transactivation of this promoter. Furthermore, the role of SF2/ASF transactivation by E2 has yet to be demonstrated in the context of the natural life cycle of HPV-16.

4 Regulation of splicing associated, SR proteins by HPV-16

4.1 Introduction

As demonstrated in the previous chapter HPV-16 E2 transcriptionally activates the expression of splicing associated SR protein, SF2/ASF. SF2/ASF is known to interact indirectly with the LRE in both HPV-16 infected undifferentiated and differentiated cells (McPhillips et al. 2004). However, SF2/ASF is only one member of a family of SR proteins involved in splicing regulation during RNA processing. To date, no assays have been performed to determine whether other SR proteins associate with the LRE. Therefore, it is possible that SR proteins other than SF2/ASF may have a role in control of late gene expression. Thirteen distinct transcripts are produced by the virus in differentiated cells and five splice acceptor and six splice donor sites have been identified (Milligan et al., 2006). Furthermore, a number of RNA elements have been found within the virus late region (Kennedy et al. 1990; Kennedy et al. 1991; Collier et al. 1998; Cumming et al. 2003; Oberg et al. 2003; Zhao et al. 2004; Rush et al. 2005; Zhao et al. 2005). Each element is proposed to act via interactions with cellular RNA processing factors; however, upon differentiation of uninfected epithelial cells, there is a general downregulation of RNA processing proteins (McPhillips 2002). Therefore, it is hypothesised that some of these factors are regulated by HPV-16 to ensure correct processing of virus transcripts at the appropriate stage of differentiation. SR proteins have roles in constitutive and alternative splicing, mRNA export and translation (Caceres et al. 1998; Huang et al. 2003; Sanford et al. 2004). As late gene expression can potentially be controlled at any or all of these stages, HPV-16 mediated regulation of SR proteins would be likely. Therefore, the work in this chapter was undertaken to determine what effect, if any, episomal infection of HPV-16 had upon SR protein levels and localisation, during the viral life cycle.

To fully understand the relevance of this chapter, the roles of a sub-set of SR proteins within human cells will be summarised. SR proteins are comprised of two regions, the N-terminus containing one or two RNA recognition motifs (RRMs) and the C-terminus containing an arginine/serine rich domain (RS domain) of variable length. SRp20, SC35 and 9G8 all contain one RRM, whereas SF2/ASF, SRp40, SRp55 and SRp75 contain two (Figure 4.1). Whilst the RS domain is involved in protein-protein interactions among splicing factors (Wu and Maniatis 1993; Cao

and Garcia-Blanco 1998), the RRM(s) interact with RNA at specific sequences known as exonic splicing enhancers (ESEs) (Kanopka et al. 1996). SR proteins contribute to constitutive splicing by bridging U1 snRNP at the 5' splice site and U2AF at the 3' splice site, to form the spliceosome and contribute to the various stages of splicing (reviewed by Reed and Palandjian 1997). During alternative splicing however, SR proteins associate with ESEs to facilitate splicing at the most proximal splice sites (Caceres et al. 1994; Eperon et al. 2000). In contrast, another family of RNA binding proteins, hnRNPs, are known to associate with a second type of RNA element, known as exonic splicing silencers (ESSs). This interaction directs splicing to more distal splice sites, allowing for regulation of splice patterns through the use of competing ESEs and ESSs and varying levels of antagonistic SR and hnRNP proteins. Within the late region of the HPV-16 genome, and particularly within the long exonic sequence encoding the L1 capsid protein, there are a number of predicted ESEs and ESSs (Figures 4.2). At least some of these are likely involved in directing appropriate splicing of late transcripts depending on the differentiation status of the cell. Most predicted ESEs for SRp20 and SRp40, are unlikely to be authentic, due to their high frequency. However, potential SF2/ASF, 9G8, SC35 and SRp55 ESEs are less frequent and are more likely to be real sites, which may be acted upon by the relevant SR protein. In addition, Zhao et al. have revealed an ESS within the 5' region of L1 transcripts, which inhibits use of a 3' splice site immediately upstream in undifferentiated cells, via interactions with hnRNP A1, confirming the computer modelling, shown in figure 4.2 (Zhao et al. 2004). Furthermore, an ESE within the E4 ORF enhances splicing from a weak 3' splice site within the E2 ORF (Rush et al. 2005). However, proteins regulating this element have so far not been identified. A sub-set of SR proteins also have roles in post-transcriptional regulation of gene expression, other than splicing. For instance, SF2/ASF, SRp20 and 9G8 are able to shuttle to between the nucleus and the cytoplasm (Caceres et al. 1998). This shuttling activity is thought to help link splicing to mRNA export, and all three proteins are shown to interact with TAP, the major nuclear export adaptor of cellular mRNA (Huang et al. 2003). In addition, SF2/ASF is shown to regulate translation in the cytoplasm (Sanford et al. 2004). As discussed, splicing-associated proteins show general downregulation during differentiation of uninfected epithelial cells.

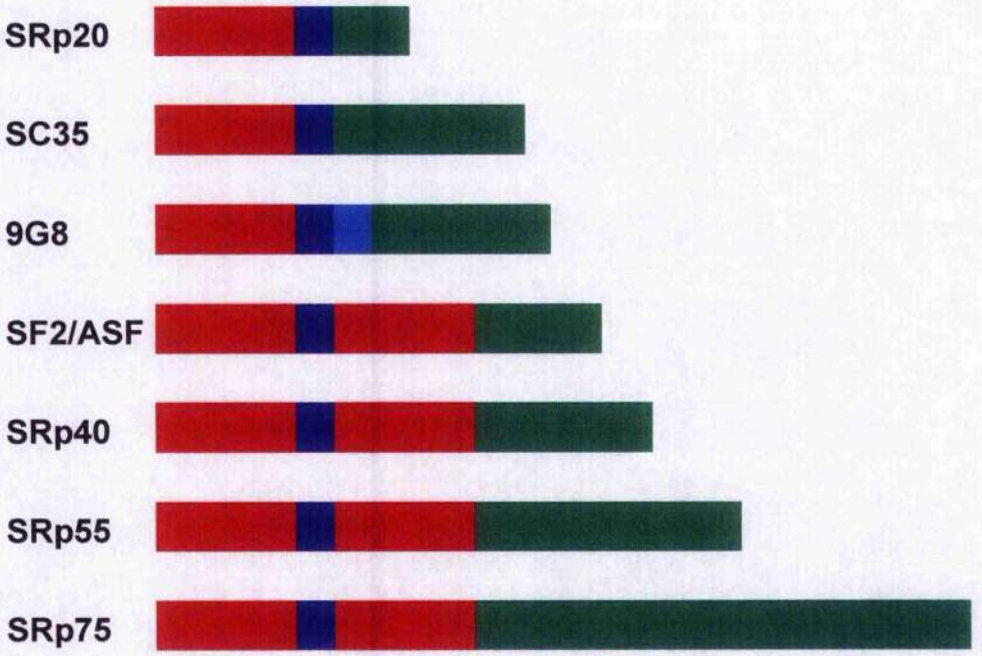


Figure 4.1: **SR protein structure**

Schematic diagram of domain structure of a sub-set of SR proteins. RNA recognition motifs are shown in red and RS domains of variable length are shown in green. The dark blue region depicts a glycine, arginine or proline rich region adjoining the domains. In the case of 9G8 the light blue region indicates a zinc knuckle, unique to this SR protein.

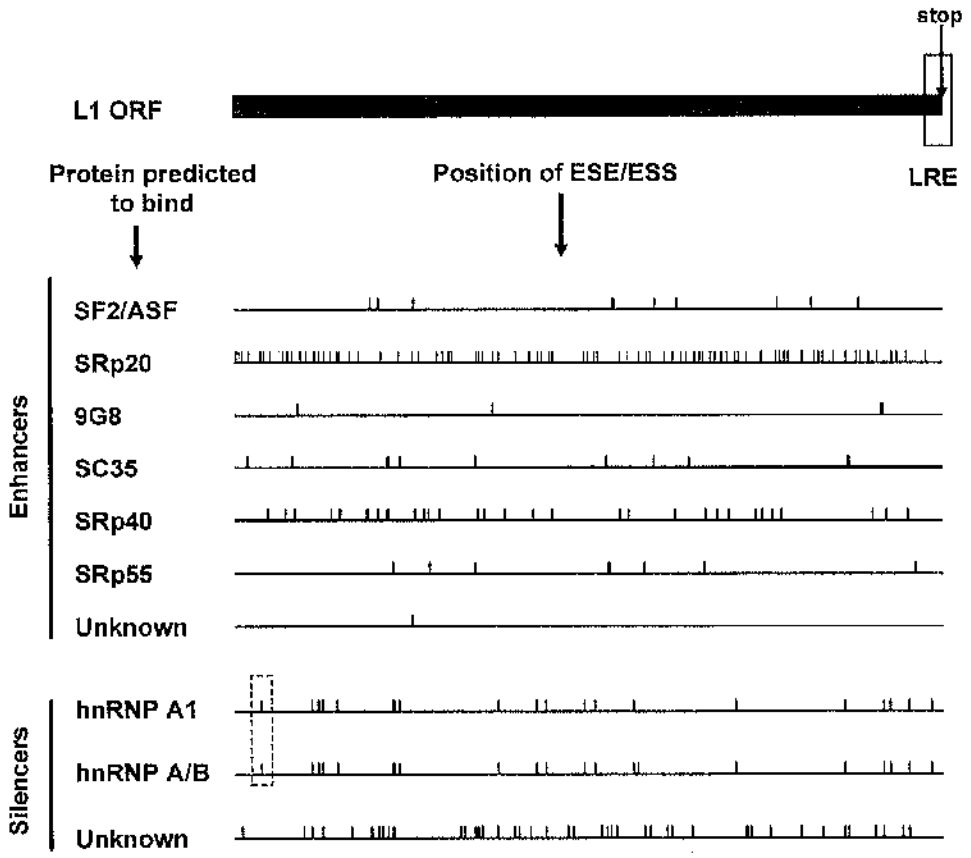


Figure 4.2: **A number of potential ESEs and ESSs are found within the HPV-16 L1 ORF**

Potential ESEs and ESSs within the HPV-16 L1 ORF were determined using RegRNA (http://regrna.mbc.nctu.edu.tw/index.html). The L1 ORF is shown in blue at the top, with the LRE shown as an open rectangle and stop codon by a vertical arrow. Potential ESEs and ESSs are depicted by vertical black tabs, in relation to the L1 ORF, below. The proteins predicted to bind each element are shown to the left along with whether they are potential ESEs or ESSs. The hnRNP A1 responsive ESS discovered by Zhao et al. is highlighted by a perforated rectangle.

Therefore, a least a sub-set of RNA processing proteins must be regulated by the virus to ensure correct processing, export and translation of viral transcripts in differentiated cells. SR proteins would provide a useful target for HPV-16 as they have multiple roles during post-transcriptional regulation of gene expression.

The work in this chapter focused on regulation of splicing-related SR proteins during the HPV-16 life cycle. Western blots were used to determine changes in SR protein expression levels upon differentiation of W12E cells. SR protein levels were also assayed in cells stably expressing E2, as E2 is involved in upregulation of SF2/ASF protein levels (McPhillips et al. 2004). Furthermore, localisation of SR proteins was also determined using immunofluorescence. As SF2/ASF is found to be hyperphosphorylated in differentiated W12E cells, HPV-16 mediated regulation of SR protein kinase 1 (SRPK1), which is a major kinase of SF2/ASF, was also determined using western blotting and immunofluorescence. In addition, co-localisation of SR proteins and SRPK1, with E1^E4 and E2 was determined, as these are the major viral proteins which are likely to regulate SR proteins during HPV-16 infection. There are a number of reasons for this. For example, expression of both proteins is elevated in differentiated W12E cells (Doorbar et al. 1997; Maitland et al. 1998). HPV-16 E2 is involved in transcriptional regulation of SF2/ASF, as shown in the previous chapter. It is also possible that E2 acts to regulate cellular localisation and/or phosphorylation of SR proteins, via regulation of SRPK1. SRPK1 may be expected to be regulated by HPV-16 in a similar manner to SR proteins, and E2 may regulate SRPK1 abundance, which in turn causes hyperphosphorylation of SF2/ASF, and possible other SR proteins. Consequently, SR protein phosphorylation status may regulate SR protein localisation during HPV-16 infection. Furthermore, HPV-1 E1^E4 has recently been shown to interact with SRPK1, which may also be involved in regulating phosphorylation status and/or relocalisation of SR proteins during differentiation (S. Roberts, personal communication). Although this interaction has not been determined for HPV-16 E1^E4, it is possible that it occurs and is involved in HPV-16 mediated regulation of SR proteins. Again, E1^E4-SRPK1 interaction may cause SR protein hyperphosphorylation, which may regulate cellular localisation. In contrast, E1^E4 may interact with both SRPK1 and SR proteins and regulate both phosphorylation and localisation directly. Expression of both E2 and E1^E4 and not simply one or the other, may be necessary to regulate SR protein expression, phosphorylation and localisation during the HPV-16 life cycle. Therefore, localisation of SR proteins and SRPK1 was determined in

undifferentiated and differentiated epithelial cells transiently expressing HPV-16 E2 or E1^E4. Furthermore, differentiated W12E cells were assayed for E1^E4 and SR protein or SRPK1 co-localisation by immunofluorescence. Similar experiments were not performed for E2 due to the lack of antibodies able to detect the lower level of expression of E2 in W12E cells, as compared to stable and transiently transfected cells.

4.2 SR proteins, SF2/ASF, SRp20 and SRp75 are upregulated by HPV-16 E2

Experiments were performed to determine if SR proteins, other than SF2/ASF, are regulated during the virus life cycle. To determine what effect differentiation may be having on SR protein abundance, we performed western blots comparing protein extracts from undifferentiated and differentiated W12E cells (Figure 4.3). As we have previously demonstrated, total SF2/ASF is shown to be upregulated ~4-8 fold in differentiated W12E cells. Furthermore, total SRp20 is also elevated in differentiated W12E cells, although to a lesser extent (~2-4 fold). However, total 9G8 is not upregulated in response to differentiation, suggesting this protein may not be involved in control of late gene expression. To look at a further sub-set of SR proteins, Mb104 was used, which recognises a common phospho-epitope of a number of SR proteins (Roth et al. 1991). Whilst phosphorylated SRp75 could not be detected, levels of phosphorylated SRp30s (SF2/ASF (Zahler et al. 1992), SC35 (Fu and Maniatis 1992)) and SRp20 are shown to be elevated in differentiated cells. Furthermore, levels of both phosphorylated SRp55 and SRp40 are upregulated. To ensure elevated phosphorylation of this sub-set of SR proteins is due to HPV-16 infection and not differentiation of epithelial cells, western blots comparing protein lysates from undifferentiated and differentiated HaCaT cells (uninfected epithelial cells) were performed (Figure 4.4). In contrast, to the situation in W12E cells, levels of phosphorylated SR proteins, using Mb104, and total SF2/ASF and SRp20 remained the same or were depleted upon differentiation of HaCaT cells. This suggested a sub-set of SR proteins are specifically upregulated during the HPV-16 life cycle.

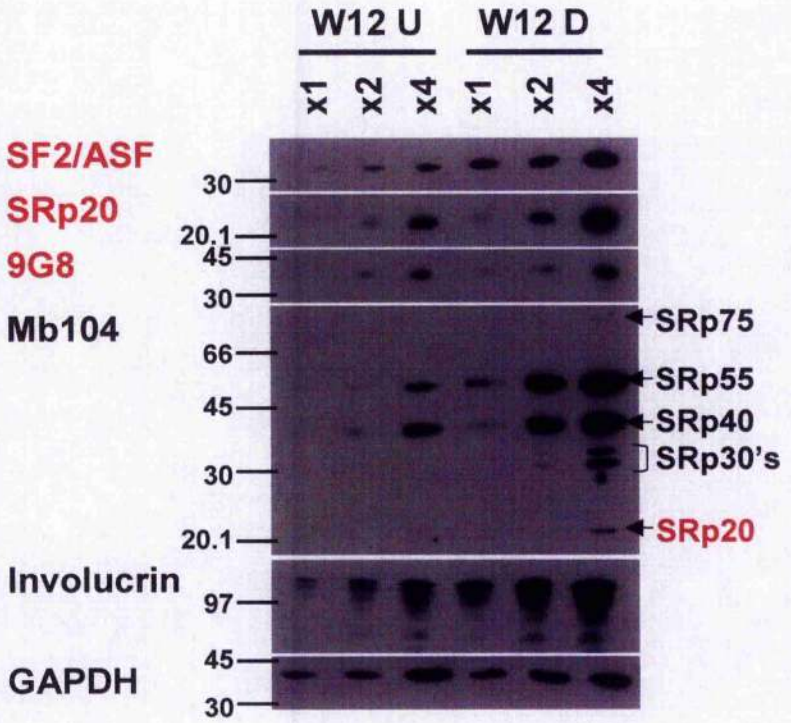


Figure 4.3: **A sub-set of SR proteins are upregulated in response to HPV-16**

Western blots using x1, x2 and x4 quantities of protein lysates from undifferentiated (W12 U) and differentiated (W12 D) W12E cells. Sizes markers in KDa are shown by horizontal lines to the left of each blot. Antibodies used in each panel are indicated on the far left and SR proteins detected using Mb104 are shown on the right. SR proteins which are able to shuttle between the nucleus and the cytoplasm are highlighted in red. Involucrin and GAPDH were used as a marker for differentiation and loading control, respectively.

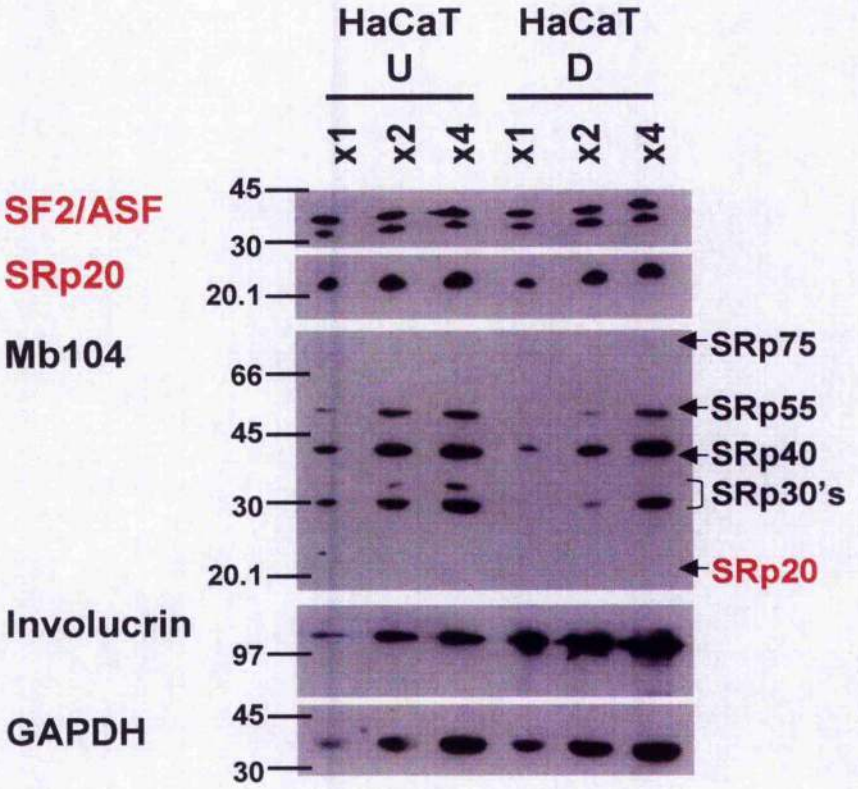


Figure 4.4: **SR proteins are down regulated during differentiation of uninfected epithelial cells**

Western blots using x1, x2 and x4 quantities of protein lysates from undifferentiated (HaCaT U) and differentiated (HaCaT D) HaCaT cells. Sizes markers in KDa are shown by horizontal lines to the left of each blot. Antibodies used in each panel are indicated on the far left and SR proteins detected using Mb104 are shown on the right. SR proteins which are able to shuttle between the nucleus and the cytoplasm are highlighted in red. Involucrin and GAPDH were used as a marker for differentiation and loading control, respectively.

Having demonstrated that HPV-16 regulates a sub-set of SR proteins, we went on to determine whether E2 was involved. This was achieved using two cell clones which stably express E2 to different levels (A4 and B1). In addition to SF2/ASF, phosphorylated levels of SRp75 and SRp20 are also elevated in cells expressing E2, when compared to controls (Figure 4.5). However, phosphorylated SRp55 and SRp40, which show some elevated levels in differentiated W12E cells, are not regulated by E2 alone. Whilst E2 is expressed at all stages of differentiation of an HPV-16 infected epithelium, the highest levels are observed in the suprabasal layers (Maitland et al. 1998). We hypothesise that E2 expression is elevated in these cells to regulate expression of cellular proteins that have functions related to the viral late life cycle, such as SR proteins.

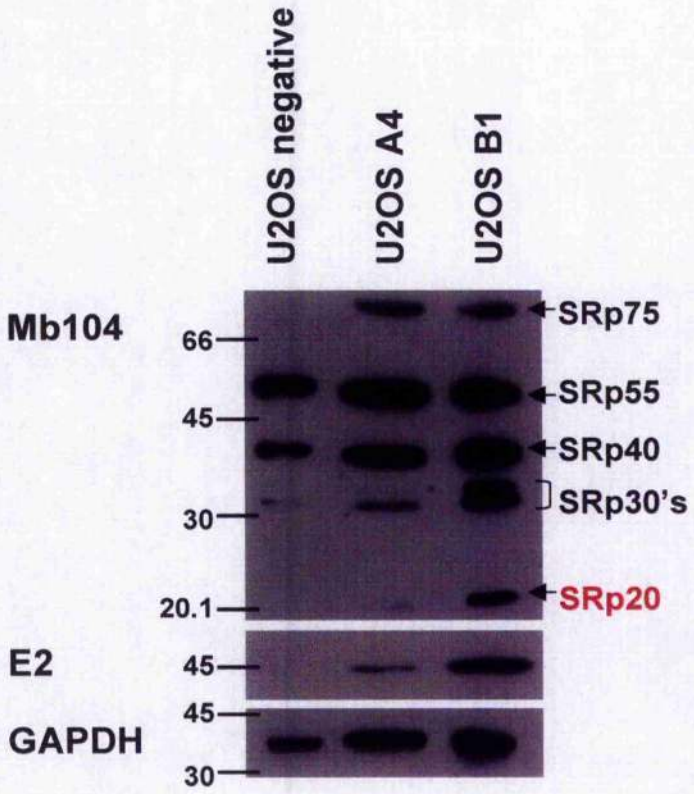


Figure 4.5: **A sub-set of SR proteins are upregulated cells expressing HPV-16 E2**

Western blots comparing equal quantities of protein lysates from non-transfected U2OS cells (negative), and two U2OS cell clones stably expressing HPV-16 E2 (A4 and B1). Sizes markers in KDa are shown by horizontal lines to the left of each blot. Blots were probed using Mb104 and the SR proteins detected are shown on the right. SR proteins which are able to shuttle between the nucleus and the cytoplasm are highlighted in red. GAPDH was used as a loading control.

4.3 Episomal HPV-16 may cause redistribution of shuttling SR proteins, SF2/ASF and SRp20 but not 9G8, to the cytoplasm of differentiated cells

Having shown that HPV-16 E2 is involved in regulation of SR proteins other than SF2/ASF, we wanted to determine the cellular localisation of the shuttling SR proteins in both undifferentiated and differentiated HPV-16 infected cells. Cellular localisation may be important to the functions of the proteins and may suggest roles in export and/or translation of late transcripts. To achieve this, immunofluorescence using undifferentiated and differentiated W12E cells was performed. As detailed above involucrin was used as a marker for differentiation, whilst SC35 was used as a non-shuttling SR protein control. Results here show increase levels of involucrin within differentiated cells as expected (Figure 4.6). Furthermore, staining for SC35 revealed the protein is localised to speckles within the nucleus, the characteristic pattern for SR proteins, in both undifferentiated and differentiated cells. This was expected as SC35 does not shuttle between the nucleus and the cytoplasm (Caceres et al. 1998). In contrast, in some experiments, whilst both SF2/ASF and SRp20 are localised predominantly to nuclear speckles within undifferentiated W12E cells, their pattern is more diffuse in the nucleus and also in the cytoplasm within differentiated cells (Figure 4.6). However, in other experiments, both of these proteins localise to nuclear speckles in both undifferentiated and differentiated cells (Figure 4.7). Whilst the reason for the difference is unclear, the method of differentiation may impact SR protein localisation. Cells differentiated in monolayer culture retain nuclear speckling of SR proteins, whilst methylcellulose differentiation seemed to result in redistribution of SF2/ASF and SRp20. Furthermore, staining for 9G8 revealed this protein to be found within nuclear speckles in both undifferentiated and differentiated cells (Figure 4.6). Although 9G8 is known to shuttle between the nucleus and the cytoplasm, its shuttling activity is not dependent upon transcription, in the way that both SF2/ASF and SRp20 are. To ensure possible shuttling of SF2/ASF and SRp20 was not a product of epithelial differentiation, immunofluorescence was performed using undifferentiated and differentiated HaCaT cell. In this case anti-9G8 was used a non-shuttling control, whilst SF2/ASF and SRp20 were monitored for redistribution. Figure 4.8 shows all three proteins localised to nuclear speckles in both undifferentiated and differentiated cells. This suggests that potential

redistribution of shuttling SR proteins; SF2/ASF and SRp20 in HPV-16 infected differentiated cells may be due to the presence of virus genomes.

4.4 SR protein kinase 1 (SRPK1) is regulated during differentiation of HPV-16 infected epithelial cells

As discussed, SF2/ASF is hyperphosphorylated in differentiated W12E cells. SRPK1 is a potential candidate for this, due to its interaction with HPV-1 E1^E4 (Roberts, personal communication). Therefore, we wanted to determine whether SRPK1 is regulated during the HPV-16 life cycle. Firstly to compare protein levels between undifferentiated and differentiated W12E cells, western blots were performed. SRPK1 is shown to be elevated ~2 fold upon differentiation of W12E cells (Figure 4.9). In contrast, in differentiated HaCaT cells, SRPK1 levels are shown to diminish. This suggests that SRPK1, like a sub-set of SR proteins, is specifically upregulated in response to HPV-16 infection. In addition to analysing protein levels, localisation of SRPK1 was assayed in undifferentiated and differentiated W12E cells. The protein shows diffuse cytoplasmic location in both undifferentiated and differentiated cells (Figure 4.10). However, in undifferentiated W12E cells, there is more of the protein in the nucleus, whilst differentiation results in redistribution to the cytoplasm. This suggests HPV-16 may regulate cellular localisation of SRPK1. To ensure that SRPK1 distribution does not change during differentiation of normal epithelial cells, HaCaT cells were used. Figure 4.9 shows no change was observed in SRPK1 localisation between undifferentiated and differentiated HaCaT cells. However, SRPK1 is found to be predominantly nuclear in HaCaT cells, suggesting a fundamental difference between W12E and HaCaT cells with respect to SRPK1 localisation. This suggests that SRPK1 protein levels are regulated during the HPV-16 life cycle, and the protein redistributes to the cytoplasm upon differentiation of HPV-16 infected cells.

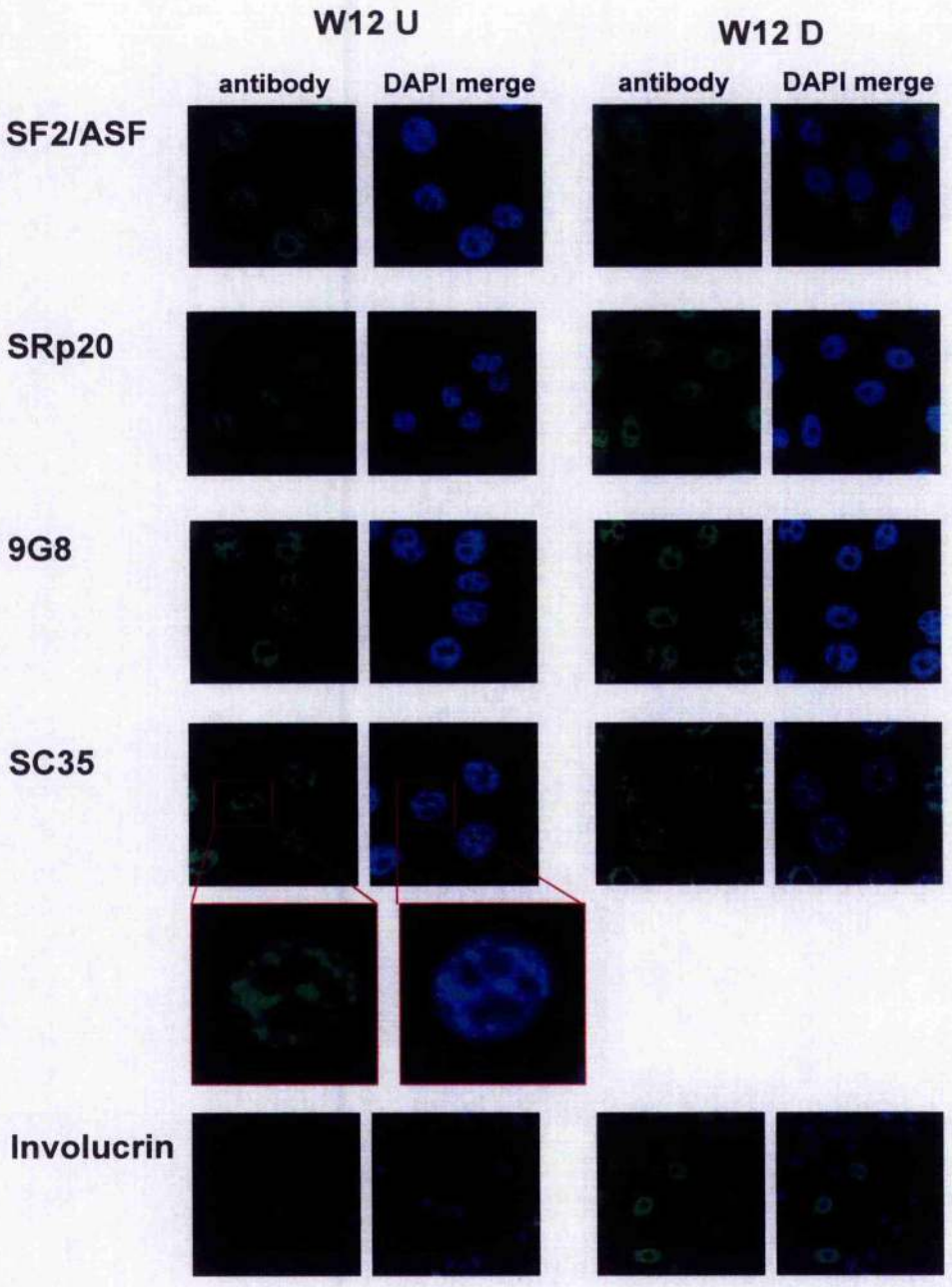


Figure 4.6: **Redistribution of SR protein, SF2/ASF and SRp20, during differentiation of W12E cells**

Immunofluorescence showing localisation of SR proteins, SF2/ASF, SRp20, 9G8 and SC35 in undifferentiated (W12 U) and methylcellulose differentiated (W12 D) W12E cells. SR proteins are shown in green and DAPI stained nuclei are shown in blue. The protein stained for is given to the left. Higher magnification of W12 U SC35 stained cells shows nuclear speckling pattern. Involucrin was used as a marker for differentiation and cells in this panel are shown at a lower magnification. Involucrin staining in W12D cells is shown at lower exposure due to the intensity of the signal

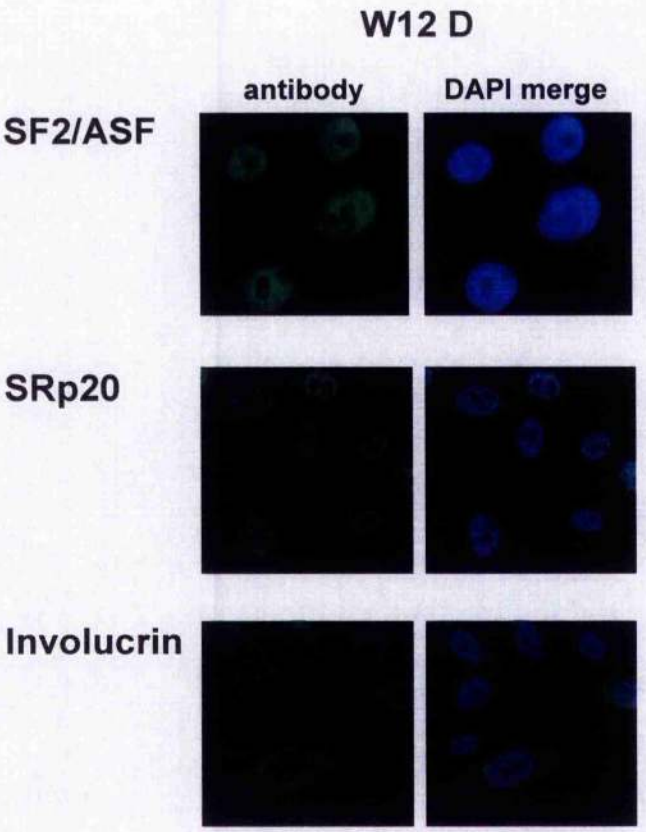


Figure 4.7: **Redistribution of SF2/ASF and SRp20, during differentiation of W12E cells may depend on genome integration and/or the extent of differentiation**

Immunofluorescence showing localisation of SR proteins, SF2/ASF and SRp20 in monolayer differentiated (W12 D) W12E. SR proteins are shown in green and DAPI stained nuclei are shown in blue. The protein stained for is given to the left. Involucrin was used as a marker for differentiation.

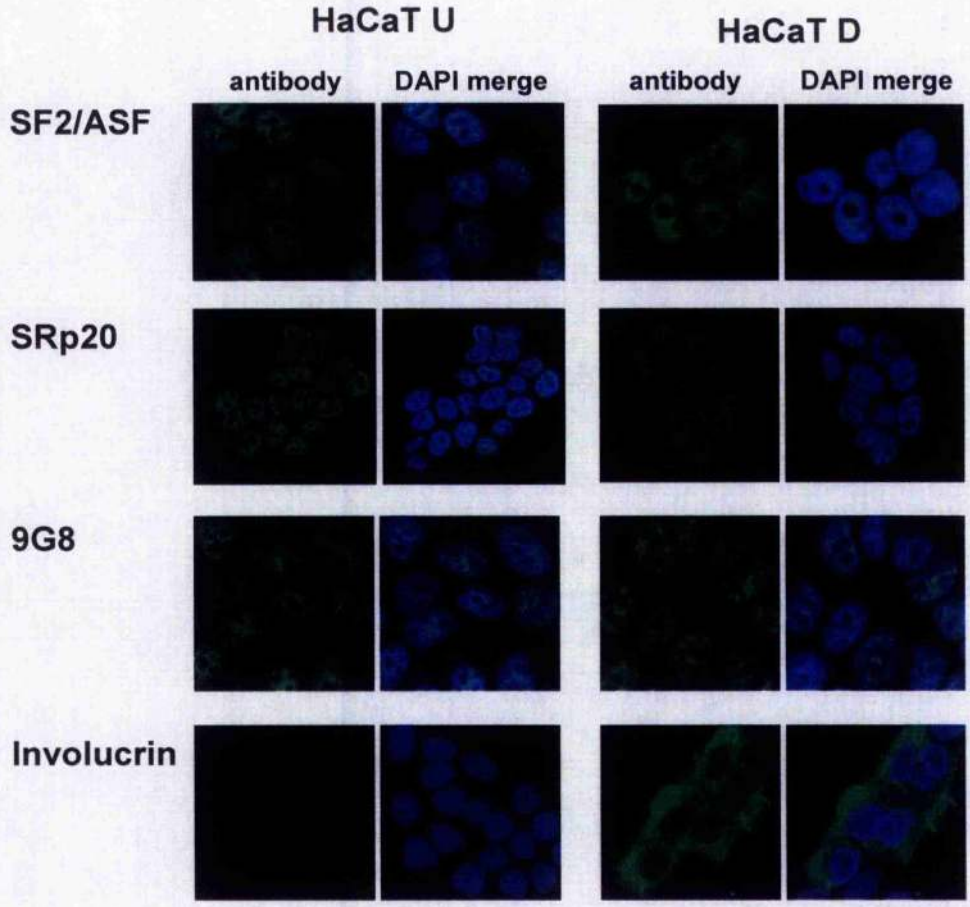


Figure 4.8: **Localisation of SR protein during differentiation of HaCaT cells**

Immunofluorescence showing localisation of SR proteins, SF2/ASF, SRp20 and 9G8 in undifferentiated (HaCaT U) and differentiated (HaCaT D) HaCaT cells. SR proteins are shown in green and DAPI stained nuclei are shown in blue. The protein stained for is given to the left. Involucrin was used as a marker for differentiation.

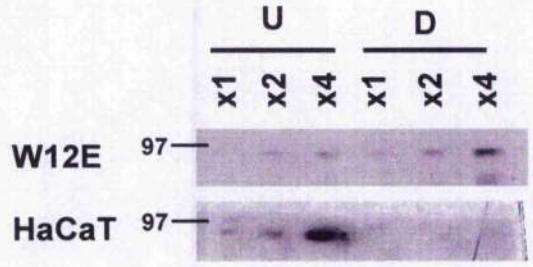


Figure 4.9: **SRPK1 is upregulated during differentiation of HPV-16 infected epithelial cells**

Western blots using x1, x2 and x4 quantities of protein lysates from undifferentiated (U) and differentiated (D) W12E and HaCaT cells, probed with anti-SRPK1. Sizes markers in KDa are shown by horizontal lines to the left of each blot. Involucrin and GAPDH, differentiation and loading control western blots are shown in figure 4.3 for W12E samples and figure 4.4 for HaCaT samples.

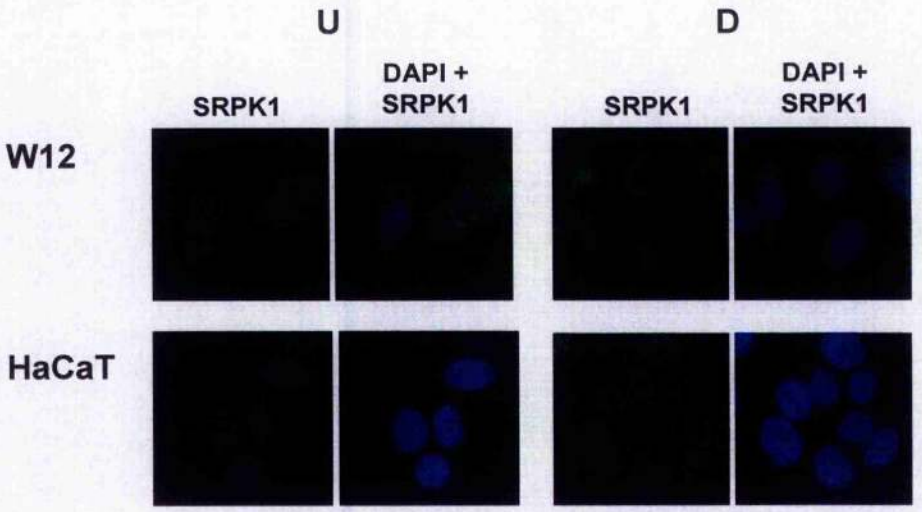


Figure 4.10: **Relocalisation of SRPK1 during differentiation of W12E cells**

Immunofluorescence showing localisation of SRPK1 in undifferentiated (U) and differentiated (D) W12E or HaCaT cells. SRPK1 is shown in green and DAPI stained nuclei are shown in blue.

4.5 SR proteins and SRPK1 do not co-localise with E2 or E1^E4 in transfected cells

To further investigate the localisation of shuttling SR proteins and SRPK1 in W12E cells, differentiated W12E and E2 or E1^E4 transfected cells were subjected to co-localisation immunofluorescence experiments. E2 and E1^E4 were chosen as potential candidates for SR protein regulation for reasons discussed in the introduction to this chapter. Therefore, stable and transiently HPV-16 E2 or E1^E4 transfected cells were assayed for SR protein and SRPK1 localisation. Figure 4.11 shows localisation of SR proteins in U2OS cells (B1), which are stably expressing HPV-16 E2. Similar results were found using U2OS A4, but expression of E2 was lower in this cell clone, so detection of E2 was more difficult. Neither cell clone was assayed for co-staining between E2 and SR protein or SRPK1. No relocalisation of SR proteins was found upon expression of E2, suggesting E2 has little effect on SR protein localisation when expressed in U2OS cells. However, SRPK1 localises to the nucleus and peri-nuclear region in E2 expressing cells, whereas the protein shows more diffuse staining throughout the cell in the absence of E2. A similar pattern in SRPK1 redistribution is observed during differentiation of W12E cell (Figure 4.10), suggesting that E2 may regulate SRPK1 localisation. A more definitive experiment to determine whether E2 co-localises with SR proteins and/or SRPK1 during HPV-16 infection, would have been to co-stain undifferentiated or differentiated W12E cells. However, antibodies against E2 did not detect the protein in either undifferentiated or differentiated W12E cells, and therefore this experiment was not possible.

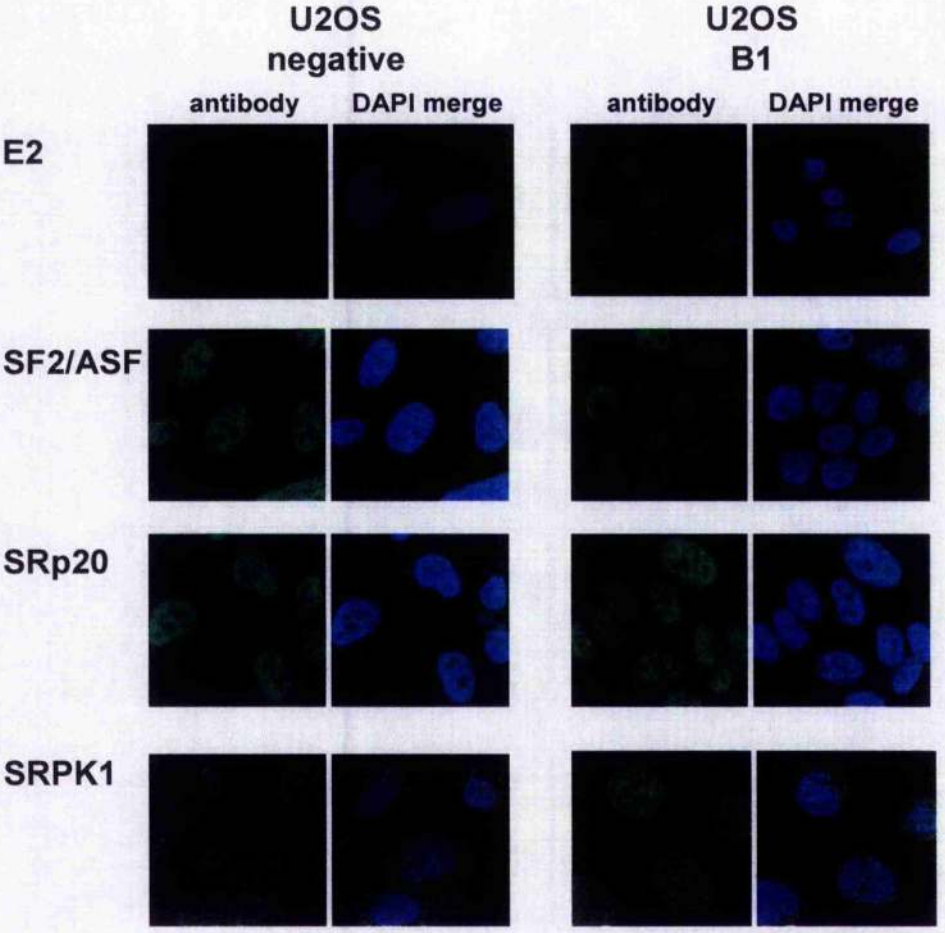


Figure 4.11: **HPV-16 E2 causes redistribution of SRPK1 when expressed in U2OS cells**

Immunofluorescence showing localisation of SF2/ASF, SRp20 and SRPK1 in in non-transfected U2OS cells (U2OS negative) or HPV-16 E2 stably expressing U2OS cell clone B1 (U2OS B1). SR proteins and SRPK1 are shown in green, whilst DAPI stained nuclei are shown in blue. Expression of E2 is also shown in the top panel, where U2OS B1 cells are shown at a lower magnification.

To determine potential co-localisation between HPV-16 E1^E4 and SR proteins or SRPK1, similar experiments were performed. Firstly, HeLa cells, as a model for undifferentiated epithelial cells, were transiently transfected with an HPV-16 E1^E4 expression vector. Cells were then co-stained using anti-E1^E4 and anti-SR protein or SRPK1 antibodies. Figure 4.12 shows no change in localisation, or any co-staining between SF2/ASF, SRp20 or SRPK1 in E1^E4 expressing cells. To determine whether epithelial differentiation or tumourigenesis had any effect on this, E1^E4 transiently transfected differentiated HaCaT cells, were subjected to co-staining experiments. Again E1^E4 did not co-stain with either SR proteins or SRPK1 and their cellular localisation was not affected by E1^E4 expression (Figure 4.13). To look at potential co-localisation during the HPV-16 life cycle, differentiated W12E cells were co-stained with anti-E1^E4 and antibodies recognising SF2/ASF, SRp20 or SRPK1. Whilst E1^E4 could not be detected in undifferentiated W12E cells, it was shown to localise to the cytoplasm in a peri-nuclear fashion in differentiated cells (Figure 4.14). However, neither SR proteins nor SRPK1 were found to obviously co-localise with E1^E4 in differentiated W12E cells. There may be some co-localisation in the cytoplasm for each protein; however, it is difficult to confirm this at the magnification shown. Cells used in these experiments were differentiated in monolayer culture and retained nuclear speckling of SR proteins upon differentiation. Therefore, we cannot discount the possibility that should SF2/ASF and SRp20 have been shown to redistribute to the cytoplasm in these experiments, they may have co-localised with E1^E4. To determine this would require that experiments be repeated using methylcellulose differentiated W12E cells.

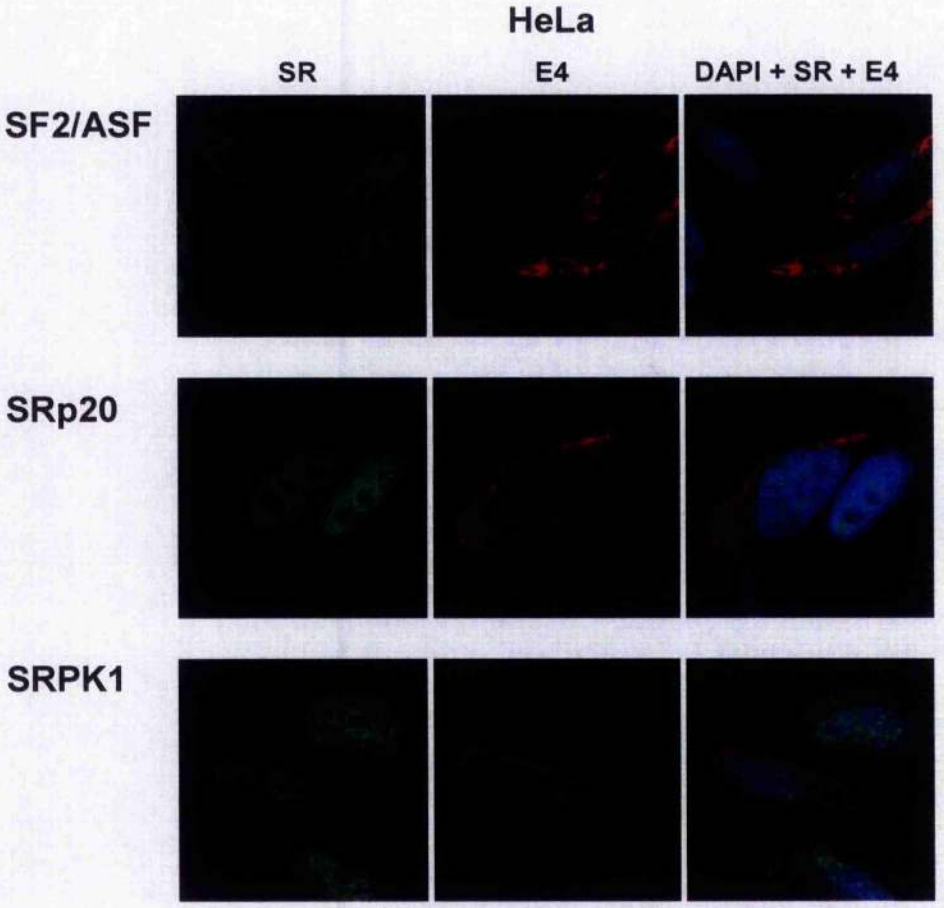


Figure 4.12: **HPV-16 E1^E4 does not co-localise with or cause redistribution of SR proteins when expressed in HeLa cells**

Immunofluorescence showing co-staining of HPV-16 E1^E4 transiently transfected HeLa cells with anti-E1^E4 and anti-SF2/ASF, anti-SRp20 or anti-SRPK1. SR proteins are shown in green, E1^E4 in red and DAPI stained nuclei are shown in blue. The SR protein stained for is given to the left. Non-transfected cells within each panel act as negative controls.

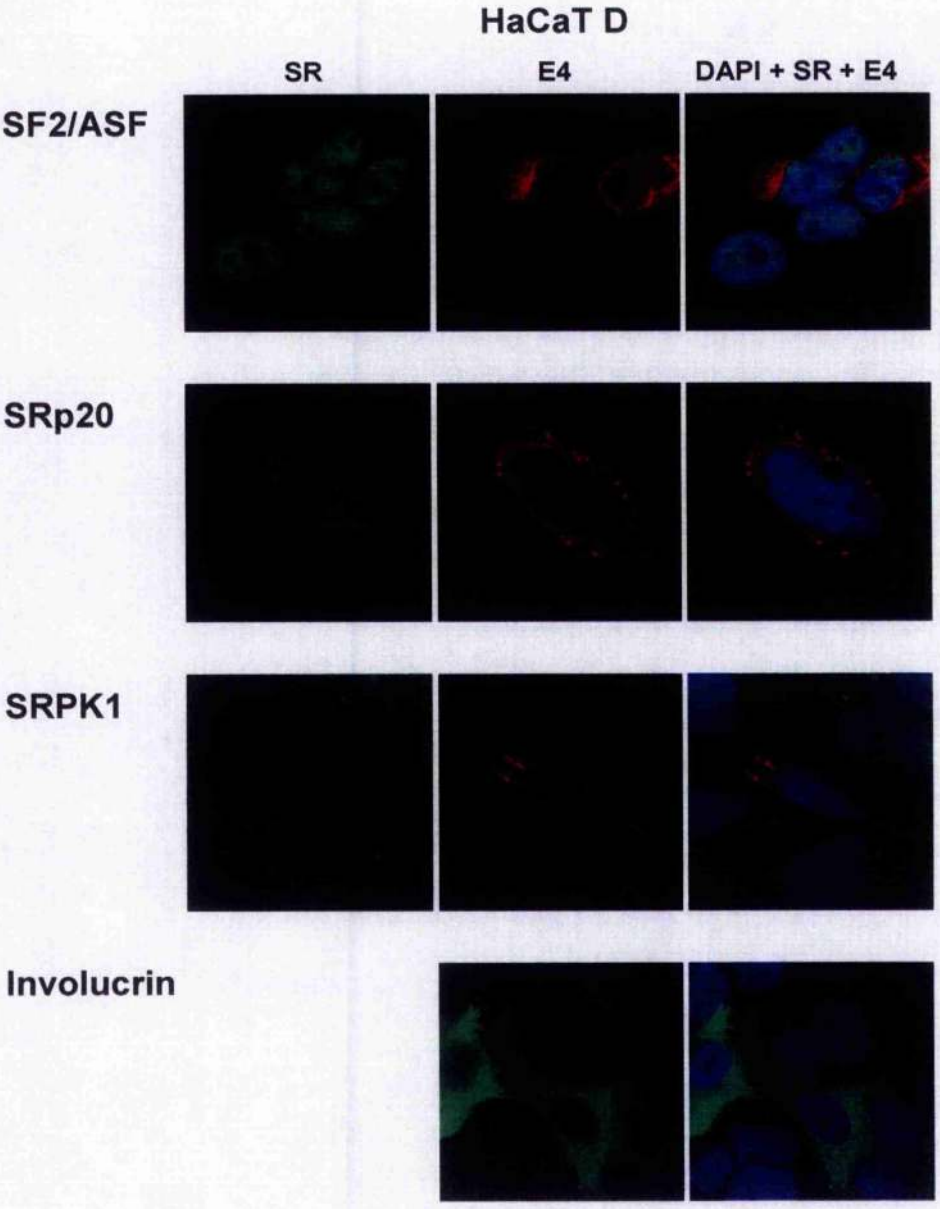


Figure 4.13: **HPV-16 E1^E4 does not co-localise with or cause redistribution of SR proteins when expressed in differentiated HaCaT cells**

Immunofluorescence showing co-staining of HPV-16 E1^E4 transiently transfected differentiated HaCaT cells with anti-E1^E4 and anti-SF2/ASF, anti-SRp20 or anti-SRPK1. SR proteins are shown in green, E1^E4 in red and DAPI stained nuclei are shown in blue. The SR protein stained for is given to the left. Involucrin is used as a marker for differentiation and staining is shown at lower exposure due to the intensity of the signal. Non-transfected cells within each panel act as negative controls.

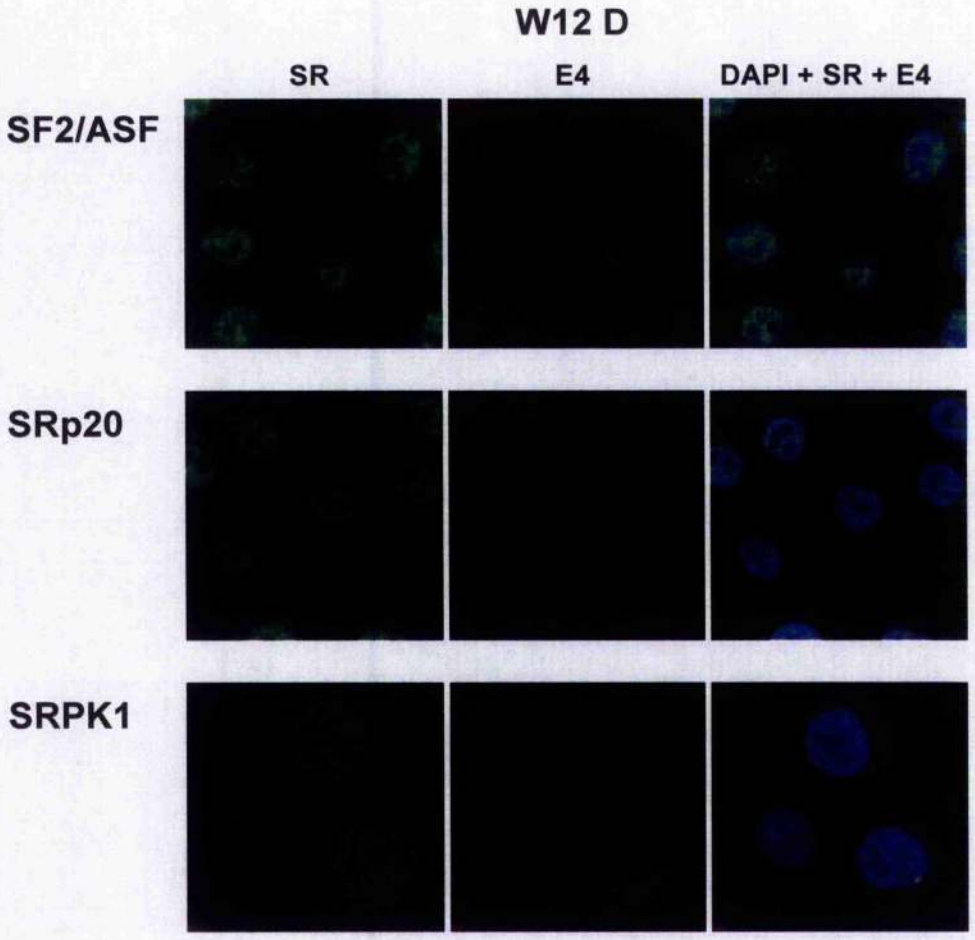


Figure 4.14: **HPV-16 E1^E4 does not co-localise with SR proteins or SRPK1 in differentiated W12E cells**

Immunofluorescence showing co-staining of HPV-16 E1^E4 and SF2/ASF, SRp20 or SRPK1 in differentiated W12E cells. SR proteins are shown in green, E1^E4 in red and DAPI stained nuclei are shown in blue. The SR protein stained for is given to the left.

4.6 Discussion

During differentiation of HPV-16 infected epithelial cells, a number of transcripts are produced by the virus, which requires extensive alternative splicing (Milligan et al., 2006). Furthermore, repression of late gene expression, which is observed in undifferentiated cells, needs to be alleviated upon differentiation. A number of *cis*-acting RNA regulatory elements are observed within the HPV-16 late region, and these are thought to function via interaction with cellular RNA regulatory proteins (Zhao et al. 2004; Rush et al. 2005). Whilst many such factors are abundant in undifferentiated epithelial cells, they are depleted upon differentiation (McPhillips et al. 2004). Therefore, it was hypothesised that HPV-16 must regulate at least a sub-set of RNA regulatory factors during differentiation, to ensure appropriate gene expression and completion of the virus life cycle. Two antagonistic factors known to be upregulated in HPV-16 infected differentiated cells are SR protein, SF2/ASF and hnRNP A1 (McPhillips et al. 2004; Veerapraditsin 2004). In contrast, these proteins are downregulated in response to differentiation of uninfected epithelial cells. Therefore, it was suggested that upregulation is a direct consequence of HPV-16 infection. Furthermore, as shown in the previous chapter, SF2/ASF is transactivated by HPV-16 E2, suggesting that this viral protein may be involved in regulating a sub-set of RNA regulatory proteins during differentiation. The work in this chapter was undertaken to determine if SR proteins other than SF2/ASF are regulated in response to HPV-16 infection, and if so, what viral proteins might be involved. As outlined above SR proteins are multifunctional proteins, with roles in splicing, mRNA export and translation, making them good targets for HPV-16 to regulate late gene expression.

Results here suggest that levels of phosphorylated SR proteins, SF2/ASF, SRp20, SRp40, SRp55 and SC35 are upregulated to differing extents in differentiated W12E cells, whereas all SR proteins are shown to maintain their phosphorylated levels or phosphorylated forms are downregulated in uninfected differentiated epithelial cells. Furthermore, stable expression of E2 in U2OS cells results in elevated levels of phosphorylated SRp75, SC35, SF2/ASF and SRp20. This suggests that HPV-16 E2 is involved in regulation of a sub-set of SR proteins during differentiation of W12E cells. To determine if these SR proteins might be regulated in a similar manner to SF2/ASF would require promoter analysis. Similar to SF2/ASF, little is known about transcriptional regulation of other SR

proteins. Therefore, a region spanning ~1kb upstream of the transcription start sites for each of the genes was analysed for homology. Each of the promoters was divergent suggesting that regulation for each may differ. In addition, each promoter was analysed using TFSearch, and like the SF2/ASF promoter, each lacks a TATA box. Furthermore, no particular TF binding site was found in abundance within any of the promoter, suggesting that SR protein expression may be regulated by a number of different TFs, potentially indicating that the promoters are found in open chromatin conformation. However, as no particular promoter elements have been identified to control E2-mediated transactivation of the SF2/ASF promoter, it was not possible to determine if similar elements were present in promoters for genes upregulated in response to E2, but absent in those that are not. The Sp1 binding site found at -575 within the SF2/ASF promoter was not found at similar position in any of the other SR protein promoters. This may suggest that either this Sp1 binding site is not necessary for E2-mediated regulation of SF2/ASF expression, or that it might act in a position independent manner to regulate E2-mediated expression of SR proteins. At least one Sp1 binding site is observed within each of the other SR protein promoters; however, this is a common TF, binding sites for which may be expected to be observed in a high proportion of promoters. In contrast, lack of Sp1 binding sites at ~-600 in each of the SR protein promoter might indicate that E2 regulates each promoter via different mechanisms. Therefore, to establish a role for E2 in transactivation of SR proteins, other than SF2/ASF, would require analysis of each promoter in turn, using similar methods as described in chapter 3.

To determine whether HPV-16 regulates localisation of SR proteins, immunofluoresence experiments using undifferentiated and differentiated W12E cells were performed. SF2/ASF, SRp20 and 9G8 are known to shuttle between the nucleus and the cytoplasm of HeLa cells (Caceres et al. 1998), and it was possible that potential redistribution of these proteins during differentiation of W12E cells may result in differential regulation of *cis*-acting elements within the HPV-16 late region. Results showed that SF2/ASF and SRp20, may redistribute from nuclear speckles to a diffuse cytoplasmic location upon differentiation. However, the results were inconsistent and may depend on the method used to differentiate cells. When cells were differentiated using methylcellulose, SF2/ASF and SRp20 redistribution was observed. In contrast, cells differentiated in monolayer culture show nuclear speckling of SR proteins in both undifferentiated and differentiated W12E cells. Methylcellulose differentiation is thought to result in

a more differentiated population of cells. Therefore, it is possible that SF2/ASF and SRp20 redistribute at a very late stage of differentiation, which is not achieved by differentiation in monolayer culture. In contrast, as the status of the viral genome was not assayed in cells used in these experiments, it is possible that the genome had integrated in some of the cells used. It is not known whether genome integration would inhibit or promote redistribution of SR proteins. Therefore, experiments would need to be repeated to determine which, if any of these possibilities, were true. Alternatively, staining patterns in raft cultured W12E tissue may give a better indication of SR protein localisation upon differentiation. The final shuttling SR, protein, 9G8, does not redistribute in any case. Shuttling of 9G8 is not dependent on continued transcription, as the protein does not get trapped in the cytoplasm when transcription is inhibited (Caceres et al. 1998). Therefore it is possible that a block to transcription is observed in differentiated W12E cells where SF2/ASF and SRp20 are redistributed to the cytoplasm. Alternatively, 9G8 is not upregulated in response to HPV-16 infection, and therefore, may simply not be a target of the virus.

An additional protein assayed for regulation by HPV-16 was SR protein kinase 1 (SRPK1). SRPK1 is shown to be upregulated in differentiated W12E cells and redistributes to the cytoplasm. In addition, SF2/ASF, SRp20, and SRPK1 were assayed for co-localisation with viral proteins E2 and E1^E4. As discussed, E2 and E1^E4 are the prime viral protein candidates for regulation of SR proteins and SRPK1, during differentiation of HPV-16 infected cells. Little apparent co-localisation was observed between E2 or E1^E4 and either SR protein or SRPK1, in transfected or W12E cells. However, SRPK1 showed distribution reminiscent of that found in differentiated W12E cells, in U2OS B1 cells, which stably express high levels of E2. This may indicate that E2 has a role in controlling SRPK1 localisation in differentiation W12E cells. For E1^E4, whilst co-localisation with both SR proteins and SRPK1 in the cytoplasm of differentiated W12E cells cannot be discounted, no redistribution of any of the three proteins was observed in transiently transfected epithelial cells. Furthermore, no redistribution of SR proteins was observed in differentiated W12E cells in co-staining experiments. Therefore, it is possible that should redistribution have been observed, so too might have greater co-localisation. However, similar experiments were not performed for E2, due to the lack of antibodies able to detect the lower expression of E2 in W12E cells, when compared with transfected cells. Therefore, whilst these experiments showed little co-localisation between SR proteins and SRPK1

and viral proteins E2 and E1^E4, it is possible that they do interact. For example, it is feasible that a low level of co-localisation exists, which is difficult to detect by immunofluorescence. Therefore, it may be useful to determine potential association of the proteins in co-immunoprecipitation experiments. This could be done using differentiated W12E cells for E1^E4, since antibodies are available which can detect the protein in these cells. Conversely, SF2/ASF, SRp20 and SRPK1 antibodies could be used in these cells, to determine association with both E2 and E1^E4. Although antibodies detecting E2 by immunofluorescence in W12E cells were not available, E2 can be detected by western blotting in W12E cells. In addition, co-immunoprecipitation between E2 or E1^E4 and SF2/ASF, SRp20 or SRPK1 in transiently transfected epithelial cells, such as differentiated HaCaTs could be determined. These experiments may indicate whether E2 and/or E1^E4 are involved in SF2/ASF, SRp20 and SRPK1 regulation during the HPV-16 life-cycle.

In summary, whilst a sub-set of levels of phosphophorylated SR protein are shown to be upregulated in response to HPV-16 E2 expression, potential redistribution of shuttling SR proteins upon differentiation of W12E cells remain inconclusive. Therefore, a number of experiments are required to determine how SR proteins are regulated during the HPV-16 life cycle, and what viral proteins, if any, are involved.

5 The role of SF2/ASF in LRE-mediated control of late gene expression

5.1 Introduction

SF2/ASF is upregulated and hyperphosphorylated in HPV-16 infected differentiated epithelial cells where capsid proteins (e.g. L1 and L2) are also produced (McPhillips et al. 2004). L1 and L2 transcripts can be detected in less differentiated cells (Milligan et al., 2006), however, *cis*-acting RNA elements within the late region of the HPV-16 genome may act to prevent their expression (Kennedy et al. 1990; Kennedy et al. 1991; Cumming et al. 2003; Oberg et al. 2003; Zhao et al. 2004; Rush et al. 2005; Zhao et al. 2005). One such element, the LRE, which resides at the 3' end of the L1 coding region and spans into the late 3' UTR, has been shown to confer negative regulatory activity upon reporter gene constructs in undifferentiated epithelial cells (Kennedy et al. 1991; Dietrich-Goetz et al. 1997; Cumming et al. 2003). As SF2/ASF is shown to associate with the LRE, in both undifferentiated and differentiated W12E cells (Veerapraditsin 2004), it is hypothesized that its upregulation and hyperphosphorylation may act to activate the LRE in differentiated cells and hence aid production of late transcripts.

The LRE is known to confer ~20 fold negative regulatory activity upon reporter gene expression in undifferentiated epithelial HeLa cells (Cumming et al. 2003). It has been proposed that this is attributable to a number of mechanisms during post-transcriptional regulation of gene expression. In HPV-16 infected undifferentiated epithelial cells (W12E), polyadenylation of late transcripts is inhibited (Milligan et al., 2006). Whilst unprocessed RNA encoding late genes L1 and E1^E4 were detected in total RNA from undifferentiated and differentiated W12E cells, polyadenylated mRNAs for L1 and E1^E4 were detected only in differentiated W12E cells. In contrast to the *in vivo* situation, no such inhibition of polyadenylation was observed *in vitro,* when comparing constructs containing and lacking the LRE (Kennedy et al. 1990). However, these experiments used HeLa extracts, and therefore do not reflect the real situation observed in W12E cells. HeLa cells are undifferentiated epithelial cells; however, they are highly transformed and express high levels of cellular RNA processing factors, which may be involved in regulation HPV-16 late polyadenylation via the LRE. Therefore, it is possible that high levels of expression of polyadenylation factors in

HeLa cell extracts allow polyadenylation mediated by the LRE, whilst lower expression levels in W12E cells may not. It is also possible that *cis*-acting RNA elements other than the LRE, which are not present in the RNA substrates used in *in vitro* experiments, conributed to inhibition of polyadenylation. However, to date no repressive sequences within the 3' region of late RNAs, other than the LRE, have been discovered. In addition to its effects on polyadenylation, the LRE has also been implicated in RNA stability. Whereas constructs lacking the LRE were stable for more than 90 minutes in *in vitro* mRNA decay assays, the half life of LRE containing transcripts was ~30 minutes (Kennedy et al. 1991). This has recently been confirmed *in vivo* (Veerapraditsin et al., manuscript in preparation). This suggests that the LRE acts as an instability element in undifferentiated epithelial cells. In addition, whilst there is no evidence to date implicating the LRE in translational regulation of late transcripts, it is possible that the LRE may enhance translation in differentiated W12E cells. The element is found within the 3'UTR, which has previously been shown to be involved in regulation of translation via a number of elements, such as the 15-lipoxygenase (LOX) DICE element (Ostareck-Lederer et al. 1994). Repeats of this element were shown to repress translation of the LOX mRNA via interactions with hnRNP K and hnRNP E1 (Ostareck et al. 1997). Furthermore, proteins associated with the LRE, such as SF2/ASF, have been shown to regulate translation (Sanford et al. 2004). Therefore, whilst there is some indication of how the LRE functions to repress late gene expression in undifferentiated W12E cells, how the element is then activated in differentiated cells is as yet unknown.

As mentioned the LRE associates with a number of cellular RNA regulatory factors and these are thought to mediate the functions described above. Most of the work in this area focused on *in vitro* assays using nuclear and cytoplasmic extracts from HeLa cells, and undifferentiated and differentiated W12E cells. Electrophorectic mobility shift assays (EMSAs) were used to determine what complexes are formed upon the LRE (Koffa et al. 2000; Cumming et al. 2003; Veerapraditsin 2004). In these experiments 3 complexes formed with nuclear extracts from HeLa, and undifferentiated and differentiated W12E cells. There was little change in the abundance of each complex upon differentiation, suggesting nuclear LRE complexes remain constant throughout differentiation. In contrast, whilst only one complex formed with HeLa cytoplasmic extracts, two formed in the presence of W12E cytoplasmic extracts (Veerapraditsin 2004). Furthermore, a shift was observed in complex abundance, where undifferentiated extracts formed mainly

the lower complex and differentiated extracts formed mainly the upper complex (Veerapraditsin 2004). In addition, proteins binding the LRE by UV cross-linking were shown to change upon W12E differentiation, particularly in the cytoplasm (Veerapraditsin 2004). Many more proteins from W12E differentiated cytoplasmic extracts bind the LRE than from undifferentiated cytoplasmic extracts. This suggests that complexes formed on the LRE in the cytoplasm of W12E cells change during differentiation, which likely affects the function of the element. In addition, if there are few late RNAs in the cytoplasm of undifferentiated W12E cells, complexes formed *in vitro* with W12E undifferentiated cytoplasmic extract may be of little consequence to LRE function.

The first protein found associating with the LRE was U2AF^{65}, using HeLa nuclear extracts in UV cross-linking experiments (Dietrich-Goetz et al. 1997). This was found to associate with the 3'GU rich region of the LRE, which shows similarity to the natural U2AF^{65} binding site, B2P2. B2P2 could compete for LRE binding of a 65KDa protein, suggesting this protein was indeed U2AF^{65}. Furthermore, U2AF^{65} did not cross-link with the 5' region of the LRE, suggesting it interacts specifically with the 3'GU rich region (Cumming et al. 2003). As discussed previously, U2AF^{65} is a component of the spliceosome which associates with the polypyrimidine tract upstream of a 3' splice site. Other factors involved in the spliceosome include components of the U1 snRNP complex, which associates with the 5' splice site and SR proteins, which bridge 5' and 3' splice site complexes by protein-protein interactions. Using affinity chromatography, components of U1 snRNP were found associating, predominantly with the 5' LRE, which contains 4 weak 5' splice sites (Cumming et al. 2003). These include U1A and Sm proteins, which associated via the 5' splice sites. Anti-Sm antibody was also shown to supershift complexes formed with HeLa nuclear extracts in EMSA experiments. In addition, U1 snRNA bound the LRE; however, U1-70K, which has previously been shown to interact with an inhibitory element in the BPV-1 late 3'UTR and inhibit polyadenylation (Gunderson et al. 1998), could not be detected in association with the LRE (Cumming et al. 2003). This suggests that the HPV-16 LRE does not inhibit polyadenylation via the same mechanism as the BPV-1 late 3'UTR element. However, a U1 snRNP like complex, containing, U1A and Sm proteins, and U1 snRNA, associates with the 5' LRE.

In addition to U1 snRNP and U2AF^{65}, SR protein, SF2/ASF was also shown to associate with the LRE in HeLa nuclear extracts using affinity chromatography

(McPhillips et al. 2004). Experiments showed association between the 3' LRE and SF2/ASF, via U2AF^{65}. Supershifting was observed when nuclear and cytoplasmic HeLa extracts were pre-incubated with anti-SC35 antibody, which cross-reacts with SF2/ASF (Veerapraditsin 2004). Whilst the antibody probably supershifted SF2/ASF in the cytoplasmic extracts, as SC35 is confined to the nucleus, nuclear supershifting could have been a result of SF2/ASF or SC35 recognition. Transient transfection of HeLa cells with LRE containing constructs resulted in increased co-immunoprecipitation between SF2/ASF and U2AF^{65} (McPhillips et al. 2004). This suggests that U2AF^{65} and SF2/ASF bind the 3' LRE in complex with each other. Anti-U2AF^{65} also co-immunoprecipitated SF2/ASF more efficiently in differentiated W12E nuclear extract, when compared with undifferentiated nuclear extracts (Veerapraditsin 2004). Therefore, as a U1 snRNP like complex associates with the 5' region and U2AF^{65} interacts with the 3' LRE, this provides the potential for formation of a splicing-like complex over the LRE in the nucleus of infected cells. Furthermore, as complexes which form on the LRE are unchanged during differentiation of W12E cells (Veerapraditsin 2004), it is suggested that this splicing-like complex forms in both undifferentiated and differentiated cells. In contrast, no interaction between SF2/ASF and U2AF^{65} is observed in cytoplasmic extracts from undifferentiated or differentiated W12E cells (Veerapraditsin 2004). This suggests the splicing-like complex is formed only in the nucleus and that SF2/ASF may interact with the LRE via a different mechanism in the cytoplasm.

In addition to components of the splicing machinery, the LRE also interacts with cellular factors involved in other aspects of RNA regulation. CstF-64, a sub-unit of the cleavage stimulating factor CstF, which is known to stabilise the formation of polyadenylation and cleavage complexes, associates with the LRE (Koffa et al. 2000). Pre-incubation of HeLa nuclear extracts with anti-CstF-64 antibody resulted in disruption of only the largest complex forming on the LRE. Interaction is mediated by the CstF-64 RNA binding domain, as shown in EMSAs using GST tagged truncated CstF-64, containing only the RNA binding domain. In addition, the full CstF complex was shown to bind to sense but not antisense LRE in UV cross-linking experiments, suggesting CstF-64 is involved in binding CstF to the LRE (Koffa et al. 2000). HuR, which is involved in stabilisation and transport of mRNA from the nucleus to the cytoplasm, also interacts the LRE (Veerapreditsin et al., manuscript in preparation). Affinity chromatography and EMSAs, using HeLa extracts showed this interaction required the 3' LRE, but HuR was also able to bind the 5'LRE, although with lower affinity. Furthermore, immunofluoresence

of HuR in raft cultured W12E tissue suggested the protein localised to both the nucleus and the cytoplasm of undifferentiated and differentiated W12E cells. Overexpression of HuR in differentiated epithelial cells also resulted in production of more L1 protein (Veerapraditsin et al., manuscript in preparation). Therefore, it is possible that HuR may act to stabilise LRE containing transcripts in the cytoplasm of differentiated W12E cells.

A further protein associating with the LRE in the cytoplasm and possibly the nucleus is hnRNP A1 (Veerapraditsin 2004). hnRNP A1 is known to antagonise the function of SF2/ASF via interaction with *cis*-acting exonic splicing silencers (ESSs) (Eperon et al. 2000). ESS-hnRNP A1 interactions are involved in inhibiting binding of U1snRNP to proximal 5' splice sites. The protein can also shuttle between the nucleus and the cytoplasm and is known to affect mRNA export and stability in the cytoplasm (Hamilton et al. 1997; Izaurralde et al. 1997; Mili et al. 2001). Furthermore, a binding site for hnRNP A1 is found in the middle of the LRE, spanning the 5' and 3' regions and GST-tagged hnRNP A1 interacts directly with the LRE in EMSAs (Veerapraditsin 2004). Anti-hnRNP A1 antibody was able to shift complexes formed with undifferentiated and differentiated W12E cytoplasmic extract. However, it has not yet been determined whether a similar shift is obtained using nuclear extracts due to ribonuclease contamination of the anti-hnRNP A1 antibody solution. In addition, expression of hnRNP A1 increased upon differentiation of W12E cells (Veerapraditsin 2004). This suggests hnRNP A1 may be an important cellular factor involved in LRE activation in differentiated W12E cells.

As levels of SF2/ASF were shown to increase upon W12E differentiation (McPhillips et al. 2004), this protein is suggested to be vital in the switch from repression to activation of late gene expression by the LRE. In addition, more hyperphosphorylated SF2/ASF was detected in differentiated W12E cells, when compared with undifferentiated extracts (McPhillips et al. 2004). SF2/ASF may also redistribute from nuclear speckles, where it is predominantly found in undifferentiated W12E cells, to a more diffuse cytoplasmic distribution in differentiated W12E cells. Therefore, this change in abundance, phosphorylation status and/or localisation is hypothesised to contribute to LRE de-repression in differentiated W12E cells. SF2/ASF has roles not only during splicing but is also able to shuttle between the nucleus and the cytoplasm (Caceres et al. 1998), can regulate shuttling (Cazalla et al. 2002) and is also able to regulate translation

(Sanford et al. 2004). In undifferentiated W12E cells, where SF2/ASF is predominantly nuclear, and is found in lower abundance and in hypophosphorylated form, it is suggested that formation of the splicing-like complex over the LRE in the nucleus is involved in repression of polyadenylation (Figure 5.1A). Presence of a 5' splice site within the 3' most exon has previously been shown to repress polyadenylation *in vitro* and *in vivo* (Niwa et al. 1992) and a number of components of the splicing machinery are also known to associate with polyadenylation and cleavage factors (Lutz et al. 1996; Gunderson et al. 1997; Gunderson et al. 1998; Vagner et al. 2000). For BPV-1, binding of U1 snRNP to a 5' splice site within the late 3'UTR resulted in inhibition of polyadenylation in undifferentiated cells (Furth et al. 1994; Gunderson et al. 1998). Whilst SF2/ASF may also contribute to repression of export of LRE containing RNAs from the nucleus to the cytoplasm, it is also possible that this is a result of inhibition of polyadenylation, as non-polyadenylated mRNAs are usually not exported. However, SF2/ASF is a shuttling protein, which is thought to be transported to the nucleus in hyperphosphorylated form (Caceres et al. 1998). Hypophosphorylated SF2/ASF is thought to reside predominantly in nuclear speckles. Therefore, late transcripts may be retained in the nucleus via interaction with SF2/ASF, due to the lower abundance of the hyperphosphorylated form, and the predominant nuclear localisation of the protein, within undifferentiated W12E cells.

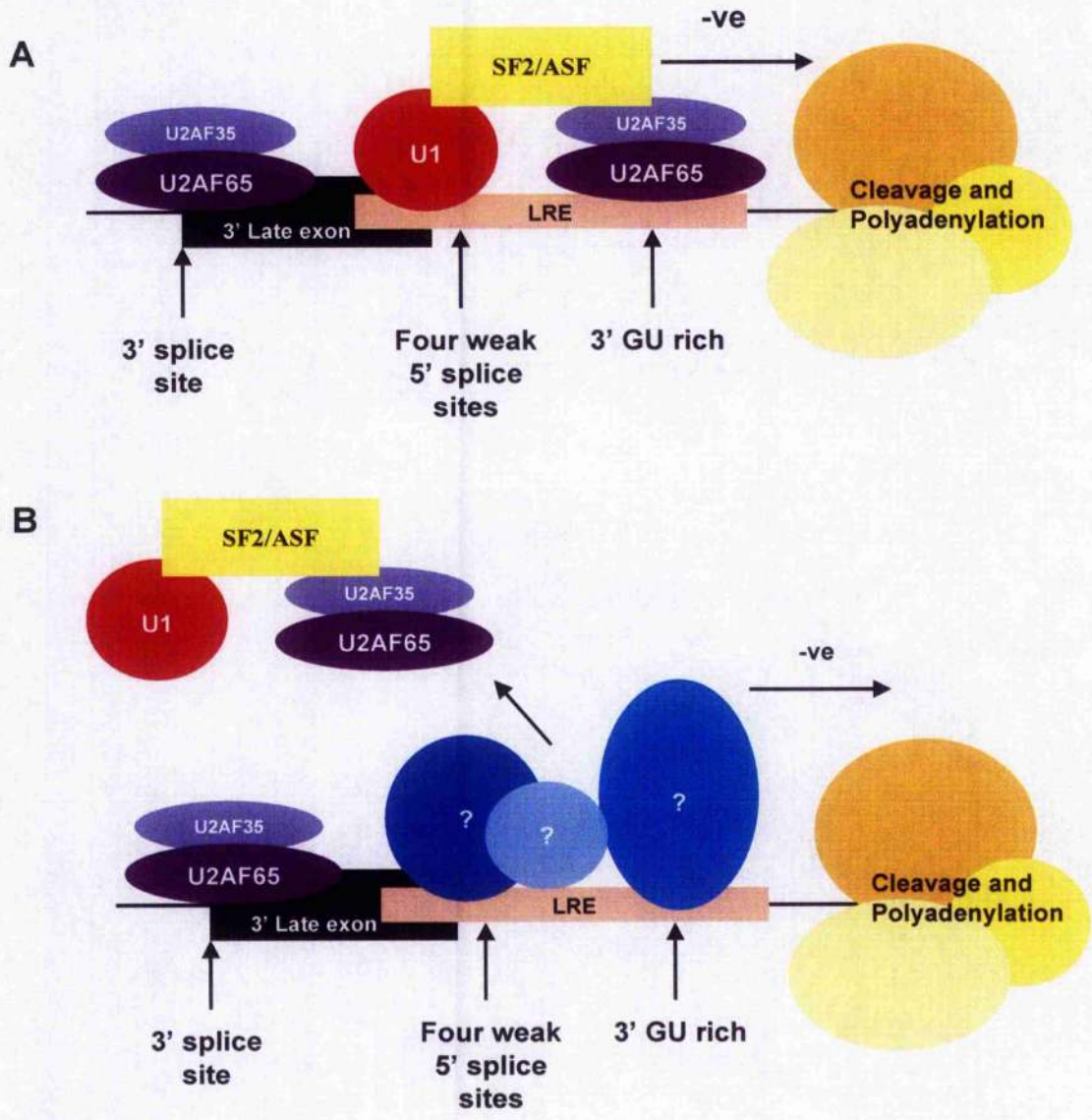


Figure 5.1: **Potential roles of SF2/ASF during repression of the LRE in undifferentiated W12E cells**

A) In undifferentiated cells SF2/ASF, in association with the splicing-like complex may cause repression of cleavage and polyadenylation by the LRE. B) Other cellular LRE binding proteins may compete with the splicing-like complex for LRE binding and this may inhibit polyadenylation.

Upregulation and hyperphosphorylation of SF2/ASF, as well as potential relocalisation, may contribute to late gene expression via the LRE in differentiated W12E cells by several mechanisms. Firstly, it is possible that the change in phosphorylation status and protein levels causes alleviation of inhibition of polyadenylation. In this instance, binding of SF2/ASF to the LRE via the splicing-like complex in the nucleus may be required. In addition, this complex may increase the efficiency of splicing of the terminal late exon (Figure 5.2A). The final HPV-16 L1 exon is long and contains a number of potential exonic splicing enhancers and silencers (ESEs and ESSs) (Figure 4.1). In the absence of additional *cis*-acting RNA signals, splicing of this exon, via terminal exon definition, may be difficult. Presence of a splicing-like complex on the LRE, may provide an additional signal required to enhance terminal exon definition, via protein-protein interactions between factors binding the LRE and those associated with final 3' splice site. Phosphorylation of SF2/ASF is known to affect protein-protein interactions (Xiao and Manley 1997; Xiao and Manley 1998). Therefore, increased hyperphosphorylation in differentiated W12E cells may enhance interactions between proteins binding the terminal 3' splice site and the splicing-like complex binding the LRE, hence increasing the efficiency of terminal exon definition. In addition, potential relocalisation of SF2/ASF in differentiated W12E cells may indicate a role for the protein in export of late transcripts. Furthermore, as discussed, SF2/ASF does not bind the LRE in association with $U2AF^{65}$ in the cytoplasm of W12E cells. Therefore, formation of an as yet identified complex over the LRE may be involved in regulating translation of late transcripts in the cytoplasm of differentiated W12E cells (Figure 5.2C).

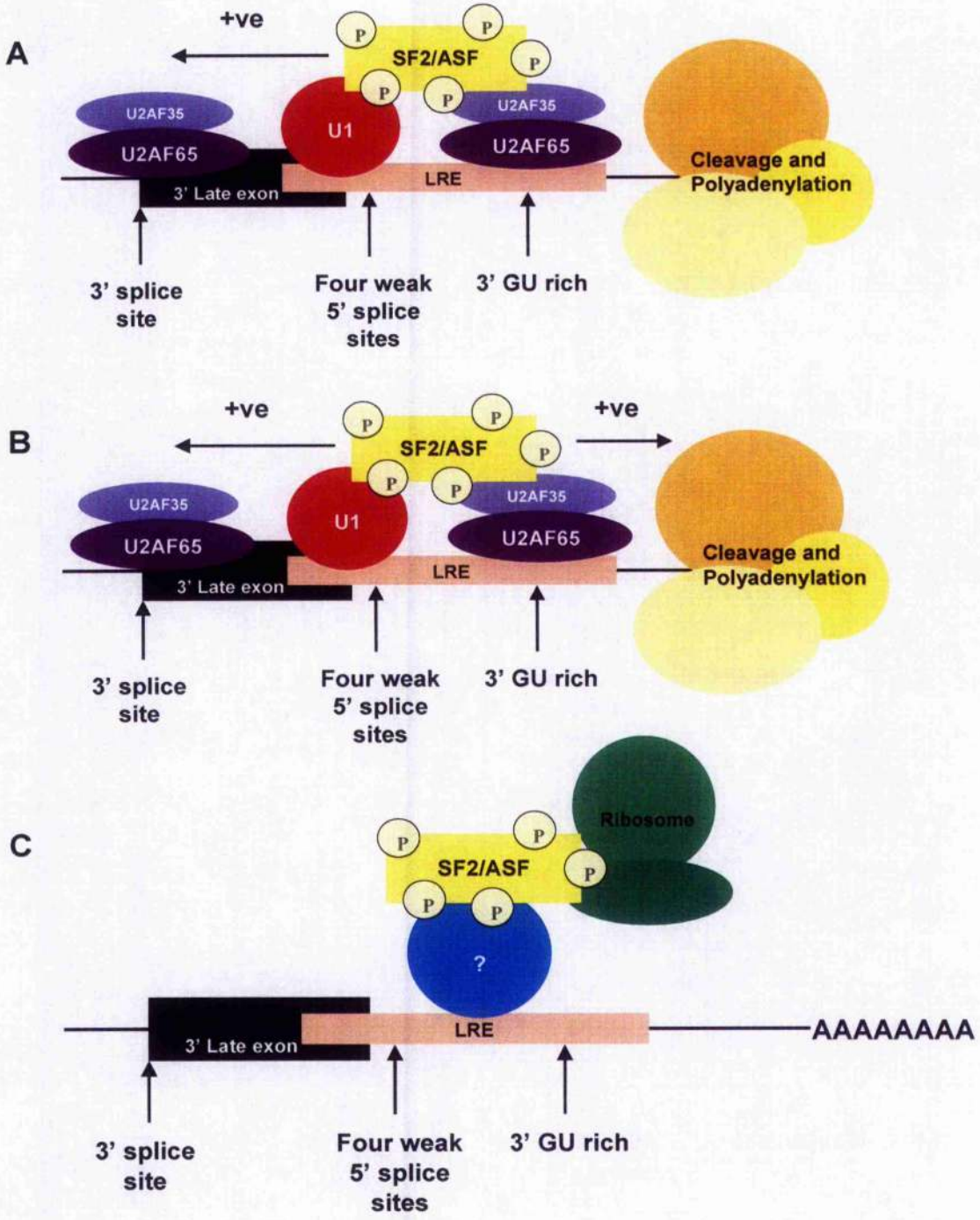


Figure 5.2: **Potential roles of SF2/ASF during de-repression of the LRE in differentiated W12E cells**

A) In differentiated cells the splicing-like complex may relieve repression of polyadenylation and positively affect terminal exon definition of the final long L1 exon. B) In addition to enhancing terminal exon definition, the LRE-bound splicing-like complex may also promote polyadenylation. C) SF2/ASF may act via a different complex in the cytoplasm to enhance translation of LRE containing transcripts

An alternative hypothesis is that the LRE-bound splicing-like complex acts only to enhance expression from LRE containing transcripts. It is possible that formation of the complex may be infrequent in undifferentiated W12E cells, and therefore may have little effect on LRE function. In these cells, other cellular proteins, which may have inhibitory effects, may out compete the splicing-like complex for LRE binding (Figure 5.1B). In contrast, elevated SF2/ASF in differentiated W12E cells may increase formation of the splicing-like complex over the LRE. In agreement with this, co-immunoprecipitation of SF2/ASF with U2AF^{65}, via which SF2/ASF associates with the LRE, is increased in differentiated W12E cells (Veerapraditsin 2004). In this case, it is suggested that the LRE-bound splicing-like complex would act not only to enhance terminal exon definition, as discussed above, but may also promote polyadenylation, export and translation (Figure 5.2B). Conversely, SF2/ASF may be elevated in differentiated W12E cells to bind *cis*-acting RNA elements, other than the LRE, within the HPV-16 late region. In this instance, the primary function of the splicing-like complex on the LRE may be to inhibit polyadenylation in undifferentiated cells.

From the data outlined above, the LRE is suggested to be one of the major *cis*-acting RNA elements regulating HPV-16 late gene expression. Furthermore, as it is regulated during HPV-16 infection, SF2/ASF is hypothesised to play an important role in LRE control. Therefore, experiments were preformed in which SF2/ASF was overexpressed in undifferentiated epithelial cells to determine if this had any affect on LRE function. Whilst the most appropriate system in which to carry out experiments would be W12E cells, this was problematic. Culturing of W12E cells is complex, they are difficult to transfect and they remain undifferentiated only at low density. However, these experiments are being optimised and can be attempted in the future. Therefore, as model systems, HeLa cells and undifferentiated HaCaT cells were used. To investigate a role for SF2/ASF in LRE control, reporter constructs fused to the HPV-16 late 3'UTR were transiently transfected into these cells, along with SF2/ASF expression vectors. Reporter protein activity, as well as total and polyadenylated RNA levels were measured and compared between cells overexpressing SF2/ASF and those expressing endogenous levels. It was hypothesised that elevated levels of SF2/ASF would enhance polyadenylation of LRE containing transcripts, which would potentially result in increased protein expression.

5.2 Expression from reporter constructs containing the LRE is not affected by SF2/ASF overexpression in undifferentiated epithelial cells

To investigate the possibility that upregulation of SF2/ASF expression is involved in LRE activation in differentiated W12E cells, SF2/ASF was overexpressed in undifferentiated epithelial cells. Although this does not recreate the W12E differentiated cell environment, it does allow us to assess whether SF2/ASF alone can regulate the LRE, simply via expression levels. However, it should be noted that SF2/ASF is already expressed in HeLa to levels similar to those found in differentiated W12E cells (McPhillips et al. 2004), suggesting that SF2/ASF regulated activation of the LRE may already occur within these cells to some extent. Reporter constructs containing the HPV-16 late 3'UTR, either including or lacking the LRE, were used to establish if overexpression of SF2/ASF could affect regulation by the LRE. These constructs are derived from pLW2, a CAT expression vector driven by the Herpes simplex virus immediate early (HSVIE) 5 promoter and containing and SV40 polyadenylation signal. The SV40 poly (A) signal was removed and replaced with the 3'UTR from HPV-16, either including (pLW2+LRE) or lacking the LRE (pLW2-LRE) (Figure 5.3). The pLW2-LRE construct lacks the 5' region of the late 3'UTR, up to the SspI restriction site, which contains the LRE. These constructs were then co-transfected into HeLa cells with pCG, pCG-SF2 or pCG-T7-SF2. The latter two constructs express SF2/ASF and T7 tagged SF2/ASF, respectively, whilst pCG is an empty vector. Both SF2/ASF and T7 tagged SF2/ASF were used as they express different levels of phospho-forms of SF2/ASF (Figure 5.4B). Protein expression from pCG-SF2 resulted in both hypo and hyperphosphorylated forms in roughly equal quantities, whereas the T7 tagged form of SF2/ASF seemed to result in expression of predominantly hypophosphorylated forms. As SF2/ASF is hyperphosphorylated upon differentiation of W12E cells, it was thought the use of these two expression vectors might result in differences with respect to LRE activation. Furthermore, CAT activity from cells transfected with pBluescript and pLW2 were used as negative and positive controls respectively (data not shown). As shown in figure 5.4A, loss of the LRE resulted in ~40 fold upregulation of reporter gene activity, as expected. However, CAT activity did not differ significantly upon SF2/ASF overexpression, either from constructs containing or lacking the LRE.

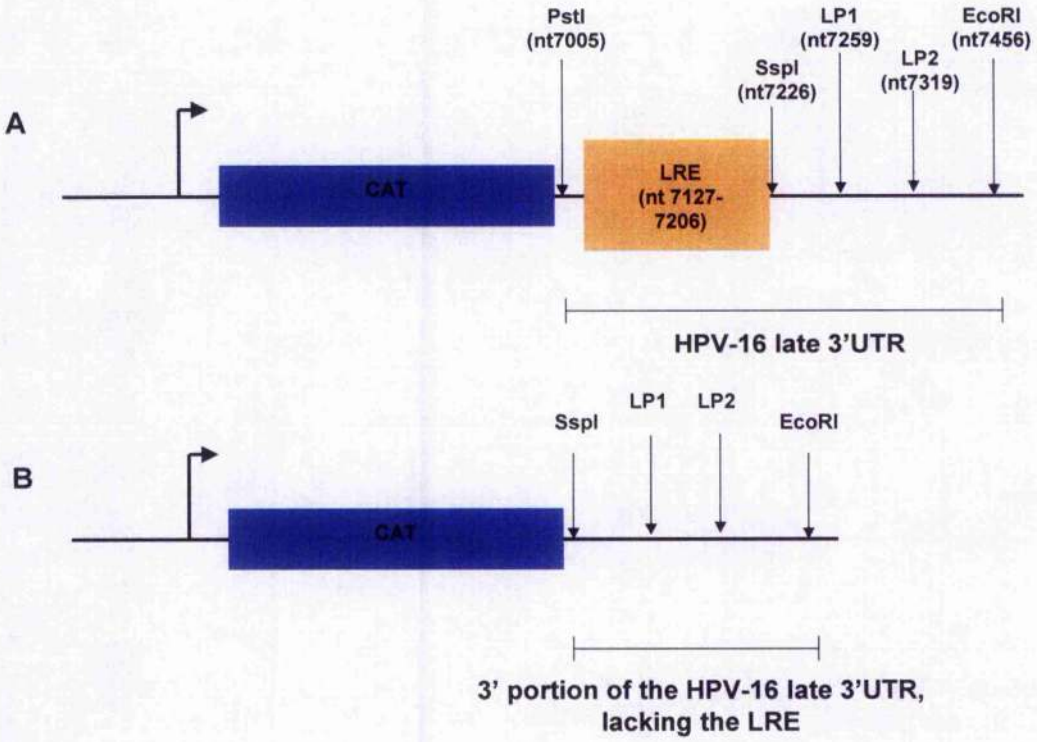


Figure 5.3: **pLW2+/-LRE**

Schematic diagrams of the CAT reporter constructs containing and lacking the LRE. A) 3'UTR from HPV-16 from nt 7005-7456, between PstI and EcoRI restriction sites, cloned into pLW2. This region contains the LRE (orange rectangle) and the two late polyadenylation signals (LP1 and LP2). B) 3'UTR from HPV-16 from nt 7226-7456, between SspI and EcoRI restriction sites, cloned into pLW2. This region contains the two late polyadenylation signals but lacks the LRE.

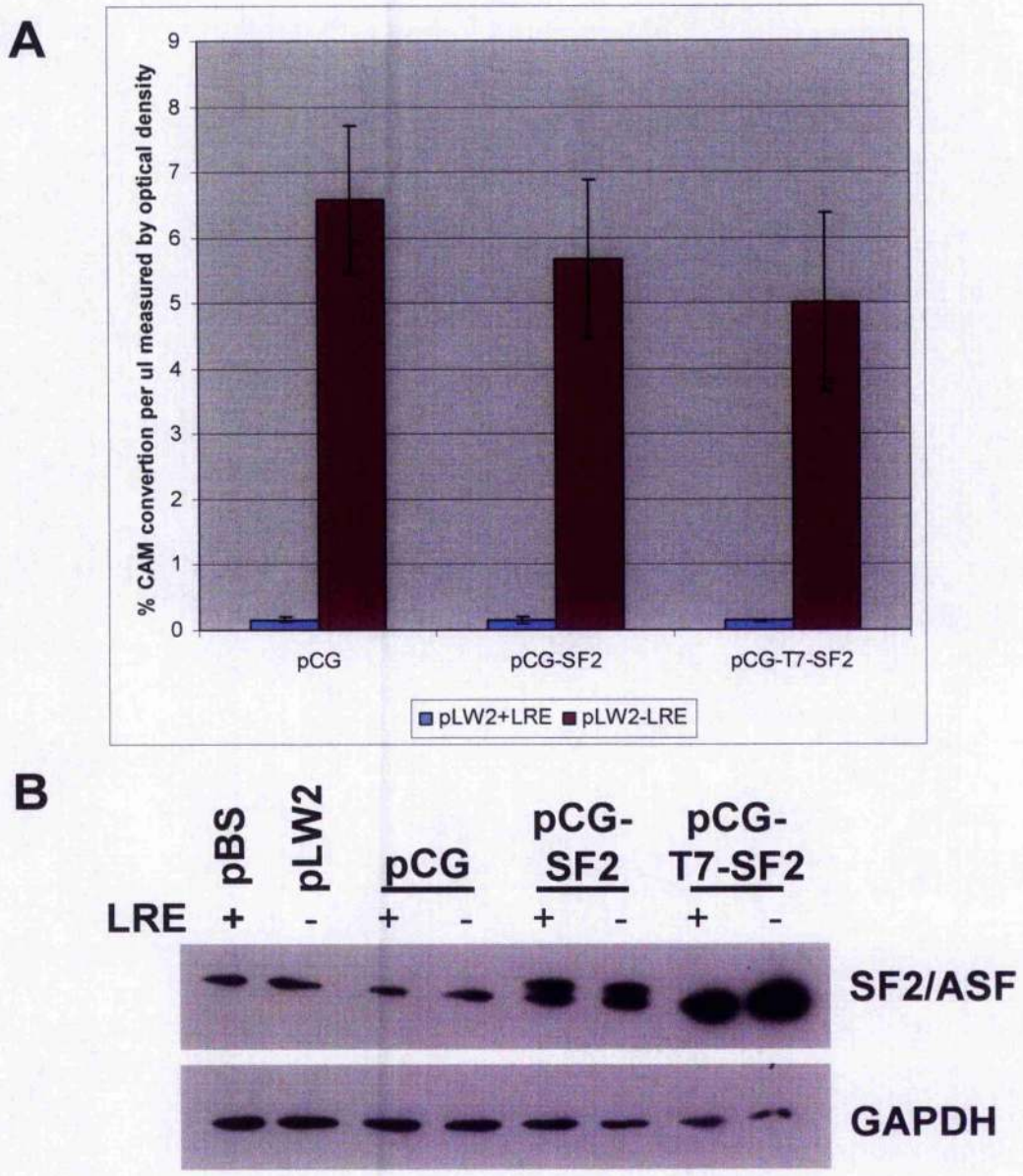


Figure 5.4: **Reporter gene activity from constructs fused to the HPV-16 3'UTR, containing the LRE, is not affected by overexpression of SF2/ASF**

A) Protein lysates were produced from HeLa cells co-transfected with 400ng pLW2+/-LRE and 250ng pCG, pCG-SF2 or pCG-T7-SF2 and CAT assays were performed. Transfections were performed in triplicate and the graph represents mean and standard deviation about the mean from three separate experiments. B) Western blots using equal quantities of protein lysate from HeLa cells transfected with pCG, pCG-SF2 or pCG-T7-SF2 and either pLW2+/-LRE probed using anti-SF2/ASF and anti-GAPDH antibodies. GAPDH acts as a loading control.

Although there was slight downregulation of CAT activity upon overexpression of SF2/ASF from both constructs, this was found to be statistically insignificant with SF2/ASF (t=1.45) and between p=0.01 and p=0.05 for T7 tagged SF2/ASF (t=2.34). This suggests SF2/ASF overexpression has little effect on protein expression from constructs containing the HPV-16 late 3'UTR in HeLa cells as model undifferentiated epithelial cells.

Although LRE-containing constructs used in the above experiments contain the late 3'UTR from HPV-16, they lack other processing signals such as introns, which is uncommon for eukaryotic genes. Furthermore, as discussed in the introduction to this chapter it is hypothesised that LRE complexes are involved in terminal exon definition of the final long L1 exon. Therefore, a chimaeric β-globin intron (taken from pCI-neo – Promega) was inserted within the 5'UTR of CAT expression constructs containing the HPV-16 late 3'UTR (pLW2(intron)+LRE and pLW2(intron)-LRE) (Figure 5.5). This time, the LRE was precisely deleted from the pLW2(intron)-LRE construct. Addition of the intron caused increased CAT expression and smaller amounts of each plasmid were used in transfections due to saturation of CAT activity. This was not unexpected, as transcripts containing introns are expressed more efficiency due to their association with cellular RNA regulatory factors. However, expression from LRE containing constructs, was still reduced ~10-15 fold in comparison with constructs lacking the LRE (Figure 5.6A). The extent of repression by the LRE was alleviated slightly by addition of the intron. CAT expression from pLW2-LRE is already very high, and addition of the intron had little effect. This suggests that transcripts expressed from constructs lacking the LRE may be translated at close to maximal rate, and production and/or export of more transcripts may make little difference. Expression of SF2/ASF from constructs pCG-SF2 and pCG-T7-SF2 gave different patterns of phosphorylation compared with previous experiments (compare figure 5.4B with figure 5.6B). Although this may reflect differing phosphorylation patterns, it could also reflect degradation of SF2/ASF in these samples. Furthermore, overexpression of SF2/ASF resulted in increased CAT activity from both constructs. This was not unexpected, as SF2/ASF will associate with intron complexes, allowing more efficient RNA processing and possibly translation of the reporter constructs. Greater CAT activity was observed from both LRE containing and lacking constructs when T7 tagged SF2/ASF was expressed and the increase from pLW2(intron)-LRE was found to be significant between p=0.01 and p=0.001

(t=3.07). However, results show a general increase was observed with constructs containing or lacking the LRE, suggesting SF2/ASF is not acting via the LRE to increase CAT activity.

To ensure the LRE could not be activated by SF2/ASF overexpression, similar experiments were performed in undifferentiated HaCaT cells. HaCaT cells are not transformed and therefore, are more akin to undifferentiated W12E cells, in terms of SF2/ASF expression, in which the natural pattern of HPV-16 infection is observed, than HeLa cells. As discussed in the introduction to this chapter, undifferentiated W12E cells were not used due to difficulties in transfecting them. Furthermore, HeLa cells express large quantities of SF2/ASF, similar to those observed in differentiated W12E cells (McPhillips et al. 2004). Therefore, it was possible that no affect was observed during overexpression of SF2/ASF in HeLa cells as endogenous levels were already above the critical threshold, and the LRE was already being activated to some extent. In contrast, HaCaT cells express lower levels of SF2/ASF compared with HeLa and differentiated W12E cells, and expression closely resembles that observed in undifferentiated W12E cells (McPhillips et al. 2004). Figure 5.7 shows co-transfection experiments of HaCaT cells with pLW2(intron)+/-LRE and SF2/ASF expression constructs. Whilst CAT activity was inhibited ~20 fold from the construct containing the LRE, CAT activity from constructs containing and lacking the LRE is elevated upon overexpression of T7 tagged SF2/ASF. It is possible non-tagged overexpressed SF2/ASF did not have the same affect due to the abundance of the hyperphosphorylated form, compared with expression from the T7 tagged version. Expression of T7-SF2/ASF seemed to shift to a more hyperphosphorylated form when co-transfected with pLW2-LRE (Figure 5.7B). However, this is more likely due to the way the gel has run, as this sample was loaded at the edge. When this sample was run within the centre of the gel, T7-SF2/ASF expression did not differ between pLW2+/-LRE co-transfected samples (data not shown). Upregulation of CAT activity was statistically significant between p=0.01 and p=0.001 for pLW2+LRE (t=9.18) and above p=0.01 for pLW2-LRE (t=16.70) using ANOVA. However, using student's t tests, significance was below p=0.001 in both cases (pLW2+LRE, t=0.15; pLW2-LRE, t=3.08). This again suggests that T7-SF2/ASF is acting on regions of the construct other than the LRE. Therefore, we conclude from the above experiments that overexpression of SF2/ASF in undifferentiated epithelial cells has no LRE dependent effect on protein production from constructs containing the HPV-16 late 3'UTR, using uninfected undifferentiated epithelial cells as a model.

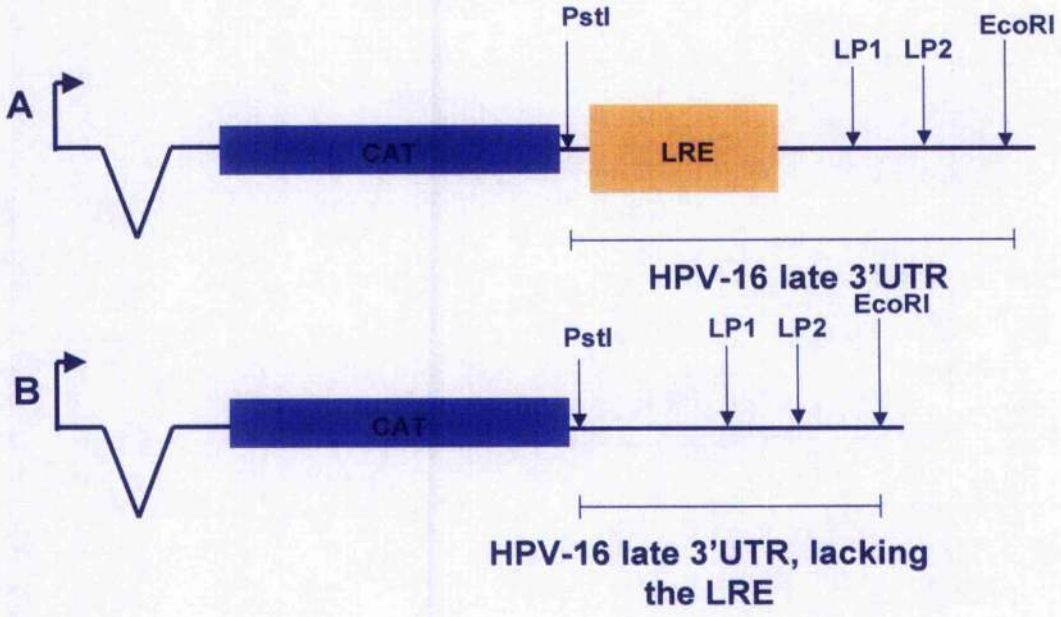


Figure 5.5: **pLW2(intron)+/-LRE**

Schematic diagrams of the CAT reporter constructs containing and lacking the LRE, with the addition of an intron in the 5'UTR. A) 3'UTR from HPV-16 from nt 7005-7456, between PstI and EcoRI restriction sites, cloned into pLW2. This region contains the LRE (orange rectangle) and the two late polyadenylation signals (LP1 and LP2). B) 3'UTR from HPV-16 from nt 7005-7456, between PstI and EcoRI restriction sites, with nt 7127-7206, encompassing the LRE precisely deleted, cloned into pLW2. This region contains the two late polyadenylation signals but lacks the LRE.

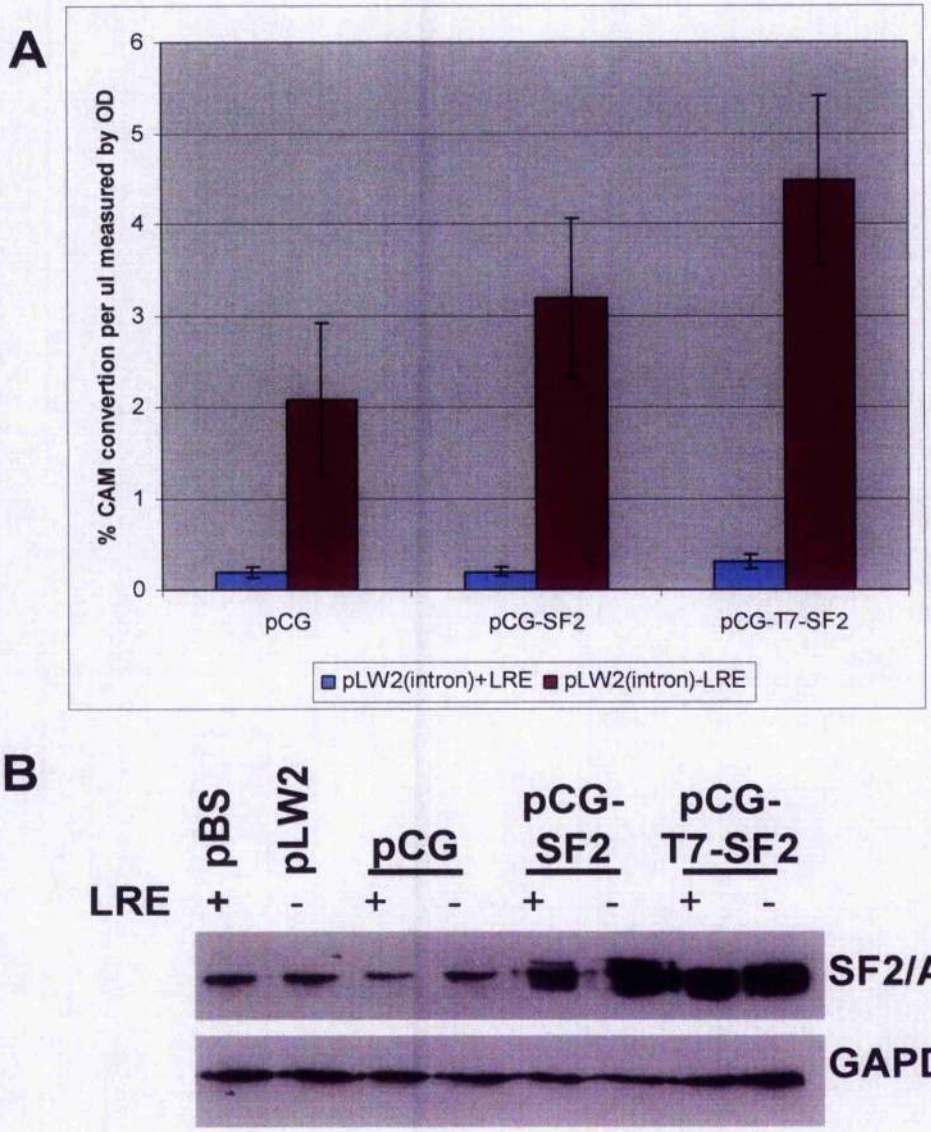


Figure 5.6: **Insertion of an intron into the 5'UTR of reporter constructs containing the LRE does not alter the affect of SF2/ASF in HeLa cells**

A) Protein lysates were produced from HeLa cells co-transfected with 150ng pLW2(intron)+/-LRE and 250ng pCG, pCG-SF2 or pCG-T7-SF2 and CAT assays were performed. Transfections were performed in triplicate and the graph represents mean and standard deviation about the mean from three separate experiments. B) Western blots using equal quantities of protein lysate from HeLa cells transfected with pCG, pCG-SF2 or pCG-T7-SF2 and either pLW2(intron)+/-LRE probed using anti-SF2/ASF and anti-GAPDH antibodies. GAPDH acts as a loading control.

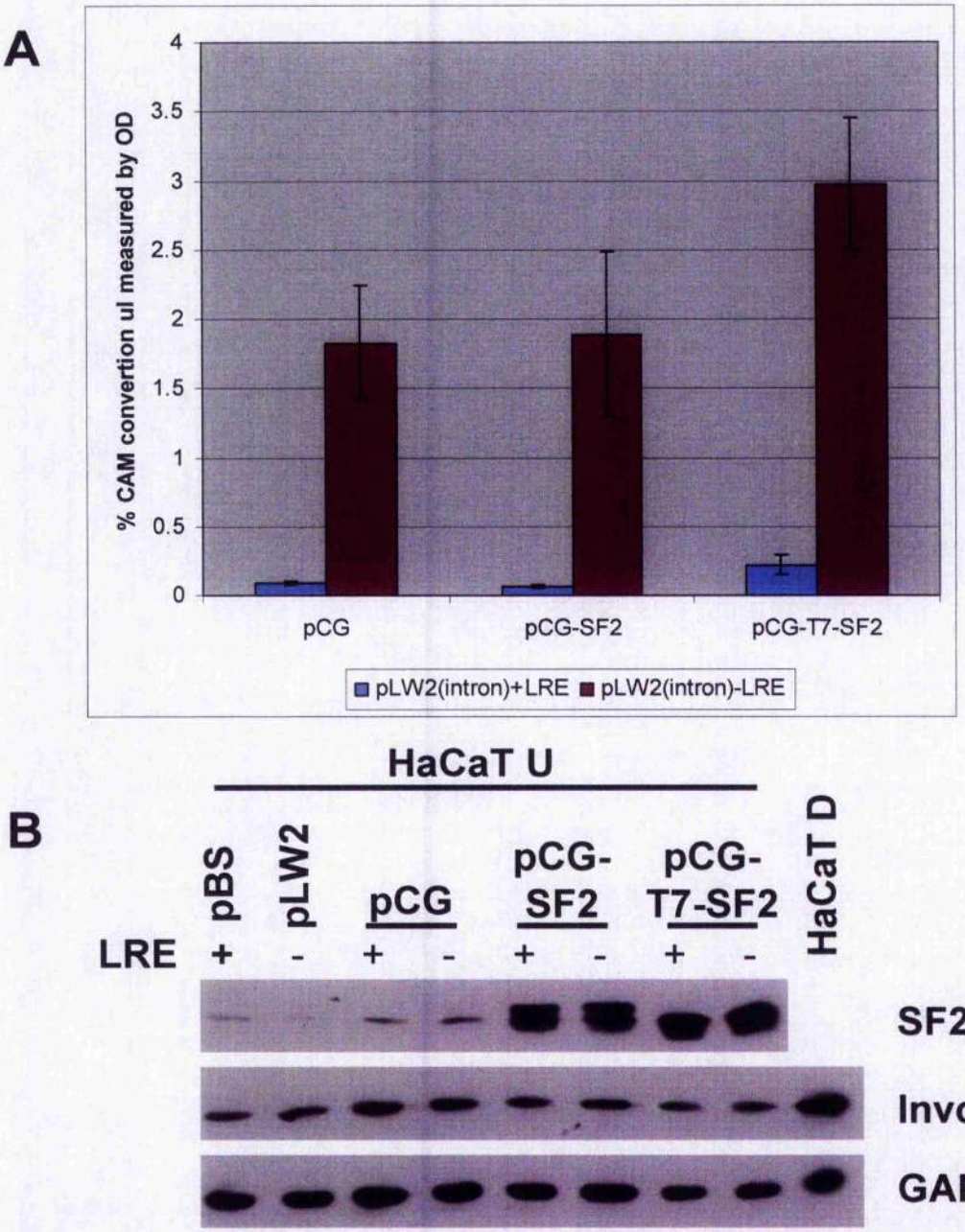


Figure 5.7: **SF2/ASF overexpression affects expression from both constructs containing and lacking the LRE in undifferentiated epithelial cells**

A) Protein lysates were produced from undifferentiated HaCaT cells co-transfected with 150ng pLW2+/-LRE and 250ng pCG, pCG-SF2 or pCG-T7-SF2 and CAT assays were performed. Transfections were performed in triplicate and the graph represents mean and standard deviation about the mean from three separate experiments. B) Western blots using equal quantities of protein lysate from undifferentiated HaCaT cells transfected with pCG, pCG-SF2 or pCG-T7-SF2 and either pLW2+/-LRE probed using anti-SF2/ASF and anti-GAPDH antibodies. GAPDH acts as a loading control.

5.3 The LRE may affect RNA stability and polyadenylation, as well as export and/or translation

Although no response was observed during SF2/ASF overexpression from LRE containing transcripts at the level of reporter protein activity, it is possible that SF2/ASF may affect RNA processing without affecting protein production. As discussed there are a number of RNA regulatory proteins associated with the LRE, each of which may have roles during differentiation at different post-transcriptional stages. For instance, it is possible that LRE containing transcripts are unstable in SF2/ASF overexpressing undifferentiated epithelial cells. In addition, upregulation of SF2/ASF may allow more efficient polyadenylation of late transcripts; however, protein expression may not be elevated due to inhibition of other post-transcriptional processes, such as export and/or translation. Therefore, to look at post-transcriptional regulation of LRE containing transcripts in a bit more detail, levels of polyadenylated and total CAT RNA were determined in HaCaT cells co-transfected with SF2/ASF expression constructs and pLW2(intron)+/-LRE. To do this, total RNA was extracted from cells and RT-PCR was carried out using either random primers for total RNA, or oligo(dT) for polyadenylated (polyA+) RNA. The primers used in PCRs amplify an~200bp fragment within the 3' region of the CAT gene (CAT3' F & R). In reactions, GAPDH was used as an internal control, to ensure equal levels of RNA were added from each sample. Following standard RT-PCR, quantitative RT-PCR (q-RT-PCR) was performed to accurately compare RNA levels between treatments. Figures 5.8 and 5.9 show the results, with –RT reactions being performed to control for the effects of DNA contamination.

Firstly, looking at total RNA levels in cells expressing only endogenous SF2/ASF, figure 5.8 shows little difference between constructs including or lacking the LRE. This suggests transcription and potentially RNA stability are unaffected by the LRE. However, upon overexpression of SF2/ASF, total CAT RNA produced from pLW2(intron)-LRE is highly elevated (~15 fold), whereas, total CAT RNA produced from pLW2(intron)+LRE is elevated only modestly (~5 fold). This suggests that SF2/ASF affects either transcription rate and/or stability of these transcripts and that the LRE may act to counteract this. In contrast to total RNA levels, polyA+ RNA increased ~3.2 fold in cells transfected with pLW2(intron)-LRE, when compared with pLW2(intron)+LRE (Figure 5.9). This suggests the LRE may inhibit

polyadenylation from the late HPV-16 polyadenylation signal. However, the modest increase in polyA+ RNA levels from constructs lacking the LRE does not account for the ~20 fold increase in protein production shown above (Figure 5.7). Therefore, it is likely the LRE also acts at other processing stages, such as during export or translation. Interestingly when SF2/ASF is overexpressed, polyA+ RNA is elevated from both constructs (~5-7 fold), although to a greater extent from pLW2(intron)+LRE when T7-tagged SF2/ASF is expressed (~14 fold) (Figure 5.9). However, looking at pLW2(intron)+LRE only, the difference between the two SF2/ASF expression vectors may simply reflect the amount of total RNA, as the percentage of total RNA to be polyadenylated is roughly the same from each (Table 5.1). Furthermore, the percentage of total RNA produced from pLW2(intron)+LRE which is polyadenylated is increased ~3-4 fold upon SF2/ASF overexpression. This suggests that elevated levels of SF2/ASF may enhance polyadenylation at the HPV-16 late polyadenylation signal via the LRE. In addition, whilst polyA+ RNA levels are seen to increase upon SF2/ASF overexpression from pLW2(intron)-LRE, the percentage of total RNA which is polyadenylated decreases by ~60% (Table 5.1). This may suggest that the level of polyA+ RNA produced from pLW2(intron)-LRE is almost at saturation and the majority of total RNA is not polyadenylated or used for protein production. The increase in polyA+ RNA produced from pLW2(intron)-LRE is reflected in results at the protein level, where there is an increase in CAT activity upon SF2/ASF overexpression.

Transfection	pCG	pCG-SF2	pCG-T7-SF2
pLW2(intron)+LRE	5.4	20.3	18.1
pLW2(intron)-LRE	14.6	5.7	6.2

Table 5.1: Percentage of total RNA that is polyadenylated for each co-transfection.

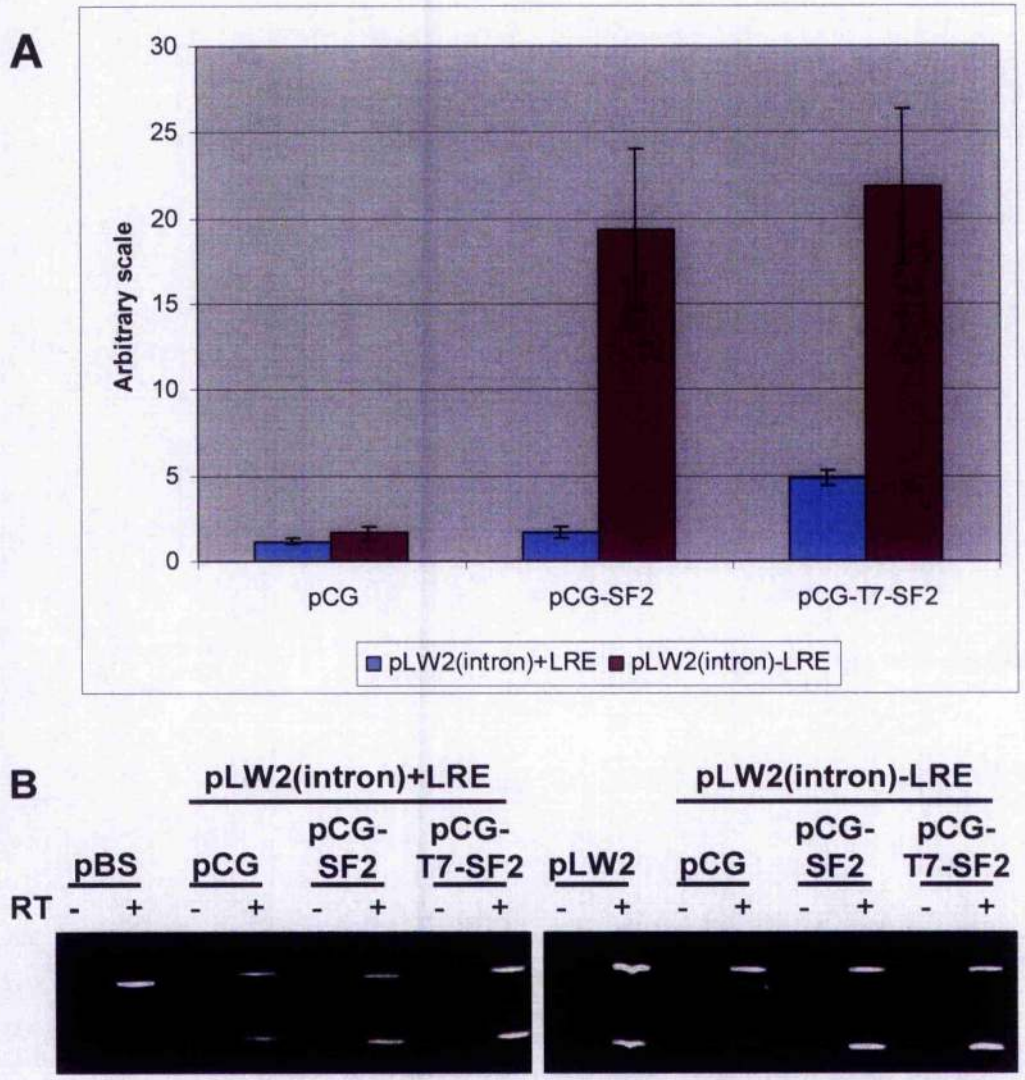


Figure 5.8: **Levels of total reporter gene RNA upon overexpression of SF2/ASF**

A) Total RNA was produced from undifferentiated HaCaT cells co-transfected with pLW2+/-LRE and pCG, pCG-SF2 or pCG-T7-SF2 and total levels of CAT RNA were assayed using quantitative RT-PCR. Transfections were performed in duplicate and the graph represents mean and standard deviation about the mean from three separate experiments. Results are normalised against GAPDH. B) Semiquantitative RT-PCR using total RNA samples from undifferentiated HaCaT cells transfected with pCG, pCG-SF2 or pCG-T7-SF2 and either pLW2+/-LRE. Primers amplify within CAT (lower band) and GAPDH (upper band) transcripts.

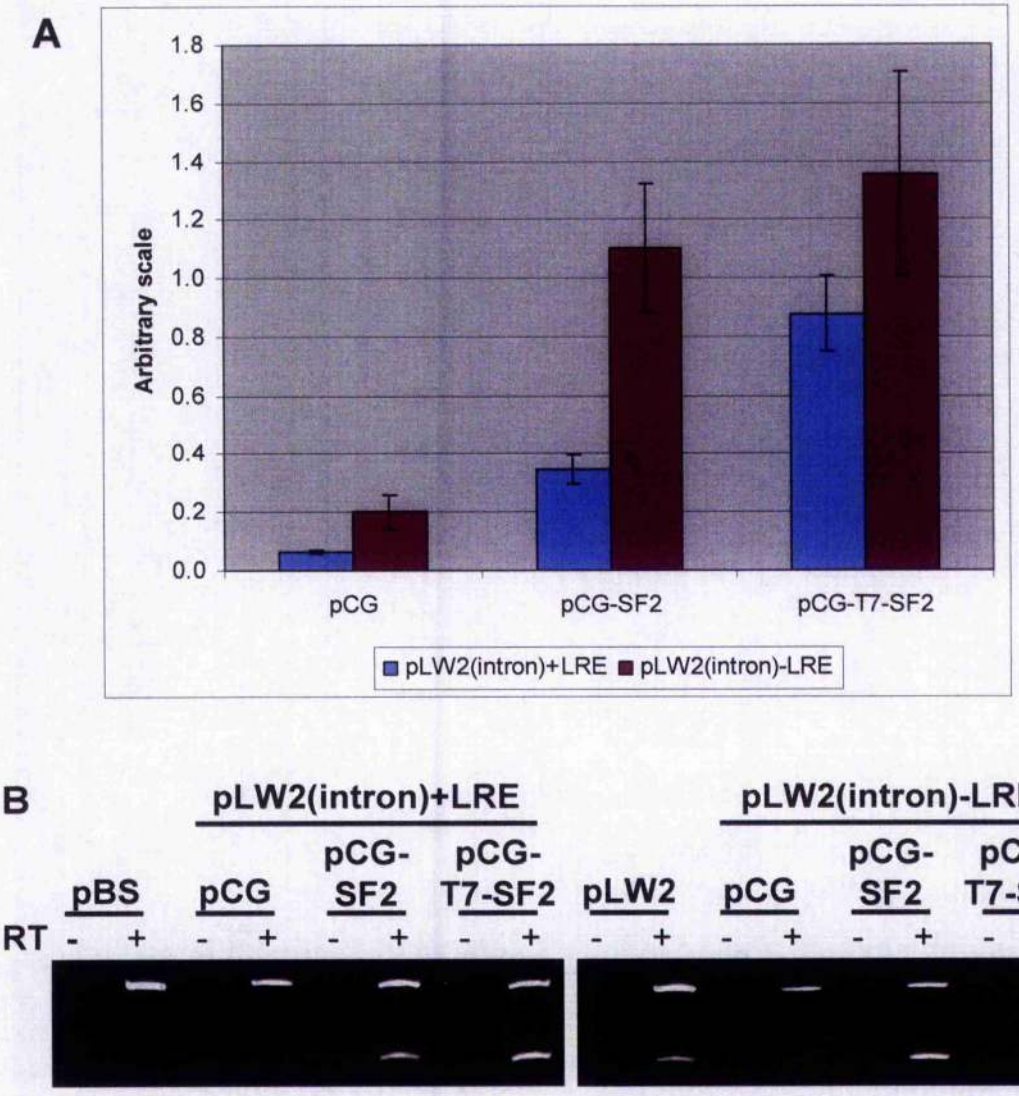


Figure 5.9: **Levels of polyadenylated reporter gene RNA upon overexpression of SF2/ASF**

A) Total RNA was produced from undifferentiated HaCaT cells co-transfected with pLW2+/-LRE and pCG, pCG-SF2 or pCG-T7-SF2 and poly(A)+ levels of CAT RNA were assayed using quantitative RT-PCR. Transfections were performed in duplicate and the graph represents mean and standard deviation about the mean from three separate experiments. Results are normalised against GAPDH. B) Semiquantitative RT-PCR using total RNA samples from undifferentiated HaCaT cells transfected with pCG, pCG-SF2 or pCG-T7-SF2 and either pLW2+/-LRE. Primers amplify within CAT (lower band) and GAPDH (upper band) transcripts.

5.4 Discussion

There are several proteins involved in RNA metabolism which are known to associate with the LRE in undifferentiated and differentiated epithelial cells. Of particular interest is SF2/ASF, as this protein is found to be elevated and hyperphosphorylated in differentiated HPV-16 infected cells, and it has many roles during RNA metabolism. Due to its upregulation, SF2/ASF is thought to be important to regulation of the LRE in differentiated W12E cells. It is hypothesised that elevated SF2/ASF acts to de-repress the LRE by several mechanisms, such as enhancing polyadenylation, export and/or translation. However, it is also possible that upregulated SF2/ASF is necessary to stimulate other cis-acting RNA elements within the HPV-16 late region. Therefore, it could be suggested that SF2/ASF association with the LRE may negatively affect late protein expression and that the splicing-like complex, containing SF2/ASF, may inhibit polyadenylation, for example. It has previously been reported that U1 snRNP, via U1-70K, is able to repress polyadenylation, when associated with an LRE in the late 3'UTR of BPV-1 in undifferentiated cells (Gunderson et al. 1998), indicating that the splicing-like complex associated with the HPV-16 LRE may act in a similar manner. To analyse this hypothesis, SF2/ASF could be depleted from undifferentiated HaCaT cells using SF2/ASF specific siRNAs, followed by transfection with reporter constructs containing the LRE. Enhanced polyadenylation would be observed if the LRE-bound splicing-like complex were involved in its inhibition. However, SF2/ASF is an essential cellular protein and depletion of SF2/ASF may result in death of undifferentiated HaCaT cells. However, more than 50% reduction in levels of splicing related protein, polypyrimidine tract binding protein (PTB) using siRNA can be tolerated in HeLa cells (Sauliere et al. 2006). This suggests that downregulation of SF2/ASF may also be achievable in HaCaT cells. In addition, the splicing-like complex, which forms on the LRE, is thought to be present in both undifferentiated and differentiated cells (Koffa et al. 2000; Cumming et al. 2003). Therefore, change in abundance of SF2/ASF may have some affect on this, possibly to increase the frequency of its formation. In this case, it is hypothesised that the splicing-like complex positively regulates expression from LRE containing transcripts in differentiated cells. Therefore, in this study overexpression of SF2/ASF in undifferentiated epithelial cells was used to determine if this has any affect on LRE function. Results show that protein production from reporter constructs fused to

the HPV-16 late 3'UTR, which contains the LRE, is not affected by SF2/ASF overexpression. However, production of reporter total and polyadenylated RNA from the same constructs does show variation when SF2/ASF levels are elevated. This suggests that whilst steady state CAT activity produced from transcripts containing the LRE are not affected by SF2/ASF alone, SF2/ASF may have an important role during RNA metabolism of such transcripts. In addition, the data suggests the LRE may act as an instability element and may regulate export and/or translation of transcripts containing it, independently of SF2/ASF in undifferentiated epithelial cell model systems.

SF2/ASF is not only an essential cellular protein, but has also been shown to regulate gene expression from other viruses such as HIV and Adenovirus. It is shown to repress tat pre-mRNA splicing from HIV-1 (Wang et al. 1998) and is also involved in Rev mediated splicing via interactions with the Rev response element (Powell et al. 1997). Furthermore, SF2/ASF plays a part in prevention of the switch from early to late gene expression during the adenovirus life cycle, by interacting with an intronic repressor element and inhibiting use of the IIIa 3 ' splice site within the L1 unit (Kanopka et al. 1996; Molin and Akusjarvi 2000). No role during splicing was determined for LRE bound SF2/ASF in these experiments; however, I cannot rule out such a role. One hypothesis is that LRE-bound SF2/ASF acts to enhance splicing of the terminal L1 exon in differentiated W12E cells. As discussed, the L1 exon is long and its definition may be difficult due to the distance between the final 3' splice site and the polyadenylation signal. SF2/ASF and/or components of the splicing-like complex binding the LRE may complex with splicing factors at the final 3' splice site, creating a spliceosome-like complex, and hence aid terminal exon definition. In addition a number of potential ESEs and ESSs are observed within the L1 exon, which may act in concert with the LRE to enhance appropriate splicing of the final exon. To determine this it may be useful to employ *in vitro* splicing assays using probes encompassing the HPV-16 late region. Splice patterns would then be examined in the presence and absence of SF2/ASF. Furthermore, splice patterns could be assayed *in vivo*, using similar experiments as performed here. Again using undifferentiated epithelial cells; constructs containing the L1 and L2 ORFs and late 3'UTR would be transiently transfected into cells, along with SF2/ASF expressing vectors and RNA would be purified. Primers within the late region could then be used in RT-PCR experiments to detect if there was an increase in use of the final 3' splice site upon SF2/ASF overexpression. However, as further cis-acting elements are found

within the HPV-16 late region, any change in splice patterns could not be confirmed to be solely attributable to the LRE.

Formation of a splicing-like complex over the LRE has previously been suggested to affect polyadenylation of late transcripts. There are two polyadenylation signals in the late 3'UTR, and both sites are used during the HPV-16 life cycle (Milligan et al. 2006). As hypothesised, it is thought that SF2/ASF may regulate LRE function via formation of an early splicing-like complex over the LRE. During BPV-1 infection, an element within the late 3'UTR is shown to inhibit polyadenylation in undifferentiated cells, via interaction with U1 snRNP. Whilst U1-70K, the component of U1 snRNP which is directly involved in repression of polyadenylation by this element, is not found associated with the HPV-16 LRE, it is suggested that the splicing-like complex forming over the LRE may also inhibit polyadenylation in undifferentiated HPV-16 infected cells. However, results here suggest that overexpression of SF2/ASF enhances polyadenylation from LRE containing transcripts in undifferentiated epithelial cells. Therefore, elevated SF2/ASF in differentiated W12E cells may either alleviate the repressive activity of the splicing-like complex upon polyadenylation, or the splicing-like complex may itself enhance polyadenylation. As SR proteins are involved in bringing together splice components over splice junctions, it is possible that increased levels of SF2/ASF cause formation of the splicing-like complex with greater frequency. This could prevent binding of other cellular factors that may inhibit polyadenylation. Alternatively, formation of the splicing-like complex itself may regulate polyadenylation via protein-protein interactions, with cleavage and polyadenylation factors. Furthermore, the extent of polyadenylation from LRE containing transcripts (~20% of total RNA) upon SF2/ASF overexpression is high in comparison to normal cellular polyadenylation levels (<10%). This suggests enhancement of polyadenylation rather than removal of repressive activity. However, this may simply reflect increased productivity of reverse transcription reactions using oligo d(T), rather than random primers.

In vitro assays are an obvious route to further investigate the role of SF2/ASF in polyadenylation of LRE containing transcripts. However, Kennedy et al. showed that polyadenylation is not inhibited from LRE containing probes *in vitro* (Kennedy et al. 1990). Therefore, to investigate the role of SF2/ASF in LRE regulated polyadenylation would require further *in vivo* assays. For example, if siRNA mediated downregulation of SF2/ASF were to result in inhibition of polyadenylation

from LRE containing constructs in transfected cells, this would suggest SF2/ASF to be necessary for LRE mediated regulation of polyadenylation. However, it would not indicate whether SF2/ASF is the vital component that directly enhances polyadenylation, or whether it is simply necessary to promote formation of the splicing-like complex, which in turn regulates polyadenylation. In the latter case, increased formation of the splicing-like complex may be achieved via overexpression of an SR protein, other than SF2/ASF. If polyadenylation were shown to be enhanced upon overexpression of another SR protein this would suggest that either the necessity for SF2/ASF in this reaction is to promote splicing-like complex formation, or that other SR proteins can substitute for SF2/ASF. In contrast, no change in polyadenylation would indicate that SF2/ASF is essential and directly involved in enhancing polyadenylation from LRE containing transcripts. One further possibility is that SF2/ASF acts to cause displacement of polyadenylation inhibitory proteins from the LRE. This again may be caused by increased formation of the splicing-like complex. However, to investigate this further would probably require such inhibitory factors to be determined.

Aside from the function of SF2/ASF in LRE control, the element may function to cause instability and may also regulate export and/or translation of transcripts containing it. It is shown that whilst protein levels are inhibited ~20 fold from constructs expressing LRE containing transcripts, production of polyA+ RNA is inhibited only ~3 fold. This indicates that the LRE acts to inhibit late gene expression at stages after RNA processing. Furthermore, polyadenylation of LRE containing transcripts seems to increase upon SF2/ASF overexpression but no such increase is observed in protein production. It has previously been reported that reporter constructs containing the LRE are predominant retained in the nucleus (Koffa et al. 2000). This suggested that the LRE does inhibit export; however, non-polyadenylated RNAs, which the majority of LRE containing transcripts are, should not be exported from the nucleus. Therefore, previous results showing inhibition of export may simply be a product of inefficient polyadenylation. Therefore, nuclear:cytoplasmic ratio of LRE containing transcripts in undifferentiated epithelial cells co-transfected with SF2/ASF expression vectors, to enhance polyadenylation, would be assayed. Nuclear and cytoplasmic RNA would be extracted from cells and subjected to RT-PCR, to compare levels of total and polyadenylated RNA in both fractions. Lack of RNA from LRE containing transcripts in the cytoplasm, even upon SF2/ASF

overexpression would indicate that the LRE inhibits export. However, presence of transcripts in the cytoplasm would indicate that lack of export of LRE containing transcripts in the presence of endogenous levels of SF2/ASF might be a product of inefficient polyadenylation. Alternatively, this might suggest that SF2/ASF is involved in promoting export of LRE containing transcripts.

To examine the possible role of the LRE during translation, direct transfection of *in vitro* synthesised LRE containing transcripts into the cytoplasm of undifferentiated epithelial cells, followed by reporter assays would be performed. Should reporter activity be reduced from LRE containing transcripts, this would suggest that it is involved in inhibition of translation. However, lack of translational stimulation may simply suggest that cellular proteins involved in this process may not be present in the cytoplasm. As mention previously SF2/ASF may redistribute from nuclear speckles to a more diffuse cytoplasmic location upon differentiation of W12E cells. Whilst this is not confirmed, it may be involved in cytoplasmic activities of the LRE, such as a possible role in translation. However, in the model epithelial system used in the assays performed here, SF2/ASF is predominantly nuclear. Therefore, it is possible that elevated SF2/ASF is necessary for functions of LRE in the cytoplasm of differentiated W12E cells, but the assays described here are unable to detect this. To look at this in more detail, it might be useful to use mutated versions of SF2/ASF that localise to the cytoplasm. Transfection of cells expressing this with either LRE containing reporter constructs or *in vitro* synthesised RNAs might indicate whether cytoplasmic SF2/ASF can act to enhance protein production from LRE containing transcripts, via increased translation. Should protein production be observed to increase this would indicate that SF2/ASF plays an important cytoplasmic role during activation of the LRE. In contrast, no change might suggest that SF2/ASF does not have cytoplasmic roles with respect to LRE function. However, it must be taken into account that only regulation of SF2/ASF upon differentiation of W12E cells is modelled in these assays and that the cells used are undifferentiated. Therefore, lack of translational stimulation by cytoplasmic SF2/ASF may suggest that other proteins required for this process are not present in the cytoplasm of undifferentiated epithelial cells.

In summary, whilst SF2/ASF is known to associate with the HPV-16 LRE in both undifferentiated and differentiated epithelial cells, how it acts to regulate LRE function is largely unknown. Results here suggests that the elevated levels of SF2/ASF, which are observed in differentiated HPV-16 infected epithelial cells,

may be involved in regulating polyadenylation of LRE containing transcripts. In addition, the LRE itself may act as an instability element, and may inhibit export and/or translation due to the discrepancies observed between protein production and poly(A)+ RNA, when comparing transcripts containing and lacking the LRE. Whilst no firm conclusions can be drawn from this data, it does provide further insight into the possible mechanisms governing LRE function.

6 General discussion

6.1 Perspectives

Regulation of gene expression of eukaryotic genes is highly dependent on post-transcriptional events. For example, most eukaryotic genes are encoded on multiple exons separated by introns, which are often larger. It is thought that up to 75% of human genes are spliced, suggesting that splicing plays a vital role during gene expression. Furthermore, alternative splicing is often involved in production of different protein isoforms, which may be required at different times throughout development or by different cell types. For example, sex determination in *Drosophila* is highly dependent on differential splicing of a number of genes in males and females (reviewed by Black 2003). One instance of this is the alternative splicing of *drosophila* doublesex (dsx) mRNA. In males, a truncated non-functional version of Transformer (Tra), an RS domain containing splicing regulatory protein, is produced due to the presence of a stop codon in exon 2 (reviewed by Black 2003). In the absence of Tra, splicing of dsx follows the default pattern that results in exon 4 skipping (Figure 6.1A). This is due to the weak exon 4 3' splice site, which is not recognised by U2AF and results in production of the male specific dsx protein containing exons, 1, 2, 3, 5 and 6. However, in females Transformer (Tra) protein is produced, due to removal of exon 2 and the premature stop codon (reviewed by Black 2003). Tra, along with non-sex specific proteins RBP1 (an SR protein) and Tra-2 (an SR-related protein), binds to an ESE within exon 4 and promotes U2AF recruitment to the weak exon 4 3' splice site (Figure 6.1B). In this case a splice variant containing exons 1, 2, 3 and 4 is produced which encodes the female specific dsx. This provides one example of how alternate splicing can be used to produce different protein in different cells, and is therefore very important to genetic diversity and gene expression.

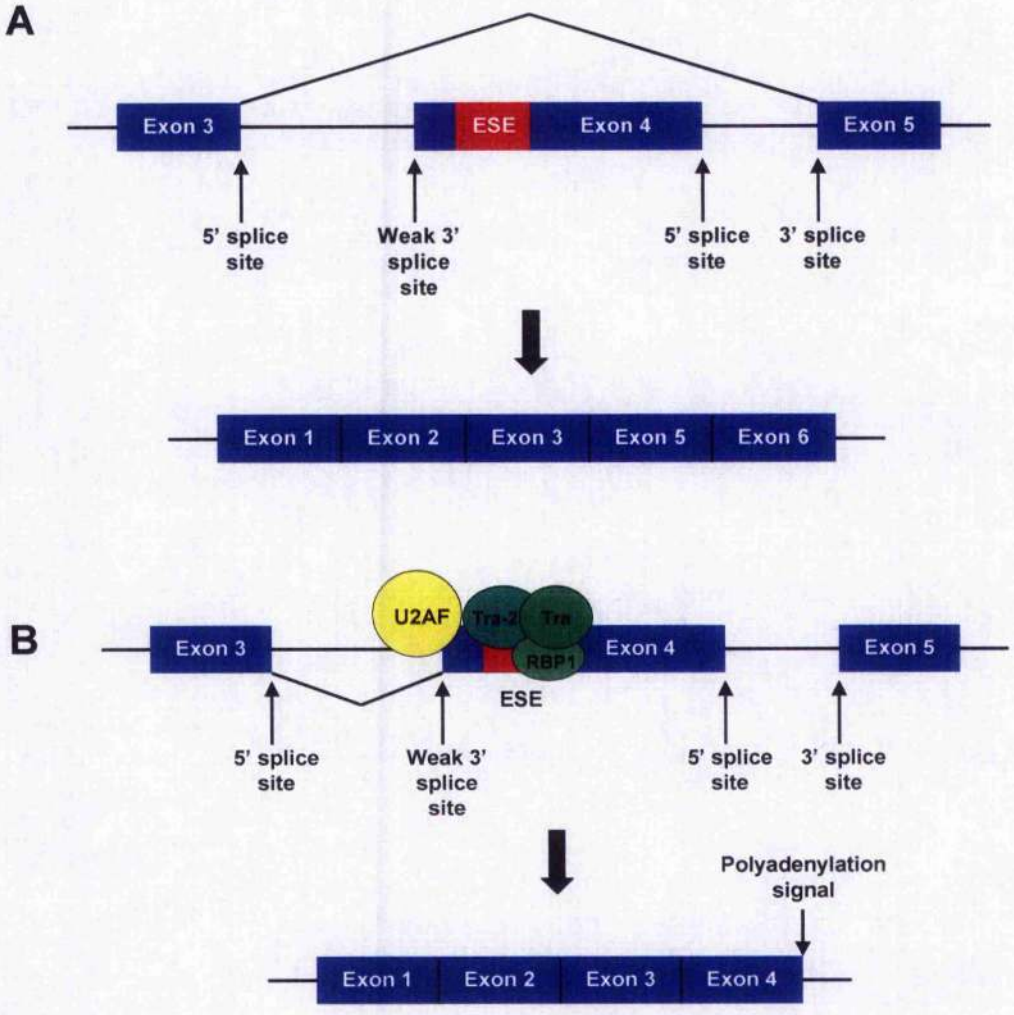


Figure 6.1: *Drosophila* doublesex splicing

Schematic diagrams of splicing of the *Drosophila* doublesex (dsx) gene in males and females during sex determination (not to scale). A) In males, when Tra is not present, skipping of exon 4 occurs as the U2AF complex is not recruited to the weak exon 4 3' splice site. This results in production of the male specific dsx protein which lacks exon 4. B) In females, where Tra is produced, a complex containing Tra, Tra-2 and RBP1 binds to an ESE within exon 4 and recruits the U2AF complex to the weak exon 4 3' splice site. This results in production of the female specific protein which contains a earlier polyadenylation signal on exon 4.

Further highlighting the importance of splicing during gene expression, ~15% of disease causing mutations result in splicing defects (reviewed by Faustino and Cooper 2003). For example, mutations within the *ATP7A* gene are known to cause Menkes disease, a lethal disorder of copper metabolism, or less commonly, occipital horn syndrome (OHS), a milder form of the disease. Mutation within the exon 6 5' splice donor site were found to cause skipping of this exon in the majority of mRNAs (Moller et al. 2000). However, Menkes disease is caused when no functional protein is produced due to incorrect splicing of the remaining small proportion of mRNAs, which do contain exon 6. This is due to mutation of two highly conserved nucleotides within the splice donor site. In contrast, with mutations in the least conserved site within the donor site, a small amount of correctly spliced mRNA could be detected in samples from OHS patients (Moller et al. 2000). Therefore, a small amount of functional protein is produced, which decreases disease severity markedly. This example shows how inappropriate splicing can lead to different degrees of disease depending on how splicing is affected. In addition to having a vital role during eukaryotic gene expression, a number of viruses also control protein production via post-transcriptional processes. These include a number of viruses, such as herpes simplex virus and Epstein-Barr virus. The two examples to be discussed in detail are the early to late shifts in the adenovirus and HIV-1 life cycles. The reason for this is they are well-studied systems, and the role of SR proteins during the life cycle of each has been the subject of much research. As regulation of SR proteins and their potential roles during the life cycle of HPV-16 are the focus of this thesis, the role of these factors in regulation of adenovirus and HIV-1 gene expression will be discussed. In addition, whilst adenovirus provides a good example of how regulation of SR proteins during the life cycle of a virus can induce an early to late shift in gene expression, the HIV-1 example shows the importance of different combinations of SR proteins to splice patterns, and how ESEs and ESSs contribute to this.

The early to late switch in the adenovirus life cycle is, in part, controlled by alternative RNA processing. For example, splicing of the L1 gene changes between the early and late phase of infection. Via the use of one 5' and two 3' splice sites, two L1 mRNA species are produced: 52,55K and IIIa (Figure 6.2) (Kanopka et al. 1996). IIIa is the predominant form late in infection, and splicing to the IIIa 3' splice site is inhibited by exogenous SR proteins, both *in vitro* and in transient transfection assays (Kanopka et al. 1996). Inhibition was found to be

attributable to an intronic repressor element, termed 3RE, upstream of the IIIa branch point site, to which SR proteins were shown to bind. In addition, removal of 3RE from the L1 unit induces IIIa splicing *in vitro* (Kanopka et al. 1998). However, no change in abundance of SR proteins during adenovirus infection, which may have caused the shift from early to late 3' splice site usage, could be determined (Kanopka et al. 1998). In contrast, SR proteins purified from late adenovirus infected cells were unable to repress IIIa splicing of the L1 gene *in vitro*. SR proteins purified from these cells were also found to be dephosphoshorylated when compared with SR proteins from uninfected HeLa cells (Kanopka et al. 1998). Therefore, it was thought that virus induced dephosphorylation of SR proteins was involved in the switch between 52,55K and IIIa splicing of the L1 gene during adenovirus infection. The virally encoded protein E4-ORF4 was previously shown to associate with serine and arginine specific protein phosphatase 2A (PP2A), and Kanopka et al. showed that addition of E4-ORF4 to *in vitro* assays containing SR proteins purified from uninfected HeLa cells, resulted in phosphate release (Kanopka et al. 1998). Furthermore, this could be repressed by inhibition of PP2A. In addition, transient expression of E4-ORF4 in HeLa cells infected with L1 constructs resulted in increased IIIa splice site usage, a function that was dependent on the 3RE (Kanopka et al. 1998).

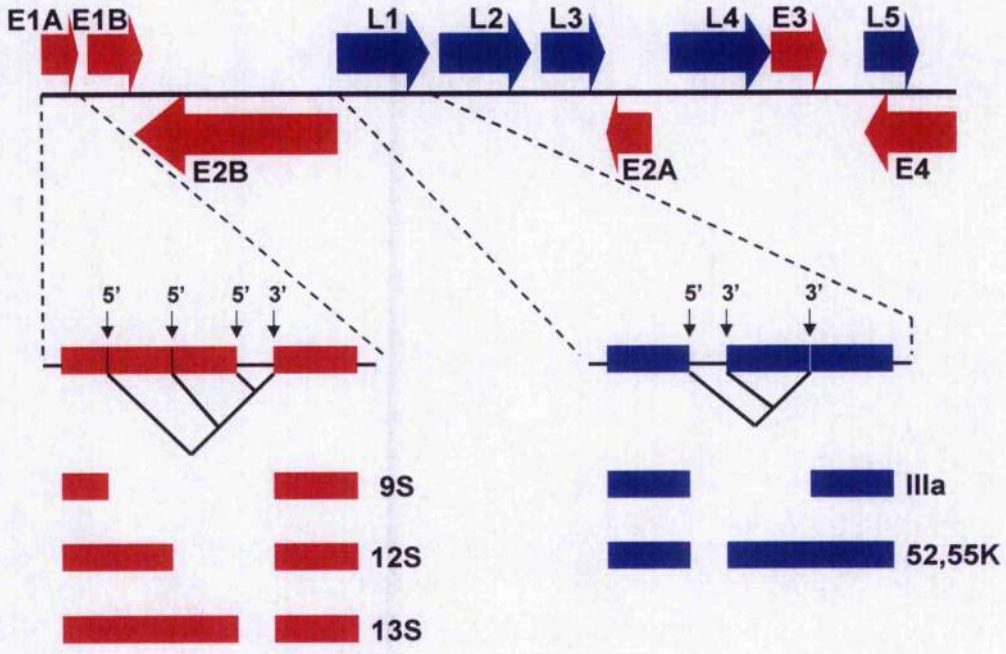


Figure 6.2: The adenovirus genome

A schematic diagram of the adenovirus genome (not to scale) composed of 11 genes. Early genes are shown in red, and late genes are shown in blue, with the arrowhead indicating the direction in which they are transcribed. Splicing of E1A and L1 genes is shown below, with 5' and 3' splice sites indicated by vertical arrows, either 5' or 3' respectively, above each transcript. mRNAs produced from each transcript are shown below with the name of each given to the right.

To build on data gathered using *in vitro* and transient transfection assays, recombinant adenovirus genomes were produced, which were able to inducibly express SF2/ASF. When SF2/ASF was overexpressed in cells infected with this recombinant virus there was a marked reduction in L1 IIIa mRNA splicing (Molin and Akusjarvi 2000). In addition, splicing from genes within the early region of the genome was also affected. The E1A gene is spliced via the use of three alternate 5' splice site and one 3' splice site to produce 13S, 12S and 9S mRNAs (Figure 6.2). 13S is abundant early in infection, whereas 9S accumulates at late times. SF2/ASF had previously been shown to activate 13S splicing both *in vitro* (Harper and Manley 1992) and in transient transfection assays (Wang and Manley 1995). During infection with wild type adenovirus, overexpression of SF2/ASF was shown to inhibit 9S splicing at later time, and the predominant E1A mRNA was 13S (Molin and Akusjarvi 2000). In addition, overexpression of SF2/ASF was shown to impair viral DNA replication and virus particle formation, suggesting that this protein, and potentially other SR proteins, play a vital role in the adenovirus life cycle.

HIV-1 gene expression is also separated into early late phases depending on production of either regulatory proteins, or structural, enzymatic and ancillary proteins, respectively. Regulatory proteins are expressed from the 2kb mRNA species produced from the virus genome, which depends on a degree of alternative splicing. There are 4 5' splice sites and 8 3' splice sites, use of which relies on *cis*-acting RNA regulatory elements within the HIV-1 genome (Figure 6.3). Different splicing patterns were thought to be partially regulated by different combinations of SR proteins. Ropers et al. used *in vitro* splicing and *ex vivo* assays to determine usage of which 3' splice sites were enhanced by which SR proteins (Ropers et al. 2004). *In vitro* assays using truncated regions of the HIV-1 genome containing 5' splice donor site D1 and one of 3' splice acceptors sites A1, A2, A3, A4a, A4b, A4c or A5, suggested that different SR proteins promote splicing from each site with differing efficiencies. SF2/ASF and SC35 activated site A1 to a similar extent, whilst SF2/ASF was a stronger activator of site A2, than SC35. SRp40 and SC35 both activated site A3 usage and 9G8 showed modest stimulatory activity from all sites assayed (Ropers et al. 2004).

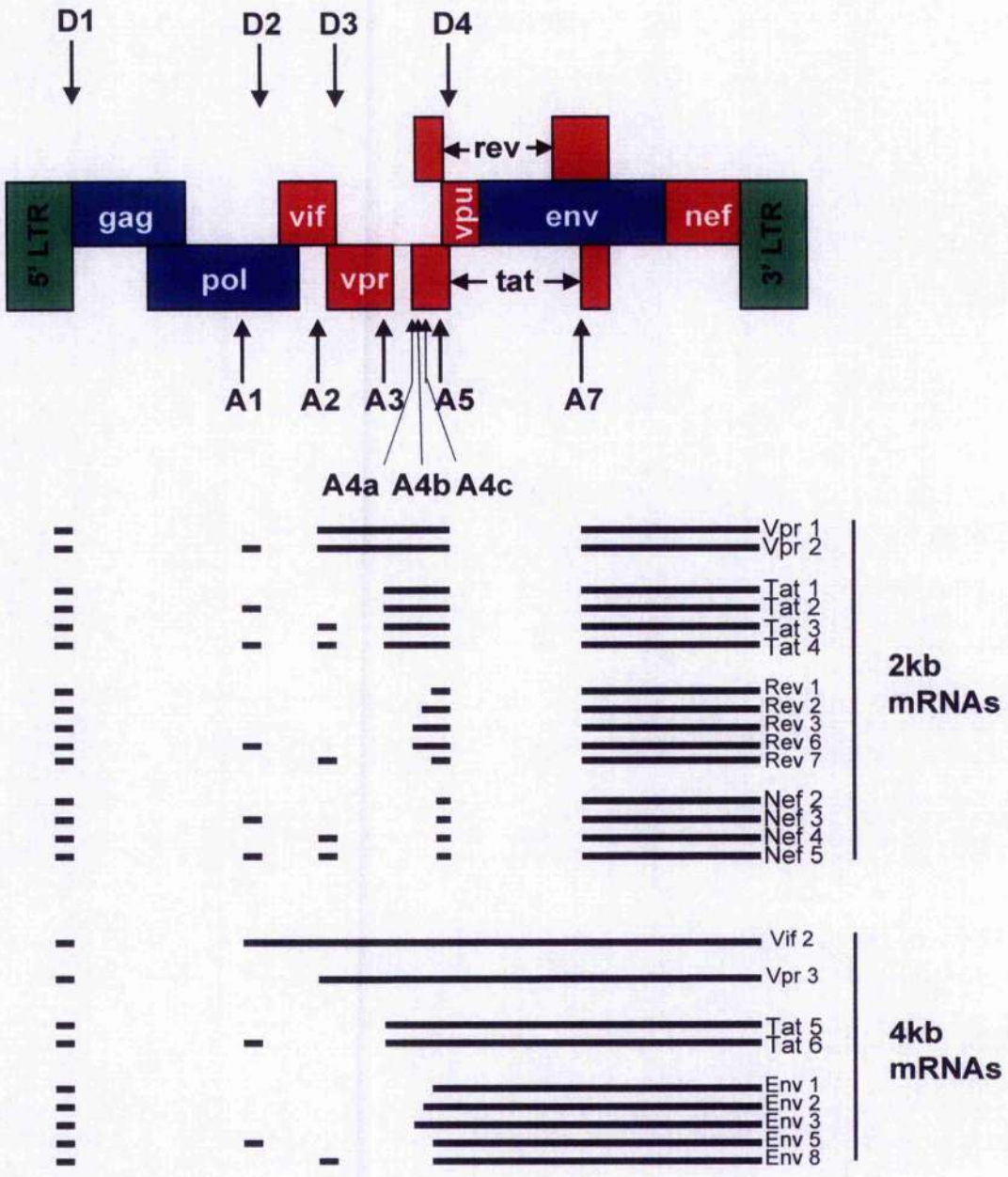


Figure 6.3: The HIV-1 genome

A schematic diagram of the HIV-1 genome (not to scale) composed of 9 genes encompassed by two long terminal repeat regions (LTRs), shown in green. Regulatory genes are shown in red, and structural, enzymatic and ancillary genes are shown in blue. 5' splice donor sites (D1-D4) are shown as vertical arrows above, and 3' splice acceptor sites (A1-A3, A4a-c, A5 and A7) are shown as vertical arrows below. Transcripts produced by the virus are shown by horizontal lines below the genome, along with the proteins they encode to the right and the size species they fall into.

When further probes containing the 5' splice donor site D4 and the 3' acceptor sites A3, A4a, A4b, A4c and A5 were subjected to *in vitro* splicing assays, site A5 was preferentially used. In contrast, addition of SF2/ASF to assays enhanced splicing from sites A4a and A4b, and to a lesser extent A3, whilst SRp40 and SC35 promoted site A3 usage and inhibited use of site A5. These observations were confirmed in *ex vivo* experiments using HeLa cells transfected with HIV-1 DNA and SR protein expression vectors (Ropers et al. 2004). Following RNA purification, splice patterns were determined using RT-PCR. As shown *in vitro*, when no SR proteins were overexpressed, site A5 was preferentially used, suggesting this is the default splice pattern for HIV-1. In addition, overexpression of SF2/ASF resulted in preferential use of 3' splice site A2, and overexpression of SRp40 or SC35 gave strong D1 to A3 splicing.

Whilst the above experiments gave an indication of how splice patterns may be affected by expression of different SR proteins, no detail was given as to how this may be achieved. To this affect, two *cis*-acting RNA regulatory elements within the HIV-1 genome will be discussed. Firstly, an ESS within tat exon 2, named ESS2, which had previously been shown to inhibit usage of splice site A3 via interactions with hnRNP A1, was more recently been found to interact with SC35 *in vitro* (Zahler et al. 2004). Furthermore, SC35 binding was inhibited by hnRNP A1. This region was found to contain an SC35 dependent ESE, using a *Drosophila* dsx probe, which is not spliced *in vitro*, in the absence of an enhancer due to a weak 3' splice site. As discussed, dsx is alternately spliced in males and females, due to presence of Tra protein in females (reviewed by Black 2003). Tra enhances splicing from the exon 4 weak 3' splice site via an ESE in exon 4, causing inclusion of the exon in females. The probe used in these experiments has the ESE removed so that splicing to the weak 3' splice site is inefficient. However, fusion of a region containing an ESE, 3' of the 3' splice site will induce splicing from this site. Addition of the ESS2 region to this probe induced splicing in the presence of SC35, suggesting this region contains an SC35 responsive ESE (Zahler et al. 2004). SC35 and hnRNP A1 binding sites were found to overlap, suggesting that hnRNP A1 bound to ESS2 inhibits splicing from site A3, by inhibiting SC35 binding to an overlapping ESE (Zahler et al. 2004). In support of this, SC35 overexpression was found to enhance usage of site A3 in HeLa cells (Ropers et al. 2004). A further ESE, named GAR, was found within exon 5, which acted in a bi-directional fashion, potentially upon upstream splice acceptor sites A4a, A4b, A4c and A5 and downstream splice donor D4 (Caputi et al. 2004). This

element was shown to enhance splicing when placed upstream of the dsx probe described above in the presence of SF2/ASF or SRp40, but not SC35, SRp50 or SRp70. Furthermore, mutation of SF2/ASF and SRp40 binding sites within the GAR element, decreased splicing activation in this assay (Caputi et al. 2004). In agreement with a role for this element in regulation of upstream splice acceptor sites, SF2/ASF was shown by Ropers et al. to enhance use of acceptor sites A4a and A4b (Ropers et al. 2004). However, SRp40 was shown to inhibit A5 site usage *in vitro*, suggesting that this protein may regulate use of other acceptor site within the region, or may be involved in inhibition of site A5 via interactions with GAR. In addition, transient transfection using the *env* region of the HIV-1 genome, indicated that GAR mediates use of splice donor site D4 (Caputi et al. 2004). Production of *env* is dependent upon D4 usage, and *env* levels fell when GAR was mutated within the SF2/ASF and SRp40 binding sites. Furthermore, tethering of SF2/ASF or SRp40 to the GAR region using the bacteriophage MS2 coat protein and binding site resulted in increased *env* expression when MS2 was fused to either SF2/ASF or SRp40 compared to MS2 protein alone (Caputi et al. 2004). This suggests that the GAR elements functions to regulate HIV-1 splicing in a bi-directional manner.

In addition to roles of SR proteins during regulation of HIV-1 splicing, the virus is also thought to regulate activity of SR proteins. For example, SF2/ASF interacts with a major *cis*-acting RNA regulatory element of the virus, the Rev response element (RRE) (Powell et al. 1997). Rev interaction with the RRE inhibits viral RNA splicing and activates nuclear export of incompletely spliced viral mRNAs. Furthermore, once in the cytoplasm, Rev is involved in enhancing translation of these mRNAs to ensure production of structural, enzymatic and ancillary proteins, which are expressed from the 4kb single spliced and 9kb unspliced RNAs, at later stages of infection. SF2/ASF was shown to interact with the RRE in a Rev dependent manner (Powell et al. 1997). It was suggested that association of SF2/ASF with the RRE interferes with spliceosome formation and hence inhibits viral splicing. Therefore, it was hypothesised that this would be overcome by overexpression of SF2/ASF. A CAT construct which is spliced so that the CAT gene is removed was used to investigate this. Whilst CAT activity was minimal from constructs lacking the RRE, addition of the RRE resulted in a ~25 fold increase in CAT activity due to inhibition of splicing. However, increasing levels of SF2/ASF overexpression reduced CAT activity, suggesting that inhibition of splicing was overcome (Powell et al. 1997). In addition, overexpression of

SF2/ASF also inhibited HIV-1 replication *in vivo*, as assayed by accumulation of the replication protein, Gag. This suggests that interaction of SF2/ASF to the RRE, in a Rev dependent manner, is important to inhibit HIV-1 splicing, and hence allow progression of the virus life cycle.

Having discussed the importance of SR proteins to gene expression from two other viruses, I will now focus on the potential role of SR proteins in the HPV-16 life cycle, and how the virus may regulate these factors. Although preliminary studies were performed to determine changes in SR protein abundance and localisation upon differentiation of HPV-16 infected epithelial cells, so far no more sophisticated experiments to look at how specific SR proteins may affect splice patterns, and therefore protein production have been performed. Chapter 4 shows that a sub-set of SR proteins are upregulated during differentiation of HPV-16 infected epithelial cells. However, little is known about expression of SR proteins in normal systems, although they are thought to be constitutively expressed in proliferating and some terminally differentiated cells. Sanford and Bruzik showed that whilst SR proteins are expressed throughout nematode early embryogenesis, their splicing activity is developmentally regulated (Sanford and Bruzik 1999). In addition, splicing activity correlated with dephosphorylation of SR proteins. This suggests that regulation of SR protein phosphorylation is one mechanism by which the function of these proteins is controlled. This is exemplified during the adenovirus early to late switch in gene expression, where SR proteins are dephosphorylated to ensure a change in splice patterns (Kanopka et al. 1998). In that case, dephosphorylation is used to inactivate SR proteins as alternative splicing regulators during the late stages of the adenovirus life cycle. However, results presented here suggest that HPV-16 upregulates SR proteins during the later stages of its life cycle. In contrast to adenovirus-infected cells, expression of SR proteins would be lost from HPV-16-infected cells, if the virus did not regulate them. This suggests that HPV-16 upregulates expression of SR proteins as they are vital to completion of the virus life cycle.

As discussed previously, phosphorylation status of SR proteins is important to their function. Therefore, it would have been useful to determine the phosphorylation status of SR proteins during differentiation of W12E cells. SF2/ASF is known to become hyperphosphorylated in differentiated W12E cells (McPhillips et al. 2004); however, nothing is known about the phosphorylation status of other SR proteins. Mb104 antibody recognises a common phospho-

epitope of several SR proteins (Roth et al. 1991). Therefore, as there is an abundance of SR proteins shown using this antibody, particularly SRp55 and SRp40, it is possible that the majority of each protein is phosphorylated in W12E cells. Indeed, McPhillips et al. found no detectable non-phosphorylated SF2/ASF in either undifferentiated or differentiated W12E cells (McPhillips et al. 2004). In contrast, little SRp20 and no SRp75 are recognised using Mb104. Whilst this is possibly due to low abundance in the case of SRp75, SRp20 is readily detected using anti-SRp20 specific antibody. Therefore, it is possible that SRp20 exists in predominantly non-phosphorylated form in W12E cells. Alternatively, Mb104 may simply not recognise this protein as well as the other SR proteins. Therefore, more detailed analysis of the phosphorylation status of SR proteins during W12E differentiation would have been beneficial. To have fully analysed this, samples could have been taken at more time points from the onset of differentiation. That way, we could determine whether there was a rapid increase in SR protein abundance and/or hyperphosphorylation at a specific time point during differentiation, or whether it was more gradual. Furthermore, use of antibodies specific to each SR protein would have given a better indication of changes in abundance of each. Should SR proteins be found in predominantly hyperphosphorylated form upon differentiation, this would suggest that they might act to promote alternative splicing in differentiated cells. This is in contrast to both adenovirus and HIV-1, which deactivate SR protein mediated regulation of splicing at later times during infection, albeit by different mechanisms (Powell et al. 1997; Kanopka et al. 1998). The reason for this difference may be a result of protein abundance in differentiated epithelial cells. Adenovirus and HIV-1 infected cells have constant levels of SR proteins throughout the life cycle and expression of SR proteins is vital to cell viability. Therefore, these viruses must find other mechanisms to inhibit the default functions of SR proteins late in infection. In contrast, differentiated epithelial cells do not require SR proteins, and protein expression is lost as the cells differentiate. Therefore, HPV-16 can regulate these factors for its own means, without the necessity to deal with SR protein produced by endogenous expression, or the effects of SR proteins on cellular gene expression. As SR proteins are multifunctional, use of them rather than inhibition of their function makes more sense as they can affect post-transcriptional gene expression at many stages. Therefore, HPV-16 may simply regulate SR proteins to aid late gene expression because of the cells it infects, whereas adenovirus and HIV-1 are unable to do this.

In addition, a further sub-set of SR proteins, SF2/ASF and SRp20, may redistribute from nuclear speckles to a diffuse cytoplasmic localisation in differentiated W12E cells. However, as discussed this was inconclusive and may have benefited from a more detailed analysis of the cells, with respect to the status of the genome (whether it was integrated or episomal) and the extent of differentiation. To repeat these experiments would be important as nuclear and cytoplasmic roles for SR proteins during HPV-16 late gene expression are likely to differ. In the nucleus, SR proteins likely act to regulate appropriate splicing, and potentially polyadenylation of late transcripts in both undifferentiated and differentiated W12E cells. However, if SF2/ASF and SRp20 were found to relocate to the cytoplasm in differentiated W12E cells, this may suggest that they predominantly have roles during export and/or translation of late transcripts in these cells. As suggested for analysing protein abundance and phosphorylation status in more detail, it may be useful to look at more time points following the onset of differentiation. If redistribution were observed only at very late stages of differentiation it is possible that SR proteins are involved in appropriate processing of late transcripts in the nucleus of less differentiated cells. SF2/ASF and SRp20 may also be involved in regulating export and/or translation at later stages of differentiation. These two proteins may associate with the RNA within the nucleus and mediate it's export to the cytoplasm. In the cytoplasm, it is possible that SF2/ASF enhances translation of late transcripts; however, the protein is found predominantly in hyperphosphorylated form, whilst hypophosphorylation is associated with translational regulation by SF2/ASF (Sanford et al. 2005). Therefore, it is likely that if SF2/ASF were involved in regulating translation of late transcripts, this would only encompass a small proportion of the protein within differentiated cells.

Whilst the exact nature of SR protein regulation during W12E cell differentiation is yet to be determined, it is clear that phosphorylated SF2/ASF, SRp20, SC35 and SRp75 are upregulated in cells expressing HPV-16 E2. As this was shown using mb104 antibody, it is not certain that this represents total SR protein abundance; therefore, it would be important to use anti-SR protein specific antibodies to determine this. However, from the data present here, it is hypothesised that E2 regulates expression of these SR proteins during W12E differentiation. In addition to general regulation of SR proteins, the role of HPV-16 E2 in transactivation of SF2/ASF was investigated. Whilst E2 was shown to transactivate and associate with the SF2/ASF promoter *in vivo*, no mode of interaction was determined. In

addition, whilst transactivation of the SF2/ASF promoter in CAT assays was highest in U2OS clone A4, endogenous SF2/ASF expression is greater in clone B1 (comparing figure 3.4 with figure 4.4). The reasons for this are unclear, but may reflect differences in E2-mediated transactivation of the promoter contained on the plasmid and the endogenous promoter. E2 expression is also higher in B1 clonal cells, so perhaps this has some repressive effect on the plasmid promoter, which is not contained within the cellular chromatin environment, and therefore may be more accessible to E2 binding. In addition, lower expression of E2 in A4 clonal cells may access the endogenous SF2/ASF promoter with lower frequency than in B1 cells, resulting in lower transactivation of endogenous SF2/ASF. In contrast, during clonal selection of clone A4, endogenous expression of SF2/ASF may have been compromised, resulting in the lower levels observed within this cell clone. Conversely endogenous SF2/ASF expression may have been elevated during selection of clone B1.

E2 potentially interacts with the SF2/ASF promoter directly via a weak association, or may be tethered to it using cellular proteins. Sp1, a known cellular binding partner of E2, may interact with the promoter via the same region to which E2 may bind weakly. HPV-18 E2 is known to regulate the hTERT promoter via interactions with Sp1, which tethers it to the promoter (Lee et al. 2002). However, although E2 association with this region is stronger in the presence of nuclear extract *in vitro*, the shift obtained in EMSAs does not change in size. Therefore, it is possible that presence of Sp1 within transcription complexes in this region may not tether E2 to the promoter but may strengthen its binding, perhaps by changing DNA structure, making transactivation more likely (Figure 6.4A). However, association of Sp1 with the promoter *in vivo* has not been determined. In contrast, TBP was shown in many ChIP assays to associate with the SF2/ASF promoter. As discussed, HPV-16 E2 interacts with TFIIB (Benson et al. 1997), a component of the pre-initiation complex (PIC), which interacts with a recognition sequence (BRE) upstream of the TATA-box. TFIIB interaction with the promoter has not been tested, but would be expected, as it is necessary for the recruitment of RNA polymerase II. The SF2/ASF promoter does not contain a consensus TATA-box, but does contain a potential BRE, ~35nt downstream from the initiation site. Binding of TFIIB to this element may recruit TBP to the promoter, and hence cause assembly of the PIC (Figure 6.4B).

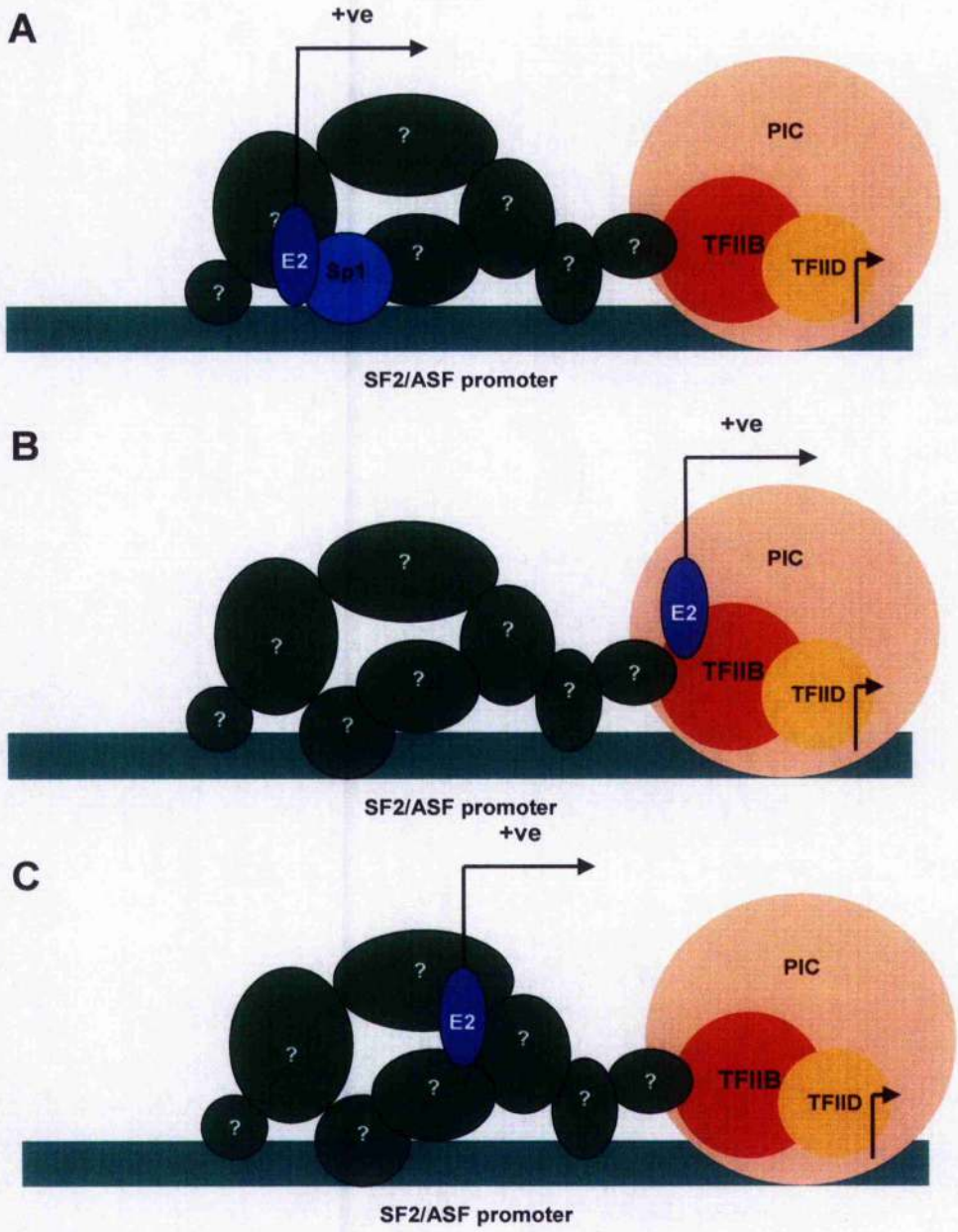


Figure 6.4: Potential models of E2-mediated transactivation of the SF2/ASF promoter

HPV-16 E2 associates with the SF2/ASF promoter *in vivo*; however, no mode of interaction has been determined *in vitro*. Protein and protein complexes are depicted as filled circles, whilst the transcription start site is shown with an arrow. A) E2 may associate with the promoter using Sp1, via protein-protein interactions, or Sp1, or another cellular TF may alter the DNA structure so that E2 can bind more readily, and promote transactivation. B) E2 may interact directly with TFIIB, a component of the PIC. C) E2 may associate via an unknown cellular factor.

If E2 were to be recruited to the SF2/ASF promoter by TFIIB, it would be expected that E2 could still mediate transactivation, even with only a short region of the promoter covering the initiation site and the potential BRE present. In contrast, should Sp1 be involved in E2-mediated transactivation, this would require the region spanning nt –684 to –482, to which Sp1 and E2 may bind. In addition, E2 may associate with the SF2/ASF promoter via an as yet unknown mechanism (Figure 6.4C). Therefore, fragmentation of the promoter and use of these reporter constructs in CAT assays would have been useful to determine which region was important for E2-mediated transactivation. This along with experiments described in section 3.8, would have given considerable more information as to the nature of E2 transactivation of the SF2/ASF promoter.

From results shown in chapter 5, upregulation of SF2/ASF during W12E cell differentiation is not the sole reason for de-repression of the LRE in these cells. However, it is hypothesised that high levels of expression of SF2/ASF, potentially via formation of a splicing-like complex over the LRE, enhances polyadenylation of late transcripts, as discussed in detail in section 5.4. This is in contrast to previous *in vitro* data, which suggests that polyadenylation is not inhibited from probes containing the LRE (Kennedy et al. 1990). More recently, Milligan et al. showed that polyadenylated HPV-16 late RNAs can be detected only in differentiated W12E cells, suggesting that polyadenylation is inhibited in undifferentiated cells (Milligan et al., 2006). Whilst this could be achieved via the mechanism of other *cis*-acting regulatory RNA elements within the HPV-16 late region, data presented here suggests that polyadenylation is likely to be regulated, at least partly by the LRE. However, polyadenylated RNA from LRE containing constructs is repressed only ~3 fold, compared with constructs lacking the LRE, when expressed in undifferentiated HaCaT cells. As protein levels are repressed ~20 fold from LRE containing transcripts, this suggests that inhibition of polyadenylation is not the main mechanism by which the LRE represses gene expression in undifferentiated epithelial cells. It is possible that the LRE works in concert with other HPV-16 *cis*-acting RNA regulatory elements, perhaps within the L1 ORF, to inhibit polyadenylation, and is inefficient at doing so when isolated from these.

The LRE has also been shown to cause RNA instability. The half-life of transcripts containing it is ~30 minutes *in vitro*, compared with over 90 minutes for those lacking it (Kennedy et al. 1991). However, no such instability was observed here, with the amount of total RNA produced from constructs containing and lacking the

LRE being roughly equal. It is possible that proteins involved in stabilising late transcripts via the LRE in differentiated W12E cells, such as potentially HuR, are expressed at critical levels in undifferentiated HaCaT cells, to allow stabilisation. In contrast upon overexpression of SF2/ASF, total RNA from constructs lacking the LRE increased ~15 fold, whilst expression from LRE containing constructs increased only ~5 fold. This suggests that SF2/ASF somehow increases RNA production from these constructs, a process that may be inhibited by the LRE. In this case, due to the increased amount of LRE containing transcripts, the level of stabilising protein(s) may not be sufficient to stabilise all the RNA. In addition, SF2/ASF seems unlikely to be involved in LRE-mediated stability of transcripts.

6.2 Future aims

This thesis provides evidence that SR proteins are regulated during the life cycle of HPV-16. However, whilst there is preliminary data to suggest that SF2/ASF may enhance polyadenylation of late transcripts when complexed with the LRE, nothing is known about the potential functions of other SR proteins, which are regulated by the virus. Therefore, the future aims of this investigation would be to not only to investigate specifically how HPV-16 regulates each SR protein, but also to determine why they are regulated and how they affect late gene expression from HPV-16 during differentiation. Firstly, as discussed, more work is necessary to confirm how E2 transactivates expression of SF2/ASF. Furthermore, it would be interesting to determine if the sub-set of SR proteins, which are upregulated in HPV-16 infected differentiated epithelial cells, are transactivated in the same way. It would be expected that HPV-16 would use a common mechanism to upregulate expression of all SR proteins; however, this would need confirmation as differences in endogenous control of expression of different SR proteins may prevent the virus from doing this. As discussed this would involve following a similar procedure as used in chapter 3 for each promoter to be studied.

To study the roles of specific SR proteins during the HPV-16 life cycle would require considerably more work. This may involve a more intensive study of potential ESEs and ESSs within the HPV-16 late region, particularly within the L1 ORF. As discussed there are a number of potential ESEs within the L1 ORF (Figure 4.1), many of which are likely to contribute to late gene expression. Specific predicted elements could be cloned upstream of the dsx weak 3' splice

site, followed by *in vitro* splicing assays. In this way, it could be determined whether the predicted ESE was able to enhance use of the weak dsx 3' splice site, and in response to which SR proteins. Some work has already been performed in this area by the Schwartz lab (Zhao et al. 2004; Rush et al. 2005). Although they provide evidence that elements within the late region prevent late gene specific splicing in undifferentiated epithelial cell, there is nothing known about elements that might enhance late gene expression upon differentiation. Furthermore, these studies use a synthetic system in which a CMV promoter drives expression of the HPV-16 late region with LRE deleted. Although this provides a more ammenable system in which to assay splice patterns and *cis*-acting RNA regulatory elements, it may not reflect the natural system for a number of reasons. Expression from constructs produced by the Schwatrz lab results in production of much higher levels of transcripts than would be expected using the natural HPV-16 promoters, which may force aberrant splicing not found in real infections. Furthermore, we hypothesise that the LRE itself is involved in regulation of splicing of late transcripts. Therefore, deletion of this element may also result in the production of transcripts not observed during natural infection. Alternatively, loss of the LRE may cause ESE or ESSs within the late region to behave differently, as they may act in concert with the LRE. Therefore, confirmation that a given predicted ESE or ESS is bona fide, would require that this element be mutated in the context of the virus, followed by transfection into epithelial cells. However, these experiments would be very challenging, due to the nature of the HPV-16 life cycle and the difficulty in producing HPV-16 stably transfected cells lines. An alternative approach would be to determine global changes in splicing from HPV-16 in response to specific SR proteins. This could follow a similar procedure to that used by Ropers et al. when determining the effects of SR protein on HIV-1 splicing (Ropers et al. 2004). In particular, transfection of undifferentiated W12E cells with exogenous expression vectors for a specific SR protein, followed by RT-PCR analysis of the resulting transcripts, may indicate how this protein alters splice patterns. Whilst these experiments would provide an indication of the role of specific SR proteins during splicing of HPV-16 transcripts, they would not determine if and when this was important during the viral life cycle.

In conclusion, it is hypothesised that SF2/ASF may regulate late gene expression via the LRE by enhancing polyadenylation of late transcripts. Although to date there is no evidence to suggest that SF2/ASF may regulate stability, export and/or translation of LRE containing transcripts, the possibility cannot be discounted. As

discussed in section 5.1, SF2/ASF may be multifunctional in LRE regulation, and assays performed during the course of this PhD, were not sufficient to investigate this fully. However, it is clear that HPV-16 regulates expression of SF2/ASF, and potentially other SR proteins, a process which may be involved in control of late gene expression, possible throughout infection.

References

Antson, A. A., J. E. Burns, O. V. Moroz, D. J. Scott, C. M. Sanders, I. B. Bronstein, G. G. Dodson, K. S. Wilson and N. J. Maitland (2000). "Structure of the intact transactivation domain of the human papillomavirus E2 protein." Nature **403**(6771): 805-9.

Apt, D., R. M. Watts, G. Suske and H. U. Bernard (1996). "High Sp1/Sp3 ratios in epithelial cells during epithelial differentiation and cellular transformation correlate with the activation of the HPV-16 promoter." Virology **224**(1): 281-91.

Armstrong, D. J. and A. Roman (1997). "The relative ability of human papillomavirus type 6 and human papillomavirus type 16 E7 proteins to transactivate E2F-responsive elements is promoter- and cell-dependent." Virology **239**(1): 238-46.

Arroyo, M., S. Bagchi and P. Raychaudhuri (1993). "Association of the human papillomavirus type 16 E7 protein with the S-phase-specific E2F-cyclin A complex." Mol Cell Biol **13**(10): 6537-46.

Ashmole, I., P. H. Gallimore and S. Roberts (1998). "Identification of conserved hydrophobic C-terminal residues of the human papillomavirus type 1 E1E4 protein necessary for E4 oligomerisation in vivo." Virology **240**(2): 221-31.

Ashrafi, G. H., D. R. Brown, K. H. Fife and M. S. Campo (2006). "Down-regulation of MHC class I is a property common to papillomavirus E5 proteins." Virus Res **120**(1-2): 208-11.

Ashrafi, G. H., M. Haghshenas, B. Marchetti and M. S. Campo (2006). "E5 protein of human papillomavirus 16 downregulates HLA class I and interacts with the heavy chain via its first hydrophobic domain." Int J Cancer **119**(9): 2105-12.

Ashrafi, G. H., M. R. Haghshenas, B. Marchetti, P. M. O'Brien and M. S. Campo (2005). "E5 protein of human papillomavirus type 16 selectively downregulates surface HLA class I." Int J Cancer **113**(2): 276-83.

Ashrafi, G. H., E. Tsirimonaki, B. Marchetti, P. M. O'Brien, G. J. Sibbet, L. Andrew and M. S. Campo (2002). "Down-regulation of MHC class I by bovine papillomavirus E5 oncoproteins." Oncogene **21**(2): 248-59.

Balsitis, S., F. Dick, N. Dyson and P. F. Lambert (2006). "Critical Roles for Non-pRb Targets of Human Papillomavirus Type 16 E7 in Cervical Carcinogenesis." Cancer Res **66**(19): 9393-400.

Balsitis, S., F. Dick, D. Lee, L. Farrell, R. K. Hyde, A. E. Griep, N. Dyson and P. F. Lambert (2005). "Examination of the pRb-dependent and pRb-independent functions of E7 in vivo." J Virol **79**(17): 11392-402.

Balsitis, S. J., J. Sage, S. Duensing, K. Munger, T. Jacks and P. F. Lambert (2003). "Recapitulation of the effects of the human papillomavirus type 16 E7 oncogene on mouse epithelium by somatic Rb deletion and detection of pRb-independent effects of E7 in vivo." Mol Cell Biol **23**(24): 9094-103.

Barbosa, M. S., D. R. Lowy and J. T. Schiller (1989). "Papillomavirus polypeptides E6 and E7 are zinc-binding proteins." J Virol **63**(3): 1404-7.

Bastien, N. and A. A. McBride (2000). "Interaction of the papillomavirus E2 protein with mitotic chromosomes." Virology **270**(1): 124-34.

Baxter, M. K., M. G. McPhillips, K. Ozato and A. A. McBride (2005). "The mitotic chromosome binding activity of the papillomavirus E2 protein correlates with interaction with the cellular chromosomal protein, Brd4." J Virol **79**(8): 4806-18.

Bechtold, V., P. Beard and K. Raj (2003). "Human papillomavirus type 16 E2 protein has no effect on transcription from episomal viral DNA." J Virol **77**(3): 2021-8.

Becker, K. A., L. Florin, C. Sapp, G. G. Maul and M. Sapp (2004). "Nuclear localization but not PML protein is required for incorporation of the papillomavirus minor capsid protein L2 into virus-like particles." J Virol **78**(3): 1121-8.

Becker, K. A., L. Florin, C. Sapp and M. Sapp (2003). "Dissection of human papillomavirus type 33 L2 domains involved in nuclear domains (ND) 10 homing and reorganization." Virology **314**(1): 161-7.

Benson, J. D., R. Lawande and P. M. Howley (1997). "Conserved interaction of the papillomavirus E2 transcriptional activator proteins with human and yeast TFIIB proteins." J Virol **71**(10): 8041-7.

Bentley, D. (2002). "The mRNA assembly line: transcription and processing machines in the same factory." Curr Opin Cell Biol **14**(3): 336-42.

Berg, M. and A. Stenlund (1997). "Functional interactions between papillomavirus E1 and E2 proteins." J Virol **71**(5): 3853-63.

Berget, S. M. (1995). "Exon recognition in vertebrate splicing." J Biol Chem **270**(6): 2411-4.

Bernard, B. A., C. Bailly, M. C. Lenoir, M. Darmon, F. Thierry and M. Yaniv (1989). "The human papillomavirus type 18 (HPV18) E2 gene product is a repressor of the HPV18 regulatory region in human keratinocytes." J Virol **63**(10): 4317-24.

Bernard, H. U. (2005). "The clinical importance of the nomenclature, evolution and taxonomy of human papillomaviruses." J Clin Virol **32 Suppl 1**: S1-6.

Bienroth, S., W. Keller and E. Wahle (1993). "Assembly of a processive messenger RNA polyadenylation complex." Embo J **12**(2): 585-94.

Blachon, S., S. Bellanger, C. Demeret and F. Thierry (2005). "Nucleo-cytoplasmic shuttling of high risk human Papillomavirus E2 proteins induces apoptosis." J Biol Chem **280**(43): 36088-98.

Black, D. L. (2003). "Mechanisms of alternative pre-messenger RNA splicing." Annu Rev Biochem **72**: 291-336.

Boner, W., E. R. Taylor, E. Tsirimonaki, K. Yamane, M. S. Campo and I. M. Morgan (2002). "A Functional interaction between the human papillomavirus 16 transcription/replication factor E2 and the DNA damage response protein TopBP1." J Biol Chem **277**(25): 22297-303.

Bonne-Andrea, C., F. Tillier, G. D. McShan, V. G. Wilson and P. Clertant (1997). "Bovine papillomavirus type 1 DNA replication: the transcriptional activator E2 acts in vitro as a specificity factor." J Virol **71**(9): 6805-15.

Bordeaux, J., S. Forte, E. Harding, M. S. Darshan, K. Klucevsek and J. Moroianu (2006). "The l2 minor capsid protein of low-risk human papillomavirus type 11 interacts with host nuclear import receptors and viral DNA." J Virol **80**(16): 8259-62.

Both, G. W., A. K. Banerjee and A. J. Shatkin (1975). "Methylation-dependent translation of viral messenger RNAs in vitro." Proc Natl Acad Sci U S A **72**(3): 1189-93.

Boukamp, P., R. T. Petrussevska, D. Breitkreutz, J. Hornung, A. Markham and N. E. Fusenig (1988). "Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line." J Cell Biol **106**(3): 761-71.

Bousarghin, L., A. L. Combita-Rojas, A. Touze, S. El Mehdaoui, P. Y. Sizaret, M. M. Bravo and P. Coursaget (2002). "Detection of neutralizing antibodies against human papillomaviruses (HPV) by inhibition of gene transfer mediated by HPV pseudovirions." J Clin Microbiol **40**(3): 926-32.

Bouvard, V., A. Storey, D. Pim and L. Banks (1994). "Characterization of the human papillomavirus E2 protein: evidence of trans-activation and trans-repression in cervical keratinocytes." Embo J **13**(22): 5451-9.

Brannon, A. R., J. A. Maresca, J. D. Boeke, M. A. Basrai and A. A. McBride (2005). "Reconstitution of papillomavirus E2-mediated plasmid maintenance in Saccharomyces cerevisiae by the Brd4 bromodomain protein." Proc Natl Acad Sci U S A **102**(8): 2998-3003.

Braunstein, T. H., B. S. Madsen, B. Gavnholt, M. W. Rosenstierne, C. Koefoed Johnsen and B. Norrild (1999). "Identification of a new promoter in the early region of the human papillomavirus type 16 genome." J Gen Virol **80 (Pt 12)**: 3241-50.

Breitburd, F., J. Salmon and G. Orth (1997). "The rabbit viral skin papillomas and carcinomas: a model for the immunogenetics of HPV-associated carcinogenesis." Clin Dermatol **15**(2): 237-47.

Brennan, C. M., I. E. Gallouzi and J. A. Steitz (2000). "Protein ligands to HuR modulate its interaction with target mRNAs in vivo." J Cell Biol **151**(1): 1-14.

Brown, D. R., J. M. Schroeder, J. T. Bryan, M. H. Stoler and K. H. Fife (1999). "Detection of multiple human papillomavirus types in Condylomata acuminata lesions from otherwise healthy and immunosuppressed patients." J Clin Microbiol **37**(10): 3316-22.

Brown, K. M. and G. M. Gilmartin (2003). "A mechanism for the regulation of pre-mRNA 3' processing by human cleavage factor Im." Mol Cell **12**(6): 1467-76.

Bryan, J. T. and D. R. Brown (2001). "Transmission of human papillomavirus type 11 infection by desquamated cornified cells." Virology **281**(1): 35-42.

Caceres, J. F. and A. R. Krainer (1997). Mammalian pre-mRNA splicing factors. *Eukaryotic mRNA processing*. A. R. Krainer. New York, IRL Press, Cold Spring Harbour: 174-203.

Caceres, J. F., G. R. Screaton and A. R. Krainer (1998). "A specific subset of SR proteins shuttles continuously between the nucleus and the cytoplasm." Genes Dev **12**(1): 55-66.

Caceres, J. F., S. Stamm, D. M. Helfman and A. R. Krainer (1994). "Regulation of alternative splicing in vivo by overexpression of antagonistic splicing factors." Science **265**(5179): 1706-9.

Canavan, T. P. and N. R. Doshi (2000). "Cervical cancer." Am Fam Physician **61**(5): 1369-76.

Cao, W. and M. A. Garcia-Blanco (1998). "A serine/arginine-rich domain in the human U1 70k protein is necessary and sufficient for ASF/SF2 binding." J Biol Chem **273**(32): 20629-35.

Cao, W., S. F. Jamison and M. A. Garcia-Blanco (1997). "Both phosphorylation and dephosphorylation of ASF/SF2 are required for pre-mRNA splicing in vitro." Rna **3**(12): 1456-67.

Caputi, M., M. Freund, S. Kammler, C. Asang and H. Schaal (2004). "A bidirectional SF2/ASF- and SRp40-dependent splicing enhancer regulates human immunodeficiency virus type 1 rev, env, vpu, and nef gene expression." J Virol **78**(12): 6517-26.

Carlsson, A. and S. Schwartz (2000). "Inhibitory activity of the human papillomavirus type 1 AU-rich element correlates inversely with the levels of the elav-like HuR protein in the cell cytoplasm." Arch Virol **145**(3): 491-503.

Carpenter, G. (2003). "ErbB-4: mechanism of action and biology." Exp Cell Res **284**(1): 66-77.

Carrillo, E., E. Garrido and P. Gariglio (2004). "Specific in vitro interaction between papillomavirus E2 proteins and TBP-associated factors." Intervirology **47**(6): 342-9.

Cazalla, D., J. Zhu, L. Manche, E. Huber, A. R. Krainer and J. F. Caceres (2002). "Nuclear export and retention signals in the RS domain of SR proteins." Mol Cell Biol **22**(19): 6871-82.

Chen, S. L., S. T. Lin, T. C. Tsai, W. C. Hsiao and Y. P. Tsao (2007). "ErbB4 (JM-b/CYT-1)-induced expression and phosphorylation of c-Jun is abrogated by human papillomavirus type 16 E5 protein." Oncogene **26**(1): 42-53.

Chew, S. L., H. X. Liu, A. Mayeda and A. R. Krainer (1999). "Evidence for the function of an exonic splicing enhancer after the first catalytic step of pre-mRNA splicing." Proc Natl Acad Sci U S A **96**(19): 10655-60.

Choe, J., P. Vailiancourt, A. Stenlund and M. Botchan (1989). "Bovine papillomavirus type 1 encodes two forms of a transcriptional repressor: structural and functional analysis of new viral cDNAs." J Virol **63**(4): 1743-55.

Chong, T., D. Apt, B. Gloss, M. Isa and H. U. Bernard (1991). "The enhancer of human papillomavirus type 16: binding sites for the ubiquitous transcription factors oct-1, NFA, TEF-2, NF1, and AP-1 participate in epithelial cell-specific transcription." J Virol **65**(11): 5933-43.

Clavel, C., J. P. Bory, S. Rihet, M. Masure, I. Duval-Binninger, I. Putaud, M. Lorenzato, C. Quereux and P. Birembaut (1998). "Comparative analysis of human papillomavirus detection by hybrid capture assay and routine cytologic screening to detect high-grade cervical lesions." Int J Cancer **75**(4): 525-8.

Clavel, C., M. Masure, J. P. Bory, I. Putaud, C. Mangeonjean, M. Lorenzato, R. Gabriel, C. Quereux and P. Birembaut (1999). "Hybrid Capture II-based human papillomavirus detection, a sensitive test to detect in routine high-grade cervical lesions: a preliminary study on 1518 women." Br J Cancer **80**(9): 1306-11.

Clower, R. V., J. C. Fisk and T. Melendy (2006). "Papillomavirus E1 protein binds to and stimulates human topoisomerase I." J Virol **80**(3): 1584-7.

Clower, R. V., Y. Hu and T. Melendy (2006). "Papillomavirus E2 protein interacts with and stimulates human topoisomerase I." Virology **348**(1): 13-8.

Collier, B., L. Goobar-Larsson, M. Sokolowski and S. Schwartz (1998). "Translational inhibition in vitro of human papillomavirus type 16 L2 mRNA mediated through interaction with heterogenous ribonucleoprotein K and poly(rC)-binding proteins 1 and 2." J Biol Chem **273**(35): 22648-56.

Collier, B., D. Oberg, X. Zhao and S. Schwartz (2002). "Specific inactivation of inhibitory sequences in the 5' end of the human papillomavirus type 16 L1 open reading frame results in production of high levels of L1 protein in human epithelial cells." J Virol **76**(6): 2739-52.

Colwill, K., T. Pawson, B. Andrews, J. Prasad, J. L. Manley, J. C. Bell and P. I. Duncan (1996). "The Clk/Sty protein kinase phosphorylates SR splicing factors and regulates their intranuclear distribution." Embo J **15**(2): 265-75.

Conger, K. L., J. S. Liu, S. R. Kuo, L. T. Chow and T. S. Wang (1999). "Human papillomavirus DNA replication. Interactions between the viral E1 protein and two subunits of human dna polymerase alpha/primase." J Biol Chem **274**(5): 2696-705.

Cripe, T. P., T. H. Haugen, J. P. Turk, F. Tabatabai, P. G. Schmid, 3rd, M. Durst, L. Gissmann, A. Roman and L. P. Turek (1987). "Transcriptional regulation of the human papillomavirus-16 E6-E7 promoter by a keratinocyte-dependent enhancer, and by viral E2 trans-activator and repressor gene products: implications for cervical carcinogenesis." Embo J **6**(12): 3745-53.

Cueille, N., R. Nougarede, F. Mechali, M. Philippe and C. Bonne-Andrea (1998). "Functional interaction between the bovine papillomavirus virus type 1 replicative helicase E1 and cyclin E-Cdk2." J Virol **72**(9): 7255-62.

Cullen, B. R. (2003). "Nuclear RNA export." J Cell Sci **116**(Pt 4): 587-97.

Culp, T. D., L. R. Budgeon, M. P. Marinkovich, G. Meneguzzi and N. D. Christensen (2006). "Keratinocyte-secreted laminin 5 can function as a transient receptor for human papillomaviruses by binding virions and transferring them to adjacent cells." J Virol **80**(18): 8940-50.

Culp, T. D. and N. D. Christensen (2004). "Kinetics of in vitro adsorption and entry of papillomavirus virions." Virology **319**(1): 152-61.

Cumming, S. A., M. G. McPhillips, T. Veerapraditsin, S. G. Milligan and S. V. Graham (2003). "Activity of the human papillomavirus type 16 late negative regulatory element is partly due to four weak consensus 5' splice sites that bind a U1 snRNP-like complex." J Virol **77**(9): 5167-77.

Cumming, S. A., C. E. Repellin, M. McPhillips, J. C. Radford, J. B. Clements and S. V. Graham (2002). "The human papillomavirus type 31 late 3' untranslated region contains a complex bipartite negative regulatory element." J Virol **76**(12): 5993-6003.

Danos, O., M. Katinka and M. Yaniv (1982). "Human papillomavirus 1a complete DNA sequence: a novel type of genome organization among papovaviridae." Embo J **1**(2): 231-6.

Dao, L. D., A. Duffy, B. A. Van Tine, S. Y. Wu, C. M. Chiang, T. R. Broker and L. T. Chow (2006). "Dynamic localization of the human papillomavirus type 11 origin binding protein E2 through mitosis while in association with the spindle apparatus." J Virol **80**(10): 4792-800.

Darshan, M. S., J. Lucchi, E. Harding and J. Morolanu (2004). "The l2 minor capsid protein of human papillomavirus type 16 interacts with a network of nuclear import receptors." J Virol **78**(22): 12179-88.

Davy, C. E., M. Ayub, D. J. Jackson, P. Das, P. McIntosh and J. Doorbar (2006). "HPV16 E1--E4 protein is phosphorylated by Cdk2/cyclin A and relocalizes this complex to the cytoplasm." Virology **349**(1): 230-44.

Davy, C. E., D. J. Jackson, K. Raj, W. L. Peh, S. A. Southern, P. Das, R. Sorathia, P. Laskey, K. Middleton, T. Nakahara, Q. Wang, P. J. Masterson, P. F. Lambert, S. Cuthill, J. B. Millar and J. Doorbar (2005). "Human papillomavirus type 16 E1 E4-induced G2 arrest is associated with cytoplasmic retention of active Cdk1/cyclin B1 complexes." J Virol **79**(7): 3998-4011.

Davy, C. E., D. J. Jackson, Q. Wang, K. Raj, P. J. Masterson, N. F. Fenner, S. Southern, S. Cuthill, J. B. Millar and J. Doorbar (2002). "Identification of a G(2) arrest domain in the E1 wedge E4 protein of human papillomavirus type 16." J Virol **76**(19): 9806-18.

Day, P. M., C. C. Baker, D. R. Lowy and J. T. Schiller (2004). "Establishment of papillomavirus infection is enhanced by promyelocytic leukemia protein (PML) expression." Proc Natl Acad Sci U S A **101**(39): 14252-7.

Day, P. M., D. R. Lowy and J. T. Schiller (2003). "Papillomaviruses infect cells via a clathrin-dependent pathway." Virology **307**(1): 1-11.

Day, P. M., R. B. Roden, D. R. Lowy and J. T. Schiller (1998). "The papillomavirus minor capsid protein, L2, induces localization of the major capsid protein, L1, and the viral transcription/replication protein, E2, to PML oncogenic domains." J Virol **72**(1): 142-50.

de Villiers, E. M., C. Fauquet, T. R. Broker, H. U. Bernard and H. zur Hausen (2004). "Classification of papillomaviruses." Virology **324**(1): 17-27.

Dell, G., K. W. Wilkinson, R. Tranter, J. Parish, R. Leo Brady and K. Gaston (2003). "Comparison of the structure and DNA-binding properties of the E2 proteins from an oncogenic and a non-oncogenic human papillomavirus." J Mol Biol **334**(5): 979-91.

Demeret, C., C. Desaintes, M. Yaniv and F. Thierry (1997). "Different mechanisms contribute to the E2-mediated transcriptional repression of human papillomavirus type 18 viral oncogenes." J Virol **71**(12): 9343-9.

Demeret, C., A. Garcia-Carranca and F. Thierry (2003). "Transcription-independent triggering of the extrinsic pathway of apoptosis by human papillomavirus 18 E2 protein." Oncogene **22**(2): 168-75.

Demers, G. W., S. A. Foster, C. L. Halbert and D. A. Galloway (1994). "Growth arrest by induction of p53 in DNA damaged keratinocytes is bypassed by human papillomavirus 16 E7." Proc Natl Acad Sci U S A **91**(10): 4382-6.

Deng, W., B. Y. Lin, G. Jin, C. G. Wheeler, T. Ma, J. W. Harper, T. R. Broker and L. T. Chow (2004). "Cyclin/CDK regulates the nucleocytoplasmic localization of the human papillomavirus E1 DNA helicase." J Virol **78**(24): 13954-65.

Desaintes, C., C. Demeret, S. Goyat, M. Yaniv and F. Thierry (1997). "Expression of the papillomavirus E2 protein in HeLa cells leads to apoptosis." Embo J **16**(3): 504-14.

Desaintes, C., S. Goyat, S. Garbay, M. Yaniv and F. Thierry (1999). "Papillomavirus E2 induces p53-independent apoptosis in HeLa cells." Oncogene **18**(32): 4538-45.

Dietrich-Goetz, W., I. M. Kennedy, B. Levins, M. A. Stanley and J. B. Clements (1997). "A cellular 65-kDa protein recognizes the negative regulatory element of human papillomavirus late mRNA." Proc Natl Acad Sci U S A **94**(1): 163-8.

DiMaio, D. and D. Mattoon (2001). "Mechanisms of cell transformation by papillomavirus E5 proteins." Oncogene **20**(54): 7866-73.

Dixon, E. P., G. L. Pahel, W. J. Rocque, J. A. Barnes, D. C. Lobe, M. H. Hanlon, K. A. Alexander, S. F. Chao, K. Lindley and W. C. Phelps (2000). "The E1 helicase of human papillomavirus type 11 binds to the origin of replication with low sequence specificity." Virology **270**(2): 345-57.

Doorbar, J. (2005). "The papillomavirus life cycle." J Clin Virol **32 Suppl 1**: S7-15.

Doorbar, J., I. Coneron and P. H. Gallimore (1989). "Sequence divergence yet conserved physical characteristics among the E4 proteins of cutaneous human papillomaviruses." Virology **172**(1): 51-62.

Doorbar, J., R. C. Elston, S. Napthine, K. Raj, E. Medcalf, D. Jackson, N. Coleman, H. M. Griffin, P. Masterson, S. Stacey, Y. Mengistu and J. Dunlop (2000). "The E1E4 protein of human papillomavirus type 16 associates with a putative RNA helicase through sequences in its C terminus." J Virol **74**(21): 10081-95.

Doorbar, J., S. Ely, J. Sterling, C. McLean and L. Crawford (1991). "Specific interaction between HPV-16 E1-E4 and cytokeratins results in collapse of the epithelial cell intermediate filament network." Nature **352**(6338): 824-7.

Doorbar, J., C. Foo, N. Coleman, L. Medcalf, O. Hartley, T. Prospero, S. Napthine, J. Sterling, G. Winter and H. Griffin (1997). "Characterization of events during the late stages of HPV16 infection in vivo using high-affinity synthetic Fabs to E4." Virology **238**(1): 40-52.

Doorbar, J., E. Medcalf and S. Napthine (1996). "Analysis of HPV1 E4 complexes and their association with keratins in vivo." Virology **218**(1): 114-26.

Dostatni, N., P. F. Lambert, R. Sousa, J. Ham, P. M. Howley and M. Yaniv (1991). "The functional BPV-1 E2 trans-activating protein can act as a repressor by preventing formation of the initiation complex." Genes Dev **5**(9): 1657-71.

Dowanhick, J. J., A. A. McBride and P. M. Howley (1995). "Supression of cellular proliferation by papillomavirus E2 protein." Journal of Virology **69**: 7791-7799.

Drain, P. K., K. K. Holmes, J. P. Hughes and L. A. Koutsky (2002). "Determinants of cervical cancer rates in developing countries." Int J Cancer **100**(2): 199-205.

Duensing, S., A. Duensing, E. R. Flores, A. Do, P. F. Lambert and K. Munger (2001). "Centrosome abnormalities and genomic instability by episomal expression of human papillomavirus type 16 in raft cultures of human keratinocytes." J Virol **75**(16): 7712-6.

Duensing, S., L. Y. Lee, A. Duensing, J. Basile, S. Piboonniyom, S. Gonzalez, C. P. Crum and K. Munger (2000). "The human papillomavirus type 16 E6 and E7 oncoproteins cooperate to induce mitotic defects and genomic instability by uncoupling centrosome duplication from the cell division cycle." Proc Natl Acad Sci U S A **97**(18): 10002-7.

Duensing, S. and K. Munger (2002). "The human papillomavirus type 16 E6 and E7 oncoproteins independently induce numerical and structural chromosome instability." Cancer Res **62**(23): 7075-82.

Duensing, S. and K. Munger (2003). "Human papillomavirus type 16 E7 oncoprotein can induce abnormal centrosome duplication through a mechanism independent of inactivation of retinoblastoma protein family members." J Virol **77**(22): 12331-5.

Durst, M., F. X. Bosch, D. Glitz, A. Schneider and H. zur Hausen (1991). "Inverse relationship between human papillomavirus (HPV) type 16 early gene expression and cell differentiation in nude mouse epithelial cysts and tumors induced by HPV-positive human cell lines." J Virol **65**(2): 796-804.

Embers, M. E., L. R. Budgeon, T. D. Culp, C. A. Reed, M. D. Pickel and N. D. Christensen (2004). "Differential antibody responses to a distinct region of human papillomavirus minor capsid proteins." Vaccine **22**(5-6): 670-80.

Enzenauer, C., G. Mengus, A. Lavigne, I. Davidson, H. Pfister and M. May (1998). "Interaction of human papillomavirus 8 regulatory proteins E2, E6 and E7 with components of the TFIID complex." Intervirology **41**(2-3): 80-90.

Eperon, I. C., O. V. Makarova, A. Mayeda, S. H. Munroe, J. F. Caceres, D. G. Hayward and A. R. Krainer (2000). "Selection of alternative 5' splice sites: role of U1 snRNP and models for the antagonistic effects of SF2/ASF and hnRNP A1." Moi Cell Biol **20**(22): 8303-18.

Estmer Nilsson, C., S. Petersen-Mahrt, C. Durot, R. Shtrichman, A. R. Krainer, T. Kleinberger and G. Akusjarvi (2001). "The adenovirus E4-ORF4 splicing enhancer protein interacts with a subset of phosphorylated SR proteins." Embo J **20**(4): 864-71.

Evander, M., I. H. Frazer, E. Payne, Y. M. Qi, K. Hengst and N. A. McMillan (1997). "Identification of the alpha6 integrin as a candidate receptor for papillomaviruses." J Virol **71**(3): 2449-56.

Fang, L., L. R. Budgeon, J. Doorbar, E. R. Briggs and M. K. Howett (2006). "The human papillomavirus type 11 E1ΛE4 protein is not essential for viral genome amplification." Virology **351**(2): 271-9.

Faustino, N. A. and T. A. Cooper (2003). "Pre-mRNA splicing and human disease." Genes Dev **17**(4): 419-37.

Fay, A., W. H. t. Yutzy, R. B. Roden and J. Moroianu (2004). "The positively charged termini of L2 minor capsid protein required for bovine papillomavirus infection function separately in nuclear import and DNA binding." J Virol **78**(24): 13447-54.

Ferris, D. G., T. C. Wright, Jr., M. S. Litaker, R. M. Richart, A. T. Lorincz, X. W. Sun, L. Borgatta, H. Buck, L. Kramer and R. Rubin (1998). "Triage of women with ASCUS and LSIL on Pap smear reports: management by repeat Pap smear, HPV DNA testing, or colposcopy?" J Fam Pract **46**(2): 125-34.

Fife, K. H., H. M. Cramer, J. M. Schroeder and D. R. Brown (2001). "Detection of multiple human papillomavirus types in the lower genital tract correlates with cervical dysplasia." J Med Virol **64**(4): 550-9.

Flaherty, S. M., P. Fortes, E. Izaurralde, I. W. Mattaj and G. M. Gilmartin (1997). "Participation of the nuclear cap binding complex in pre-mRNA 3' processing." Proc Natl Acad Sci U S A **94**(22): 11893-8.

Flores, E. R. and P. F. Lambert (1997). "Evidence for a switch in the mode of human papillomavirus type 16 DNA replication during the viral life cycle." J Virol **71**(10): 7167-79.

Foster, S. A., G. W. Demers, B. G. Etscheid and D. A. Galloway (1994). "The ability of human papillomavirus E6 proteins to target p53 for degradation in vivo correlates with their ability to abrogate actinomycin D-induced growth arrest." J Virol **68**(9): 5698-705.

Frattini, M. G. and L. A. Laimins (1994). "Binding of the human papillomavirus E1 origin-recognition protein is regulated through complex formation with the E2 enhancer-binding protein." Proc Natl Acad Sci U S A **91**(26): 12398-402.

Frisch, M. and M. T. Goodman (2000). "Human papillomavirus-associated carcinomas in Hawaii and the mainland U.S." Cancer **88**(6): 1464-9.

Fu, X. D. and T. Maniatis (1992). "Isolation of a complementary DNA that encodes the mammalian splicing factor SC35." Science **256**(5056): 535-8.

Fuchs, E. (1990). "Epidermal differentiation." Curr Opin Cell Biol **2**(6): 1028-35.

Fuchs, E. (1993). "Epidermal differentiation and keratin gene expression." J Cell Sci Suppl **17**: 197-208.

Fuchs, E. and C. Byrne (1994). "The epidermis: rising to the surface." Curr Opin Genet Dev **4**(5): 725-36.

Funk, J. O., S. Waga, J. B. Harry, E. Espling, B. Stillman and D. A. Galloway (1997). "Inhibition of CDK activity and PCNA-dependent DNA replication by p21 is blocked by interaction with the HPV-16 E7 oncoprotein." Genes Dev **11**(16): 2090-100.

Furth, P. A. and C. C. Baker (1991). "An element in the bovine papillomavirus late 3' untranslated region reduces polyadenylated cytoplasmic RNA levels." J Virol **65**(11): 5806-12.

Furth, P. A., W. T. Choe, J. H. Rex, J. C. Byrne and C. C. Baker (1994). "Sequences homologous to 5' splice sites are required for the inhibitory activity of papillomavirus late 3' untranslated regions." Mol Cell Biol **14**(8): 5278-89.

Furuichi, Y. and A. J. Shatkin (2000). "Viral and cellular mRNA capping: past and prospects." Adv Virus Res **55**: 135-84.

Gammoh, N., H. S. Grm, P. Massimi and L. Banks (2006). "Regulation of human papillomavirus type 16 E7 activity through direct protein interaction with the E2 transcriptional activator." J Virol **80**(4): 1787-97.

Gamzu, R., B. Almog, I. Levin, O. Fainaru, J. Niv, J. B. Lessing and A. Bar-Am (2002). "Clinical and economic implications of adding HPV tests to the routine cytology follow-up and management of patients with histologically defined cervical intraepithelial neoplasia grade 1." Gynecol Oncol **86**(2): 129-33.

Gardiol, D., C. Kuhne, B. Glaunsinger, S. S. Lee, R. Javier and L. Banks (1999). "Oncogenic human papillomavirus E6 proteins target the discs large tumour suppressor for proteasome-mediated degradation." Oncogene **18**(40): 5487-96.

Gatfield, D. and E. Izaurralde (2002). "REF1/Aly and the additional exon junction complex proteins are dispensable for nuclear mRNA export." J Cell Biol **159**(4): 579-88.

Gauthier, J. M., J. Dillner and M. Yaniv (1991). "Structural analysis of the human papillomavirus type 16-E2 transactivator with antipeptide antibodies reveals a high mobility region linking the transactivation and the DNA-binding domains." Nucleic Acids Res **19**(25): 7073-9.

Gingras, A. C., B. Raught and N. Sonenberg (1999). "eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation." Annu Rev Biochem **68**: 913-63.

Giroglou, T., L. Florin, F. Schafer, R. E. Streeck and M. Sapp (2001). "Human papillomavirus infection requires cell surface heparan sulfate." J Virol **75**(3): 1565-70.

Giuliano, A. R., R. Harris, R. L. Sedjo, S. Baldwin, D. Roe, M. R. Papenfuss, M. Abrahamsen, P. Inserra, S. Olvera and K. Hatch (2002). "Incidence, prevalence, and clearance of type-specific human papillomavirus infections: The Young Women's Health Study." J Infect Dis **186**(4): 462-9.

Glaunsinger, B. A., S. S. Lee, M. Thomas, L. Banks and R. Javier (2000). "Interactions of the PDZ-protein MAGI-1 with adenovirus E4-ORF1 and high-risk papillomavirus E6 oncoproteins." Oncogene **19**(46): 5270-80.

Gloss, B., T. Chong and H. U. Bernard (1989). "Numerous nuclear proteins bind the long control region of human papillomavirus type 16: a subset of 6 of 23 DNase I-protected segments coincides with the location of the cell-type-specific enhancer." J Virol **63**(3): 1142-52.

Goodman, A. (2000). "Role of routine human papillomavirus subtyping in cervical screening." Curr Opin Obstet Gynecol **12**(1): 11-4.

Goodwin, E. C., E. Yang, C. J. Lee, H. W. Lee, D. DiMaio and E. S. Hwang (2000). "Rapid induction of senescence in human cervical carcinoma cells." Proceedings of the National Academy for Science, USA **97**: 10978-10983.

Gopalakrishnan, V. and S. A. Khan (1994). "E1 protein of human papillomavirus type 1a is sufficient for initiation of viral DNA replication." Proc Natl Acad Sci U S A **91**(20): 9597-601.

Grassmann, K., B. Rapp, H. Maschek, K. U. Petry and T. Iftner (1996). "Identification of a differentiation-inducible promoter in the E7 open reading frame of human papillomavirus type 16 (HPV-16) in raft cultures of a new cell line containing high copy numbers of episomal HPV-16 DNA." J Virol **70**(4): 2339-49.

Graveley, B. R. (2000). "Sorting out the complexity of SR protein functions." Rna **6**(9): 1197-211.

Grm, H. S., P. Massimi, N. Gammoh and L. Banks (2005). "Crosstalk between the human papillomavirus E2 transcriptional activator and the E6 oncoprotein." Oncogene **24**(33): 5149-64.

Gui, J. F., H. Tronchere, S. D. Chandler and X. D. Fu (1994). "Purification and characterization of a kinase specific for the serine- and arginine-rich pre-mRNA splicing factors." Proc Natl Acad Sci U S A **91**(23): 10824-8.

Gunderson, S. I., K. Beyer, G. Martin, W. Keller, W. C. Boelens and L. W. Mattaj (1994). "The human U1A snRNP protein regulates polyadenylation via a direct interaction with poly(A) polymerase." Cell **76**(3): 531-41.

Gunderson, S. I., M. Polycarpou-Schwarz and I. W. Mattaj (1998). "U1 snRNP inhibits pre-mRNA polyadenylation through a direct interaction between U1 70K and poly(A) polymerase." Mol Cell **1**(2): 255-64.

Gunderson, S. I., S. Vagner, M. Polycarpou-Schwarz and I. W. Mattaj (1997). "Involvement of the carboxyl terminus of vertebrate poly(A) polymerase in U1A autoregulation and in the coupling of splicing and polyadenylation." Genes Dev **11**(6): 761-73.

Hadaschik, D., K. Hinterkeuser, M. Oldak, H. J. Pfister and S. Smola-Hess (2003). "The Papillomavirus E2 protein binds to and synergizes with C/EBP factors involved in keratinocyte differentiation." J Virol **77**(9): 5253-65.

Ham, J., G. Steger and M. Yaniv (1994). "Cooperativity in vivo between the E2 transactivator and the TATA box binding protein depends on core promoter structure." Embo J **13**(1): 147-57.

Hamilton, B. J., C. M. Burns, R. C. Nichols and W. F. Rigby (1997). "Modulation of AUUUA response element binding by heterogeneous nuclear ribonucleoprotein A1 in human T lymphocytes. The roles of cytoplasmic location, transcription, and phosphorylation." J Biol Chem **272**(45): 28732-41.

Hammell, C. M., S. Gross, D. Zenklusen, C. V. Heath, F. Stutz, C. Moore and C. N. Cole (2002). "Coupling of termination, 3' processing, and mRNA export." Mol Cell Biol **22**(18): 6441-57.

Han, Y., Y. M. Loo, K. T. Militello and T. Melendy (1999). "Interactions of the papovavirus DNA replication initiator proteins, bovine papillomavirus type 1 E1 and simian virus 40 large T antigen, with human replication protein A." J Virol **73**(6): 4899-907.

Hanamura, A., J. F. Caceres, A. Mayeda, B. R. Franza, Jr. and A. R. Krainer (1998). "Regulated tissue-specific expression of antagonistic pre-mRNA splicing factors." Rna **4**(4): 430-44.

Harper, D. M., E. L. Franco, C. Wheeler, D. G. Ferris, D. Jenkins, A. Schuind, T. Zahaf, B. Innis, P. Naud, N. S. De Carvalho, C. M. Roteli-Martins, J. Teixeira, M. M. Blatter, A. P. Korn, W. Quint and G. Dubin (2004). "Efficacy of a bivalent L1 virus-like particle vaccine in prevention of infection with human papillomavirus types 16 and 18 in young women: a randomised controlled trial." Lancet **364**(9447): 1757-65.

Harper, J. E. and J. L. Manley (1992). "Multiple activities of the human splicing factor ASF." Gene Expr **2**(1): 19-29.

Harwood, C. A., T. Surentheran, P. Sasieni, C. M. Proby, C. Bordea, I. M. Leigh, F. Wojnarowska, J. Breuer and J. M. McGregor (2004). "Increased risk of skin cancer associated with the presence of epidermodysplasia verruciformis human papillomavirus types in normal skin." Br J Dermatol **150**(5): 949-57.

Hebner, C. M. and L. A. Laimins (2006). "Human papillomaviruses: basic mechanisms of pathogenesis and oncogenicity." Rev Med Virol **16**(2): 83-97.

Hegde, R. S. and E. J. Androphy (1998). "Crystal structure of the E2 DNA-binding domain from human papillomavirus type 16: implications for its DNA binding-site selection mechanism." J Mol Biol **284**(5): 1479-89.

Helt, A. M. and D. A. Galloway (2001). "Destabilization of the retinoblastoma tumor suppressor by human papillomavirus type 16 E7 is not sufficient to overcome cell cycle arrest in human keratinocytes." J Virol **75**(15): 6737-47.

Hines, C. S., C. Meghoo, S. Shetty, M. Biburger, M. Brenowitz and R. S. Hegde (1998). "DNA structure and flexibility in the sequence-specific binding of papillomavirus E2 proteins." J Mol Biol **276**(4): 809-18.

Hoppe-Seyler, F. and K. Butz (1992). "Activation of human papillomavirus type 18 E6-E7 oncogene expression by transcription factor Sp1." Nucleic Acids Res **20**(24): 6701-6.

Hou, S. Y., S. Y. Wu, T. Zhou, M. C. Thomas and C. M. Chiang (2000). "Alleviation of human papillomavirus E2-mediated transcriptional repression via formation of a TATA binding protein (or TFIID)-TFIIB-RNA polymerase II-TFIIF preinitiation complex." Mol Cell Biol **20**(1): 113-25.

Howley, P. (1996). *Papillomavirinae*: The Viruses and Their Replication. Fields Virology. D. M. Fields, P. M. Knipe and P. Howley, Lippincott-Raven: 2045-2076.

Hsu, C. Y., F. Mechali and C. Bonne-Andrea (2006). "Nucleo-cytoplasmic shuttling of bovine papillomavirus E1 helicase down regulates viral DNA replication in S phase." J Virol.

Hu, J., C. S. Lutz, J. Wilusz and B. Tian (2005). "Bioinformatic identification of candidate cis-regulatory elements involved in human mRNA polyadenylation." Rna **11**(10): 1485-93.

Huang, S. M. and D. J. McCance (2002). "Down regulation of the interleukin-8 promoter by human papillomavirus type 16 E6 and E7 through effects on CREB binding protein/p300 and P/CAF." J Virol **76**(17): 8710-21.

Huang, Y., R. Gattoni, J. Stevenin and J. A. Steitz (2003). "SR splicing factors serve as adapter proteins for TAP-dependent mRNA export." Mol Cell **11**(3): 837-43.

Huang, Y. and J. A. Steitz (2001). "Splicing factors SRp20 and 9G8 promote the nucleocytoplasmic export of mRNA." Mol Cell **7**(4): 899-905.

Hudson, E. (1990). "Cervical cytology." Bmj **300**(6736): 1353-4.

Huibregtse, J. M., M. Scheffner and P. M. Howley (1991). "A cellular protein mediates association of p53 with the E6 oncoprotein of human papillomavirus types 16 or 18." Embo J **10**(13): 4129-35.

Huibregtse, J. M., M. Scheffner and P. M. Howley (1993). "Localization of the E6-AP regions that direct human papillomavirus E6 binding, association with p53, and ubiquitination of associated proteins." Mol Cell Biol **13**(8): 4918-27.

Hummel, M., J. B. Hudson and L. A. Laimins (1992). "Differentiation-induced and constitutive transcription of human papillomavirus type 31b in cell lines containing viral episomes." J Virol **66**(10): 6070-80.

Hwang, S. G., D. Lee, J. Kim, T. Seo and J. Choe (2002). "Human papillomavirus type 16 E7 binds to E2F1 and activates E2F1-driven transcription in a retinoblastoma protein-independent manner." J Biol Chem **277**(4): 2923-30.

Izaurralde, E. and S. Adam (1998). "Transport of macromolecules between the nucleus and the cytoplasm." Rna **4**(4): 351-64.

Izaurralde, E., A. Jarmolowski, C. Beisel, I. W. Mattaj, G. Dreyfuss and U. Fischer (1997). "A role for the M9 transport signal of hnRNP A1 in mRNA nuclear export." J Cell Biol **137**(1): 27-35.

Jenny, A., H. P. Hauri and W. Keller (1994). "Characterization of cleavage and polyadenylation specificity factor and cloning of its 100-kilodalton subunit." Mol Cell Biol **14**(12): 8183-90.

Jeon, S., B. L. Allen-Hoffmann and P. F. Lambert (1995). "Integration of human papillomavirus type 16 into the human genome correlates with a selective growth advantage of cells." J Virol **69**(5): 2989-97.

Jeon, S. and P. F. Lambert (1995). "Integration of human papillomavirus type 16 DNA into the human genome leads to increased stability of E6 and E7 mRNAs: implications for cervical carcinogenesis." Proc Natl Acad Sci U S A **92**(5): 1654-8.

Jones, D. L., R. M. Alani and K. Munger (1997). "The human papillomavirus E7 oncoprotein can uncouple cellular differentiation and proliferation in human keratinocytes by abrogating p21Cip1-mediated inhibition of cdk2." Genes Dev **11**(16): 2101-11.

Kalantari, M. and H. U. Bernard (2006). Gene Expression of Papillomaviruses. Papillomavirus Research. M. S. Campo. Norfolk, Caister Academic Press: 41-52.

Kamper, N., P. M. Day, T. Nowak, H. C. Selinka, L. Florin, J. Bolscher, L. Hilbig, J. T. Schiller and M. Sapp (2006). "A membrane-destabilizing peptide in capsid protein L2 is required for egress of papillomavirus genomes from endosomes." J Virol **80**(2): 759-68.

Kanopka, A., O. Muhlemann and G. Akusjarvi (1996). "Inhibition by SR proteins of splicing of a regulated adenovirus pre-mRNA." Nature **381**(6582): 535-8.

Kanopka, A., O. Muhlemann, S. Petersen-Mahrt, C. Estmer, C. Ohrmalm and G. Akusjarvi (1998). "Regulation of adenovirus alternative RNA splicing by dephosphorylation of SR proteins." Nature **393**(6681): 185-7.

Katahira, J., K. Straesser, T. Saiwaki, Y. Yoneda and E. Hurt (2002). "Complex formation between Tap and p15 affects binding to FG-repeat nucleoporins and nucleocytoplasmic shuttling." J Biol Chem **277**(11): 9242-6.

Kawana, Y., K. Kawana, H. Yoshikawa, Y. Taketani, K. Yoshiike and T. Kanda (2001). "Human papillomavirus type 16 minor capsid protein l2 N-terminal region containing a common neutralization epitope binds to the cell surface and enters the cytoplasm." J Virol **75**(5): 2331-6.

Keene, J. D. (1999). "Why is Hu where? Shuttling of early-response-gene messenger RNA subsets." Proc Natl Acad Sci U S A **96**(1): 5-7.

Keller, W. (1995). 3'-End Cleavage and Polyadenylation of Nuclear Messenger RNA Precursors. Pre-mRNA Processing. A. I. Lamond. Austin, R.G. Landes Company: 113-134.

Kennedy, I. M., J. K. Haddow and J. B. Clements (1990). "Analysis of human papillomavirus type 16 late mRNA 3' processing signals in vitro and in vivo." J Virol **64**(4): 1825-9.

Kennedy, I. M., J. K. Haddow and J. B. Clements (1991). "A negative regulatory element in the human papillomavirus type 16 genome acts at the level of late mRNA stability." J Virol **65**(4): 2093-7.

Kidd, J. G. and P. Rous (1940). "A transplantable rabbit carcinoma originating in a virus-induced papilloma and containing the vius in masked or altered form." Journal of Experimental Medicine **71**: 813-837.

Kim, K., P. A. Garner-Hamrick, C. Fisher, D. Lee and P. F. Lambert (2003). "Methylation patterns of papillomavirus DNA, its influence on E2 function, and implications in viral infection." J Virol **77**(23): 12450-9.

Kiyono, T., A. Hiraiwa, M. Fujita, Y. Hayashi, T. Akiyama and M. Ishibashi (1997). "Binding of high-risk human papillomavirus E6 oncoproteins to the human homologue of the Drosophila discs large tumor suppressor protein." Proc Natl Acad Sci U S A **94**(21): 11612-6.

Kjaer, S. K., B. Chackerian, A. J. van den Brule, E. I. Svare, G. Paull, J. M. Walboomers, J. T. Schiller, J. E. Bock, M. E. Sherman, D. R. Lowy and C. L. Meijer (2001). "High-risk human papillomavirus is sexually transmitted: evidence from a follow-up study of virgins starting sexual activity (intercourse)." Cancer Epidemiol Biomarkers Prev **10**(2): 101-6.

Klucevsek, K., J. Daley, M. S. Darshan, J. Bordeaux and J. Moroianu (2006). "Nuclear import strategies of high-risk HPV18 L2 minor capsid protein." Virology **352**(1): 200-8.

Knight, G. L., J. R. Grainger, P. H. Gallimore and S. Roberts (2004). "Cooperation between different forms of the human papillomavirus type 1 E4 protein to block cell cycle progression and cellular DNA synthesis." J Virol **78**(24): 13920-33.

Koffa, M. D., S. V. Graham, Y. Takagaki, J. L. Manley and J. B. Clements (2000). "The human papillomavirus type 16 negative regulatory RNA element interacts with three proteins that act at different posttranscriptional levels." Proc Natl Acad Sci U S A **97**(9): 4677-82.

Koutsky, L. (1997). "Epidemiology of genital human papillomavirus infection." Am J Med **102**(5A): 3-8.

Koutsky, L. A., K. A. Ault, C. M. Wheeler, D. R. Brown, E. Barr, F. B. Alvarez, L. M. Chiacchierini and K. U. Jansen (2002). "A controlled trial of a human papillomavirus type 16 vaccine." N Engl J Med **347**(21): 1645-51.

Krajewski, S., M. Krajewska and J. C. Reed (1996). "Immunohistochemical analysis of in vivo patterns of Bak expression, a proapoptotic member of the Bcl-2 protein family." Cancer Res **56**(12): 2849-55.

Kumar, A., Y. Zhao, G. Meng, M. Zeng, S. Srinivasan, L. M. Delmolino, Q. Gao, G. Dimri, G. F. Weber, D. E. Wazer, H. Band and V. Band (2002). "Human papillomavirus oncoprotein E6 inactivates the transcriptional coactivator human ADA3." Mol Cell Biol **22**(16): 5801-12.

Kuo, S. R., J. S. Liu, T. R. Broker and L. T. Chow (1994). "Cell-free replication of the human papillomavirus DNA with homologous viral E1 and E2 proteins and human cell extracts." J Biol Chem **269**(39): 24058-65.

Laimins, L. A. (1996). "Human papillomaviruses target differentiating epithelia for virion production and malignant conversion." Seminars in Virology **7**: 305-313.

Lambert, P. F. (1991). "Papillomavirus DNA replication." J Virol **65**(7): 3417-20.

Lambert, P. F., B. A. Spalholz and P. M. Howley (1987). "A transcriptional repressor encoded by BPV-1 shares a common carboxy-terminal domain with the E2 transactivator." Cell **50**(1): 69-78.

Le Hir, H., E. Izaurralde, L. E. Maquat and M. J. Moore (2000). "The spliceosome deposits multiple proteins 20-24 nucleotides upstream of mRNA exon-exon junctions." Embo J **19**(24): 6860-9.

Lechner, M. S. and L. A. Laimins (1994). "Inhibition of p53 DNA binding by human papillomavirus E6 proteins." J Virol **68**(7): 4262-73.

Lechner, M. S., D. H. Mack, A. B. Finicle, T. Crook, K. H. Vousden and L. A. Laimins (1992). "Human papillomavirus E6 proteins bind p53 in vivo and abrogate p53-mediated repression of transcription." Embo J **11**(8): 3045-52.

Lee, C. and L. A. Laimins (2004). "Role of the PDZ domain-binding motif of the oncoprotein E6 in the pathogenesis of human papillomavirus type 31." J Virol **78**(22): 12366-77.

Lee, D., S. G. Hwang, J. Kim and J. Choe (2002). "Functional interaction between p/CAF and human papillomavirus E2 protein." J Biol Chem **277**(8): 6483-9.

Lee, D., H. Z. Kim, K. W. Jeong, Y. S. Shim, I. Horikawa, J. C. Barrett and J. Choe (2002). "Human papillomavirus E2 down-regulates the human telomerase reverse transcriptase promoter." J Biol Chem **277**(31): 27748-56.

Lee, D., B. Lee, J. Kim, D. W. Kim and J. Choe (2000). "cAMP response element-binding protein-binding protein binds to human papillomavirus E2 protein and activates E2-dependent transcription." J Biol Chem **275**(10): 7045-51.

Lee, S. S., B. Glaunsinger, F. Mantovani, L. Banks and R. T. Javier (2000). "Multi-PDZ domain protein MUPP1 is a cellular target for both adenovirus E4-ORF1 and high-risk papillomavirus type 18 E6 oncoproteins." J Virol **74**(20): 9680-93.

Li, R., J. D. Knight, S. P. Jackson, R. Tjian and M. R. Botchan (1991). "Direct interaction between Sp1 and the BPV enhancer E2 protein mediates synergistic activation of transcription." Cell **65**(3): 493-505.

Li, Y. and B. J. Blencowe (1999). "Distinct factor requirements for exonic splicing enhancer function and binding of U2AF to the polypyrimidine tract." J Biol Chem **274**(49): 35074-9.

Lin, B. Y., A. M. Makhov, J. D. Griffith, T. R. Broker and L. T. Chow (2002). "Chaperone proteins abrogate inhibition of the human papillomavirus (HPV)

E1 replicative helicase by the HPV E2 protein." Mol Cell Biol **22**(18): 6592-604.

Liu, D., R. Ishima, K. I. Tong, S. Bagby, T. Kokubo, D. R. Muhandiram, L. E. Kay, Y. Nakatani and M. Ikura (1998). "Solution structure of a TBP-TAF(II)230 complex: protein mimicry of the minor groove surface of the TATA box unwound by TBP." Cell **94**(5): 573-83.

Liu, J. S., S. R. Kuo, A. M. Makhov, D. M. Cyr, J. D. Griffith, T. R. Broker and L. T. Chow (1998). "Human Hsp70 and Hsp40 chaperone proteins facilitate human papillomavirus-11 E1 protein binding to the origin and stimulate cell-free DNA replication." J Biol Chem **273**(46): 30704-12.

Liu, W. J., Y. M. Qi, K. N. Zhao, Y. H. Liu, X. S. Liu and I. H. Frazer (2001). "Association of bovine papillomavirus type 1 with microtubules." Virology **282**(2): 237-44.

Longman, D., I. L. Johnstone and J. F. Caceres (2000). "Functional characterization of SR and SR-related genes in Caenorhabditis elegans." Embo J **19**(7): 1625-37.

Loo, Y. M. and T. Melendy (2004). "Recruitment of replication protein A by the papillomavirus E1 protein and modulation by single-stranded DNA." J Virol **78**(4): 1605-15.

Lowe, S. W., T. Jacks, D. E. Housman and H. E. Ruley (1994). "Abrogation of oncogene-associated apoptosis allows transformation of p53-deficient cells." Proc Natl Acad Sci U S A **91**(6): 2026-30.

Lutz, C. S. and J. C. Alwine (1994). "Direct interaction of the U1 snRNP-A protein with the upstream efficiency element of the SV40 late polyadenylation signal." Genes Dev **8**(5): 576-86.

Lutz, C. S., K. G. Murthy, N. Schek, J. P. O'Connor, J. L. Manley and J. C. Alwine (1996). "Interaction between the U1 snRNP-A protein and the 160-kD subunit of cleavage-polyadenylation specificity factor increases polyadenylation efficiency in vitro." Genes Dev **10**(3): 325-37.

Ma, T., N. Zou, B. Y. Lin, L. T. Chow and J. W. Harper (1999). "Interaction between cyclin-dependent kinases and human papillomavirus replication-initiation protein E1 is required for efficient viral replication." Proc Natl Acad Sci U S A **96**(2): 382-7.

MacDonald, C. C., J. Wilusz and T. Shenk (1994). "The 64-kilodalton subunit of the CstF polyadenylation factor binds to pre-mRNAs downstream of the cleavage site and influences cleavage site location." Mol Cell Biol **14**(10): 6647-54.

Madison, K. C. (2003). "Barrier function of the skin: "la raison d'etre" of the epidermis." J Invest Dermatol **121**(2): 231-41.

Maitland, N. J., S. Conway, N. S. Wilkinson, J. Ramsdale, J. R. Morris, C. M. Sanders, J. E. Burns, P. L. Stern and M. Wells (1998). "Expression patterns of the human papillomavirus type 16 transcription factor E2 in low- and high-grade cervical intraepithelial neoplasia." J Pathol **186**(3): 275-80.

Maloney, E. M., E. R. Unger, R. A. Tucker, D. Swan, K. Karem, N. W. Todd and W. C. Reeves (2006). "Longitudinal measures of human papillomavirus 6 and 11 viral loads and antibody response in children with recurrent respiratory papillomatosis." Arch Otolaryngol Head Neck Surg **132**(7): 711-5.

Mantovani, F. and L. Banks (2001). "The human papillomavirus E6 protein and its contribution to malignant progression." Oncogene **20**(54): 7874-87.

Massimi, P., N. Gammoh, M. Thomas and L. Banks (2004). "HPV E6 specifically targets different cellular pools of its PDZ domain-containing tumour suppressor substrates for proteasome-mediated degradation." Oncogene **23**(49): 8033-9.

Massimi, P., D. Pim, C. Bertoli, V. Bouvard and L. Banks (1999). "Interaction between the HPV-16 E2 transcriptional activator and p53." Oncogene **18**(54): 7748-54.

Masterson, P. J., M. A. Stanley, A. P. Lewis and M. A. Romanos (1998). "A C-terminal helicase domain of the human papillomavirus E1 protein binds E2 and the DNA polymerase alpha-primase p68 subunit." J Virol **72**(9): 7407-19.

Matsumoto, Y., S. Nakagawa, T. Yano, S. Takizawa, K. Nagasaka, K. Nakagawa, T. Minaguchi, O. Wada, H. Ooishi, K. Matsumoto, T. Yasugi, T. Kanda, J. M. Huibregtse and Y. Taketani (2006). "Involvement of a cellular ubiquitin-protein ligase E6AP in the ubiquitin-mediated degradation of extensive substrates of high-risk human papillomavirus E6." J Med Virol **78**(4): 501-7.

McBride, A. A., J. C. Byrne and P. M. Howley (1989). "E2 polypeptides encoded by bovine papillomavirus type 1 form dimers through the common carboxyl-terminal domain: transactivation is mediated by the conserved amino-terminal domain." Proc Natl Acad Sci U S A **86**(2): 510-4.

McIntyre, M. C., M. N. Ruesch and L. A. Laimins (1996). "Human papillomavirus E7 oncoproteins bind a single form of cyclin E in a complex with cdk2 and p107." Virology **215**(1): 73-82.

McMillan, N. A., E. Payne, I. H. Frazer and M. Evander (1999). "Expression of the alpha6 integrin confers papillomavirus binding upon receptor-negative B-cells." Virology **261**(2): 271-9.

McPhillips, M. G., J. G. Oliveira, J. E. Spindler, R. Mitra and A. A. McBride (2006). "Brd4 is required for e2-mediated transcriptional activation but not genome partitioning of all papillomaviruses." J Virol **80**(19): 9530-43.

McPhillips, M. G., K. Ozato and A. A. McBride (2005). "Interaction of bovine papillomavirus E2 protein with Brd4 stabilizes its association with chromatin." J Virol **79**(14): 8920-32.

McPhillips, M. G., T. Veerapraditsin, S. A. Cumming, D. Karali, S. G. Milligan, W. Boner, I. M. Morgan and S. V. Graham (2004). "SF2/ASF binds the human papillomavirus type 16 late RNA control element and is regulated during differentiation of virus-infected epithelial cells." J Virol **78**(19): 10598-605.

McPhillips, M.G. (2004). "Regulation of Human Papillomavirus Type 16 Late Protein EXpression During Epithelial Differentiation." Submitted thesis.

McPhillips, M. G., T. Veerapraditsin, S. A. Cumming, D. Karali, S. G. Milligan, W. Bonner, I. M. Morgan, P. Cordano, M. S. Campo and S. V. Graham (Unpublished). "SF2/ASF binds the human papillomavirus type 16 late RNA control element and is regulated during differentiation of virus-infected epitehlial cells."

Melsheimer, P., S. Vinokurova, N. Wentzensen, G. Bastert and M. von Knebel Doeberitz (2004). "DNA aneuploidy and integration of human papillomavirus type 16 e6/e7 oncogenes in intraepithelial neoplasia and invasive squamous cell carcinoma of the cervix uteri." Clin Cancer Res **10**(9): 3059-63.

Mercer, J., Mahmoudi, M. and Bennet, M. (2007). "DNA damage, p53, apoptosis and vascular disease." Mutat Res.

Merle, E., R. C. Rose, L. LeRoux and J. Moroianu (1999). "Nuclear import of HPV11 L1 capsid protein is mediated by karyopherin alpha2beta1 heterodimers." J Cell Biochem **74**(4): 628-37.

Mermoud, J. E., P. T. Cohen and A. I. Lamond (1994). "Regulation of mammalian spliceosome assembly by a protein phosphorylation mechanism." Embo J **13**(23): 5679-88.

Middleton, K., W. Peh, S. Southern, H. Griffin, K. Sotlar, T. Nakahara, A. El-Sherif, L. Morris, R. Seth, M. Hibma, D. Jenkins, P. Lambert, N. Coleman and J.

Doorbar (2003). "Organization of human papillomavirus productive cycle during neoplastic progression provides a basis for selection of diagnostic markers." J Virol **77**(19): 10186-201.

Mili, S., H. J. Shu, Y. Zhao and S. Pinol-Roma (2001). "Distinct RNP complexes of shuttling hnRNP proteins with pre-mRNA and mRNA: candidate intermediates in formation and export of mRNA." Mol Cell Biol **21**(21): 7307-19.

Milligan, S. G., T. Veerapraditsin, B. Ahamet, S. Mole and S. V. Graham (2006). "Analysis of novel human papillomavirus type 16 late mRNAs in differentiated W12 cervical epithelial cells." Virology.

Minvielle-Sebastia, L. and W. Keller (1999). "mRNA polyadenylation and its coupling to other RNA processing reactions and to transcription." Curr Opin Cell Biol **11**(3): 352-7.

Modis, Y., B. L. Trus and S. C. Harrison (2002). "Atomic model of the papillomavirus capsid." Embo J **21**(18): 4754-62.

Mohr, I. J., R. Clark, S. Sun, E. J. Androphy, P. MacPherson and M. R. Botchan (1990). "Targeting the E1 replication protein to the papillomavirus origin of replication by complex formation with the E2 transactivator." Science **250**(4988): 1694-9.

Molin, M. and G. Akusjarvi (2000). "Overexpression of essential splicing factor ASF/SF2 blocks the temporal shift in adenovirus pre-mRNA splicing and reduces virus progeny formation." J Virol **74**(19): 9002-9.

Moller, L. B., Z. Tumer, C. Lund, C. Petersen, T. Cole, R. Hanusch, J. Seidel, L. R. Jensen and N. Horn (2000). "Similar splice-site mutations of the ATP7A gene lead to different phenotypes: classical Menkes disease or occipital horn syndrome." Am J Hum Genet **66**(4): 1211-20.

Morgan, I. M., G. J. Grindlay and M. S. Campo (1998). "Epithelial specific transcriptional regulation of the bovine papillomavirus 4 promoter by E2." J Gen Virol **79 (Pt 3)**: 501-8.

Morgan, I. M., G. J. Grindlay and M. S. Campo (1999). "The bovine papillomavirus type 4 long control region contains an epithelial specific enhancer." J Gen Virol **80 (Pt 1)**: 23-7.

Muhlemann, O., B. G. Yue, S. Petersen-Mahrt and G. Akusjarvi (2000). "A novel type of splicing enhancer regulating adenovirus pre-mRNA splicing." Mol Cell Biol **20**(7): 2317-25.

Muller, A., A. Ritzkowsky and G. Steger (2002). "Cooperative activation of human papillomavirus type 8 gene expression by the E2 protein and the cellular coactivator p300." J Virol **76**(21): 11042-53.

Muller, F., Y. S. Seo and J. Hurwitz (1994). "Replication of bovine papillomavirus type 1 origin-containing DNA in crude extracts and with purified proteins." J Biol Chem **269**(25): 17086-94.

Munger, K., J. R. Basile, S. Duensing, A. Eichten, S. L. Gonzalez, M. Grace and V. L. Zacny (2001). "Biological activities and molecular targets of the human papillomavirus E7 oncoprotein." Oncogene **20**(54): 7888-98.

Murthy, K. G. and J. L. Manley (1995). "The 160-kD subunit of human cleavage-polyadenylation specificity factor coordinates pre-mRNA 3'-end formation." Genes Dev **9**(21): 2672-83.

Muthukrishnan, S., G. W. Both, Y. Furuichi and A. J. Shatkin (1975). "5'-Terminal 7-methylguanosine in eukaryotic mRNA is required for translation." Nature **255**(5503): 33-7.

Nakagawa, S. and J. M. Huibregtse (2000). "Human scribble (Vartul) is targeted for ubiquitin-mediated degradation by the high-risk papillomavirus E6 proteins and the E6AP ubiquitin-protein ligase." Mol Cell Biol **20**(21): 8244-53.

Nakahara, T., A. Nishimura, M. Tanaka, T. Ueno, A. Ishimoto and H. Sakai (2002). "Modulation of the cell division cycle by human papillomavirus type 18 E4." J Virol **76**(21): 10914-20.

Nakahara, T., W. L. Peh, J. Doorbar, D. Lee and P. F. Lambert (2005). "Human papillomavirus type 16 E1circumflexE4 contributes to multiple facets of the papillomavirus life cycle." J Virol **79**(20): 13150-65.

Nakielny, S. and G. Dreyfuss (1999). "Transport of proteins and RNAs in and out of the nucleus." Cell **99**(7): 677-90.

Narahari, J., J. C. Fisk, T. Melendy and A. Roman (2006). "Interactions of the cellular CCAAT displacement protein and human papillomavirus E2 protein with the viral origin of replication can regulate DNA replication." Virology **350**(2): 302-11.

Negrini, B. P., M. H. Schiffman, R. J. Kurman, W. Barnes, L. Lannom, K. Malley, L. A. Brinton, G. Delgado, S. Jones, J. G. Tchabo and et al. (1990). "Oral contraceptive use, human papillomavirus infection, and risk of early cytological abnormalities of the cervix." Cancer Res **50**(15): 4670-5.

Nelson, L. M., R. C. Rose and J. Moroianu (2002). "Nuclear import strategies of high risk HPV16 L1 major capsid protein." J Biol Chem **277**(26): 23958-64.

Nelson, L. M., R. C. Rose and J. Moroianu (2003). "The L1 major capsid protein of human papillomavirus type 11 interacts with Kap beta2 and Kap beta3 nuclear import receptors." Virology **306**(1): 162-9.

Nguyen, M. L., M. M. Nguyen, D. Lee, A. E. Griep and P. F. Lambert (2003). "The PDZ ligand domain of the human papillomavirus type 16 E6 protein is required for E6's induction of epithelial hyperplasia in vivo." J Virol **77**(12): 6957-64.

Niwa, M. and S. M. Berget (1991). "Mutation of the AAUAAA polyadenylation signal depresses in vitro splicing of proximal but not distal introns." Genes Dev **5**(11): 2086-95.

Niwa, M., C. C. MacDonald and S. M. Berget (1992). "Are vertebrate exons scanned during splice-site selection?" Nature **360**(6401): 277-80.

Niwa, M., S. D. Rose and S. M. Berget (1990). "In vitro polyadenylation is stimulated by the presence of an upstream intron." Genes Dev **4**(9): 1552-9.

Oberg, D., B. Collier, X. Zhao and S. Schwartz (2003). "Mutational inactivation of two distinct negative RNA elements in the human papillomavirus type 16 L2 coding region induces production of high levels of L2 in human cells." J Virol **77**(21): 11674-84.

O'Connor, M. J., W. Stunkel, H. Zimmermann, C. H. Koh and H. U. Bernard (1998). "A novel YY1-independent silencer represses the activity of the human papillomavirus type 16 enhancer." J Virol **72**(12): 10083-92.

O'Connor, M. J., S. H. Tan, C. H. Tan and H. U. Bernard (1996). "YY1 represses human papillomavirus type 16 transcription by quenching AP-1 activity." J Virol **70**(10): 6529-39.

Oh, S. T., M. S. Longworth and L. A. Laimins (2004). "Roles of the E6 and E7 proteins in the life cycle of low-risk human papillomavirus type 11." J Virol **78**(5): 2620-6.

Okoye, A., P. Cordano, E. R. Taylor, I. M. Morgan, R. Everett and M. S. Campo (2005). "Human papillomavirus 16 L2 inhibits the transcriptional activation function, but not the DNA replication function, of HPV-16 E2." Virus Res **108**(1-2): 1-14.

Oldak, M., H. Smola, M. Aumailley, F. Rivero, H. Pfister and S. Smola-Hess (2004). "The human papillomavirus type 8 E2 protein suppresses beta4-integrin expression in primary human keratinocytes." J Virol **78**(19): 10738-46.

Ostareck, D. H., A. Ostareck-Lederer, M. Wilm, B. J. Thiele, M. Mann and M. W. Hentze (1997). "mRNA silencing in erythroid differentiation: hnRNP K and hnRNP E1 regulate 15-lipoxygenase translation from the 3' end." Cell **89**(4): 597-606.

Ostareck-Lederer, A., D. H. Ostareck, N. Standart and B. J. Thiele (1994). "Translation of 15-lipoxygenase mRNA is inhibited by a protein that binds to a repeated sequence in the 3' untranslated region." Embo J **13**(6): 1476-81.

Ozbun, M. A. and C. Meyers (1998). "Temporal usage of multiple promoters during the life cycle of human papillomavirus type 31b." J Virol **72**(4): 2715-22.

Pagano, M., M. Durst, S. Joswig, G. Draetta and P. Jansen-Durr (1992). "Binding of the human E2F transcription factor to the retinoblastoma protein but not to cyclin A is abolished in HPV-16-immortalized cells." Oncogene **7**(9): 1681-6.

Parazzini, F., C. La Vecchia, E. Negri, L. Fedele, S. Franceschi and L. Gallotta (1992). "Risk factors for cervical intraepithelial neoplasia." Cancer **69**(9): 2276-82.

Parish, J. L., A. Kowalczyk, H. T. Chen, G. E. Roeder, R. Sessions, M. Buckle and K. Gaston (2006). "E2 proteins from high- and low-risk human papillomavirus types differ in their ability to bind p53 and induce apoptotic cell death." J Virol **80**(9): 4580-90.

Park, P., W. Copeland, L. Yang, T. Wang, M. R. Botchan and I. J. Mohr (1994). "The cellular DNA polymerase alpha-primase is required for papillomavirus DNA replication and associates with the viral E1 helicase." Proc Natl Acad Sci U S A **91**(18): 8700-4.

Patel, D., S. M. Huang, L. A. Baglia and D. J. McCance (1999). "The E6 protein of human papillomavirus type 16 binds to and inhibits co-activation by CBP and p300." Embo J **18**(18): 5061-72.

Patterson, N. A., J. L. Smith and M. A. Ozbun (2005). "Human papillomavirus type 31b infection of human keratinocytes does not require heparan sulfate." J Virol **79**(11): 6838-47.

Peng, S. S., C. Y. Chen, N. Xu and A. B. Shyu (1998). "RNA stabilization by the AU-rich element binding protein, HuR, an ELAV protein." Embo J **17**(12): 3461-70.

Petersen-Mahrt, S. K., C. Estmer, C. Ohrmalm, D. A. Matthews, W. C. Russell and G. Akusjarvi (1999). "The splicing factor-associated protein, p32, regulates RNA splicing by inhibiting ASF/SF2 RNA binding and phosphorylation." Embo J **18**(4): 1014-24.

Pim, D., M. Thomas, R. Javier, D. Gardiol and L. Banks (2000). "HPV E6 targeted degradation of the discs large protein: evidence for the involvement of a novel ubiquitin ligase." Oncogene **19**(6): 719-25.

Poljak, M., A. Brencic, K. Seme, A. Vince and I. J. Marin (1999). "Comparative evaluation of first- and second-generation digene hybrid capture assays for detection of human papillomaviruses associated with high or intermediate risk for cervical cancer." J Clin Microbiol **37**(3): 796-7.

Powell, D. M., M. C. Amaral, J. Y. Wu, T. Maniatis and W. C. Greene (1997). "HIV Rev-dependent binding of SF2/ASF to the Rev response element: possible role in Rev-mediated inhibition of HIV RNA splicing." Proc Natl Acad Sci U S A **94**(3): 973-8.

Proudfoot, N. (1991). "Poly(A) signals." Cell **64**(4): 671-4.

Raj, K., S. Berguerand, S. Southern, J. Doorbar and P. Beard (2004). "E1 empty set E4 protein of human papillomavirus type 16 associates with mitochondria." J Virol **78**(13): 7199-207.

Rank, N. M. and P. F. Lambert (1995). "Bovine papillomavirus type 1 E2 transcriptional regulators directly bind two cellular transcription factors, TFIID and TFIIB." J Virol **69**(10): 6323-34.

Reed, R. and L. Palandjian (1997). Spliceosome Assembly. *Eukaryotic mRNA processing*. A. R. Krainer. New York, IRL Press, Cold Spring Harbour: 103-129.

Roberts, S., I. Ashmole, L. J. Gibson, S. M. Rookes, G. J. Barton and P. H. Gallimore (1994). "Mutational analysis of human papillomavirus E4 proteins: identification of structural features important in the formation of cytoplasmic E4/cytokeratin networks in epithelial cells." J Virol **68**(10): 6432-45.

Roberts, S., I. Ashmole, G. D. Johnson, J. W. Kreider and P. H. Gallimore (1993). "Cutaneous and mucosal human papillomavirus E4 proteins form intermediate filament-like structures in epithelial cells." Virology **197**(1): 176-87.

Roberts, S., I. Ashmole, S. M. Rookes and P. H. Gallimore (1997). "Mutational analysis of the human papillomavirus type 16 E1--E4 protein shows that the C terminus is dispensable for keratin cytoskeleton association but is involved in inducing disruption of the keratin filaments." J Virol **71**(5): 3554-62.

Rodrigues, J. P., M. Rode, D. Gatfield, B. J. Blencowe, M. Carmo-Fonseca and E. Izaurralde (2001). "REF proteins mediate the export of spliced and unspliced mRNAs from the nucleus." Proc Natl Acad Sci U S A **98**(3): 1030-5.

Ropers, D., L. Ayadi, R. Gattoni, S. Jacquenet, L. Damier, C. Branlant and J. Stevenin (2004). "Differential effects of the SR proteins 9G8, SC35, ASF/SF2, and SRp40 on the utilization of the A1 to A5 splicing sites of HIV-1 RNA." J Biol Chem **279**(29): 29963-73.

Roscigno, R. F. and M. A. Garcia-Blanco (1995). "SR proteins escort the U4/U6.U5 tri-snRNP to the spliceosome." Rna **1**(7): 692-706.

Rosenstierne, M. W., J. Vinther, C. N. Hansen, M. Prydsoe and B. Norrild (2003). "Identification and characterization of a cluster of transcription start sites located in the E6 ORF of human papillomavirus type 16." J Gen Virol **84**(Pt 11): 2909-20.

Ross, J. (1995). "mRNA stability in mammalian cells." Microbiol Rev **59**(3): 423-50.

Rossi, F., E. Labourier, T. Forne, G. Divita, J. Derancourt, J. F. Riou, E. Antoine, G. Cathala, C. Brunel and J. Tazi (1996). "Specific phosphorylation of SR proteins by mammalian DNA topoisomerase I." Nature **381**(6577): 80-2.

Roth, M. B., A. M. Zahler and J. A. Stolk (1991). "A conserved family of nuclear phosphoproteins localized to sites of polymerase II transcription." J Cell Biol **115**(3): 587-96.

Rous, P. and J. G. Kidd (1936). "The carcinogenic effect of a virus upon tarred skin." Science **83**: 468-469.

Rush, M., X. Zhao and S. Schwartz (2005). "A splicing enhancer in the E4 coding region of human papillomavirus type 16 is required for early mRNA splicing and polyadenylation as well as inhibition of premature late gene expression." J Virol **79**(18): 12002-15.

Ryner, L. C., S. F. Goodwin, D. H. Castrillon, A. Anand, A. Villella, B. S. Baker, J. C. Hall, B. J. Taylor and S. A. Wasserman (1996). "Control of male sexual behavior and sexual orientation in Drosophila by the fruitless gene." Cell **87**(6): 1079-89.

Sachs, A. B., P. Sarnow and M. W. Hentze (1997). "Starting at the beginning, middle, and end: translation initiation in eukaryotes." Cell **89**(6): 831-8.

Sanchez-Perez, A. M., S. Soriano, A. R. Clarke and K. Gaston (1997). "Disruption of the human papillomavirus type 16 E2 gene protects cervical carcinoma cells from E2F-induced apoptosis." J Gen Virol **78 (Pt 11)**: 3009-18.

Sanford, J. R. and J. P. Bruzik (1999). "Developmental regulation of SR protein phosphorylation and activity." Genes Dev **13**(12): 1513-8.

Sanford, J. R., J. D. Ellis, D. Cazalla and J. F. Caceres (2005). "Reversible phosphorylation differentially affects nuclear and cytoplasmic functions of splicing factor 2/alternative splicing factor." Proc Natl Acad Sci U S A **102**(42): 15042-7.

Sanford, J. R., N. K. Gray, K. Beckmann and J. F. Caceres (2004). "A novel role for shuttling SR proteins in mRNA translation." Genes Dev **18**(7): 755-68.

Sasieni, P. and J. Adams (2001). "Changing rates of adenocarcinoma and adenosquamous carcinoma of the cervix in England." Lancet **357**(9267): 1490-3.

Sauliere, J., A. Sureau, A. Expert-Bezancon and J. Marie (2006). "The polypyrimidine tract binding protein (PTB) represses splicing of exon 6B from the beta-tropomyosin pre-mRNA by directly interfering with the binding of the U2AF65 subunit." Mol Cell Biol **26**(23): 8755-69.

Scheffner, M., J. M. Huibregtse, R. D. Vierstra and P. M. Howley (1993). "The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53." Cell **75**(3): 495-505.

Scheffner, M., B. A. Werness, J. M. Huibregtse, A. J. Levine and P. M. Howley (1990). "The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53." Cell **63**(6): 1129-36.

Schneider-Gadicke, A. and E. Schwarz (1986). "Different human cervical carcinoma cell lines show similar transcription patterns of human papillomavirus type 18 early genes." Embo J **5**(9): 2285-92.

Schwartz, S. (1998). "*Cis*-Acting Negative RNA Elements on Papillomavirus Late mRNAs." Seminars in Virology **8**: 291-300.

Schweiger, M. R., J. You and P. M. Howley (2006). "Bromodomain protein 4 mediates the papillomavirus E2 transcriptional activation function." J Virol **80**(9): 4276-85.

Sen, E., S. Alam and C. Meyers (2004). "Genetic and biochemical analysis of cis regulatory elements within the keratinocyte enhancer region of the human papillomavirus type 31 upstream regulatory region during different stages of the viral life cycle." J Virol **78**(2): 612-29.

Seo, Y. S., F. Muller, M. Lusky, E. Gibbs, H. Y. Kim, B. Phillips and J. Hurwitz (1993). "Bovine papilloma virus (BPV)-encoded E2 protein enhances binding of E1 protein to the BPV replication origin." Proc Natl Acad Sci U S A **90**(7): 2865-9.

Shafti-Keramat, S., A. Handisurya, E. Kriehuber, G. Meneguzzi, K. Slupetzky and R. Kirnbauer (2003). "Different heparan sulfate proteoglycans serve as cellular receptors for human papillomaviruses." J Virol **77**(24): 13125-35.

Shah, K. V. and P. Howley (1996). Papillomaviruses. New York, Raven Press.

Shatkin, A. J. and J. L. Manley (2000). "The ends of the affair: capping and polyadenylation." Nat Struct Biol **7**(10): 838-42.

Shen, H. and M. R. Green (2004). "A pathway of sequential arginine-serine-rich domain-splicing signal interactions during mammalian spliceosome assembly." Mol Cell **16**(3): 363-73.

Shen, H., J. L. Kan and M. R. Green (2004). "Arginine-serine-rich domains bound at splicing enhancers contact the branchpoint to promote prespliceosome assembly." Mol Cell **13**(3): 367-76.

Shope, R. (1933). "Infectious papillomatosis of rabbits." Journal of Experimental Medicine **58**: 607-624.

Sibbet, G., C. Romero-Graillet, G. Meneguzzi and M. S. Campo (2000). "alpha6 integrin is not the obligatory cell receptor for bovine papillomavirus type 4." J Gen Virol **81**(Pt 2): 327-34.

Sibbet, G. J. and M. S. Campo (1990). "Multiple interactions between cellular factors and the non-coding region of human papillomavirus type 16." J Gen Virol **71 (Pt 11)**: 2699-707.

Skiadopoulos, M. H. and A. A. McBride (1998). "Bovine papillomavirus type 1 genomes and the E2 transactivator protein are closely associated with mitotic chromatin." J Virol **72**(3): 2079-88.

Slebos, R. J., M. H. Lee, B. S. Plunkett, T. D. Kessis, B. O. Williams, T. Jacks, L. Hedrick, M. B. Kastan and K. R. Cho (1994). "p53-dependent G1 arrest involves pRB-related proteins and is disrupted by the human papillomavirus 16 E7 oncoprotein." Proc Natl Acad Sci U S A **91**(12): 5320-4.

Smith, C. W. and J. Valcarcel (2000). "Alternative pre-mRNA splicing: the logic of combinatorial control." Trends Biochem Sci **25**(8): 381-8.

Smotkin, D. and F. O. Wettstein (1986). "Transcription of human papillomavirus type 16 early genes in a cervical cancer and a cancer-derived cell line and identification of the E7 protein." Proc Natl Acad Sci U S A **83**(13): 4680-4.

Sokolowski, M., H. Furneaux and S. Schwartz (1999). "The inhibitory activity of the AU-rich RNA element in the human papillomavirus type 1 late 3' untranslated region correlates with its affinity for the elav-like HuR protein." J Virol **73**(2): 1080-91.

Sokolowski, M., W. Tan, M. Jellne and S. Schwartz (1998). "mRNA instability elements in the human papillomavirus type 16 L2 coding region." J Virol **72**(2): 1504-15.

Sokolowski, M., C. Zhao, W. Tan and S. Schwartz (1997). "AU-rich mRNA instability elements on human papillomavirus type 1 late mRNAs and c-fos mRNAs interact with the same cellular factors." Oncogene **15**(19): 2303-19.

Solheim, J. C. (1999). "Class I MHC molecules: assembly and antigen presentation." Immunol Rev **172**: 11-9.

Song, S., G. A. Gulliver and P. F. Lambert (1998). "Human papillomavirus type 16 E6 and E7 oncogenes abrogate radiation-induced DNA damage responses in vivo through p53-dependent and p53-independent pathways." Proc Natl Acad Sci U S A **95**(5): 2290-5.

Song, S., A. Liem, J. A. Miller and P. F. Lambert (2000). "Human papillomavirus types 16 E6 and E7 contribute differently to carcinogenesis." Virology **267**(2): 141-50.

Stanley, M. A., H. M. Browne, M. Appleby and A. C. Minson (1989). "Properties of a non-tumorigenic human cervical keratinocyte cell line." Int J Cancer **43**(4): 672-6.

Steele, B. K., C. Meyers and M. A. Ozbun (2002). "Variable expression of some "housekeeping" genes during human keratinocyte differentiation." Anal Biochem **307**(2): 341-7.

Steger, G. and S. Corbach (1997). "Dose-dependent regulation of the early promoter of human papillomavirus type 18 by the viral E2 protein." J Virol **71**(1): 50-8.

Steger, G., J. Ham, O. Lefebvre and M. Yaniv (1995). "The bovine papillomavirus 1 E2 protein contains two activation domains: one that interacts with TBP and another that functions after TBP binding." Embo J **14**(2): 329-40.

Steger, G., C. Schnabel and H. M. Schmidt (2002). "The hinge region of the human papillomavirus type 8 E2 protein activates the human p21(WAF1/CIP1) promoter via interaction with Sp1." J Gen Virol **83**(Pt 3): 503-10.

Sterling, J. C. (2005). "Human papillomaviruses and skin cancer." J Clin Virol **32 Suppl 1**: S67-71.

Sterlinko Grm, H., M. Weber, R. Elston, P. McIntosh, H. Griffin, L. Banks and J. Doorbar (2004). "Inhibition of E6-induced degradation of its cellular substrates by novel blocking peptides." J Mol Biol **335**(4): 971-85.

Straight, S. W., B. Herman and D. J. McCance (1995). "The E5 oncoprotein of human papillomavirus type 16 inhibits the acidification of endosomes in human keratinocytes." J Virol **69**(5): 3185-92.

Straight, S. W., P. M. Hinkle, R. J. Jewers and D. J. McCance (1993). "The E5 oncoprotein of human papillomavirus type 16 transforms fibroblasts and effects the downregulation of the epidermal growth factor receptor in keratinocytes." J Virol **67**(8): 4521-32.

Swindle, C. S., N. Zou, B. A. Van Tine, G. M. Shaw, J. A. Engler and L. T. Chow (1999). "Human papillomavirus DNA replication compartments in a transient DNA replication system." J Virol **73**(2): 1001-9.

Talis, A. L., J. M. Huibregtse and P. M. Howley (1998). "The role of E6AP in the regulation of p53 protein levels in human papillomavirus (HPV)-positive and HPV-negative cells." J Biol Chem **273**(11): 6439-45.

Tan, S. H., B. Gloss and H. U. Bernard (1992). "During negative regulation of the human papillomavirus-16 E6 promoter, the viral E2 protein can displace Sp1 from a proximal promoter element." Nucleic Acids Res **20**(2): 251-6.

Tan, S. H., L. E. Leong, P. A. Walker and H. U. Bernard (1994). "The human papillomavirus type 16 E2 transcription factor binds with low cooperativity to two flanking sites and represses the E6 promoter through displacement of Sp1 and TFIID." J Virol **68**(10): 6411-20.

Tan, W. and S. Schwartz (1995). "The Rev protein of human immunodeficiency virus type 1 counteracts the effect of an AU-rich negative element in the human papillomavirus type 1 late 3' untranslated region." J Virol **69**(5): 2932-45.

Taura, T., M. C. Siomi and H. Siomi (2005). The Molecular Mechanisms of mRNA Export. *Nuclear Import and Export in Plants an Animals*. T. Tzfira and V. Citovsky, Kluwer Academic/Plenum Publishers: 161-174.

Terhune, S. S., W. G. Hubert, J. T. Thomas and L. A. Laimins (2001). "Early polyadenylation signals of human papillomavirus type 31 negatively regulate capsid gene expression." J Virol **75**(17): 8147-57.

Thain, A., O. Jenkins, A. R. Clarke and K. Gaston (1996). "CpG methylation directly inhibits binding of the human papillomavirus type 16 E2 protein to specific DNA sequences." J Virol **70**(10): 7233-5.

Thierry, F. and P. M. Howley (1991). "Functional analysis of E2-mediated repression of the HPV18 P105 promoter." New Biol **3**(1): 90-100.

Thomas, J. T., W. G. Hubert, M. N. Ruesch and L. A. Laimins (1999). "Human papillomavirus type 31 oncoproteins E6 and E7 are required for the maintenance of episomes during the viral life cycle in normal human keratinocytes." Proc Natl Acad Sci U S A **96**(15): 8449-54.

Thomas, M. and L. Banks (1998). "Inhibition of Bak-induced apoptosis by HPV-18 E6." Oncogene **17**(23): 2943-54.

Thomas, M., R. Laura, K. Hepner, E. Guccione, C. Sawyers, L. Lasky and L. Banks (2002). "Oncogenic human papillomavirus E6 proteins target the MAGI-2 and MAGI-3 proteins for degradation." Oncogene **21**(33): 5088-96.

Tommasino, M., J. P. Adamczewski, F. Carlotti, C. F. Barth, R. Manetti, M. Contorni, F. Cavalieri, T. Hunt and L. Crawford (1993). "HPV16 E7 protein associates with the protein kinase p33CDK2 and cyclin A." Oncogene **8**(1): 195-202.

Trofatter, K. F., Jr. (1997). "Diagnosis of human papillomavirus genital tract infection." Am J Med **102**(5A): 21-7.

Vagner, S., C. Vagner and I. W. Mattaj (2000). "The carboxyl terminus of vertebrate poly(A) polymerase interacts with U2AF 65 to couple 3'-end processing and splicing." Genes Dev **14**(4): 403-13.

Vance, K. W., M. S. Campo and I. M. Morgan (1999). "An enhanced epithelial response of a papillomavirus promoter to transcriptional activators." J Biol Chem **274**(39): 27839-44.

Vance, K. W., M. S. Campo and I. M. Morgan (2001). "A novel silencer element in the bovine papillomavirus type 4 promoter represses the transcriptional response to papillomavirus E2 protein." J Virol **75**(6): 2829-38.

Veerapraditsin, T. (2004). "Interaction between the HPV-16 negative regulatory element and cellular proteins during epithelial cell differentiation." Submitted thesis.

Veerapraditsin, T., S. A. Cumming, S. Mole and S. V. Graham (unpublished). "The human papillomavirus type 16 late regulatory element controls mRNA stability in vivo and binds HuR and hnRNP A1."

Venkataraman, K., K. M. Brown and G. M. Gilmartin (2005). "Analysis of a noncanonical poly(A) site reveals a tripartite mechanism for vertebrate poly(A) site recognition." Genes Dev **19**(11): 1315-27.

Venturoli, S., M. Cricca, F. Bonvicini, F. Giosa, F. R. Pulvirenti, C. Galli, M. Musiani and M. Zerbini (2002). "Human papillomavirus DNA testing by PCR-ELISA and hybrid capture II from a single cytological specimen: concordance and correlation with cytological results." J Clin Virol **25**(2): 177-85.

Venturoli, S., M. Zerbini, M. La Placa, Jr., A. D'Antuono, M. Negosanti, G. Gentilomi, G. Gallinella, E. Manaresi and M. Musiani (1998). "Evaluation of immunoassays for the detection and typing of PCR amplified human papillomavirus DNA." J Clin Pathol **51**(2): 143-8.

Villa, L. L., R. L. Costa, C. A. Petta, R. P. Andrade, K. A. Ault, A. R. Giuliano, C. M. Wheeler, L. A. Koutsky, C. Malm, M. Lehtinen, F. E. Skjeldestad, S. E. Olsson, M. Steinwall, D. R. Brown, R. J. Kurman, B. M. Ronnett, M. H. Stoler, A. Ferenczy, D. M. Harper, G. M. Tamms, J. Yu, L. Lupinacci, R. Railkar, F. J. Taddeo, K. U. Jansen, M. T. Esser, H. L. Sings, A. J. Saah and E. Barr (2005). "Prophylactic quadrivalent human papillomavirus (types 6, 11, 16, and 18) L1 virus-like particle vaccine in young women: a randomised double-blind placebo-controlled multicentre phase II efficacy trial." Lancet Oncol **6**(5): 271-8.

Wahle, E. (1991). "A novel poly(A)-binding protein acts as a specificity factor in the second phase of messenger RNA polyadenylation." Cell **66**(4): 759-68.

Wahle, E. (1995). "Poly(A) tail length control is caused by termination of processive synthesis." J Biol Chem **270**(6): 2800-8.

Wahle, E. and W. Keller (1996). "The biochemistry of polyadenylation." Trends Biochem Sci **21**(7): 247-50.

Walboomers, J. M., M. V. Jacobs, M. M. Manos, F. X. Bosch, J. A. Kummer, K. V. Shah, P. J. Snijders, J. Peto, C. J. Meijer and N. Munoz (1999). "Human papillomavirus is a necessary cause of invasive cervical cancer worldwide." J Pathol **189**(1): 12-9.

Wang, H. Y., W. Lin, J. A. Dyck, J. M. Yeakley, Z. Songyang, L. C. Cantley and X. D. Fu (1998). "SRPK2: a differentially expressed SR protein-specific kinase involved in mediating the interaction and localization of pre-mRNA splicing factors in mammalian cells." J Cell Biol **140**(4): 737-50.

Wang, J. and J. L. Manley (1995). "Overexpression of the SR proteins ASF/SF2 and SC35 influences alternative splicing in vivo in diverse ways." Rna **1**(3): 335-46.

Wang, J., A. Sampath, P. Raychaudhuri and S. Bagchi (2001). "Both Rb and E7 are regulated by the ubiquitin proteasome pathway in HPV-containing cervical tumor cells." Oncogene **20**(34): 4740-9.

Wang, J., S. H. Xiao and J. L. Manley (1998). "Genetic analysis of the SR protein ASF/SF2: interchangeability of RS domains and negative control of splicing." Genes Dev **12**(14): 2222-33.

Wang, Q., H. Griffin, S. Southern, D. Jackson, A. Martin, P. McIntosh, C. Davy, P. J. Masterson, P. A. Walker, P. Laskey, M. B. Omary and J. Doorbar (2004). "Functional analysis of the human papillomavirus type 16 E1=E4 protein provides a mechanism for in vivo and in vitro keratin filament reorganization." J Virol **78**(2): 821-33.

Wassarman, D. A. and J. A. Steitz (1992). "Interactions of small nuclear RNA's with precursor messenger RNA during in vitro splicing." Science **257**(5078): 1918-25.

Wassarman, K. M. and J. A. Steitz (1993). "Association with terminal exons in pre-mRNAs: a new role for the U1 snRNP?" Genes Dev **7**(4): 647-59.

Webster, K., J. Parish, M. Pandya, P. L. Stern, A. R. Clarke and K. Gaston (2000). "The human papillomavirus (HPV) 16 E2 protein induces apoptosis in the absence of other HPV proteins and via a p53-dependent pathway." J Biol Chem **275**(1): 87-94.

Weiss, E. A., G. M. Gilmartin and J. R. Nevins (1991). "Poly(A) site efficiency reflects the stability of complex formation involving the downstream element." Embo J **10**(1): 215-9.

Weiss, I. M. and S. A. Liebhaber (1995). "Erythroid cell-specific mRNA stability elements in the alpha 2-globin 3' nontranslated region." Mol Cell Biol **15**(5): 2457-65.

Werness, B. A., A. J. Levine and P. M. Howley (1990). "Association of human papillomavirus types 16 and 18 E6 proteins with p53." Science **248**(4951): 76-9.

White, A. E., E. M. Livanos and T. D. Tlsty (1994). "Differential disruption of genomic integrity and cell cycle regulation in normal human fibroblasts by the HPV oncoproteins." Genes Dev **8**(6): 666-77.

Wiegand, H. L., G. A. Coburn, Y. Zeng, Y. Kang, H. P. Bogerd and B. R. Cullen (2002). "Formation of Tap/NXT1 heterodimers activates Tap-dependent nuclear mRNA export by enhancing recruitment to nuclear pore complexes." Mol Cell Biol **22**(1): 245-56.

Wilson, R., F. Fehrmann and L. A. Laimins (2005). "Role of the E1--E4 protein in the differentiation-dependent life cycle of human papillomavirus type 31." J Virol **79**(11): 6732-40.

Wilusz, J., T. Shenk, Y. Takagaki and J. L. Manley (1990). "A multicomponent complex is required for the AAUAAA-dependent cross-linking of a 64-kilodalton protein to polyadenylation substrates." Mol Cell Biol **10**(3): 1244-8.

Winokur, P. L. and A. A. McBride (1992). "Separation of the transcriptional activation and replication functions of the bovine papillomavirus-1 E2 protein." Embo J **11**(11): 4111-8.

Wu, J. Y. and T. Maniatis (1993). "Specific interactions between proteins implicated in splice site selection and regulated alternative splicing." Cell **75**(6): 1061-70.

Wu, S. Y., A. Y. Lee, S. Y. Hou, J. K. Kemper, H. E. Bromage, P. Tempst and C. M. Chiang (2006). "Brd4 links chromatin targeting to HPV transcriptional silencing." Genes Dev **20**(17): 2383-96.

Wu, X. and A. J. Levine (1994). "p53 and E2F-1 cooperate to mediate apoptosis." Proc Natl Acad Sci U S A **91**(9): 3602-6.

Xiao, S. H. and J. L. Manley (1997). "Phosphorylation of the ASF/SF2 RS domain affects both protein-protein and protein-RNA interactions and is necessary for splicing." Genes Dev **11**(3): 334-44.

Xiao, S. H. and J. L. Manley (1998). "Phosphorylation-dephosphorylation differentially affects activities of splicing factor ASF/SF2." Embo J **17**(21): 6359-67.

Yang, R., P. M. Day, W. H. t. Yutzy, K. Y. Lin, C. F. Hung and R. B. Roden (2003). "Cell surface-binding motifs of L2 that facilitate papillomavirus infection." J Virol **77**(6): 3531-41.

Yang, R., W. H. t. Yutzy, R. P. Viscidi and R. B. Roden (2003). "Interaction of L2 with beta-actin directs intracellular transport of papillomavirus and infection." J Biol Chem **278**(14): 12546-53.

Yoon, C. S., K. D. Kim, S. N. Park and S. W. Cheong (2001). "alpha(6) Integrin is the main receptor of human papillomavirus type 16 VLP." Biochem Biophys Res Commun **283**(3): 668-73.

You, J., J. L. Croyle, A. Nishimura, K. Ozato and P. M. Howley (2004). "Interaction of the bovine papillomavirus E2 protein with Brd4 tethers the viral DNA to host mitotic chromosomes." Cell **117**(3): 349-60.

You, J., M. R. Schweiger and P. M. Howley (2005). "Inhibition of E2 binding to Brd4 enhances viral genome loss and phenotypic reversion of bovine papillomavirus-transformed cells." J Virol **79**(23): 14956-61.

Zahler, A. M., C. K. Damgaard, J. Kjems and M. Caputi (2004). "SC35 and heterogeneous nuclear ribonucleoprotein A/B proteins bind to a juxtaposed exonic splicing enhancer/exonic splicing silencer element to regulate HIV-1 tat exon 2 splicing." J Biol Chem **279**(11): 10077-84.

Zahler, A. M., W. S. Lane, J. A. Stolk and M. B. Roth (1992). "SR proteins: a conserved family of pre-mRNA splicing factors." Genes Dev **6**(5): 837-47.

Zerbini, M., S. Venturoli, M. Cricca, G. Gallinella, P. De Simone, S. Costa, D. Santini and M. Musiani (2001). "Distribution and viral load of type specific HPVs in different cervical lesions as detected by PCR-ELISA." J Clin Pathol **54**(5): 377-80.

Zerfass-Thome, K., W. Zwerschke, B. Mannhardt, R. Tindle, J. W. Botz and P. Jansen-Durr (1996). "Inactivation of the cdk inhibitor p27KIP1 by the human papillomavirus type 16 E7 oncoprotein." Oncogene **13**(11): 2323-30.

Zhao, X., D. Oberg, M. Rush, J. Fay, H. Lambkin and S. Schwartz (2005). "A 57-nucleotide upstream early polyadenylation element in human papillomavirus type 16 interacts with hFip1, CstF-64, hnRNP C1/C2, and polypyrimidine tract binding protein." J Virol **79**(7): 4270-88.

Zhao, X., M. Rush and S. Schwartz (2004). "Identification of an hnRNP A1-dependent splicing silencer in the human papillomavirus type 16 L1 coding region that prevents premature expression of the late L1 gene." J Virol **78**(20): 10888-905.

Zhou, Z., M. J. Luo, K. Straesser, J. Katahira, E. Hurt and R. Reed (2000). "The protein Aly links pre-messenger-RNA splicing to nuclear export in metazoans." Nature **407**(6802): 401-5.

Zimmermann, H., R. Degenkolbe, H. U. Bernard and M. J. O'Connor (1999). "The human papillomavirus type 16 E6 oncoprotein can down-regulate p53 activity by targeting the transcriptional coactivator CBP/p300." J Virol **73**(8): 6209-19.

Zimmermann, H., C. H. Koh, R. Degenkolbe, M. J. O'Connor, A. Muller, G. Steger, J. J. Chen, Y. Lui, E. Androphy and H. U. Bernard (2000). "Interaction with CBP/p300 enables the bovine papillomavirus type 1 E6 oncoprotein to downregulate CBP/p300-mediated transactivation by p53." J Gen Virol **81**(Pt 11): 2617-23.

Zolotukhin, A. S., W. Tan, J. Bear, S. Smulevitch and B. K. Felber (2002). "U2AF participates in the binding of TAP (NXF1) to mRNA." J Biol Chem **277**(6): 3935-42.

Zuo, P. and T. Maniatis (1996). "The splicing factor U2AF35 mediates critical protein-protein interactions in constitutive and enhancer-dependent splicing." Genes Dev **10**(11): 1356-68.

zur Hausen, H. (1996). "Papillomavirus infections--a major cause of human cancers." Biochim Biophys Acta **1288**(2): F55-78.

zur Hausen, H. and E. M. de Villiers (1994). "Human papillomaviruses." Annu Rev Microbiol **48**: 427-47.

GLASGOW UNIVERSITY LIBRARY