Regulation of Human Papillomavirus Type 16 Late Protein Expression During Epithelial Differentiation

by

Maria G. McPhillips

A Thesis Presented for the Degree of Doctor of Philosophy

in

The Faculty of Science at the University of Glasgow

Division of Virology
IBLS, University of Glasgow
Church St
Glasgow G11 5JR

April 2002

ProQuest Number: 13818491

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 13818491

Published by ProQuest LLC (2018). 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:
12667
COPY 1

Abstract

Human papillomaviruses type 16 (HPV-16) is one of a number of HPV types specifically associated with the development of carcinoma in the anogenital tract and HPV-16 is the most commonly found subtype in cervical carcinomas worldwide. The early HPV proteins are expressed throughout epithelial differentiation, but expression of the late proteins is tightly linked to the differentiation status of the host cell, being found only in cells in the process of keratinisation in the granular layer of stratified epithelia. For HPV-16, a 79 nt negative regulatory element (NRE), overlapping the 3' end of the L1 stop codon and extending into the late 3' untranslated region, is an important post-transcriptional regulator of viral late gene expression. Unsurprisingly, the HPV-16 NRE has previously been shown to bind a range of RNA processing factors *in vitro*, including the auxiliary splicing factor $U2AF^{65}$, the polyadenylation factor CstF-64, and the *elav*-like HuR protein.

I carried out saturating site-directed mutagenesis of the HPV-16 NRE and found that no individual short sequences are responsible for the activity of this inhibitory element, or its ability to interact with cellular factors. I have used an affinity-chromatography based RNA binding assay to show that the NRE can also bind the splicing-related (SR) protein, ASF/SF2, a member of the Sm protein family, and the U1A subunit of U1snRNP. Furthermore, using a novel monolayer culture differentiation system and organotypic rafts to generate undifferentiated and differentiating populations of HPV-16 positive epithelial cell lines, I have shown by Western blot analysis that the level of expression of the NRE-binding proteins is differentially regulated, and is influenced by the status of the viral genome in the infected cell. I suggest that the inhibitory activity of the HPV-16 NRE is overcome as a result of the upregulation of cellular factors that bind the NRE.

Acknowledgements

I would like to acknowledge the following people who have helped to bring this thesis to completion:

Professor R. Elliott for giving me the opportunity to conduct this study in the Division of Virology.

My supervisor, Dr. Sheila V. Graham, for her patience, encouragement and helpful advice throughout the project and for critically reading this manuscript. Dr Craig Meyers and the members of his laboratory at the Hershey Medical Centre for making me feel so welcome during my visit and training me in 'raftology'.

Past and present members of lab A406 especially Sarah and Kirsty for helpful discussions and frequently steering me in the right direction.

Megan and Iain for making the daily process of scientific endeavour more enjoyable. Helen, a fine mentor and friend, and purveyor of many stimulating conversations.

My friends in Glasgow, Belfast, and beyond for always being there.

Finally, my family, your unfaltering support and encouragement are appreciated more than you can know.

The author was in receipt of a Medical Research Council studentship. Unless otherwise stated, the author carried out the work presented herein.

Contents

Abstract	i
Acknowledgements	ii
Table of contents	iii
List of figures	x
List of tables	xiii
Abbreviations	xiv

Chapter 1: Introduction

1.1.1 History	1
1.1.2 Taxonomy	3
1.1.3 Sites of HPV infection	4
1.1.4 Differentiation of normal epithelia	4
1.1.5 HPV-infection of epithelia	5
1.2.1 HPV-associated disease	8
Skin	8
Respiratory tract	9
Genital tract	11
1.2.2 Development of cervical carcinoma	12
1.2.3 Integration of vial DNA into the host genome	13
1.2.4 Epidemiology	14
1.2.5 Risk factors	16
1.2.6 Infection with multiple HPV types	18
1.2.7 Immune response to HPV infection	19
1.2.8 Latency	20
1.2.9 HPV diagnosis	21
1.2.10 HPV vaccines	22
1.3.1 Genomic organisation	24
1.3.2 Early viral proteins	24
E1	25

	E2	25
	E5	26
	E6	26
	E7	28
	Productive infections	28
	Non-productive infections	29
1.3.3	Late viral proteins	30
	E4	30
	L1 and L2	31
1.3.4	Viral promoters	32
	Early promoter	32
	Late promoter	33
1.3.5	Viral polyadenylation signals	34
1.3.6	HPV-16 splicing patterns	36
1.4.1	Regulation of HPV-16 late gene expression	38
1.4.2	RNA processing	39
	Splicing	39
	Cleavage and polyadenylation	40
	Nuclear export	41
1.4.3	Negative regulatory elements within the viral untranslated region	42
	BPV-1	42
	HPV-1	43
	HPV-31	43
1.4.4	Mapping of the HPV-16 NRE	44
1.4.5	The HPV-16 NRE acts as a general regulator of polyadenylation	45
1.4.6	Protein binding to the NRE	46
1.4.7	Inhibitory elements within the viral coding region	48
	HPV-16 L1 and L2	48
	HPV-31 L2	49

1.4.8 *In vitro* study of papillomaviruses 50
History 50
Calcium 51
Suspension in semi-solid medium 52
Organotypic raft culture 52

1.5 Aims and objectives 55

Chapter 2: Materials and Methods
2.1 Materials 56
2.1.1 Enzymes 56
2.1.2 Radiochemicals 56
2.1.3 Plasmids/Vectors 56
2.1.4 Antibodies 57
2.1.5 Bacterial culture 58
2.1.6 DNA probes 58
2.1.7 Cell lines 58
2.1.8 Common reagents and solutions 59
2.2 Methods 63
2.2.1 Tissue culture 63
2.2.1.1 Growth and maintenance of cell lines 63
2.2.1.2 Preparation of cell stocks 65
2.2.1.3 Establishment and maintenance of organotypic raft cultures 65
2.2.1.4 Preparation of cell extracts 66
(i) Protein extracts from monolayer culture 66
(ii) Protein extracts from organotypic raft culture 66
(iii) Nuclear extracts 66
2.2.1.5 Estimation of protein concentration 67
2.2.1.6 Transfection of cells 67

	(i) Lipofection with Lipofectamine	67
	(ii) Electroporation of primary keratinocytes	68
2.2.2	CAT assay	68
2.2.3	Manipulation of DNA	69
	2.2.3.1 Preparation and transformation of competent bacterial cells	69
	(i) DH5α cells	69
	(ii) XL1 blue cells	70
	2.2.3.2 Phenol chloroform extraction and ethanol precipitation	70
	2.2.3.3 Purification of plasmid DNA	71
	(i) Alkaline lysis	71
	(ii) Caesium chloride	71
	2.2.3.4 Restriction enzyme digestion of DNA	72
	2.2.3.5 DNA ligation	72
	2.2.3.6 Gel electrophoresis of DNA and recovery	73
	(i) Agarose gel electrophoresis	73
	(ii) Acrylamide gel electrophoresis	73
	2.2.3.7 Oligonucleotide synthesis and purification	74
	2.2.3.8 Quantification of DNA, RNA and oligonucleotides	75
2.2.4	PCR mediated site-directed mutagenesis	75
2.2.5	Southern blotting	77
	2.2.5.1 Hirt extraction of DNA from monolayers	77
	2.2.5.2 Total DNA extraction from rafts	77
	2.2.5.3 Electrophoresis and transfer	78
	2.2.5.4 Random priming of dsDNA	78
	2.2.5.4 Hybridisation and washing	79
2.2.6	SDS-PAGE	80
	2.2.6.1 Silver staining	81
	2.2.6.2 Western blotting	81
	2.2.6.3 Stripping membranes for reprobing	82

2.2.7 Immunohistochemistry 83
2.2.7.1 Counterstaining 83
2.2.8 RNA binding assay 84
2.2.8.1 Large-scale *in vitro* transcription of RNA 84
2.2.8.2 Linking RNA to agarose beads 84
2.2.8.3 RNA binding reaction 85
2.2.9 UV cross-linking 86
2.2.9.1 *In vitro* transcription of radiolabelled RNA 86
2.2.9.2 Cross-linking binding reaction 87
2.2.10 RNase protection assay 87
2.2.10.1 Extraction of sample RNA 87
2.2.10.2 Preparation of riboprobe template 88
2.2.10.3 *In vitro* transcription of a radiolabelled riboprobe 88
2.2.10.4 Hybridisation of the riboprobe and sample RNA 90
2.2.10.5 RNase digestion of hybridised probe and sample RNA 90

Chapter 3 - Characterization of HPV-16 positive epithelial cell lines
Introduction 91
3.1 The HPV-16 genome is episomal 93
3.2.1 Protein expression in monolayer cultures 93
3.2.2 L1 expression in monolayer cultures 94
3.2.3 Protein expression in monolayer cultures of HaCaT cells 96
3.2.4 Viral late protein expression in monolayer cultures of epithelial cells 97
3.2.5 Protein expression in untreated and phorbol ester-treated monolayer cultures 98
3.3.1 Organotypic raft cultures of HPV-16 epithelial cell lines 99
3.3.2 Protein expression in HPV-16 raft tissue 101
3.3.3 Viral late protein expression in organotypic raft cultures 103

3.3.4 L1 mRNA expression in organotypic rafts 105
3.3.5 Viral DNA amplification in organotypic rafts 106
3.4 Discussion 107

Chapter 4 – Site-directed mutagenesis of the HPV-16 NRE
Introduction 111
4.1.1 Saturating site-directed mutagenesis of the NRE 114
4.1.2 Mutagenesis does not affect the inhibitory activity of the NRE 114
4.1.3 Mutagenesis does not affect protein binding to the NRE 115
4.2 Deletion of the NRE in the context of the viral genome 117
4.3 Discussion 121

Chapter 5 - Protein-binding to the HPV-16 NRE
Introduction – Part 1 126
5.1.1 Binding of U2AF^{65}, CstF-64 and HuR to the HPV-16 NRE 127
5.1.2 Identification of NRE-binding proteins using a novel RNA binding assay 128
5.1.3 The HPV-16 NRE can bind U2AF^{65}, CstF-64 and HuR 130
5.1.4 The HPV-16 NRE can bind ASF/SF2 and Sm proteins 130
5.1.5 The HPV-16 NRE does not bind the 70kDa subunit of U1 snRNP 131
5.1.6 Binding of the U1 snRNP-A protein to the HPV-16 NRE 132
5.1.7 Comparison of protein binding to the full-length and half NRE constructs 134
Introduction – Part 2 136
5.2.1 Differential expression of U2AF^{65} in monolayer cultures of epithelial cells 138
5.2.2 Differential expression of CstF-64 in monolayer cultures of epithelial cells 139

5.2.3	Differential expression of HuR in monolayer cultures of epithelial cells	139
5.2.4	Differential expression of ASF/SF2 in monolayer cultures of epithelial cells	140
5.2.5	Differential expression of Sm proteins in monolayer cultures of epithelial cells	140
5.2.6	Differential expression of U1A in monolayer cultures of epithelial cells	141
5.3.1	Expression of NRE-binding proteins in organotypic raft cultures	141
5.3.2	Expression of CstF-64 and U2AF^{65} in CIN 612 9E organotypic rafts	143
5.4	Discussion	145

Chapter 6: General Discussion 155

Appendix 1

References I

List of Figures

1.1 Phylogenetic tree containing sequences of 103 Papillomaviruses
1.2 Epithelial differentiation
1.3 Progression to dysplasia
1.4 Malignant progression
1.5 Genomic map of HPV-16
1.6 HPV oncogenes and the cell cycle
1.7 HPV-16 mRNA species and coding potentials
1.8 Exon definition and splicing
1.9 Cleavage and polyadenylation
1.10 *Pst* I/*Eco* R1 fragment
1.11 Organotypic raft culture system

2.1 Site-directed mutagenesis of the NRE

3.1 Southern blot analysis of HPV-16 DNA
3.2 Analysis of protein extracts from W12 cells grown in monolayer culture
3.3 Western blot analysis of HPV-16 L1 expression
3.4 Analysis of protein extracts from monolayer cultures of W12 cells
3.5 Analysis of protein extracts from monolayer cultures of HaCaT cells
3.6 Western blot analysis of viral late protein expression in monolayer cultured cells
3.7 Comparison of untreated and C8-treated monolayer cultured cells
3.8 H & E staining of organotypic rafts
3.9 Analysis of protein extracts prepared from organotypic raft tissue
3.10 Immunohistochemical staining of differentiation markers
3.11 Western blot analysis of late viral protein expression in organotypic rafts

3.12 Localisation of HPV-16 late proteins
3.13 Ribonuclease protection assays quantifying the levels of L1 transcripts
3.14 Southern blot analysis of HPV DNA in raft tissue

4.1a P/E 445nt fragment
4.1b Site-directed mutagenesis of the NRE
4.2 pLW1 expression vector
4.3 Functional analysis of NRE mutants
4.4 UV cross-linking analysis of protein binding to the NRE and site-directed mutants at 60mM KCl
4.5 UV cross-linking analysis of protein binding to the NRE and site-directed mutants at 125mM KCl
4.6 UV cross-linking analysis of protein binding to the NRE and site-directed mutants at 250mM KCl
4.7 NRE-deletion mutant
4.8 PCR products for Δ52 mutant
4.9 Generation of the NRE-deletion mutant
4.10 Restriction enzyme analysis of Δ52 mutants

5.1 Western blot analysis of proteins UV cross-linked to a wild-type NRE RNA probe
5.2 Comparison of the protein binding profiles of NRE RNA probes
5.3 Western blot analysis of proteins binding to the NRE following an RNA binding assay
5.4 The HPV-16 NRE can bind ASF/SF2 and SM proteins
5.5 Binding of U1 snRNP subunits to the NRE
5.6 Binding of U1A following UV cross-linking
5.7 Western blot analysis of U1A binding to the NRE following UV cross-linking

5.8 Comparison of protein binding to full-length and half-NRE RNA constructs
5.9 Binding of HuR and ASF/SF2 to full-length and half-NRE RNA constructs
5.10 Comparison of monolayer protein extracts by Coomasssie blue staining
5.11 Western blot analysis of involucrin expression
5.12 Western blot analysis of $U2AF^{65}$ expression in monolayer cultured cells
5.13 Western blot analysis of CstF-64 expression in monolayer cultured cells
5.14 Western blot analysis of HuR expression in monolayer cultured cells
5.15 Western blot analysis of ASF/SF2 expression in monolayer cultured cells
5.16 Western blot analysis of Sm protein expression in monolayer cultured cells
5.17 Western blot analysis of U1A expression in monolayer cultured cells
5.18 Analysis of protein expression in C8-treated and untreated raft material by Western blotting
5.19 Protein expression in CIN 612 9E organotypic raft cultures
5.20 Model of protein binding to the NRE

List of Tables

1.1 Sites of HPV-infection and associated disease

2.1 Antibodies used in the course of this work

2.2 Sequences of the primer pairs used to mutate the NRE

5.1 Summary of differential expression of NRE-bound proteins

Abbreviations

ABC reagent	avidin and biotinylated horseradish peroxidase macromolecular complex
Ad	Adenovirus
A_E	early polyadenylation site
A_L	late polyadenylation site
APS	ammonium persulphate
ARE	AU-rich element
ASF/SF2	alternative splicing factor / splicing factor 2
ATP	adenosine triphosphate
BES	N, N-bis[2-Hydroxethyl]-2-amino-ethanesulfonic acid
bp	base pair
BPV	bovine papillomavirus
BSA	bovine serum albumin
C8	1,2-dioctanoyl-sn-glycerol
CAT	chloramphenicol acetyltransferase
CF	cleavage factor
CMV	cytomegalovirus
CIN	cervical intraepithelial neoplasia
cpm	counts per minute
CPSF	cleavage and polyadenylation specificity factor
CRPV	cottontail rabbit papillomavirus
CsCl	caesium chloride
CstF	cleavage stimulation factor
CTD	carboxy terminal domain
CTP	cytosine triphosphae
dATP	2'–deoxyadenosine-5'-triphosphate
DAB	diaminobenzidine tetrahydochloride
DCC	desquamated cornified cells

DCS	donor calf serum
dCTP	2'–deoxycytidine-5'-triphosphate
DEPC	diethyl pyrocarbonate
dGTP	2'–deoxyguanosine-5'-triphosphate
DMEM	Dulbecco's modified eagles medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DSE	downstream sequence element
DTT	dithiothreitol
dTTP	2'–deoxythymidine-5'-triphosphate
dUTP	2'–deoxyuridine-5'-triphosphate
E medium	epithelial medium
E	early
EBV	Epstein Barr virus
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetra-acetic caid
EtBr	ethidium bromide
EtOH	ethanol
EV	epidermodysplasia verruciforms
FCS	foetal calf serum
FEH	focal epithelial hyperplasia
g	gram
GTP	guanosine triphosphate
h	hour
HC	Hybrid capture
HEPES	N-2-hydroxyethyl piperazine-N'-2-ethane sulphonic acid
HFK	human foreskin keratinocyte
HGF	human growth factor
HIV	human immunodeficiency virus
HLA	human leukocyte antigen

hnRNPs	heterogeneous nuclear riboproteins
HPV	human papillomavirus
HR	high risk
HSV	herpes simplex virus
HTLV	human T-cell leukaemia virus
HuR	human antigen R
IGF-1	insulin-like growth factor 1
IgG	immunoglobulin G
IPTG	isopropyl thiogalactopyrnoside
k	keratin
kb	kilobase
kDa	kilodalton
KGF	keratinocyte growth factor
KGM	keratinocyte growth medium
L	late
M	molar
mCi	millicurie
ml	millilitre
min	minute
mM	millimolar
mRNA	messenger ribonucleic acid
nm	nanometers
NMSC	non-melanoma skin cancer
NRE	negative regulatory element
ORF	open reading frame
P	promoter
PAB II	poly(A) binding protein II
PAGE	polyacrylamide gel electrophoresis
Pap	Papanicolaou
PAP	poly(A) polymerase
PBS	phosphate buffered saline

PBS-T	phosphate buffered saline plus Tween
PCR	polymerase chain reaction
PKC	protein kinase C
PMA	12-O-tetradecanoylphorbol-13-acetate
PMSF	phenylmethylsulfonyl fluoride
pRb	Retinoblastoma protein
PV	papillomavirus
PVDF	polyvinylidene difluoride
RPA	RNase protection assay
rpm	revolutions per minute
RNA	ribonucleic acid
RRP	recurrent respiratory papillomatosis
SCC	squamous cell carcinoma
SDM	site-directed mutagenesis
SDS	sodium dodecyl sulphate
sec	second
sm	smith antigen
snRNA	small nuclear RNA
snRNP	small nuclear ribonucleoprotein
SSC	standard saline citrate
SV40	simian virus 40
TAE	Tris-acetate buffer
TBE	Tris-borate buffer
TE	Tris-EDTA buffer
TEMED	N, N, N',N'-tetramethyletylenediamine
TPA	12-0-tetradeconoylphorbol-13-acetate
TTP	tyrosine triphosphate
μ	micro
U	units
U2AF	U2 associated factor
USE	upstream sequence element

UTR	untranslated region
UTP	uridine triphosphate
UV	ultraviolet
UVR	ultraviolet radiation
VLP	virus-like particle
v/v	volume per volume
w/v	weight per volume
Xgal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside

CHAPTER 1: INTRODUCTION

1.1.1 History

The infectious nature of animal and human warts was established in the 1890s but it was not until 1933 that the first papillomavirus was described by Shope, who determined that cutaneous papillomatosis in the cottontail rabbit was caused by the cottontail rabbit papillomavirus (CRPV) (Shope, 1933, reviewed in Howley, 1996, zur Hausen *et al.*, 1994). Shope showed that inoculation of a wart extract onto scarified skin of cottontail or domestic rabbits resulted in transmission of the disease (Shope, 1933, Breitburd *et al.*, 1997). Wart regression occurs in most infected animals within 6 months, however, malignant transformation of certain warts occurs after this time in 25% of animals, and metastatic tumours are observed in the majority of these malignancies (Breitburd *et al.*, 1997). CRPV was one of the first experimental examples of a mammalian cancer virus and of an oncogenic DNA virus. Since then it has emerged that papillomaviruses are widespread in nature and, while they have been described primarily in higher vertebrates, papillomavirus infection in lower vertebrates has also been reported (reviewed in Howley, 1996).

Papillomavirus virion production requires full epithelial differentiation, consequently, studies on papillomaviruses have, until recently, been hampered by the lack of a suitable *in vitro* tissue culture system. Initial studies, therefore, focused on bovine papillomavirus (BPV) since this virus, unlike most HPV-types, causes large productive lesions, in the natural host, from which large quantities of BPV virions can be isolated. BPV induces fibropapillomas and is the only papillomavirus known to cross the species barrier, giving rise to sarcoids in horses, although these lesions do not produce infectious virions (Olson *et al.*, 1951). BPV-4 infects the upper gastrointestinal tract and induces squamous epithelial papillomas. However, in cattle grazing on bracken fern these papillomas can transform and

progress to squamous cell carcinoma (Beniston *et al.*, 2001). Bracken fern contains immunosuppressants and a number of mutagens, including quercetin. BPV virions are also capable of transforming a variety of rodent cells in tissue culture and by the late 1970s a quantitative transformation assay had been developed for BPV-1 that opened the way for the analysis of viral genes involved in the induction of cellular proliferation (Dvoretzky *et al.*, 1980, reviewed in Howley, 1996). This development, together with the successful cloning of the BPV-1 genome in bacteria in 1980 (Howley *et al.*, 1980), led to extensive study of this virus, which has served as the molecular prototype of the papillomaviruses, particularly in the study of viral DNA replication and transcriptional regulation. Interest in the study of BPV and other PVs has recently been renewed by the development of anti-papillomavirus vaccines in naturally occurring animal models including cattle, rabbits and dogs. A number of anti-HPV vaccines, based on vaccines developed in these animal models, are now in clinical trials (Stanley *et al.*, 1997, Morgan *et al.*, 2000, Nicholls *et al.*, 2000).

Gradual improvements in molecular cloning and cell culture techniques made the direct study of HPVs less problematic, and by the late 1970s the plurality of human papillomavirus (HPV) types was beginning to emerge (reviewed by zur Hausen *et al.*, 1994, Howley, 1996). The major stimulus for the interest in HPVs at this time originated from the discovery of their causal relationship with a common form of human cancer, carcinoma of the cervix. Surprisingly, this relationship had not been considered prior to 1974 despite the fact that CRPV was known to be carcinogenic in rabbits, and BPV could malignantly transform rodent cells in tissue culture and infected cells *in vivo* in association with bracken fern (zur Hausen *et al.*, 1994). By 1983 however, specific HPV types had been isolated directly from cervical cancer biopsies and a preponderance of evidence now supports a causal link between human papillomavirus infection and cervical neoplasia (Durst *et al.*, 1983, Boshart *et al.*, 1984, Canavan *et al.*, 2000).

1.1.2 Taxonomy

Until very recently, the papillomaviruses were grouped together with the polyomaviruses to form the papovavirus family on the basis of small size, a non-enveloped virion, an icosahedral capsid, a double-stranded circular DNA genome, and the nucleus as a site of replication (Howley, 1996). However, the papovavirus family has now been divided into two families, the *Polyomaviridae* and the *Papillomaviridae*, as a result of the fundamental differences in the genomic organisation, size and biology of these viruses (de Villiers, 2001): Polyomaviruses are generally smaller in size than papillomaviruses (40nm versus 55nm), with a smaller genome (5000 bp versus 8000 bp). In addition, the molecular biology of polyomaviruses differs considerably; for example, both strands of the genome serve as templates for transcription (Dimmock and Primrose, 1994). The taxonomic classification of papillomaviruses, based on the degree of genome relatedness has been a controversial issue, and has to date, not been accepted by the International Committee on the Taxonomy of Viruses (ICTV) (de Villiers, 2001). However, 86 complete genomes of the human papillomaviruses and 16 animal papillomaviruses have been characterized so far and more than 120 putative novel HPV types have been partially characterized (de Villiers, 2001). The definition of a new papillomavirus type has changed over the years as more information from newly characterized types has become known. Since 1995, a new papillomavirus type has been defined as one sharing less than 90% DNA homology within only the L1 open reading frame (ORF) of the closest related type (reviewed in Howley, 1996, de Villiers, 2001). Based on nucleotide sequences the *Papillomaviridae* can be divided into at least five subgroups, classified as *Papillomavirinae A* to *E,* and it is likely that additional subfamilies will be identified in the future. *Papillomavirinae A* to *C* are further divided into genera A1 to A14, B1 and B2, and C1 to C3 respectively (de Villiers, 2001)

(Fig. 1.1). Demarcation of genera within the remaining subfamilies is not possible at present due to a lack of information.

1.1.3 Sites of HPV-Infection

Thus far, all types of HPV identified appear to be strictly epitheliotropic; they infect epithelial cells either of the skin or of the anogenital and oropharyngeal mucosa (reviewed in Beutner *et al.*, 1997). Because of this, papillomaviruses have historically been grouped according to tissue tropism, i.e. the location of the lesion from which the virus was originally isolated and subsequently detected in. Therefore, HPV types are generally grouped as either mucosal/genital or cutaneous groups, however, this division is not absolute. Infection with HPV, whether mucosal or cutaneous, is associated with a wide variety of clinical manifestations ranging from laryngeal papillomatosis to intra-epithelial neoplasia (reviewed by Shah *et al.*, 1996) Table 1.1. This table clearly demonstrates that while papillomaviruses have a strict tropism for epithelial cells, this tropism extends beyond merely host and tissue specificity to an affinity for specific anatomical sites, HPV-1 is commonly found on the soles of the feet or the fingers and hands, whereas HPV-16 is generally associated with the anogenital region. This preference for anatomical sites is most likely related to differences in transcriptional requirements of different HPV types as well as the mechanisms of transmission (Meyers *et al.*, 1994a).

1.1.4 Differentiation of normal epithelia

The productive viral life cycle of HPVs is tightly linked to epithelial differentiation (Stanley, 1994). In normal epithelia, only the basal cells in the lower layers are mitotically active and divide to produce two daughter cells, one of which may migrate from the basal layer and become committed to differentiation (Fuchs *et al.*, 1994). If this happens, this daughter cell moves from the basal layer to the spinous layer, in which cells are postmitotic but

metabolically active, and devote much of their protein synthesizing activity to manufacturing two keratins, K1 and K10 (Fuchs *et al.*, 1993). In addition, cells in the upper spinous layer and granular layer make lysine-rich envelope proteins such as involucrin, which are deposited on the inner surface of the plasma membrane of each cell (reviewed in Fuchs, 1990). As cells reach the upper granular layers, they stop generating keratin proteins and switch to production of loricrin, which is a glycine-rich insoluble protein, and a major component of the cornified envelope. Filaggrin, a matrix protein for keratin filaments is also expressed at this time (Fuchs *et al.*, 1994). During the transition from the granular to the cornified layer there is a rather abrupt onset of intracellular degradation leading to the loss of nuclei and cellular organelles. By the time the daughter cell reaches the skin surface it has undergone a series of biochemical and morphological changes that culminate in the production of dead, flattened, enucleated squames, which are sloughed from the surface and continually replaced by inner cells differentiating outward. This is a continuous, life-long process in humans which for skin takes approximately 3 to 4 weeks for completion (Fuchs *et al.*, 1994) (Fig. 1.2). Non-keratinizing epithelia such as the cervix may have a more rapid turnover time but this is unlikely to be less than 7-10 days (Fuchs *et al.*, 1993).

1.1.5 HPV-Infection of Epithelia

It is generally accepted that papillomavirus growth requires the infection of cells in the basal layer of the epithelium, since these contain the necessary transcription machinery for viral protein expression and replication. The most likely route of infection by papillomaviruses is through tiny abrasions and microtraumas in the epithelial architecture (Meyers *et al.*, 1994a). Only the primary target cells of cottontail rabbit papillomavirus (CRPV) have been identified to date, and are thought to be present in hair follicles, where they colocalize with a subpopulation of keratinocytes with properties of epidermal

stem cells (Schmitt *et al.*, 1996). However, unlike CRPV, a number of PVs specifically target epithelial sites that lack hair follicles, such as mucosal epithelium. Nevertheless, cells with features of stem cells have been identified in discrete areas of mucosal epithelium, for example, at intervals in the basal layer of the pluristratified epithelium of the cervix, and so these stem-like cells may serve as target cells for this subset of PVs (Stanley, 1994). However, the question remains as to whether these target cells carry a specific receptor molecule for PV or alternatively provide unique transcription machinery for PV gene expression. BPV-1 virions and HPV-11 virus-like particles (VLPs) have been shown to bind both to human keratinocytes and cell lines of epithelial and fibroblast origin suggesting that the PV receptor molecule(s) are ubiquitously expressed (Roden, *et al.*, 1994). Despite this, the identity of the cell surface receptor(s) for PVs remains controversial and several candidates have been proposed to date. The integrin alpha-six beta-four protein complex (α_6 integrin) is expressed primarily during wound healing which makes it a logical candidate, and while no functional studies have yet shown it to mediate HPV entry, a recent report using HPV-16 VLPs indicated that the degree of VLP binding was directly proportional to the level of expression of α_6 integrin (Yoon *et al.*, 2001). In contrast, it has been shown that α_6 integrin is not the obligatory cell receptor for BPV-4, which, like all HPVs, is strictly epitheliotropic. BPV-4 virions were found to bind cells lacking α_6 integrin to similar, saturatable levels as normal keratinocytes (Sibbet *et al.*, 2000). Nor does it appear that α_6 integrin is involved in HPV-33 pseudovirion infection (Giroglou *et al.*, 2001). Additional reports have implicated the ubiquitous polysaccharide, heparan sulphate, as the cell surface receptor for PV (Combita *et al.*, 2001, Giroglou *et al.*, 2001). A recent study has also presented evidence suggesting that heparan sulphate may be the initial attachment point, and this is followed by association with a secondary receptor complex prior to internalisation, similar to the situation for certain herpes and adenoviruses, as well as dengue, Sindbis and vaccinia viruses (Giroglou *et al.*, 2001). Moreover, it has been

postulated that this secondary receptor may be L2-specific; L2 is the minor viral capsid protein and results suggest that an interaction between a conserved N-terminal domain of L2 and an, as yet unidentified, cell surface protein plays an important role in papillomavirus infection (Kawana *et al.*, 2001). It has been well established that the incorporation of L2 increases the infection efficiency of pseudovirions and that antibodies to L2 can be neutralising, indicating that L2 is important for infection (Cornelison, 2000).

Following entry into the basal cell, HPV genomes are established as extrachromosomal elements in the nucleus and copy number is increased to approximately 20-100 copies per cell, with viral DNA replication occuring in synchrony with DNA replication in the infected cell (Stanley, 1994). As infected cells divide, viral DNA is distributed between both daughter cells. One daughter cell may withdraw from the cell cycle, migrate from the basal layer and initiate a programme of differentiation. The remaining daughter cell continues to divide in the basal layer and provides a reservoir of viral DNA for further cell divisions, but the virus continues to maintain its genome at a low-level in these cells. Production of mature virus is restricted to suprabasal cells, consequently, the cells in the basal layer are not lysed by virion production but continue to proliferate and can persist for extensive periods. As the differentiating infected daughter cell migrates upward, viral early proteins continue to be expressed in all layers of the epithelium. However, expression of late viral proteins and activation of productive viral replication is blocked very effectively until the infected cells undergo terminal differentiation (Meyers, *et al.*, 1994b). This is an unusual infection strategy since, in order to replicate its genome in the unfavourable environment of the differentiated cell, HPV must reactivate the quiescent cellular DNA replication machinery, similar to the polyomaviruses and adenoviruses. It has been reported that HPV-16 can switch replication mode during its life cycle. In the mitotically active basal cells, the virus replicates bidirectionally, via theta structures, and is highly dependent on host cellular factors,

however in terminally differentiated cells, the virus switches to a rolling circle mode of replication which is unidirectional, and one initiation event leads to the generation of multiple copies of the genome and so fewer cellular factors are required (Lambert *et al.*, 1991, Flores *et al.*, 1997). Mature virus particles are observed in the granular layers of the epithelium but little is known about papillomavirus assembly or release, although the virus does not appear to be cytolytic. It seems highly likely that virions are transmitted from the layers of desquamated cornified cells (DCCs), which are continuously shed from the stratum corneum, the uppermost epithelial layer. DCCs normally have a very durable structure, and therefore virion release from these cells would require the cornified cell envelope to break apart. Interestingly, it has previously been reported that the envelope of DCCs from HPV-11-infected genital epithelium are unusually thin and fragile and it has since been shown that DCCs serve as vehicles for the efficient, concentrated delivery of virions in an HPV-11 infection (Bryan *et al.*, 2001). If other HPVs use a similar mechanism for virion release then it seems highly likely that virion transmission and subsequent infection is a local event.

1.2.1 HPV- associated Disease

For the purposes of this discussion I have divided HPV-associated diseases on the basis of anatomical site of infection i.e. skin, respiratory tract and anogenital tract, as shown in Table 1.1:

Skin

The soles of the feet and the hands appear to be the favoured sites for wart formation and specific HPV types are associated with each location and wart morphology; for example, common warts are generally found on the hands and are associated with HPV-2 and –4, while deep plantar warts, associated with HPV-1, are more commonly found on the soles of the feet. Butcher's warts are associated with HPV-7 and might be considered an occupational

hazard since they are found almost exclusively on the hands of butchers and meat and fish handlers. The majority of warts regress spontaneously within 2 years and are rarely associated with the development of malignancy (reviewed in Shah *et al.*, 1996).
Epidermodysplasia verruciforms (EV) is a rare genetic skin condition involving a lifelong predisposition to HPV-infection, leading to chronic nongenital HPV lesions on sun-exposed sites, which can develop into squamous cell carcinomas over time (reviewed in Shah *et al.*, 1996). Two types of warts occur in EV patients, flat warts and reddish-brown macular plaques and although HPV types –5, -8, -17 and –20 are most frequently found in EV patients, a large number of other HPV-types have also been associated with EV lesions including –3, -9, -10, -12, -14, -15, -17, -19, -20 and –25 (reviewed in Shah *et al.*, 1996).
There has been a great deal of data published recently regarding the potential role of human papillomavirus infection in the genesis and progression of non-melanoma skin cancers (NMSC), which are the most commonly diagnosed carcinomas among Caucasians worldwide (de Villiers *et al.*, 1999). The importance of ultraviolet radiation (UVR) in the development of these cancers has previously been well established and is now considered the most important etiologic factor. However, a growing body of evidence suggests that certain types of HPV, similar to those associated with EV lesions, either alone or in conjunction with UVR, may play a role in the pathogenesis of these cancers (reviewed in Kiviat, 1999).

HPV infection has also been implicated in the development of head and neck squamous cell carcinoma as a result of the presence of HPV DNA, particularly HPV-16, in tumour tissue. However, the results of various retrospective and prospective case studies have been inconclusive and it remains possible that the development of cancer simply improves the detection of virus in these lesions (Mork *et al.*, 2001).

Respiratory Tract

The best-characterised HPV infections of the respiratory tract are laryngeal papillomas, also known as recurrent respiratory papillomatosis (RRP) (Mounts *et al.*, 1984). These papillomas result from transmission of HPV-6 and –11, commonly found in genital warts, to the respiratory tract and so in most cases, virus transmission is believed to occur vertically during the birthing process (reviewed in Shah *et al.*, 1996). In addition, it has been suggested that viral transmission may occur *in utero*, but this appears to be an infrequent event (Fredericks *et al.*, 1993). A striking characteristic of laryngeal papillomas is the frequency of recurrence following surgery or treatment. Recurrence most likely results from the reactivation of latent viral DNA in histologically normal epithelium adjacent to the papillomas (Ferenczy *et al.*, 1985).

Two HPVs appear to exclusively infect the oral cavity, HPV-13 and –32, and both are associated with the well-characterised condition, focal epithelial hyperplasia (FEH), which, although it has a worldwide distribution, is particularly common in the indigenous populations of Central and South America and of Alaska and Greenland (reviewed in Shah *et al.*, 1996). FEH is characterised by self-limiting multiple nodules on the oral mucosa, which do not undergo malignant transformation.

HPV infection is also frequently detected in tonsillar carcinoma; however, unlike the examples mentioned above, some reports suggest that the presence of HPV in these cases is a prognostically favourable factor for patient survival and also for low risk of relapse by making the tumours more susceptible to radiation treatment (Friesland *et al.*, 2001). However, a previous study reported the presence of integrated HPV-6a DNA in a tonsillar carcinoma; this is very unusual since HPV-6 is generally considered a low-risk virus and rarely integrates into the host genome (Bercovich *et al.*, 1991).

Genital Tract

One of the most well studied sites for HPV infections is the genital tract. Approximately 40 different HPV types are known to infect the anogenital tract where infection can give rise to a range of clinical outcomes from benign genital warts through to cancer of the cervix, penis and vulva. Papillomaviruses were first recognised as the etiologic agents of genital warts in the late 1960s and further study led to the recognition of the causal role of HPV-infection in carcinomas of the genital region (reviewed in zur Hausen *et al.*, 1994).

Genital warts are the most commonly recognized clinical lesions of genital HPV infections and are typically transmitted through sexual contact. HPV-6 and HPV-11 are most commonly associated with condyloma acuminata and these warts generally regress either spontaneously or following treatment and rarely progress to malignancy. Therefore, HPV types –6 and –11 are generally regarded as low-risk types due to their lack of oncogenic potential Beutner *et al.*, 1997). Conversely, a subset of genital HPVs are frequently detected in anogenital cancer biopsy specimens: The main representatives are HPV types –16, -18, -31, -33, -39, -45, -52, -58, and –69, which specifically possess cell-immortalising and transforming properties and are considered as high-risk types (reviewed in Kaufman *et al.*, 2000). These high-risk viruses are associated with carcinoma at a number of lower genital sites, however, the most extensively studied anogenital cancer is cervical carcinoma, which is second only to breast cancer in terms of the number of women affected worldwide (Canavan *et al.*, 2000). The high incidence of invasive cervical cancer as compared to the lower incidence of cancer at other sites in the female genital tract is ascribed to the high susceptibility of the cervical transformation zone to carcinogens (Shah *et al.*, 1996). The transformation zone is located at the lower end of the cervix where the columnar cells of the endocervix meet the stratified squamous epithelium of

the ectocervix; cells in this zone are subject to rapid turnover (reviewed in Stanley *et al.*, 1992).

1.2.2 Development of Cervical Carcinoma

The natural history of invasive squamous cell carcinoma of the cervix has been well documented, and it is generally accepted that the majority of invasive neoplasms arise from a focus of intraepithelial neoplasia that may have been present within the cervix for many years (reviewed in Stanley, 1994). For example, detectable HPV-16 DNA persists in the female genital tract for a mean time of approximately a year, and the median time to develop an anti-HPV-16 capsid antibody response in serum is also approximately 1 year (Galloway *et al.*, 1996). Following detection of viral DNA, the earliest pre-invasive changes, which are squamous intraepithelial lesions, are asymptomatic and can develop in as little time as 2 years, although additional co-factors may influence this time interval (Galloway *et al.*, 1996). Currently it is assumed that squamous cell carcinoma of the cervix develops through a continuum of progressive cervical intraepithelial neoplasia (CIN) lesions (Stanley, 1994) (Fig. 1.3). These lesions are graded according to the proportion of the epithelium occupied by undifferentiated cells of basal type: In CIN grade I, the cells in the upper and middle third of the epithelium undergo differentiation. In CIN grade II, cells with an undifferentiated basal phenotype extend beyond the lower third of the epithelium but not into the upper third and some abnormal mitotic figures are also present in the lower two thirds. In CIN grade III, undifferentiated nonstratified cells occupy more than two thirds, and often the full thickness, of the epithelium. These abnormal cells remain intraepithelial but if invasion occurs, the neoplastic cells penetrate the underlying basement membrane and invade the stroma with the potential for widespread dissemination (reviewed in Stanley, 1994) .

1.2.3 Integration of Viral DNA into the Host Genome

Increasing CIN grade is often accompanied by integration of the viral genome into the host DNA (Durst *et al.*, 1985). This integration event frequently disrupts the early viral coding region, resulting in the loss of expression of all the viral proteins with the exception of the viral oncoproteins E6 and E7, which actually become overexpressed (Durst *et al.*, 1991). However, given that the average age of patients with CIN III in the United States is 29, and the median age of invasive cervical cancer patients is 49, the additional changes required for invasion and metastasis appear to be acquired slowly over time and may not be a direct consequence of the overexpression of viral oncoproteins (reviewed in Galloway *et al.*, 1996). A substantial body of evidence now exists which supports the hypothesis that while high-risk oncogenes are necessary for cell immortalisation and tumourigenesis, they are not sufficient for the development of the malignant phenotype, which requires the interruption of host cell genes that normally suppress the tumorigenicity of immortalised cells. For example, somatic cell hybridisation of individual clones of HPV-immortalised human keratinocytes frequently results in senescence of the cell hybrids despite the continued expression of viral oncogenes (reviewed in zur Hausen, 1999). Moreover, a number of specific chromosomal aberrations have been observed in HPV-immortalised cells and in cervical carcinomas; cytogenetic analyses have frequently reported changes in chromosome 3 as well as chromosome 18, corresponding to loss of the fragile histidine triad (FHIT) gene (Butler *et al.*, 2000) and the putative tumour suppressor gene, deleted in colon cancer (DCC) (Klingelhutz *et al.*, 1995), respectively. Surprisingly, however, HPV genomes do not appear to integrate at any specific locus on the host genome, for example close to tumour suppressor genes or proto-oncogenes, as observed for other oncogenic viruses such as certain retroviruses and hepatitis B viruses. Therefore, it appears that in addition to expression of high-risk HPV oncogenes, other factors contribute to specific alterations

within the host-cell DNA, which collectively result in malignant progression (Fig. 1.4).

1.2.4 Epidemiology

In 1995 the International Agency for Research on Cancer (IARC) stated that there was no doubt that infection with certain types of HPV is the main cause of cervical cancer (reviewed in Munoz, 2000). The IARC also concluded that there was sufficient evidence to categorize HPV types –16 and –18 as human carcinogens but the existing evidence was limited or inadequate to allow a similar categorization of other HPV types (Munoz, 2000). However, subsequent extensive worldwide studies have led to the recognition of a large number of additional carcinogenic HPV types including HPV-31, -33, -35, -39, -45, -51, -52, -54, -56, -58, -59, -66, –69 and –70 (Munoz, 2000). A comparison of 1000 cases of histologically confirmed invasive cervical carcinomas from 22 countries worldwide revealed that 99.7% of cases were HPV-positive: 53% HPV-16, 15% HPV-18, 9% HPV-45, 6% HPV-31 and 3% HPV-33 (reviewed in Munoz, 2000). HPV-16 was found to be the most prominent type world wide although there were geographical variations; in Indonesia and South East Asia, in general, HPV-18 was the most common type, while in Western Africa HPV 45 was frequently found, whereas HPV types -33, -39 and -59 were found more often in Central and South America than in other regions (reviewed in Munoz, 2000). Higher rates of HPV prevalence in certain geographical regions also correlates well with the differences in cervical cancer incidences worldwide. African countries and Latin America have a high HPV prevalence and large numbers of cervical cancer cases, other countries such as Thailand and the Philippines report an intermediate prevalence of HPV infection and intermediate numbers of cervical carcinoma patients, while most European countries have a much lower HPV prevalence and fewer cases of cervical carcinoma (Bosch, 1999).

The mean age of cervical cancer patients worldwide is 52 years and the distribution of cases is biphasic with peaks at 35-39 and 60-64 years, although a recent study in Argentina surprisingly revealed no second peak of incidence (Munoz, 2000). The majority of all cervical carcinomas are squamous cell carcinoma (85-90%) and the second most common type is adenocarcinoma (10-15%), which arises from the columnar epithelium, lining the endocervical canal and glands (Tjiong *et al.*, 2001). Adenocarcinoma is generally more rapid and aggressive that squamous cell carcinoma (SCC) and is more frequently associated with HPV-18 than HPV-16 (reviewed in Beutner *et al.*, 1997).

Most HPV infections are transient in immunocompetent woman and the prevalence of infection is age-related, such that in woman aged 20-25 years the reported HPV prevalence is 20-46% while in women over 30 this decreases to 6% (Melkert *et al.*, 1993). Moreover, it appears that in most women the immune system can successfully overcome an HPV infection even when cervical intraepithelial neoplasia (CIN) has developed; data from a number of retrospective studies indicates that only 1 % of CIN I progress to invasive lesions, this increases to 5% for CIN II and to 12% for CIN III lesions (Arends *et al.*, 1998), which are widely believed to be the immediate precursors of invasive carcinoma. Clearly, cervical cancer is a multi-step process as indicated by the low number of high-risk HPV infected woman that develop cervical cancer and the low rate of progression from high-grade cervical lesions to invasive carcinoma. Further viral and non-viral factors must play a role in cervical carcinogenesis.

1.2.5 Risk Factors

HPV-associated risk factors for invasive cervical carcinoma include infection with certain high-risk types particularly HPV-16, -18, -45, and –56 (reviewed in Canavan *et al.*, 2000). Most genital HPV infections are cleared within 6 to 12 months of infection, and infections not cleared within this time are considered to be persistent and only persistent infections progress to neoplasia (Einstein *et al.*, 2001). Naturally occurring HPV variants may also be important co-factors. Variants are defined as having less than 2% variation in their genome. For example, infection with Non-European HPV-16 variants is more likely to indicate an increased risk of cervical cancer development compared to infection with European variants (reviewed in Nindl, 2002). It is likely that naturally occurring variants such as these have different biological and biochemical properties that result in differences in viral pathogenicity.

Sexual activity is considered the most important non-viral risk factor. Genital HPV infection is primarily a sexually transmitted disease and therefore factors such as the age at first intercourse (Parazzini *et al.*, 1992), the number of sexual partners and the frequency of sexual intercourse influence the risk of acquiring such an infection (Tijong *et al.*, 2001). However, it has been argued that a lower age at first intercourse is not a true co-factor but instead exerts its effect by influencing the lifetime number of sex partners (reviewed in Koutsky, 1997). Individuals, who have previously been infected with a sexually transmitted disease, including HPV, also appear to be at greater risk of acquiring a persistent HPV infection (Parazzini *et al.*, 1992).

The use of oral contraceptives has long been the subject of great controversy with several reports presenting contradictory data including one report suggesting that while oral contraceptive use had no effect on the incidence of low-grade cervical lesions, the risk of high-grade lesions did increase, implying that oral contraceptives, possibly by varying hormone

levels, act by altering disease progression rather than by increasing the risk of HPV infection (Negrini *et al.*, 1990). The hormonal and physiological changes that occur during pregnancy might also explain the higher rates of HPV infection detected in pregnant women, in some studies (Schneider *et al.*, 1990). Reports relating to the influence of cigarette smoking are also contradictory and less convincing, partly because it is difficult to establish if this type of activity influences the risk of acquiring infection or simply improves the efficiency of detection of latent infections (reviewed in Koutsky, 1997).

A number of reports have also suggested that women with HLA class II DQw3 are at an increased risk of developing cervical cancer (Wank *et al.*, 1991), however, a further report disputed this and the matter remains unresolved (Glew *et al.*, 1993). A similar impasse has been reached with regards to the influence of the p53 codon 72 polymorphism on progression of cervical neoplasia, with some reports suggesting that an arginine/arginine genotype at codon 72 in the p53 gene predisposes individuals to tumour progression (Storey *et al.*, 1998), and yet others convincingly arguing that there is no correlation between HPV infection, p53 codon 72 arginine polymorphism and the development of squamous cell carcinoma (O'Connor *et al.*, 2001).

In contrast, the immune status of the host appears to have a significant impact on the ability to detect genital HPV infection and on the progression of HPV-induced lesions. In immunocompromised individuals, such as, human immunodeficiency virus (HIV)-infected women and organ transplant recipients, the presence of HPV infections and anogenital neoplasia is higher than in the general population. In one study persistent HPV infections were detected in 24% of HIV-seropositive women but only in 4% of the control group (Sun *et al.*, 1997). Moreover, approximately 50% of symptomatic HIV-infected women in a number of studies appeared to have abnormal cervical

cytology suspect for squamous intraepithelial lesions (Schafer *et al.*, 1991). In a comparison of immunosuppressed renal transplant patients and normal controls, a 17-fold greater incidence of genital HPV infection and a 9-fold increase in HPV-related CIN lesions were found in the immunosuppressed group (Halpert *et al.*, 1986). Immunosuppression could result in a higher prevalence of HPV infection either by increasing the risk of infection or by impairing the body's ability to suppress latent infection.

1.2.6 Infection with multiple HPV types

A recent study comparing HPV-types in NMSC lesions of immunocompetent and immunosuppressed patients revealed that 59.1% of transplant tumours compared to 13.04% of immunocompetent tumours contained multiple HPV-types (Harwood *et al.*, 2000). This observation also extends to anogenital warts. Christensen *et al.*, (1997) reported several genital HPV types detected within a single anal wart from a renal transplant patient, however, localisation by *in situ* hybridisation suggested that each HPV type maintained regional separation within the lesion. Similarly Unger *et al.*, (1997) reported the presence of multiple HPV types clustered in geographically distinct areas within anogenital warts in immunocompromised HIV-positive patients. Coinfection with multiple types of genital HPV in immunocompetent individuals has also been reported, but the frequency of occurrence and whether prior infection with specific HPV types inhibits subsequent infection by related types is not known. A recent report found that concurrent acquisition of multiple HPV types occurred more often than expected by chance, however, no two types were more or less likely to be acquired concurrently, and prior infection with a phylogenetically related type did not decrease the risk of subsequent infection with a new type (Thomas *et al.*, 2000). Fife *et al.*, (2001) have suggested that the number of HPV-types in a multiple infection correlates with increasing cervical dysplasia.

1.2.7 Immune Response to HPV-Infection

The reasons for the enormous diversity of HPV types as well as the restriction of viral propagation to external skin or specific internal mucosal sites are presently unknown. The possibility exists however, that both of these observations are interrelated. The replication of viral DNA and the subsequent particle formation in external differentiated epithelial cells of skin, anogenital, and oral mucosa may expose these infections to less immunologic interference (reviewed in zur Hausen, 2000). The absence of immunologic interference could be the major reason for reduced evolutionary restraints, permitting the adaptation of new HPV variants to different types of differentiated epithelia. It has been reported that the rate of seroconversion following infection with either HPV 16, 18 or 6 varies, such that the timing of seroconversion was markedly delayed for HPV-16 compared with HPV-6 infections and it seems likely that this is due to differences in the nature of these viral infections (Carter *et al.*, 2000). HPV-6 capsid proteins may be more immunogenic and therefore the infections are eliminated more quickly. Conversely, high-risk HPV infections employ strategies that may delay the recognition of the viral proteins such as interference with the interferon response pathways: Stat-1 and interferon genes have recently been identified as transcriptional targets of HPV-31 through microarray analysis (Chang *et al.*, 2000). HPV-16 E6 and E7 proteins have also been shown to inhibit the transcriptional activity of interferon regulatory factor 3 (IRF-3) (Ronco *et al.*, 1998). Therefore, it appears that high risk HPVs can affect both the synthesis of interferon and also the interferon response pathway.

1.2.8 Latency

HPV restricts expression of its most immunogenic proteins, the capsid proteins, to the upper layers of the skin, which not only helps efficient transmission of mature virions but may also enable the virus to establish latent infections in the lower layers of the epithelium. While it is not yet clear if papillomaviruses have a true latent phase, as has been well documented for a number of other viruses such as HSV-1, EBV or varicella zoster, evidence exists to suggest that CRPV can induce a latent infection (Stubenrauch *et al.*, 1999). Infection of rabbits with low doses of CRPV results in the development of papillomas at a small number of sites (10-20%), sites that do not develop papillomas are positive for viral DNA and can be induced to form papillomas following treatment with photosensitizers indicating that these infections are truly latent and not abortive (Amella *et al.*, 1994). In addition, several clinical observations suggest that a latent state also exists for HPV infections *in vivo.* The rapid appearance of genital and cutaneous papillomas in immunosuppressed renal transplant and HIV-positive patients, despite the absence of obvious lesions before treatment, provides support for such a state (Sillman *et al.*, 1984). In addition, the persistent reappearance of HPV-11 papillomas in the larynx of children indicates that even in immunocompetent individuals this viral type can exist latently (Ferenczy *et al.*, 1985). True HPV latency can be defined as the presence of viral DNA in the absence of differentiation-dependent virion production and therefore, the presence of HPV DNA in basal cells cannot be called a true latent state (Stubenrauch *et al.*, 1999). However, if an infected stem cell failed to divide a state of latency could be achieved, and the level of expression and activity of viral proteins such as E6 and E7 could potentially prevent the cell cycle progression of infected cells. In support of this, a comparison of HPV-11 transcripts in respiratory papillomas and latently infected tissue showed that this tissue lacked correctly processed mRNAs encoding the E6 and E7 proteins and had only low levels of

expression of the early viral proteins, E1 and E2, suggesting that a limiting amount of key viral proteins may be a factor in latent infections (Maran *et al.*, 1995). Whether a true latent state exists for these viruses remains an important question for the development of therapeutics to treat HPV infections.

1.2.9 HPV Diagnosis

In light of the recognition of the strong causal relationship between infection with certain types of HPV and the development of cervical carcinoma, accurate diagnosis of HPV infection is an important source of information. Observation together with colposcopic examination is a useful conventional method for the detection of clinically apparent infection, manifesting as distinctive anogenital warts, however due to low diagnostic specificity this approach is more useful as an investigative, rather than a diagnostic, tool (Trofatter, 1997). Moreover, since the majority of individuals have no clinical manifestation of HPV-infection, detection and diagnosis of HPV in these cases can be problematic. The most common method of cytologic testing is the Papanicolaou test, generally referred to as the Pap smear (Trofatter, 1997). Exfoliated genital cells are stained and examined for evidence of abnormal cells, such as koilocytes, which are considered characteristic of low-grade CIN. However, although the Pap smear has been very successful at reducing the incidence of cervical cancer in most countries, it has been shown that the sensitivity of this test does not exceed 70%, with a false-negative rate ranging from 20 to 30% (Fahey *et al.*, 1995).

It has been proposed that HPV testing in combination with the Pap smear may help lower the false-negative rate of the latter (Wright *et al.*, 1995, Harper *et al.*, 2001). Recent studies have shown that HPV testing combined with a Pap smear has a promising future in primary screening, particularly in women over the age of 30 (Cox *et al.*, 1995, Nobbenhuis *et al.*, 1999).

The most commonly used methods for detecting HPV in tissue are PCR and solution hybridisation, also known as Hybrid Capture. These tests have similar sensitivity and specificity but PCR is more difficult to use routinely, whereas Hybrid Capture is a rapid test and is available as a kit. PCR is considered the gold standard research-based test for HPV (reviewed in Lorincz *et al.*, 2001). Different types of primers can be used, including consensus primers, which hybridise to a common HPV nucleotide sequence and can detect many HPV-types, while type-specific primers can be used to identify the type of HPV involved. The Hybrid Capture (HC) method is the most commonly used test for HPV detection in clinical practice, (reviewed in Lorincz *et al.*, 2001). It is a simple, amplification-based chemiluminescent assay using RNA probes specific for the genomes of the 13 viral types, HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59 and –69, etiologically connected to the pathogenesis of cervical cancer (reviewed in Lorincz *et al.*, 2001). Recent studies using HC II, the current version of HC test, have consistently documented a sensitivity of about 90% or greater for the presence of high-grade CIN, (reviewed in Cuzick, 1999).

1.2.10 HPV Vaccines

As a viral disease, HPV infection is a candidate for preventative vaccination, with current efforts targeted at the development of both prophylactic and therapeutic vaccines. Prophylactic vaccines could theoretically prevent HPV infection by inducing a protective antibody response in the genital mucosa against the viral capsid proteins L1 and L2. A number of vaccines based on high-risk virus-like particles (VLPs) are currently in Phase 1 trials and the potential success of such vaccines is supported by results using animal papillomavirus models of infection, (reviewed in Cornelison, 2000, Harro *et al.*, 2001). One possible shortcoming of these VLP-vaccines, however, is that they are unlikely to have a therapeutic effect because the capsid proteins are not detected in the proliferating cells of the infected epithelia or

in cervical carcinomas. Immunisation by combination strategies using chimeric VLPs containing both early and late HPV gene products offers increased potential for both prophylaxis and therapy (reviewed in Kaufman *et al.*, 2000, Cornelison, 2000).

Two types of therapeutic vaccines are being considered: The first is aimed at suppressing the replicating virus in infected individuals, and so the early viral proteins E1 and E2 are possible immunological targets for this (Selvakumur *et al.*, 1995). The second vaccine type is aimed at counteracting established neoplastic processes and therefore the oncogenic proteins E6 and E7, and possibly E5, could potentially be used as tumour vaccine candidates, and a number of preliminary studies addressing this have given encouraging results (Liu *et al.*, 2000, Muderspach *et al.*, 2000). There has also been considerable recent interest in the development of oncolytic viruses that specifically target high-risk HPV-induced tumours, such as adenovirus mutants that efficiently replicate only in tumour cells with defective p53. High-risk E6 is constitutively expressed in cervical tumour cells and degrades p53 in these cells, therefore, mutant adenoviruses should be able to replicate in these neoplastic cells and bring about selective cell death. This work is still at an early stage but preliminary results are promising (Balague *et al.*, 2001).

1.3.1 Genomic Organisation

Papillomaviruses are small, 52-55nm in diameter, nonenveloped DNA viruses. The capsid is of icosahedral symmetry and comprises 72 capsomers and contains within it a single molecule of double-stranded circular DNA composed of approximately 8,000bp (Howley, 1996). The prototype HPV-16 sequence was first published in 1985 and is now one of more than 100 papillomaviruses that have been fully sequenced (Seedorf *et al.*, 1985, de Villiers, 2001). The genomic organization of each papillomavirus is remarkably similar: The genetic information is localized on one strand of the viral DNA and the genome can be divided into three regions: the early region (E), the late region (L), and the non-coding region, also called the untranslated region (UTR) (Fig. 1.5). HPV genomes are transcribed into polycistronic messages through the use of multiple promoters, early and late polyadenylation signals, and extensive splicing (reviewed in Howley, 1996, Ozbun *et al.*, 1998a). Polycistronic messages are uncommon in eukaryotes yet seem to be prevalent in both high and low-risk HPV infections (Hummel *et al.*, 1992). It is known that differential splicing of early RNAs in all PVs generates a variety of polycistronic messages, which vary considerably in relative abundance (Hummel *et al.*, 1992, Barksdale *et al.*, 1995).

1.3.2 Early Viral Proteins

The early region represents approximately 45% of the genome and encodes up to eight viral proteins, although their open reading frames overlap extensively. HPV-16, like most HPVs characterised to date, expresses only the E1, E2, E4, E5, E6 and E7 proteins.

E1

E1 is the largest ORF in all PVs and is essential for viral replication (Ustav *et al.*, 1991). E1 exhibits ATPase and helicase activity and binds with low affinity to the A/T-rich origin of replication within the UTR of papillomaviruses (Hughes *et al.*, 1993). The subsequent binding of the second viral replication protein, E2, to its binding sequences flanking the origin of replication, and interactions between E1 and E2, strengthen the binding of E1 to the origin (Frattini *et al.*, 1994). E1 is responsible for recruiting the cellular DNA replication initiation machinery and mediates the formation of a stable, multimeric complex (Park *et al.*, 1994). Following the assembly of this protein complex, E2 is released from the origin and is free to fulfil its primary function as a regulator of viral transcription (Spalholz *et al.*, 1985), however, the possibility remains that the role of E2 in viral replication extends beyond the recruitment and stabilisation of E1 (Li *et al.*, 1993).

E2

The E2 protein is highly conserved among PV types and comprises three functional domains: The N-terminal 200 amino acids which function in transactivation and replication are linked by a flexible 'hinge' region to the C-terminal domain responsible for sequence-specific DNA-binding and dimerisation (McBride *et al.*, 1989). E2 binds the consensus sequence $ACCN_6GGT$ and has been shown to control early viral gene expression through E2-responsive elements located within the viral UTR, flanking the early promoter (Androphy *et al.*, 1989). E2 can therefore control expression of itself as well as the other early viral proteins and consequently modulate viral genome copy number. E2 is also required for genome maintenance and segregation and the amino terminus transactivation domain is necessary and sufficient for association of the E2 protein with mitotic chromosomes, thus ensuring efficient segregation of viral DNA to daughter nuclei (Bastien *et al.*, 2000).

E5

Transforming activity has been demonstrated for the E5 protein of BPV and some HPVs, including HPV-16 (Di Maio *et al.*, 2001). BPV E5 transforms cells by binding to and activating the platelet-derived growth factor (PDGF) β receptor in a ligand-independent fashion leading to the recruitment of cellular signalling proteins (Petti *et al.*, 1991). HPV E5 also appears to act via receptor tyrosine kinase signalling pathways and most reports have implicated the epidermal growth factor receptor (EGFR), moreover HPV-16 E5 also enhances the keratinocyte response to endothelin-1, suggesting interactions with a variety of growth factor receptors (Straight *et al.*, 1993, Venuti *et al.*, 1998). The E5 protein of both human and bovine papillomaviruses has been shown to interact with the 16kDa subunit of vacuolar H^+-ATPase (V-ATPase) and this interaction is believed to be responsible for the downregulation of gap junction intercellular communication and for the prevention of the acidification of endosomes and Golgi apparatus (Goldstein *et al.*, 1991, Conrad *et al.*, 1993). However, a recent report has suggested that the binding of E5 to V-ATPase does not affect the activity or function of this enzyme and that binding is due simply to a chaperone function of the 16kDa subunit (Ashby *et al.*, 2001). It has also been reported that HPV-16 E5 may act as a survival factor, protecting cells from apoptosis by enhancing the P13K-Akt and ERK1/2 MAPK signal pathways (Zhang *et al.*, 2002). However, the E5 open reading frame is frequently deleted in cervical carcinoma cells suggesting that the role of this hydrophobic protein may lie in the initiation of tumourigenesis (reviewed in DiMaio *et al.*, 2001). The function(s) of the E5 protein in naturally occurring infections remains poorly understood.

E6

In contrast, much more is known about the early viral proteins E6 and E7, both of which play multiple roles during HPV-infection. However, unlike E1 and E2, the activities of E6 and E7 differ depending on whether they are

expressed from low-risk or high-risk HPVs (reviewed in Howley, 1996). Both proteins are invariably retained and expressed in anogenital tumours and high-risk E6 and E7 can transform keratinocytes *in vitro*, and are now recognised as oncogenes (reviewed in Mantovani *et al.*, 2001, Munger *et al.*, 2001). High-risk HPV E6, (HR E6), can bind the cellular protein p53 and mediate the degradation of this protein through the ubiquitin-ligase pathway in conjunction with the E6-associated protein (E6-AP) (Werness *et al.*, 1990, Scheffner *et al.*, 1990). The removal of p53 in this way allows cells to bypass the G_1/S checkpoint, which normally serves as a control for DNA-damaged cells. High-risk E6 also mediates the degradation of another proapoptotic protein, Bak, a member of the Bcl-2 family (Thomas *et al.*, 1998, Thomas and Banks, 1999). Moreover, high-risk E6 has also been shown to activate host-cell telomerase (Klingelhutz *et al.*, 1996). The ability to complex with p53 is a feature of other viral oncoproteins including the SV40 T Ag and the adenovirus (Ad) E1B 55k protein, however unlike E6, these viral proteins inactivate p53, at least in part, by sequestering it into stable complexes (Sarnow *et al.,* 1982, reviewed by Howley, 1996). The E6 protein of low-risk HPVs cannot bind p53 and is unable to mediate the degradation of this cellular protein. Moreover, low-risk E6 is unable to transform keratinocytes, although it does appear to have similar transcriptional activation properties to its high-risk counterpart (Scheffner *et al.*, 1990, Sedman *et al.*, 1991). Another characteristic HR E6, not shared with the low-risk protein, is the ability to inhibit terminal differentiation of epithelial cells (reviewed in Mantovani *et al.*, 2001). Although this mechanism is not fully understood, HR E6 can interact with the calcium-binding protein ERC 55 (Chen *et al.*, 1995), the focal adhesion protein paxillin (Tong *et al.*, 1997), and the human homologue of the *Drosophila* discs large tumour suppressor protein, which is required for regulation of cell adhesion and proliferation in epithelial tissues (Kiyono *et al.*, 1997, Mantovani *et al.*, 2001). Each of these interactions could potentially interfere with epithelial differentiation.

E7

E7 was the first oncogene of high-risk HPVs to be discovered (Bedell *et al.*, 1989). In addition to functional similarities, HPV-16 E7 shares amino acid sequence similarity with portions of two other oncogenic viral proteins, adenovirus (Ad) 12S E1A product and the SV40 large T Ag (reviewed by Howley, 1996). A key observation for the function of E7 was the demonstration of its binding to the retinoblastoma protein, pRb (Dyson *et al.*, 1989), and retinoblastoma protein (Rb)-related pocket proteins, p107 and p130 (Dyson *et al.*, 1992), an observation that has also been made for Ad 12S E1A and SV40 T Ag (Dyson *et al.*, 1992, DeCaprio *et al.,* 1988, reviewed in Howley, 1996). This binding results in phosphorylation of pRb, leading to its degradation by ubiquination, and the subsequent release of transcription factors of the E2F family from the pRb complex, thereby activating transcription of genes regulating cell proliferation and permitting progression into the S phase of the cell cycle (Boyer *et al.*, 1996). Low-risk E7 binds pRb with a ten-fold lower efficiency that high-risk E7 (Gage *et al.*, 1990). Surprisingly, this reduced binding efficiency has been attributed to a single amino acid difference between high and low-risk E7 proteins (Sang *et al.*, 1992). High-risk E7 can also inactivate the cyclin-dependent kinase inhibitors $p21^{CIP-1}$ and $p27^{KIP-1}$ thus uncoupling cdk activity and cdk inhibitors, and contributing to the increased growth of HPV-infected cells (reviewed in zur Hausen, 2000). High-risk E7 proteins have also been identified in cyclin A and cyclin E complexes and this interaction may allow HPV-positive cells to bypass the G_1/S checkpoint, independent of the E6 mediated bypass of this checkpoint (Zerfass *et al.*, 1995).

Productive Infections

The amount of E6 and E7 proteins produced during a productive viral infection is very low, and in the case of E7 in particular this is a consequence of its relatively short half-life of less than 2h (Munger *et al.*, 2001). In the course of a productive HPV-infection, the replicative phase is confined to the

unfavourable environment of differentiated cells. The ability of both high and low-risk E7 to interact with pRb, albeit with reduced affinity for the latter, is thought to be important in preventing the cell cycle exit of HPV-infected cells upon differentiation, thereby permitting vegetative viral DNA amplification in suprabasal cells, a process that is tightly controlled both in low and high-risk viruses (reviewed in Howley, 1996). Moreover, the combined action of E6, E7 and possibly E5 is believed to result in a delay of differentiation leading to a lateral expansion of the infected cell in the basal layer giving rise to a clonal population, consequently resulting in numerous virus-producing cells at the surface of the developing lesion following differentiation (reviewed in zur Hausen *et al.*, 1994). Studies on high-risk HPV-31 and low-risk HPV-11 have also suggested that E6 and E7 are required for the stable maintenance of viral episomes and act to modify the cellular environment in such a way as to prevent the removal of these extrachromosomal elements, an activity that is independent of their role in immortalisation, in the case of high-risk viruses (Thomas *et al.*, 1999, 2001). Thus, both low and high-risk E6 and E7 work cooperatively during a productive infection to create a favourable environment for viral replication whilst bypassing the cellular checkpoints that would normally prevent such re-initiation of DNA replication in differentiating cells.

Non-productive Infections

In light of this, however, it is not difficult to imagine how overexpression of these cellular proteins in a non-productive infection could initiate processes that lead to immortalisation and ultimately to full transformation of the cell. Integration of high-risk viral genomes, as seen in many cervical tumours, leads to increased steady state levels of viral mRNAs transcribed from the E6 and E7 oncogenes (Jeon *et al.*, 1995b, Desaintes *et al.*, 1996). This increased mRNA stability is believed to result in part from the replacement of the viral early 3'UTR, containing an mRNA instability element, with cellular sequences (Jeon *et al.*, 1995b). The combined action of the E6 and E7

proteins of high-risk HPVs in a non-productive infection essentially result in deregulated cellular growth and genomic instability and allow the infected cells to bypass the cell cycle checkpoints, avoid apoptosis and proceed to malignancy (Fig. 1.6). It should be clear, however, that this is not the preferred outcome of an HPV infection, representing instead the most unfortunate result of a progressive and multifunctional process that occurs only on some rare occasions.

1.3.3 Late Viral Proteins

E4

The E4 ORF is located within the early coding region but is considered to encode a late protein by virtue of the differentiation-inducible promoter from which it is transcribed, see below (reviewed in Howley, 1996). A transcript containing a few codons spliced from the beginning of the E1 ORF to E4 appears to be the major RNA in HPV-11, -16 and –31-induced lesions (Nasseri *et al.*, 1987, Doorbar *et al.*, 1990, Hummel *et al.*, 1995, Ozbun *et al.*, 1997). E1^E4 has been found in cells expressing L1 in the upper layers of the epithelium both in natural lesions and athymic mouse grafts of HPV-11 and HPV-16 and its appearance coincides closely with the onset of vegetative viral amplification (Doorbar *et al.*, 1989, Crum *et al.*, 1990, Brown *et al.*, 1994, Doorbar *et al.*, 1997). E1^E4 has also been found to co-localise with cytokeratins, and in the case of human keratinocytes expressing HPV-16 E1^E4, this interaction results in the collapse of the keratin network, at least *in vitro*, suggesting a potential role for HPV-16 E1^E4 in viral egress (Doorbar *et al.*, 1991, Doorbar *et al.*, 1996). HPV-16 E1^E4 has also been implicated in the posttranscriptional control of viral gene expression as a result of its interaction with a DEAD box helicase, potentially involved in regulating mRNA stability and degradation (Doorbar *et al.*, 2000). Recently, expression of HPV-16 E1^E4 in a HPV-16 immortalised line, SiHa, appeared to arrest the cells in the G2 phase of the cell cycle. E1^E4, through its

interaction with the cytokeratin network, was found to sequester components of the mitosis promoting complex, specifically cyclin B and Cdc2, and prevent the shuttling of these factors between the nucleus and cytoplasm thereby effectively stalling the cells in G2 (Davy *et al.*, 2001). The authors of this work propose that this E1^E4 induced G2 arrest, coupled with E6/E7 mediated bypass of the G_1/S checkpoint, will create a pseudo S-phase environment conducive to viral genome amplification (Davy *et al.*, 2001) (Fig. 1.6).

<u>The capsid proteins, L1 and L2</u>

The designated late coding region accounts for approximately 40% of the viral genome and encodes two viral proteins, L1 and L2. The major capsid protein L1, has a molecular weight of approximately 57kDa, and comprises more than 95% of the capsid; this protein is highly conserved among papillomaviruses (Kaufman *et al.*, 2000). The minor capsid protein, L2, forms less than 5% of the capsid, its molecular weight is approximately 70kDa, and portions of L2, namely 200 amino acids in the N-terminus and 30 amino acids in the C-terminus are greatly conserved (Kaufman *et al.*, 2000). The icosahedral capsid of papillomaviruses is composed of 72 capsomers arranged in a T=7 symmetry (reviewed in Howley, 1996). The capsomers are pentamers of the L1 protein, and while L2 represents only a small proportion of the total viral capsid protein, its exact position in the capsid remains unknown (Baker *et al.*, 1991). However, a recent three-dimensional reconstruction of the BPV capsid suggested that L2 could be located in the centre of the capsomeres at the 12 pentavalent vertices (Trus *et al.*, 1997). The tight association between late gene expression and terminal differentiation of epithelial cells has hampered the *in vitro* study of PV late proteins. Nevertheless, a number of groups have demonstrated the formation of HPV-like particles (VLPs) by expressing either recombinant L1, or L1 and L2, using the baculovirus, vaccinia virus or yeast expression systems (Zhou, *et al.*, 1991, Kirnbauer *et al.*, 1993). These studies showed

that L1 is sufficient for the formation of VLPs, which are bound and internalised efficiently and appear morphologically and immunologically similar to mature virions. However, further studies in BPV-1 and HPV-18 indicated that L2, through its N-terminal DNA-binding domain, is required for the encapsidation of viral DNA, although there appears to be no requirement for specific DNA sequences, nor is the size of DNA critical (Roden *et al.*, 1996, Stauffer, *et al.*, 1998). VLPs have been shown to induce levels of neutralizing antibodies that are comparable to those induced by authentic virions and this, together with the ability to produce preparative amounts in non-mammalian cells, has led to the use of VLPs in several vaccine trials (Kirnbauer *et al.*, 1992, Pastrana *et al.*, 2001).
The L2 capsid protein is also reportedly targeted to distinct nuclear bodies known as promyelocytic leukaemia oncogenic domains (PODs) or ND10 domains, and has been shown to recruit L1 and E2 to these structures (Day *et al.*, 1998). Initially, it was suggested that this co-localization might enhance encapsidation by bringing together viral DNA and the capsid proteins. Recently, however, HPV-33 L2 expression has been shown to result in a complete loss of the cellular factor Sp100 from PODs and a redistribution of an additional cellular protein, Daxx, exclusively to PODs, both in cell culture and in naturally occurring lesions; potentially indicating that these L2-induced cellular changes may be involved in viral DNA encapsidation (Florin *et al.*, 2001).

1.3.4 Viral Promoters

Early promoter

Transcripts encoding the early proteins of HPV-16 are initiated from an early promoter within the E6 ORF, which has been mapped to position 97, P_{97} (Smotkin *et al.*, 1986, Doorbar *et al.*, 1990). The early promoter of a number of other genital HPVs has been mapped to a similar position e.g. P_{105} in HPV-18 and P_{99} in HPV-31 (Schneider-Gadicke *et al.*, 1986, Ozbun *et al.*,

1998a). Previous extensive transcription studies in BPV-1 identified 7 promoters, six of which appeared to be active throughout the viral life cycle and could be grouped together as early promoters (Baker *et al.*, 1987b). Recent studies on the temporal usage of promoters during the life cycle of HPV-31b identified 4 viral promoters including the major early promoter P_{99}, a novel minor early promoter P_{77}, the previously identified late promoter P_{742}, and another novel promoter P_{3320} (Ozbun *et al.*, 1998a). With the exception of P_{742}, each of the promoters appeared to be constitutively active during the viral life cycle (Ozbun *et al.*, 1998a, Ozbun *et al.*, 1999). RNA initiation sites analogous to P_{77} have been described for HPV-16 also, although they have not yet been accurately mapped (Grassmann *et al.*, 1996). Therefore, it seems likely that additional early promoters exist within the HPV-16 genome. HPV-16, like the other genital papillomaviruses, contains four E2-binding sites in conserved relative positions, flanking the early promoter, P_{97} (Romanczuk *et* al., 1990). Specific binding of E2 to one or more of these sites results in the transcriptional repression of the early viral promoter, probably by interfering with the assembly of the transcription pre-initiation complex (McBride *et al.*, 1991). The level of early mRNAs initiated at the early promoter does not appear to be affected by differentiation and so early proteins are expressed throughout the strata of the differentiating epithelium in a productive infection (Frattini *et al.*, 1994, Desaintes *et al.*, 1996, Ozbun *et al.*, 1998a). However, in many non-productive infections integration of the viral genome leads to loss of E2-mediated transcriptional repression of the early viral promoter and an overexpression of the oncogenic E6 and E7 viral proteins (Jeon *et al.*, 1995a and b).

Late promoter

The level of expression of the HPV-16 late viral proteins E1^E4, L1 and L2 increases substantially in the suprabasal layers of the epithelium and the activation of a differentiation-inducible late promoter, mapped to position 670 within the E7 ORF, P_{670}, is partly responsible for this (Grassmann *et al.*,

1996). Differentiation-inducible promoters have also been identified in BPV-1, P_{7250}, and other HPVs, including P_{742} in HPV-31 and in HPV-11 an exact nucleotide has not yet been mapped (Baker *et al.*, 1987b, Hummel *et al.*, 1992, Stubenrauch *et* al., 1999). Unlike the early promoter, this late region lacks a consensus TATA box and it has previously been shown that many genes, particularly housekeeping genes which lack TATA sequences are regulated during differentiation (Azizkhan *et al.*, 1993, reviewed in Smale, 1997). Upstream of P_{670} lies a potential binding site for the CCAAT enhancer-binding protein, (C/EBP), which promotes terminal differentiation of adipocytes and may also be important in differentiation of other tissues including skin, through the regulated activation of differentiation-specific genes (Johnson *et* al., 1989, Umek *et* al., 1991). Moreover, this CCAAT motif appears to be conserved in PVs and is present upstream of the differentiation-inducible promoter of HPV-6, -11, and –31 and BPV-1 (Baker *et* al., 1987b, Hummel *et al.*, 1992, Ozbun *et al.*, 1998a, Ai *et al.*, 1999, Zhao 1999).

The mechanisms regulating activation of the late viral promoter and consequently late viral gene transcription are not yet understood. However, in HPV-31 it appears that upon differentiation the region surrounding the late promoter within the E7 ORF becomes accessible to DNase I, indicating that chromatin remodelling in this region makes the late promoter available for transcription factor binding following differentiation, (Del Mar Pina *et al.*, 2001). Moreover, Kukimoto *et al.*, (2001) have shown that a differentiation-specific transcription factor, hSkn-1a, can displace YY1 from the late promoter region of HPV-16, thus relieving the repression of this late promoter in differentiated cells (reviewed in Desaintes *et al.*, 1996).

1.3.5 Viral Polyadenylation Signals

It is widely believed that the majority of late messages are terminated at a late polyadenylation signals within the late 3' UTR, however, some

transcripts, e.g. E4-coding transcripts, may be terminated at as yet unidentified poly(A) sites (Higgins *et al.*, 1992, Hummel *et al.*, 1992, 1995, Ozbun *et al.*, 1998a, Terhune *et al.*, 1999). Nonetheless, since these late transcripts initiate at a promoter within the early region they must read-through the early polyadenylation site in order to terminate at a poly(A) site in the late 3'UTR. This phenomenon has mostly been studied in HPV-31 and it has been determined that, while there is significant read-through both in undifferentiated and differentiated cells, read-through increases by 50% following differentiation (Terhune *et al.*, 1999). This difference has been attributed to the weakness of the HPV-31 early polyadenylation site for binding of the polyadenylation factor CstF, since insertion of a high-affinity CstF binding site into the early poly(A) signal significantly reduced read-through (Terhune *et al.*, 1999, 2001). By extension, the early polyadenylation signal of HPV-16 is also probably very weak due to the lack of a good consensus downstream GU/U-rich region. In contrast, the HPV-16 late polyadenylation region has a good consensus downstream GU/U-rich region and would therefore be predicted to bind CstF strongly and act as a strong polyadenylation signal (Seedorf *et al.*, 1985, Kennedy *et al.*, 1990); however, this has not yet been established experimentally for HPV-16. Nevertheless, the tandem arrangement of early and late polyadenylation signals, as seen in most papillomaviruses, suggests that a read-through mechanism may be conserved in this family (Baker *et al.*, 1987a, Kennedy *et al.*, 1990).

However, unlike HPV-31 or any other sequenced HPV, analysis of the prototype HPV-16 sequence indicates that there are three putative late polyadenylation signals in the 3' untranslated region of this virus: LP1 at nt7260, LP2 at nt7330, and LP3 at nt7660 (Kennedy *et al.*, 1985, Seedorf *et al.*, 1990). Yet, while each of the putative sites contains the conserved AAUAAA hexanucleotide, the downstream GU-rich element, characteristic of many 3' processing signals is weak for both LP1 and LP3, whereas downstream of LP2 the consensus GU-rich sequence, YGUGUUYY, where

Y is a pyrimidine, is present (McLauchlan *et al.*, 1985, Seedorf *et al.*, 1985). In light of this, it is unsurprising that only the LP2 polyadenylation site was found to function efficiently in basal epithelial cells, *in vitro*, and while LP1 functioned with low efficiency, LP3 did not function at all (Kennedy *et al.*, 1990). However, when the GU-rich element downstream of the LP1 site, TGTGTAAC, was altered to resemble the LP2 GU-rich sequence, TGTGTTGT, the first polyadenylation signal was found to function as efficiently as LP2, indicating that lack of utilization of LP1 and LP3 is most likely due to weak downstream GU-rich elements (Kennedy *et al.*, 1990). It remains possible though, given the small differences in the LP1 and LP2 GU-rich elements, that the virus uses this tandem arrangement of late polyadenylation signals to facilitate differential usage in specific cell types in order to control viral late protein expression. In support of this, it has recently been shown that both LP1 and LP2 are functional, *in vivo*, in the context of genuine late transcripts and are in fact utilised in a differentiation stage-specific manner, such that LP2 appears to be the site of choice in undifferentiated cells while poly(A) site usage switches to LP1 upon differentiation (McGuire *et al.*, 2002).

1.3.6. HPV-16 Splicing Patterns

In most HPV-16 positive cervical carcinomas and cell lines derived from such tumours, such as the SiHa and CaSki lines, the viral genome is integrated into the host DNA, the E1/E2 coding region is frequently disrupted and while the virus remains transcriptionally active, transcription is limited to the E6 and E7 ORFs (Smotkin *et al.*, 1986, Durst *et al.*, 1987, Meissner, 1999). Two major and one minor type of E6/E7 transcripts have been identified in cervical carcinomas, all of which are fused at their 3' ends with cellular sequences (Schwarz *et al.*, 1985, Smotkin *et al.*, 1986). This observation lends support to the proposed role of high-risk E6 and E7 proteins in the establishment and maintenance of a malignant phenotype.

Information about the HPV-16 splicing pattern in pre-cancerous lesions was limited until the derivation of the W12 line, which was established from a low-grade cervical lesion and maintains up to 100 copies of the viral genome extrachromosomally (Stanley *et al.*, 1989). Analyses of cDNAs obtained from these cells led to the identification of 6 novel mRNA species (Doorbar *et al.*, 1990) (Fig. 1.7). A number of previously uncharacterised splice donor and acceptor sites were also identified which may be involved in the generation of HPV-16 late proteins (Doorbar *et al.*, 1990). This study suggested that the episomal state of the viral DNA in the W12 cell line dictated a different programme of gene transcription than that previously observed in cervical carcinomas. Furthermore, it was suggested that alterations in the splicing patterns of specific mRNAs might be related to tumour stage and the physical state of the viral genome. Surprisingly however, while no mRNAs encoding the late proteins of HPV-16 were detected in high-grade lesions or the majority of carcinomas, the frequency and size of early transcripts did not appear to be related to tumour stage or physical state of the viral genome (Sherman *et al.*, 1992). Viral anti-sense RNAs, corresponding to sequences in the untranslated region, and the early and late coding regions, were detected in a number of cervical carcinoma samples but not in any pre-malignant lesions (Vormwald-Dogan *et al.*, 1992). This suggests that integration of the viral genome into transcriptionally active regions of the host genome, as frequently observed in invasive carcinomas, most likely results in fusion transcripts, initiated from a cellular promoter, which could potentially give rise to anti-sense viral mRNAs (Vormwald-Dogan *et al.*, 1992). It is probable that such anti-sense transcripts by binding to complementary viral transcripts could inhibit gene expression but the possible consequences of this for tumour progression remains unknown.

1.4.1 Regulation of HPV-16 late gene expression

Several groups have reported the presence of late viral transcripts in undifferentiated HPV-16 infected cells. Stoler *et al.*, (1989) noted the presence of HPV-16 L1 and L2 transcripts by *in situ* hybridisation in the nuclei of less differentiated cells in the lower layers of squamous intraepithelial lesions. HPV-16 late gene transcripts were also detected by PCR in monolayer cultures of W12 cells by Doorbar *et al.*, (1990) and again using Northern blot analysis of undifferentiated monolayer cultures of W12 cells, Koffa *et al.*, (2000) reported the nuclear localisation of HPV-16 late mRNAs, which appeared to be fully processed. Nevertheless, despite the presence of late viral transcripts in undifferentiated cells, HPV-16 late proteins are detected exclusively in differentiating keratinocytes (Sterling *et al.*, 1991, Sterling *et al.*, 1993, Doorbar *et al.*, 1997). These observations strongly suggest that viral late gene expression is regulated post-transcriptionally, possibly via a number of RNA processing mechanisms, such as mRNA splicing, polyadenylation, nuclear export, and cytoplasmic mRNA stability (Kennedy *et al.*, 1991, Dietrich-Goetz *et al.*, 1997, Koffa *et al.*, 2000, McGuire *et al.*, 2002). Control mechanisms such as these most likely depend on interactions between cellular or viral *trans*-acting factors that are responsive to cell differentiation and act on viral *cis*-acting DNA and RNA elements (Schwartz *et al.*, 1999, Koffa *et al.*, 2000). Given the small size of papillomavirus genomes, and their limited coding potential, it seems highly likely that the virus has evolved in such a way as to exploit the differential changes in the cellular environment in order to help control viral late gene expression. Positive factors that antagonise the inhibitory activity of elements within late protein mRNAs may be upregulated with differentiation; alternatively, negative factors mediating the inhibitory activity might be downregulated during differentiation (Tan *et al.*, 1995b, Koffa *et al.*, 2000).

1.4.2 RNA Processing – a summary:

In order to understand more fully the mechanisms potentially involved in the post-transcriptional control of HPV-16 late gene expression I will briefly discuss the steps involved in RNA processing. Processing of pre-mRNA transcripts involves a number of steps: pre-mRNAs must be spliced to remove introns, polyadenylated to confer stability, and then when processing is complete, the mRNAs must be efficiently exported from the nucleus to the cytoplasm for translation.

<u>Splicing</u>

Pre-mRNAs are spliced in a two-step pathway; in the first step, the pre-mRNA is cleaved at the 5' splice site, generating a linear first exon RNA species and an intron-second exon RNA species in a lariat configuration. The second step involves cleavage at the 3' splice site followed by ligation of the two exons and release of the intervening intron. Splicing occurs in a large multicomponent complex called the spliceosome; formation of a commitment complex between a pair of splice sites triggers the sequential interaction of splicing factors that collectively make up the spliceosome (reviewed in Green, 1991). Critical to this assembly process is the initial recognition of the 5' and 3' splice sites and it appears that the exon acts as the unit of recognition, hence this model is termed exon definition (Niwa *et al.*, 1992) (Fig 1. 8). U2AF^{65} initially recognises a branch site upstream of the exonic 3' splice site, leading to the recruitment of U2 small nuclear ribonucleoprotein (U2 snRNP), followed by U1snRNP (reviewed in Zhao *et al.*, 1999). U1 snRNP is primarily involved in the recognition of 5' splice sites and binding of this protein to the 3' splice site poises U1 snRNP to search across the exon in a 3' to 5' direction for a downstream 5' splice site (reviewed in Green, 1991, Zhao *et al.*, 1999). Interactions between splicing factors bound at the 5' and 3' splice sites are believed to be mediated by

serine-arginine (SR) proteins, through a bridging interaction between $U2AF^{35}$ and the U1 snRNP 70K protein subunit (reviewed in Chabot, 1996). In the case of a terminal exon, i.e. an exon beginning with a 3' splice site and ending in a polyadenylation signal, this model predicts that factors bound at the 3' splice site can interact with the polyadenylation complex, and an increasing body of evidence exists to support a direct physical linkage between the processes of splicing and polyadenylation (Niwa *et al.*, 1992, Lutz *et al.*, 1996, reviewed in Zhao *et al.*, 1999).

Cleavage and Polyadenylation

In mammalian cells, three elements define the core polyadenylation signal: The highly conserved AAUAAA hexanucleotide located 10 to 30 nts upstream of the cleavage site, a less well conserved GU/U-rich element located downstream of the cleavage site, and the cleavage site itself, which since it becomes the point of poly(A) addition, is generally referred to as the poly(A) site (reviewed in Wahle *et al.*, 1999) (Fig.1.9). Formation of 3' ends in pre-mRNAs is also a two-step reaction. The cleavage and polyadenylation specificity factor (CPSF) recognizes the AAUAAA hexanucleotide and binding of this factor is greatly enhanced by a cooperative interaction with the cleavage-stimulating factor (CstF), which binds to the downstream GU/U-rich region (reviewed in Zhao *et al.*, 1999). While it seems that CPSF is involved both in polyadenylation and cleavage, CstF and the cleavage factors I_M and II_M are involved only in cleavage; it also appears that the carboxyl- terminal domain (CTD) of RNA polymerase II (pol II) is required for cleavage and may act by stabilizing the cleavage complex through interactions with CPSF and CstF, alternatively, the CTD of RNA pol II may exert its effect through the donation of CstF or CPSF to the assembling polyadenylation complex (Hirose *et al.*, 1998, Hirose *et al.*, 2000). Following endonucleatic cleavage at the poly(A) site, adenosine residues are added to the 3' end of the fragment to form a poly(A) tail through the action of poly (A) polymerase (PAP) (reviewed in Colgan *et al.*, 1997). Rapid elongation and

control of poly(A) tail length are mediated by the poly(A)-binding protein II (PAB II) (reviewed in Colgan *et al.*, 1997). Additional less well defined sequence elements, acting in a position and orientation-dependent manner, have been shown to regulate polyadenylation efficiency in a number of viral poly(A) signals (Gilmartin *et al.*, 1995, Lutz *et al.*, 1994, Moreira *et al.*, 1998). The action of these upstream sequence elements (USE) is often dependent on the binding of RNA processing factors (Gilmartin *et al.*, 1995, Lutz *et al.*, 1996, Moreira *et al.*, 1998). A stable polyadenylation complex is the consequence of multiple, cooperative interactions between the basic cleavage and polyadenylation factors, an arrangement which affords many targets for regulation.

RNA Nuclear Export

Only after the pre-mRNA has been fully processed can it be exported from the nucleus to the cytoplasm. It has been shown that formation of a correct 3' end appears to be important for export (reviewed in Wahle *et al.*, 1999), and it is possible that the spliceosome or polyadenylation complex retains pre-mRNAs in the nucleus by preventing interactions with the nuclear pore, alternatively, binding of the pre-mRNA by the spliceosome and/or polyadenylation machinery may prevent binding of transport factors to specific export signals on pre-mRNAs (Green, 1991). Several reports have suggested that RNA binding proteins, collectively known as heterogenous ribonucleoproteins (hnRNPs), are actually deposited onto pre-mRNAs during 3' end formation. Many hnRNPs have been shown to actively shuttle between the nucleus and cytoplasm, suggesting that RNA transcripts are passively exported through the binding of these shuttling proteins (Zhao *et al.*, 1999). Binding of shuttling proteins which facilitate the efficient nuclear export of mRNAs are believed to be important in overcoming the negative effects of destabilising elements found throughout many mRNAs (reviewed in Ross, 1995).

1.4.3 Negative Regulatory Elements within the Viral Untranslated Region

A *cis*-acting negative regulatory element (NRE) has been identified in the late 3' UTR of HPV-16, upstream of the LP2 signal; this element can repress reporter gene expression following transient transfection in HeLa cells, used as a model for basal epithelial cells (Kennedy *et al.*, 1990). Similar inhibitory sequences have also been identified in the late 3' UTRs of BPV-1, HPV-1, and HPV-31 and it has been proposed that these elements, like the HPV-16 NRE, may regulate HPV expression in a differentiation dependent manner, such that the inhibitory activity is alleviated as the infected cells undergo terminal differentiation (Furth *et al.*, 1991, Tan *et al.*, 1995b, Cumming *et al.*, 2002a). However, while these inhibitory elements share some degree of sequence homology, reports to date suggest that these regions act via distinctly different mechanisms (Kennedy *et al.*, 1991, Furth *et al.*, 1994, Sokolowski *et al.*, 1997).

BPV-1

A 53nt sequence located immediately upstream of the poly(A) signal in the BPV-1 late 3'UTR acts in an orientation-dependent manner to reduce the levels of cytoplasmic, polyadenylated mRNAs (Furth *et al.*, 1991). The inhibitory activity of this element is determined by a 9nt sequence, AAGGUAAGU, with perfect homology to a consensus 5' splice site. This sequence binds U1 small nuclear riboprotein (U1snRNP), effectively uncoupling the processes of splicing and polyadenylation as a result of the direct interaction of the 70kDa subunit of U1snRNP with poly(A) polymerase, (PAP), thus reducing the 3' end processing efficiency of late mRNA transcripts (Furth *et al.*, 1994, Gunderson *et al.*, 1998).

HPV-1

A negative regulatory RNA element located within the HPV-1 late 3' UTR acts in an orientation- dependent manner to reduce late mRNA levels in the nucleus and cytoplasm (Tan *et al.*, 1995a, Zhao *et al.*, 1996, Sokolowski *et al.*, 1997). This 57nt element contains 97% A+U residues, and can be divided into two parts: the 5' half contains two AUUUA motifs and the 3' half contains three UUUUU motifs, characteristic of mRNA instability elements (Sokolowski *et al.*, 1997). AU-rich RNA elements (AREs) in mRNAs generally contain AUUUA pentamers in an AU-rich environment and target these messages for rapid degradation, probably as a result of their interaction with cellular factors (Ross, 1995). The HPV-1 element can bind a number of cellular proteins including hnRNP C1 and C2 and the shuttling protein, HuR (Sokolowski *et al.*, 1997, Sokolowski *et al.*, 1999). It has been proposed that HPV-1 late messages are retained in the nucleus and prematurely degraded in undifferentiated cells as a consequence of hnRNP C1 and C2 binding; a differential increase in the expression of HuR, however, overcomes this negative effect by stabilising late messages and mediating their export to the cytoplasm (Schwartz *et al.*, 1999).

HPV-31

Recently an element within the late 3' UTR of HPV-31 has been identified that inhibits late gene expression in undifferentiated epithelial cells (Cumming *et al.*, 2002a). Unlike the negative elements described previously in HPV-1, -16 and BPV-1 this element is surprisingly complex, comprising a major 130nt inhibitory element upstream of the late polyadenylation signal and a novel minor element of 110nt downstream of the poly(A) site (Cumming *et al.*, 2002a). A portion of the sequence of the major element closely resembles the HPV-16 NRE and not surprisingly, both elements bind the same cellular proteins *in vitro*, including $U2AF^{65}$, CstF-64 and HuR (Koffa *et al.*, 2000, Cumming *et al.*, 2002a). While the minor element also binds a number of cellular proteins these differ to those bound by the major element.

It has been proposed that these elements act together, possibly via a number of post-transcriptional mechanisms, to disrupt polyadenylation at the late site in undifferentiated cells (Cumming *et al.*, 2002a).

1.4.4 Mapping of the HPV-16 NRE

The HPV-16 NRE was mapped by cloning fragments of the 3' UTR downstream of a chloramphenicol acetyltransferase, (CAT), reporter gene under the control of the HSV 1 immediate early reporter in a plasmid lacking polyadenylation signals called pLW1 (Kennedy *et al.*, 1990). Mutational analysis initially mapped this inhibitory region to within nts 7008 and 7226 of the HPV-16 UTR and showed that, while it had a negative effect on the level of expression of CAT mRNA, it did not appear to affect 3' end processing, at least *in vitro* (Kennedy *et al.*, 1990) (Fig 1.10). Further mutation studies mapped this negative regulatory element (NRE) specifically from nts 7128 to 7206, with the 5' end lying just upstream of the L1 stop codon and the 3' end extending into the late 3' UTR (Kennedy *et al.*, 1991). This *cis*-acting 79nt RNA element can be divided into two portions: The 5' half portion, nts 7128 to 7176, comprises a putative stem loop structure and four weak 5' splice sites. The 3' portion, nts 7177 to 7206, contains a GU-rich region (Kennedy *et al.*, 1991) (Fig. 1.10, Fig.4.1B). However, additional studies carried out in another laboratory mapped the HPV-16 NRE to the first 51nts of the NRE (nt 7128-7178) (Furth *et al.*, 1994). Functional tests of this element were carried out in the context of the SV40 early polyadenylation site, and in this context, the second of the four weak 5' splice sites appeared to be important for the inhibitory activity of this element (Furth *et al.*, 1994). However, this shorter element lacks most of the GU-rich region, which has now been shown to have independent inhibitory activity (Cumming *et al.*, 2002b). Moreover, when the NRE is functionally tested in the context of its own late polyadenylation region it appears that the four 5' splice sites actually act

cooperatively to mediate the inhibitory activity of the 5' portion of this negative element (Cumming *et al.*, 2002b).

1.4.5 The HPV-16 NRE acts as a general regulator of polyadenylation

Work published in the early 1990's reported that the inhibitory effect of the NRE was neither species, tissue nor cell-type specific and suggested that this element may act to destabilise polyadenylated RNA, *in vitro* at least (Kennedy *et al.*, 1991). However, *in vivo* studies have so far failed to support this observation and it now appears that the NRE may in fact act as a general regulator of polyadenylation (McGuire *et al.*, 2002). A recent report has shown that the NRE can act as an upstream efficiency element (USE) to control polyadenylation site usage in a position and orientation-dependent manner, both in the context of the HPV-16 late 3'UTR and also in a heterologous poly(A) competition assay utilising a tandem arrangement of weak and strong poly(A) sites, similar to the arrangement of LP1 and LP2 in HPV-16 (McGuire *et al.*, 2002). Furthermore, it appears that, unlike the situation *in vitro*, both the LP1 and LP2 poly(A) sites are functional *in vivo*, and that a large proportion of the late transcripts induced upon differentiation are polyadenylated at the LP1 site (McGuire *et al.*, 2002). This observation would appear to support the hypothesis that differential usage of the tandem late polyadenylation sites in HPV-16 helps to control the expression of late viral proteins, with the NRE acting as a possible differential regulator of poly(A) site choice (McGuire *et al.*, 2002).

Moreover, it seems that the NRE can further influence polyadenylation by acting as DNA element and pausing transcription (McGuire *et al.*, 2002). Transcription pause sites are generally located downstream of the polyadenylation signal and it is conceivable that since the NRE lies upstream of the late poly(A) signals that stalling transcription at this point in the exon would result in the rapid degradation of late messages due to the lack of a suitable upstream polyadenylation site (Yonaha *et al.*, 1999, McGuire *et al.*,

2002). This would be an extremely efficient mechanism of preventing late protein expression in undifferentiated cells. This type of inhibition could be overcome with differentiation as a result of the increased rate of transcription through the late region leading to efficient 3' end processing.

1.4.6 Protein binding to the NRE

The 79nt NRE has also been shown to bind a range of cellular proteins, three of which have been identified as proteins specifically involved in RNA processing: The auxillary splicing factor $U2AF^{65}$, the 64kDa subunit of the cleavage stimulation factor, CstF-64, and the *elav*-like shuttling protein HuR (Dietrich-Goetz *et al.*, 1997, Koffa *et al.*, 2000). Furthermore, the level of expression of these cellular factors appears to be differentially regulated such that $U2AF^{65}$ levels decrease upon differentiation, CstF-64 expression is upregulated with differentiation, and HuR undergoes a differential redistribution from the nucleus to the cytoplasm (Koffa *et al.*, 2000).

The presence of weak 5' splice sites in the 5' portion of the HPV-16 NRE together with the ability of this element to interact with $U2AF^{65}$ has lead to analogies being drawn between the HPV-16 NRE and the negative element in the late 3'UTR of BPV-1 (Furth *et al.*, 1994, Dietrich-Goetz *et al.*, 1997). However, it appears that, unlike BPV-1, the four weak 5' splice sites in the HPV-16 NRE act together to exert a negative effect (Cumming *et al.*, 2002b). Binding of U1snRNP to the NRE has not been reported, although our data strongly suggests that U1 snRNP-associated proteins do bind to the NRE (Chapter 5). However, it remains to be determined if the HPV-16 NRE regulates 3' end processing via a mechanism similar to that used by BPV-1. Alternatively, the NRE may fulfil a function similar to that of the upstream efficiency element (USE) in the late polyadenylation region of SV40 (Lutz *et al.*, 1994). Binding of the U1A subunit of U1 snRNP to this USE actually serves to enhance polyadenylation of late messages by coupling

polyadenylation and splicing through the direct interaction of U1A with the 160kDa subunit of the cleavage-polyadenylation specificity factor (CPSF) (Lutz *et al.*, 1996).

The polyadenylation protein CstF-64 normally binds to the GU/U-rich region downstream of a polyadenylation signal, stabilising formation of the polyadenylation and cleavage complex (Takagaki *et al.*, 1990, Takagaki *et al.*, 1997). The GU-rich 3' region of the NRE may compete for CstF binding with the downstream GU/U-rich region, thereby inhibiting efficient polyadenylation of late messages in undifferentiated cells. Differential changes in the level of expression of CstF-64 could promote binding of this cleavage factor to the stronger GU/U binding site, downstream of the poly(A) sites, and improve the efficiency of late mRNA polyadenylation upon differentiation (Terhune *et al.*, 1999). As mentioned previously, the HPV-16 NRE acts as an USE to regulate differential late poly(A) site usage (McGuire *et al.*, 2002). In this scenario, CstF-64 binding to the NRE in differentiated cells could promote use of the NRE-proximal LP1 site, similar to the situation in the C2 complement polyadenylation region where binding of CstF-64 to a GU-rich region upstream of the poly(A) signal stimulates cleavage and polyadenylation at this site (Moreira *et al.*, 1998).

Unlike the negative element of HPV-1, the HPV-16 NRE lacks the AUUUA tandem repeat characteristic of AU-rich elements, however, it does contain a GUUUG tandem repeat, which may be sufficient for HuR-binding (Seedorf *et al.*, 1985, Koffa *et al.*, 2000). HuR binding to the HPV-16 NRE might promote nuclear export of late mRNAs and improve the cytoplasmic stability of these late messages, similar to the situation in HPV-1 (Sokolowski *et al.*, 1999).

1.4.7 Inhibitory Elements within the Viral Coding Region

While the inhibitory elements described above in BPV-1, HPV-1, -16 and -31 are located within the non-coding region of the virus, *cis*-acting negative elements have also been identified within the HPV-16 L1 and L2 coding regions as well as the L2 coding region of HPV-31 (Tan *et al.*, 1995a, Sokolowski *et al.*, 1998, Terhune *et al.*, 2001, Collier *et al.*, 2002). At present, negative elements within late coding sequences have been identified only in these two high-risk viruses, both of which produce small numbers of mature virions in productive infections, leading to the suggestion that intragenic negative elements determine the levels of virus produced from specific HPV types (Schwartz *et al.*, 1999).

<u>HPV-16 L1 and L2</u>

The HPV-16 L1 element is located within the 5' end of the ORF (nt 5813-6150) and acts in an orientation dependent manner to post-transcriptionally inhibit L1 expression (Tan *et al.*, 1995a, Collier *et al.*, 2002). The inhibitory region in the HPV-16 L1 coding sequence contains seven AUUUA pentanucleotides, which are a general feature of mRNA instability elements and have been identified in several cellular mRNAs also under post-transcriptional regulation, including c-fos and c-myc (Chen *et al.*, 1995, Ross, 1995). The HPV-16 L2 coding region also contains strong negative elements that act in an orientation-dependent manner to reduce the levels of L2 encoding mRNAs in the nucleus and the cytoplasm (Sokolowski *et al.*, 1998). A region within the 5' half of the L2 ORF appears to act in *cis* to reduce mRNA half life, however, while this region does not contain any AUUUA or UUUUU motifs it does have a 60% A+U content. A weaker element has also been identified in the 3' end of the L2 coding sequence and may affect the efficiency of utilisation of L2 mRNAs. This element has been shown to bind a number of cellular proteins, including the polyC binding proteins 1 and 2 (PCBP-1 and –2) and hnRNP K (Collier *et al.*, 1998).

Interestingly, the 5' element of HPV-16 L1 also specifically binds these factors, suggesting that both late proteins are subject to the same control mechanisms; however, the role of these cellular proteins in the virus life cycle remains to be investigated (Collier *et al.*, 1998).

HPV-31 L2

The negative element of HPV-31 L2 is located within the first 800nt of the ORF and may function either as a single large element or cooperatively through multiple redundant elements (Terhune *et al.*, 2001). Disruption of this region in the context of the whole viral genome results in a significant reduction in genome replication and loss of stable maintenance in primary foreskin keratinocytes (Terhune *et al.*, 2001). These observations tend to suggest that the mechanism of action of the HPV-31 L2 negative element differs to that of the HPV-16 L1 and L2 elements, which appear to act primarily at the level of mRNA instability.

The regulation of viral late-gene expression caused by negative regulatory elements within protein coding regions appears to be a common strategy used by many viruses. For example, human immunodeficiency virus type 1 (HIV-1) contains inhibitory sequences in the Gag, Pol and Env coding regions (Cochrane *et al.*, 1991, Maldarelli *et al.*, 1991, Schwartz *et al.*, 1992), and human T-cell leukaemia virus type 1 (HTLV-1) contains inhibitory sequences in the 5' UTR and in the Env and Pol coding regions (Seiki *et al.*, 1990, Saiga *et al.*, 1997). Hepatitis B virus also contains a *cis*-acting RNA element shown to regulate the expression of surface gene transcripts (Huang *et al.*, 1994). The presence of inhibitory sequences on mRNAs encoding structural proteins may allow tight regulation of virus production, which probably contributes to the ability of HIV-1, HTLV-1 hepatitis B and papillomaviruses to persist in their hosts even in the presence of specific immune responses against the infection.

1.4.8 *In Vitro* Study of Papillomaviruses

History

Until relatively recently it has been virtually impossible to study the life-cycle of PVs outwith the natural host, due to the lack of a reproducible cell culture system permissive for vegetative viral replication. A number of characteristic features of papillomavirus biology have hampered the development of such an *in vitro* system: Given the tight association between keratinocyte differentiation and expression of the late viral proteins, the biggest hurdle has been finding a system that can faithfully mimic the epithelial differentiation programme as seen *in vivo.* Furthermore, human papillomaviruses have a specific tropism for epithelial cells, and a predilection for certain anatomical sites, thus precluding the use of animal models for HPV-infection.

However, since the mid-1980s xenografting in the athymic mouse system has been successfully used to recreate HPV infections outwith the natural host. Kreider *et al.*, (1985) first grafted normal cervical tissue infected with HPV-11, i.e. condyloma acuminata-derived material, onto the flanks of athymic mice and showed that the resulting lesions displayed morphological transformation similar to that of low-grade cervical lesions. Furthermore, when neonatal foreskin epithelium, infected with HPV-11 DNA extracted from human vulvar condyloma acuminata, was grafted under the renal capsule of athymic mice and organotypic raft cultures, the xenografts and rafts were able to support the vegetative replication of papillomaviruses, and the virions produced were infectious (Kreider *et al.*, 1986, Dollard *et al.*, 1992). In the case of the former, this was the first time a HPV type had been produced in the laboratory.

Following this, focus shifted to the derivation of HPV-positive cell lines from low-grade cervical lesions, in an attempt to recreate a productive HPV-infection in an *in vitro* tissue culture system. Eventually after a number of failed attempts in which the viral genome consistently became integrated, a

HPV-16 line which maintained episomal copies of the viral genome was reported and was called W12 (Stanley *et al.*, 1989). Grafting of this line onto granuloma beds in the flanks of nude mice resulted, with time, in the formation of a stratified epithelium that displayed morphological characteristics of low-grade lesions (Sterling *et al.*, 1990). In the upper layers of the structure, viral DNA amplification was detected together with capsid protein expression and formation of mature virions (Sterling *et al.*, 1991, Sterling *et al.*, 1993). However, for reasons that remain unknown, the mature virions shed from the epithelium were not infectious.

HPV-positive keratinocyte cell lines are also amenable to study in monolayer culture. Rheinwald and Green first reported in 1975 that a feeder layer of irradiated mouse 3T3 fibroblast cells supported the clonal growth of keratinocytes but not other cell types (Rheinwald & Green, 1975). 3T3 cells treated in this way are no longer able to replicate but continue to secrete soluble factors such as insulin-like growth factor I (IGF-1), human growth factor (HGF) or keratinocyte growth factor (KGF), which sustain keratinocyte proliferation (Taichman, *et al.*, 1986, Parkinson, *et al.*, 1992, Meyers *et al.*, 1994a). This system is very useful for the clonal growth and serial cultivation of epithelial cell lines, however, while the feeder support system does allow limited stratification, keratinocytes grown using this technique do not undergo complete differentiation, therefore this system cannot support the complete life cycle of HPV-infected cell lines.

Calcium

Keratinocytes can be induced to differentiate in the absence of a fibroblastic feeder layer. One factor that regulates many aspects of epidermal differentiation, including desmosome formation, stratification and transglutamine activation, is extracellular calcium. In medium with a calcium concentration below 0.1mM, keratinocytes maintain a basal cell phenotype, while an increase in extracellular calcium to 1.2mM leads to differentiation;

cells stratify within 1-2 days and terminally differentiate with cell sloughing by 4 days (Hennings *et al.*, 1980).

Suspension in Semi-Solid Medium

In 1977 Green *et al.*, first demonstrated that suspension of keratinocytes in semisolid medium resulted in their differentiation (Green *et al.*, 1977). Suspension of HPV-positive keratinocytes in semisolid medium, such as methylcellulose, is a simplified differentiation method, which allows for the analysis of differentiation-dependent activities as a function of time. A recent study reported that, following 24 h in methylcellulose, approximately 25% of HPV-31-positive cells expressed high levels of the late viral protein E1^E4 and appeared to have amplified levels of viral DNA. However, synthesis of the viral capsid protein L1 could not be detected in these cells, suggesting that this method does not induce faithful terminal differentiation (Ruesch *et al.*, 1998a and b). Nevertheless, suspension of HPV-16 positive cells in methylcellulose has been used to show that the mechanism of viral DNA replication switches upon differentiation (Flores *et al.*, 1997), and this method continues to be used extensively, particularly in studies addressing differential changes in HPV-promoter usage and polyadenylation (Terhune *et al.*, 1999, Terhune *et al.*, 2001).

Organotypic Raft Culture

It appears that, *in vitro*, the complete keratinocyte differentiation programme can best be obtained by culturing keratinocytes on organotypic raft cultures (Fig. 1. 11). This system has been proven to accurately mimic the *in vivo* physiology of the epidermis. Epithelial cells are placed on top of a dermal equivalent, grown to sub-confluence, and raised to an air-liquid interface for growth and differentiation (Meyers *et al.*, 1994a). The dermal equivalent used most widely now for the cultivation of HPV-infected lines was first described by Bell *et al.*, in 1979 and further modified by Asselineau and Prunieras in 1984 (Bell *et al.*, 1979, Asselineau *et al.*, 1984, Asselineau *et*

al., 1986). In this system, living mesenchymal cells, e.g. murine 3T3 cells, are incorporated into a collagen matrix, which solidifies to form the dermal equivalent. Epithelial cells are plated on top of the collagen-fibroblast matrix, allowed to attach, and grown to confluence while remaining submerged. Air exposure is generally accomplished by placing the matrix on metal grids so that the medium feeds the confluent culture with nutrients and growth factors through the collagen and ventral surface of the epithelium. The rafts are incubated in this way usually for 2-3 weeks during which time the epidermal cells stratify and differentiate (Bell *et al.*, 1979, Asselineau *et al.*, 1984, 1986, Meyers*et al.*, 1994a).

This system has been used to successfully propagate a number of HPV-positive cell lines. The CIN 612 9E line was derived from a low-grade cervical lesion and maintains episomal copies of the HPV-31 genome (Bedell*et al.*, 1991). When this cell line was applied to the organotypic raft culture system it stratified and differentiated efficiently, resulting in a lesion similar to the one from which the line was originally derived (Bedell*et al.*, 1991). However, in agreement with previous reports, the differentiation of the CIN 612 reconstructed epithelium appeared incomplete and this was believed to account for the low level of detection of viral capsid transcripts and the lack of mature virion production in these cultures (Asselineau *et al.*, 1986, Bedell*et al.*, 1991). Treatment of CIN 612 raft cultures with an activator of the protein kinase C (PKC) pathway, 12-0-tetradecanoylphorbol-13-acetate (TPA) resulted in a more complete differentiation programme as indicated by an increase in expression of the differentiation markers keratin 10 and filaggrin (Meyers*et al.*, 1992). Moreover, late viral capsid proteins were detected in the suprabasal layers of TPA-treated CIN 612 rafts and mature, infectious HPV-31 virions were produced (Meyers*et al.*, 1992, Meyers *et al.*, 1994). This was the first demonstration of the production of mature virions from an *in vitro* system using a biopsy-derived continuous cell line.

Recent modifications of the organotypic raft culture system have involved the use of epithelial lines containing cloned viral DNA. Cloned, recircularlised HPV-11, -18, and –31 DNA was co-transfected with a neomycin resistance plasmid (pSV$_2$neo) into monolayers of normal human foreskin keratinocytes (HFKs) (Frattini*et al.*, 1996, Frattini *et al.*, 1997, Meyers*et al.*, 1997, Thomas*et al.*, 2001). In each case, clones that maintained episomal copies of the viral DNA were expanded, seeded onto organotypic rafts, and allowed to differentiate at the air-liquid interface. For the HPV-18 and –31 lines mature virions were produced, while in the HPV-11 line, late viral protein expression and viral DNA amplification was detected in the suprabasal layers and it should be noted that phorbol ester treatment was not required (Frattini *et al.*, 1996, Meyers *et al.*, 1997, Thomas *et al.*, 2001). The establishment of epithelial lines containing cloned viral DNA permits genetic manipulation of the viral genome and allows more complete genetic and biochemical studies of HPV than previously afforded by biopsy-derived cell lines. However, working with HFKs in cell culture can be problematic due to their limited lifespan, which can make it difficult to select for stable transfectants before they senesce. Therefore, the recent identification of a spontaneously immortalised HFK cell line called BC-1-Ep/SL may prove to be more useful in these types of studies (Flores *et al.*, 1999). BC-1-Ep/SL cells are primarily diploid but contain a duplication in the small arm of chromosome 8. Nevertheless, they exhibit many characteristics of normal HFKs and differentiate well on the organotypic raft culture system (Flores *et* al., 1999, Allen-Hoffmann *et* al., 2000). These cells are capable of maintaining episomal copies of HPV-16 and –31 DNA following stable transfection and can support the productive viral life cycle when seeded onto raft cultures (Flores*et al.*, 1999, Allen-Hoffmann *et al.*, 2000).

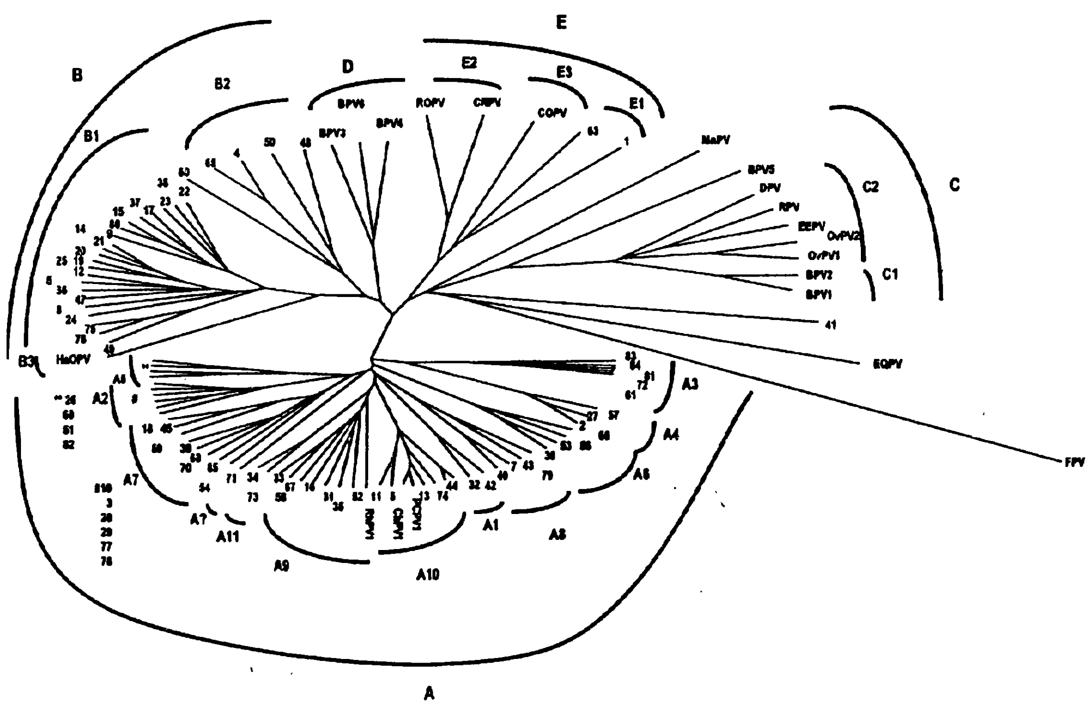
1.5 Aims and Objectives

The main objective of this work was to understand the molecular mechanisms regulating the tight linkage of viral late gene expression and epithelial differentiation.

The biopsy-derived HPV-16 positive W12 cell line contains episomal copies of the HPV-16 genome and is considered to be operationally equivalent to a basal epithelial cell latently infected with HPV-16, this line can also be induced to differentiate. The W12 cell line therefore represents a good model system in which to study the expression of HPV-16 late proteins in response to epithelial differentiation, however, this line has remained relatively uncharacterised. The first aim of this project, therefore, was to characterise the growth of HPV-16 infected epithelial cell lines both in monolayer culture and on the organotypic raft culture system.

The negative regulatory element (NRE) of HPV-16 is believed to play a central role in the post-transcriptional control of HPV-16 late gene expression. Therefore, the second aim of this project was to attempt to elucidate the mechanisms through which the NRE exerts its inhibitory effect in undifferentiated cells, and conversely, establish how the virus overcomes NRE-mediated inhibition of late gene expression upon differentiation. In order to do this, saturating site-directed mutagenesis of the NRE was carried out. Each NRE mutant was tested both for its ability to bind nuclear proteins and also its ability to repress reporter gene expression, compared to a wildtype control. Furthermore, I attempted to identify cellular factors that interact with this negative element and ascertain the effects of epithelial differentiation, both in the presence and absence of HPV-16 infection, on the level of expression of these NRE-bound proteins.

Fig. 1.1. Phylogenetic tree containing sequences of 103 papillomaviruses. Adapted from de Villiers, 2001



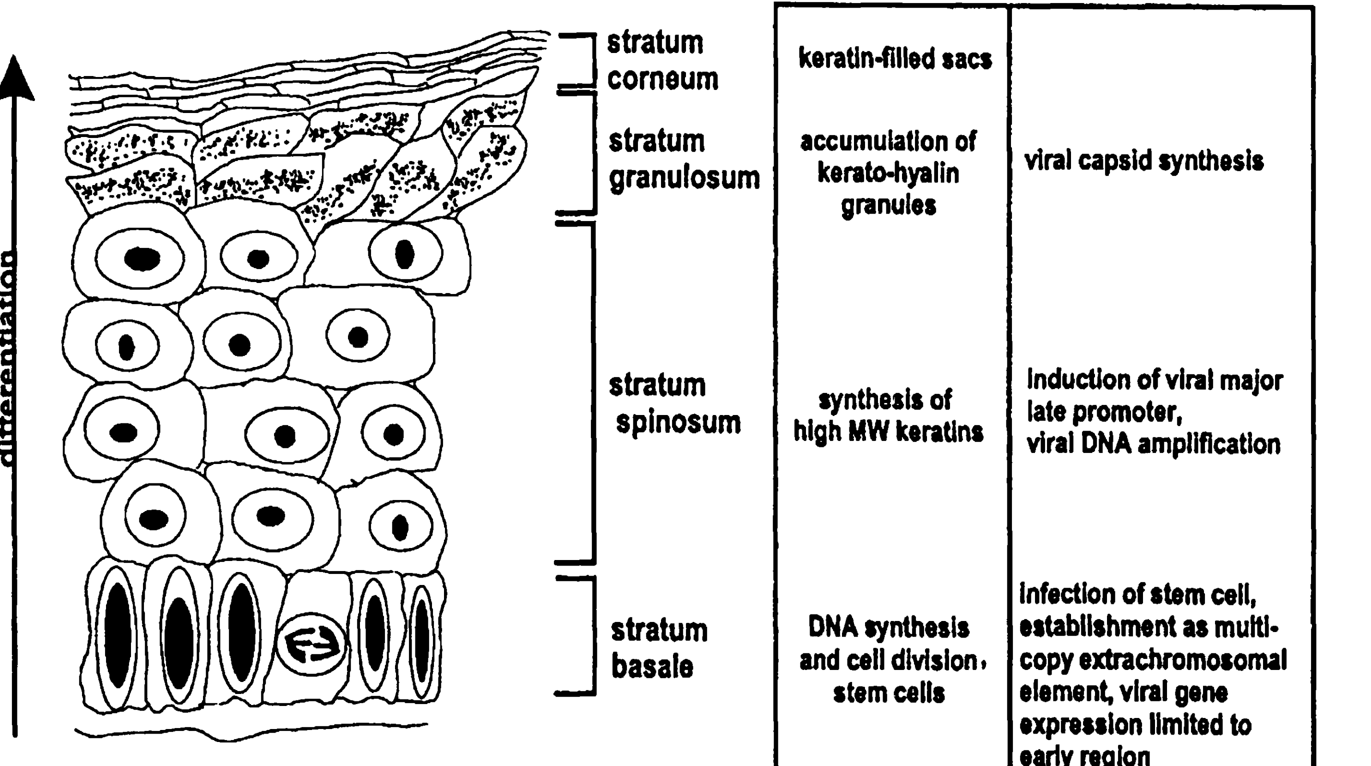
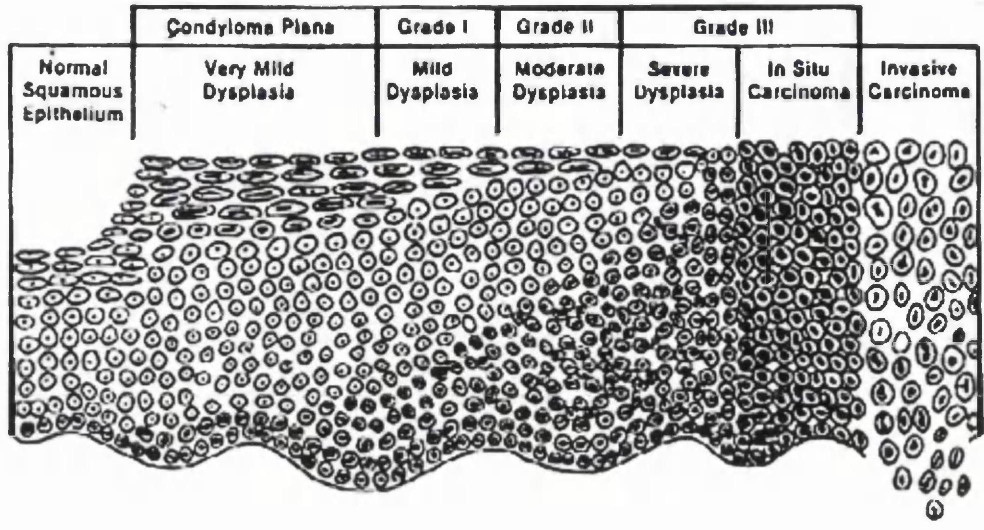


Fig. 1. 2. Epithelial differentiation. Schematic diagram showing the differentiation-dependent changes in normal and HPV-infected epithelial cells. Adapted from Stubenrauch *et al.*, 1996.

Fig. 1.3. Progression to dysplasia. The histological changes that occur during the develop of invasive carcinoma. Adapted from Bennett Jenson *et al.*, 1990.



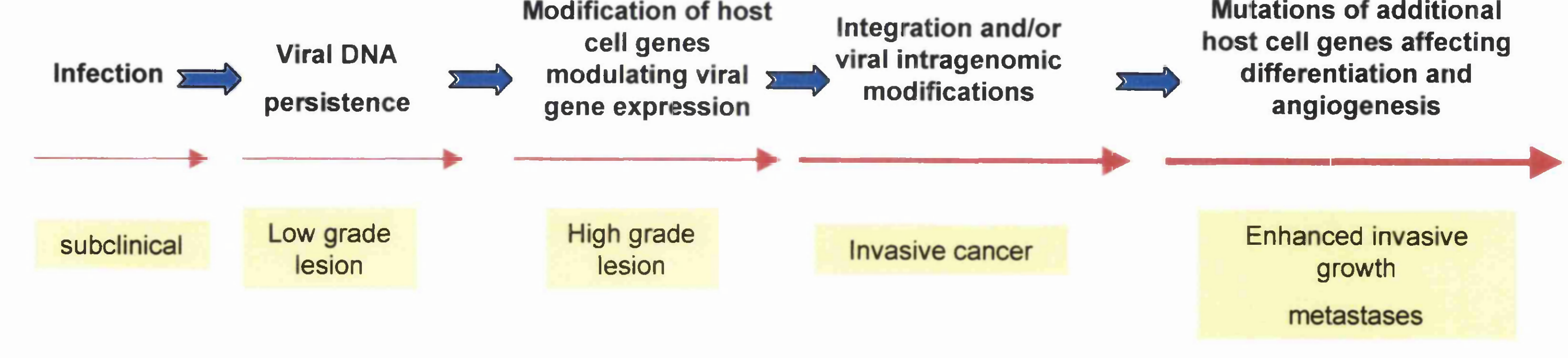


Fig. 1.4. Malignant progression. Tentative scheme of papillomavirus-induced pathogenesis resulting in cervical cancer. Adapted from zur Hausen, 1999.

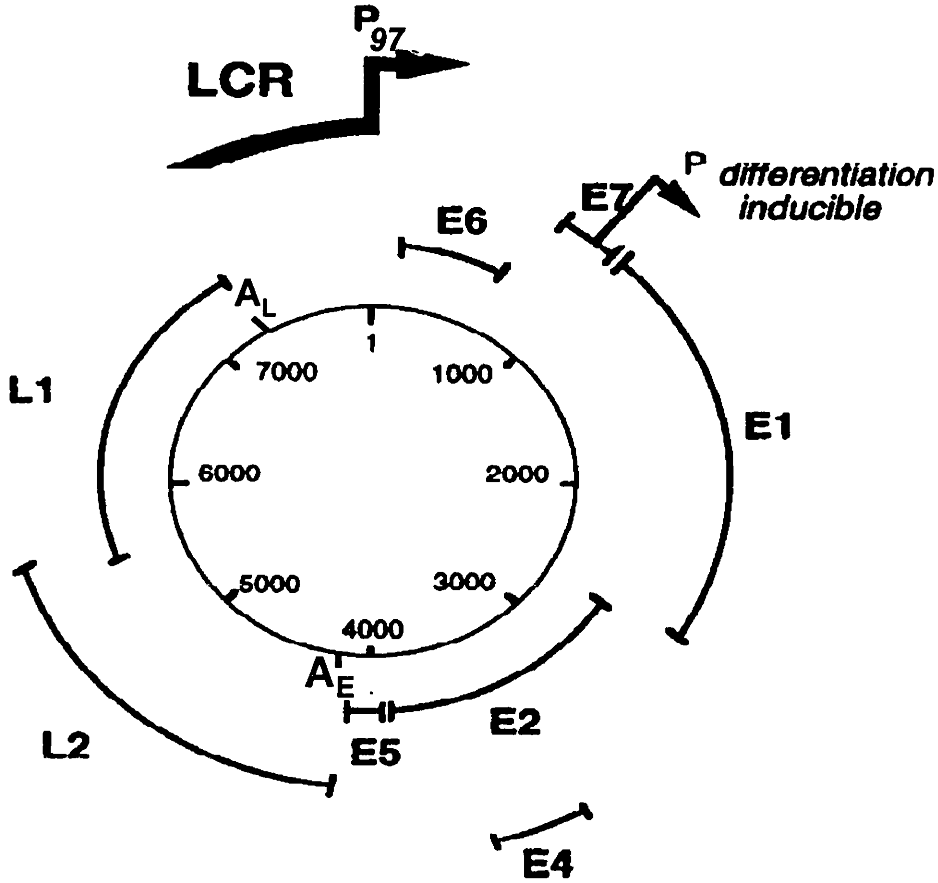


Fig. 1.5. Genomic map of HPV-16. The individual ORFs of the early (E) and late (L) regions are shown as areas outside the double-stranded circular genome. LCR designates the long control region. The early promoter, P_{97}, and late promoter, $P_{\text{differentiation inducible}}$, are indicated. A_E and A_L denotes the early and late polyadenylation sites, respectively. Adapted from Desaintes *et al.*, 1996.

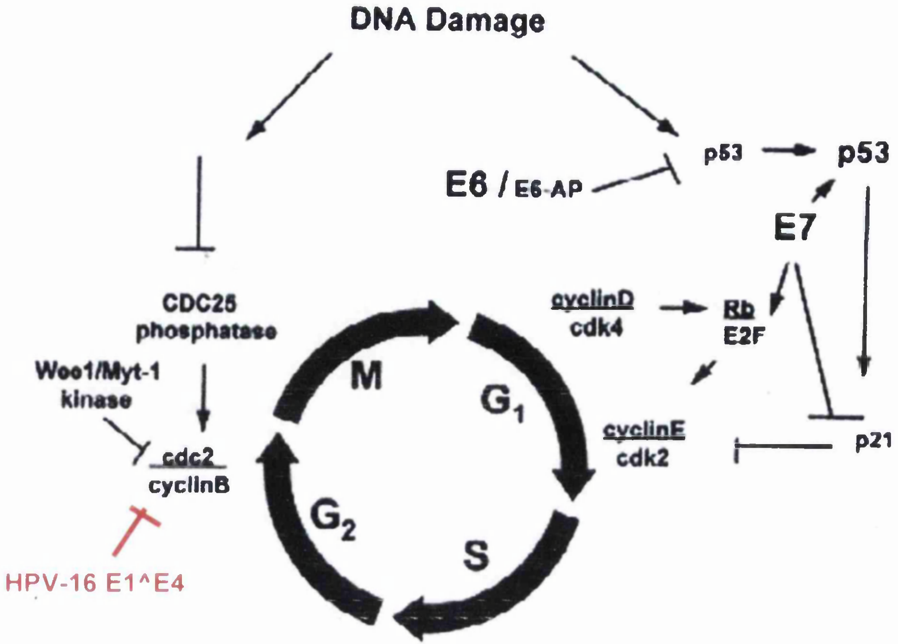


Fig. 1.6. HPV oncogenes and the cell cycle. In normal cells DNA damage induces cell cycle arrest in G1 and G2. In HPV-infected cells, E6 bypasses G1 arrest by degrading p53 and also gradually attenuates the G2 checkpoint. Rb is inactivated by E7 which also inhibits the cdk inhibitor, p21, and bypasses the G1 checkpoint. Also shown is the putative inhibition of cyclin B by HPV-16 E1^E4, resulting in G2 arrest. Adapted from Galloway and McDougall, 1996.

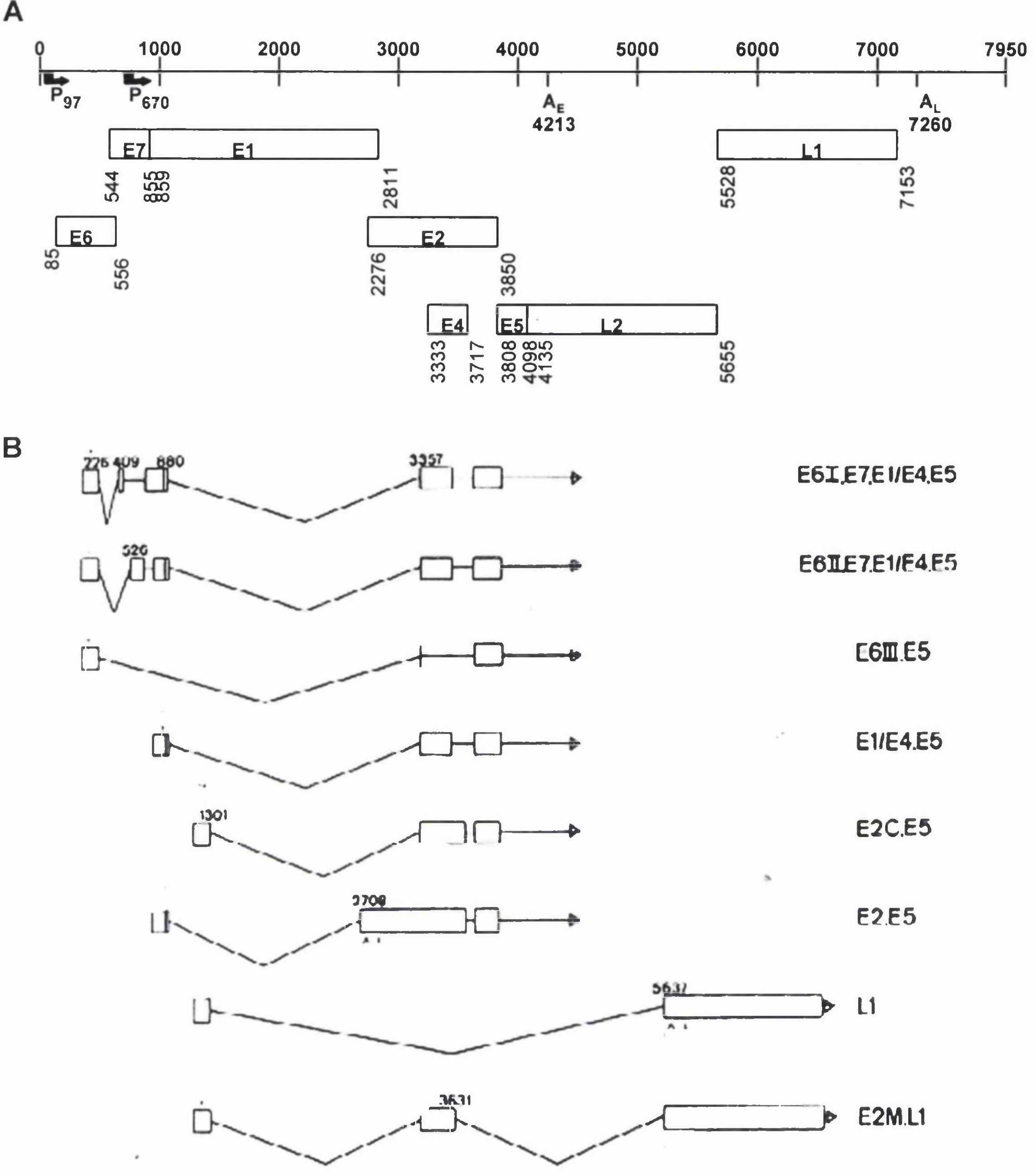


Fig.1. 7. HPV-16 mRNA species and coding potentials. A. Linear diagram of the HPV-16 genome and positions of open reading frames. The nucleotide positions of the 5' and 3' ends of each cDNA clone are indicated. Also shown are the positions of the early, P_{97}, and late, P_{670}, promoters and early, A_E, and late, A_L, polyadenylation sites. **B.** RNA species isolated from HPV-16 cervical carcinoma and the W12 cell line (Smotkin *et al.*, 1989, Doorbar *et al.*, 1990). Open boxes represent potential coding regions; coding capacity of each species is indicated on the right. Positions of common splice sites are shown. Adapted from Sherman *et al.*, 1992, Vormwald-Dogan *et al.*, 1992.

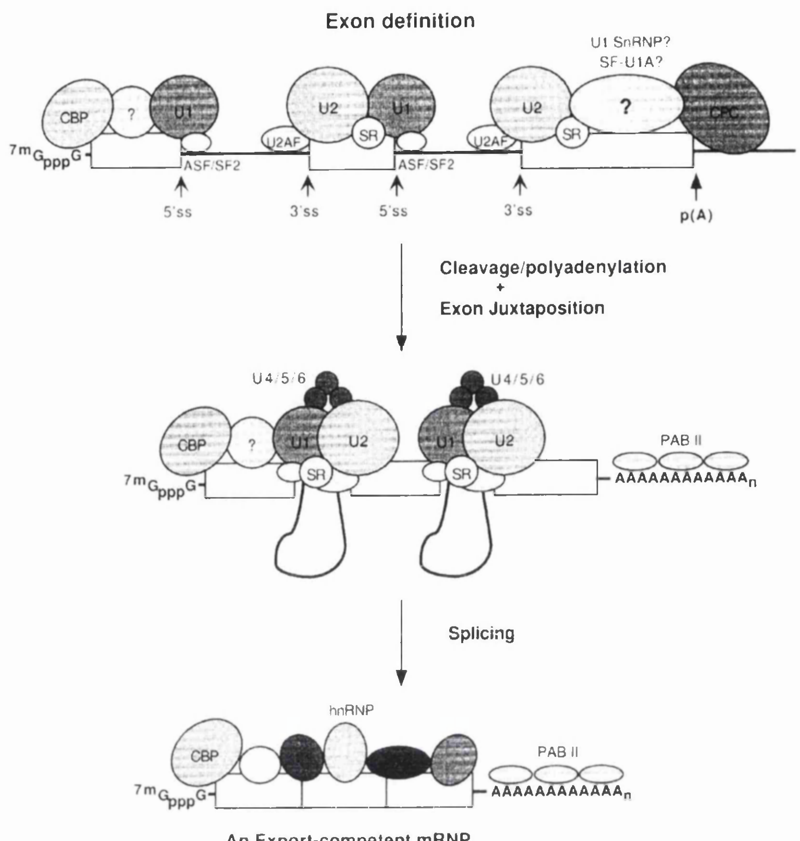
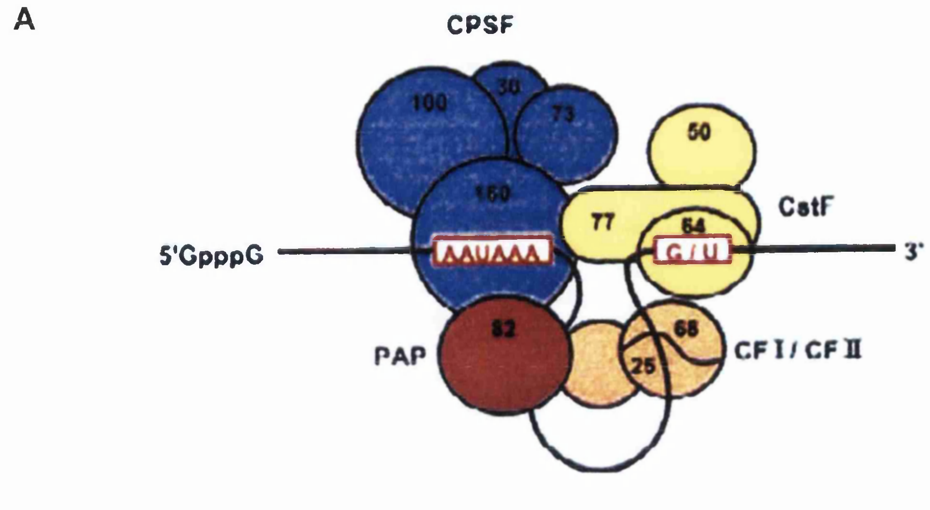


Fig. 1.8. Exon definition and splicing leading to the formation of an export-competent mRNA. CBP, cap-binding proteins: U1, U2, the U1 and U2 snRNPs: SR; members of the serine and arginine-rich family of splicing factors; U2AF, U2 snRNP auxiliary factor; SF-U1A, snRNP-free U1A complex; CPC, cleavage/polyadenylation complex; PAB II, poly (A)-binding protein II. The identity of factors directly contacting splicing factors during the bridging of terminal exons is not yet certain. Adapted from Zhao *et al* ., 1999.



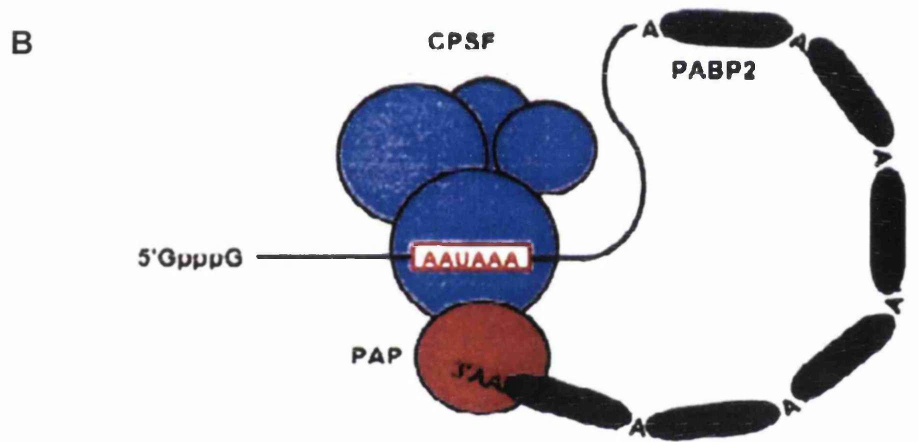


Fig. 1.9. Cleavage and polyadenylation. A. A model of the cleavage complex. CPSF recognises the AAUAAA hexanucleotide while CstF binds downstream at the GU/U-rich region. The positions of CF I, CF II and PAP in the complex are not yet known. **B.** A model of the polyadenylation complex. CPSF remains bound to the AAUAAA sequence while PAP2 binds the poly(A) tail. Adapted from Wahle *et al.*, 1999.

Fig. 1.10. P/E Fragment. The sequence of a 445 nt *Pst* I-*Eco* RI fragment from the L1 ORF/3' UTR of the HPV-16 genome. Features are highlighted and listed above.

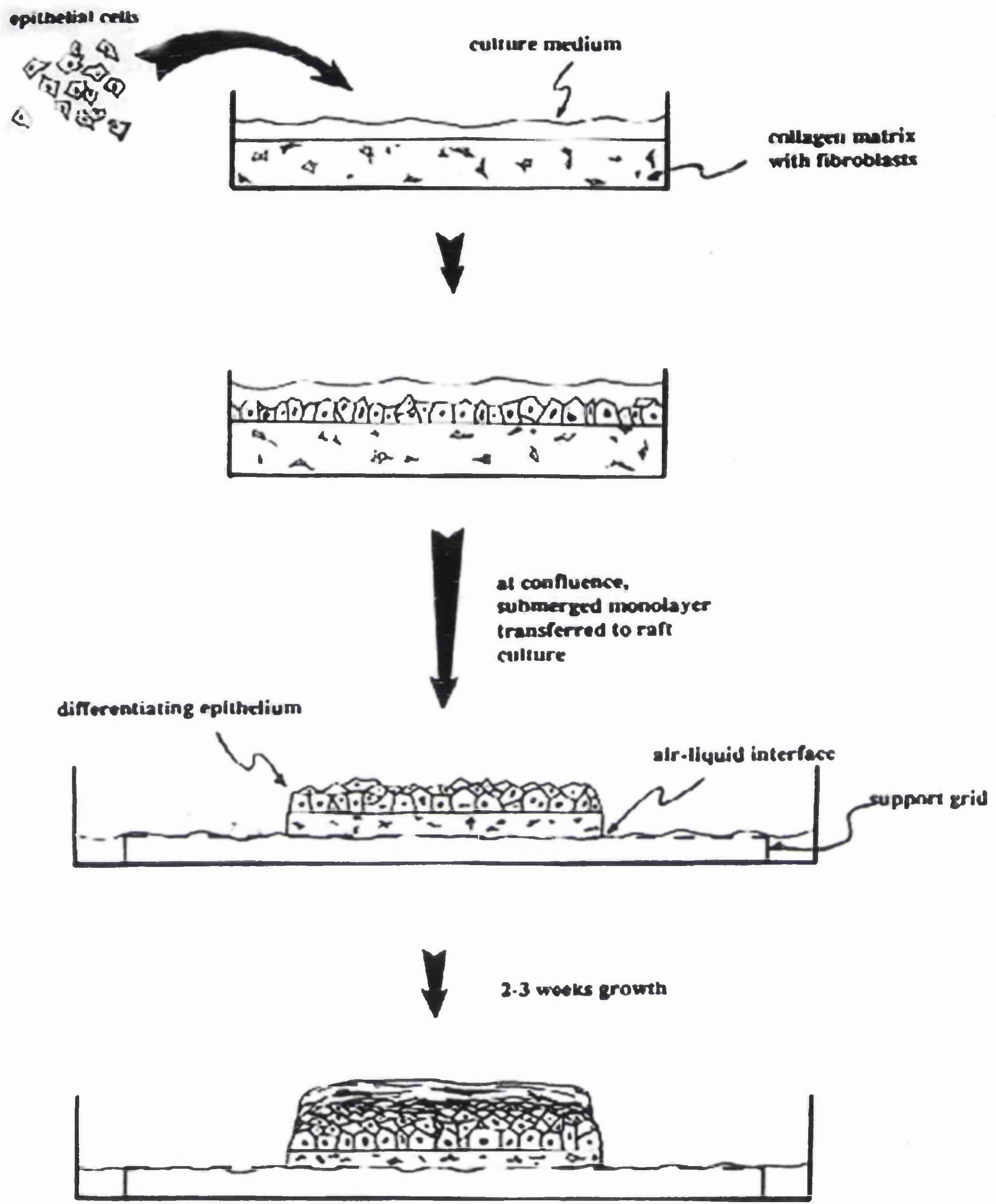


Fig. 1.11. Organotypic raft culture system. Epithelial cells are plated onto a submerged collagen matrix containing fibroblast feeders in the presence of medium. When cells reach confluence the matrix is raised onto a metal grid at the air-liquid interface and subsequent feeding occurs from underneath. Cells stratify and differentiate over a 2-week period. Adapted from Meyers *et al.*, 1994.

Site	HPV-associated disease	HPV Types
Skin	Wart	1, 2, 3, 4, 7, 10, 26, 27, 28, 29, 41, 48, 60, 63, 65, 75, 76, 77, ...
	Epidermodysplasia verruciforms (EV)	5,8,9,12,14,15,17,19,20,21, 22,23,24,25,36,37,38,46, 47,50,...
Respiratory tract	Laryngeal papillomatosis	6, 11
	Focal epithelial hyperplasia (FEH)	13, 32, 57, 72, 73
Genital tract	Genital warts	6, 11
	Intraepithelial neoplasia	34, 40, 42, 43, 44, 53, 55, 61, 62, 64, 67, 68, 71,74
	Intraepithelial neoplasia and invasive carcinoma	16, 18, 31, 33, 35, 39, 45, 51, 52, 54, 56, 58, 66, 69, 70

Table 1. 1: Sites of HPV-infection and associated diseases.

Adapted from Howley, 1996 and zur Hausen, 1996.

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

2.1.1. Enzymes

All restriction enzymes were purchased from New England Biolabs. T4 DNA ligase, T3 RNA polymerase, T7 RNA polymerase, Klenow polymerase, RNasin, and RNase-free DNase 1 were purchased from Promega. TAQ polymerase was purchased from ABgene.

2.1.2. Radiochemicals

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

α - [^{35}S] dATP	12.5mCi/ml	Amersham
α - [^{32}P]dATP	3000Ci/mmol	DuPont NEN
α - [^{32}P]UTP	800Ci/mol	DuPont NEN
[^{3}H] chloramphenicol	58.5mCi/mmol	Amersham

2.1.3. Plasmids/Vectors

pGem®-T Vector – a cloning vector used to clone PCR products. The vector was supplied pre-cut with *Eco*R V and with a 3' terminal thymidine added to both ends thus providing a compatible overhang for PCR products generated using Taq polymerase, which adds an additional deoxyadenosine residue to the 3' ends of amplified fragments. Insertion of the PCR product within the α-peptide coding region of the β-galactosidase enzyme causes inactivation of this enzyme and allows blue/white selection of recombinant clones on Xgal/IPTG plates (Promega). Map in Appendix 1.

pLW1 – an expression vector containing a chloramphenicol acetyltransferase (CAT) reporter gene under the control of the Herpes Simplex Virus type 2 (HSV-2) immediate early promoter. This vector lacks 3' end processing signals. Inserts were cloned downstream of the reporter gene between the *Pst* I/*Hin*d III sites (Gaffney *et al.*, 1985) (Fig. 4.2).

pBS – a cloning vector derived from a pUC plasmid that contains bacteriophage T3 and T7 promoters into which the M13 origin of replication has been inserted in opposite orientations (Stratagene). Map in Appendix 1.

pBS PE445 – this plasmid contains a 445nt *Pst* I-*Eco* RI (PE) fragment from the 3' coding region of HPV-16 L1 extending into the late 3'UTR (7008nt to 7453nt), Fig. 1.10, (McGuire *et al.*, 2002).

pBS 114K/ pBS 114B – full-length HPV-16 DNA derived from a cervical condyloma acuminatum cloned into the *Bam* HI site of pBS (Kirnbauer *et al.*, 1993).

pBS HPV31EcoRI – full-length HPV-31 DNA from the CIN 612 9E cell line cloned into the *Eco* RI site of pBS (Ozbun *et al.*, 1997).

2.1.4. Antibodies

Name, source and dilution of all antibodies used in this study are listed in Table 2.1.

Primary Antibody and type	Western blot dilution	Immunocyto chemistry dilution	Source and Reference
HPV-16 L1 CamVir 1 Mouse	1:10000	1:250	Abcam McClean *et al.*, 1990
HPV-16 E1^E4 TVG 402 Mouse	1:500	1:50	J. Doorbar Doorbar *et al.*, 1992
Involucrin SY5 clone mouse	1:5000	1:100	Sigma
K10 K8.60 clone Mouse	1:500	1:100	Sigma
U2AF^{65} MC3 mouse	1:100	Not attempted	M. Carmo-Fonseca Gama-Carvalho *et al.*, 1997
CstF-64 α64k mouse	1:100	Not attempted	J. Manley Takagaki *et al.*, 1990
HuR 16A5 mouse	1:250	Not attempted	H. Ferneaux Levy *et al.*, 1998
ASF/SF2 αSF2 96 mouse	1:1000	Not attempted	A. R. Krainer Hanamura *et al.*, 1998
Sm Y12 mouse	1:1000	Not attempted	Lab Vision Lerner *et al.*, 1981
U1A Anti-U1A antiserum rabbit	1:1000	Not attempted	I. M. Mattaj
U1 70K H111 mouse	1:1000	Not attempted	Euro Diagnostica Kastner *et al.*, 1992
GAPDH 6CS mouse	1:1000	Not attempted	Biodesign International

Table 2.1. Antibodies used in the course of this work.

2.1.5. Bacterial Culture

The E. Coli strains DH5α and XL1 Blue were used for maintenance and propagation of plasmid DNA. Both strains were grown in L-Broth (10g NaCl, 16g Bactopeptone, 5g yeast extract in 1l water, pH 7.5). Agar plates were made with 1.5% (w/v) agar in L-Broth. When necessary, L-Broth and LB agar plates were supplemented with 100μg/ml ampicillin.

2.1.6. DNA Probes

Full-length HPV-16 genomic probes were prepared by linearising plasmid pBS114K by digestion with *Bam* HI.
Full-length HPV-31 genomic probes were prepared by digesting plasmid pBS HPV31Eco RI with *Eco* RI to release the viral genome.

2.1.7. Cell Lines

HaCaT – Spontaneously immortalized aneuploid human keratinocyte cell line (Boukamp *et al.*, 1988)
HeLa – HPV-18-positive epithelial line derived from an aggressive cervical lesion (Griffiths, 1953)
J2 3T3 – J2 clone of random-bred Swiss mouse 3T3 cells (Rheinwald *et al.*, 1975)
NHFK – Pooled normal human epidermal keratinocytes (neonatal skin) (Clonetics/BioWhittaker)
W12 – HPV-16-positive cervical epithelial cell line established from a low-grade lesion (Stanley *et al.*, 1989)
20863 – Subclone of the parental W12 cell line harbouring extrachromosomal copies of HPV-16 (Jeon *et al.*, 1995a)
20861 - Subclone of the parental W12 cell line harbouring integrated copies of HPV-16 (Jeon *et al.*, 1995a)

2W12 – Subclone of 20863 cell line harbouring episomal HPV-16 (Ozbun and Meyers, unpublished data)
CIN 612 9E - HPV-31-positive cervical epithelial cell line established from a low-grade lesion (Bedell *et al.*, 1991)

2.1.8. Common Reagents, Chemicals and Solutions

30% Acrylamide	29% (w/v) acrylamide, 1% (w/v) N, N'-methylene bis-acrlyamide
40% Acrylamide	38% acrylamide (w/v), 2% bis-acrylamide
5x agarose gel loading buffer	1x TAE, 1% SDS (w/v), 50% glycerol (v/v), 1mg/ml bromophenol blue
Coomassie Blue stain	0.02% (w/v) Coomassie Brilliant Blue, 50% (v/v) methanol, 43% (v/v) water, 7% (v/v) acetic acid
50x Denhardts solution	5g Ficoll, 5g polyvinyl-pyrrolidone, 5g bovine serum albumin, H_2O to 500ml. Filter and aliquot at -20°C
Diethyl Pyrocarbonate (DEPC) treated dH_20	300μl DEPC added to 500ml dH_2O, Incubated overnight at room temperature and baked overnight at 80°C to inactivate DEPC
Destain	5% (v/v) methanol, 7% (v/v) acetic acid, 88% (v/v) water

HEPES extract buffer	50mM HEPES, 50mM NaCl, 0.1% (v/v) NP40, pH7.5
PBS	170mM NaCl, 3.4mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4, pH7.2
PBS-Tween	PBS plus 0.05% (v/v) Tween 20
11.1x PCR buffer	4.5mM TrisCl pH8.8, 11mM $(NH_4)_2SO_4$, 4.5mM $MgCl_2$, 6.7mM β-mercaptoethanol, 4.4mM EDTA, 100mM dATP, dCTP, dGTP, dTTP, 113μg/ml BSA
7x Protease Inhibitor Cocktail	One tablet (Roche) dissolved in 1.5ml distilled water
3x Protein gel loading buffer	150mM Tris HCl, 300mM dithiothreitol, 6% (w/v) SDS, 0.3% (w/v) bromophenol blue, 30% (v/v) glycerol, pH 6.8
Protein gel running buffer	50mM Tris base, 50mM glycine, 0.1% (w/v) SDS
20x SSC (standard saline citrate)	3M NaCl, 300mM trisodium citrate
50x TAE	200mM Tris base, 50mM EDTA (pH8.0), bring to pH8.0 with acetic acid
10x TBE	890mM Tris, 10mM EDTA, 890mM boric acid

TE buffer	10mM Tris HCl, 1mM EDTA, pH8.0
Towbin Blotting buffer	25mM Tris base, 192mM glycine, 20% (v/v) methanol
Trypsin	0.25% (w/v) trypsin, in Tris-saline Containing phenol red, adjusted to pH 7.5 with $NaHCO_3$
UV X-Linking binding buffer	60mM KCl, 20mM HEPES pH7.6, 1mM $MgCl_2$, 10% glycerol
Versene	0.6mM EDTA in PBS, 0.002% phenol red
Water	Sterile distilled water obtained from a "Milli-Ro 60 plus" deioniser (Millipore, USA) and sterilised by autoclaving

All chemicals and reagents were purchased from BDH Chemicals UK or from Sigma Chemical Co. unless otherwise stated in this section or in the methods.

Amersham	ECL Western blotting reagents, nitrocellulose membrane, PVDF membrane, nylon membrane, Rainbow molecular weight markers
Beecham Research	Ampicillin sodium B.P. (Penbritin®)

Bio-Rad	Ammonium persulphate, Bradfords reagent, Coomassie blue
Joseph Mills Ltd.	Ultra pure ethanol, methanol
Prolabo	Butanol, glacial acetic acid, glycerol, isopropanol, xylene

2.2 Methods

2.2.1 Tissue Culture

2.2.1.1 Growth and maintenance of cell lines

HeLa cells were grown in Dulbecco's Modified Eagles Medium (DMEM), supplied by Life Technologies, supplemented with 10% foetal calf serum (FCS) and 5% L-glutamine (10mg/ml), also supplied by Life Technologies. HaCaT cells were maintained under non-differentiating conditions in calcium-free DMEM supplemented with 10% FCS. For the purposes of differentiation, HaCaT cells were grown in E medium (see below).
Primary human foreskin keratinocytes were isolated from newborn circumcisions, pooled, and supplied by Clonetics, BioWhittaker, UK Ltd. Primary cells were maintained in Keratinocyte Growth Medium (KGM) supplemented with 50μg gentamycin sulfate, 50ng amphotericin, 0.1ng human epidermal growth factor, 30μg bovine pituitary extract, 5μg bovine insulin, and 0.5μg hydrocortisone, supplied by Clonetics, BioWhittaker, UK Ltd.
J2 3T3 cells were maintained in DMEM supplemented with 10% donor calf serum (DCS) and 5% L-glutamine (10mg/ml).
HPV-positive cell lines (W12, 20863, 20861, 2W12, CIN 612 9E) were maintained in monolayer culture in W12 medium (DMEM supplemented with 10% FCS, 0.1nM cholera toxin and 0.4μg/ml hydrocortisone). These human epidermal keratinocyte cell lines were cultivated with a feeder layer of J2 3T3 cells; this method has been shown to support the clonal growth of keratinocytes only (Rheinwald and Green, 1975). The 3T3 feeder layer was prepared by first treating the feeder cells with mitomycin C (4μg/ml) for 2-5 h at 37°C to irreversibly inhibit proliferation (Rheinwald and Green, 1975). Following treatment, the 3T3 cells were washed 3 times with PBS, trypsinized and re-plated in W12 medium. The plating density of the feeder

cells is dependent on the desired seeding density of the keratinocyte cell lines since the optimal ratio is 5:1 of J2 3T3 cells to keratinocytes. Generally, cell stocks of the HPV-positive lines were prepared by growing the lines in W12 medium, with 0.5ng/ml epidermal growth factor (EGF) added 24h after plating. For the purposes of differentiation in monolayer culture and on organotypic rafts, cells were cultured in epithelial (E) medium without EGF. E medium was prepared in 5l vats as follows (Meyers, 1996):

dH_2O	4.0625l
DMEM with 4500mg/l D-glucose &L-Glutamine, no sodium pyruvate/sodium bicarbonate	3.75l
F-12 nutrient mixture with L-Glutamine, no sodium bicarbonate	1.25l
Sodium bicarbonate	15.34g
Adenine	18mM
Insulin	5μg/ml
Transferrin	5μg/ml
3, 3', 5-Triiodo-L-Thyronine (T3)	2pM
Penicillin	500U
Streptomycin	500μg/ml
Hydrocortisone	2μg/ml
Cholera enterotoxin	10nM
Conc. HCl	1.56ml

The solution was mixed well and the pH adjusted to pH 7.1 with conc. HCl. Finally the solution was filter-sterilised using a low protein 0.2μM filter, aliquoted into sterile1l bottles and stored at 4°C.

2.2.1.2 Preparation of cell stocks

Following trypinisation, cells were counted and resuspended in the appropriate growth medium at $1x10^6$ cells/ml and aliquoted to screw cap tubes. Dimethylsulphoxide (DMSO) was added to a final concentration of 10% (v/v) and the cells were stored under liquid nitrogen.
This procedure was followed for all cell lines grown.

2.2.1.3 Establishment and maintenance of organotypic raft cultures

This method has been adapted from a previously published protocol (Meyers, 1996). The dermal equivalent was prepared by mixing non-mitocycin C treated J2 3T3 fibroblasts ($6.25x10^5$ cells/raft) with a collagen mix (2.5ml/raft) composed of 80% type IV collagen (generously provided by Dr. Mike Edward, Dept. of Dermatology), 10% 10xDMEM and 10% reconstitution buffer (2.2% (w/v) $NaHCO_3$, 4.77% (w/v) HEPES in 0.062N NaOH) and 0.024N NaOH. The dermal equivalent was aliquoted to each well of a six well dish and incubated at 37°C for 30 min to set. Epithelial cells were seeded onto the dermal equivalent at $1.0x10^6$cells/raft. The collagen matrixes were incubated overnight at 37°C to allow attachment of the epithelial cells to the dermal equivalent and growth to confluence. On the following day, the epithelia cells were brought to the air-liquid interface by raising the collagen matrixes onto wire grids. The epithelial cells continued to be fed by diffusion through the collagen matrix from the surrounding E medium for up to 12 days; medium was changed every second day. Once at the air-liquid interface, the rafts were treated with 10μM 1,2-dioctanoyl-sn-glycerol (C8:O; Sigma) on alternate days to induce differentiation, or remained untreated as a negative control for C8:O treatment.

2.2.1.4 Preparation of cell extracts

(i) Protein extracts from monolayer culture

Cells were washed twice in cold PBS then harvested in 5ml of PBS by scraping. Cells were pelleted by centrifugation at 1000rpm for 5 min at 4°C (Sorvall RT 6000B, refrigerated benchtop centrifuge) and resuspended in 800μl ice cold Buffer E (100mM Tris-HCl pH8.0, 100mM NaCl, 2mM EDTA, 2mM EGTA, 1% (v/v) NP40, 0.5% (v/v) sodium deoxycholate, 0.5mM PMSF) and chilled on ice for 15 min. Following cell lysis, chromosomal DNA was sheared by passing the solution through a 23 gauge needle attached to a 2ml syringe, 5 to 10 times. Cell debris was pelleted by micro-centrifugation at 12000rpm at 4°C for 15 min (Hettich EBA 12R, refrigerated microcentrifuge). The soluble protein extract was removed and stored at –70°C.

(ii) Protein extracts from organotypic raft culture

Tissue harvested from organotypic raft culture was washed twice with ice-cold PBS and pelleted by centrifugation at 1000rpm for 5 min at 4°C (Sorvall RT 6000B, refrigerated benctop centrifuge). The pelleted tissue was resuspended in 5 volumes of ice-cold suspension buffer (0.1M NaCl, 0.01M Tris HCl pH7.6, 0.001M EDTA pH8.0, 1μg/ml aprotinin, 100μg/ml PMSF. An equal volume of 2x SDS gel-loading buffer was added and the samples were placed in a boiling water bath for 10 min. The chromosomal DNA was sheared by sonication in a soni-bath (Kerry Ultrasonic Bath) for 30-60 sec and the cell debris was pelleted by centrifugation at 10,000rpm for 10min (Eppendorf Microcentrifuge 5415 C). The soluble protein extract was removed and stored at –70°C.

(iii) Nuclear Extracts

Nuclear extracts were prepared from cells grown in monolayer culture by resuspending harvested cells in three packed cell volumes of CE buffer (10mM HEPES pH7.8, 1mM EDTA, 60mM KCl, 1mM PMSF, 0.1% (v/v)

NP40, 25% (v/v) glycerol, 4% (v/v) protease inhibitor cocktail) and chilled on ice for 4 min. Nuclei were pelleted at 14,000rpm for 10 sec (Eppendorf 5415 C, microcentrifuge) and resuspended in three packed cell volumes of CW buffer (10mM HEPES pH7.8, 1mM EDTA, 60mM KCl, 1mM PMSF) followed by 10-20 strokes in a Dounce homogeniser. Nuclei were pelleted again at 14,000rpm for 60 sec and resuspended in two packed cell volumes of NE buffer (20mM Tris HCl pH8.0, 420mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 0.5mM PMSF, 25% (v/v) glycerol, 4% (v/v) protease inhibitor cocktail) and chilled on ice for 10 min. The suspension was centrifuged at 14,000rpm for 10 min at 4°C (Hettich EBA 12R, refrigerated microcentrifuge). The supernatant was removed as the nuclear extract and stored at –70°C. This method was used in preference to the method of Dignam (as described in Wahle *et al.*, 1994) because it was faster, gave good nuclear / cytoplasmic separation and the final salt concentration of the extract was quite high (210mM NaCl) which was useful because I wanted to use these extracts to identify nuclear proteins that bind the HPV-16 NRE with high affinity.

2.2.1.5 Estimation of protein concentration

10μl of protein sample was diluted in 90μl of TE buffer and mixed with 300μl of Bradford's reagent (BioRad). Following mixing the absorbency of the solution was measured at 620nm (Anthos htII plate reader) and converted to mg protein by comparison with a standard curve produced using known quantities of bovine serum albumin.

2.2.1.6 Transfection of cells

(i) Lipofection with Lipofectamine (Gibco, BRL)

HeLa cells were seeded at $1x10^5$ cells per well of a six well dish and incubated overnight at 37°C. On the following day, 2μg of the DNA to be transfected was diluted into 100μl of serum-free medium and 2μl of Lipofectamine reagent, per transfection, was added to 100μl of serum-free

medium. The two solutions were combined, mixed and allowed to incubate at room temperature for 30 min, to allow DNA-liposome complexes to form. The cells to be transfected were rinsed with 2ml of serum-free medium and 800μl of serum-free medium was added to the DNA-Lipofectamine mix which was then applied to the pre-rinsed cells. The cells were incubated at 37°C for 5 h and 1ml of growth medium with 2x serum was added and incubation continued overnight. The medium was replaced the following day with fresh, complete medium and the cells were harvested 48 h after the start of transfection.

(ii) Electroporation of primary keratinocytes

Primary foreskin keratinocytes were electroporated with linearized HPV-16 DNA released by digestion with *Bam* H1 from the pBS vector backbone of either plasmid 114K or plasmid 114B (gifts from Matthias Durst, Kirnbauer *et al.*, 1993). For each electroporation, 10μg HPV-16 DNA was mixed with 4.25μg boiled salmon sperm DNA. Approximately $5x10^6$ primary cells, resuspended in 250μl of E media plus 5mM BES pH7.2, were incubated with the DNA mixture at room temperature for 10 min. The solution was transferred to an electroporation cuvette (0.4cm BIO-RAD Gene Pulser Cuvette) and electroporated at 210v/960μFd (BIO-RAD Gene Pulser) followed by a 10 min incubation at room temperature. Each electroporation sample was layered onto 10ml E media and centrifuged at 1,000rpm for 5 min (Sorvall RT 6000B refrigerated benchtop centrifuge), the pellet was resuspended in 6ml of E media and seeded onto freshly prepared mitomycin C treated J2 3T3 fibroblast feeders.

2.2.2 Chloramphenicol acetlytransferase (CAT) assay

Transfected cells were harvested by scraping and pelleted by centrifugation at 1000rpm for 5 min (Sorvall RT 6000B refrigerated benchtop centrifuge). The cell pellet was resuspended in 1ml of PBS, centrifuged at 14,000rpm for

25 sec (Eppendorf 5415C microcentrifuge) and the supernatant was discarded. The pellet was resuspended in 160μl of prechilled resuspension buffer (0.11M Tris-HCl pH7.8, 1mM EDTA, 1mM DTT) and the cells were lysed by freeze thawing three times in a dry ice/ethanol mix and at 37°C. Cell debris was pelleted by centrifugation at 14,000rpm for 3 min at 4°C (Hettich EBA 12R refrigerated microcentrifuge), the supernatant was retained and incubated at 65°C for 6 min to inactivate internal acetylases. Each sample was incubated at 37°C for 2 h with 20μl 0.5mM chloramphenicol, 2μl ^{3}H-chloramphenicol (58.4mCi/mmol) and 2μl butyryl coenzyme A (25mM). The reaction was stopped by adding 400μl xylene, mixed by vortexing and micro-centrifuged at 14,000rpm for 2 min (Eppendorf 5415C). The upper phase was retained and 200μl of xylene extraction buffer (10mM Tris-HCl pH7.5, 1mM EDTA) added, mixed by vortexing and centrifuged as before, again the upper phase was retained and the extraction procedure was repeated three further times. Following the final extraction, one tenth of the volume of the remaining upper aqueous phase was added to 2ml Ecoscint and counted in a liquid scintillation counter (Beckman LS 5000CE).

2.2.3 Manipulation of DNA

2.2.3.1 Preparation and transformation of competent bacterial cells

(i) DH5α cells

A 1ml overnight culture was inoculated into 100ml L-Broth and grown for approximately two hours at 37°C with shaking until the OD_{600} was 0.6. The culture was then chilled on ice for 15 min and the cells pelleted by centrifugation at 3,000rpm for 15 min at 4°C in a Sorvall RT 6000B refrigerated benchtop centrifuge. The pellet was resuspended in 30ml cold TfB I (30mM potassium acetate, 50mM manganese chloride, 100mM potassium chloride pH 5.8, 10mM calcium chloride, 15% glycerol) and chilled

on ice for 1 h. Cells were pelleted as before and resuspended in 8ml of cold TfB II (10mM sodium MOPS pH 7.0, 75mM calcium chloride, 10mM potassium chloride, 15% glycerol). Cells were chilled overnight at 4°C prior to use or aliquoted and stored at –70°C until required.

(ii) XL1 blue cells

Competent XL1 blue cells were prepared as described above for the DH5α cells but following centrifugation the cell pellet was resuspended in 20ml of sterile cold 100mM $CaCl_2$ and chilled on ice for 30 min. The suspension was centrifuged at 2,500rpm for 5 min at 4°C in a Sorvall RT 6000B refrigerated benchtop centrifuge and the pellet was resuspended in 10ml of sterile cold 100mM $CaCl_2$ and chilled on ice for 15 min followed by centrifugation as before. The pellet was then resuspended in 5ml sterile cold 100mM $CaCl_2$ and chilled overnight at 4°C prior to use or aliquoted and 10% glycerol (v/v) added and stored at –70°C until required.

200μl aliquots of bacterial cells were thawed gently on ice prior to transformation. Approximately 10ng of DNA (5-10μl) was added to the cells and chilled on ice for 30 min, the mix was then heat shocked by incubating at 42°C for 45 sec and chilled on ice for 2 min. 500μl of L-Broth was added to the cells which were then incubated at 37°C with shaking for 1 h before plating onto LB plates containing ampicillin (100μg/ml). Plates were incubated overnight at 37°C.

2.2.3.2 Phenol:Chloroform extraction and ethanol precipitation

Generally, the reaction volume was increased to 200μl with TE buffer and an equal volume of phenol:chloroform was added, mixed by vortexing and microcentrifuged for 5 min at maximum speed (Eppendorf 5415C); (phenol pH 7.9 for DNA; phenol pH 4.5 for RNA). The upper phase was transferred

to a clean eppendorf tube, mixed thoroughly with an equal volume of chloroform and centrifuged as before. Again the upper phase was transferred to a clean tube and 1/10 of the volume of 3M sodium acetate pH 5.2 (DNA) or 7.5M ammonium acetate (RNA) was added together with 2.5 volumes of ice-cold 100% ethanol. The solution was mixed by inversion and chilled on ice for 30 min followed by centrifugation at maximum speed for 10 min (Eppendorf 5415C microcentrifuge) to pellet the nucleic acid. The pellet was washed in 70% ethanol, centrifuged for 5 min, air-dried and resuspended in DEPC-treated dH_2O (RNA) or TE buffer (DNA).

2.2.3.3 Purification of plasmid DNA

(i) Small scale purification of plasmid DNA: alkaline lysis

1ml of bacterial culture containing the plasmid DNA was microcentrifuged for 1 min at maximum speed (Eppendorf 5415C) and the pellet was resuspended in 100μl of solution I (0.5M TrisCl pH8.0, 10mM EDTA) and mixed by vortexing. The cells were lysed by adding 200μl of freshly prepared solution II (1% SDS, 0.2N NaOH), mixed by inversion and incubated on ice for 5 min. 150μl of neutralizing solution III (3M potassium acetate, 11.5% acetic acid) was added to the samples and mixed gently by inversion. Cell debris was pelleted by centrifugation at 14,000rpm for 10 min (Eppendorf 5415C microcentrifuge). The supernatant was transferred to a clean eppendorf tube and contaminating proteins were removed by phenol:chloroform extraction. Plasmid DNA was ethanol precipitated, pelleted, washed with 70% ethanol and resuspended in TE buffer.

(ii) Large scale caesium chloride purification of plasmid DNA

500ml of overnight bacterial culture was pelleted by centrifugation at 6000rpm for 10 min at 4°C (Sorvall GSA rotor, lowspeed centrifuge). The pellet was resuspended in 8ml solution I (0.5M TrisCl pH8.0, 10mM EDTA, 1mg lysozyme) and chilled on ice for 15 min. The cells were lysed by adding

16ml of fresh solution II (1% SDS, 0.2N NaOH), mixed by inversion and incubated on ice for 10 min. The sample was neutralised by the addition of 12ml of solution III (3M potassium acetate, 11.5% acetic acid), mixed by inversion and chilled on ice for 15 min to precipitate the protein complexes. Cell debris was pelleted by centrifugation at 13,000rpm for 20 min (Sorvall SS34 rotor, lowspeed centrifuge), the supernatant was filtered through muslin and the plasmid DNA isopropanol precipitated from the filtrate. The DNA pellet was allowed to air dry and was resuspended in 4.5ml of TE buffer with 5g CsCl and 200μl ethidium bromide (10mg/ml). This solution was transferred to an ultracentrifuge tube and centrifuged at 48,000 rpm for 18h at 4°C (Beckman Ti70 rotor, ultracentrifuge). Following centrifugation the plasmid DNA band was removed by side-puncturing the tube using a syringe and needle and transferred to a 15ml tube. Ethidium bromide was removed from the plasmid DNA by repeated extraction with water-saturated butan-2-ol. The DNA was precipitated with ethanol and finally resuspended in TE buffer.

2.2.3.4 Restriction enzyme digestion of DNA

Small scale restriction enzyme digestion of DNA was used to identify recombinant plasmids. Digests were generally carried out in 20μl reaction volumes using 10 units of restriction enzyme per μg of DNA and 1x enzyme specific buffer. Reactions were incubated at 37°C usually for 1-2 h then agarose gel loading buffer was added prior to electrophoresis.

2.2.3.5 DNA Ligation

Vector and DNA fragments were prepared by restriction enzyme digest, gel-purified and ligated, usually, at a 1:3 ratio. Reactions were carried out in 20μl volumes containing 1 x ligase buffer and 2 units of T4 DNA ligase with 1mM ATP and were incubated overnight at 15°C or at room temperature for at

least 4 h. PCR products were ligated into the pGEM-T vector following the manufacturer's instructions.

2.2.3.6. Gel electrophoresis of DNA and recovery

(i) Agarose gel electrophoresis

DNA fragments produced by restriction enzyme digestion or PCR were fractionated by agarose gel electrophoresis. 1% horizontal slab gels were prepared with Agarose LE Analytical grade (Promega) in 1xTBE buffer and electrophoresied at 100V. 100bp DNA ladder standard size markers (Gibco, BRL) were used where appropriate. Following electrophoresis, gels were stained in an ethidium bromide solution (0.5μg/ml) for 10 min. DNA was visualised either under short-wave UV light, or under long UV light for preparative gels, and photography was carried using an Ultra Violet Products Gel Documentation System, Image Store 7500, Version 7.2.
Agarose gel slices containing the appropriate DNA fragments were excised from gels under long UV light and the DNA recovered using a Millipore Ultrafree-DA kit. The gel slice was placed in the Gel Nebulizer and microcentrifuged at 5,000 rpm for 10 min (Eppendorf 5415C) causing the resultant gel slurry to be sprayed onto the sample filter cup. The membrane retained the gel matrix and the DNA passed through into the filtrate vial.

(ii) Acrylamide gel electrophoresis

Small DNA fragments produced by PCR were analysed on 6% non-denaturing acrylamide gels prepared as follows:

30% acrylamide/ bisacrylamide mix	3ml
10 x TBE	1.5ml
10% APS	100μl
TEMED	12μl
dH_2O	10.4ml

Electrophoresis was carried out at 130V for 45 min and gels were stained in ethidium bromide (0.5μg/ml) for 10 min, visualised under UV light and photographed using an Ultra Violet Products Gel Documentation System. Acrylamide gel slices containing the desired DNA fragment were excised under long UV light and the DNA was eluted overnight at 4°C in 400μl of 0.5M NaCl. Eluted DNA was ethanol precipitated, pelleted, washed with 70% ethanol and resuspended in TE buffer.

2.2.3.7 Oligonucleotide Synthesis and Purification

Oligonucleotide synthesis was preformed on an automated Cruachem PS250 by cyanoethyl phosphoramidite chemisty on controlled pore glass (CPG) columns. The first nucleoside of the 3' end of the oligonucleotide was attached to the CPG surface via an ester linkage and the completed oligonucleotide was removed by passing 1.5ml of ammonia across the matrix, cleaving the ester bond and generating a free 3'OH. The base-protecting dimethoxytrityl groups were removed by incubation at 55°C in ammonia for 5 h and the DNA was lyophilised under vacuum followed by phenol:chloroform extraction. Oligonucleotides were ethanol precipitated and resuspended in dH_2O.

2.2.3.8 Quantification of DNA, RNA and Oligonucleotides

A spectrophotometer (Eppendorf BioPhotometer) was used to determine the concentration of DNA, RNA and oligonucleotides based on the following calculations:

Double-stranded DNA	1 A_{260} = 50μg/ml
Single-stranded oligonucleotides	1 A_{260} = 30μg/ml
RNA	1 A_{260} = 40μg/ml

In addition, the purity of nucleic acid was determined by comparing readings at 260nm and 280nm (A_{260}/A_{280}), assuming a ratio of approximately 1.8 indicated that the samples were relatively free of protein.

2.2.4 PCR Mediated Site-Directed Mutagenesis

The HPV-16 negative regulatory element (NRE) was divided into fifteen non-overlapping groups of five nucleotides from the 5' end through to the 3'end. Within each group PCR-mediated site-directed mutagenesis (SDM) was used to change purine bases to pyrimidines and *vice-versa* (Fig. 4.1B). A PCR based protocol (Matthews *et al.*, 1994) was used to generate the site-directed mutants (Fig. 2.1). Plasmid pBS PE445, which consists of the 445nt *Pst* I/*Eco* RI fragment from the HPV-16 late 3' untranslated region (Fig 1.10) inserted into the multiple cloning site of pBS, was used as the wild-type DNA template. Fifteen sets of complimentary primers were designed that contained each of the five mismatched bases flanked by 10-17 bp of wild-type 5' sequence and 10-17 bp of wild-type 3' sequence (Table 2.2). The mutagenesis protocol is summarized in Fig 2.1A and B. In each case, the mutant forward primer was used together with a reverse primer encoding the baceriophage T3 promoter, designated T3 (Fig. 2.1C), to mutate and amplify *Sac* 1 linearized pBS PE445. *Kpn* 1 linearized pBS PE445 provided the

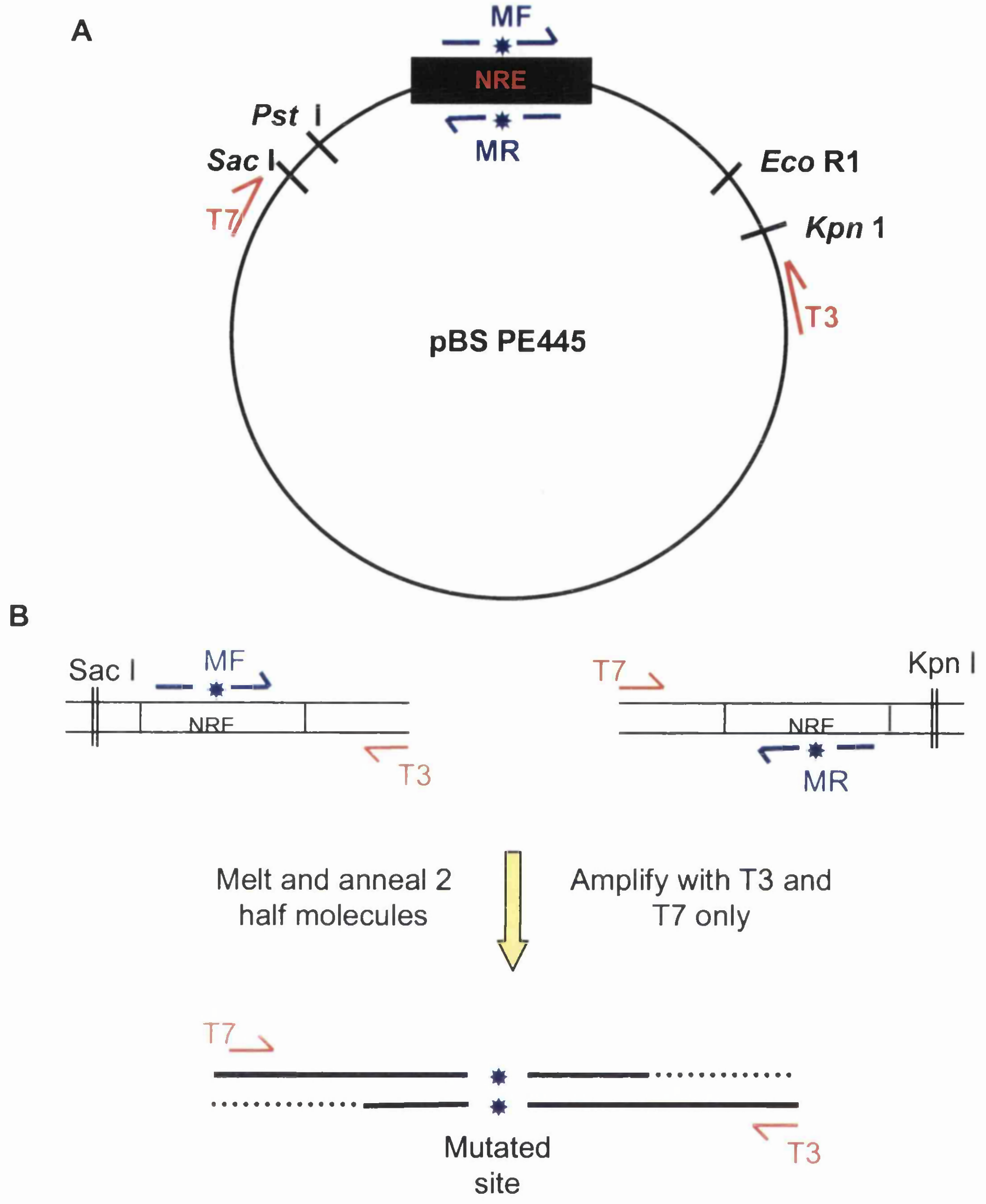
template for a separate PCR reaction involving the complimentary mutant reverse primer and a forward primer encoding the bacteriophage T7 promoter, designated T7 (Fig. 2.1C). The result of these separate PCR reactions was two complimentary half molecules containing the desired mutations, which could then be annealed, and a further round of fill-in PCR using the T3 and T7 primers generated a full-length, double-stranded product (Fig. 2.1B). All PCR products generated in this way were subsequently cloned into the pGem-T vector and sequenced to ensure only the desired mutations were present.

The following PCR conditions were used:

Stage	Temperature	Time	Cycles
1	95°C	3 min	1
2	95°C 55°C 72°C	30 sec 1 min 30 sec	30
3	55°C 72°C	5 min 10 min	1

Mutant	Forward Primer (5' - 3')	Reverse Primer (3' - 5')
M1	CATCTACCTCTACAACT**TAGTC**ACGCAAAAAACGTAAGC	GCTTACGTTTTTTGCGT**GACTA**AGTTGTAGAGGTAGATG
M2	CCTCTACAACTGCTAA**TATGC**AAAAACGTAAGCTGTAAG TATTG	CAATACTTACAGCTTACGTTTTT**GCATA**TTAGCAGTTGTA GAGG
M3	CAACTGCTAAACGCAAAAAA**GTACT**GCTFTAAGTATTGTA TG	CATACAATACTTACAGC**AGTAC**TTTTTTGCGTTTAGCAGT TG
M4	CTGCTAAACGCAAAAAACGTAA**TAGCA**AAGTATTGTATG TATGTTG	CAACATACATACAATACTT**TGCTA**TTACGTTTTTTGCGTTT AGCAG
M5	CGTAAGCTGT**CTCAG**TTGTATGTATGTTG	CAACATACATACAA**CTGAG**ACAGCTTACG
M6	CGTAAGCTGTAAGTA**GATGC**TGTATGTTGAATTAG	CTAATTCAACATACA**CGATC**TACTTACAGCTTACG
M7	CGTAAGCTGTAAGTATTGTA**GTACG**GTTGAATTAGTGTT GTTG	CAAACAACACAATTCAAC**CGTAC**TACAATACTTACAGCTT ACG
M8	GTATGTAT**TAGTC**ATTAGTGTTGTTTGTTGTG	CACAACAAACAACACTAAT**GACTA**ATACATAC
M9	GTATGTTGA**CGATC**TGTTGTTTGTTGTGTATATG	CATATACACAACAAACAACA**GATCG**TCAACATAC
M10	GAATTAG**CTGAT**TTTGTTGTGTATATGTTTG	CAAACATATACACAACAAA**ATCAG**CTAATTC
M11	GTGTTG**GAGTA**AGTGTATATGTTTGTATGTGC	GCACATACAAACATATACACT**TACTC**CAACAC
M12	GTGTTGTTTGT**ATACG**ATATGTTTGTATGTGCTTGTATG	CATACAAGCACATACAAACATAT**CGTAT**ACAAACAACAC
M13	GTTTGTTGTGT**CGTAC**TTTGTATGTGCTTGTATTG	CACATACAAGCACATACAAA**GTACG**ACACAACAAAC
M14	GTGTATATG**GAGCA**ATGTGCTTGTATGTGCTTG	CAAGCACATACAAGCACAT**TGCTC**CATATACAC
M15	GTATATGTTTGT**CATG**GCTTGTATGTGCTTGTAAAT	ATTTACAAGCACATACAAGC**CATG**ACAAACATATAC

Table 2.2. Sequences of the primer pairs used to mutate the NRE. The mutations introduced in each group are in bold.



C

T7: CCAAGCTTC<u>TAATACGACTCACTATAG</u>GGATATCATTTACAAGCACATACAAGC

T3: CAGAGATG<u>CAATTAACCCTCACTAAAGGG</u>AGAATTCCCTCATCTACCTCTACAACT

Fig. 2.1. Site-directed mutagenesis of the NRE. A. Cartoon of pBS PE445 showing the relevant restriction enzyme sites and positions of the primers used to mutate the NRE. **B.** Two complimentary half molecules, containing the NRE mutation, were PCR amplified, melted, annealed and amplified again to generate a full-length, double-stranded molecule. **C.** Sequence of the primers designated T7 and T3, bacteriophage RNA polymerase promoter sequences are underlined. Sequences of the mutagenesis primers are listed in Table 2.2.

2.2.5. Southern Blotting

2.2.5.1 Hirt extraction of DNA from monolayers

Monolayer cells were washed in PBS, trypsinized and pelleted at 1,000rpm for 5 min (Sorvall RT 6000B, refrigerated benchtop centrifuge). Cell pellets were resuspended in 3ml of Hirt extraction buffer (400mM NaCl, 10mM TrisCl pH 7.4, 10mM EDTA) and RNase A, Proteinase K, and SDS was added to final concentrations of 50μg/ml, 50μg/ml, and 0.2% (v/v), respectively. Solutions were incubated at 55°C for 3 h with vortexing every hour followed by the addition of 1.25M NaCl and overnight incubation at 4°C. The following day, cell debris was pelleted by centrifugation at 17,000rpm for 1 h at 4°C (Sorvall SS34 rotor, lowspeed centrifuge). The supernatant was extracted twice with phenol:chloroform and once with chloroform. The DNA was ethanol precipitated from the supernatant, washed with 70% ethanol and resuspended in TE buffer. The concentration of DNA was determined by spectrophotometry at A_{260nm}.

2.2.5.2 Total DNA extraction from rafts

Rafts were harvested into 15ml polypropylene tubes and washed with PBS. 3ml of Hirt extraction buffer (400mM NaCl, 10mM TrisCl pH 7.4, 10mm EDTA) was added to each tube together with 50μg/ml RNase A and 0.2% (v/v) SDS and incubated overnight at 37°C on a rotating wheel. On the following day, 50μg/ml of Proteinase K was added and the solution was incubated for 1h at 37°C on a rotating wheel. DNA was sheared by passing the solution through an 18-gauge needle 10 times. Contaminants were removed from the solution by extraction with phenol:chloroform and the DNA was precipitated with ethanol overnight at –20°C. On the following day, the DNA was pelleted by centrifugation at 10,000rpm for 30 min at 4°C (Sorvall

SS34 rotor, lowspeed centrifuge), washed with 70% ethanol and resuspended in TE buffer. The concentration of DNA was determined by spectrophotometry at A_{260nm}.

2.2.5.3 Electrophoresis and transfer

10μg samples of total genomic DNA and Hirt DNA were digested with *Bam* H1 to linearize HPV-16 or *Eco* RI to linearize HPV-31 prior to electrophoresis on 0.8% agarose gels at 80V for 2 h. Following electrophoresis, the gel was photographed and the distance of the DNA markers was measured. The gel was then soaked in 100ml of 0.25M HCl with gentle shaking for 15 min until the bromophenol blue front turned yellow indicating that the acid treatment was complete. After a 5 min wash in dH_2O the gel was soaked in 0.4N NaOH for 30 min. Meanwhile, a nylon membrane (Hybond–N, Amersham), similar in size to the gel, was washed briefly in dH_2O and then soaked in 0.4N NaOH for 20 min. Following soaking, a blot was assembled such that the DNA side of the gel was in direct contact with the nylon membrane which was overlaid with 10 sheets of 3MM paper, a glass plate and a weight; O.4N NaOH was used as transfer buffer. Following an overnight transfer, the blot was dissembled, the membrane allowed to air-dry, and the DNA irreversibly bound to the membrane by UV cross-linking at $1.25x10^5$ μJoules for 2 min in a Stratagene UV Stratalinker.

2.2.5.4 Random priming of dsDNA

A random primer method was used to synthesize radioactively labelled HPV-16 and HPV-31 DNA probes. Firstly, DNA templates were generated by releasing the HPV-16 genome from the plasmid pBS 114K by digestion with *Bam* H1; The HPV-31b viral genome was freed from the plasmid pBS HPV31 by digestion with *Eco* R1. DNA templates were resuspended in TE buffer at 1.25μg/ml and the labelling reactions were set up as follows:

20ng template DNA	16μl
Rxn (A)*	5μl
BSA	1μl
α [^{32}P] dATP (800Ci/mmol)	2.5μl
Klenow DNA polymerase	0.5μl

*Rxn (A):

Solutions A, B, and C were mixed in the following ratio: 100:250:150

Solution A = 1ml solutionφ (1.25M TrisCl pH 8.0, 0.125M $MgCl_2$) + 18μl β-Mercaptoethanol + 5μl of 100mM dCTP, dGTP, dTTP

Solution B = 2M HEPES pH 6.6

Solution C = pN_6 (Promega)

Reactions were incubated overnight at room temperature. Unincorporated nucleotides were removed using Mini Quick Spin Columns (Roche). The sephadex matrix in the column was first resuspended, the lid removed and the bottom tip broken off. The column was centrifuged at 6,500rpm for 1 min (Eppendorf 5415C, microcentrifuge) and transferred to a clean eppendorf tube. The radiolabelled reaction was added drop-wise directly onto the matrix and the column was centrifuged at 6,500rpm for 4 min and the eluate retained. The activity of the probes was calculated by Cerenkov counting in a Beckman liquid scintillation counter.

2.2.5.5 Hybridisation and washing

The nylon membrane was washed briefly in 2 x SSC prior to prehybridisation in 10ml of Pall membrane hybridisation buffer (5 x Denhardts solution, 5 x SSC, 0.2% SDS (v/v), 0.5μg/ml salmon sperm DNA) for 2 h at 65°C. Radioactively labelled DNA probe (5 x 10^6cpm) was added to the

hybridisation buffer and hybridisation continued overnight at 65°C. On the next day, the membrane was removed from the hybridisation buffer and washed with shaking at 65°C for 15 min in each of the following solutions: Wash A (2 x SSC, 0.1% SDS), Wash B (0.5 x SSC, 0.1% SDS), Wash C (0.1 x SSC, 0.1% SDS) and Wash D (0.1 x SSC, 1% SDS). The membrane was allowed to air dry and then exposed to Kodak X-OMAT S film.

2.2.6 SDS-PAGE

Proteins were separated by electrophoresis through polyacrylamide gels with SDS using Bio-Rad mini gel electrophoresis tanks (Laemmli, 1970). The gel apparatus and glass plates were assembled according to the manufacturer's instructions. Gel mixes were prepared as follows:

	12% resolving gel	15% resolving gel	Stacking gel
dH_2O	3.3ml	2.3ml	2.95ml
30% acrylamide/ bisacrylamide mix	4ml	5ml	650μl
1.5M Tris pH 8.8	2.5ml	2.5ml	-
1M Tris pH 6.8	-	-	2.5ml
10% SDS	100μl	100μl	50μl
10% APS	100μl	100μl	50μl
Temed	12μl	12μl	12μl

The resolving gel was mixed well and poured between the glass plate sandwich leaving a 1.5cm space at the top for the stacking gel. The resolving gel was overlaid with water to occlude air bubbles while the gel polymerised, following polymerisation the water was removed and the stacking gel was poured on top of the resolving gel and a comb inserted. The gel was placed in the electrophoresis apparatus, the comb removed and the tank filled with SDS-PAGE running buffer. Samples in protein loading

buffer were boiled for 5 min prior to loading onto the gel, 2μl Rainbow marker was also loaded onto each gel. Gels were run at 130v until the bromophenol blue front reached the bottom of the gel. Gels were removed from the glass plate sandwich and either stained with Comassie blue stain for 30 min and then destained in protein gel destain, or subjected to Silver Staining, or transferred electrophoretically to nitrocellulose membrane for Western blot analysis.

2.2.6.1 Silver Staining

The Bio-Rad Silver Stain kit was used to stain SDS-PAGE gels to detect proteins present at a low concentration. Gels were incubated, with shaking, in fixative (40% methanol, 10% acetic acid) for at least 30 min then transferred to oxidizer (contains $K_2Cr_2O_7$ and HNO_3) for a further 5 min. The gel was then washed extensively in large volumes of water with regular changes of wash for 15 min. A 0.1% solution of silver reagent was prepared by dissolving 0.1g of silver nitrate in 100ml dH_2O and the gel was incubated in this freshly prepared solution for 20 min. The gel was then washed briefly in water for 30sec and transferred to the developer solution (contains Na_2CO_3 and paraformaldehyde) for 30 sec then the solution was removed and fresh developer added and incubated for 5 min. The developer was changed every 5 min until the desired staining intensity was obtained. The staining reaction was stopped by incubating the gel in stop solution (5% acetic acid) for 15 min.

2.2.6.2 Western Blotting

For Western blot analysis, proteins fractionated on SDS-PAGE gels were transferred to nitrocellulose or polyvinylidene fluoride (PVDF) membranes (Hybond-C or Hybond-P, Amersham) using a Bio-Rad transblot cell (Towbin *et al.*, 1979). A blotting stack was assembled in which the gel was in direct

contact with the membrane while sandwiched between two sheets of 3MM paper, which in turn were sandwiched between sponges (provided by the manufacturer). Electroblotting was carried out for 1h at room temperature at 250mA or overnight at 4°C at 50mA. Membranes (Hybond-C or Hybond-P, Amersham) were blocked in PBS with 5% dried milk overnight at 4°C or for 1h at room temperature with gentle shaking. Following 3 washes of the membrane in PBS with 0.05% Tween (PBS-T), the membrane was incubated in primary antibody (diluted in PBS-T with 1% dried milk) for 2 h at room temperature with shaking. Membranes were washed 3 further times in PBS-T then incubated with secondary antibody for 1 h at room temperature with shaking. Horseradish peroxidase conjugated anti-mouse IgG or protein A was diluted 1:1000 in PBS-T to detect monoclonal and polyclonal primary antibodies, respectively. Membranes were washed extensively in PBS-T then subjected to enhanced chemiluminescence (ECL) using the Amersham ECL system. Reagents 1 and 2 were mixed in equal proportion and the total volume poured directly onto the membrane and incubated for 1 min. Excess ECL reagent was drained from the membrane, which was then wrapped in clingfilm and exposed to Kodak X-OMAT S film for 5 sec to 10 min.

2.2.6.3 Stripping membranes for reprobing

Bound primary and secondary antibodies were removed from membranes by incubating the membrane in stripping buffer (0.288mM β-Mercaptoethanol, 2% v/v SDS, 62.5mM Tris-Cl pH 6.7) at 50°C for 30 min with shaking followed by 3 x 10 min washes in PBS-T. Membranes could then be blocked and reprobed as previously described.

2.2.7 Immunohistochemistry

Immunohistochemistry was carried out using the Vectastain® ABC Kit (Vector Laboratories). 4μm cross sections of paraffin-embedded rafts were dewaxed in xylene for 5 min and rehydrated in a graded series of alcohol washes (10 dips each in 100%, 100%, 95%, 70%, and 50% ethanol). Slides were washed in running tap water for 5 min followed by a 20 min incubation in 0.3% hydrogen peroxidase to quench endogenous peroxidase activity. Slides were then washed for 5 min in PBS-B (10mM sodium phosphate pH 7.5, 0.9% saline) and incubated for 20 min in normal blocking serum. Excess serum was removed from the sections and the slides were incubated in the appropriate dilution of primary antibody for 2 h. Following a 5 min wash in PBS the slides were incubated with diluted biotinylated secondary antibody for 30 min, then washed again for 5 min in PBS-B buffer. Pre-mixed ABC reagent (avidin and biotinylated horseradish peroxidase macromolecular complex) was incubated on the sections for 30 min and this was followed by a 5 min wash in PBS-B. Diaminobenzidine tetrahydrochloride (DAB) chromogen was used to localize peroxidase in the tissue sections by incubating the slides in a solution of DAB (with or without nickel chloride) for 10 min. DAB was removed and the slides were washed thoroughly in running tap water prior to counterstaining.

2.2.7.1 Counterstaining

Sections were counterstained in hematoxylin (Gill's Formula, Vector Laboratories) for 5 min, rinsed in tap water, and dipped 10 times in acid rinse solution (2% glacial acetic acid) to clear background staining. This was followed by a 1 min incubation in Scott's tap water substitute (0.04M $NaHCO_3$, 0.16M $MgSO_4$) to enhance the development of the hematoxylin colour. The slides were then washed in tap water and dehydrated in a graded series of alcohol washes (10 dips each in 50%, 70%, 95%, 100%

and 100% ethanol) and incubated in xylene prior to mounting with Pertex (Vector Laboratories).

2.2.8 RNA Binding Assay

2.2.8.1 Large-Scale *In Vitro* Transcription of RNA

Reactions were assembled at room temperature in the following order:

10x transcription buffer	20μl
100mM ATP, CTP, GTP, UTP	20μl
100uM DTT	10μl
RNasin (40U)	1μl
DNA template (≅500pmol RNA *)	χμl
T3 RNA polymerase (200U)	4μl
Diethyl pyrocarbonate (DEPC) dH_20 to 200μl	

* 309 x length of template (nt) = molecular weight of template

Concentration of template (pmol/ μl) = conc. of template (μg/ml) /molecular weight of template

Reactions were incubated at 37°C for 2 h followed by acid phenol:chloroform extraction and ethanol precipitation.

2.2.8.2 Linking RNA to Agarose Beads

In vitro transcribed RNA was covalently linked to adipic acid dihydrazide agarose beads by first incubating 500pmol of substrate RNA with 100mM sodium acetate (pH 5.0) and 5mM sodium periodate in the dark at room temperature for 1 h. The oxidised RNA was ethanol precipitated and resuspended in 500μl of 0.1M sodium acetate pH5.0. A 400μl aliquot of

adipic acid dihydrazide agarose bead 50% slurry (Sigma) was washed four times in 10ml of 0.1M sodium acetate pH5.0 and pelleted after each wash at 300rpm for 3 min (Heraeus Multifuge 3, refrigerated benchtop centrifuge). The washed beads were resuspended in 300μl of 0.1M sodium acetate pH5.0 and the periodate-treated RNA was added to the slurry and incubated for 12 h at 4°C on a rotating wheel. The RNA bound beads were pelleted at 300 rpm for 3 min as before and then washed three times in 1ml of 2M NaCl and a further 3 times in 1ml of buffer D (20mM HEPES-KOH pH7.6, 5% glycerol, 0.1M KCl, 0.2mM EDTA, 0.5mM DTT) with centrifugation after each wash at 300rpm for 3 min. A 400μl aliquot of Ag Poly(U) Type 6 linked beads (Pharmacia Biotech) were washed in 2M NaCl and buffer D as described above.

2.2.8.3 RNA Binding Reaction

The beads with the bound RNA and the Poly(U) beads were incubated separately in 650μl binding reactions containing 250μl of HeLa nuclear extract for 20 min at 30°C. The beads were pelleted by centrifugation at 1,000rpm for 3 min (Hettich EBA 12R, refrigerated microcentrifuge) and unbound proteins were removed by washing the beads four times in 1ml of buffer D with 4mM $MgCl_2$ and spinning at 1,000rpm for 3 min after each wash. Proteins bound to the RNA were eluted into 60μl of protein loading buffer by heating the beads to 90°C for 5 min. Eluted proteins were then fractionated by SDS-PAGE.

2.2.9 UV Cross-Linking

2.2.9.1 *In Vitro* Transcription of Radiolabelled RNA

An *in vitro* transcription kit (Stratagene) was used to generate radiolabelled RNA transcripts. Reactions were assembled in the following order at room temperature:

5x transcription buffer	2μl
2.5mM ATP, CTP, GTP	2μl
100uM UTP	1.2μl
100mM DTT	1μl
RNasin	0.5μl
linearized DNA template (150ng)	1μl
T7 RNA polymerase	0.5μl
α [^{32}P]-UTP (800 mCi/mmol)	2.5μl
	Final volume 10.2μl

Reactions were incubated at 37°C for 2 h. Unincoporated NTPs were removed from the transcription reaction by passing the reaction through an RNA mini quick spin column (Roche Diagnostics). The specific activity of the radiolabelled RNA was measured by Cerenkov counting in a Beckman liquid scintillation counter.

2.2.9.2 Cross-linking binding reaction

Radiolabelled RNA probes were incubated with HeLa nuclear extracts in 96 well dishes at room temperature in the following order:

2x UV X-linking binding buffer	10µl
(60mM KCl, 20mM HEPES pH7.6,	
1mM $MgCl_2$, 10% glycerol)	
Baker's yeast tRNA (10mg/ml)	2µl
^{32}P labelled RNA probe ($5x10^6$cpm)	χµl
Hela nuclear extract	2µl
Diethyl pyrocarbonate (DEPC) treated dH_2O to 20µl	

Reactions were incubated at room temperature for 20 min. UV cross-linking was performed using a Stratagene UV Stratalinker at a setting of $2.5x10^5$ µJoules. Unbound RNA was digested by the addition of 1µl RNase A (10µg/ml) and incubation at 37°C for 15 min. Proteins with bound RNA were eluted into protein loading buffer and separated by SDS-PAGE. Gels were exposed overnight using Kodak X-OMAT S film and for some experiments the separated proteins were transferred to a nitrocellulose membrane and Western blotted as previously described.

2.2.10 RNase Protection Assay (RPA)

2.2.10.1 Extraction of sample RNA

Total RNA was extracted from monolayer cells and raft tissue using TRIzol™ reagent (Gibco BRL) based on a modification of a previously published protocol (Ozbun *et al.*, 1997). Raft tissue was homogenized in 2ml of TRIzol using a Dounce homogenizer. For monolayer cells 2ml of TRIzol was added to a 100mm dish and the lysate passed several times through a 5ml pipette.

Following extraction in TRIzol, the homogenized samples were incubated at room temperature for 5 min and 0.2 volumes of chloroform were added. The samples were mixed vigorously for 15 sec by shaking and incubated at room temperature for 3 min. The phases were separated by centrifugation at 3,500rpm for 15 min at 4°C (Sorvall RT 6000B, refrigerated benchtop centrifuge) and the upper aqueous phase was transferred to a clean tube. The RNA was precipitated by the addition of 0.833 volumes of isopropanol followed by a 10 min incubation at room temperature. The samples were centrifuged at 9,500rpm (Sorvall SS34 Rotor, lowspeed centrifuge) for 10 min at 4°C to pellet the RNA. The pellet was then washed with 1ml of 70% ethanol, mixed by vortexing and centrifuged again at 8000rpm at 4°C for 15 min and finally resuspended in dH_2O. The quality of the RNA was checked by agarose gel electrophoresis to verify the presence of undegraded 28s and 18s ribosomal RNA.

2.2.10.2 Preparation of Riboprobe Template

Digesting 10μg of the plasmid pHPV16-RL1 with *Ssp*I generated a antisense HPV-16 L1 riboprobe template; map in appendix 1. Digesting the plasmid pGOZ31-L1 with *Hin*d III generated a antisense HPV-31 L1 template, map in appendix 1. The linearized plasmids were incubated with 150μg/ml of Proteinase K for 30 min at 50°C followed by phenol:chloroform extraction and ethanol preciptation.

2.2.10.3 *In vitro* transcription of a Radiolabelled Riboprobe

An *in vitro* transcription kit (Stratagene) and Ambion RPA II kit were used to generate radiolabelled RNA riboprobes by mixing the following components, in order, at room temperature:

10x Transcription Buffer	1μl
200mM DTT	0.5μl
10mM ATP, CTP, GTP	1.5μl
RNasin (12.5U)	0.5μl
DNA Riboprobe Template (500ng)	1μl
α [^{32}P] UTP	2.5μl
T7 RNA polymerase (10U)	0.5μl
Diethyl pyrocarbonate (DEPC) dH_2O to	20μl

Reactions were incubated at 37°C for 60 min and for a further 15 min following the addition of 1μl of RNase-free DNase I (2U/μl). The reaction was stopped by adding an equal volume of gel loading buffer (80% v/v formamide, 2mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol) and heating the sample at 90°C for 3 min. The full-length riboprobe was excised from a denaturing 5% acrylamide/8M urea gel (see below) following electrophoresis at 200v for 40 min and eluted overnight at room temperature in 350μl of elution buffer (0.5M NH_4OAc, 1mM EDTA, 0.1% SDS). The activity of the riboprobe was determined by Cerenkov counting in a liquid scintillation counter (Beckman LS 5000 CE).

5% acrylamide/8M urea gels (60ml) were prepared as follows:

Urea	28.8g
10xTBE	6ml
40% acrylamide	7.6ml
dH_2O	11.6ml
10% APS	480μl
TEMED	50μl

The solution was mixed thoroughly and incubated at 37°C to allow the urea to dissolve prior to the addition of APS and TEMED.

2.2.10.4 Hybridization of the riboprobe and sample RNA

For each sample, $8x10^4$cpm of ^{32}P labelled probe was mixed with 10μg of RNA. Control reactions consisted of the riboprobe mixed with 10μg of torulla yeast RNA (Ambion). Each mixture was ethanol precipitated and the pellet resuspended in 20μl of Hybridization buffer (80% v/v formamide, 0.1M $C_6H_5Na_3O_7$ pH6.4, 0.3M NaOAc pH6.4, 1mM EDTA) and incubated for 16 h at 45°C.

2.2.10.5 RNase digestion of hybridised probe and sample RNA

200μl of RNase solution (1:100 dilution of RNase mix (250U/ml RNase A; 10,000U/ml RNase T1) in RNase digestion buffer (proprietary) was added to each sample including one yeast RNA control reaction, designated Y1, the other yeast RNA control reaction, designated Y2, received only RNase digestion buffer. Samples were incubated at 37°C for 30 min and the reaction stopped by adding 300μl Solution Dx (RNase inactivation/precipitation solution; proprietary) to each tube. Protected fragments were precipitated by freezing at –20°C for 15 min and pelleted by centrifugation at maximum speed for 15 min at 4°C (Hettich EBA 12R, refrigerated microcentrifuge). Pellets were resuspended in 8μl of gel loading buffer, heated at 90°C for 3 min, and electrophoresied through a denaturing 5% polyacrylamide / 8M urea at 200v for 2 h. Gels were exposed overnight to Kodak X-OMAT S film to detect the protected fragments.

CHAPTER 3 – Characterisation of HPV-16 positive epithelial cell lines

All HPVs have a strict tissue tropism and are absolutely dependent on terminally differentiating keratinocytes for viral late protein expression, such that, only basal keratinocytes capable of undergoing terminal differentiation can support the complete viral life cycle (Meyers *et al.*, 1994a). Early attempts at establishing HPV-positive lines using clinical material consistently resulted in the production of cell lines with integrated viral DNA, which could not complete the viral life cycle (Friedl *et al.*, 1970, Pattillo *et al.*, 1976, Durst *et al.*, 1986, Pirisi *et al.*, 1987). Therefore, the establishment of the W12 cell line was a very important development in the study of HPV-16 gene expression. This cell line was derived from a HPV-16 positive, low-grade cervical lesion and is an immortalised but not fully transformed line (Stanley *et al.*, 1989). Approximately 100 copies of the HPV-16 genome are stably maintained in an episomal form in these cells, although, with continued growth *in vitro,* the cells have developed an aneupolid karyotype (Stanley *et al.*, 1989). However, while the W12 cells have acquired an extended life span they remain responsive to cholera toxin and epidermal growth factor (EGF) similar to normal cervical keratinocytes cultured *in vitro*, indicating that the W12 cell line is operationally equivalent to a basal epithelial cell latently infected with HPV-16 (Sterling *et al.*, 1990). In monolayer culture, W12 cells exhibit typical basal keratinocyte morphology, undergo limited stratification and express basal cell specific keratins but not markers of terminal differentiation (Greenfield and Stanley, unpublished data; Chapter 3). However, when these cells are transplanted into the flank of the athymic mouse, an epithelial lesion develops with many histological features in common with the lesion from which the line was derived. Numerous cells in the granular layer are positive for the late viral proteins L1 and E1^E4, and HPV-16 viral particles are observed in the degenerating nuclei in the uppermost layers of the graft (Sterling *et al.*, 1990, Sterling *et al.*, 1993). This implies that, under appropriate conditions, this cell line can

undergo a productive HPV-16 life cycle and therefore represents an invaluable tool in the study of HPV-16 late gene expression in response to epithelial differentiation.

Following the derivation of the W12 line, another group found that, in their hands, the HPV-16 genome was unstable in this line and rapidly became integrated with continued *in vitro* growth (Jeon *et al.*, 1995a). To overcome this, clonal populations of the W12 line were derived which harboured multiple copies of extrachromosomal viral DNA which appeared less inclined to integrate; this clonal derivative was called the 20863 line (Jeon *et al.*, 1995a). The 20863 line has also been shown to grow well both in monolayer culture and when applied to the organotypic raft system. The L1 capsid protein is readily detectable in the granular layers of these rafts, although the pattern of epidermal differentiation appears slightly perturbed (Flores *et al.*, 1999). The 20863 line was further sub-cloned by another group to yield a line called 2W12 which, like the parental line, maintains the HPV-16 genome episomally, however, little is known about this line and it will discussed only briefly in this chapter (Ozbun and Meyers, unpublished data).

The aim of the work presented here was to study the regulation of HPV-16 late gene expression during epithelial cell differentiation and therefore, we wished to use the W12 and 20863 cell lines as a model system of a productive HPV-16 infection since these cells are considered to be representative of the situation in a pre-invasive lesion and seem to be capable of completing the viral life cycle when transplanted onto nude mice or applied to the organotypic raft culture system. However, neither of these lines has been particularly well characterised and so we set about characterising the growth of these lines both in monolayer culture and on organotypic rafts whilst paying particular attention to the pattern of late gene expression.

3.1. The HPV-16 genome is episomal

Total genomic and/or Hirt DNA extracts were routinely prepared from monolayer and organotypic raft cultures of W12 and 20863 cells and analysed by Southern blotting to verify the state of the viral DNA in the cell lines. Figure 3.1 shows a typical blot of episomal viral DNA probed with a full-length HPV-16 DNA probe. Bands representing open circular and supercoiled viral DNA, and full-length linearized HPV-16 DNA, are indicated in Fig. 3.1A and B, lanes 2 and 4, and lanes 3 and 5 respectively.
This experiment was repeated regularly throughout the course of this work to ensure the HPV-16 genome remained episomal in the cell lines used (data not shown). Contrary to the report by Jeon *et al.*, (1995a) we found that the HPV-16 genome was stably maintained as episomes in the W12 line, at least to passage number 19.

3.2.1. Protein expression in W12 monolayer cultures

An earlier key study characterising the growth of the biopsy-derived HPV-31 cell line CIN 612, described the use of phorbol esters to facilitate the completion of the HPV-31 life cycle and production of infectious virions in organotypic raft culture (Meyers *et al.*, 1992). Phorbol esters, such as PMA and C8:0, are thought to bring about a more complete differentiation programme through the activation of the PKC pathway. Although the exact mechanism of action remains unclear, enhanced expression of the differentiation markers K10 and filaggrin has been reported (Meyers *et al.*, 1992). Phorbol ester treatment of W12 monolayer cultures has been shown to relieve the inhibitory effects of a negative regulatory element located within the 3'UTR of HPV-16 (Dietrich-Goetz *et al.*, 1997). Also, PMA-treatment appears to affect the level of expression and distribution of a number of RNA processing factors known to interact with this element (Dietrich-Goetz *et al.*, 1997, Koffa *et al.*, 2000). Initially, I wished to establish

the effects, if any, of prolonged growth in monolayer culture on viral late protein expression in the absence of phorbol esters. This had not previously been addressed, although an earlier studying using C8:0-treated monolayer cultures of CIN 612 cells reported no detectable HPV-31 late protein expression (Hummel *et al.*, 1995). Protein extracts prepared on consecutive days from W12 and 20863 cells grown for 5 to 10 days were analysed by Coomassie blue staining, on the basis of total protein in μg (as calculated by Bradford assay) (Fig. 3.2A), or as equivalent numbers of cells (Fig. 3.2B). Comparing figures 3.2A and B, it is clear that analysing protein extracts in terms of total protein allows a more accurate comparison than that resulting from analysis based on similar cell counts. While the extracts analysed in Fig. 3.2B have been prepared from similar numbers of cells, the amount of protein appears to decrease steadily from day 5 to day 10; a possible explanation for this might be that the level of expression of non-essential cellular proteins is downregulated with differentiation. This experiment was repeated at least three times using different protein extracts and similar results were obtained each time (data not shown).

3.2.2. L1 expression in monolayer cultures

Total protein extracts were prepared from 20863 and W12 cells grown for 5 to 10 days in monolayer culture and, based on the results shown in Fig. 3.2, we chose to analyse 10μg of each extract for HPV-16 L1 protein expression. Surprisingly, Western blotting with the CamVir 1 antibody (anti-HPV-16 L1) identified a specific band of approximately 59kDa in each extract (Fig. 3.3A, lanes 1 to 6). However, the intensity of the band appeared to increase from day 5 to day 7 and this intensity was maintained until day 10. Although the quality of this blot could be better, if the level of L1 expression is compared between days 5 and 10 only, lanes 1 and 6, it is clear that while there is some L1 protein in day 5 monolayer cultures of 20863 cells, the level of expression of this viral protein increases substantially in cells cultured for 10

days. One shortcoming of this experiment, however, is that the cells used had previously been grown to sub-confluence, for approximately 5 to 7 days, prior to use in this experiment. It is not yet known if the differentiation programme in W12 or 20863 cells is reset when these lines are serially passaged. If this is not the case, then the extracts used in Fig. 3.3A are further along the differentiation pathway than if the cells were freshly recovered from frozen specifically for the purposes of this experiment. This might help explain why L1 expression is detected even after only 5 days in monolayer culture (lane 1) and the level of late protein expression does not appear to increase from day 7 to day 10 (lanes 3 to 6). To test this, W12 cells were recovered from liquid nitrogen and the time course begun immediately. Protein extracts were prepared following 5 days and 10 days growth and analysed again for L1 expression; J2 3T3 extract was included as a negative control. It appears that under these experimental conditions only W12 cells cultured for 10 days express detectable levels of L1 protein (Fig. 3.3B, lane 2). This experiment was repeated using freshly prepared extracts on at least three occasions and the results were very similar (data not shown). This observation suggests that W12 or 20863 cells harvested after only 5 days growth in monolayer culture are predominantly undifferentiated whereas growing these cells for 10 days, even in the absence of phorbol esters, results in the initiation of differentiation, as indicated by an increase in the level of expression of the capsid protein, L1.

The protein profile of W12 day 5 and day 10 extracts was compared following Coomassie blue staining (Fig. 3.4A) and appears to be very similar, at least at this level of sensitivity. One might argue that the apparent expression of L1 protein in monolayer culture is an artefact of the HPV-16 cell lines, or the culturing methods used in our laboratory. I therefore attempted to ascertain if these epithelial cells were undergoing differentiation, since the tight linkage between viral late protein expression and keratinocyte differentiation has been well documented. The level of

expression of involucrin, a known marker of differentiation that is generally expressed in the spinous and granular layers of the epithelium, was compared between day 5 and day 10 extracts by Western blotting with SY5, an anti-involucrin antibody. Involucrin synthesis is a specific marker of squamous differentiation in keratinocytes, since no other cell types produce this protein, at least in any significant quantity (Fuchs *et al.*, 1994). The level of this protein is dramatically increased in day 10 extracts compared to day 5, supporting the hypothesis that while day 5 cells are relatively undifferentiated, day 10 cells are indeed differentiating (Fig. 3.4B). Western blotting the W12 day 5 and day 10 extracts with the 6CS antibody (anti-GAPDH) indicates that similar amounts of protein were loaded in each lane (Fig. 3.4C lanes 1 and 2).

Taken together these results suggest that W12 and 20863 cells begin to differentiate following prolonged growth in monolayer culture even in the absence of phorbol ester treatment. This observation contrasts with several previous reports and also raises the possibility that previous studies involving a comparison of "undifferentiated" and differentiated cell populations following phorbol ester treatment were perhaps comparing differentiating samples and slightly more differentiated samples instead (Hummel *et al.*, 1995, Ozbun *et al.*, 1997, Koffa *et al.*, 2000).

3.2.3. Protein expression in monolayer cultures of HaCaT cells

In the previous experiment the J2 3T3 line was used as a negative control, however, this murine fibroblast line cannot be induced to differentiate and is therefore not the most suitable control for this type of experiment. Attention therefore was directed to the HaCaT cell line for use as a potential control for the differentiation of epithelial cells, since although this line is immortalised, it has retained the ability to differentiate (Boukamp *et al.*, 1988, Schoop *et al.*, 1999). HaCaT cells were grown in monolayer culture in a similar manner to that used for W12 and 20863 cells and protein extracts were prepared from

cells harvested following 5 days and 10 days in culture. Coomassie blue staining was used to compare the protein profile of these extracts, however, there appeared to be no significant differences between these extracts (Fig. 3.5A). Immunoblotting day 5 and day 10 HaCaT extracts with an anti-involucrin antibody, SY5, revealed that, similar to the situation in W12 cells, the level of expression of this differentiation marker is significantly increased between days 5 and 10 (Fig. 3.5B). Western blotting with 6CS, the anti-GAPDH antibody, confirmed that similar amounts of protein had been loaded for each sample (Fig. 3.5C). This result indicates that epithelial cells, which lack HPV-16, can also be induced to differentiate through prolonged growth in monolayer culture. Differentiating day 10 HaCaT cells are therefore, I believe, a more suitable negative control in experiments designed to analyse the differential expression of viral late proteins.

3.2.4. Viral late protein expression in monolayer cultures of epithelial cells

Protein extracts prepared from day 5 and day 10 W12 cells and day 10 HaCaT cells were Western blotted with CamVir 1, an antibody directed against HPV-16 L1, to ensure that the previous result (Fig. 3.3B) could be repeated in the presence of a more suitable control. From the blot shown in Fig. 3.6A, it appears that, as expected, HaCaT cells do not express HPV-16 L1. However, as shown previously, day 5 W12 cells express a small amount of L1 protein, while the level of expression of this late viral protein is increased in day 10 W12 cells (Fig. 3.6A). This experiment was repeated at least three times with fresh extracts and similar results were recorded each time. In order to ensure that similar amounts of protein had been analysed for each sample, this blot was stripped and reprobed with an antibody directed against GAPDH, 6CS (Fig. 3.6B) and it appears that each sample on this blot contains a similar amount of protein.

Extracts similar to those used in the above experiments were immunoblotted using an antibody directed against HPV-16 E1^E4, TVG 402. I found that when 10μg of each extract were analysed, only a very faint band could be detected in W12 day 10 samples following a long exposure (data not shown). The amount of extract was then increased to 20μg in order to improve the detection of E1^E4. As shown in Fig. 3.6C lane 3, a band of approximately 10kDa could be readily detected in W12 day 10 extracts, but day 5 W12 extracts appeared to express only a low level of this viral protein (Fig. 3.16C lane 2). Day 10 HaCaT cells did not express HPV-16 E1^E4 (Fig. 3.6B lane 3). When this blot was stripped and reprobed with the 6CS antibody each lane appeared to contain a comparable amount of protein as indicated by the level of expression of GAPDH (Fig. 3.6D). The apparent absence of expression of the late viral proteins, L1 and E1^E4, in day 5 W12 extracts further supports the hypothesis that these cells are relatively undifferentiated, while day 10 W12 cells appear to be differentiating, as indicated by the enhanced expression of involucrin and the presence of late viral proteins.

3.2.5. Protein expression in untreated and phorbol ester-treated monolayer cultures

Having established that W12 and 20863 cells begin to differentiate if grown in monolayer cultures for extended periods of time, I next wanted to compare extracts prepared from 20863 and W12 cells grown for 5 and 10 days in the presence and absence of the phorbol ester, C8:0. A Coomassie stain of these extracts did not suggest that there were any significant differences between the protein profiles of C8:0 treated and untreated cells (Fig. 3.7A). The extracts were then analysed by Western blotting with the SY5 antibody, directed against involucrin (Fig. 3.7B). As before, the level of expression of this cellular protein increased between day 5 and day 10 cells, however, C8:0 treatment did not appear to have any significant effect on the

expression of involucin (Fig. 3.7B, lanes 3 and 4). When the same samples were immunoblotted with the CamVir 1, ab anti-HPV-16 L1 antibody, a small, but similar, amount of L1 protein was detected in treated and untreated day 5 extracts (Fig. 3.7C, lanes 1 and 2). However, C8-treated day 10 samples appeared to express slightly more L1 protein than the corresponding untreated sample (Fig. 3.7C, lanes 3 and 4). These observations tend to suggest that while phorbol-ester treatment of W12 and 20863 monolayer cultures does not affect the level of expression of the differentiation marker, involucrin, C8:0 treatment may have a positive effect on the expression of the late viral protein, L1.

3.3.1. Organotypic raft cultures of HPV-16 epithelial cell lines

The organotypic raft culture system is a very useful *in vitro* system for the propagation of HPV since it is capable of reproducing the keratinocyte differentiation programme seen *in vivo*, and allows viral late protein expression (Meyers *et al.*, 1994a, b). Keratinocyte lines transformed by HPV-16 or HPV-18 appear to undergo abnormal differentiation, comparable to the *in vivo* situation, when cultivated on rafts (McCance *et al.*, 1988, Rader *et al.*, 1990, Merrick *et al.*, 1992). However, when cell lines containing episomal viral genomes are applied to the raft culture system they stratify, differentiate and express viral late proteins in the upper layers of the epithelium (Rader *et al.*, 1990, Bedell *et al.*, 1991, Meyers *et al.*, 1992, Frattini *et al.*, 1997, Meyers *et al.*, 1997). In addition, treatment of raft cultures of the HPV-31 cell line, CIN 612, with an activator of the protein kinase C (PKC) pathway was found to result in production of mature infectious virions (Meyers *et al.*, 1992). However, more recent reports using HPV-11, -18 and -31 cell lines, established from cloned viral DNA, present evidence of the complete viral life cycle in the absence of phorbol ester treatment (Frattini *et al.*, 1996, 1997, Meyers *et al.*, 1997, Thomas *et al.*, 2001). It has previously been reported that phorbol ester-treatment of W12 raft cultures leads to a strong induction

of the capsid proteins, L1 and L2 (Higgins and Stanley, unpublished data). However, although mature HPV-16 virions are produced from these raft tissues they do not appear to be infectious (Meyers and Ozbun, personal communication). The reason for this is not yet understood, although it may be an artefact of the W12 line. Nevertheless, organotypic raft cultures of the W12 and 20863 lines are invaluable tools in studying the differential control of HPV-16 late protein expression and so we have used this system to cultivate the W12, 20863 and 2W12 lines as well as the HPV-31 cell line, CIN 612 9E, in our laboratory. Rafts were harvested following 12 days at the air-liquid interface and histochemically stained using hematoxylin and eosin (H&E). Figure 3.8 shows typical H&E stains of C8-treated W12 and 2W12 raft tissues; variations in colour are due to differences in the depth of counterstaining. Although I have not shown the H&E stains of the 20863 and CIN 612 raft sections, each line tested appeared to form stratified layers and closely resembled tissue from a CIN I lesion. Although the thickness of the stratified epithelium differed between the lines, in general, the lower third of the epithelium contained basal-like cells, whereas differentiation occurred in the upper epithelium. Overall, I generally observed that the basal layers appeared as an organised arrangement of cuboidal cells with large nuclei and little cytoplasm. Moving upwards, the cell shape changes gradually to flattened, enucleated squames in the uppermost epithelial layers as a result of differentiation and stratification (Fig. 3.8A and B). Unlike normal cervical tissue, these HPV-positive lines form cornified layers as indicated in Fig. 3.8. In addition, some abnormal mitotic figures were observed in cells beyond the basal layer (Fig. 3.8 A and B). There appeared to be little morphological difference between untreated and C8-treated organotypic rafts cultivated using each of the cell lines tested (data not shown). This is unsurprising because although phorbol-ester treatment is believed to result in a more complete differentiation programme it is unlikely that these subtle differences would be visible using this staining technique (Meyers *et al.*, 1992). The cornified layer of C8-treated 2W12 organotypic rafts appears to be thinner

than that of the C8-reated W12 samples and also seems ruptured in places perhaps suggesting that virus-containing cells have been shed from this superficial layer. However, this might also be an artefact of the procedure used to prepare the section for staining. However, as with the W12 line, while this 2W12 line produces mature virions on organotypic rafts they are not believed to be infectious (Ozbun and Meyers, personal communication.). The 2W12 line was sub-cloned from the 20863 line which in turn was derived from the parental W12 line. It is possible therefore that the selective pressures used to isolate this line have led to the outgrowth of cells harbouring episomal HPV and a high proportion of integrated viral DNA and thus bare more resemblance to a CIN II lesion. Due to time constraints this line was not characterised any further.

3.3.2. Protein expression in HPV-16 raft tissue

Protein extracts were prepared from C8-treated or untreated W12 or 20863 organotypic raft cultures and compared following Coomassie blue staining (Fig. 3.9A). Not surprisingly the protein profile of both samples was very similar, although the band at approximately 60kDa appears stronger in the C8:0-treated sample compared to the untreated sample. The overall protein profile from the raft material was comparable to the profile generated from monolayer cultures, although the intensity of some bands, possibly keratins, is stronger. The raft extracts were analysed for terminal differentiation by Western blotting with SY5, the anti-involucrin antibody and both samples contained a large amount of involucrin protein and it was difficult to establish if there was any difference between untreated and C8-treated samples (Fig. 3.9B lanes 1 and 2). This result is not unexpected given the fact that the organotypic raft system is capable of accurately recreating the epithelial differentiation programme. The raft extracts were then immunoblotted with the anti-GAPDH antibody 6CS (Fig. 3.9C), which indicated that each sample contained a similar amount of protein.

Immunohistochemical staining of harvested raft tissue was performed using antibodies directed against the epidermal differentiation markers keratin type 10 (K10) and involucrin. In normal epithelia, K10 is expressed in the suprabasal layers beginning in the spinous layer, while involucrin is found only in the upper spinous and granular layers of human epidermis (Fuchs *et al.*, 1994). This experiment was repeated several times for each line and a representative result is shown of the C8-treated W12 line (Fig. 3.10A). Cells positive for K10 expression are stained brown and while some basal layer cells appear stained, the majority of positive cells are in the suprabasal layers. A similar result was observed for each of the HPV-lines tested and also the HaCaT cell line, which was included as a control for epidermal differentiation (data not shown). In agreement with previous studies the HaCaT rafts that I generated did not appear to form a cornified layer and this would tend to suggest that HaCaT rafts are unable to complete the full terminal differentiation programme (Boelsma *et al.*, 1999, Schoop *et al.*, 1999). In Fig. 3.10B, C8-treated W12 cells expressing involucrin are stained brown and it is clear that these cells are found predominantly in the upper spinous and granular layers, in agreement with the pattern of involucrin expression in normal differentiated epidermis. Each of the HPV-positive lines and the HaCaT cell line yielded similar results (data not shown).

3.3.3. Viral late protein expression in organotypic raft cultures

Since it appeared that the epithelial differentiation programme had been recreated using HPV-16 cell lines on the raft system, I next attempted to establish if the viral late proteins, L1 and E1^E4, were expressed and determine the effects, if any, of C8:0 treatment on the level of expression of these proteins. Protein extracts prepared from untreated and C8-treated W12 raft tissue were Western blotted with CamVir 1, an anti-HPV-16 L1 antibody, and it appeared that C8-treated rafts expressed slightly more L1 protein than untreated rafts (Fig. 3.11A, lanes 1 and 2). This observation is in agreement with the result generated using untreated and C8:0-treated W12 day 10 monolayer samples (Fig. 3.7C, lanes 3 and 4). This blot was stripped and reprobed with the anti-GAPDH antibody, 6CS, and the level of GAPDH protein in both samples appears very similar (Fig. 3.11A, lanes 3 and 4). The raft samples were also Western blotted with the TVG 402 antibody, directed against HPV-16 E1^E4. Similar to the situation with L1, the level of expression of E1^E4 appeared to be slightly upregulated in C8-treated rafts compared to untreated samples (Fig. 3.11B, lanes 1 and 2). As before, this blot was stripped and reprobed with 6CS and the result indicates that comparable amounts of protein had been loaded for each sample (Fig. 3.11B). These experiments were repeated at least three times with different extracts and the results were consistently similar. These results suggest that while both untreated and C8:0-treated W12 rafts produce a readily detectable amount of HPV-16 late proteins, treatment with phorbol ester increases the level of expression of the viral proteins L1 and E1^E4.

The rafts were further analysed for viral late protein expression by carrying out immunohistochemical staining using antibodies raised against the HPV-16 late proteins E1^E4 and L1. Previous studies detected the non-structural E1^E4 protein in the upper layers of the epidermis, and experiments using the TVG 402 antibody indicated that this viral protein is localized to the

expression due to the presence of background staining. In addition, it appears that the anti HPV-16 E1^E4 antibody, TVG 402, that I used in this experiment does not work well on paraffin-embedded material, and since the sections that I used originated from paraffin-embedded raft tissue, it is possible that this may have affected the efficiency of epitope recognition. An additional HPV-16 E1^E4 antibody, TVG 405, generated by the same group is reported to be more suitable for paraffin-embedded tissue (Doorbar *et al.*, 1997), unfortunately due to a lack of time this experiment could not be repeated using this antibody. The anti-HPV-16 L1 antibody, CamVir 1, has previously been used to examine HPV-16 positive clinical material and nude mouse grafts of the W12 line; in each case, L1 protein expression was confined to the upper granular layers of the epithelium (McClean *et al.*, 1990, Sterling *et al.*, 1990, Sterling *et al.*, 1993). Cells staining positively for L1 expression following immunohistochemistry of W12 raft sections are blue/black in colour, as shown in the representative section in Fig. 3.12A. A number of L1-positive cells are present in the granular layer of this section but no such expression was apparent in the HaCaT control (data not shown). While there appears to be a distinct patch of positively-stained cells in the W12 raft section shown, there also appears to be some diffuse staining in the upper granular layer, particularly to the right of the positively stained patch, and this observation is similar to previous reports utilising rafts containing cells harbouring HPV-16, HPV-18 and HPV-31 (Frattini *et al.*, 1996, Frattini *et al.*, 1997, Flores *et al.*, 1999). Cells in the granular layer are undergoing nuclear breakdown and this might help to explain this staining pattern.

3.3.4. L1 mRNA expression in organotypic rafts

Since C8-treatment appears to have a slight positive effect on the level of expression of the L1 protein, I next attempted to determine the effect of C8:0 treatment on the level of HPV-16 and HPV-31 L1 mRNAs using RNase protection assays (RPAs). Late viral transcripts have previously been detected in undifferentiated HPV-positive cells, and C8:0 treatment of rafts of the HPV-31 cell line. CIN 612, results in an increase in expression of both L1 and L2 mRNAs (Hummel *et al.*, 1995, Ozbun *et al.*, 1997). However, the effect of C8 treatment on HPV-16 L1 mRNA expression has not yet been examined. The level of HPV-31 L1 mRNAs appears to increase with C8:0 treatment of CIN 612 organotypic rafts, in agreement with previous reports (Fig. 3.13A). When HPV-16 L1 mRNA levels were compared between treated and untreated rafts it also appeared that C8:0 treatment resulted in a slight increase in HPV-16 L1 mRNA expression, although, the difference between untreated and treated mRNA samples was not as pronounced as previously observed for HPV-31. Nonetheless, this experiment was repeated with fresh extracts on a number of occasions and similar results were obtained.

3.3.5. Viral DNA amplification in organotypic rafts

To further investigate the extent of the effect of phorbol-ester treatment on rafts containing episomal HPV genomes, the level of viral DNA amplification was compared, by Southern blot analysis, using DNA prepared from untreated and C8-treated W12 and 20863 raft tissue containing HPV-16 genomes and CIN 612 raft tissue containing HPV-31 genomes (Fig. 3.14). In the uncut samples, bands representing open-circular and supercoiled viral DNA are arrowed, while in the restriction enzyme digested samples, linearised viral DNA is shown, running at approximately 8kb, thus confirming that these rafts contain episomal viral DNA. For both HPV-16 and HPV-31 there appears to be little difference in the amount of open circular and supercoiled viral DNA between untreated and C8-treated samples (Fig. 3.14 A and B, lanes 1 and 3). However, when the amount of linearized viral DNA is compared between treated and untreated samples it is obvious that C8-treatment of both W12, HPV-16, and CIN 612, HPV-31, containing rafts, results in a upregulation of viral DNA amplification (Fig. 3.14 A and B, lanes 2 and 4). While this C8:0-dependent increase in HPV-31 viral DNA amplification in the CIN 612 line has been previously reported (Meyers *et al.*, 1992, Ozbun *et al.*, 1998b), this is the first time, to our knowledge, that a similar effect has been reported using raft tissue containing episomal HPV-16 genomes.

3.4. Discussion

The study of the HPV life cycle in tissue culture has been hampered by the lack of a cell-culture system that stably maintains HPV DNA episomally and the difficulty in recreating the three dimensional structure of the epithelium to induce the full HPV life cycle. However, the derivation of non-malignant cell lines, which maintain HPV genomes episomally, and the cultivation of these keratinocyte lines on the organotypic raft culture system has opened the way for a fuller understanding of the HPV life cycle.

The central aim of this thesis was to study the regulation of HPV-16 late gene expression during epithelial cell differentiation. Initially, I set about characterising the growth of the W12 and 20863 cell lines. Both these lines contain extrachromosomal copies of the HPV-16 genome and, under differentiating conditions, the virus is capable of completing a productive life cycle, although the mature virions are not infectious (Sterling *et al.*, 1990, Sterling *et al.*, 1993, Flores *et al.*, 1999). In monolayer culture it appears that prolonged growth of these cell lines results in an induction of differentiation, even in the absence of phorbol ester treatment. W12 or 20863 cells harvested following 10 days in monolayer culture, express a high level of the differentiation marker involucrin. *In vivo*, the level of involucrin expression is directly related to the degree of cervical keratinocyte differentiation (Fuchs, 1990). Day 10 W12 or 20863 cells also express detectable levels of the HPV-16 late proteins E1^E4 and L1. Previous studies have reported the presence of late protein transcripts in monolayer cultures (Stoler *et al.*, 1989, Doorbar *et al.*, 1990, Hummel *et* al., 1992, Pray *et* al., 1995, Ozbun *et al.*, 1997, Koffa *et al.*, 2000); however, to our knowledge, this is the first report of the detection of HPV-16 late viral proteins in monolayer culture, although Ozbun *et al.*, (1997) suggested that late proteins may be synthesized in monolayer cultures but that they are present at levels below the sensitivity of currently used techniques. One major implication of our results is that it raises the possibility that previous experiments comparing phorbol ester-

treated and untreated cell samples as differentiated and undifferentiated populations were effectively comparing two differentiating samples, since it appears that phorbol ester treatment, at least of the W12 and 20863 lines, is not necessary for the induction of differentiation. Conversely, analysis of W12 and 20863 cells following 5 days in monolayer culture reveals a low level of expression of involucrin and little or no late viral protein expression. This is an important observation, particularly in light of the results obtained with day 10 cells, since it means that we now have a reliable source of undifferentiated cells at our disposal, which are capable of mimicking the non-productive stage of the viral life cycle. When monolayer cultures of W12 and 20863 cells were treated with the phorbol ester C8:0, it emerged that C8-treatment enhanced the level of expression of HPV-16 L1 but did not appear to effect the expression of involucrin, following 10 days in monolayer culture. Taken together, these results suggest that while non-treated W12 or 20863 cells grown in monolayer culture for 5 days are undifferentiated, after 10 days these epithelial cells have begun to differentiate and C8:0-treatment generates slightly more differentiated cells. Therefore, both untreated and phorbol-ester treated monolayer cultures of W12 and 20863 cells are a valuable resource in the study of differentiation-dependent viral late gene expression.

However, while monolayer cultures are useful, their use is somewhat limited by the fact that they are unable to support complete terminal differentiation. To this end, the organotypic raft culture system is an invaluable technique since it can mimic the epithelial differentiation programme similar to that found in intact tissue. I have established this tissue culture system in our laboratory and have successfully grown a number of HPV-positive cell lines on organotypic raft cultures. Histological staining indicates that the rafts resemble low-grade cervical lesions and immunohistochemical analysis using antibodies against the cellular differentiation markers K10 and involucrin indicates that differentiation is proceeding as expected. Protein extracts prepared from raft cultures following 12 days at the air-liquid

interface expressed readily detectable levels of the late viral proteins E1^E4 and L1 in untreated rafts, and in agreement with the situation in monolayer cultures, C8:0 treatment moderately increases the expression of these proteins. Immunohistochemical staining using antibodies directed against these viral proteins revealed that they are expressed exclusively in the upper granular layers of the epithelium, similar to the situation in clinical material. Moreover, analysis of HPV-16 and HPV-31 L1 mRNA transcripts in total RNA samples prepared from harvested raft cultures indicated that, in agreement with previously published results using the CIN 612 HPV-31 line, C8:0 treatment results in enhanced transcription of this late message both in HPV-31 and HPV-16 rafts. Furthermore, C8:0 treatment also increases the rate of amplification of episomal viral DNA in both HPV-16 and HPV-31 containing raft cultures. It has been well documented that phorbol ester treatment of organotypic raft cultures of the CIN 612 line brings about a more complete differentiation programme, and is necessary for the productive stage of the HPV-31 life cycle (Meyers *et al.*, 1992, Ozbun *et al.*, 1997, 1998a, 1998b). However, to our knowledge, this is the first comprehensive analysis of the effects of C8:0 treatment on HPV-16 containing monolayer cultures and organotypic rafts. It has previously been proposed that HPV-31 late gene expression is controlled, to some extent, by post-transcriptional mechanisms dependent on C8:0 activation of the protein kinase C (PKC) pathway (Hummel *et al.*, 1995). However, it appears that this proposition does not extend to all HPVs. I have shown that while C8:0 treatment enhances the level of HPV-16 late protein expression, it is not necessary, since late viral proteins were detectable both in untreated monolayer cultures and organotypic rafts of the biopsy-derived HPV-16 positive lines, W12 and 20863. In addition, infectious HPV-18 virions have recently been produced using the organotypic raft culture system in the absence of C8:0 treatment (Meyers *et al.*, 1997), and an HPV-11 cell line, recently established using cloned viral DNA, was also reported to undergo a productive life cycle in semisolid media, in the absence of phorbol esters (Thomas *et al.*, 2001).

It appears that HPV-31 positive cell lines that maintain transfected viral DNA episomally can also complete a productive life cycle when grown on untreated rafts (Frattini *et al.*, 1996, 1997). These observations tend to suggest that the requirement for PKC-induced differentiation changes to facilitate virion synthesis is a particular property of the biopsy-derived HPV-31 cell line, CIN 612.

Previously published reports have included images of histological sections of nude mice grafts of the W12 line (Sterling *et al.*, 1990, Sterling *et al.*, 1993). Pictures of the W12 line cultivated on the organotypic raft culture system have, until now, not been published. As well as generating a large amount of useful information regarding the growth, differentiation and pattern of gene expression in HPV-16 infected cells, this extensive characterisation of the W12 and 20863 lines has also provided us with a wide range of material from undifferentiated monolayer cultures through to terminally differentiated raft tissue for use in the study of HPV-16 late gene expression in response to epithelial differentiation.

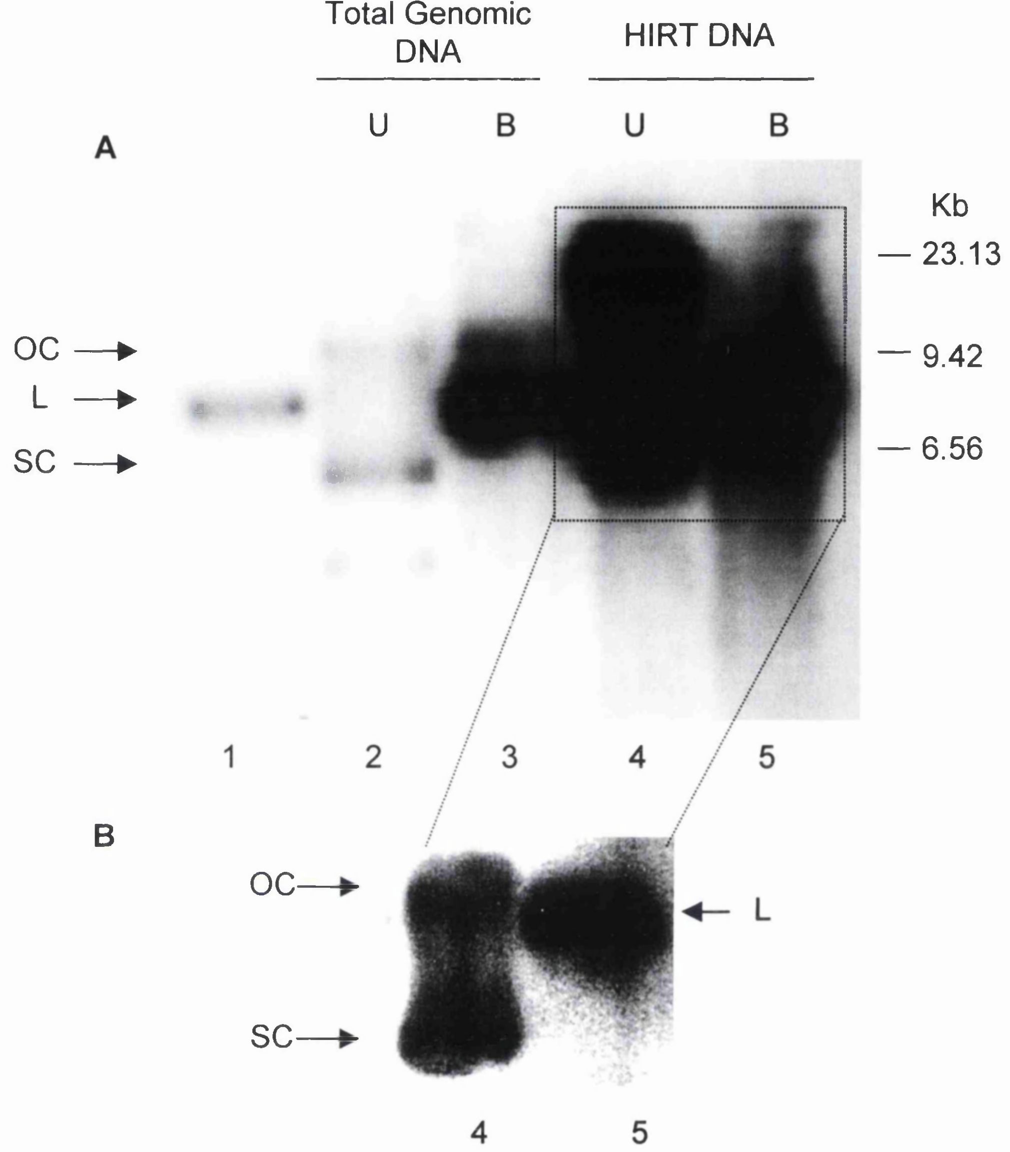
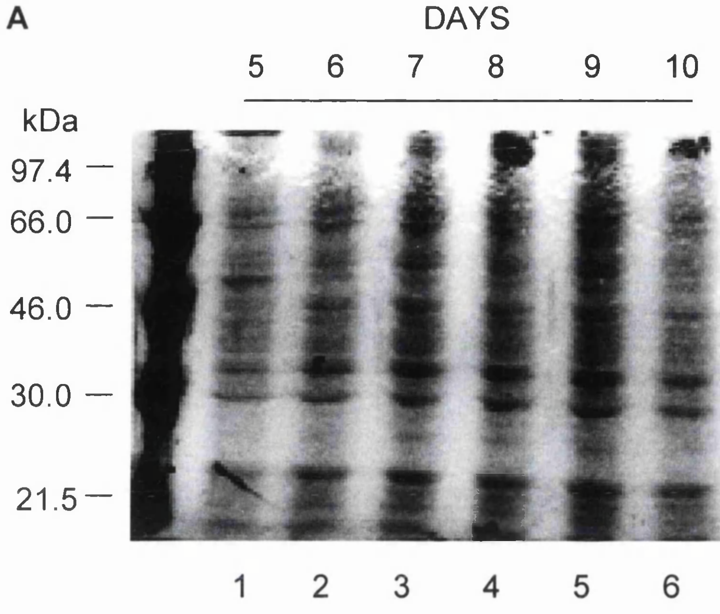


Fig. 3.1. Southern blot analysis of HPV-16 DNA. The blot was hybridized with a full-length HPV-16 DNA probe, prepared from the insert of pBS 114K. **A.** Lane 1 is a copy number control showing 100 copies of *Bam* H1 linearized, full-length, HPV-16 DNA. Total genomic DNA (lanes 2 and 3), and Hirt DNA (lanes 4 and 5) was extracted from 20863 cells grown to subconfluence in monolayer culture. Undigested (U) DNA (lanes 2 and 4) contains open circular (OC) and supercoiled (SC) HPV-16 DNA. The extracted DNA was linearized (L) by digestion with *Bam* H1 (B), (lanes 3 and 5). **B.** Shorter exposure of lanes 4 and 5 with open circular, supercoiled and linearized HPV-16 DNA indicated.



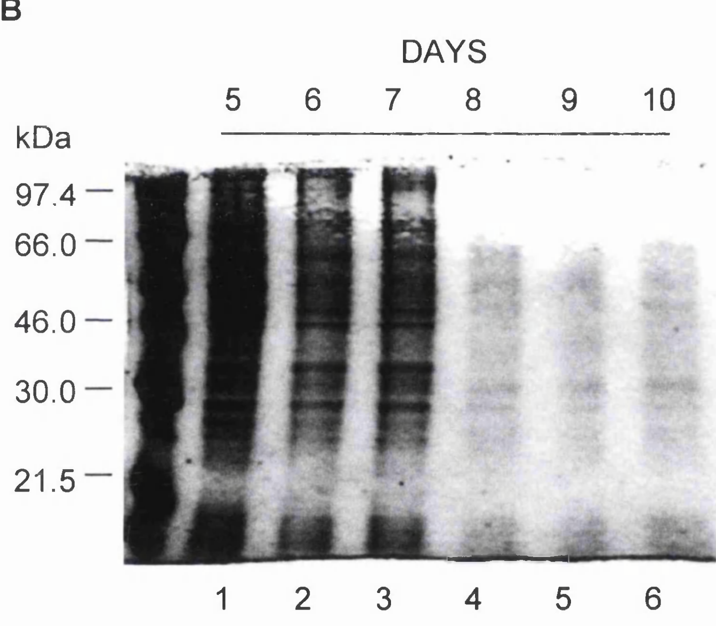
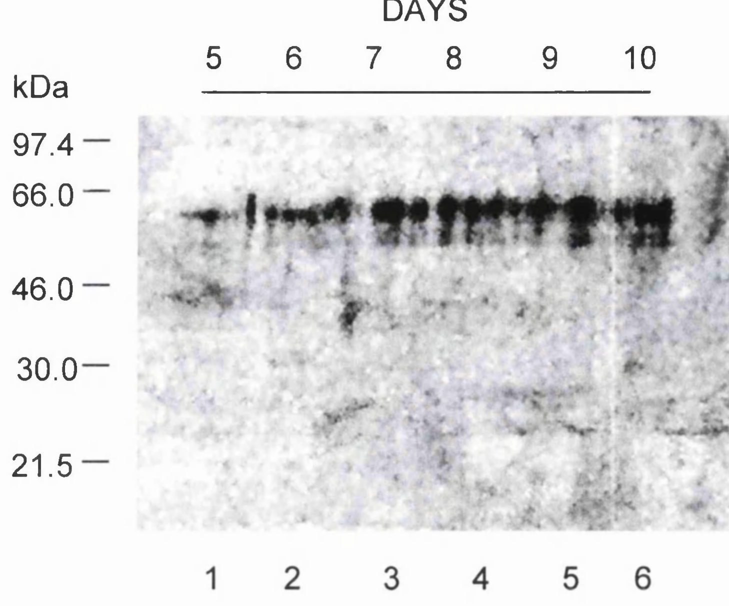


Fig. 3.2. Analysis of protein extracts from W12 cells grown in monolayer culture. Protein extracts were prepared from cells grown for 5, 6, 7, 8, 9 and 10 days in monolayer culture (lanes 1-6 respectively). **A.** 10μg of each protein stained with Coomassie blue. **B.** Protein extracts from each sample, equivalent to 2×10^4 cells, stained with Coomassie blue.



B

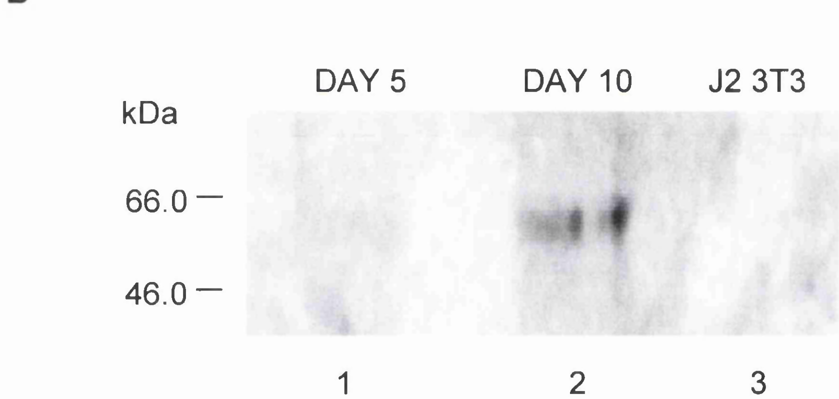


Fig. 3.3. Western blot analysis of HPV-16 L1 expression. **A.** 10μg of protein extracts prepared from 20863/P16 cells grown in monolayer culture for 5, 6, 7, 8, 9, and 10 days (lanes 1-6 respectively) Western blotted with the CamVir 1 antibody, directed against HPV-16 L1. **B.** 10μg of protein extract prepared from 20863/P16 cells grown for 5 (lane 1) and 10 days in monolayer culture (lane 2), together with 10μg of J2 3T3 extract (lane 3), immunoblotted with CamVir 1.

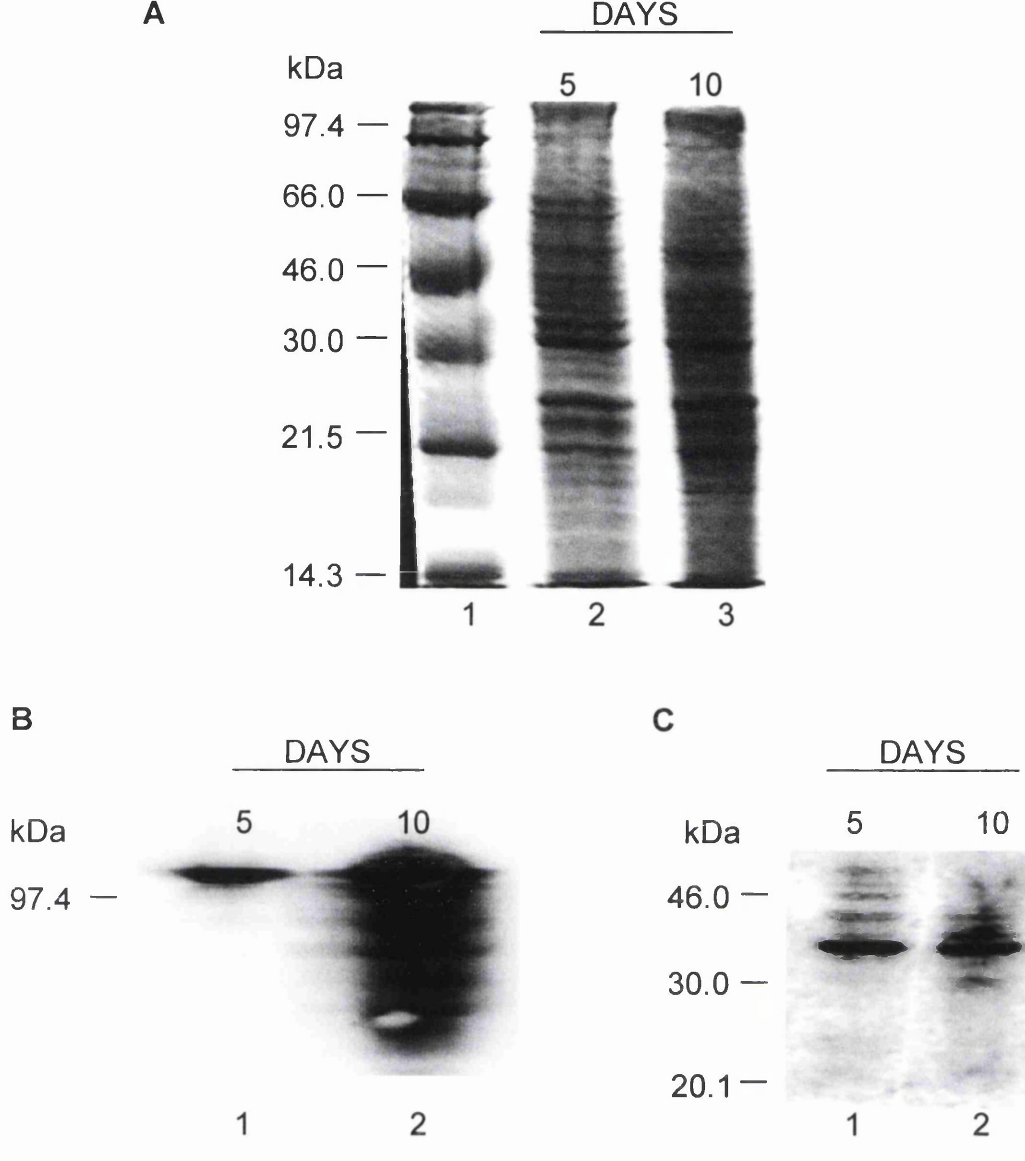
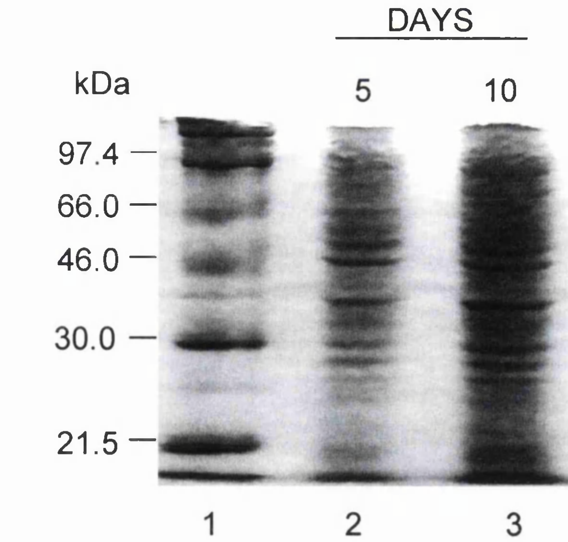
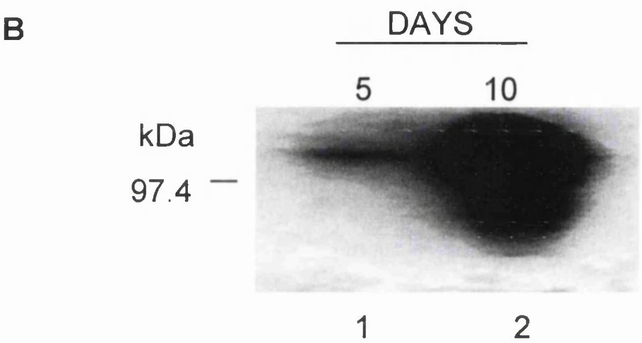


Fig. 3.4. Analysis of protein extracts from monolayer cultures of W12 cells. 10μg of protein extracts prepared from W12/P17 cells grown in monolayer culture for 5 days, (lane 2), or 10 days (lane 3), and rainbow marker (lane 1). **A.** Staining with Coomassie blue. **B.** Immunoblotting with an anti-involucrin antibody, SY5. **C.** Western blotting with 6CS, an anti-GAPDH antibody.





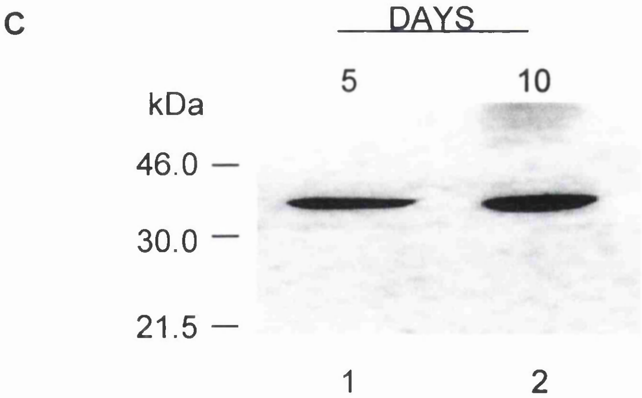
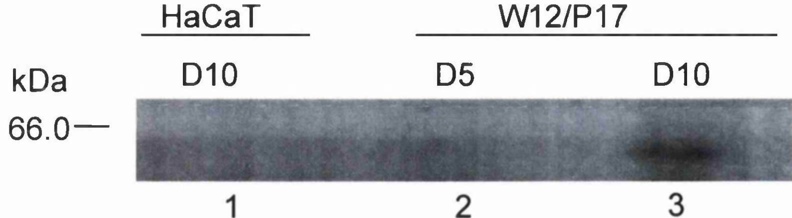
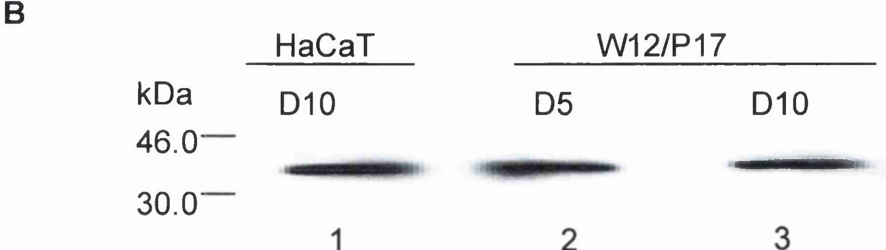


Fig. 3.5. Analysis of protein extracts from monolayer cultures of HaCaT cells. 10μg of protein extract prepared from HaCaT cells grown in monolayer culture for 5 days, (lane 2), or 10 days (lane 3), and rainbow marker (lane 1). **A.** Staining with Coomassie blue. **B.** Immunoblotting with SY5, an anti-involucrin antibody. **C.** Western blotting with 6CS, an anti-GAPDH antibody.





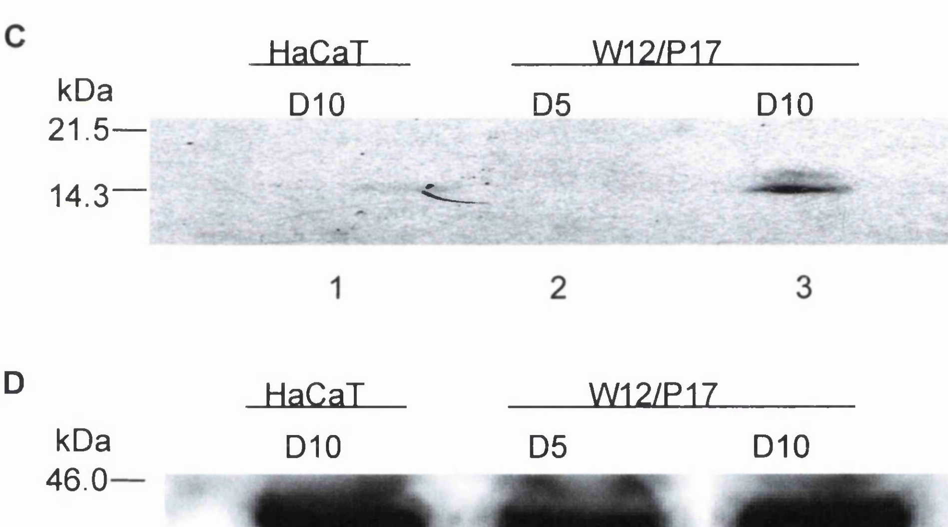


Fig. 3.6. Western blot analysis of viral late protein expression in monolayer cultured cells. Protein extracts were prepared from W12/P17 cells grown for 5 days (D5) and 10 days (D10) in monolayer culture (lanes 2 and 3), and HaCaT cells grown in monolayer culture for 10 days (lane 1). **A.** 10μg of each protein extract Western blottted with CamVir 1,an antibody directed against HPV-16 L1. **B.** 10μg of each extract was Western blotted with 6CS, an anti-GAPDH antibody. **C.** 20μg of each protein extract was Western blotted with TVG 402, an anti HPV-16 E1^E4 antibody. **D.** 20μg of each extract was Western blotted with 6CS, an anti-GAPDH antibody.

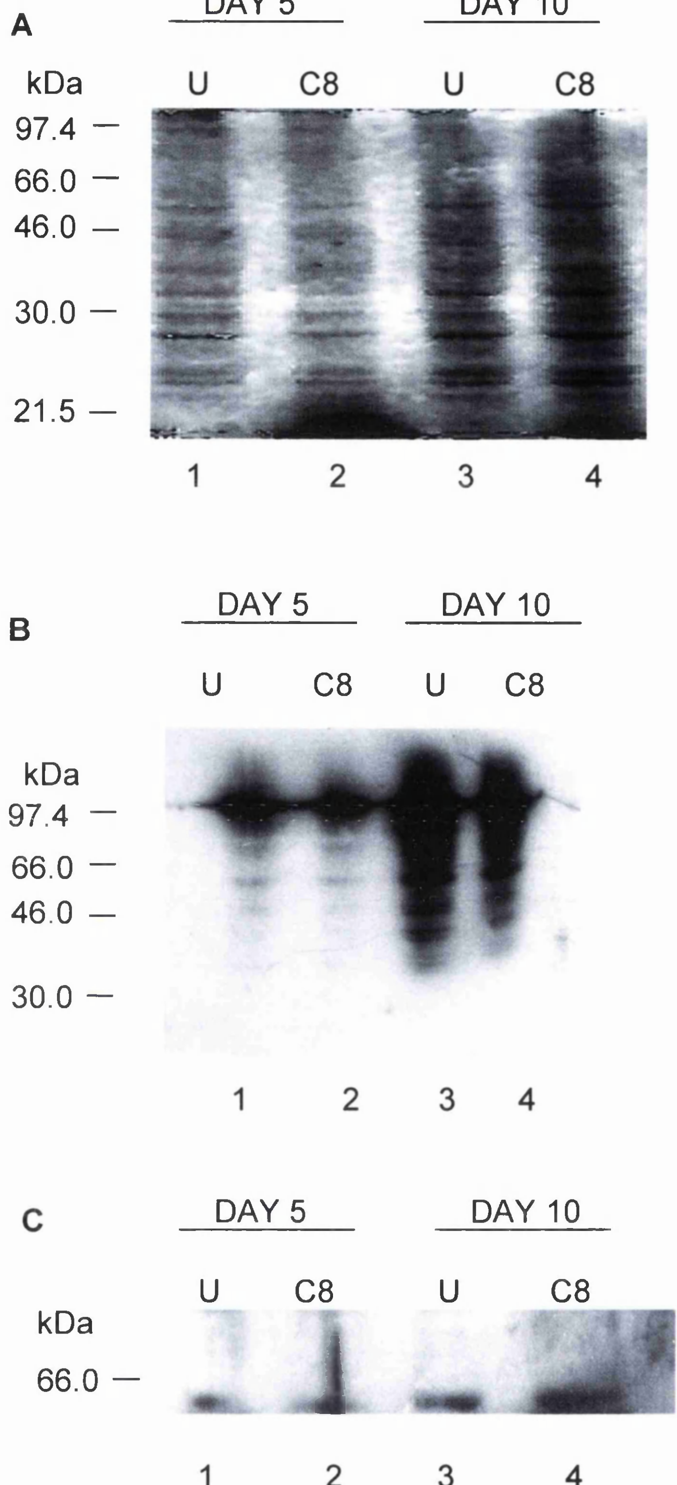


Fig. 3.7. Comparison of untreated and C8–treated monolayer cultured cells. 10μg of protein extract prepared from W12/P18 cells grown in monolayer culture for 5 days (lanes 1, 2), or 10 days (lanes 3, 4). Cells were untreated (U), (lanes 1, 3), or were treated with C8:O (C8), (lanes 2, 4), on alternate days. **A.** Staining with Coomassie blue. **B.** Western blotting with an anti-involucrin antibody, SY5. **C.** Immunoblotting with the anti-HPV-16 L1 antibody, CamVir 1.

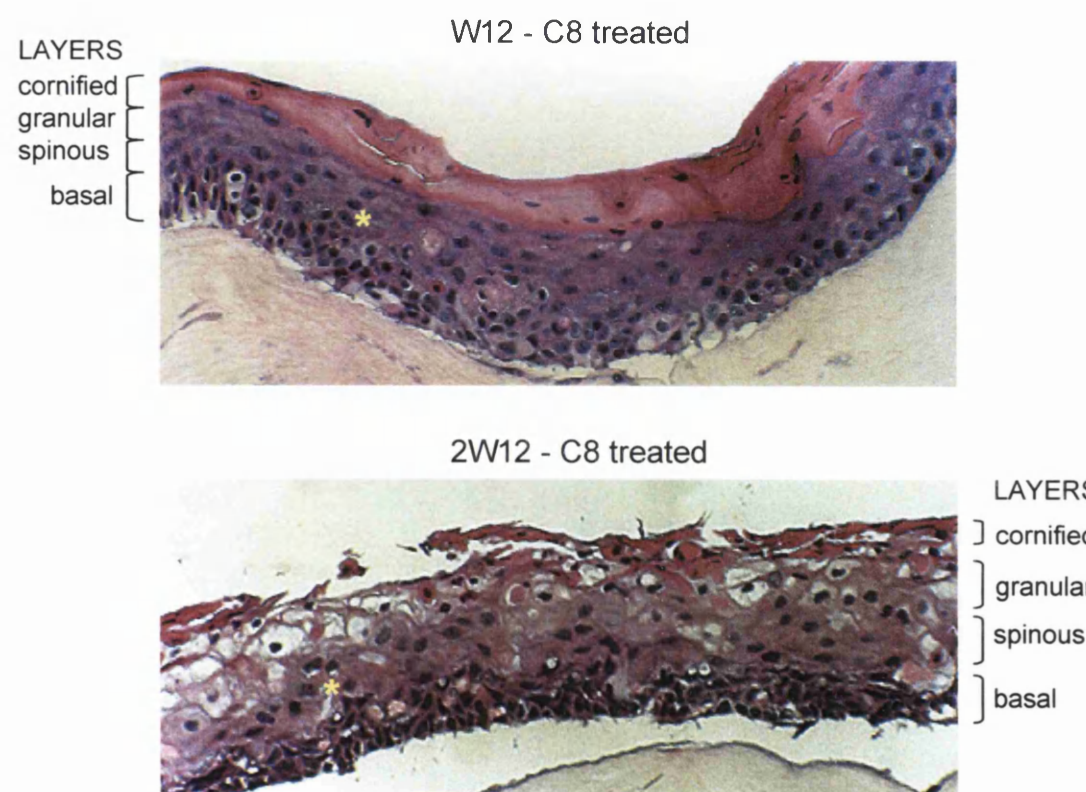
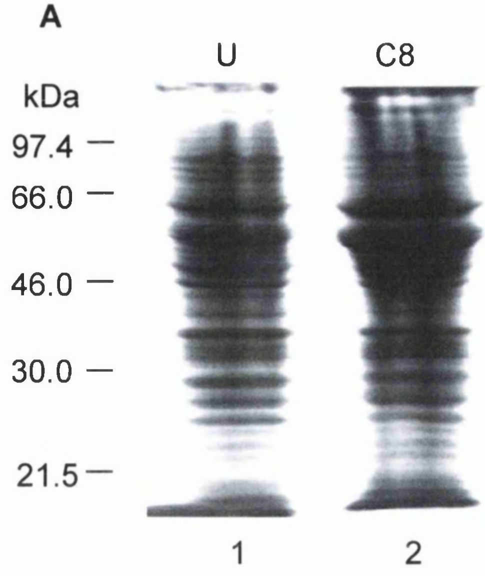


Fig. 3.8. H&E staining of organotypic raft cultures. A. W12 cells and **B.** 2W12 cells were grown on organotypic raft cultures at the air-liquid interface for 12 days; 10µM C8:0 was added with fresh media on alternate days.

* Abnormal mitotic figures.



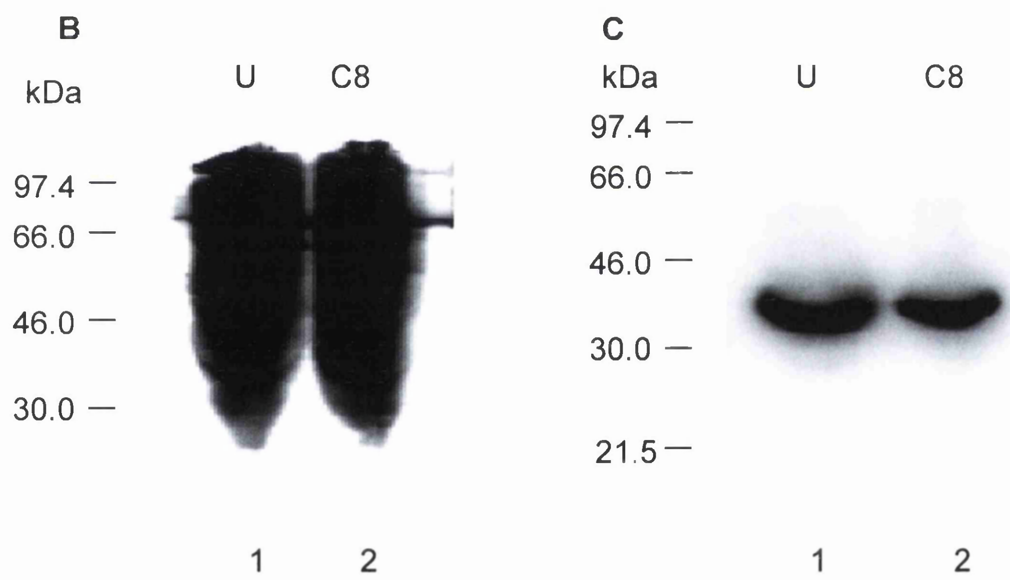


Fig. 3.9. Analysis of protein extracts prepared from organotypic raft tissue. 10μl of protein extracts prepared from untreated (U), (lane 1), or C8:O treated (C8), (lane 2), W12/P17 organotypic raft cultures. **A.** Staining with Coomassie blue. **B.** Immunoblotting with SY5, an antibody against involucrin. **C.** Immunoblotting with 6CS, an antibody directed against GAPDH.

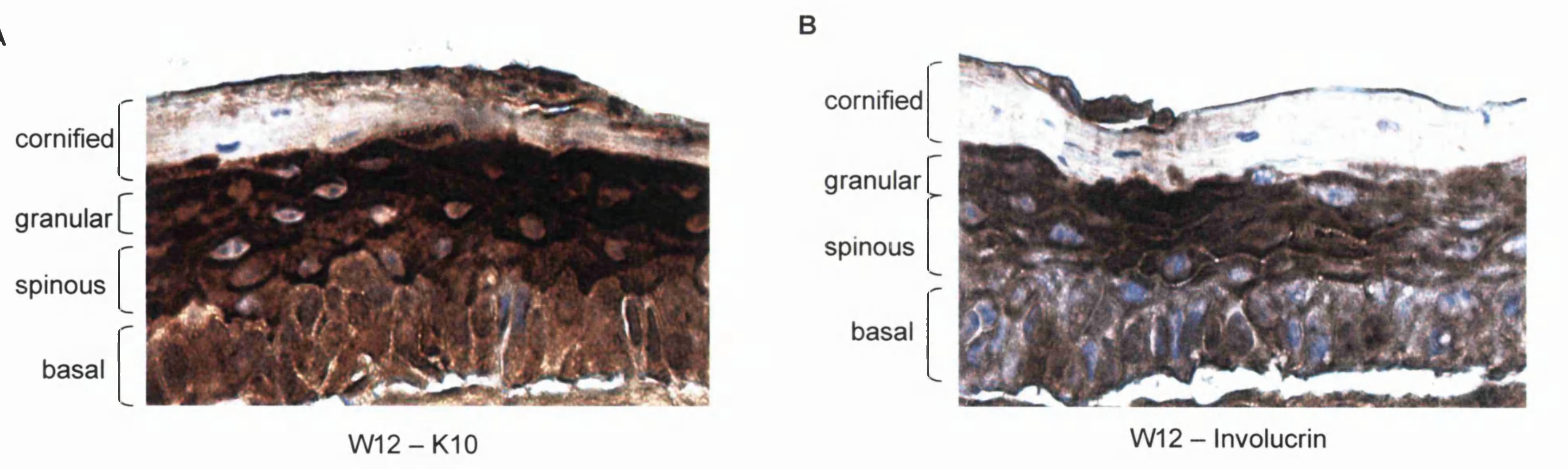


Fig. 3.10. Immunohistochemical staining of differentiation markers. W12 rafts grown at the air-liquid interface for 12 days. **A.** Localisation of K10 expression. **B.** Localisation of involucrin expression.

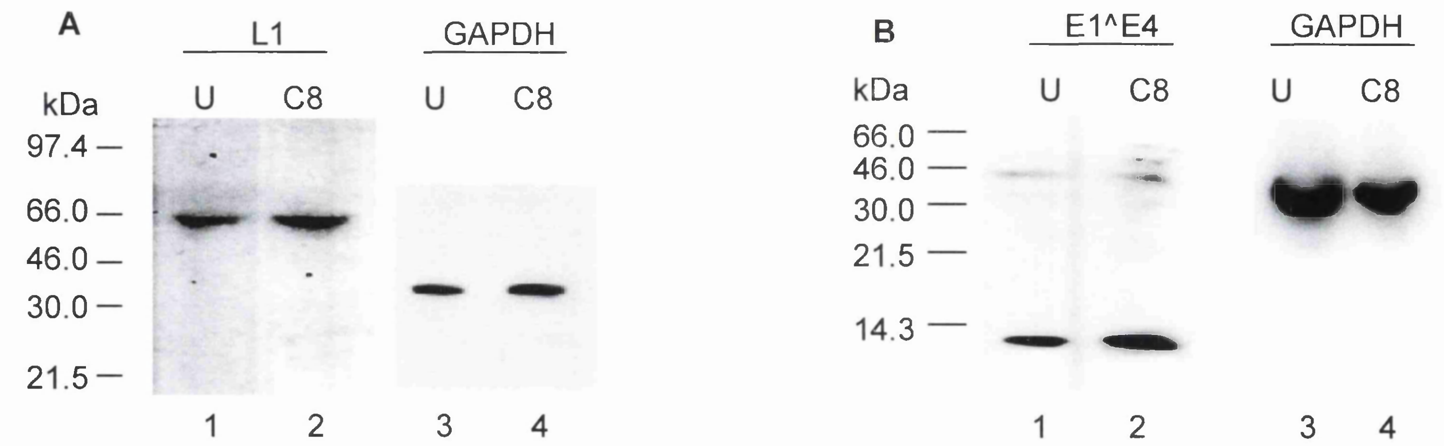


Fig. 3.11. Western blot analysis of late viral protein expression in organotypic rafts. Protein extracts were prepared from W12/P17 cells cultured on organotypic rafts for 12 days, either untreated (U), or treated with 10μg C8:O (C8). **A**. Western blotting with an antibody directed against HPV-16 L1, CamVir 1, (lanes 1 and 2), stripped blot reprobed with 6CS, anti-GAPDH, (lanes 3 and 4). **B.** Western blotting with TVG 402, an antibody directed against HPV-16 E1^E4, (lanes 1 and 2), blot stripped and reprobed with 6CS, anti-GAPDH (lanes 3 and 4).

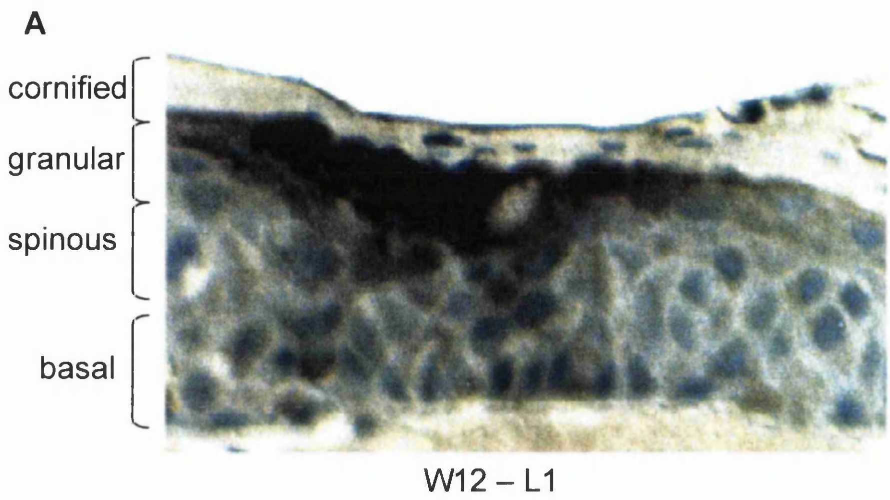


Fig. 3.12. Localisation of HPV-16 L1 protein. A. W12 rafts grown at the air-liquid interface for 12 days and stained for HPV-16 L1 expression.

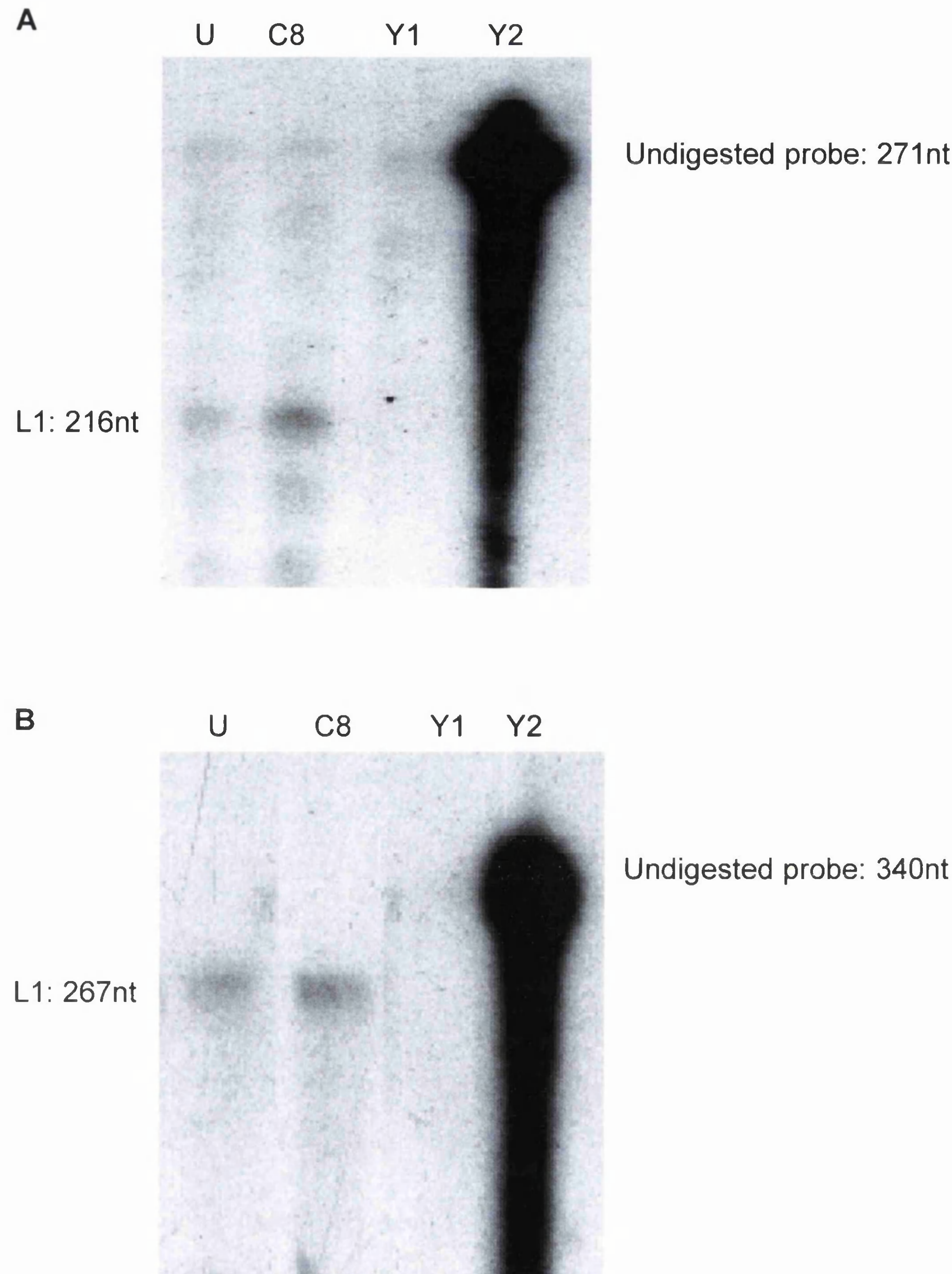
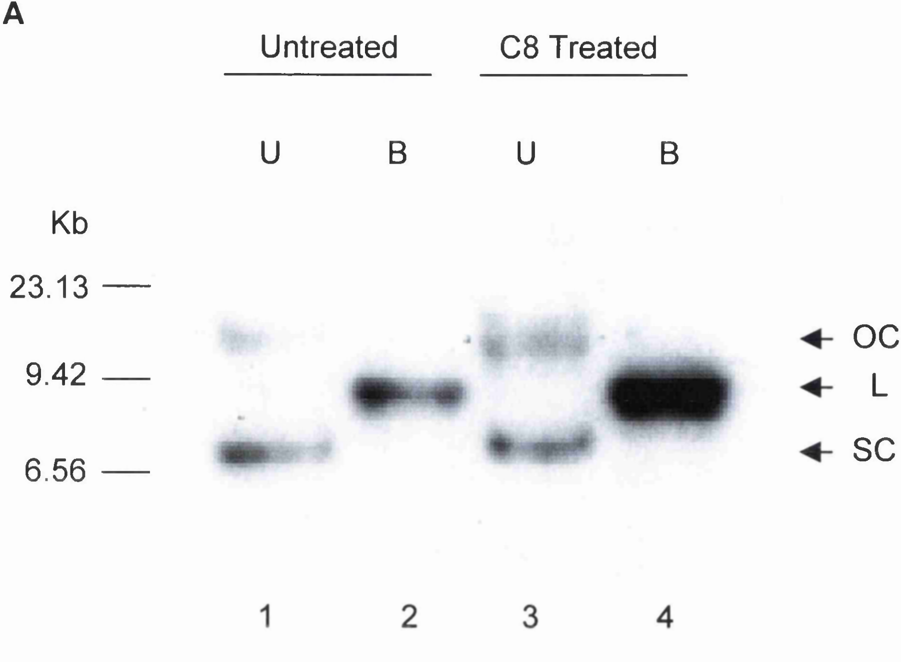


Fig. 3.13. Ribonuclease protection assays (RPA) assessing the levels of L1 transcripts. CIN-612 9E cells and 20863 cells were cultured on organotypic rafts, either untreated (U), or treated with 10μM C8:O (C8), for 12 days at the air-liquid interface. 10μg samples of total RNA or yeast RNA (Y) were hybridised with the appropriate L1 riboprobe. Two yeast RNA samples were included as controls: Y1 was RNase digested to show probe specificity, Y2 was not treated with RNase to indicate the size of the input probe. **A.** Hybridisation with a riboprobe specific to the 3' internal **L1** ORF of HPV-31 followed by RNase digestion. **B.** Hybridisation with a riboprobe specific to the 3' L1 ORF of HPV-16 followed by RNase digestion.



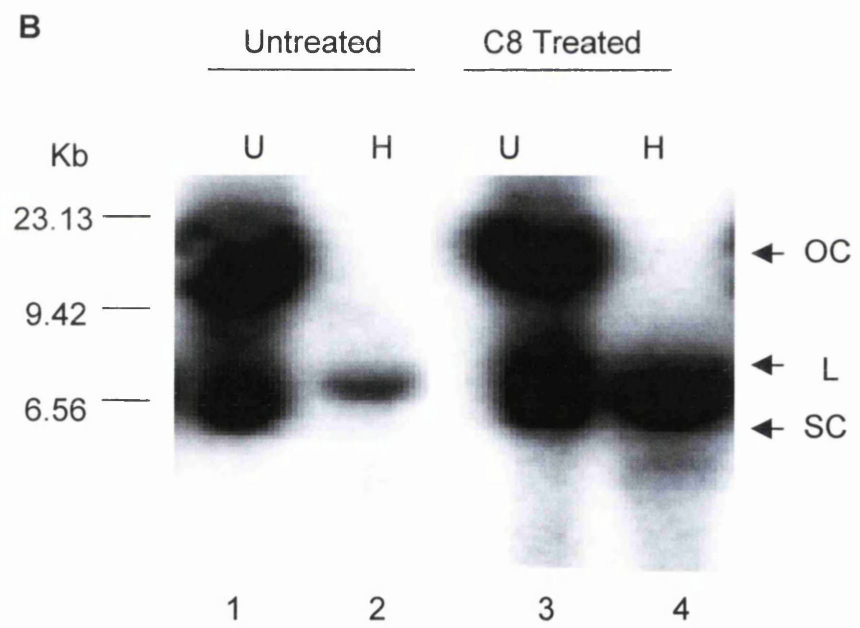


Fig. 3.14. Southern blot analysis of HPV DNA from raft tissue. Total genomic DNA was prepared from 20863 cells (A), and CIN 612 9E cells (B), cultured on organotypic rafts which were either untreated (lanes 1, 2), or treated with C8:O, (lanes 3, 4). **A.** The blot was hybridised with a full-length HPV-16 DNA probe, prepared from the insert of pBS 114K. Undigested DNA (U), contains open circular (OC), and supercoiled (SC), HPV-16 DNA. Extracted DNA was linearised (L) with *Bam* H1 (B). **B.** The blot was hybridisied with a full-length HPV-31b DNA probe, prepared from the insert of pBS HPV31. Undigested DNA (U), contains open circular (OC), and supercoiled (SC), HPV-16 DNA. Extracted DNA was linearised (L) with *Hind* III (H).

CHAPTER 4 – Site-directed mutagenesis of the HPV-16 NRE

Previous studies on the regulation of papillomavirus late gene expression have suggested that viral late protein expression is most likely controlled through interactions between cellular factors and viral *cis*-acting inhibitory elements (reviewed in Schwartz, 1998). In this scenario, the inhibitory effects of these *cis*-acting elements could be overcome through differentiation-dependent changes in the level of expression, activity or sub-cellular distribution of the factors with which they interact. Negative factors mediating the inhibitory activity may be downregulated with differentiation, or positive factors might be upregulated upon differentiation (Tan *et al.*, 1995b). In support of this, a number of *cis*-acting negative regulatory elements have been identified within the coding region of viral late proteins and also in the viral late 3' untranslated regions (UTR) (reviewed in Schwartz *et al.*, 1999). Inhibitory elements within the HPV-16 L1 and L2 open reading frames contain features of mRNA instability elements and are believed to control the levels of late mRNAs in the nucleus and cytoplasm, and may also affect the efficiency of translation of these messages (Tan *et al.*, 1995a, Collier *et* al., 1998, Sokolowski *et al.*, 1998). Conversely, an inhibitory element within the L2 ORF of HPV-31 is thought to regulate genome replication and stability (Terhune *et al.*, 2001).
Negative regulatory elements within viral late 3' UTRs have been identified in BPV-1, HPV-1, HPV-16, and recently, HPV-31 (Furth *et al.*, 1991, Kennedy *et al.*, 1990, Tan *et al.*, 1995a, Cumming *et al.*, 2002a). While these elements have some sequence similarities, they appear to exert their negative effects via distinctly different mechanisms. Each element acts in a position and orientation-dependent manner to reduce the levels of polyadenylated late mRNAs in the nucleus and cytoplasm in undifferentiated cells.

The HPV-16 negative regulatory element (NRE) is located upstream of the late polyadenylation signal in the 3' UTR. This element reduced the expression of a chloramphenicol acetytransferase (CAT) reporter gene by 99%, following transient transfection in basal epithelial cells (Kennedy *et al.*, 1990, Dietrich-Goetz *et al.*, 1997). Using a series of deletion mutants, the minimal NRE was mapped to a 79-nucleotide (nt) region from nt 7128 to 7206 (Kennedy *et al.*, 1991). This element can be roughly divided in two parts and comprises a putative stem loop structure and four weak 5' splice sites in the 5' portion and a GU-rich region in the 3'portion. Studies to date have found no role for the putative stem loop structure in NRE inhibitory activity (Clements, unpublished data, Cumming *et al.*, 2002b), however, the functional importance of the weak 5' splice sites and the GU-rich region has been the subject of two contradictory reports. Dietrich-Goetz *et al.*, (1997) used the full-length 79nt NRE to demonstrate that the GU-rich 3' region was the major determinant of NRE function, and deletion of the first and second of the four weak splice sites accounted for only 13% of the NRE-mediated while further deletion of the remaining two splice sites, including 2 nt of the GU-rich region, resulted in a 50% reduction in CAT activity. In contrast, deletion studies carried out by Furth *et al.*, (1994) mapped the HPV-16 NRE to a 51nt region (nt 7128-7178). This shorter element lacks most of the GU-rich region of the 79nt NRE. When this 51nt NRE was tested in an SV40-based CAT reporter gene vector it resulted in a 6-fold inhibition of CAT activity and the second of the four splice sites was found to be critical for this inhibitory activity (Furth et al., 1994). However, it should be noted that the constructs used by Dietrich-Goetz *et al.*, contained the endogenous HPV-16 late polyadenylation signals whereas Furth *et al.*, used constructs containing the SV40 early polyadenylation site. It is possible that the HPV-16 NRE exerts its inhibitory activity through interactions with the late polyadenylation signal, therefore, the use of a heterologous poly(A) site may disrupt this cross-talk and indirectly reduce the activity of the NRE. These differences have recently been resolved in our laboratory following extensive

mutagenesis studies (Cumming *et al.*, 2002b). Using a CAT reporter gene vector similar to that used by Dietrich-Goetz *et al.,* and a series of deletion mutants of the 79nt NRE, in the context of the HPV-16 late polyadenylation region, it has been shown that the four weak 5' splice sites act together to mediate the full inhibitory activity of the 5' portion of the NRE and, while the 3' GU-rich region also has independent inhibitory activity, the full 79nt NRE is required for maximal inhibition (Cumming *et al.*, 2002b).

UV cross-linking analysis using the minimal 79nt NRE as the target RNA has revealed that this element can bind a range of cellular proteins, the most prominent of which is a 65kDa nuclear protein band, possibility representing more than one protein, and a less prominent 40kDa protein band (Dietrich-Goetz *et al.*, 1997, Koffa *et al.*, 2000). Work to date has identified three of the cellular factors with which the NRE interacts and these are the auxiliary splicing factor $U2AF^{65}$, the cleavage-stimulating factor CstF-64 and the shuttling protein HuR (Koffa *et al.*, 2000). Moreover, the level of expression and distribution of these cellular factors was shown to alter following epithelial differentiation, $U2AF^{65}$ being downregulated, CstF-64 upregulated and HuR redistributing from the nucleus to the cytoplasm (Koffa *et al.*, 2000).

The aim of the work presented here was two-fold; firstly, I wanted to determine which specific short segments of the 79nt NRE are functionally important for the inhibitory activity of this element. Secondly, I wished to map the NRE sequences that bind cellular proteins, since subsequent mutations of sequence motifs with which NRE-binding proteins interact could reveal clues as to the RNA-based mechanisms by which the NRE functions.

4.1.1. Saturating site-directed mutagenesis of the NRE

I attempted to identify important NRE sequences by introducing point mutations throughout the element using an approach similar to that used to abolish the inhibitory activity of the HPV-1 AU-rich element (Sokolowski *et al.*, 1997), and to map the 53nt inhibitory element of BPV-1 (Furth et al., 1994). A 445nt fragment (nt 7008 to 7453) of the HPV-16 late 3' UTR that encompasses the NRE was used as a template for PCR (Fig. 1.10). The NRE was divided into fifteen non-overlapping groups of five nucleotides from the 5' end through to the 3' end of the element, M1 to M15, as shown in Fig. 4.1B. Within each group PCR mediated site-directed mutagenesis was used to change purines to pyrimidines and *vice versa* (Fig. 4.1B). Each of the fifteen mutants was cloned into the pGem-T vector and sequenced (either manually or using automated sequencing) to ensure only the desired mutations were present. Each of the weak 5' splice sites were disrupted individually with random point mutations and differed significantly from the consensus 5' splice site (AAG/GUAAGU) as indicated in Fig. 4.1B, M3 (AAG/TACTGC), M4 (TAG/CAAAGU), M6 (AGA/UGCUGU) and M7 (UAG/UACGGU).
The putative stem loop structure was not mutated in this study since this motif was the subject of another mutagenesis study in the laboratory designed to specifically disrupt the secondary structure of this feature. Previous extensive work in the laboratory of Barklie Clements, using point mutations to disrupt the loops and bulges of the structure, had failed to establish a role for this predicted stem loop (Clements, unpublished data).

4.1.2. Mutagenesis does not affect the inhibitory activity of the NRE

Following sequencing, the mutants were cloned immediately downstream of the chloramphenicol acetyltransferase (CAT) reporter gene, between the *Pst* I/*Hin*d III sites, in the expression vector, pLW1 (Fig. 4.2). The CAT gene is

under the control of the HSV-2 immediate early promoter and the plasmid lacks poly(A) signals (Fig. 4.2). However, the NRE mutants were constructed in the context of the *Pst* I/*Eco* RI 445nt fragment from the 3' UTR of HPV-16 and so they contain the HPV-16 late polyadenylation sites (Fig. 4.1A). Each mutant NRE plasmid construct was transiently transfected into HeLa cells, used as a model for basal epithelial cells, and reporter gene activity was assayed. Transfections were carried out in duplicate on at least three occasions for each mutant. Results, in counts per minute (cpm), were averaged and the standard deviation calculated (Fig. 4.3). The total number of counts differed between experiments, although the pattern of CAT activity was consistent, and so in order to allow a useful comparison of the results, the average CAT activity of each mutant was calculated as a percentage of the activity (in cpm) of the positive control plasmid I1, which lacks the region containing the NRE between the *Pst* I and *Ssp* I sites (Fig. 1.10) and was therefore assigned 100% CAT activity in each experiment. Plasmid F11 contains the full-length, wild type NRE and was included as a negative control. From the graph it is clear that none of the mutated forms of the NRE, including the splice site mutants M3, M4, M6 and M7, were capable of significantly relieving the inhibitory activity of this element, as compared to the wild type control F11 (Fig. 4.3).

4.1.3. Mutagenesis does not affect protein binding to the NRE

Radiolabelled riboprobes corresponding to each of the fifteen NRE mutants were synthesised by *in vitro* transcription. The affect of the NRE mutations on the ability of the element to interact with nuclear proteins was assayed by UV cross-linking analysis and compared to the protein binding profile generated using a wild type NRE riboprobe. Initially, UV cross-linking was carried out in 60mM KCl and as indicated in Fig. 4.4, none of the mutations introduced into the NRE appeared to affect the ability of this element to interact with HeLa nuclear proteins; the band intensity and overall profile was

very similar between mutants and the wild type control. As shown in Figs. 4.4, 4.5 and 4.6, the NRE can bind a number of nuclear proteins of various sizes, the most prominent of which is approximately 65kDa, although a 40kDa protein also binds the element quite strongly. The UV cross-linking experiment was repeated and this time the salt concentration was increased to 125mM in an attempt to determine if minor differences exist between the mutant probes under more stringent conditions, i.e. higher salt. Again, there appeared to be no significant differences between the series of NRE mutants and the wild type control (Fig. 4.5). The concentration of salt was further raised to 250mM and UV cross-linking was repeated. At this increased salt concentration the protein binding profiles showed some minor differences, (Fig. 4.6). The intensity of the 40kDa band and some smaller bands, which might possibly represent serine-arginine (SR) proteins on the basis of size, appeared to decrease slightly, particularly in mutants clustered at the end of the element (M13, M14, M15) and also in mutants 7 and 8. Each of these mutants contains fewer GU residues than in the respective wild type sequences, suggesting that efficient binding of the 40kDa and smaller proteins require GU-rich sequences. In this experiment the M12 riboprobe appears to have degraded slightly as indicated by the decrease in intensity of the 65 and 40kDa bands and the increase in smaller protein bands. However, when this experiment was repeated the M12 protein profile was similar to that of the other mutants and the wild type control, as indicated in the additional panel in Fig. 4.6. Nevertheless, even at this relatively high salt concentration the overall protein binding profile of each mutant was similar to that of the wild type control and also to the profiles generated at 60mM KCl and 125mM KCl, suggesting that the majority of nuclear proteins that bind to the NRE do so with high affinity, at least *in vitro*. However, in order to establish this conclusively I would have had to carry out UV cross-linking at higher salt concentrations, for example 500mM and 1M KCl, as nucleic acid binding proteins, e.g.TATA-binding protein (TBP), are generally highly charged basic proteins which bind tightly to their target sequences even in

high salt (Pugh, 2000); unfortunately, due to time constraints, this was not attempted. UV cross-linking was repeated at least three times for each NRE mutant at increasing salt concentrations and any minor differences in the protein binding profiles of mutants were consistent.

4.2. Deletion of the NRE in the context of the viral genome

Since the mutations introduced into the NRE did not appear to affect the inhibitory activity of this element, or its ability to bind nuclear proteins, I next attempted to delete the NRE in the context of the whole viral genome. I planned to establish a cell line in which the NRE-null viral genome was maintained episomally by transfecting normal human foreskin keratinocytes (NHFK) with the mutant virus construct. At the same time, I also wanted to establish a wild type HPV-16 line using viral DNA, known as 114/K, derived from a low-grade cervical lesion (Kirnbauer *et al.*, 1993). This is a relatively new technique and has been used in recent years to establish stable cell lines expressing a number of HPV DNAs extracted from cervical samples (Frattini *et al.*, 1996, Frattini *et al.*, 1997, Meyers *et al.*, 1997, Thomas *et al.*, 2001). The advantage of this approach is that it permits molecular manipulation of the cloned viral DNA prior to transfection. If an NRE-deletion mutant were viable then characterisation of the growth of this mutant virus both in monolayers and on the organotypic raft culture would provide valuable information on the role(s) of this element in the virus life cycle. I was particularly interested in studying the possible changes in the timing of viral late gene expression in this line, given the proposed central role of the NRE in the post-transcriptional control of late gene expression. The NRE deletion mutant was constructed using a PCR-based mutagenesis protocol similar to that used to generate the NRE site-directed mutants. Since the NRE straddles the L1 stop codon I decided to delete only the portion of the NRE lying immediately downstream of this site and therefore maintain the integrity of the L1 coding region in the mutant virus. Forward and reverse primers

were designed that lacked 52nt of the NRE immediately downstream of the L1 stop codon from position 7155 to 7207, flanked on either side by 12-15 bp of wild type sequence, and were designated Δ52F and Δ52R. Wild type viral-specific forward and reverse primers were designed; 6818F anneals upstream of the *Eco N*I site and 7440R anneals downstream of the *Pml* I site, as shown in Fig. 4.7. Two complimentary half molecules were generated by PCR-amplifying *Pml* I linearized pBS 114K with Δ52R and 6818F and *Eco N*I linearized pBS 114K with Δ52F and 7440R (Fig. 4.8A and B). These half molecules were annealed and a further round of fill-in PCR using the 6818F and 7440R primers generated a full-length, double stranded product (Fig. 4.8C), which lacked the NRE. From the agarose gels shown in Fig. 4.8 it appears that the sizes of the PCR products differ slightly from the predicted sizes, however, subsequent sequencing of the NRE-deletion mutant gave the expected sequence (see below). The filled-in PCR product, lacking the NRE, was cloned into the pGem-T vector and clones were subsequently digested with *Eco* RI and *Ssp* I to verify the presence of the mutated PCR product (data not shown). A number of positive clones were checked by automated sequencing to ensure the NRE had been deleted and no random mutations had been introduced; at least two of the sequenced clones were correct and were designated pGem Δ52 [3] and pGem Δ52 [6]. I then attempted to sub-clone the *Pml* I/*Eco* NI digested PCR product from these pGem constructs into pBS 114K from which the wild type *Pml* I/*Eco* NI fragment had been removed (Fig. 4.9B). However, despite numerous attempts including varying the vector to insert ratio and using 100x T4 DNA ligase, as recommended for *Eco* NI ligations (New England Biolabs), I was unable to ligate the wild type vector and mutant insert and the reason for this remains unknown.

As an alternative approach I digested pBS 114K with *Bam* HI to release the viral genome from the vector backbone. The HPV-16 genome was recircularised and digested with *Eco* RI, which cuts at positions 6819 and

7453, to remove a 634nt fragment that includes the wild type NRE. The pGem Δ52 mutants, 3 and 6, were also digested with *Eco* RI to generate a 583nt NRE-null insert, which was subsequently ligated with the *Eco* RI digested viral genome (Fig. 4.9C). Following ligation, 114K Δ52, 3 and 6, were recloned into the *Bam* HI site of pBS. The ligation of pBS and 114K Δ52 [6] was repeatedly less efficient than pBS and 114K Δ52 [3] and so clones from this ligated product only were digested with *Bam* HI and *Ssp* I to check the presence and orientation of the insert; if the Δ52 fragment was ligated in the correct orientation into the 114K backbone then this digest would produce a number of fragments including one at 1076nt. Approximately fifty clones were analysed in this way and only one appeared to contain the insert in the correct orientation (Fig. 4.10A, lane 4). However, routine digests of this plasmid pBS 114K Δ52 [3-4], following caesium chloride preparation, gave unexpected results when compared to the wild type control pBS 114K (Fig. 4.10B). Undigested pBS 114K (Fig. 4.10B lane 1) runs as a large single band at the top of the gel. In addition to a similar large band, undigested pBS 114K Δ52 [3-4], (Fig. 4.10B lane 4), contains a number of bands, the most predominant of which is approximately 3kb in size. Digestion of pBS 114K with *Eco* RI (Fig. 4.10B, lane 2) and *Bam* HI (Fig. 4.10B, lane 3) gave the predicted number and size of products based on the published HPV-16 sequence (Seedorf *et al.*, 1985). However, when pBS 114K Δ52 [3-4] was similarly digested with *Eco* RI (Fig. 4.10B, lane 5) and *Bam* HI (Fig. 4.10B, lane 6) the only product was a band of approximately 3kb similar to that observed in undigested pBS 114K Δ52 [3-4] (Fig. 4.10B, lane 4). On the basis of size I suspect this band may be pBS, although I did not attempt to identify the DNA, but if this were correct then it might suggest that the ligation of pBS and 114K Δ52 resulted in an unfavourable recombination event that led to the loss of 114K Δ52. In an attempt to overcome this problem, DNA from the mini-prep shown in lane 4 Fig. 4.10A, pBS 114K Δ52 [3-4], was introduced into a number of strains of

competent bacteria, including in-house preparations of DH5α and XL1 Blue cells and commercial preparations of XL10-Gold ultracompetent cells (Stratagene). Unfortunately, this did not solve the recombination problems and due to time constraints I was unable to pursue this work any further. Further to this, a number of alternative approaches are currently being pursued in the laboratory in an attempt to successfully clone an NRE-null HPV-16 virus genome.

Fortunately, the establishment of a wild type HPV-16 line was rather more straightforward. Following electroporation of the 114/K DNA into freshly established monolayer cultures of normal human foreskin keratinocytes, the transfected cells were grown for a number of passages then cyroperserved prior to characterisation. Preliminary Southern blot analysis of DNA extracted from transfected cells at the first passage indicated that the cells contained episomal viral DNA, although since the cells were at such a low passage they contained very little viral DNA (data not shown). However, as a result of the problems encountered with the establishment of the NRE-deletion mutant line, this wild type line was not used in this study and was not characterised any further.

4.3. Discussion

The differentiation-dependent control of HPV late protein expression is regulated at both transcriptional and post-transcriptional levels. The identification of negative regulatory RNA sequences both in HPV-16 and in a number of other papillomavirus late 3' UTRs suggests that these sequences, and the cellular factors with which they interact, play an important role in controlling the pattern of late protein expression. For example, an AU-rich inhibitory element has been mapped to a 57-nt sequence located immediately downstream of the L1 stop codon in HPV-1 (Sokolowski *et al.*, 1997). Multiple nuclear and cytoplasmic HeLa cell proteins interact with the HPV-1 AU-rich inhibitory sequence, and of particular functional importance are the shuttling proteins hnRNP C1 and C2 and the *elav*-like HuR protein (Sokolowski *et al.*, 1997, Sokolowski *et al.*, 1999). It has been suggested that binding of hnRNP C1 and C2 to sequences in HPV-1 late mRNAs may result in premature degradation of late messages in undifferentiated cells but binding of HuR to these elements in differentiated cells bypasses the degradation pathway (Schwartz *et al.*, 1999). Also, a 53-nt sequence located immediately upstream of the poly(A) signal in the bovine papillomavirus type 1 (BPV-1) late 3'UTR has been shown to reduce the efficiency of late mRNAs polyadenylation in undifferentiated cells (Furth et al., 1994). The inhibitory activity of this sequence has been attributed to a 5' splice site within the element which has been shown to interact with U1 snRNP; polyadenylation of late messages is inhibited as a result of the direct interaction of the U1 snRNP subunit, U1 70K, and poly (A) polymerase (Furth et al., 1994, Gunderson *et al.*, 1998).

The subject of the work presented in this chapter is the 79nt negative regulatory element of HPV-16, which straddles the L1 stop codon and extends into the late 3'UTR (Kennedy *et al.*, 1991). This element comprises a number of interesting features: The 5' portion contains a putative stem loop

structure and four weak 5' splice sites, while the 3' portion contains a GU-rich region (Kennedy *et al.*, 1991). Following the identification of this element, it has been shown to interact with a range of cellular proteins, including possibly a number of 65kDa nuclear proteins, two of which have previously been identified as $U2AF^{65}$ and CstF-64, and a less prominent 40kDa protein, believed to be HuR (Dietrich-Goetz *et al.*, 1997, Koffa *et al.*, 2000). This element has also been implicated in a number of RNA processing events including mRNA splicing, polyadenylation, nuclear export and cytoplasmic mRNA stability and the NRE is thought to be responsible for controlling expression of late viral proteins through its involvement in one or more of these post-transcriptional mechanisms (Kennedy *et al.*, 1991, Furth et al., 1994, Koffa *et al.*, 2000, McGuire *et al.*, 2002).

I hypothesized that identifying short sequence elements that relieved the inhibitory activity of the NRE and using this information to correlate loss of NRE-function with changes in the pattern of protein binding to the NRE might give some insight as to the nature of the RNA processing mechanisms involved in the control of viral late gene expression. I generated a series of site-directed mutations throughout the NRE and compared the inhibitory activity of each mutant to a wild type NRE control in a reporter gene assay. Surprisingly, however, I found that none of the mutations introduced into the NRE relieved the inhibitory activity of this element, suggesting that the inhibitory activity of the NRE is mediated by larger sequence elements. The ability of the NRE mutants to interact with HeLa nuclear proteins was also compared with a wild type NRE control following UV cross-linking at increasing salt concentrations, but the pattern of protein binding to this element remained unchanged. This observation suggests that the NRE-binding proteins may make contact with the element at more than one position and therefore, loss of one binding site, through mutation, does not significantly affect the overall ability of the NRE to bind protein complexes

and so the protein-binding profile remains similar. It should be noted that nucleic acid-binding proteins are usually part of complexes.

Additionally, each of the weak 5' splice sites was disrupted with random point mutations, however, as indicated above, none of these appeared to relieve NRE inhibition. Initially, this might suggest that none of the splice sites, including the second site, which had been previously suggested to be critical for NRE activity, are functionally important for NRE repression. However, since the NRE was mutated in non-overlapping 5nt groups this means that only one splice site was altered at any time and each of the mutated splice site constructs M3, M4, M6 and M7 contained three undisrupted splice sites and a wild type GU-rich region, which may be sufficient to maintain NRE-mediated repression of reporter gene expression.

Thus it appears that, in contrast to the situation in HPV-1 and BPV-1, short sequence elements in the HPV-16 NRE do not determine the inhibitory activity of this element, or its ability to interact with a range of HeLa nuclear proteins. This observation is supported by additional NRE deletion mutagenesis studies carried out in our laboratory (Cumming *et al.*, 2002b). As an alternative approach to identifying important sequence elements within the NRE, NRE-deletion mutants were tested in reporter gene assays similar to those used to functionally test the site-directed NRE-mutants. This approach revealed that although the full 79nt element is required for maximal inhibition, both the 5' and 3' portions of the NRE have independent inhibitory activity: While deletion of the 3' portion resulted in a 3-fold increase in the level of reporter gene expression, removal of the 5' portion of the NRE relieved the inhibition on reporter gene expression 10-fold (Cumming *et al.*, 2002b). Moreover, further mutational studies have indicated that the four weak 5' splice sites act together and are responsible for the inhibitory activity of this portion of the NRE; the putative stem loop structure was not required for inhibition (Cumming *et al.*, 2002b). However, while the 5' portion is

functionally more important than the 3' portion, it appears to bind less protein than the GU-rich region (Cumming *et al.*, 2002b). Surprisingly, however, the pattern of protein binding to the 5' and 3' portions was very similar, albeit with variations in band intensity, despite a lack of any sequence similarity between these constructs. Competition experiments using up to a 16-fold molar excess of unrelated RNA did not affect the NRE protein binding profile, confirming that protein binding to the NRE is specific (Cumming *et al.*, 2002b).

Each of the NRE-binding proteins identified to date, U2AF^{65}, CstF-64 and HuR would be predicted to interact predominantly with the GU-rich 3' region of the NRE by virtue of the U-rich nature of their consensus binding sites (Koffa *et al.*, 2000). Obviously, it is possible that the 5' portion of the NRE binds different cellular proteins and any similarities in size are purely coincidental. Alternatively, UV cross-linking may irreversibly fix an interaction that is normally transient *in vivo.* However, this is an unlikely explanation since increasing salt concentrations and competition assays did not disrupt the protein binding profile. A more plausible explanation is that the 5' and 3' parts of the NRE, despite lacking sequence similarity, can act in a cooperative manner to mediate the formation of a dynamic, NRE-bound, multi-protein complex. One could imagine that a number of cellular proteins, interacting specifically with the 5' and 3' regions of the NRE, in turn recruit additional factors, eventually forming a complex held together by extensive RNA-protein and protein-protein interactions.

Given the fact that the cellular environment changes with differentiation, and the potential effects of these changes on the activity and distribution of cellular proteins (Fuchs, 1990), it is highly likely that the components of any putative NRE-bound multi-protein complex would be influenced by differentiation. I have not directly tested this hypothesis but a recent report by Koffa *et al.*, and results presented in chapter 5, do suggest that the level

of expression of NRE-bound proteins is influenced by differentiation. It is tempting to speculate that these differentiation-dependent changes in NRE-bound proteins may be partly responsible for the relief of the inhibitory activity of this element in terminally differentiated keratinocytes, thereby facilitating viral late protein expression in these cells. Interestingly, Dietrich-Goetz *et al.*, (1997) reported that PMA treatment of HPV-16 positive W12 cells, transiently transfected with NRE constructs, relieves the inhibitory activity of this element . PMA acts as an activator of the protein kinase C pathway and can induce epithelial differentiation (Meyers *et al.*, 1992). PMA treatment of the W12 line was also shown to bring about a differential decrease in binding of the 65kDa protein to the NRE and an increase in binding of a predominantly cytoplasmic 40kDa protein, possibly HuR; indicating that protein binding to the NRE is subject to differential changes (Dietrich-Goetz *et al.*, 1997).

In summary, analysis of substitution and deletion mutants of the HPV-16 NRE has revealed that, while no short sequences alone determine the inhibitory activity of this element, the 5' and 3' regions each contribute independently to the negative effect, and the full-length 79nt element is required for maximal inhibition.

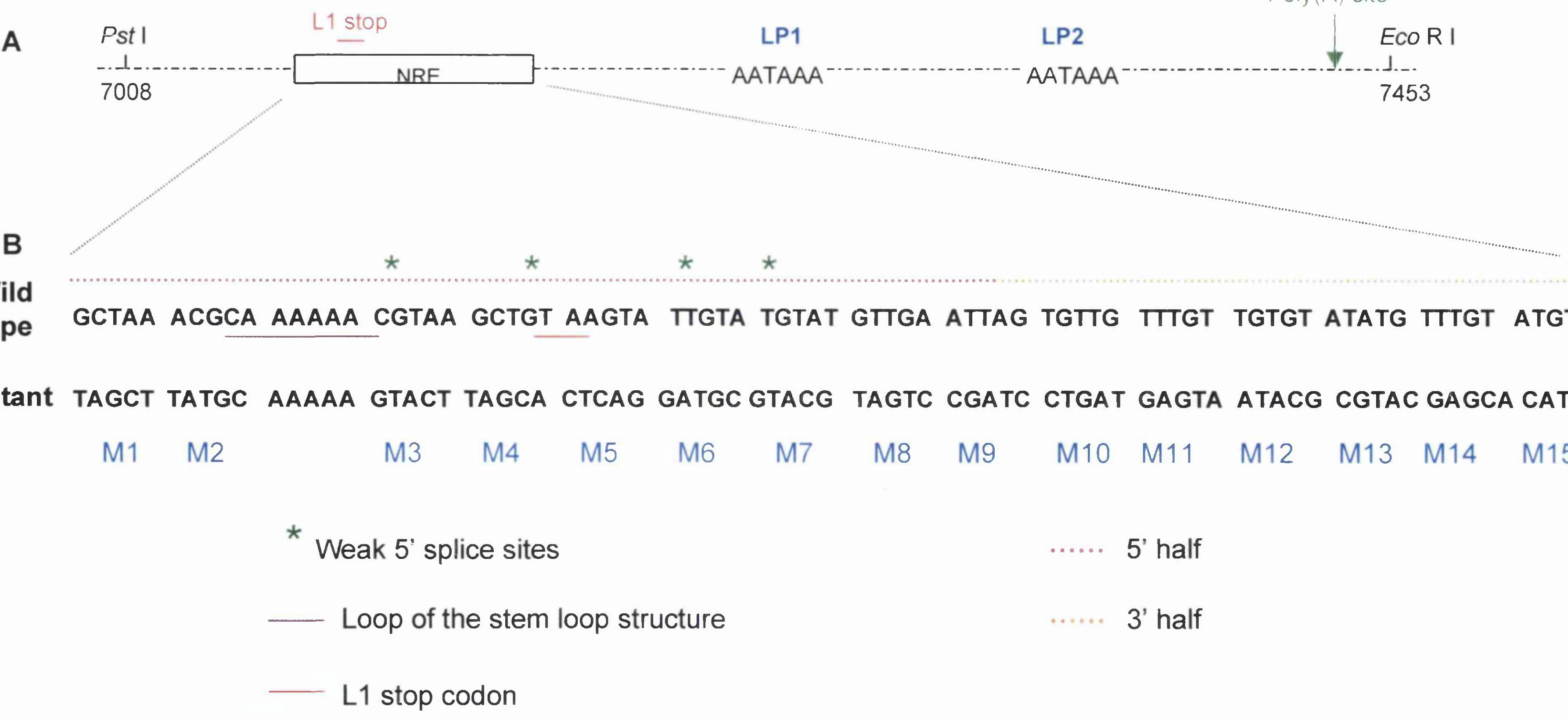


Fig. 4.1 A. P/E 445nt fragment. Diagram of the 445nt *Pst* I/*Eco* RI fragment from the 3'UTR of HPV-16 showing the position of the NRE, the LP1 and LP2 polyadenylation signals and the poly(A) site.

------ Indicates intervening sequence. **B. Site-directed muatgenesis of the NRE.** Structure and sequence of the wild type 79nt negative regulatory element located in the late 3'UTR of HPV-16. The element was divided into non-overlapping groups of 5nt and each nucleotide was mutated as shown (M1-M15).

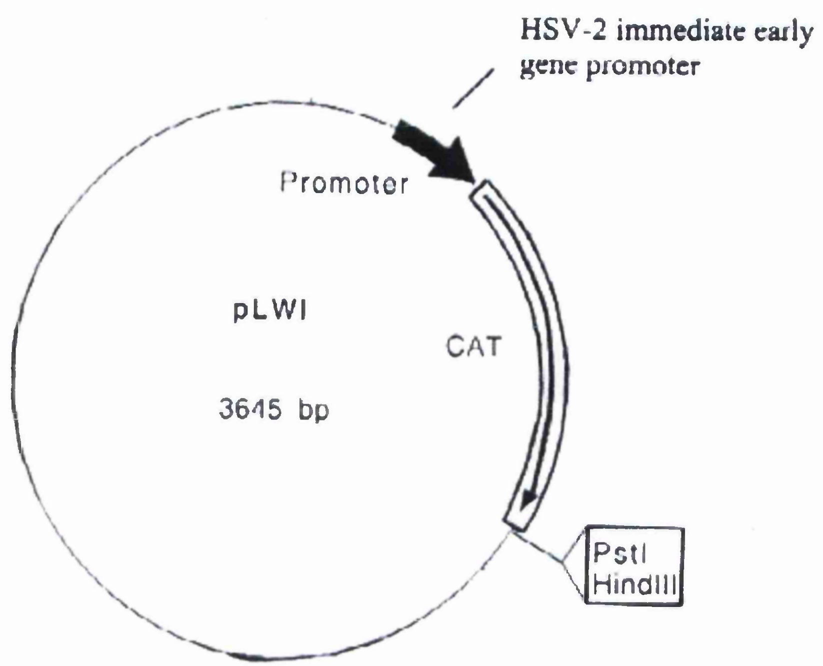


Fig. 4.2. pLW1 expression vector. Circular map of the pLW1 expression vector showing the chloramphenicol acetyltransferase (CAT) reporter gene under the control of the HSV-2 immediate early promoter. Wild type and mutant NRE constructs were inserted downstream of the reporter gene between the *Pst* 1/*Hin*d 111 sites. Gift from J. B. Clements (Gaffney *et al.*, 1985)

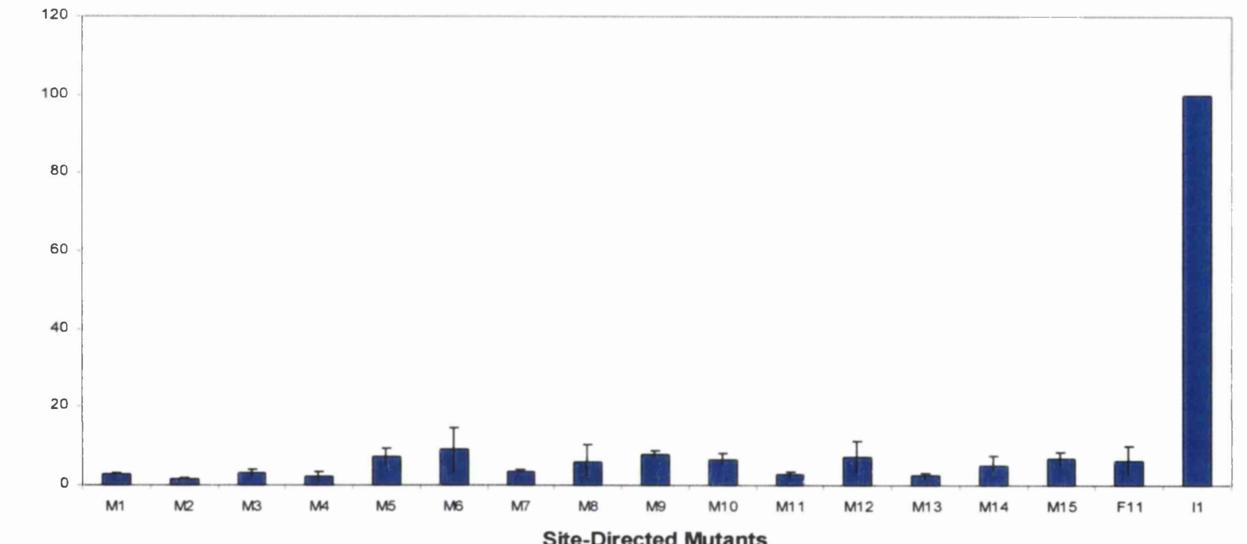


Fig. 4.3. Functional analysis of NRE mutants. The graph shows the mean and standard deviation from the mean of CAT activity in counts per minute (cpm) as a percentage of I1 for each of the 15 site-directed NRE mutants (M1-M15) based on duplicate assays from at least three separate transient transfection experiments in HeLa cells. The controls used were F11, a plasmid containing the HPV-16 L1 and overlapping late 3' UTR sequences; and plasmid I1 which lacks the *Pst* I/*Ssp* I region of the late 3' UTR of HPV-16, including the NRE.

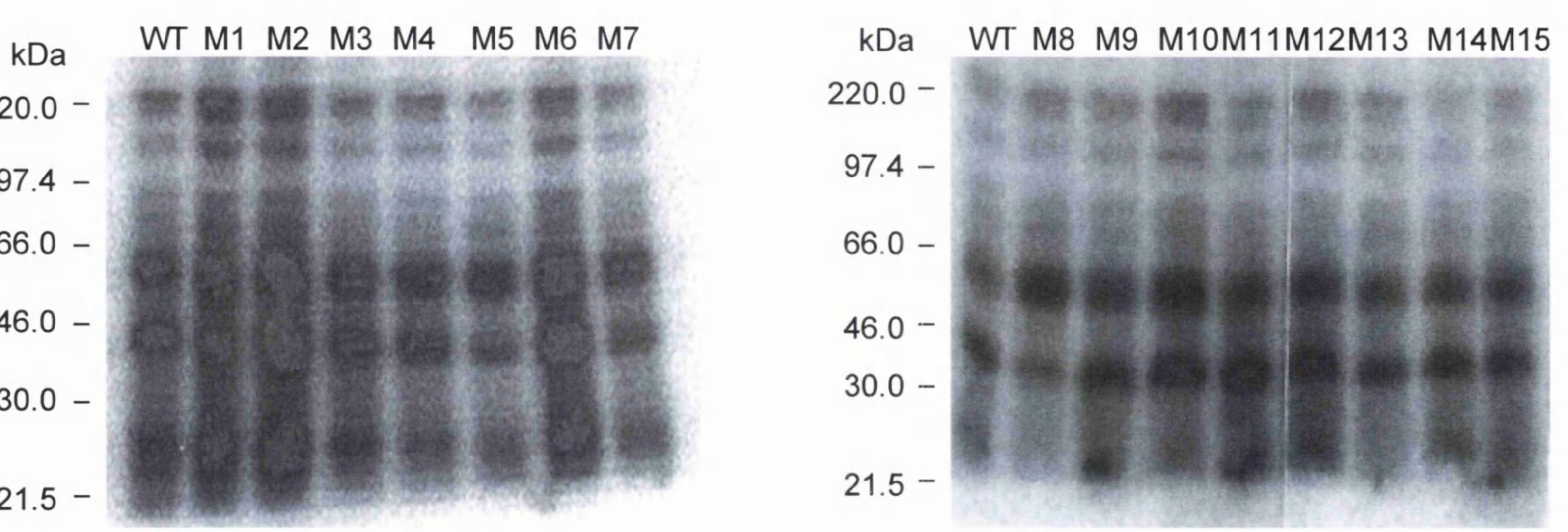


Fig. 4.4. UV cross-linking analysis of protein binding to the NRE and site-directed mutants at 60mM KCl. Radiolabelled RNA probes corresponding to the wild-type NRE (WT) and each of the 15 site-directed mutants (M1-M15) were prepared by *in vitro* transcription and UV cross-linked to HeLa nuclear extracts in binding buffer containing 60mM KCl. Bound proteins were fractionated on 12% SDS-PAGE gels and visualised by autoradiography using Kodak X-OMAT S film.

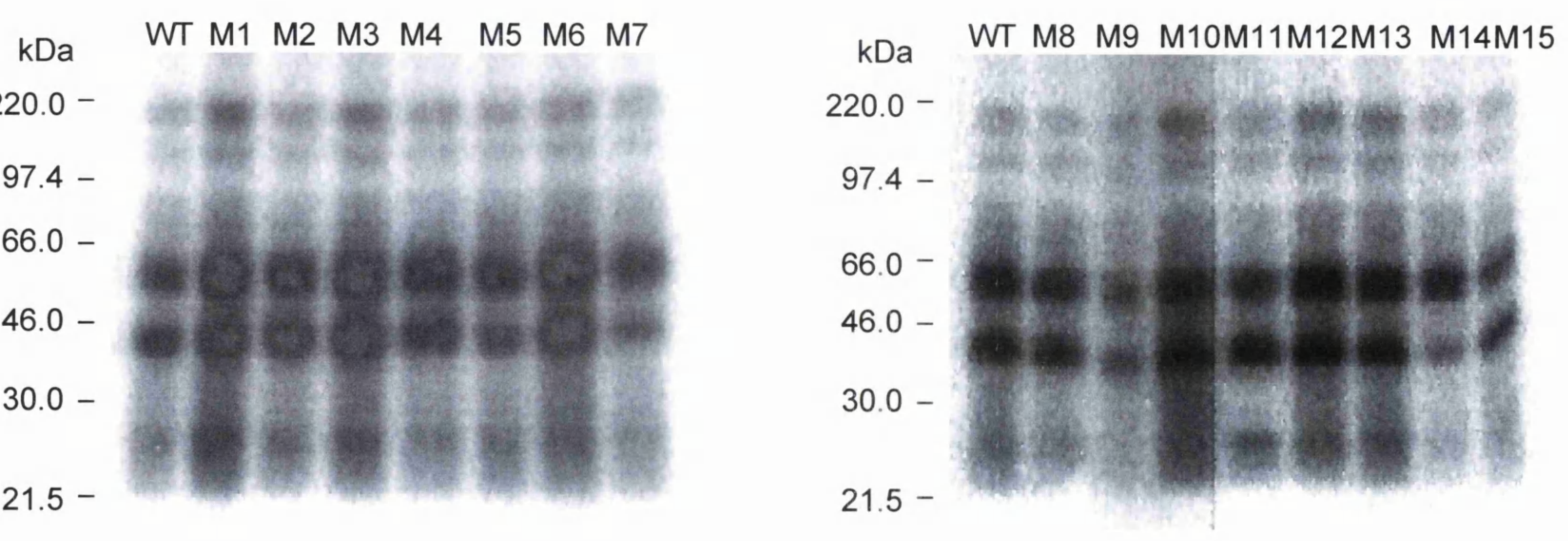


Fig. 4.5. UV crosslinking analysis of protein binding to the NRE and site-directed mutants at 125mM KCl. Radiolabelled RNA probes corresponding to the wild-type NRE (WT) and each of the 15 site-directed mutants (M1-M15) were prepared by *in vitro* transcription and UV cross-linked to HeLa nuclear extracts in binding buffer containing 125mM KCl. Bound proteins were fractionated on 12% SDS-PAGE gels and visualised by autoradiography using Kodak X-OMAT S film.

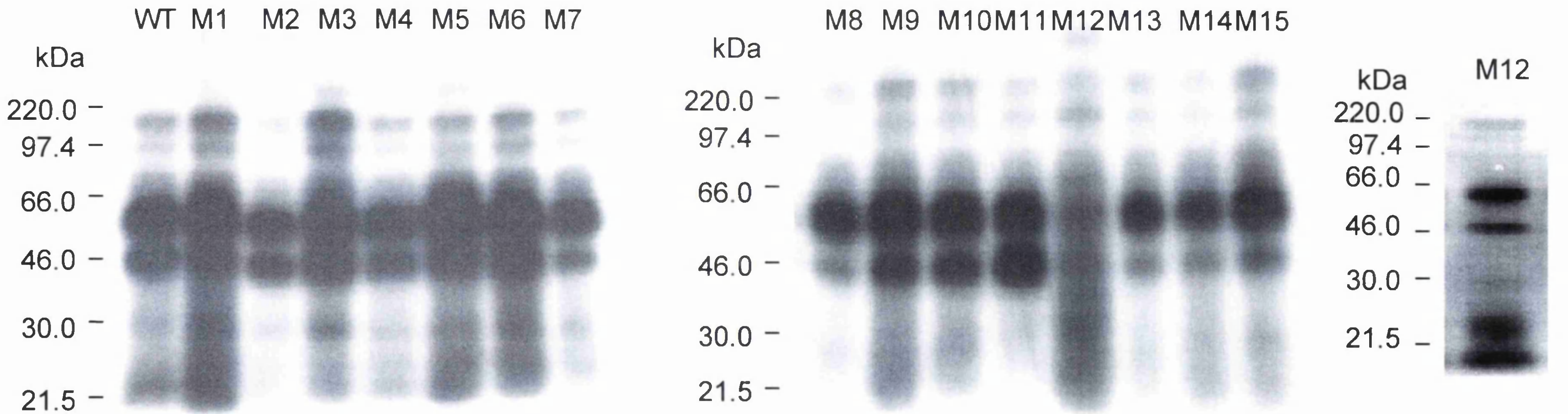
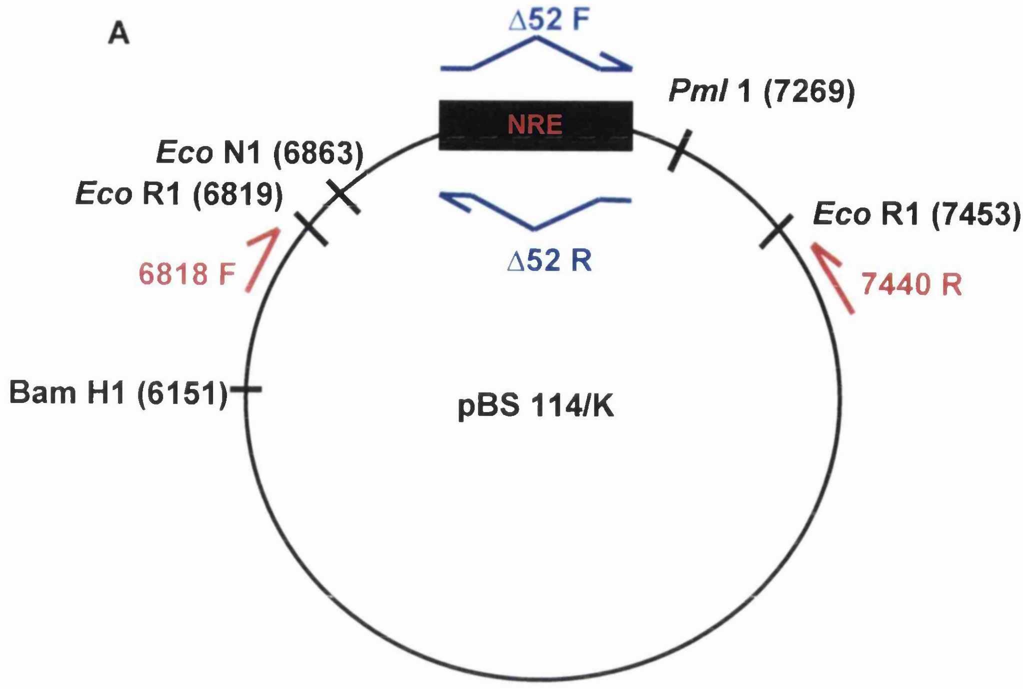


Fig. 4.6. UV cross-linking analysis of protein binding to the NRE and site-directed mutants at 250mM KCl. Radiolabelled RNA probes corresponding to the wild-type NRE (WT) and each of the 15 site-directed mutants (M1-M15) were prepared by *in vitro* transcription and UV cross-linked to HeLa nuclear extracts in binding buffer containing 250mM KCl. Bound proteins were fractionated on 12% SDS-PAGE gels and visualised by autoradiography using Kodak X-OMAT S film. Also included is an additional example of mutant M12 following UV cross-linking at 250mM KCl.



B

Δ52 F: CGTAAGCTGTAAGCTTGTATGTGCTTG

Δ52 R: CAAGCACATACAAGCTTACAGCTTACG

6818 F: GAATTCCACTATTTTGGAGGACTG

7440 R: CGGTTGAAGCTACAAAATGGG

Fig. 4.7. NRE deletion mutant. A. Cartoon of pBS114/K showing the relevant restriction enzyme sites and positions of the primers used to delete the NRE. **B.** Sequences of the primers used to delete the NRE.

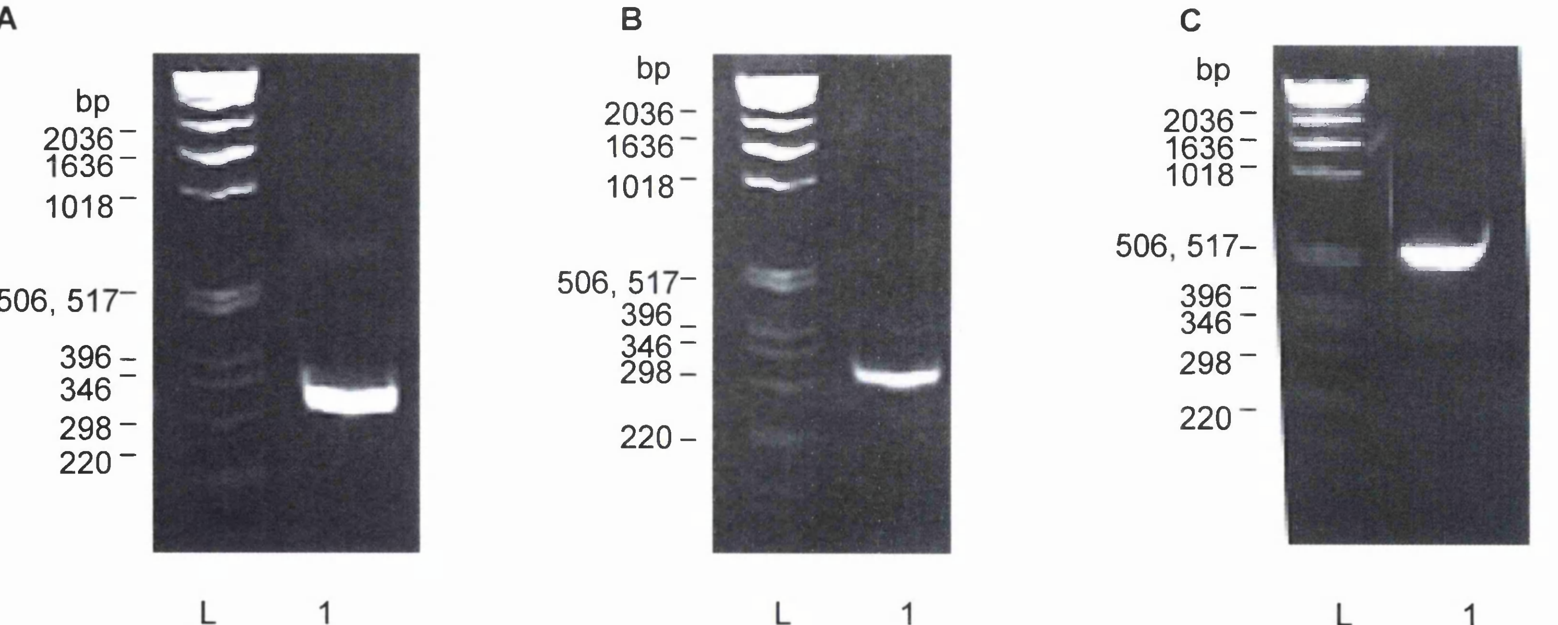


Fig. 4.8. PCR products for Δ52 mutant. Complimentary half molecules: **A.** Lane 1 contains the PCR1 product (351 bp) generated using the *Pml* 1 linearised template together with the 6818F primer and Δ52R primer. **B.** Lane 1 indicates the PCR1 product (258 bp) generated using the *Eco* N1 linearised template and the 7440R primer with the Δ52F primer. **C.** Lane 1, the complimentary half molecules were annealed and filled in by PCR using the 6818F and 7440R primers to generate a full-length, double-stranded product (583 bp). L indicates the 1Kbp DNA marker.

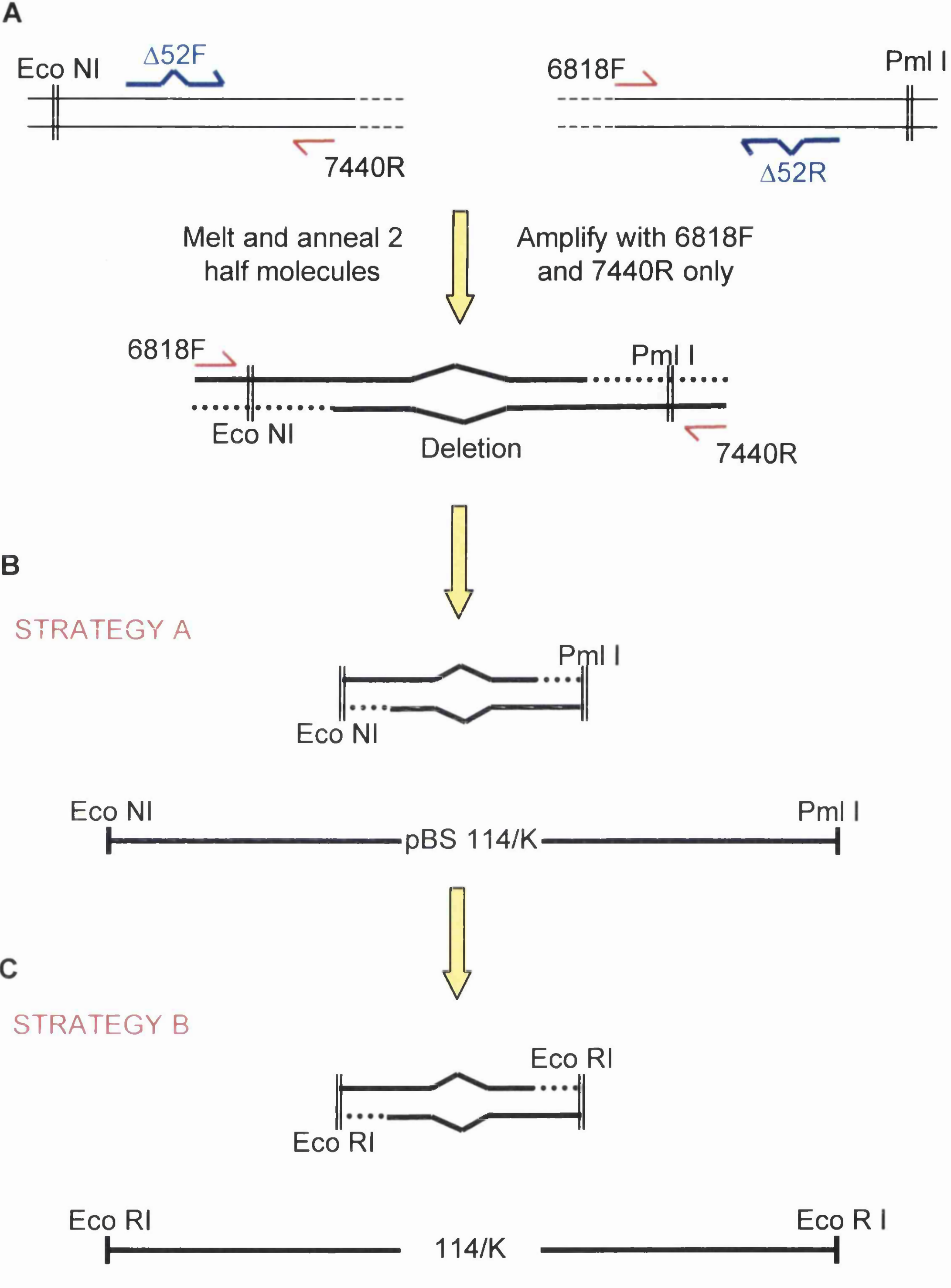


Fig. 4.9. Generation of the NRE-deletion mutant. A. Two complimentary half molecules, lacking the NRE, were PCR amplified, melted, annealed and amplified again to generate a full-length, double-stranded molecule. **B, C.** Two cloning strategies were used to religate the mutated NRE and the wild type viral genome.

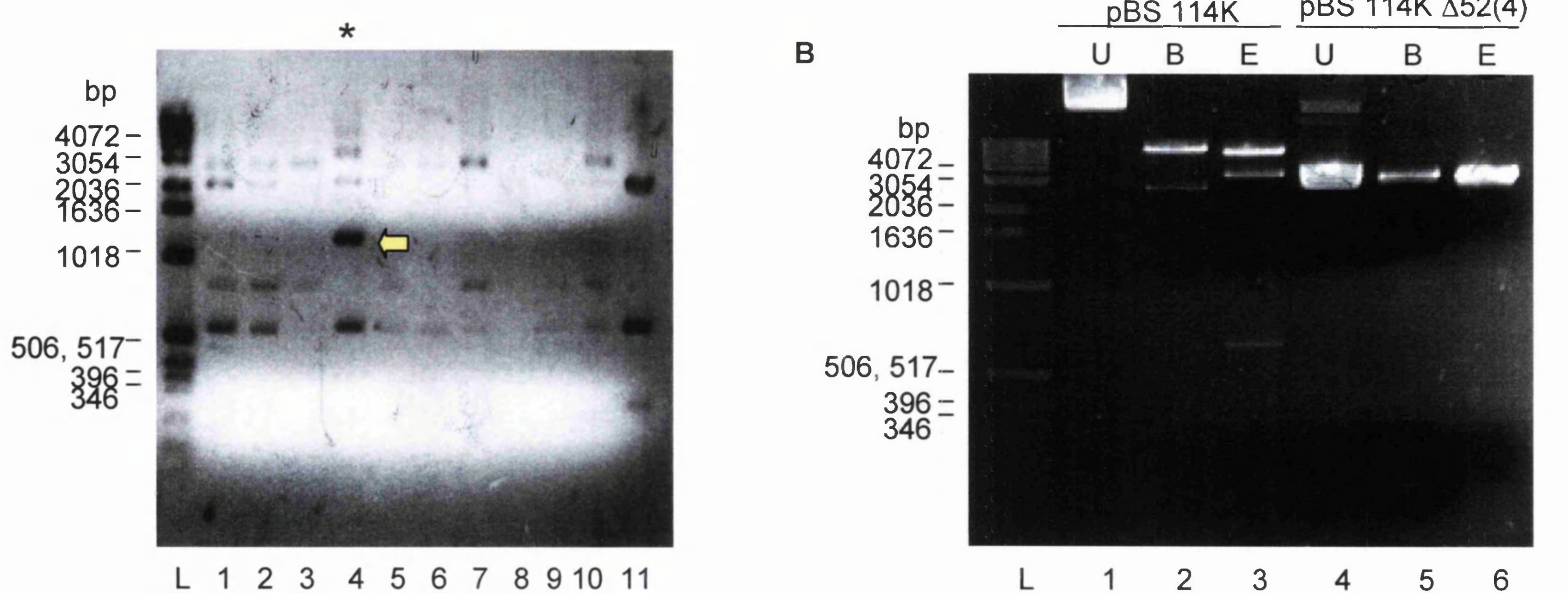


Fig. 4.10. Restriction enzyme analysis of Δ52 mutants. A. Plasmid mini-preps of the ligated DNA (pBS 114K + Δ52), lanes 1-10, were digested for 2h at 37°C with *Bam* H1 and *Ssp* 1 to check the presence and correct orientation of the Δ52 insert. A 1086nt fragment (as seen in lane 4) indicates that the insert is present in the correct orientation. Lane 11 contains a *Bam* H1/*Ssp* 1 digest of pBS as a control. **B.** Caesium chloride preps of wild-type pBS 114K and pBS 114K Δ52 (#4) were analysed as undigested DNA (U) or following digestion with either *Bam* H1 (B) or *Eco* R1 (E) for 2 h at 37°C. L in each gel indicates a 1Kb DNA marker.

CHAPTER 5 – Protein binding to the NRE

The expression of late HPV proteins is tightly controlled and despite several reports of the presence of late viral transcripts in undifferentiated cells, late viral proteins are detected only in terminally differentiating keratinocytes (Stoler *et al.*, 1989, Doorbar *et al.*, 1990, Ozbun *et al.*, 1997). The switch from early (P_{97}) to late promoter (P_{670}) usage in differentiating cells is partly responsible for controlling late gene expression (Grassmann *et al.*, 1996). However, post-transcriptional RNA processing mechanisms including alternative splicing, polyadenylation, nuclear export, and cytoplasmic stability, are also believed to play an important role in preventing translation of any late transcripts present in undifferentiated cells. The HPV-16 negative regulatory element (NRE), located in the late 3' untranslated region, was first identified due to its ability to repress reporter gene expression in undifferentiated epithelial cells (Kennedy *et al.*, 1990). Further to this, the NRE has been implicated in mRNA instability, disruption of terminal exon definition, nuclear retention and poly(A) site selection (Kennedy *et al.*, 1991, Furth et al., 1994, Koffa *et al.*, 2000, McGuire *et al.*, 2002). The coding potential of the HPV-16 genome is limited to 8 open reading frames, and therefore it is likely that the NRE is dependent on interactions with cellular factors in order to exert its inhibitory effects. In support of this, the element has been shown to bind a number of cellular proteins both by UV crosslinking and RNA affinity chromatography; a number of these proteins have been identified and include the auxiliary splicing factor $U2AF^{65}$, the polyadenylation factor CstF-64 and the shuttling protein HuR (Dietrich-Goetz *et al.*, 1997, Koffa *et al.*, 2000). Taken together this evidence suggests that the NRE has a central role to play in the post-transcriptional control of viral late gene expression, however, the exact role of the NRE remains unclear. In an attempt to address this problem, I decided to look at the involvement of the NRE in RNA processing, and more specifically, the ability of the NRE to interact with cellular factors involved in splicing. I chose to concentrate on

splicing, since removal of introns from pre-mRNAs is a crucial step in 3'end processing, and alternative splicing is known to play an important role in developmentally regulated and tissue-specific patterns of gene expression in a range of organisms including *Drosophila*, *C. elegans*, and vertebrates (reviewed in Wang *et al.*, 1997). Moreover, alternative splicing in viruses may facilitate the expression of a larger number of proteins from a relatively small genome. The aim of this work was two-fold; firstly I wanted to confirm that the NRE could bind the auxiliary splicing factor, $U2AF^{65}$, the polyadenylation factor, CstF-64, and the *elav*-like, shuttling protein HuR, as previously reported and secondly I hoped to identify other cellular proteins capable of interacting with the NRE, specifically cellular factors involved in splicing.

5.1.1 Binding of $U2AF^{65}$, CstF-64 and HuR to the HPV-16 NRE

Binding of $U2AF^{65}$, CstF-64 and HuR to the HPV-16 NRE had previously been demonstrated by UV cross-linking experiments and electrophoretic mobility shift assays (EMSA) (Dietrich-Goetz *et al.*, 1997, Koffa *et al.*, 2000). The NRE can bind several cellular proteins in UV cross-linking experiments using nuclear extracts from HeLa cells, the most abundant of which is a 65kDa protein band (Fig. 5.1A, lane 1), which is believed to contain $U2AF^{65}$ and CstF-64. A protein band of approximately 40kDa is also clearly visible within this protein binding profile and may be HuR. In order to confirm that the NRE could bind these cellular proteins UV cross-linking was carried out using a radiolabelled NRE probe incubated with HeLa nuclear extract (Fig. 5.1A, lane 1), nuclear extract only (Fig. 5.1A, lane 2), and NRE probe only (Fig. 5.1A, lane 3). HeLa cells are a useful source of basal cell-like proteins despite their highly transformed nature. Western blot analysis of the UV cross-linked separated proteins using the MC3 antibody, directed against $U2AF^{65}$, identified a single protein band at approximately 65kDa (Fig. 5.1B lanes 1,2) which when overlaid on the NRE protein binding profile (Fig. 5.1A,

lane 1) was found to co-migrate with the strong 65kDa protein band indicating that U2AF^{65} can be cross-linked to the NRE and is most likely one of the 65kDa proteins bound by this element. Similarly, Western blotting with α64k, an antibody directed against CstF-64, pulled out a single band when the NRE probe was incubated with nuclear extract (Fig. 5.1C, lane 1) which also appeared to co-migrate with the strong 65kDa protein band (Fig. 5.1A, lane 1) indicating that CstF-64 can bind the NRE and is also one of the 65kDa proteins interacting with the NRE. On this occasion, a CstF-64 specific band was not detected in the nuclear extract lane; this may indicate that not enough nuclear extract was loaded since a strong CstF-64 band was usually detected in nuclear extracts (Fig. 5.3B). Western blotting with 16A5, an anti-HuR antibody, indicated that the 40kDa protein band identified in the UV cross-linking protein binding profile quite possibly is HuR since the HuR specific protein band (Fig. 5.1D, lanes 1,2) could be overlaid directly onto this band, demonstrating that the NRE can bind HuR.

5.1.2 Identification of NRE binding proteins using a novel RNA binding assay

In order to further confirm that U2AF^{65}, CstF-64 and HuR can bind the HPV-16 NRE and to identify other cellular proteins that can interact with this element I developed an alternative method based on an RNA binding assay that had previously been used to identify cellular proteins required for the inhibition of HIV-1 pre-mRNA splicing (Caputi *et al.*, 1999). This technique is considered to be an improvement on the biotin/streptavidin binding assay since there is no longer a risk of Western blot contamination with streptavidin washed from the column. Also, the secondary structure of the target RNA is not disrupted in this method since the covalent link to the agarose beads involves the 3' end of the RNA only, similar to the poly(A) tailing approach. The RNA binding assay is based on an affinity chromatography approach and involves *in vitro* transcribing RNA corresponding to the full-length NRE

or first and second half NRE constructs (Fig. 1.10). This RNA is then covalently linked to agarose beads and the beads are used to affinity-purify factors from HeLa cell nuclear extracts that can assemble onto the HPV-16 NRE. Unbound proteins are removed by washing the beads four times in buffer D (20mM HEPES-KOH pH7.6, 5% glycerol, 0.1M KCl, 0.2mM EDTA, 0.5mM DTT) with 4mM $MgCl_2$. Poly U RNA linked to sepharose beads was used as a control since this reflects the U-rich nature of the 3' half of the NRE. A number of RNA processing factors, such as $U2AF^{65}$ are known to bind strongly to U-rich sequences (Green, 1991). In addition, agarose beads that had not been linked to RNA were also incubated with nuclear extract as a negative control. Nuclear proteins that bound to the immobilized RNAs were eluted into protein loading buffer by heating the beads to 90°C for 5 min, separated on a 12% SDS-PAGE gel and visualised by silver staining (Fig. 5.2B) since Coomassie blue staining was found not to be sensitive enough (data not shown). As with UV cross-linking, this method demonstrated that the NRE is capable of interacting with a range of cellular proteins (Fig. 5.2B, lane 2). However, the protein binding profile generated by UV cross-linking (Fig. 5.2A) is strikingly different from the profile produced by the RNA affinity chromatography approach despite the fact that the NRE RNA target is identical in both methods. In particular, the strong 65kDa band seen following UV cross-linking appears not to be pulled out in the RNA binding assay. In contrast, a number of smaller protein bands, ranging in size from 25 to 40kDa, are identified by affinity chromatography. Since polyU RNA would be predicted to bind a number of cellular proteins I was surprised that silver staining did not appear to identify proteins that could interact with this target (Fig. 5.2B, lane 3) however, in practice, it seems that it is generally difficult to detect polyU-bound cellular proteins (Baker, personal communication). Few proteins appeared to be bound by the unlinked agarose beads (Fig. 5.2B, lane 4). Unlike UV cross-linking, in which only cellular proteins that directly contact the target RNA can be irreversibly bound, the affinity chromatography approach allows the identification of

proteins that touch the NRE directly, and also proteins that are constituents of NRE-bound protein complexes but do not themselves bind to the NRE. This technical difference might help to explain the contrasting protein binding profiles generated by these assays.

5.1.3. The HPV-16 NRE can bind U2AF^{65}, CstF-64 and HuR

The RNA affinity chromatography method was used to confirm that U2AF^{65}, CstF-64 and HuR could bind to the NRE by immunoblotting with monoclonal antibodies directed against these cellular proteins (Fig. 5.3 A, B and C, lane 1). The three cellular proteins could also be affinity purified from HeLa nuclear extracts using polyU RNA (Fig. 5.3 A, B, C, lane 2). It is interesting to note that despite the fact that polyU RNA bound proteins could not be visualised by silver staining (Fig. 5.2B, lane 2) each of the antibodies used in the Western blot analysis identified a strong specific band in the polyU samples. No bound proteins were identified in the agarose beads and nuclear extract sample using this technique (Fig. 5.3 A, B, C, lane 3). Nuclear extract was included as a positive control for the Western blot (Fig. 5.3 A, B, C, lane 4) and gave a strong specific band for each antibody used.

5.1.4. The HPV-16 NRE can bind ASF/SF2 and Sm proteins

Silver staining of the affinity purified proteins identified a number of small nuclear proteins that could interact with the NRE. In an attempt to identify these small proteins Western blot analysis was carried out using the monoclonal antibody, αSF2 96, directed against the 28kDa cellular protein ASF/SF2, a member of the serine-arginine (SR) protein family of pre-mRNA splicing factors. SR proteins are a family of nuclear proteins, 10 of which have been identified so far, with important roles in all aspects of pre-mRNA splicing (Zahler *et al.*, 1992). As seen in Fig. 5.4A, ASF/SF2 can bind the NRE (lane 1), is unable to bind polyU RNA (Fig. 5.4A, lane 2), and was not

purified by unlinked agarose beads (Fig. 5.4A, lane 3). Nuclear extract was included as a positive control for the Western blot and produced a strong specific band (Fig. 5.4A, lane 4).

Western blot analysis was also performed using Y12, a monoclonal antibody directed against Sm proteins, which recognizes a band of approximately 28kDa. Sm proteins range in size from 9 to 29kDa and form a complex that binds to several small nuclear RNAs (snRNAs), including the spliceosomal U1, U2, U4 and U5 snRNAs, which are essential for splicing (reviewed in Will *et al.*, 1997). The HPV-16 NRE can bind Sm proteins (Fig. 5.4B, lane 1), however, polyU RNA did not bind Sm proteins (Fig. 5.4B, lane 2) which is surprising given that, although their binding sites on each sn RNA differ, the conserved sequences of the Sm site are generally U-rich (Will *et al.*, 1997). As with ASF/SF2, unlinked agarose beads could not bind Sm proteins (Fig. 5.4B, lane 3) and again, nuclear extract was included as a positive control for the Western blot (Fig. 5.4B, lane 4). This experiment was repeated several times with each antibody and similar results were obtained.

5.1.5. The HPV-16 NRE does not bind the 70kDa subunit of U1snRNP

Previous reports have demonstrated a specific protein-protein interaction between the 70kDa subunit of U1 snRNP (U1 70k) and ASF/SF2, which in turn can interact directly with $U2AF^{35}$ (reviewed in Caceres *et al.*, 1997). The primary role of U1 snRNP is in the recognition of 5' splice sites. Western blot analysis of the RNA affinity purified proteins using H111, an antibody raised against the α70kDa subunit of U1 snRNP, was performed to establish if this protein interacts with the NRE. As shown in Fig. 5.5 A, the NRE was unable to bind this cellular protein (Fig. 5.5 A, lane 1). In addition, U1 70k does not appear to bind to poly U RNA (Fig. 5.5 A, lane 2), and the unlinked beads were also unable to bind this protein (Fig. 5.5 A, lane 3). The Western blot was successful as indicated by bands in the nuclear extract only sample

(Fig. 5.5 A, lane 4), but there are clearly two bands present in this track, running between 65 and 70kDa; I am unable to account for the presence of the two bands but I have observed this pattern on other occasions (data not shown). It has previously been reported that the HPV-16 NRE is unable to bind U1 70k (Dietrich-Goetz *et al.*, 1997). This experiment was repeated on at least three occasions with similar results (data not shown) leading us to conclude that U1 70k cannot bind to the NRE.

5.1.6. Binding of the U1 snRNP-A protein to the HPV-16 NRE

Since the NRE does not appear to bind the α70kDa subunit of U1 snRNP, attention shifted to another component of U1 snRNP, the U1 snRNP-A protein (U1A); the size of this cellular protein (32kDa) suggested that it might be a good candidate for one of the smaller nuclear proteins identified by the RNA affinity chromatography method. Western blot analysis with anti-U1A antiserum was used to determine if this protein could interact with the HPV-16 NRE. The results of this experiment were somewhat ambiguous (Fig. 5.5 B and C); on at least two occasions U1A did not appear to bind the NRE (Fig. 5.5 B, lane 1). However, in at least two separate experiments the HPV-16 NRE did appear to bind U1A (Fig. 5.5 C, lane 1) and this was further complicated by the observation that while the first half of the NRE was unable to bind this protein (Fig. 5.5 C, lane 2), the second half of the NRE bound U1A quite strongly (Fig. 5.5 C, lane 3).

In order to clarify the situation with regards to the binding of U1A to the NRE an experiment, similar to that described in section 5.1.1, was carried out. A radiolabelled NRE probe was incubated with HeLa nuclear extract and following UV cross-linking the nuclear proteins bound to the NRE RNA probe were analysed by immunoblotting with anti-U1A antiserum. Samples of nuclear extract only and NRE-probe only were included as controls. Fig. 5.6A shows the protein binding profile generated using this method, while

Fig. 5.6B indicates that U1A can be cross-linked to the HPV-16 NRE and this U1A specific band co-migrates with a protein band of approximately 35kDa in Fig. 5.6 A, lane 1.

This experiment was repeated using a radiolabelled full-length NRE and first half and second half NRE constructs (Fig. 5.7 A lanes 1, 2, 3). As controls, the full length NRE probe only (lane 4) and nuclear extract only (lane 5) were also subjected to UV irradiation. Following cross-linking, the proteins were separated on a 15% SDS-PAGE gel and Western blotted with anti-U1A antiserum. U1A could be cross-linked to the full-length and half constructs of the HPV-16 NRE (Fig. 5.7 A, lanes 1, 2, 3). U1A was also detected in the nuclear extract only sample as expected (Fig. 5.7 A, lane 5) but not in the NRE probe only sample (Fig. 5.7 A, lane 4). An additional band is visible on this blot running at approximately 25kDa but I am unsure of its identity or significance. This nitrocellulose membrane was then stripped to remove the primary and secondary antibodies and reprobed with 6CS, an antibody against the housekeeping protein, GAPDH, as a control for the amount of nuclear extract present in each sample. Since the nuclear extract sample that had been cross-linked (Fig. 5.7A, lane 5) was positive for U1A, one could argue that the presence of U1A in the samples containing the full-length or half NRE constructs was simply a result of the presence of nuclear extract. However, if the levels of GAPDH are compared in Fig. 5.7B, particularly in lanes 2, 3 and 5, it is clear that while the levels of GAPDH in these samples are very similar, the levels of U1A protein in the same samples in Fig. 5.7A differ, such that there appears to be more U1A protein binding in lanes 2 and 3 compared to lane 5 (first half and second half NRE constructs and nuclear extract only, respectively) suggesting that U1A can be cross-linked to the HPV-16 NRE. Nevertheless, it remains possible that the detection of U1A in the UV cross-linked samples containing the full-length or half-NRE constructs (Fig. 5.7A, lanes 1-3) merely reflects the presence of nuclear extract, particularly since the data generated using the RNA binding assay suggested that U1A was unable to bind to the first half of

the NRE (Fig. 5.5C, lane 2). However, I feel that despite the shortcomings of these experiments the data indicates that U1A can interact with the HPV-16 NRE, although this interaction may be of a transient nature.

5.1.7. Comparison of protein binding to the full-length and half NRE constructs

As mentioned previously, the HPV-16 NRE can be roughly divided into two halves: the first or 5' half containing the putative stem loop structure and the four weak 5' splice sites, and the second, 3' half made up of the GU rich region (Fig. 1.10). Recent work in our laboratory has demonstrated that while the second half of the NRE is important for cellular protein binding, the first half is responsible for more of the inhibitory activity of the element (Cumming *et al.*, 2002b). RNA corresponding to the full-length NRE, and the first and second halves was linked to agarose beads and incubated with HeLa nuclear extracts as previously described. Bound proteins were fractionated by SDS-PAGE and visualised by silver staining (Fig. 5.8). In agreement with previous observations, the 2nd half NRE construct appeared to bind more protein that the 1st half construct and, surprisingly, also bound more protein than the full-length NRE, although the overall profile was similar (Cumming *et al.*, 2002b). This experiment was repeated a number of times with similar results. Some proteins in particular appeared to be bound particularly strongly by the second half of the NRE compared to the full-length construct, these included a protein, or possibly two proteins as a doublet, running at approximately 35kDa. In an attempt to identify this protein, Western blot analysis was carried out on the fractionated RNA bound proteins. Two proteins in particular, HuR and ASF/SF2, were considered good candidates given the size of the protein band and immunoblotting with antibodies against these proteins revealed that the ASF/SF2 was probably one of the proteins strongly bound by the second half of the NRE. HuR appears to require the full-length NRE for efficient binding (Fig. 5.9A, lane 1), whereas

ASF/SF2 binds more strongly to the second half of the NRE than the first half or full-length constructs, (Fig. 5.9B, lanes 3, 2 and 1, respectively), possibly suggesting that efficient binding of this protein to the full-length NRE is blocked by binding of other cellular proteins.

It was recently reported that upon induction of epithelial differentiation the levels of the NRE binding proteins U2AF^{65} and CstF-64 alter such that U2AF^{65} levels decrease and levels of CstF-64 increase with differentiation. Although the levels of HuR were unaffected by differentiation, accumulation of this protein in the cytoplasm was observed (Koffa *et al.*, 2000). This work was carried out using the HPV-16 positive epithelial cell line, W12, and confluent cell monolayers were maintained in a high calcium and serum medium for 10 days to induce differentiation (Koffa *et al.*, 2000). Conversely, a previous report had indicated that the level of expression of CstF-64 decreased with differentiation in a HPV-31 positive cell line, LKP-31 (Terhune *et al.*, 1999). In this paper the authors induced differentiation by suspending the cells in semisolid methylcellulose medium for up to 24 h; this method has previously been shown to result in the activation of the viral late promoter, transcription of late genes and viral DNA amplification (Reusch *et al.*, 1998). As previously discussed, in Chapter 3, I have shown that epithelial cells differentiate spontaneously if grown in monolayer culture for 10 days. This observation might suggest that the undifferentiated cell population utilised by Koffa *et al.*, had in fact begun to differentiate and therefore they were comparing cells at different stages of differentiation rather than undifferentiated cells with differentiated cells. If this were the case then this might help explain the difference between the reports concerning the expression of CstF-64.

The aim of the work presented here was to elucidate the changes in the level of expression of the NRE-binding proteins with differentiation, by comparing undifferentiated HPV-16 genome containing cell populations with differentiating HPV-16 genome containing populations. The effect of the status of the viral genome on these differential changes was compared using subclones of the W12 cell line containing episomal HPV-16 genomes i.e. the 20863 line, and integrated copies of the HPV-16 genome i.e. the 20861 line (Jeon *et al.*, 1995a).

Previous reports have shown that integration of the HPV genome alters the differentiation programme of the infected cell (McCance *et al.*, 1988, Rader *et al.*, 1990, Merrick *et al.*, 1992). This integration event frequently disrupts the early coding region and results in the loss of expression of all viral proteins, with the exception of the oncogenic proteins, E6 and E7. The E6 and E7 proteins have many roles in the viral life cycle but their combined action can maintain the proliferative activity of infected cells, thus modifying the normal cellular differentiation programme (reviewed in Mantovani *et al.*, 2001, Munger *et al.*, 2001). The HaCaT cell line contains no papillomavirus genomes and was used as a control for the differentiation of epithelial cells since this immortalised line has retained the ability to differentiate as demonstrated in Fig. 3.5B and as previously reported (Boukamp *et al.*, 1988, Schoop *et al.*, 1999).

Epithelial cells were grown in monolayer culture for 5 days and harvested as undifferentiated cells, and cells grown for 10 days were harvested as differentiating cells. Protein extracts were prepared and 10μg of each extract, as calculated by Bradford assay, was fractionated by SDS-PAGE and the proteins visualised by Coomassie blue staining to confirm that comparable amounts of protein extract had been loaded in each lane. Typical protein extracts from day 5 (undifferentiated) and day 10 (differentiating) 20863 cells are shown in Fig. 5.10A, the protein profiles appear very alike indicating that each tract contains similar amounts of protein extract. In addition, a comparison of day 5 and day 10 protein extracts from 20863 and 20861 cells is shown in Fig. 5.10B, it appears that similar amounts of protein extract have been loaded and there does not appear to be any significant differences in the protein profile in these different lines, at least at the level of sensitivity provided by Coomassie blue staining. Day 5 and day 10 extracts from each line were analysed by Western blotting with the SY5 antibody, directed against involucrin (Fig. 5.11). From this figure we can see that in each line the level of expression of this differentiation maker increases between day 5 and day 10 extracts.

However, it appears that the increase in expression of involucrin between day 5 and day 10 extracts of the 20861 line (Fig. 5.11B) is less significant than that observed in the 20863 and HaCaT samples (Fig. 5.11A and C). This would tend to suggest that day 10 20861 cells are slightly less differentiated than similarly treated samples of 20863 and HaCaT cells, indicating that the integration of the HPV-16 genome in the 20861 line has disrupted the differentiation programme of these cells, in agreement with previous reports (McCance *et al.*, 1988, Rader *et al.*, 1990, Merrick *et al.*, 1992).

5.2.1. Differential expression of U2AF^{65} in monolayer cultures of epithelial cells

Fractionated protein extracts (10μg) prepared from day 5 and day 10 20863 cells (Fig. 5.12A), 20861 cells (Fig. 5.12B), and HaCaT cells (Fig. 5.12C) were Western blotted with MC3, an antibody directed against U2AF^{65}. In Fig. 5.12A, lanes 1 and 2, U2AF^{65} appears to be moderately upregulated with differentiation in 20863 cells, while in 20861 cells (Fig. 5.12B, lanes 1 and 2), and HaCaT cells (Fig. 5.12C, lanes 1 and 2), this cellular protein is downregulated with differentiation. This experiment was repeated at least five times using different extracts from each cell line and while the level of U2AF^{65} in differentiating 20861 cells was consistently downregulated, the level of this protein in differentiating 20863 cells and HaCaT cells varied, although the majority of blots indicated that U2AF^{65} expression increased in differentiating 20863 cells and decreased in differentiating HaCaT cells. As a control, a number of the nitrocellulose membranes were stripped after immunoblotting for U2AF^{65} and reprobed with 6CS, an anti-GAPDH antibody. A comparison of day 5 and day 10 extracts in each blot, (Fig. 5.12A, B, C, lanes 3 and 4), indicates that the samples contain similar amounts of protein and therefore the differences in the level of expression of U2AF^{65} are not a result of uneven sample loading.

5.2.2. Differential expression of CstF-64 in monolayer cultures of epithelial cells

Day 5 and day 10 protein extracts prepared from monolayer cultures of 20863, 20861 and HaCaT cell lines were analysed by Western blot using the anti-CstF-64 antibody, α64k. The level of CstF-64 protein in 20863 cells appeared to decrease with differentiation (Fig. 5.13A, lanes 1 and 2); increase with differentiation in 20861 cells (Fig. 5.13B, lanes 1 and 2); and decrease moderately in the differentiating HaCaT cell line (Fig. 5.13C, lanes 1 and 2). This experiment was repeated on at least three occasions for each cell line and with different samples. When the blots shown here where stripped and reprobed with an anti-GAPDH antibody, 6CS, each lane appeared to contain similar amounts of protein extracts (Fig. 5.13A, B and C, lanes 3 and 4), indicating that sample loading was equal. Our results tend to support the observations of Terhune *et al.*, (1999) in that CstF-64 expression appears to decrease with differentiation in cells where the papillomavirus genome remains episomal.

5.2.3. Differential expression of HuR in monolayer cultures of epithelial cells

Western blotting with 16A5, an antibody directed against the shuttling protein HuR, indicated that 20863 cell differentiation leads to a modest decrease in the level of expression of HuR (Fig. 5.14A, lanes 1 and 2). In 20861 cells HuR is up regulated with differentiation (Fig. 5.14B, lanes 1 and 2), while in HaCaT cells the level of HuR protein appears unaffected by differentiation (Fig. 5.14C, lanes 1 and 2). Different extracts from each cell line were used to repeat this experiment at least three times, (data not shown), and the blots shown in Fig. 5.14 are representative of the majority of results obtained. In addition, the GAPDH protein levels appear comparable in the day 5 and day 10 samples of each stripped and reprobed blot shown (Fig. 5.14A, B and C lanes 3 and 4).

5.2.4. Differential expression of ASF/SF2 in monolayer cultures of epithelial cells

The αSF2 96 monoclonal antibody directed against ASF/SF2 was used to determine if epithelial cell differentiation affected the level of expression of this protein and this experiment was repeated at least three times. In Fig. 5.15A, lanes 1 and 2, ASF/SF2 appears to be substantially upregulated with differentiation in 20863 cells, but appears to be downregulated in differentiating 20861 cells (Fig. 5.15B, lanes 1 and 2). In HaCaT cells, ASF/SF2 expression is slightly decreased with differentiation (Fig. 5.15C, lanes 1 and 2). Similar amounts of protein were loaded for each sample as indicated by the anti-GAPDH blots (Fig. 5.15B and C, lanes 3 and 4), however, Fig. 5.15A, lanes 3 and 4, indicates a lower level of GAPDH protein in day 10 20863 cells than the day 5 sample suggesting that the differential increase in ASF/SF2 expression may be even more pronounced than appears in Fig. 5.15A, lanes 1 and 2.

5.2.5. Differential expression of Sm proteins in monolayer cultures of epithelial cells

Based on the results obtained from three separate experiments, the level of Sm protein expression appears to be moderately upregulated with differentiation in both the HPV-16 positive cell lines, (Fig. 5.16A and B, lanes 1 and 2), but in differentiating HaCaT cells, Sm proteins are slightly downregulated (Fig. 5.16C, lanes 1 and 2), as shown by Western blot analysis using Y12, an antibody directed Sm proteins. The blots shown are representative of the repeated experiments, which gave consistent results. When the levels of GAPDH were analysed in the stripped blots similar amounts of protein were found in day 5 and day 10 lanes in the 20861 and HaCaT blots (Fig. 5.16B and C, lanes 3 and 4), however, GAPDH levels in day 10 20863 cells were slightly higher than in the day 5 track (Fig. 5.16A, lanes 3 and 4) but I feel that this difference is not large enough to account for

the differential increase in the level of Sm proteins observed in this line (Fig. 5.17A, lanes 1 and 2).

5.2.6. Differential expression of U1A in monolayer cultures of epithelial cells

The effect of differentiation on the level of expression of U1A was analysed on three occasions by immunoblotting with rabbit antiserum raised against the protein. The level of U1A in 20863 and 20861 cells does not appear to be affected by differentiation, (Fig. 5.17A and B, lanes 1 and 2), and this was a consistent observation. However, differentiation of HaCaT cells results in a down regulation of this protein (Fig. 5.17C, lanes 1 and 2). The membranes shown in Fig. 5.17A, B and C (lanes 1 and 2) were stripped and reprobed with 6CS, an anti-GAPDH antibody, to check that the samples on each had comparable amounts of protein (Fig. 5.17A, B and C, lanes 3 and 4).

5.3.1. Expression of NRE-binding proteins in 20863 organotypic raft cultures

Total protein extracts were prepared from 20863 cells cultivated on organotypic raft cultures to enable us to analyse the level of expression of the NRE-binding proteins in a differentiating system that closely resembles the *in vivo* situation. Rafts were untreated (U), or treated with 10μM C8:0 (C8) for 12 days at the air-liquid interface. Treatment with C8:0 (1, 2-dioctanoyl-*sn*-glycerol) is thought to facilitate a more complete differentiation programme through the activation of the protein kinase C pathway (Meyers *et al.*, 1992, Ozbun *et al.*, 1997). When the HPV-31 line, CIN 612 9E, is cultivated on the raft system, treatment with C8:0 is required for the synthesis of the late viral capsid proteins (Meyers *et al.*, 1992). Based on our observations, however, C8:0 treatment of 20863 organotypic rafts, while perhaps enhancing the production of HPV-16 capsid proteins, is not necessary, since late proteins are also clearly detected in untreated rafts (Fig. 3.11). Protein extracts were fractionated by SDS-PAGE, transferred to

membranes and immunoblotted with antibodies directed against each of the cellular proteins previously found to bind the NRE: $U2AF^{65}$, CstF-64, HuR, ASF/SF2, Sm, and U1A (Fig. 5.18 A to F respectively). These experiments were repeated at least three times for each protein, and although the results varied between blots, it eventually became apparent, through repetition and by comparing sample loading by probing for GAPDH, that the level of expression of each of these cellular proteins appeared to be comparable between the untreated and C8 treated extracts. A number of the nitrocellulose membranes were stripped and reprobed with 6CS and a typical GAPDH blot is shown in Fig. 5.18G.

At first these results were quite surprising and appeared to contradict the observations I had previously made using protein extracts prepared from the same cell line grown in monolayer culture. However, I now believe that I detected no change in the level of expression of these proteins between untreated and C8:0 treated extracts because, unlike the material harvested from monolayer culture, I was not comparing undifferentiated with differentiating but rather like with like, in that, both samples were cultivated on the raft system and had therefore a large percentage of cells has commenced terminal differentiation i.e. as a heterologous cell population there was a low percentage of basal epithelial cells. This suggests that while C8 treatment of HPV-16 organotypic rafts may increase the expression of late viral proteins, as seen when E1^E4 and L1 proteins levels were compared between untreated and C8 treated rafts (Fig. 3.11), it has no obvious effect on the expression of the cellular proteins I have analysed. I have previously shown that C8:0 treatment does not affect the level of expression of the differentiation marker, involucrin (Fig. 3.7B, 3.9B). However, I did not attempt to detect changes in the level of expression of cellular markers expressed in more differentiated epithelial layers, such as filaggrin. Tissue harvested from organotypic rafts is a heterogeneous population of undifferentiated, differentiating and terminally differentiated cells that make up the various layers of the stratifying epithelium. I attempted

to overcome this problem by soaking harvested raft tissue overnight in trypsin at 4°C to separate the uppermost cornified layer from the lower epithelial layers, this technique had previously been used successfully with biopsy tissue (David Millen, Histopathology, Western Infirmary, Glasgow personal communication). Unfortunately I succeeded only in floating the epithelial tissue from the collagen support; this experiment was repeated several times with varying concentrations of trypsin but with similar results (data not shown). Another shortcoming of this experiment is that the extracts prepared from the rafts contain so much protein that any small differences between the samples are masked, it may have been possible to identify subtle differences between untreated and C8 treated extracts if I had compared serial dilutions of the samples; unfortunately, however, I did not attempt this experiment due to a lack of time.

5.3.2. Expression of CstF-64 and U2AF^{65} in CIN 612 9E organotypic rafts

It is well established that C8:0 treatment of HPV-31, CIN 612 9E, rafts is required for efficient viral late protein production and therefore it is possible that, unlike HPV-16 rafts as discussed above, a comparison of untreated and C8 treated HPV-31 raft material would be equivalent to comparing differentiating protein extracts with terminally differentiated extracts. As HPV-31 positive cells, induced to differentiate with methylcelluose, show a differential decrease in expression of CstF-64 (Terhune *et al.*, 1999), I decided to use CIN 612 9E cells grown on organotypic rafts for 12 days at the air-liquid interface in the presence of C8:0 (C8), or without treatment (U), to further investigate the changes in CstF-64 expression. In addition, I wanted to clarify the effect of C8 treatment on the expression of another cellular protein, U2AF^{65}, in light of the observations I had made using C8-treated and untreated HPV-16 raft material. Moreover, it has recently been demonstrated that an NRE-like element (NLE) in the late 3'UTR of HPV-31 can bind the cellular proteins CstF-64, U2AF^{65}, and HuR (Cumming *et al.*,

2002a). Protein extracts prepared from HPV-31 rafts were analysed by Western blotting using α64k and MC3 antibodies against CstF-64 and $U2AF^{65}$ respectively. In agreement with Terhune *et al.*, (1999) the level of CstF-64 in C8-treated CIN 612 9E cells was found to be less than that in untreated rafts (Fig. 5.19A). This also agrees with the result I generated for HPV-16 using 20863 monolayer cultures (Fig. 5.12A). The level of $U2AF^{65}$ was also found to decrease in C8-treated CIN 612 9E cells (Fig. 5.19B). These observations imply that C8:0 treatment of CIN 612 raft cultures is important not only for efficient expression of late viral proteins but also has an appreciable difference on the level of expression of at least two cellular proteins known to interact with the HPV-31 NLE, CstF-64 and $U2AF^{65}$.

5.4. Discussion

Since the discovery of the HPV-16 NRE, efforts have been focused on identifying the cellular proteins that interact with this element in an attempt to understand more fully the role it plays in the post-transcriptional control of viral late protein expression. Previously, the cellular proteins $U2AF^{65}$, CstF-64 and HuR had been shown to bind directly to the HPV-16 NRE (Dietrich-Goetz *et al.*, 1997, Koffa *et al.*, 2000). Alternative approaches, described herein, have confirmed that these proteins do indeed bind the NRE. In addition, it has been shown that the NRE can also bind the cellular proteins ASF/SF2, Sm proteins and U1A, but in agreement with previously published work, the NRE does not appear to bind the 70kDa subunit of U1 snRNP (U1 70K) (Dietrich-Goetz *et al.*, 1997). I specifically focused on cellular factors involved RNA processing, which obviously has the potential to be an important control mechanism in differential gene expression. Following the identification of these NRE-binding proteins, changes in the level of expression of each protein during epithelial cell differentiation were compared in the 20863, 20861 and HaCaT cell lines. The HaCaT cell line was used to establish the effect of epithelial differentiation on the expression of the cellular proteins in the absence of any HPV genomes. HaCaT cells have been shown to form almost regular epidermal architecture when transplanted onto subcutaneous tissue of athymic mice and also when grown on organotypic raft cultures and are therefore a very useful paradigm for skin keratinocytes *in vitro* (Boukamp *et al.*, 1988, Schoop *et al.*, 1999). Whilst terminally differentiating keratinocytes retain metabolic activity, this is generally restricted to regulating the pattern of keratin gene expression, and cellular proteins not directly involved in this process are believed to be downregulated with differentiation (Fuchs *et al.*, 1994). As expected, the level of expression of all the cellular proteins tested decreased with differentiation in HaCaT cells, with the exception of HuR which appeared to be unaffected by differentiation. This observation is in agreement with a

previous report in which the authors observed no change in expression of HuR following differentiation of spontaneously transformed A/J mouse lung tissue (Blaxall *et al.*, 2000). A comparison of the HaCaT cell line with the HPV-16 positive 20863 and 20861 lines allows us to establish the effect of viral infection on the differential expression of the NRE-binding factors and also how the state of the viral DNA (i.e. episomal versus integrated) influences this pattern of expression (Table 5.1). Interestingly, I observed that 3 cellular proteins, with well-documented roles in splicing, U2AF^{65}, ASF/SF2 and Sm proteins, were up regulated in differentiated 20863 cells, compared to the HaCaT controls. CstF-64 and HuR were downregulated with 20863 differentiation and the levels of U1A appeared to be unaffected. In contrast to these observations, a previous study on the expression of U2AF^{65}, CstF-64 and HuR in the W12 line had reported a differential decrease in U2AF^{65}, an increase in CstF-64 and a redistribution of HuR with differentiation. There are a number of possible explanations for these differences: Firstly, the methods used to induce differentiation differ between the studies; we used prolonged growth in monolayer culture to induce differentiation, whereas differentiation was induced by increasing the calcium and serum concentration of the culture medium in the previous report. Furthermore, for the first time we were able to use a cell population that had characteristics of truly undifferentiated epithelial cells. Secondly, in the original report the state of the viral DNA was not analysed in the W12 cells. Since the cells used had reached quite a high passage number, (>40), it is likely that the HPV-16 genome had become integrated in these cells causing further disruption of the differentiation programme. I used a clonal derivative of the parental W12 line, the 20863 line, which stably maintains the virus genome as an episome when the cells are at low passage. Typically cells ranging in passage number from 15 to 18 were used and Southern blot analysis confirmed that the virus was episomal in these cell populations. When protein levels were analysed in the 20861 line, another clonal derivative of the W12 line in which the viral DNA is known to be integrated,

$U2AF^{65}$ expression decreased whereas CstF-64 expression increased with differentiation, in agreement with the original report by Koffa *et al.*, (2000) indicating that the viral genome most likely was integrated in the late passage W12 cells used in that study. HuR appeared to be increased with differentiation in the 20861 line, however, we analysed nuclear extracts only and did not investigate the subcellular distribution of this protein following differentiation.

In contrast to what was observed in 20863 cells, ASF/SF2 levels decreased in differentiating 20861 cells, however, Sm protein expression was upregulated in both day 10 20861 and 20863 cells. In the 20861 line, integration of the HPV-16 genome leads to a specific increase in the expression of CstF-64 and HuR, when compared to cells harbouring episomal virus (20863) and no virus (HaCaT). Further analysis of differential gene expression in the 20861 line might provide interesting insights on the effect of viral integration on the expression of RNA processing factors, albeit *in vitro.* Much attention has been focused recently on delineating the molecular profile of various human tumours including lung cancer and prostate cancer by comparing differential gene expression profiles of normal and cancerous tissue (Blaxall *et al.*, 2000, Dhanasekaran *et al.*, 2001). It is hoped that by identifying the so-called 'molecular signature' of individual cancers this information could be used to improve detection rates through molecular classification and also aid the design of specific anti-cancer therapies.

It appears that differentiation of 20863 cells results in a specific increase in expression of two cellular factors $U2AF^{65}$ and ASF/SF2, albeit slight for the former. Both of these proteins are known to play important roles in the regulation of splicing (reviewed in Caceres *et al.*, 1997), and it is possible that a differential increase in expression of these factors is necessary to meet the demands of the increased rate of RNA processing during the productive stage of the viral life cycle. Alternatively, these proteins may be

important in overcoming the negative effects of the NRE in differentiated cells. Either way, these results suggest that HPV-16 can circumvent the normal cellular differentiation programme to ensure these proteins are expressed when needed during the viral life cycle. A number of recent reports have noted altered expression of general splicing factors both during differentiation and also as a result of various modes of cellular transformation (Chabot *et al.*, 1992, Hanamura *et al.*, 1998, Maeda *et al.*, 1999, Shinozaki *et al.*, 1999). In addition, it has been proposed that variations in the expression and activity of splicing factors during viral infection is important for the regulated expression of viral proteins. For example, it has been demonstrated that ASF/SF2 is partially inactivated during adenovirus infection to allow the early to late shift in viral mRNA expression (Molin *et al.*, 2000). Also, the HIV Rev response element (RRE) has been shown to bind ASF/SF2 and this interaction is believed to facilitate the Rev mediated inhibition of HIV RNA splicing (Powell *et al.*, 1997). Clearly, RNA-processing events in mammalian cells are highly regulated to ensure appropriate and accurate gene expression.

Splicing is an integral event in RNA processing and involves the recognition of conserved intron sequences at the 5' splice site, the 3' splice site, and the branchpoint. The auxiliary splicing factor $U2AF^{65}$ is an essential non-snRNP splicing factor which binds specifically to the pyrimidine tract upstream of a 3' splice site, and facilitates the recruitment of U2 snRNP to the upstream branch site, thus triggering spliceosome assembly (reviewed in Green, 1991). U1 snRNP is primarily involved in the recognition of 5' splice sites, but has also been shown to bind directly or indirectly at or near 3' splice sites and may interact with U2 snRNP. Niwa *et al.*, proposed an unconventional model, referred to as exon definition, to help explain splice site selection; they suggested that the exon, not the intron, is the unit of splice site recognition and that factors binding at the 3' splice site of an upstream intron communicate across the exon with factors bound at the 5' splice site of the downstream exon, resulting in definition of the exon (Niwa *et al.*, 1992).

Interactions between factors bound at the 3' splice site and the 5' splice site are thought to be mediated by SR (serine-arginine) proteins (reviewed in Benoit, 1996). In support of this, ASF/SF2, a member of the SR proteins, has been shown to interact simultaneously with the 35kDa subunit of U2AF and the 70kDa subunit of U1 snRNP (reviewed in Chabot, 1996, Caceres *et al.*, 1997). However, the exon definition model is challenged by definition of the final exon since at the 3' end of a pre-mRNA, the terminal exon does not contain a downstream 5' splice site but instead contains a polyadenylation site; therefore, it has been proposed that the 3' terminal exon is defined by the presence of an upstream 3' splice site and a downstream polyadenylation signal (Berget, 1995). This model therefore predicts that splicing factors bound to the 3' splice site can communicate across a terminal exon with factors recognizing the polyadenylation signal and much evidence has accumulated in support of a physical linkage between splicing and polyadenylation (Zhao *et al.*, 1999). The HPV-16 NRE is located upstream of the late polyadenylation signal of the terminal exon which encodes the viral capsid protein L1 and it is tempting to speculate that binding of RNA processing factors to the NRE might somehow promote definition of this final exon and improve the efficiency of polyadenylation of late mRNAs in differentiated cells by coupling the processes of splicing and polyadenylation. U2AF^{65} has been shown to interact directly with the carboxyl terminus of poly(A) polymerase (PAP), thus providing evidence of the coupling of splicing and polyadenylation (Vagner *et al.*, 2000). Although U2AF^{65} is not normally known to bind 3' UTR sequences, the U-rich nature of the second half of the NRE may be sufficient to enable binding of the protein to this element. Binding of U2AF^{65} to the NRE, and the elevated level of expression of this protein in differentiated 20683 cells, may improve the efficiency of polyadenylation of NRE-containing late mRNAs. Binding of ASF/SF2 to pre-mRNA has been shown to result in the recruitment of U1 snRNP and facilitate enhanced binding of this factor to the target mRNA (Eperon *et al.*, 1993). An increase in the level of expression of ASF/SF2 in

differentiated 20863 cells might act to stabilize the binding of U1 snRNP in the region of the NRE, via the interaction between the 70kDa U1 snRNP subunit and ASF/SF2. In addition, ASF/SF2 can modulate the selection of alternative splice sites in a concentration-dependent manner, such that, an increase in ASF/SF2 can switch splice-site selection to the most proximal 5' splice site (Lamond, 1991), in the absence of a 5' splice site, as in the L1 terminal exon, a differential increase in ASF/SF2 might promote a switch in poly(A) site usage from LP2 in undifferentiated cells to the weaker, NRE-proximal, LP1 in differentiated cells as observed by McGuire *et al.*, (2002).

Efficient recognition of a polyadenylation signal is dependent not only on the highly conserved AAUAAA hexanucleotide but also requires RNA sequences surrounding this site, including both upstream and downstream efficiency elements (USEs and DSEs). USEs have been described in a variety of viruses including SV40, ground squirrel hepatitis virus, cauliflower mosaic virus, adenovirus type 2, and HIV 1, at a distance of 10-51nt upstream of the AAUAAA hexanucleotide, and although these elements are functionally similar, they share no apparent sequence homology apart from U-richness (Schek *et al.*, 1992). The position of the NRE relative to the late poly(A) signal and its U-rich nature suggest that this element may act as a USE (McGuire *et al.*, 2002). The U1A protein subunit of U1 snRNP has been shown to bind to an USE in the SV40 late poly(A) signal (Wassarman *et al.*, 1993, Lutz *et al.*, 1994). Binding of U1A to this USE enhances polyadenylation at this site through efficient terminal exon definition and coupling of splicing and polyadenylation brought about by the direct interaction of U1A and the 160kDa subunit of the cleavage-polyadenylation specificity factor (CPSF), (Lutz *et al.*, 1996). Further to this, it was reported that U1A increased polyadenylation of the SV40 late mRNA at lower concentrations but increasing the concentration of U1A had an inhibitory effect on polyadenylation (Lutz *et al.*, 1996). It had previously been shown that free U1A protein could inhibit polyadenylation of its own pre-mRNA

through an interaction with PAP (Boelens *et al.*, 1993). We found that the level of U1A protein expression was unaffected by differentiation in 20863 cells and it is likely that the cell avoids having an excess of free U1A in order to evade the possible negative effects of this situation on polyadenylation. U1 snRNP can also be cross-linked to a site upstream of the USE in the late poly(A) region of SV40 and it has been suggested that this pattern of binding may be functionally important for communication across large terminal exons (Wasserman *et al.*, 1993). Binding of U1 snRNP upstream of the SV40 USE brings U1A closer to the USE and thus avoids the possibility of U1A becoming dissociated from the pre-mRNA as it scans this large exon (Wasserman *et al.*, 1993). Terminal exons are generally much larger that internal exons, which have an average size of 137 nucleotides, and the L1 terminal exon is no exception at 1625nt. In the case of the HPV-16 late polyadenylation region, it is possible that the U1A protein, tethered at the 3' splice site of the terminal exon via the interaction between U1 snRNP and U2 snRNP, diffuses along the terminal exon in search of the polyadenylation complex and the NRE acts as a USE-like reassociation point to aid in U1A diffusion. If the NRE was acting as a reassociation point for U1A as it scanned the exon this would be particularly useful during vegetative viral replication when late pre-mRNAs are being transcribed at a high rate and efficient processing is vital. The binding of U1A to this type of reassociation point would, by necessity, be weak and transient in nature and this might help explain the ambiguous data we obtained when attempting to analyse U1A binding to the NRE in that, UV cross-linking, on occasion, fixed a normally fleeting interaction between U1A and the NRE.

Once the poly(A) signal has been recognised, polyadenylation proceeds as a two step process involving cleavage of the primary transcript and addition of a poly(A) tail to the free 3' end. CstF-64 is a polypeptide component of the cleavage stimulation factor (CstF) and this factor is known to bind 3'UTR sequences through its N-terminal RNA recognition motif, and this factor usually binds to U-rich or GU-rich downstream efficiency elements (DSEs) to

regulate the specificity and efficiency of polyadenylation (Wahle *et al.*, 1999). CstF-64 can also bind GU-rich elements upstream of the poly(A) site and binding of CstF upstream of the C2 complement gene AAUAAA hexanucleotide stimulates cleavage and poly(A) addition at this site (Moreira *et al.*, 1998). In addition, the level of activity or abundance of CstF-64 has been implicated in the regulation of poly(A) site selection. For example, an increase in expression of CstF-64 upon B cell activation permits preferential use of the weaker upstream poly(A) site of IgM thus promoting a switch from the membrane bound to the secreted form of IgM (Takagaki *et al.*, 1998). Conversely, during adenovirus infection the binding activity of CstF-64 decreases, resulting in a switch from the use of the weak L1 mRNA poly(A) site to preferential usage of the stronger downstream L3 poly(A) site (Mann *et al.*, 1993). In HPV-16, early viral transcripts are polyadenylated at a poly(A) site located at the end of the early region while the late viral transcripts are transcribed through this early polyadenylation site into the late polyadenylation region (Kennedy *et al.*, 1990). In HPV-31 a differential decrease in the level of CstF is thought to increase early poly(A) site read-through, allowing transcription to proceed to the stronger late polyadenylation signal (Terhune *et al.*, 1999). However, unlike HPV-31, the late poly(A) region of HPV-16 contains three potential polyadenylation signals LP1, LP2 and LP3 (Seedorf *et al.*, 1985, Kennedy *et al.*, 1990). In undifferentiated cells the stronger LP2 site is preferentially used but with differentiation polyadenylation is found to switch to the weaker, NRE-proximal, LP1 site (McGuire *et al.*, 2002). A decrease in the levels of CstF-64, as observed in differentiating day 10 20863 cells, would normally result in the preferential usage of a stronger poly(A) signal e.g. LP2. Although changes in the RNA binding activity of CstF-64 were not investigated in this experiment, it is possible that a differential decrease in CstF-64 promotes early poly(A) site read-through, as in HPV-31, and another, HPV-16 specific, possibly NRE-controlled mechanism controls the switch in late poly(A) site usage.

Following polyadenylation the message must be transported to the cytoplasm for translation. Interestingly, correct 3' end formation is thought to be important for efficient mRNA export, which is believed to occur through nuclear pores (Green, 1991). Mounting evidence has implicated RNA-binding proteins as possible mediators of export. HuR is a ubiquitously expressed member of the Hu family of RNA-binding proteins related to *Drosophilia* ELAV and selectively binds AU-rich elements (AREs) in the 3'UTR of mRNAs through one or more of its three RNA recognition motifs (reviewed in Brennan *et al.*, 2001). ARE-containing transcripts are normally rapidly degraded, however, binding of HuR to ARE-containing mRNAs stabilizes these messages, although the mechanism is not yet fully understood (Brennan *et al.*, 2001). HuR is a predominantly nuclear protein but has been found to shuttle between the nucleus and cytoplasm via a novel shuttling sequence (reviewed in Brennan *et al.*, 2001). HuR's ability to shuttle has led to the suggestion that this protein may initially bind mRNAs in the nucleus and accompany them into the cytoplasm, providing ongoing protection from the degradation machinery (Sokolowski *et al.*, 1999). The HPV-16 NRE lacks the HuR consensus binding site, an AUUUA tandem repeat, but the presence of a tandem repeat of GUUUG might be sufficient to enable the protein to bind to this element. The apparent down regulation of HuR in the nuclear extracts of differentiated 20863 cells may be the result of a differentiation-induced redistribution of this protein to the cytoplasm resulting in the efficient export of HuR-bound late mRNA transcripts which would otherwise be prematurely degraded. For example, binding of HuR, and hnRNPC1/C2, to AU-rich elements in the late mRNAs of HPV-1 stabilizes these messages and improves the efficiency of translation of these late proteins (Sokolowski *et al.*, 1999). ASF/SF2 has also been shown to shuttle continuously between the nucleus and cytoplasm, and this protein appears only to exit the nucleus when bound to RNA, thus raising the possibility that, in addition to its role in splicing, ASF/SF2 may be able to

mediate the export of mRNAs to which it is bound, such as HPV-16 late mRNAs, possibly in cooperation with HuR (Caceres *et al.*, 1998).

I did not specifically address the basis of the inhibitory effect of the NRE in undifferentiated cells, however, I feel that the expression of late viral proteins in differentiated cells, and conversely the absence of late protein expression in undifferentiated cells, is controlled by a combination of differentiation-dependent events in which the NRE plays a pivotal role. Firstly, the switch from early promoter to late promoter usage in differentiated cells is crucial and leads to an abundance of late pre-mRNAs which might serve to titrate out inhibitory factors present in undifferentiated cells that possibly prevent binding of RNA processing factors necessary for late protein expression to the NRE. Secondly, although it has not yet been demonstrated for HPV-16, it is highly likely, by analogy with HPV-31 that the early polyadenylation sequence allows read-through in undifferentiated cells, but the transcripts are either prematurely terminated or rapidly turned over. Upon differentiation, read-through increases and transcription is allowed to proceed to the late polyadenylation region and this may be mediated by a decrease in the level of expression of CstF-64, as observed in differentiated 20863 cells. The early polyadenylation signal of HPV-16 contains a very weak CstF binding site and therefore a differential decrease in CstF expression would be expected to promote a switch to the stronger late poly(A) site. In combination with the increased read-through, it is probable that late messages are polyadenylated more efficiently in differentiated cells since the NRE may be acting as a USE-like element thereby promoting efficient polyadenylation of NRE-containing late mRNAs through the coupling of NRE-bound splicing factors with the polyadenylation complex. Stabilisation and rapid export of late mRNAs to the cytoplasm, mediated by NRE-bound HuR and ASF/SF2, thus avoids the degradation machinery that possibly functions in undifferentiated cells to degrade unstable late messages and facilitates expression of late viral proteins, Fig. 5.20.

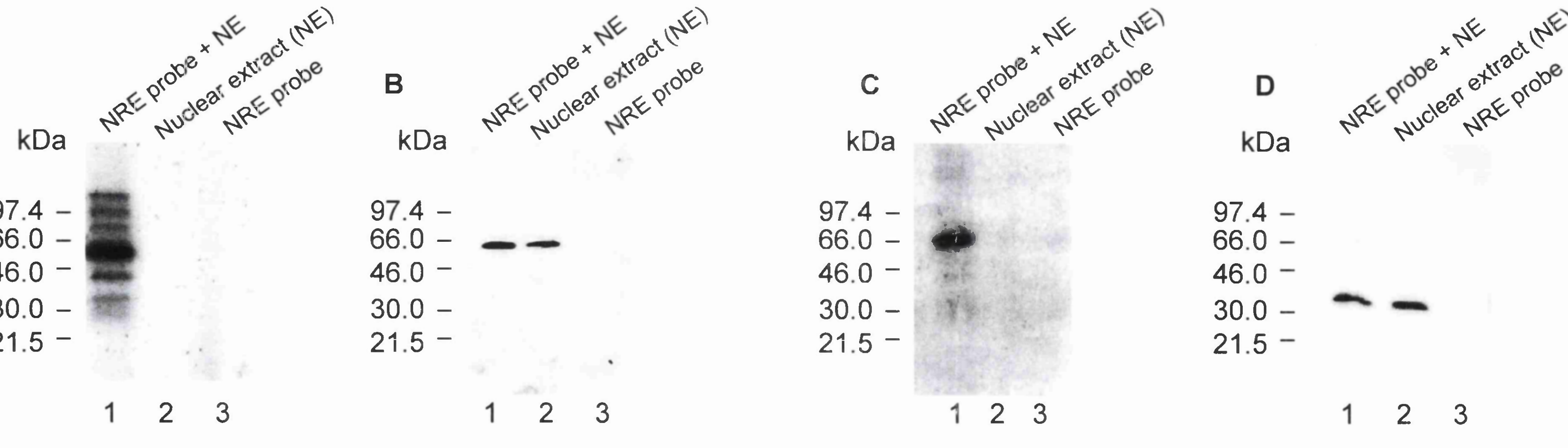


Fig. 5.1. Western blot analysis of proteins UV cross-linked to a wild-type NRE RNA probe. A radiolabelled NRE riboprobe was incubated with 2μl of HeLa nuclear extract, proteins touching the NRE were irreversibly bound by UV irradiation and then fractionated on a 12% SDS-PAGE gel and exposed to autoradiographic film (A), or transferred to nitrocellulose and Western blotted (B, C, D). **A.** Typical profile of proteins bound to the NRE following UV cross-linking. **B.** Western blotting with MC3, an antibody directed against $U2AF^{65}$. **C.** Western blotting using an antibody directed against CstF-64, α64k. **D.** Western blotting with 16A5, an anti-HUR antibody.

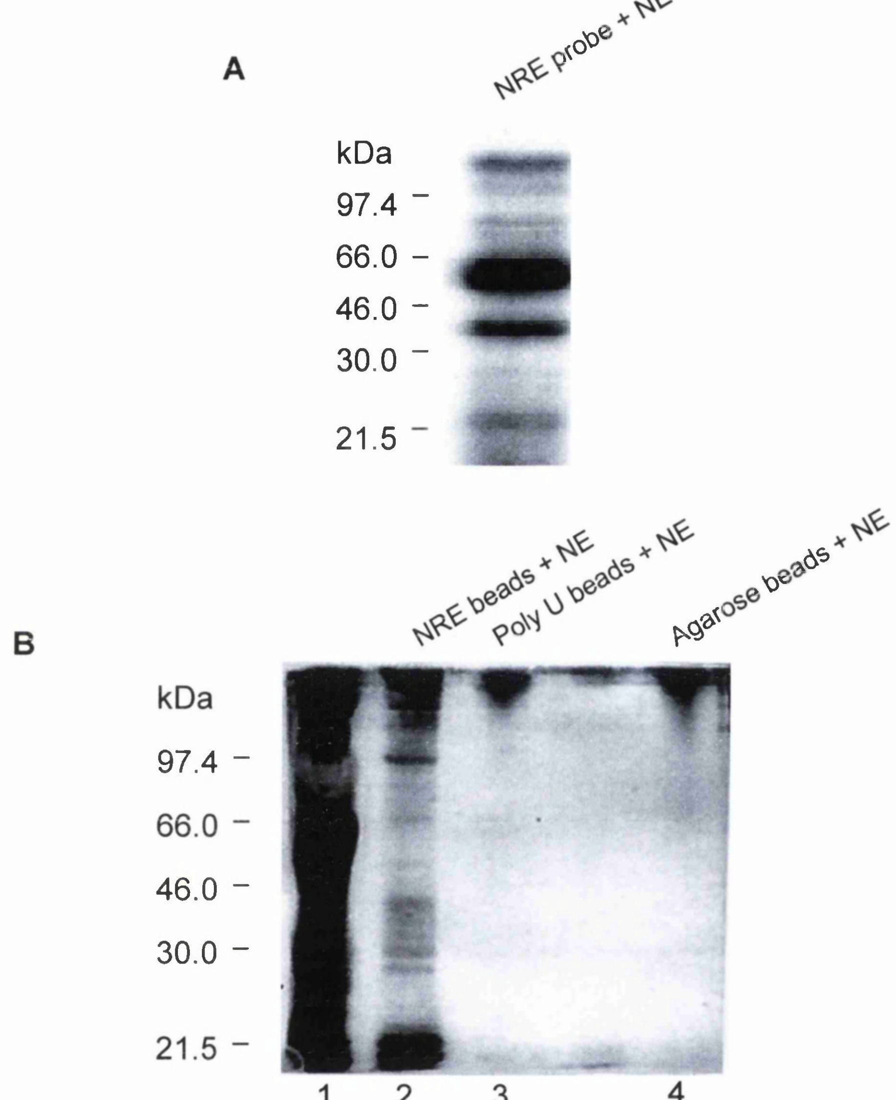
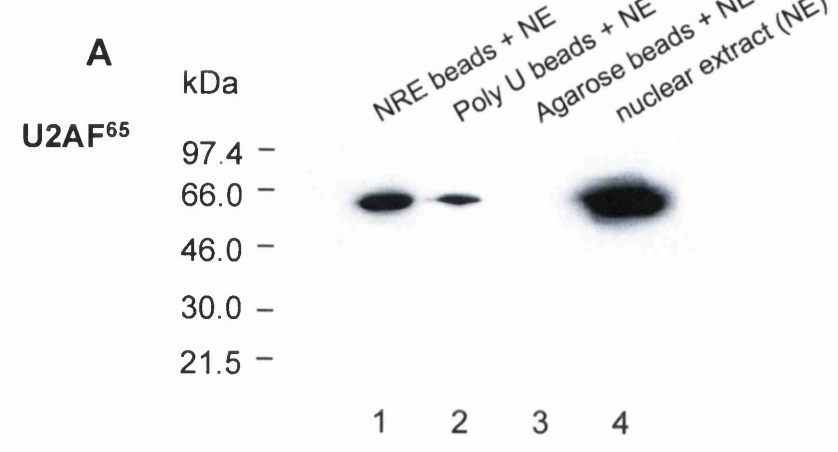
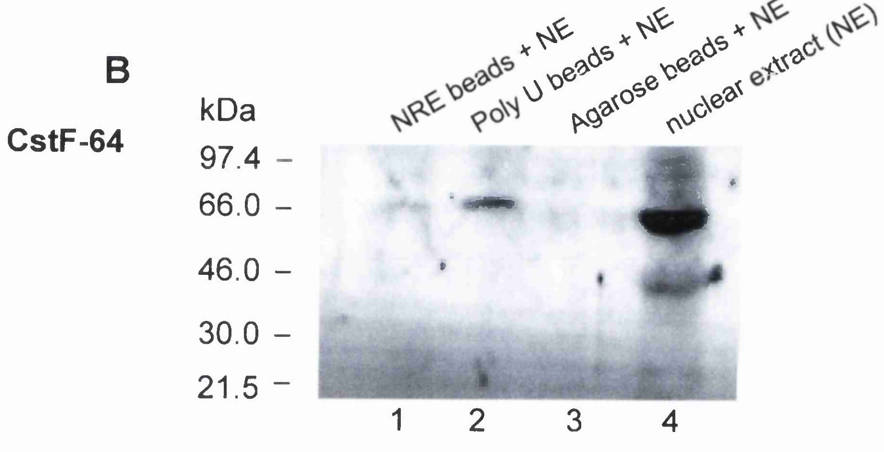
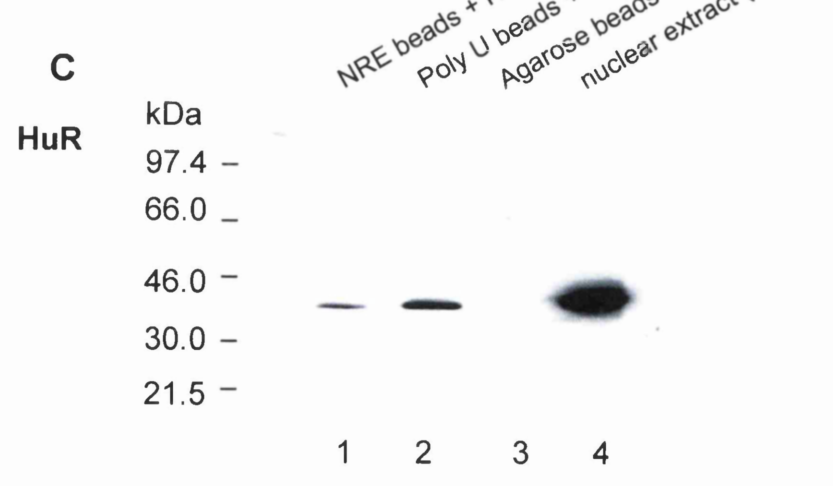


Fig. 5.2. Comparison of the protein binding profiles of NRE RNA probes using UV cross-linking and affinity chromatography. A. UV cross-linking with an NRE RNA probe and HeLa nuclear extracts. **B.** Silver staining of NRE RNA linked agarose beads (lane 2), poly U linked sepharose beads (lane 3) and agarose beads (lane 4) following incubation with HeLa nuclear extract. Lane 1 contains rainbow protein molecular weight marker.

Fig. 5.3. Western blot analysis of proteins binding to the NRE following an RNA binding assay. NRE RNA linked agarose beads (lane 1), poly U RNA linked sepharose beads (lane 2) and agarose beads (lane 3) were incubated with HeLa nuclear extract, unbound proteins were removed by washing, bound proteins were eluted and separated on a 12% SDS-PAGE gel together with nuclear extract (lane 4) and transferred to nitrocellulose membrane. **A.** Western blotting using MC3, an antibody directed against $U2AF^{65}$. **B.** Western blotting with an anti CstF-64 antibody, α64k. **C.** Western blotting with an antibody against HuR, 16A5.


A
U2AF65
kDa
97.4
66.0
46.0
30.0
21.5
NRE beads + NE
Poly U beads + NE
Agarose beads + NE
nuclear extract (NE)
1
2
3
4


B
CstF-64
kDa
97.4
66.0
46.0
30.0
21.5
NRE beads + NE
Poly U beads + NE
Agarose beads + NE
nuclear extract (NE)
1
2
3
4


C
HuR
kDa
97.4
66.0
46.0
30.0
21.5
NRE beads + NE
Poly U beads + NE
Agarose beads + NE
nuclear extract (NE)
1
2
3
4

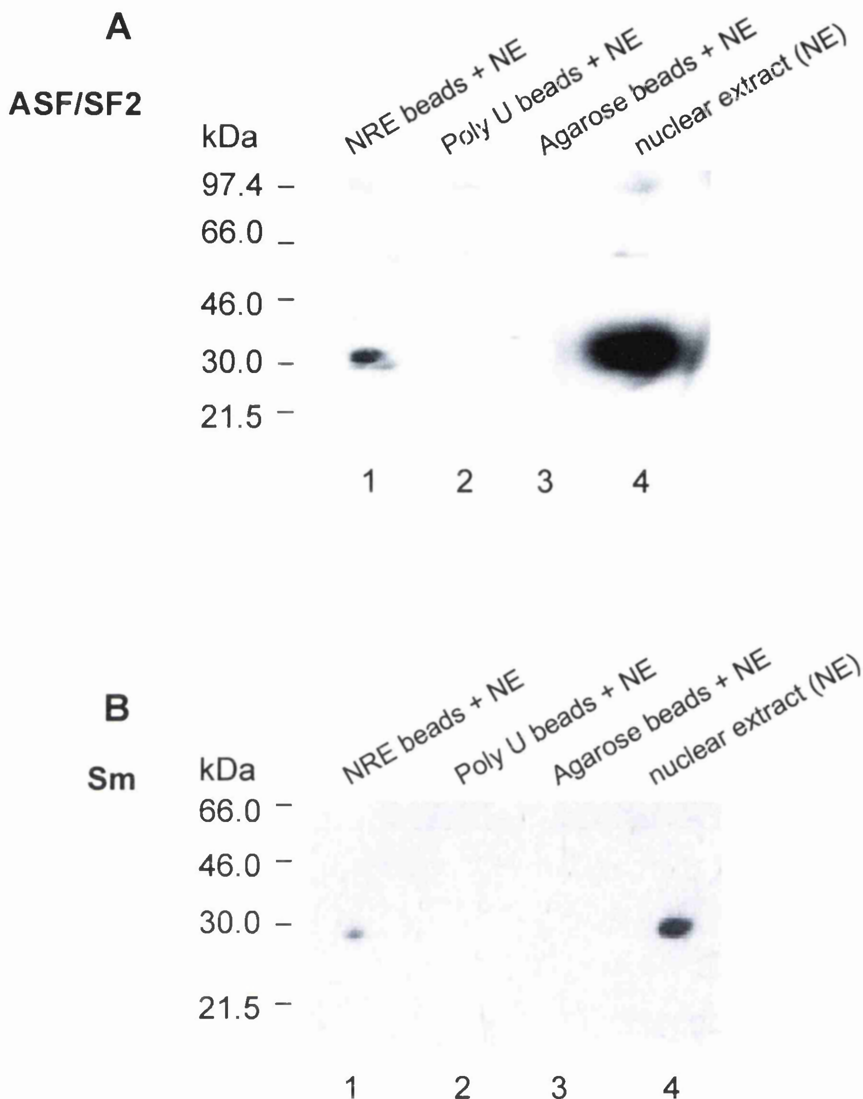
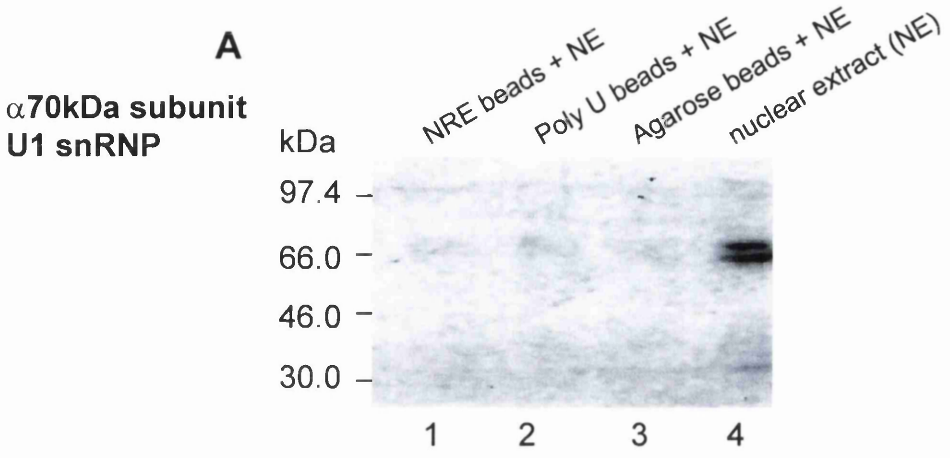
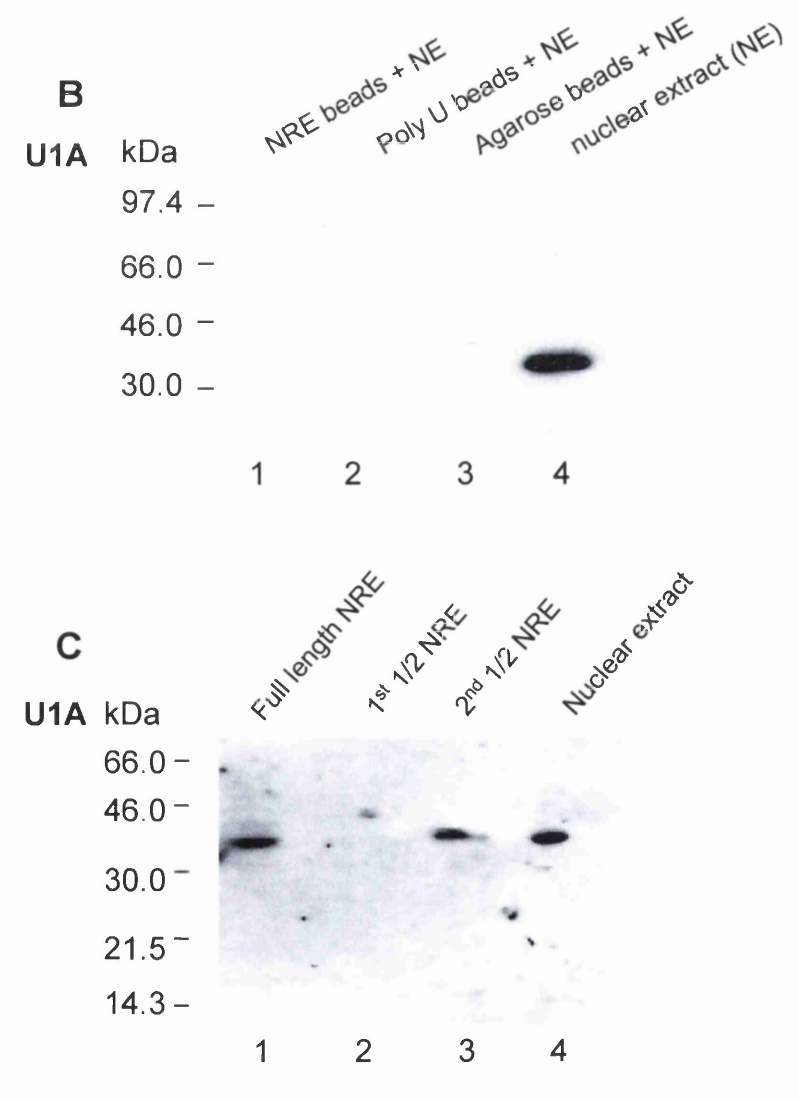


Fig. 5.4. The HPV-16 NRE can bind ASF/SF2 and Sm proteins. NRE RNA-linked agarose beads (lane 1), poly U RNA linked sepharose beads (lane 2) and agarose beads (lane 3) were incubated with HeLa nuclear extract, unbound proteins were removed by washing, bound proteins were eluted and separated by SDS-PAGE together with nuclear extract (lane 4) and transferred to nitrocellulose membrane. **A.** Western blotting with an antibody directed against ASF/SF2, αSF2 96. **B.** Western blotting with Y12, an anti-Sm protein antibody.

Fig. 5.5.Binding of U1 snRNP subunits to the NRE. NRE RNA linked agarose beads (lane 1), poly U RNA linked sepharose beads (lane 2) and agarose beads (lane 3) were incubated with HeLa nuclear extract, unbound proteins were removed by washing, bound proteins were eluted and separated by SDS-PAGE together with nuclear extract (lane 4) and transferred to nitrocellulose membrane. **A.** Western blotting with H111, an antibody directed against the α 70kDa subunit of U1 snRNP. **B.** Western blotting with anti-U1A antiserum. **C.** Agarose beads linked with full-length NRE RNA (lane 1), RNA corresponding to the 1st half of the NRE (lane 2) and RNA corresponding to the 2nd half of the NRE (lane 3) Western blotted with anti-U1A antiserum.


A
α70kDa subunit
U1 snRNP
kDa
NRE beads + NE
Poly U beads + NE
Agarose beads + NE
nuclear extract (NE)
97.4
66.0
46.0
30.0
1
2
3
4


B
U1A
kDa
NRE beads + NE
Poly U beads + NE
Agarose beads + NE
nuclear extract (NE)
97.4
66.0
46.0
30.0
1
2
3
4
C
U1A
kDa
Full length NRE
1st 1/2 NRE
2nd 1/2 NRE
Nuclear extract
66.0
46.0
30.0
21.5
14.3
1
2
3
4

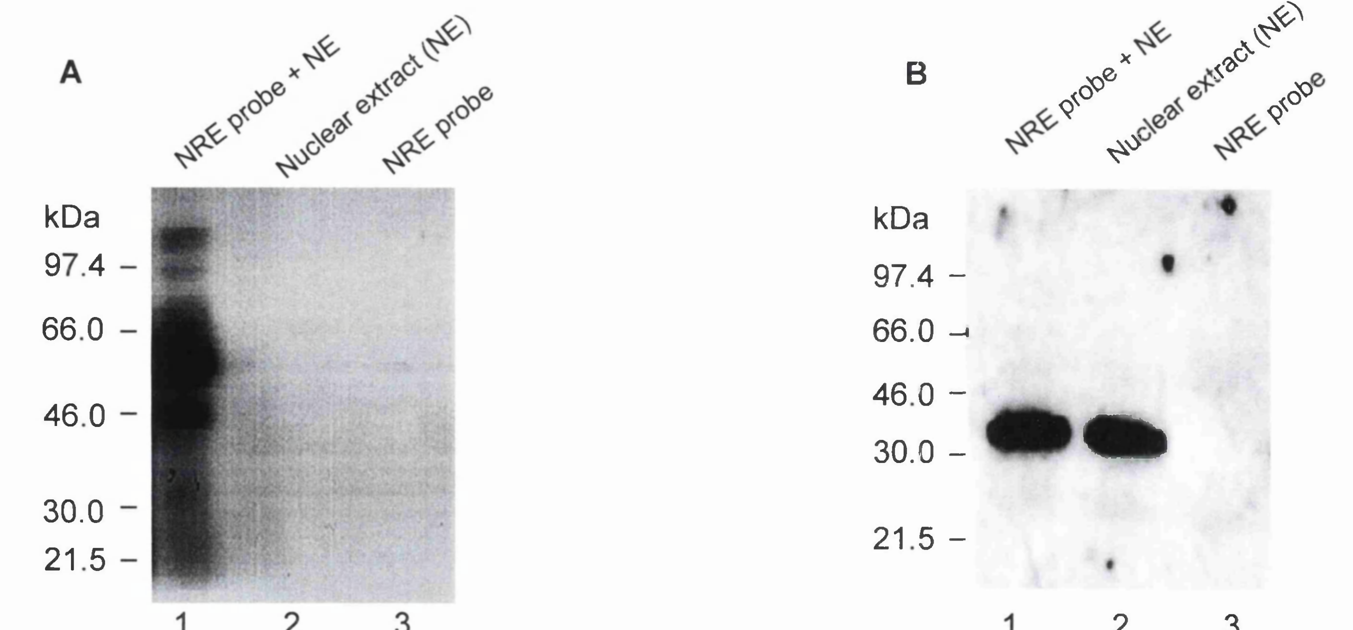
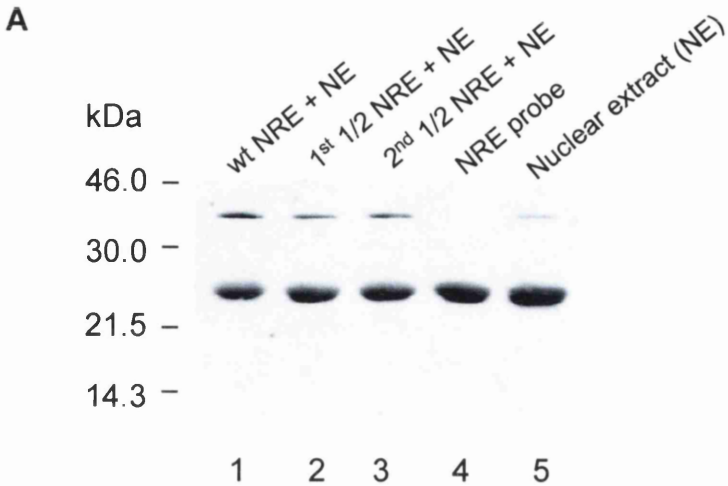


Fig. 5.6. Binding of U1A following UV cross-linking. A radiolabelled NRE riboprobe was incubated with 2μl of HeLa nuclear extract, proteins binding to the NRE were irreversibly bound by UV irradiation and then fractionated on a 12% SDS-PAGE gel and exposed to autoradiographic film (A), or transferred to nitrocellulose and Western blotted (B). **A.** Typical profile of proteins bound to the NRE following UV cross-linking. **B.** Western blotting with anti U1A antiserum.



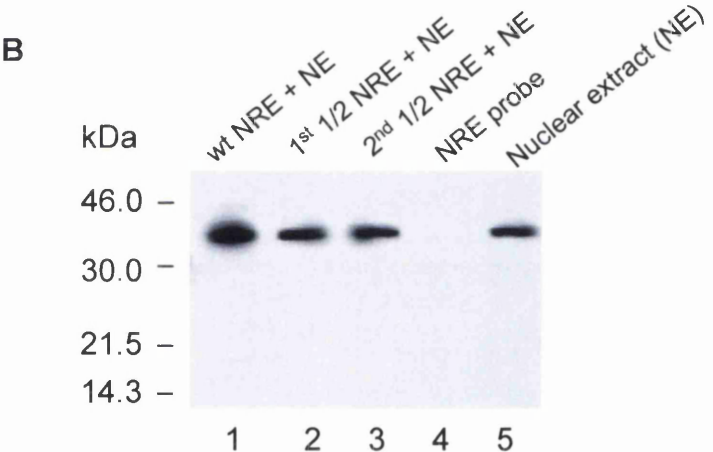


Fig. 5.7. Western blot analysis of U1A binding to the NRE following UV cross-linking. A. Radiolabelled RNA probes corresponding to the full-length NRE (lane 1), the 1st half NRE (lane 2) and the 2nd half NRE (lane 3) were incubated with 2μl of HeLa nuclear extract, UV irradiated, and separated on a 15% SDS-PAGE gel together with the NRE probe (lane 4) and HeLa nuclear extract (lane 5) and transferred to nitrocellulose membrane. **A.** Immunoblotting with anti-U1A antiserum. **B.** The nitrocellulose membrane was stripped and reprobed with 6CS, an antibody directed against GAPDH.

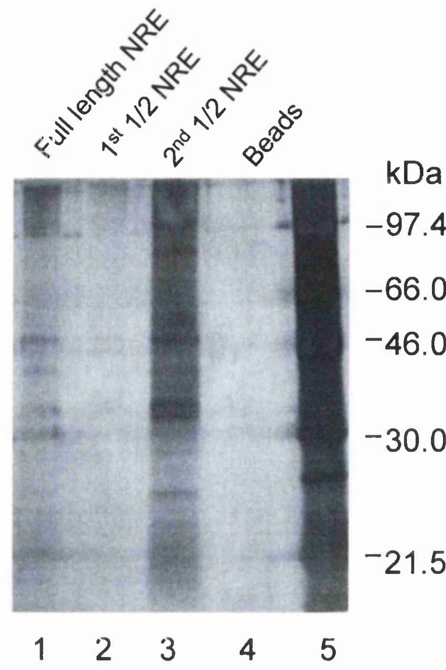


Fig. 5.8. Comparison of protein binding to full-length and half NRE RNA constructs. Full-length NRE RNA (lane 1), RNA corresponding to the 1st half of the NRE (lane 2) and RNA corresponding to the 2nd half of the NRE was linked to agarose beads and incubated with HeLa nuclear extracts, together with unlinked beads (lane 4). Bound proteins were separated by SDS-PAGE and visualised by silver staining. Lane 5 contains rainbow protein molecular weight marker.

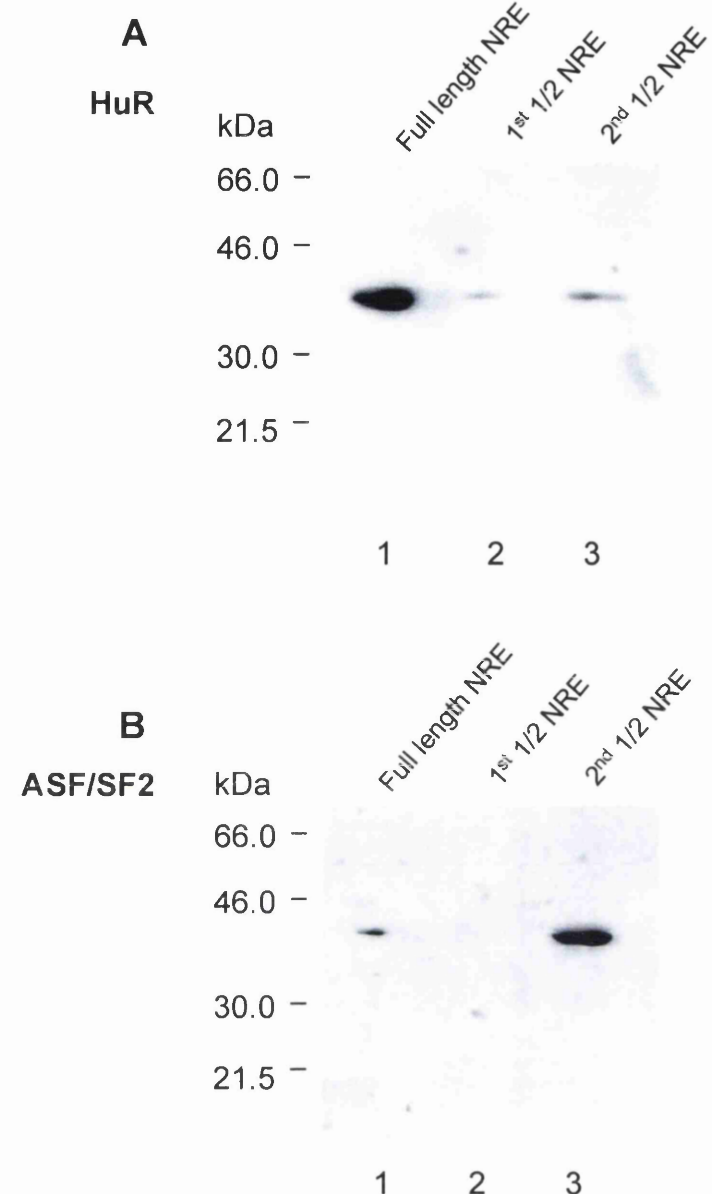


Fig. 5.9. Binding of HuR and ASF/SF2 to full-length and half NRE RNA constructs. Full-length NRE RNA (lane 1), RNA corresponding to the 1st half of the NRE (lane 2) and RNA corresponding to the 2nd half of the NRE was linked to agarose beads and incubated with HeLa nuclear extracts. **A.** Western blotting with 16A5, an antibody directed against HuR. **B.** Western blotting with an antibody directed against ASF/SF2, αSF2 96.

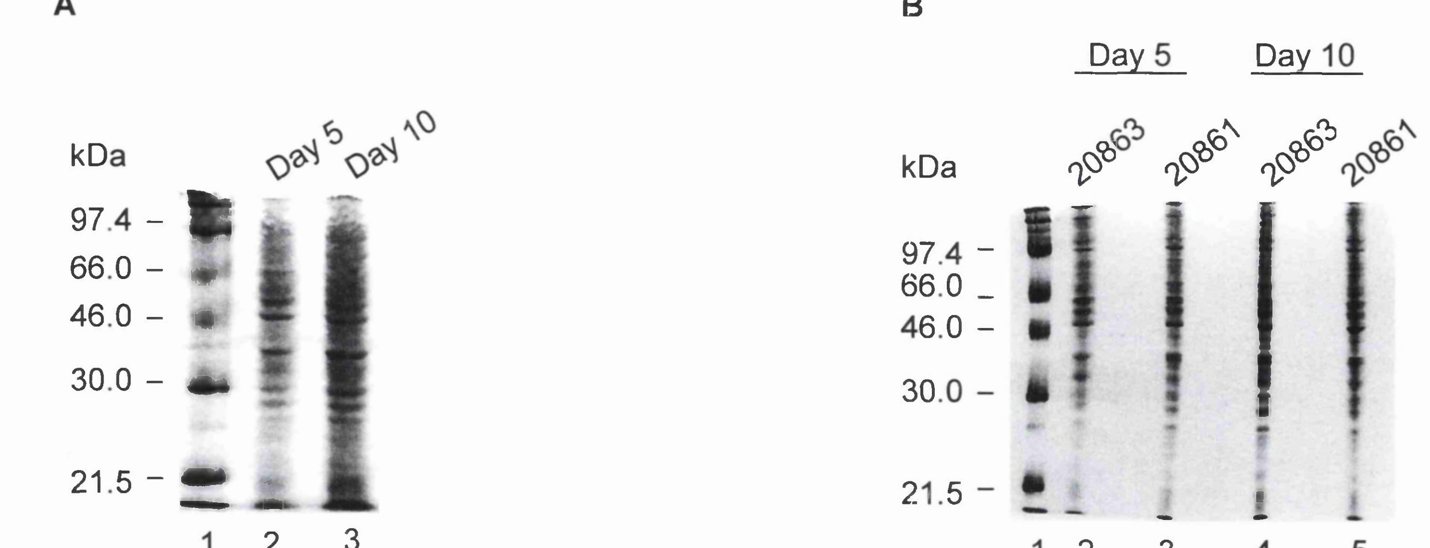


Fig. 5.10. Comparison of monolayer culture protein extracts by Coomassie blue staining. A. 10μg of protein extracts prepared from 20863 cells grown in monolayer culture for 5 days (lane 2) and 10 days (lane 3) stained with Coomassie blue. **B.** 10μg of protein extracts prepared from 20863 cells grown in monolayer culture for 5 days (lane 2) and 10 days (lane 4) and 20861 cells grown in the same way for 5 days (lane 3) and 10 days (lane 5) stained with Coomassie blue. Both membranes contain a sample of rainbow protein molecular weight marker in lane 1.

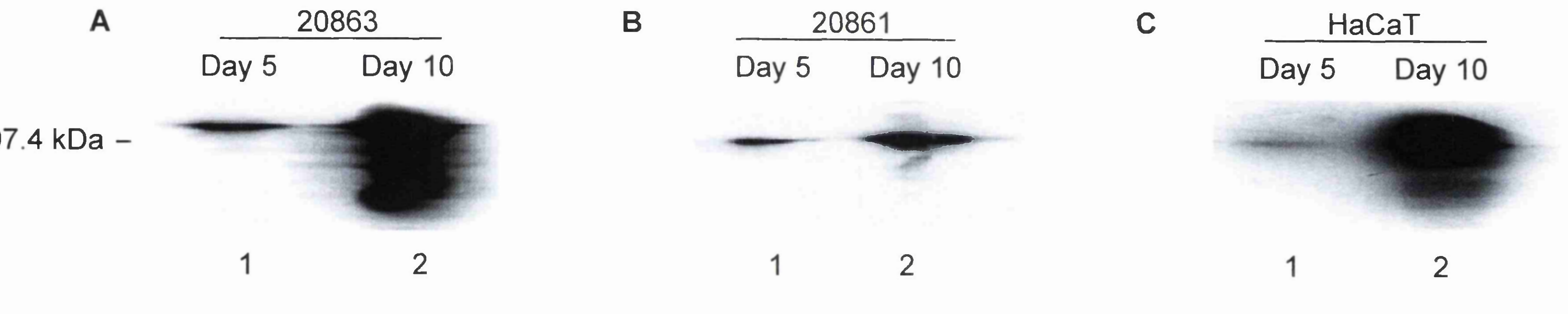


Fig. 5.11. Western blot analysis of involucrin expression. 10μg of protein extracts prepared from day 5 (lane 1) and day 10 (lane 2) monolayer cultures of: **A.** the 20863 cell line, **B.** 20861 cells, and **C**. the HaCaT cell line, Western blotted with the SY5 antibody, directed against involucrin.

Figures 5.12-5.17. Western blot analysis of protein expression in monolayer cultured cells. Protein extracts were prepared from 20863 cells **(A)**, 20861 cells **(B)**, and HaCaT cells **(C)**, grown in monolayer culture for 5 days (lanes 1 and 3) and 10 days (lanes 2 and 4); 10μg of each extract were separated by SDS-PAGE and transferred to nitrocellulose membrane:

5.12 A, B and C, lanes 1 and 2. Western blotting with MC3, an anti-$U2AF^{65}$ antibody

5.13 A, B and C, lanes 1 and 2. Western blotting with α64k, an antibody against CstF-64

5.14 A, B and C, lanes 1 and 2. Western blotting with 16A5, an anti-HuR antibody

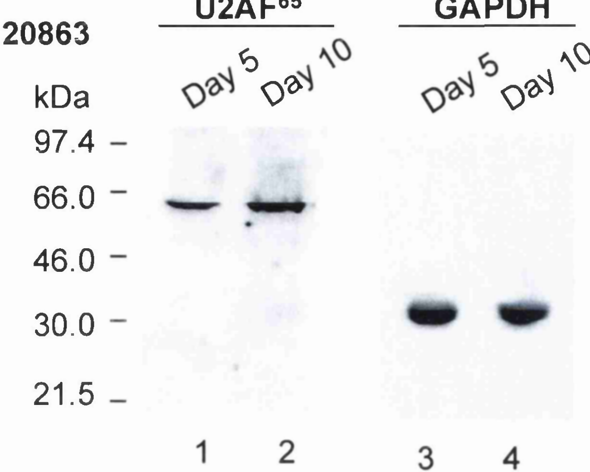
5.15 A, B and C, lanes 1 and 2. Western blotting with αSF2 96 an antibody directed against ASF/SF2

5.16 A, B and C, lanes 1 and 2. Western blotting with Y12, an antibody against Sm proteins

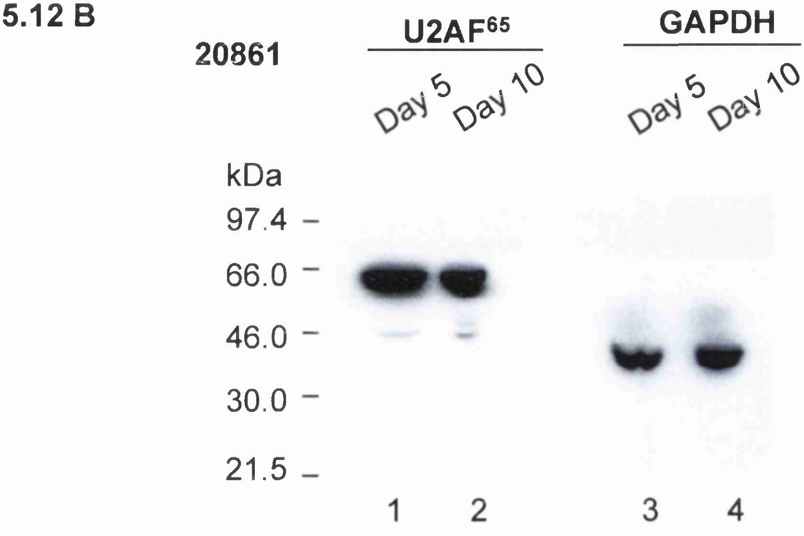
5.17 A, B and C, lanes 1 and 2. Western blotting with anti-U1A antiserum

5.12-5.17 A, B and C, lanes 3 and 4. Blots were stripped and reprobed with an antibody against GAPDH, 6CS.

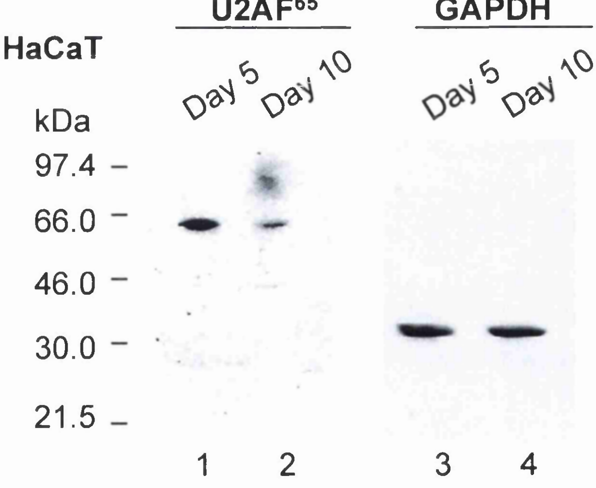
5.12 A



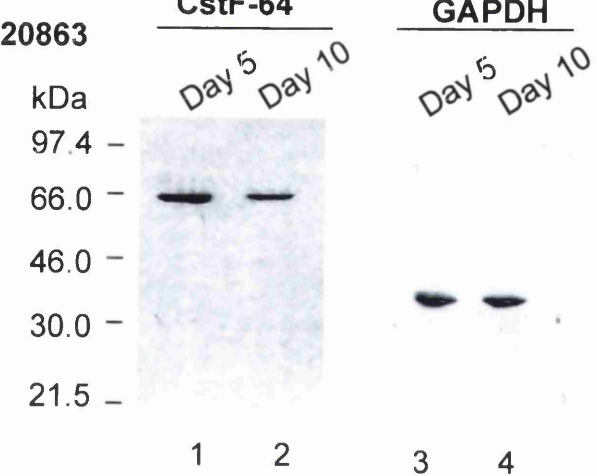
5.12 B



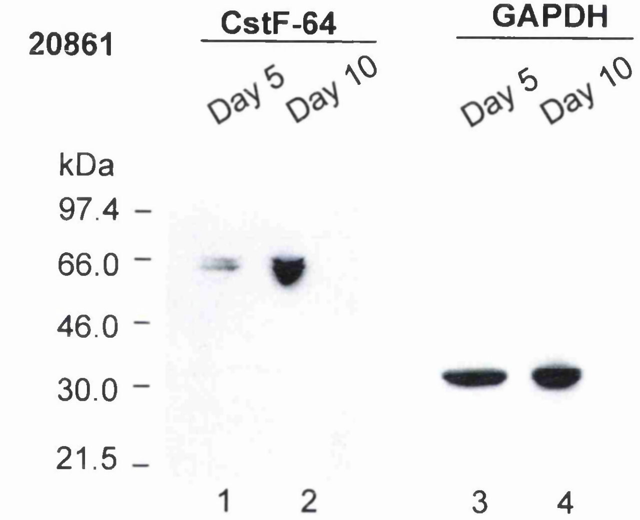
5.12 C



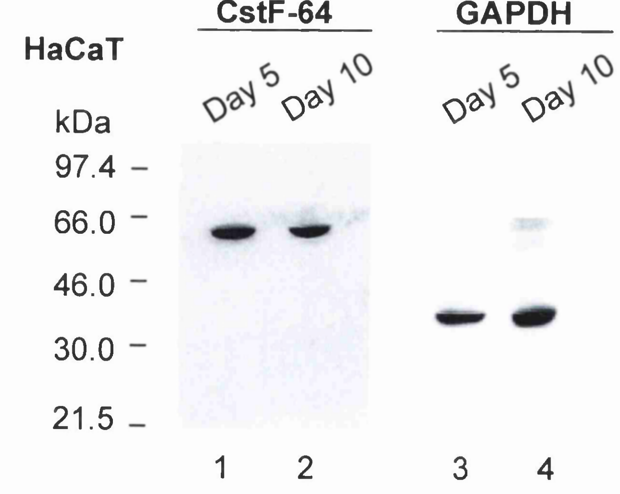
5.13 A



5.13 B



5.13 C



5.14 A

20863

HuR

GAPDH

Day 5 Day 10 Day 5 Day 10

kDa

97.4 –

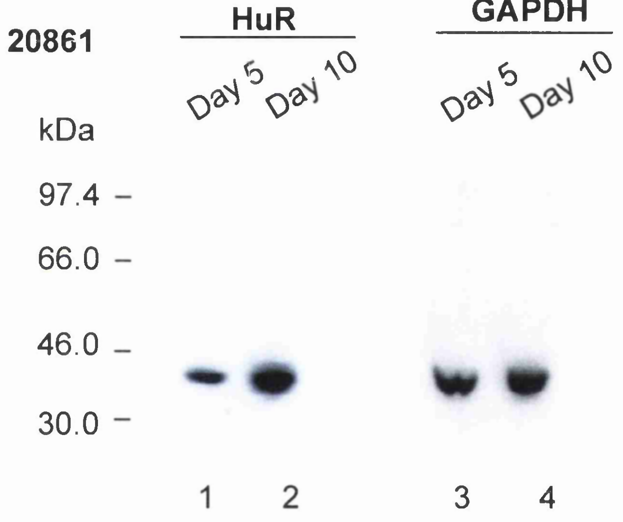
66.0 –

46.0 –

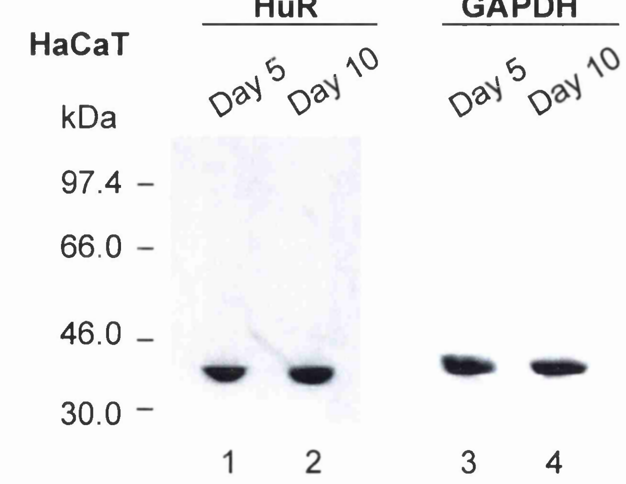
30.0 –

1 2 3 4

5.14 B



5.14 C



5.15 A

20863

ASF | GAPDH

Day 5 Day 10 | Day 5 Day 10

kDa

97.4 –

66.0 –

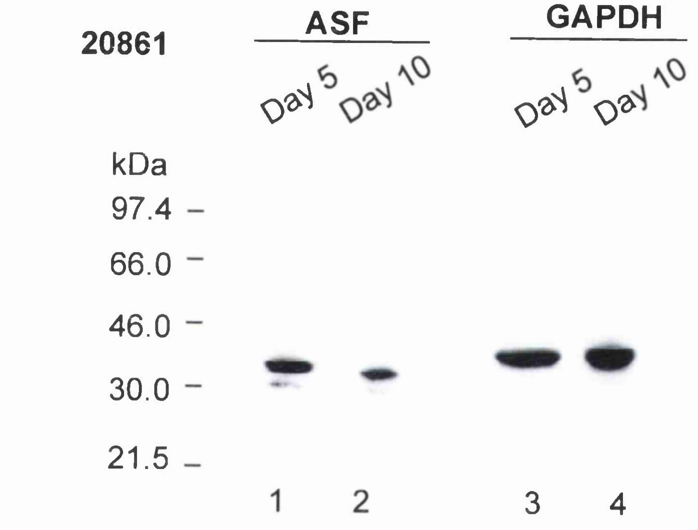
46.0 –

30.0 –

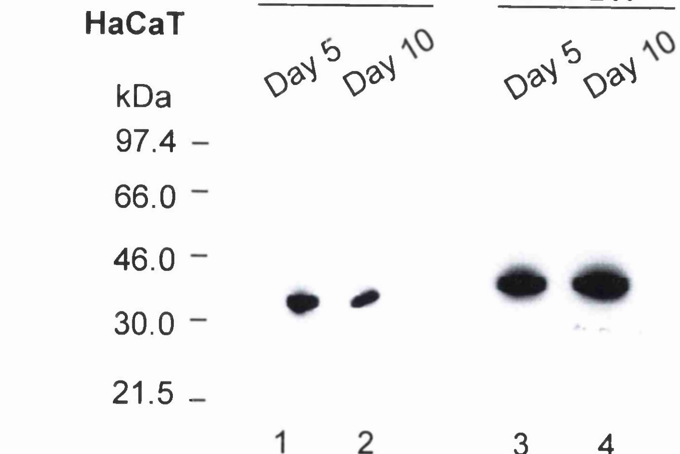
21.5 –

1 2 3 4

5.15 B



5.15 C

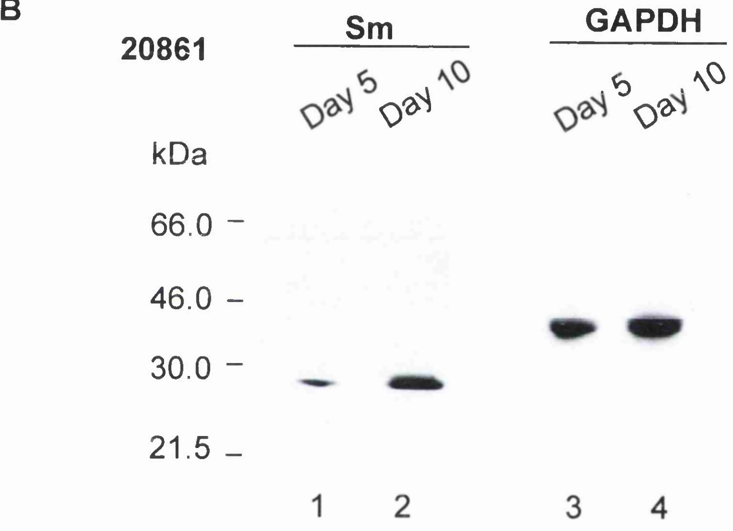


5.16 A

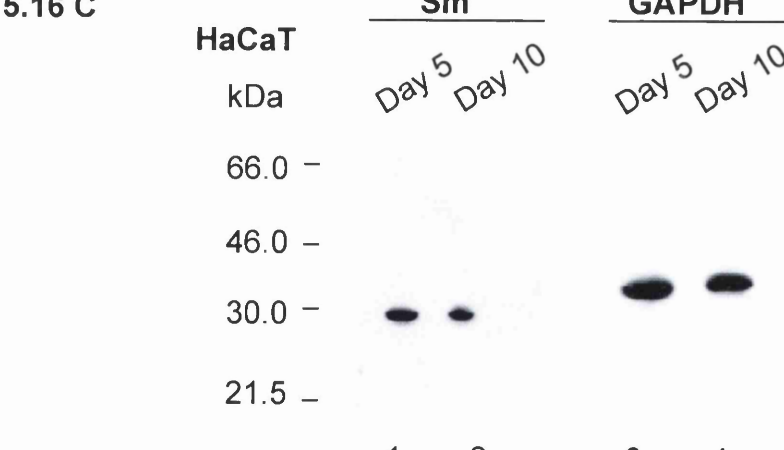
20863

	Sm		GAPDH	
kDa	Day 5	Day 10	Day 5	Day 10
66.0 –				
46.0 –				
30.0 –				
21.5 –				
	1	2	3	4

5.16 B



5.16 C



5.17 A

20863

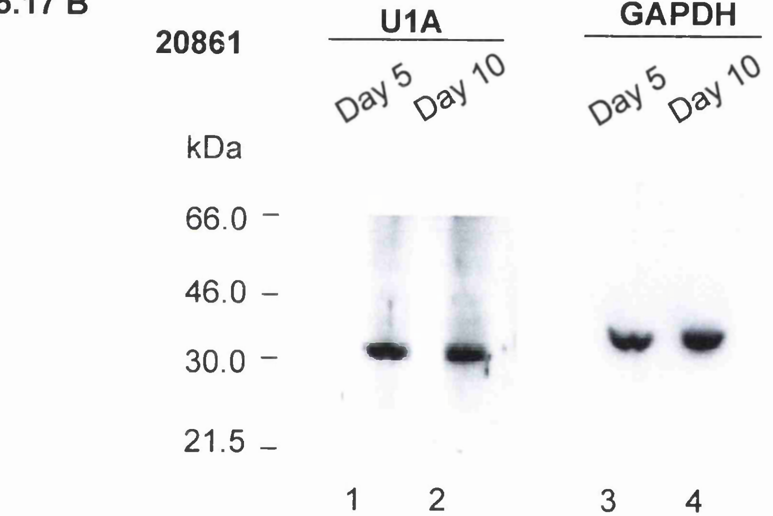
U1A — Day 5, Day 10; GAPDH — Day 5, Day 10

kDa

66.0 –
46.0 –
30.0 –
21.5 –

1 2 3 4

5.17 B



5.17 C

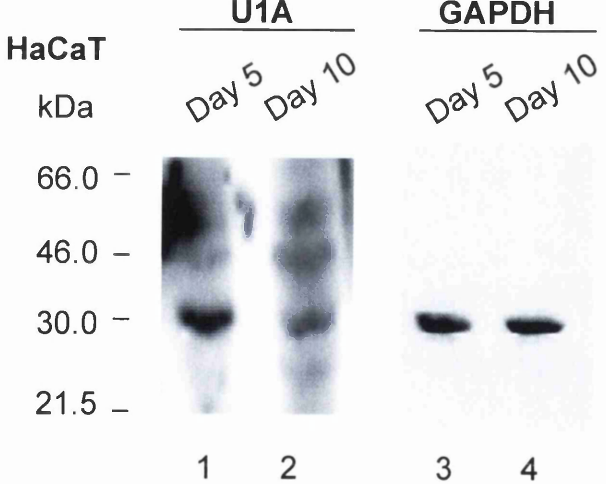
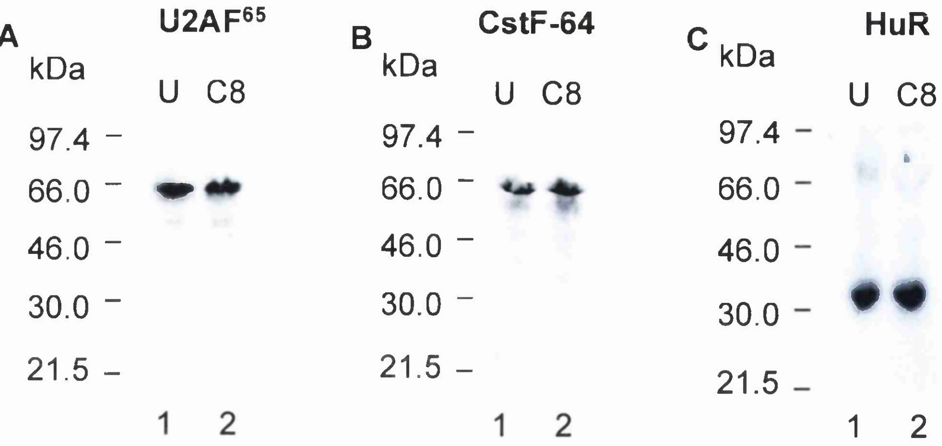
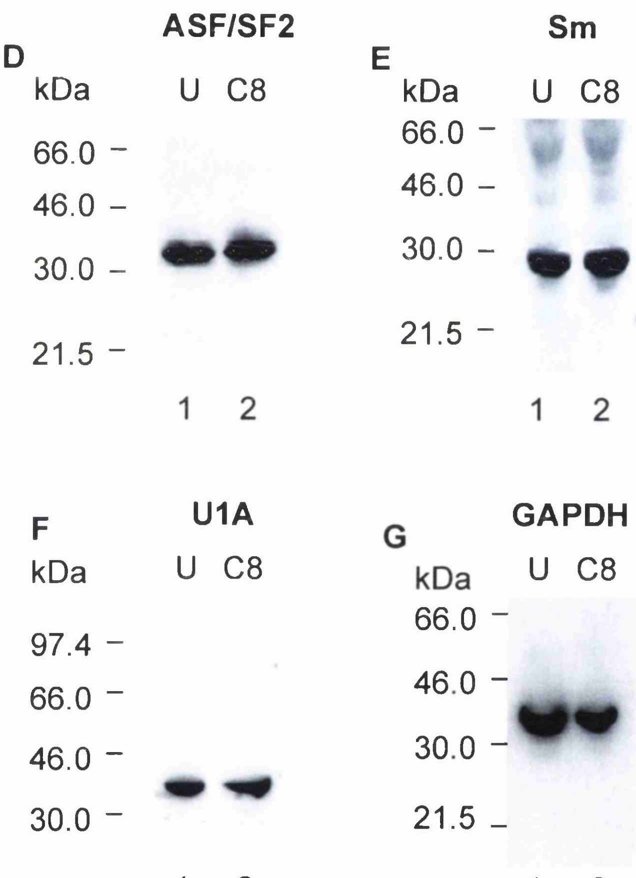


Fig. 5.18. Analysis of protein expression in C8 treated and untreated raft material by Western blotting. 10 μl of protein extracts were prepared from 20863 cells grown on organotypic rafts either untreated (U), or treated with 10μM C8:O (C8), for 12 days at the air-liquid interface. each extract was separated by SDS-PAGE and transferred to nitrocellulose membrane. **A.** Western blotting with anti-U2AF^{65,} MC3. **B.** Western blotting with α64k, an anti CstF-64 antibody. **C.** Western blotting with 16A5, anti-HuR antibody. **D.** Western blotting with αSF2 96, an anti-ASF/SF2 antibody. **E.** Western blotting with an anti-Sm antibody, Y12. **F.** Western blotting with anti-U1A antiserum **G.** A number of nitrocellulose membranes were striped and reprobed with 6CS, an antibody directed against GAPDH.


A
U2AF65
kDa
U C8
97.4
66.0
46.0
30.0
21.5
1 2
B
CstF-64
kDa
U C8
97.4
66.0
46.0
30.0
21.5
1 2
C
HuR
kDa
U C8
97.4
66.0
46.0
30.0
21.5
1 2


D
ASF/SF2
kDa
U C8
66.0
46.0
30.0
21.5
1 2
E
Sm
kDa
U C8
66.0
46.0
30.0
21.5
1 2
F
U1A
kDa
U C8
97.4
66.0
46.0
30.0
1 2
G
GAPDH
kDa
U C8
66.0
46.0
30.0
21.5
1 2

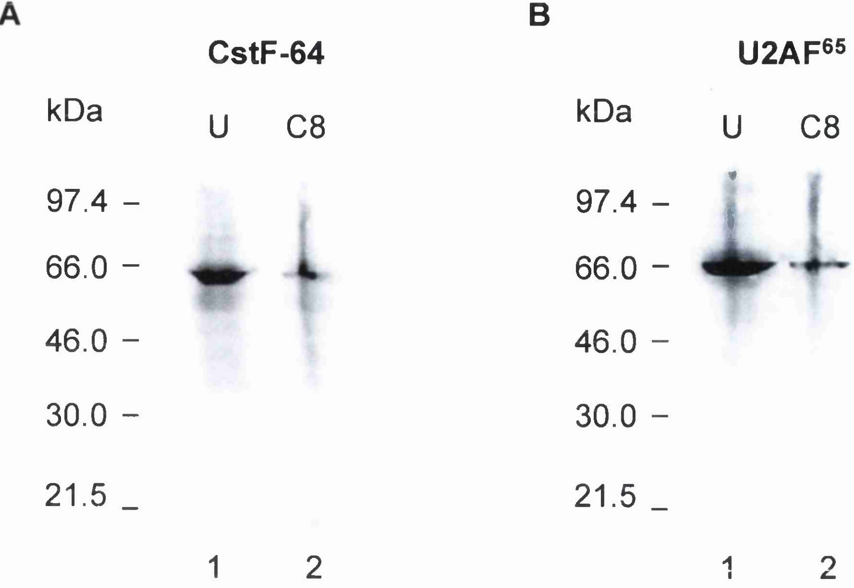


Figure 5.19. Protein expression in CIN 612 9E organotypic raft cultures. Protein extracts were prepared from CIN 612 9E cells grown on organotypic rafts either untreated (U), or treated with 10μM C8:O (C8), for 12 days at the air-liquid interface. 10 μl of each extract was separated by SDS-PAGE gel and transferred to nitrocellulose membrane. **A.** Western blotting with αCstF-64. **B.** Western blotting with MC3, an antibody directed against $U2AF^{65}$.

	U2AF^{65}	ASF/ SF2	Sm	CstF-64	HuR	U1A
20863	↑	↑	↑	↓	↓	—
20861	↓	↓	↑	↑	↑	—
HaCaT	↓	↓	↓	↓	—	↓

Table 5.1: Results of Western blot analysis of day 5 (undifferentiated) and day 10 (differentiated) protein extracts using antibodies directed against each of the NRE-binding proteins.

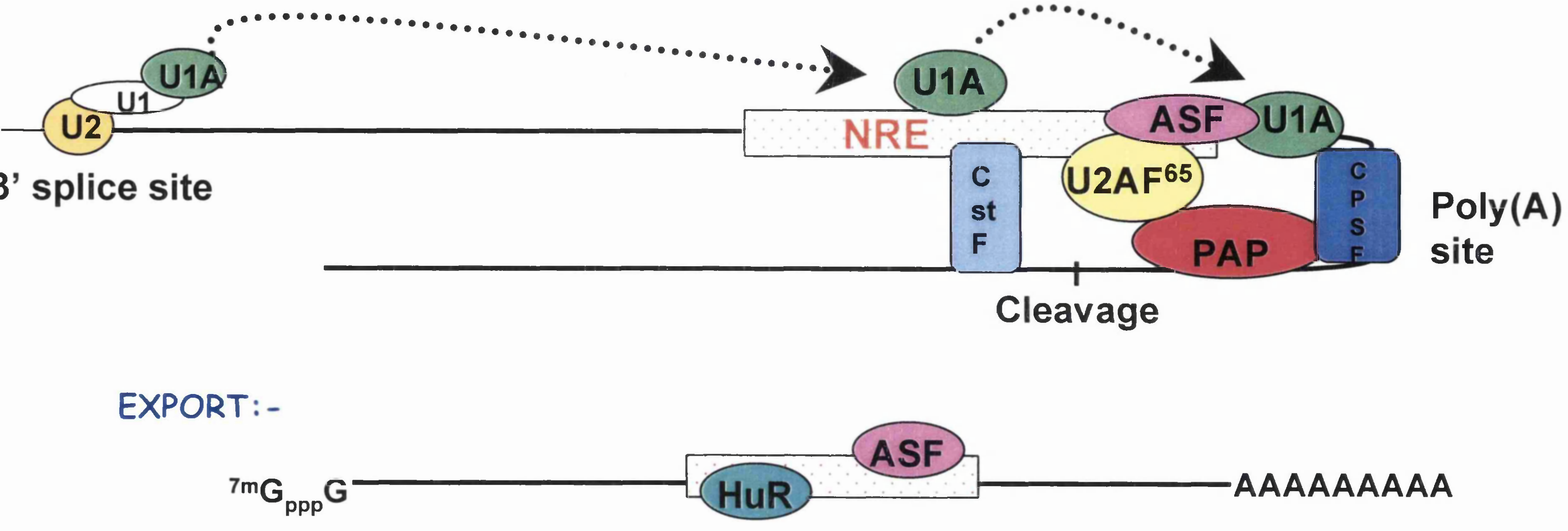


Fig. 5.20. Model of protein binding to the NRE. U2AF^{65} binds to the NRE and is also most likely bound to the downstream 3' splice site where it recruits U2 snRNP and subsequently brings U1 snRNP to this region. U1A, tethered to U1 snRNP scans the L1 terminal exon in search of the polyadenylation complex and in doing so helps define the exon. The NRE, acting as a USE-like element, provides a reassociation point for U1A as it diffuses along the terminal exon. Diffusion of U1A is halted when it reaches the assembling polyadenylation complex, downstream of the NRE. NRE-bound U2AF^{65} may be coupled to the polyadenylation machinery through its interaction with PAP and this linkage is strengthened by U1A and its interaction with the 160kDa subunit of CPSF. NRE-bound ASF/SF2 most likely plays an important supportive role in this process by maintaining communication between U2AF^{65} and U1A via interactions with U2AF^{35} and 70kDa U1 snRNP and, in the absence of a true USE, or upstream U1 snRNP binding site, possibly stabilises the interaction between U1A and the pre-mRNA. The differential increase in the expression of ASF/SF2 might also promote utilisation of the NRE-proximal L1 poly(A) site. Following polyadenylation of ASF/SF2 and HuR bound late mRNAs these messages are stabilised and rapidly exported to the cytoplasm through the combined shuttling activity of these cellular proteins

CHAPTER 6: General Discussion

In this study I have described the development of a tissue culture system in which to study the differentiation of HPV-16 positive epithelial cell lines in monolayer culture. I have found that continued growth of both the parental W12 cell line and the 20863 subclone of this line, as well as the HaCaT cell line, in monolayer cultures for up to 10 days, in the absence of phorbol ester treatment, results in the initiation of differentiation, as indicated by a substantial increase in the level of expression of the keratinocyte-specific marker of differentiation, involucrin. Furthermore, a low level of expression of the HPV-16 late viral proteins E1^E4 and L1 was detected in day 10 monolayer cultures of the W12 and 20863 lines. While several previous studies have reported the detection of late viral transcripts in monolayer cultures, this is this first report, to our knowledge, of the detection of viral capsid protein expression in such cultures. In contrast, it appears that if the W12 and 20863 lines are maintained in monolayer culture for up to 5 days they remain essentially undifferentiated, as indicated by a low level of expression of involucrin, and little or no viral late protein expression. I believe that we now have a reliable, reproducible system capable of generating undifferentiated and differentiating populations of HPV-positive cells for use in the study of the regulation of HPV-16 late protein expression in response to epithelial differentiation.

I have also characterised the growth of HPV-16 positive epithelial cells on the organotypic raft culture system. This method has been used extensively to study other HPV-positive cell lines, since this system can faithfully mimic the epithelial differentiation programme as seen *in vivo,* and is therefore a useful tissue culture system in which to study the complete viral life cycle, which is exclusively linked to the keratinocyte differentiation programme. I found that after 12 days at the air-liquid interface, HPV-16 positive tissue expressed readily detectable levels of L1 mRNA; amplification of viral DNA

was also observed by Southern blot analysis. Moreover, E1^E4 and L1 protein expression was detected both by Western blot analysis of protein extracts prepared from harvested rafts and also by immunocytochemistry on harvested raft sections. It appears that while phorbol ester treatment of the biopsy-derived HPV-16 positive W12 line, and the 20863 subclone, enhances late protein expression it is not necessary; this contrasts with previous reports concerning the biopsy-derived HPV-31 cell line, CIN 612, for which phorbol ester treatment is required for virion synthesis on organotypic rafts (Meyers *et al.*, 1992, Ozbun *et al.*, 1997). However, our observations are supported by additional reports using HPV-positive lines established using cloned viral DNA, in which completion of the viral life cycle on organotypic rafts is not dependent on PKC-induced changes in the differentiation programme (Frattini *et al.*, 1996, 1997, Meyers *et al.*, 1997, Thomas *et al.*, 2001). Taken together, the results presented herein, using both the monolayer and raft culture systems, support the concept that the productive stage of the HPV life cycle is dependent on epithelial differentiation.

The requirement of host tissue differentiation for efficient viral replication is not unique to papillomaviruses and has been described for a number of other viral systems including cytomegalovirus (Weinshenker *et al.*, 1988), Epstein-Barr virus (Li *et al.*, 1992), human immunodeficiency virus (Cullen *et al.*, 1989), measles virus (Schneider-Schaulies *et al.*, 1993), and Rift valley fever virus (Lewis *et al.*, 1989). The process by which differentiation activates the productive HPV life cycle is an area of great interest and the identification of the mechanisms controlling the differential expression of the late viral proteins has been a longstanding challenge in the study of HPV biology. In the case of HPV-16, a 79nt negative regulatory element (NRE) located within the late 3' untranslated region (UTR) is believed to play a central role in controlling the pattern of viral late protein expression (Kennedy *et al.*, 1990, 1991, Dietrich-Goetz *et al.*, 1997, Koffa *et al.*, 2000, McGuire *et*

al., 2002). This *cis*-acting element can inhibit reporter gene expression following transient transfection in basal epithelial cells (Kennedy *et al.*, 1990). Moreover, this element has been shown to act as a general regulator of polyadenylation; it appears to control the differential usage of late polyadenylation signals as well as acting as a transcriptional pause site (McGuire *et al.*, 2002). I have carried out saturating site-directed mutagenesis of the NRE and the results tend to suggest that no single short sequence is responsible for the inhibitory activity of this element or its ability to interact with a range of cellular proteins. In fact, it appears that while each half of this element has independent inhibitory activity, the full 79nt are required for maximal inhibition (Cumming *et al.*, 2002b). This contrasts with the situation in both BPV-1 and HPV-1, where the inhibitory activity of negative elements within the late 3' UTR of these viruses has been mapped to distinct short sequences (Furth *et al.*, 1994, Tan *et al.*, 1995a).

Given the putative role of the NRE in post-transcriptional control mechanisms, it is not surprising that this element has been shown to interact with a range of cellular proteins (Dietrich-Goetz *et al.*, 1997). These include the auxiliary splicing factor, $U2AF^{65}$, the polyadenylation factor CstF-64, and the *elav*-like HuR protein (Koffa *et al.*, 2000). It seems likely, given the limited coding potential of the papillomavirus genome, that the virus is dependent on interactions with cellular factors, such as these, to direct the pattern of late gene expression. In this report I have confirmed that the NRE can bind $U2AF^{65}$, CstF-64 and HuR using an affinity-chromatography based RNA binding assay. Furthermore, I have used this assay to show that the NRE can interact with three additional cellular proteins, ASF/SF2, Sm and U1A, which are primarily involved in pre-mRNA splicing: ASF/SF2 is a member of the splicing-related (SR) protein family and has also been reported to shuttle between the nucleus and cytoplasm. Sm proteins form a complex that binds to several small nuclear RNAs (snRNAs), including U1 snRNP, the primary function of which is the recognition of 5' splice sites;

U1A is a subunit of U1 snRNP (reviewed in Will *et al.*, 1997, Caceres *et al.*, 1997).

In addition, using undifferentiated and differentiating cell populations from monolayer cultures of HPV-16 positive cell lines and the HaCaT cell line, I have shown that differential changes in the level of expression of these NRE-bound cellular proteins are dependent on the status of the viral genome in the infected cell: In the absence of HPV-16 infection, each of the NRE-binding proteins is downregulated with differentiation, with the exception of HuR, which remains unchanged. However, when the HPV-16 genome is episomal, as it is in the 20863 and W12 cell lines, a specific upregulation of the splicing factors $U2AF^{65}$ and ASF/SF2 was observed upon differentiation. In contrast, when the viral genome is integrated, as is the case in the 20861 cell line, as well as many cervical carcinomas, a specific upregulation of the polyadenylation factor CstF-64, and the shuttling protein HuR, was detected following differentiation. While it is possible that a differential increase in the expression of NRE-bound splicing factors in the 20863 cell line facilitates the increase in viral RNA processing during the productive phase of the viral life cycle, I favour the hypothesis that these splicing factors are important in overcoming the negative effects of the NRE in differentiated cells. Similarly, changes in the expression and activity of splicing factors during the life cycle of other viruses, including adenovirus and HIV, regulates the expression of viral proteins in these infections (Powell *et al.*, 1997, Molin *et al.*, 2000). I propose that the differential increase in expression of these cellular splicing factors during the late stages of the HPV-16 life cycle improves the efficiency of polyadenylation of late messages through enhanced terminal exon definition, brought about by direct interactions between splicing factors bound by the NRE, and the downstream polyadenylation complex. While direct interactions between splicing and polyadenylation factors in the late 3' UTR of BPV-1 have been shown to reduce polyadenylation of late messages in this system (Furth *et al.*, 1994, Gunderson *et al.*, 1998), interactions

between the splicing and polyadenylation machinery in the late polyadenylation region of SV40 promotes 3' end processing of these messages (Lutz *et al.*, 1994, 1996). Moreover, it is possible that the late HPV-16 messages are more stable and exported more efficiently from the nucleus to the cytoplasm, in differentiating cells, as a result of the shuttling activity of NRE-bound HuR and ASF/SF2 (Caceres *et al.*, 1998, Brennan *et al.*, 2001).

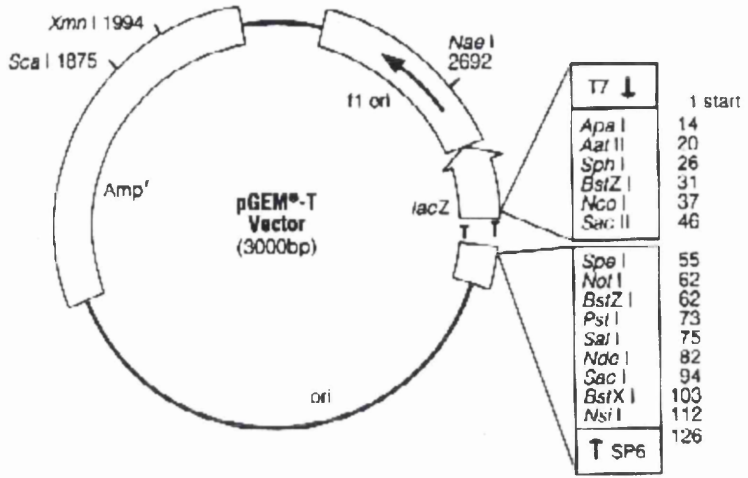
While I have reported the identification of several additional NRE-binding proteins, there are a still a large number of cellular factors capable of interacting with this element that remain unidentified. Identification of other NRE-binding proteins will help elucidate the precise mechanisms by which the NRE regulates HPV-16 late gene expression. To this end, a limited proteomics approach is currently being undertaken in our laboratory which aims to identify as many proteins as possible that bind to the NRE, both in undifferentiated and differentiating epithelial cells. This information could also help with the characterisation of protein complexes that form upon the NRE. In addition, use of the organotypic raft culture system to follow the pattern of expression of NRE-binding proteins during epithelial differentiation will be very useful in understanding the basis of the tight linkage of epithelial differentiation and viral late protein expression.

Attempts to construct an NRE-deletion mutant in the context of the viral genome were unsuccessful during the course of the work presented here, as described in section 4.2. Nevertheless, this objective is still being actively pursued in the laboratory and additional cloning strategies are currently being tested. An alternative approach to negating the NRE in the context of the viral genome might be to construct plasmids with the potential to express anti-sense NRE RNA molecules, either constitutively, or under the control of a differentiation inducible promoter. Stable transfection of these plasmids

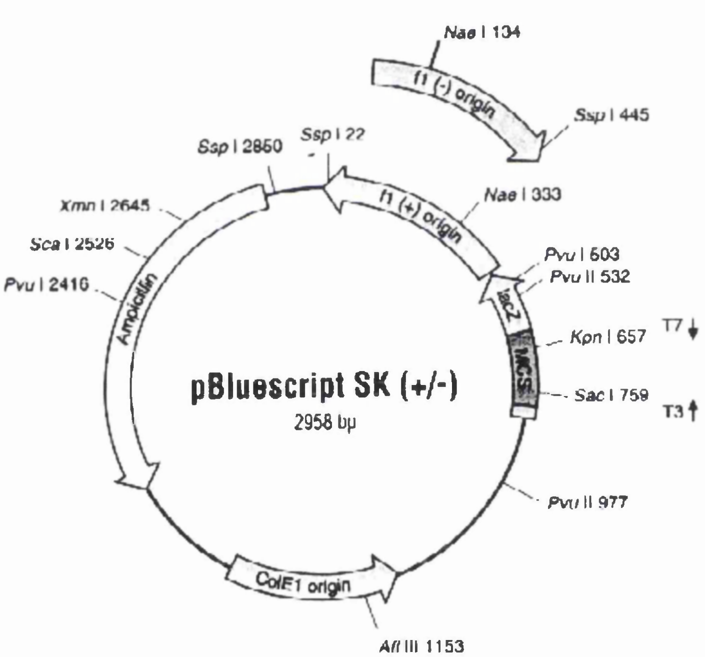
into HPV-16 epithelial cell lines might give some insight to the role of this element *in vivo*, during the epithelial differentiation programme.

At present, only a small number of groups world wide study the mechanisms controlling the tight linkage between the differentiation programme of HPV-infected cells and completion of the viral life cycle, and the role(s) of virally encoded *cis*-acting factors in this process. There are number of reasons for this, one of which is the sheer volume of research efforts currently focused on the study of the HPV oncoproteins E6, E7, and to a lesser extent, E5. The importance of these studies can not be over emphasized since the information they generate gives invaluable insights to cellular control mechanisms. Moreover, since these oncoproteins are the only viral proteins expressed in cervical carcinoma cells they can be exploited as cell-type specific targets of therapeutic cancer vaccines. However, it is also important to bear in mind that any treatment which could specifically disrupt the pattern of viral late gene expression would be a powerful tool in eliminating transmission of infectious HPV virions, thus precluding the need for therapeutic cancer vaccines. For example, if late protein expression could be induced earlier in the HPV-life cycle i.e. in the basal cells of the epithelium, this might enable the host immune system to mount a successful attack on the virus. Alternatively, if late protein expression could be blocked completely then mature virions could not assemble. Clearly, the development of either of these strategies is complex and requires a more complete understanding of the control of late viral protein expression in response to epithelial differentiation. The difficulties associated with recreating accurate skin models *in vitro*, from which populations of undifferentiated, differentiating and terminally differentiated HPV-positive cells can be produced is one of the major factors impeding progress in this field. For HPV-16 in particular, the inability to produce infectious virions from organotypic raft cultures of the HPV-16 positive epithelial lines currently available has resulted in this subtype being largely ignored in favour of, one

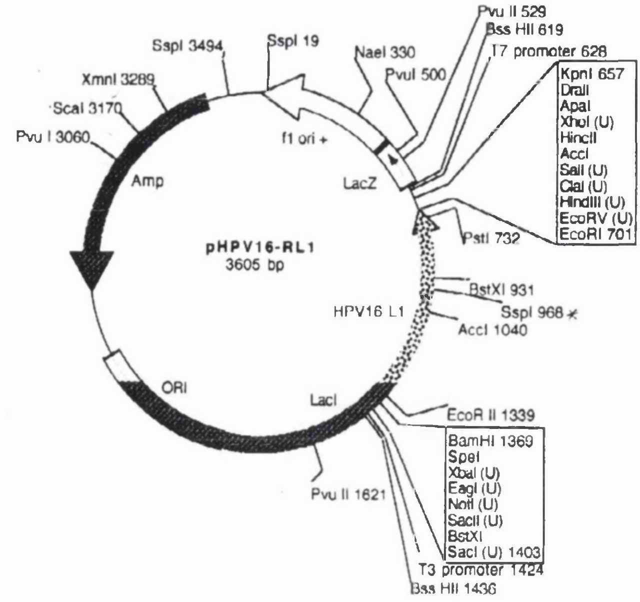
might argue, less-clinically important but more user-friendly PV types such as BPV-1, HPV-1 and HPV-31. Nonetheless, studies utilising these viral types provide much needed general information about control mechanisms that may be conserved amongst papillomaviruses. However, these studies also highlight the diversity of regulatory mechanisms employed by this viral family. Therefore, it is crucial that we continue to identify the specific methods used by individual HPV types to control the pattern of late gene expression, particularly in the clinically relevant types, such as HPV-16. Only by understanding the intricacies of the HPV life cycle more fully will we be able to design effective treatments to eliminate HPV-associated disease in the future.



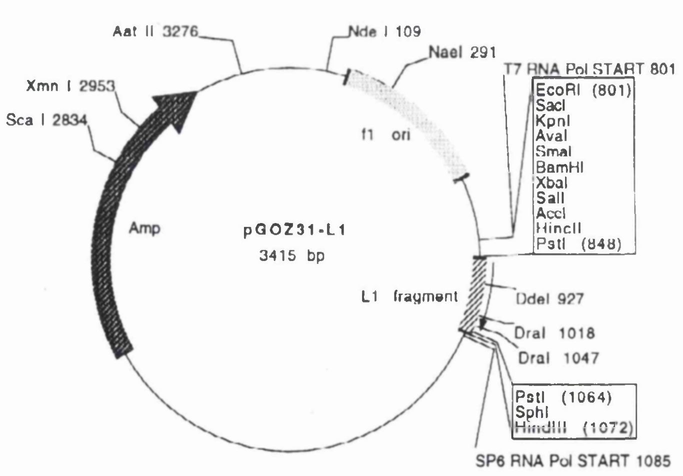
pGem-T Vector circle map and sequence reference points. (Promega)



pBluescript SK (+/-) phagemid map with sequence reference points. The SK designation indicates the polylinker is orientated such that *lacZ* transcription proceeds from *Sac I to Kpn* I. *(*Stratagene)



pHPV16-RL1 plasmid map. Digestion with *Ssp* I and transcription with T7 RNA polymerase generates a 340nt antisense probe specific for the HPV-16 L1 open reading frame. Gift from C. Meyers.



pGOZ31-L1 plasmid map. Digestion with *Hin*d III and transcription with T7 RNA polymerase generates a 271nt antisense probe specific for the HPV-31 L1 open reading frame. Gift from C. Meyers.

REFERENCES

Ai, W., Toussaint, E., Roman, A. (1999) CCAAT displacement protein binds to and negatively regulates human papillomavirus type 6 E6, E7, and E1 promoters. *J. Virol.*, **73**, 4220-4229.

Allen-Hoffmann, B.L., Schlosser, S. J., Ivarie, C. A. R., Sattler, C. A., Meisner, L. F., O'Connor, S. L. (2000) Normal growth and differentiation in a spontaneously immortalized near-diploid human keratinocyte cell line, NIKS. *J. Invest. Dermatol.*, **114**, 444-455.

Amella, C.A., Lofgren, L. A., Ronn, A. M., Nouri, M., Shikowitz, M. J., Steinberg, B. M. (1994) Latent infection induced with cottontail rabbit papillomavirus. A model for human papillomavirus latency. *Am. J. Pathol.*, **144**, 1167-1171.

Androphy, E.J., Lowy, D. R., Schiller, J. T. (1987) Bovine papillomavirus E2 transacting gene product binds to specific sites in papillomavirus DNA. *Nature*, **325**, 70-73.

Arends, M.J., Buckley, C. H., Wells, M. (1998) Aetiology, pathogenesis, and pathology of cervical neoplasia. *J. Clin. Pathol.*, **51**, 96-103.

Ashby, A.D.M., Meagher, L., Campo, M. S., Finbow, M. E. (2001) E5 transforming proteins of papillomaviruses do not disturb the activity of the vacuolar H-ATPase. *J. Gen. Virol.*, **82**, 2353-2362.

Asselineau, D., Prunieras, M. (1984) Reconstruction of 'simplified' skin: control of fabrication. *Brit. J. Dermatol.*, **111**, 219-22.

Asselineau, D., Bernard, B. A., Bailly, C., Darmon, M., Prunieras, M. (1986) Human epidermis reconstructed by culture: Is it normal?
J. Invest. Dermatol., **86**, 181-186.

Azizkhan, J.C., Jensen, D. E., Pierce, A. J., Wade, M. (1993) Transcription from TATA-less promoters: Dihydrofolate reductase as a model.
Crit. Rev. Eukayot. Gene Expr., **3**, 229-254.

Baker, C.C., Howley, P. M. (1987a) Differential promoter utilization by the papillomavirus in transformed cells and productively infected wart tissues.
EMBO J., **6**, 1027-1035.

Baker, C.C., Phelps, W. C., Lindgren, V., Bruan, M. J., Gonda, M. A., Howley, P. M. (1987b) Structural and transcriptional analysis of human papillomavirus type 16 sequences in cervical carcinoma cell lines.
J. Virol., **61**, 962-971.

Baker, T.S., Newcomb, W. W., Olson, N. H., Cowsert, L. M., Olson, C., Brown, J. C. (1991) Structures of bovine and human papillomaviruses. Analysis by cyroelectron microscopy and three-dimensional image reconstruction. *Biophys. J.*, **60**, 1445-1456.

Balague, C., Noya, F., Alemany, R., Chow, L. T., Curiel, D. T. (2001) Human papillomavirus E6E7-mediated adenovirus cell killing: Selectivity of mutant adenovirus replication in organotypic cultures of human keratinocytes.
J. Virol., **75**, 7602-7611.

Barksdale, S.K., Baker, C. C. (1995) Differentiation-specific alternative splicing of bovine papillomavirus late mRNAs.
J. Virol., **69**, 6553-6556.

Bastien, N., McBride, A. A. (2000) Interaction of the papillomavirus E2 protein with mitotic chromosomes. *Virology*, **270**, 124-134.

Bedell, M.A., Jones, K. H., Grossman, S. R., Laimins, L. A. (1989) Identification of human papillomavirus type-18 transforming genes in immortalized and primary cells. *J. Virol.*, **63**, 1247-1255.

Bedell, M.A., Hudson, J. B., Golub, T. R., Turyk, M. E., Wilbanks, G. D., Laimins, L. A. (1991) Amplification of human papillomavirus genomes in vitro is dependent on epithelial differentiation. *J. Virol.*, **65**, 2254-2260.

Bell, E., Merrill, C., Solomon, D. (1979) Characteristics of a tissue equivalent formed by fibroblasts cast in a collagen gel. *J. Cell Biol.*, **83**, 398.

Beniston, R.G., Morgan, I. M., O'Brien, V., Campo, S. M. (2001) Quercetin, E7 and p53 in papillomavirus oncogenic cell transformation. *Carcinogenesis*, **22**, 1069-1076.

Bennett, A.J., Lancaster, W. D. (1990) Association of human papillomavirus with benign, premalignant, and malignant anogenital lesions. In Pfister, H. (ed.) *Papillomaviruses and human cancer.* CRC Press, Boca Raton, pp. 12-43.

Bercovich, J.A., Centeno, C.R., Aguilar, O. G., Grinstein, S., Kahn, T. (1991) Presence and integration of human papillomavirus type 6 in a tonsillar carcinoma. *J. Gen. Virol.*, **72**, 2569-2572.

Berget, S.M. (1995) Exon recognition in vertebrate splicing. *J. Biol. Chem.*, **270**, 2411-2414.

Beutner, K.R., Tyring, S. (1997) Human papillomavirus and human disease. *Am. J.Med.*, **102**, 9-15.

Blaxall, B.C., Dwywe-Nield, L. D., Bauer, A., Bohlmeyer, T. J., Malkinson, A. M., Port, J. D. (2000) Differential expression and localization of the mRNA binding proteins, AU-rich element mRNA binding protein (AUF1) and Hu antigen R (HuR), in neoplastic lung tissue. *Mol. Carcinog.*, **28**, 76-83.

Boelens, W.C., Jansen, E. J. R., van Venrooij, W. J., Stripecke, R., Mattaj, I. W. (1993) The human U1 snRNP-specific U1A protein inhibits polyadenylation of its own pre-mRNA. *Cell*, **72**, 881-892.

Boelsma, E., Verhoeven, M. C. H., Ponec, M. (1999) Reconstruction of a human skin equivalent using a spontaneously transformed keratinocyte cell line (HaCaT). *J. Invest. Dermatol.*, **112**, 489-498.

Bosch, F.X. (1999) Presentation at the 17th International Papillomavirus Conference, Charleston, SC.

Boshart, M., Gissmann, L., Ikenberg, H., Klinheinz, A., Sheurlen, W., zur Hausen, H. (1984) A new type of papillomavirus DNA, its presence in genital cancer biopsies and in cell lines derived from cervical cancer. *EMBO J.*, **3**, 1151-1157.

Boukamp, P., Petrussevska, R. T., Breitkreutz, D., Hornung, J., Markham, A., Fusenig, N. E. (1988) Normal keratinization in a spontaneously immortalised aneuploid human keratinocyte cell line. *J. Cell Biol.*, **106**, 761-771.

Boyer, S.N., Wazer, D. E., Band, V. (1996) E7 protein of HPV-16 induces degradation of retinoblastoma protein through the ubiquitin-proteosome pathway. *Cancer Research*, **56**, 4620-4624.

Breitburd, F., Salmon, J., Orth, G. (1997) The rabbit viral skin papillomavirus and carcinomas: A model for the immunogenetics of HPV-associated carcinogenesis. *Clinics in Dermatology*, **15**, 237-247.

Brennan, C.M., Steitz, J. A. (2001) HuR and mRNA stability. *Cell. Mol. Life Sci.*, **58**, 266-277.

Brown, D.R., Fan, L., Jones, J., Bryan, J. (1994) Colocalization of human papillomavirus type 11 E1^E4 and L1 proteins in human foreskin implants grown in athymic mice. *Virology*, **201**, 46-54.

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

Butler, D., Collins, C., Mabruk, M., Walsh, C.B., Leader, M. B., Kay, E. W. (2000) Deletion of the FHIT gene in neoplastic and invasive cervical lesions is related to high-risk HPV infection but is independent of histopathological features. *J. Pathol.*, **192**, 502-510.

Caceres, J.F., Krainer, A. R. (1997) Mammalian pre-mRNA splicing factors. In Krainer, A.R. (ed.) *Eukaryotic mRNA Processing*. IRL Press, pp. 174-212.

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

Canavan, T.P., Doshi, N.R. (2000) Cervical cancer.
Am. Family Physician, **61**, 1369-76.

Caputi, M., Mayeda, A., Krainer, A. R., Zahler, A. M. (1999) hnRNP A/B proteins are required for inhibition of HIV-1 pre-mRNA splicing.
EMBO J., **18**, 4060-4067.

Carter, J.J., Koutsky, L. A., Hughes, J. P., Lee, S. K., Kuypers, J., Kiviat, N., Galloway, D. A. (2000) Comparison of human papillomavirus types 16, 18, and 6 capsid antibody responses following incident infection.
J. Infect. Dis., **181**, 1911-1919.

Chabot, B., Frappier, D., La Branche, H. (1992) Differential ASF/SF2 activity in extracts from normal W138 and transformed W138VA13 cells.
Nucleic Acid Res., **20**, 5197-5204.

Chabot, B. (1996) Directing alternative splicing: Cast and scenarios.
Trends Gen., **12**, 472-478.

Chang, Y.E., Laimins, L.A. (2000) Microarray analysis identifies interferon-inducible genes and Stat-1 as major transcriptional targets of human papillomavirus type 31. *J. Virol.*, **74**, 4174-4182.

Chen, C.-Y.A., Shyu, A-B. (1995) AU-rich elements:Characterization and importance in mRNA degradation. *Trends Biochem. Sci.*, **20**, 465-470.

Chen, J.J., Reid, C. E., Band, V., Androphy, E. J. (1995) Interaction of papillomavirus E6 oncoproteins with a putative calcium-binding protein.
Science, **269**, 529-531.

Christensen, N.D., Koltun, W. A., Cladel, N. M., Budgeon, L. R., Reed, C. A., Kreidlwee, J. W., Welsh, P. A., Patrick, S. D., Yang, H. (1997) Coinfection of human foreskin fragments with multiple human papillomavirus types produces regionally separate HPV infection within the same athymic mouse xenograft. *J. Virol.*, **71**, 7337-7344.

Cochrane, A.W., Jones, K. S., Beidas, S., Dillon, P. J., Skalka, A. M., Rosen, C. (1991) Identification and characterization of intragenic sequences which repress HIV structural gene expression. *J. Virol.*, **65**, 5305-5313.

Colgan, D.F., Manley, J. L. (1997) Mechanism and regulation of mRNA polyadenylation. *Genes Dev.*, **11**, 2755-2766.

Collier, B., Goorbar-Larsson, L., Sokolowski, M., Schwartz, S. (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**, 22648-22656.

Collier, B., Oberg, D., Zhao, X, Schwartz, S. (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**, 2739-2752.

Combita, A.L., Touze, A., Bousarghin, L., Sizaret, P. Y., Munoz, N., Coursaget, P. (2001) Gene transfer using human papillomavirus pseudovirions varies according to virus genotype and requires cell surface heparan sulphate. *FEMS Microbiol. Lett.*, **204**, 183-188.

Conrad, M., Bub, V., Schlegel, R. (1993) The human papillomavirus type 6 and 16 E5 proteins are membrane-associated proteins which associate with the 16kDa pore-forming complex. *J. Virol.*, **67**, 6170-6178.

Cornelison, T.L. (2000) Human papillomavirus genotype 16 vaccines for cervical cancer prophylaxis and treatment.
Curr. Op.Oncol., **12**, 466-473.

Cox, J.T., Lorincz, A. T., Schiffman M. H., Sherman, M. E., Cullen, A., Kurman, R. J. (1995) Human papillomavirus testing by hybrid capture appears to be useful in triaging women with a cytological diagnosis of atypical squamous cells of undetermined significance.
Am. J. Obstet. Gynecol., **172**, 946-954.

Crum, C.P., Barber, S., Symbula, M., Synder, K., Saleh, A. M., Roche, J. K. (1990) Coexpression of the human papillomavirus type 16 E4 and L1 open reading frames in early cervical neoplasia. *Virology*, **178**, 238-246.

Cumming, S.A., Repellin, C. E., McPhillips, M. G., Radford, J. C., Clements, J. B., Graham, S. V. (2002a) The HPV-31 late 3' UTR contains a complex bi-partite negative regulatory element. *J. Virol.*, **76**, In press.

Cumming, S.A., McPhillips, M. G., Clements, J. B., Graham, S. V. (2002b) Human papillomavirus type 16 late gene expression is controlled in part by weak consensus 5' splice sites in the late gene 3'untranslated region.
Submitted.

Cuzick, J. (1999) Presentation at the 17th International Papillomavirus Conference, Charleston, SC.

Davy, C., Jackson, D., Raj, K., Masterson, P., Millar, J., Cuthil, S., Doorbar, J. (2001) HPV-16 E1^E4 arrests E6/E7-expressing cells in G2 by sequestering the mitosis promoting complex onto the cytoplasmic keratin network. *DNA Tumour Virus Meeting*, Cambridge, England.

Day, P.M., Roden, R. B. S., Lowy, D. R., Schiller, J. T. (1998) The papillomavirus minor capsid protein, L2, induces localization of the major capsid protein, L1, and the viral transcription/replication, E2, to PML oncogenic domains. *J. Virol.*, **72**, 142-150.

DeCaprio, J. A., Ludlow, J. W., Figge, J., Shew, J. Y., Huang, C. M., Lee, W. H., Marsilio, E., Paucha, E., Livingston, D. M. (1988) SV40 large tumour antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. *Cell,* **54**, 275-283

de Villiers, E.M., Ruhland, A., Sekaric, P. (1999) Human papillomavirus in non-melanoma skin cancer. *Sem. Cancer Biol.*, **9**, 413-422.

de Villiers, E.M. (2001) Taxonomic classification of papillomaviruses. *Papillomavirus Report*, **12**, 57-63.

Del Mar Pina, L., Laimins, L. A. (2001) Differentiation-dependent chromatin rearrangement coincides with activation of human papillomavirus type 31 late gene expression. *J. Virol.*, **75**, 10005-10013.

Desaintes C., D., C. (1996) Control of papillomavirus DNA replication and transcription. *Sem. Cancer Biol.*, **7**, 339-347.

Dhanasekaran, S.E., Barrette, T. R., Ghosh, D., Shah, R., Varambally, S., Kurachi, K., Plenta, K. J., Rubin, M. A., Chinnaiyan, A. M. (2001) Delineation of prognostic biomarkers in prostrate cancer. *Nature*, **412**, 822-826.

Dietrich-Goetz, W., Kennedy, I. M., Levins, B., Stanley, M. A., Clements, J. B. (1997) A cellular 65kDa protein recognizes the negative regulatory element of human papillomavirus late mRNA.
Proc. Natl. Acad. Sci. USA, **94**, 163-168.

DiMaio, D., Mattoon, D. (2001) Mechanisms of cell transformation by papillomavirus E5 proteins. *Oncogene*, **20**, 7866-7873.

Dimmock, N.J., Primrose, S. B. (1994) *Introduction to Modern Virology*. Blackwell Science.

Dollard, S.C., Wilson, J. L., Demeter, L. M., Bonnez, W., Reichman, R. C., Broker, T. R., Chow, L. T. (1992) Production of human papillomavirus and modulation of the infectious program in epithelial raft cultures. *Genes Dev.*, **6**, 1131-1142.

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

Doorbar, J., Parton, A., Hartley, K., Banks, L., Crook, T., Stanley, M., Crawford, L. (1990) Detection of novel splicing patterns in a HPV-16 containing keratinocyte cell line. *Virology*, **178**, 254-262.

Doorbar, J., Ely, S., Sterling, J., McLean, C., Crawford, L. (1991) Specific interaction between HPV-16 E1^E4 and cytokeratins results in collapse of the epithelial cell intermediate filament network. *Nature*, **352**, 824-827.

Doorbar, J., Ely, N., Coleman, M., Davies, D. H., Crawford, L. (1992) Epitope-mapped monoclonal antibodies against the HPV16 E1^E4 protein. *Virology*, **187**, 353-359.

Doorbar, J., Foo, C., Coleman, N., Medcalf, E., Hartley, O., Prospero, T., Napthine, S., Sterling, J., Winter, G., Griffin, H. (1997) Characterisation of events during the late stages of HPV-16 infection *in vivo* using high-affinity synthetic Fabs to E4. *Virology*, **238**, 40-52.

Doorbar, J., Elston, R.C., Napthine, S., Raj, K., Medcalf, E., Jackson, D., Coleman, N., Griffin, H. M., Masterson, P., Stacey, S., Mengistu, Y., Dunlop, J. (2000) The E1/E4 protein of human papillomavirus type 16 associates with a putative RNA helicase through sequences in its C terminus.
J. Virol., **74**, 10081-10095.

Doorbar, J., Medcalf, E., Napthine, S. (1996) Analysis of HPV-1 E4 complexes and their association with keratins *in vivo*.
Virology, **218**, 114-126.

Durst, M., Gissmann, L., Ikenberg, H., zur Hausen, H. (1983) A new papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions.
Proc. Natl. Acad. Sci. USA, **80**, 3812-3815.

Durst, M., Kleinheinz, A., Hotz, M., Gissmann, L. (1985) The physical state of human papillomavirus type-16 DNA in benign and malignant genital tumours. *J. Gen. Virol.*, **66**, 1515-1522.

Durst, M., Gissmann, L. (1986) Transfection of primary human foreskin keratinocytes with human papillomavirus (HPV) type-16 DNA leads to escape from senescence. *European J. Cell Biol.*, **42**, 20.

Durst, M., Dzarlieva-Petrusevska, R. T., Boukamp, P., Fusenig, N. E., Gissmann, L. (1987) Molecular and cytogenetic analysis of immortalized human primary keratinocytes obtained after transfection with human papillomavirus type-16 DNA. *Oncogene*, **1**, 251-256.

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

Dvoretzky, I., Shober, R., Chattopadhyay, S. K. (1980) A quantitative *in vitro* focus assay for bovine papillomavirus. *Virology*, **103**, 369-375.

Dyson, N., Howley, P. M., Munger, K., Harlow, E. (1989) The HPV-16 E7 oncoprotein is able to bind to the retinablastoma gene product.
Science, **243**, 934-937.

Dyson, N., Guida, P., Munger, K., Harlow, E. (1992) Homologous sequences in adenovirus E1A and human papillomavirus E7 proteins mediate interaction with the same set of cellular proteins. *J. Virol.*, **66**, 6893-6902.

Einstein, M.H., Burk, R.D. (2001) Persistant human papillomavirus infection: Definitions and clinical implications. *Papillomavirus Report*, **12**, 119-123.

Eperon, I.C., Ireland, D. C., Smith, R. A., Mayeda, A., Krainer, A. R. (1993) Pathways for selection of 5' splice sites by U1 snRNPs and SF2/ASF.
EMBO J., **12**, 3607-3614.

Fahey, M.T., Irwig, L., Macaskill, P. (1995) Meta-analysis of Pap test accuracy. *Am. J. Epidemiol*, **141**, 680-689.

Ferenczy, A., Mitao, M., Nagai, N., Silverstein, S. J., Crum, C. P. (1985) Latent papillomavirus and recurring genital warts.
N. Engl. J. Med., **313**, 784-788.

Fife, K.H., Cramer, H. M., Schroeder, J. M., Brown, D. R. (2001) Detection of multiple human papillomavirus types in the lower genital tract correlates with cervical dysplasia. *J. Med. Virol.*, **64**, 550-559.

Flores, E.R., Lambert, P. F. (1997) Evidence for a switch in the mode of human papillomavirus type 16 DNA replication during the viral life cycle. *J. Virol.*, **71**, 7167-7179.

Flores, E.R., Allen-Hoffmann, B. L., Lee, D., Sattler, C. A., Lambert, P. F. (1999) Establishment of the human papillomavirus type 16 (HPV-16) life cycle in an immortalized human foreskin keratinocyte cell line. *Virology*, **262**, 344-354.

Florin, L., Schafer, F., Sapp, M., Streeck, R. E. (2001) A novel role for the papillomavirus minor capsid protein: Reorganizing nuclear domain 10. *DNA tumour virus meeting*, Cambridge, England.

Frattini, M.G., Laimins, L. A. (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. USA*, **91**, 12398-12402.

Frattini, M.G., Lim, H. B., Laimins, L. A. (1996) *In vitro* synthesis of oncogenic human papillomaviruses requires episomal genomes for differentiation-dependent late gene expression. *Proc. Natl. Acad. Sci. USA*, **93**, 3062-3067.

Frattini, M.G., Lim, H. B., Doorbar, J., Laimins, L. A. (1997) Induction of human papillomavirus type 18 late gene expression and genomic amplification in organotypic cultures from transfected DNA templates. *J. Virol.*, **71**, 7068-7072.

Fredericks, B.D., Balkin, A., Daniel, H. W., Schonrock, J., Ward, B. Frazer, I. H. (1993) Transmission of human papillomaviruses from mother to child. *Aust. N. Z. J. Obstet. Gynaecol.*, **33**, 30-32.

Friedl, F., Kimura, I., Osato, T., Ito, Y. (1970) Studies on a human cell line (SiHa) derived from carcinoma of uterus. Its establishment and morphology. *Proc. Soc. Exp. Biol. Med.*, **135**, 543-545.

Friesland, S., Mellin, H., Munck-Wikland, E., Nilsson, A., Lindholm, J., Dalianis, T., Lewensohn, R. (2001) Human papillomavirus and p53 staining in advanced tonsillar carcinoma: Relation to radiotherapy response and survival. *Anticancer Res.*, **21**, 529-534.

Fuchs, E. (1990) Epidermal differentiation: The bare essentials. *J. Cell. Biol.*, **111**, 2807-2814.

Fuchs, E., Byrne, C. (1993) Epidermal differentiation and keratin gene expression. *J. Cell Sci.*, 197-208.

Fuchs, E., Byrne, C. (1994) The epidermis: Rising to the surface. *Curr. Op. Gen. Dev.*, **4**, 725-736.

Furth, P.A., Baker, C. C. (1991) An element in the bovine papillomavirus late 3' untranslated region reduces polyadenylated cytoplasmic RNA levels. *J. Virol.*, **65**, 5806-5812.

Furth, P.A., Choe, W-T., Rex, J. H., Byrne, J. C., Baker, C. C. (1994) Sequences homologous to 5' splice sites are required for the inhibitory activity of papillomavirus late 3' untranslated regions. *Mol. Cell. Biol.*, **14**, 5278-5289.

Gaffney, D., Whilton, J. L., Lynch, M., McLauchlan, Clements, J. B. (1985) A modular system for the assay of transcriptional regulatory signals: The sequence TAATGARAT is required for herpes simplex virus immediate early gene regulation. *Nucleic Acid Res.*, **13**, 7847-7863.

Gage, J.R., Meyers, C., Wettstein, F. O. (1990) The E7 proteins of the non-oncogenic human papillomavirus type 6b and of the oncogenic HPV-16 differ in retinablastoma binding and other properties. *J. Virol.*, **64**, 723-730.

Galloway, D.A., McDougall, J. K. (1996) The disruption of cell cycle checkpoints by papillomavirus oncoproteins contributes to anogenital neoplasia. *Sem. Cancer Biol.*, **7**, 309-315.

Gama-Carvalho, M., Krauss, R. D., Chiang, L., Valcarel, J., Green, M. R., Carmo-Fonseca, M. (1997) Targeting of $U2AF^{65}$ to sites of active splicing in the nucleus. *J. Cell Biol.*, **137**, 975-987.

Gilmartin, G.M., Fleming, E. S., Oetjen, J., Graveley, B. R. (1995) CPSF recognition of an HIV-1 mRNA 3' processing enhancer: Multiple sequence contacts involved in poly(A) site definition.
Genes Dev., **9**, 72-83.

Giroglou, T., Florin, L., Schafer, F., Streeck, R. E., Sapp, M. (2001) Human papillomavirus infection requires cell surface heparan sulphate.
J. Virol., **75**, 1565-1570.

Glew, S.S., Duggan-Keen, M., Ghosh, A. K. (1993) Lack of association of HLA polymorphisms with human papillomavirus-related cervical cancer.
Hum. Immunnl., **37**, 157-164.

Goldstein, D.J., Finbow, M. E., Andresson, T., McLean, P., Smith, K. T., Bubb, V., Schlegel, R. (1991) Bovine papillomavirus E5 binds to the 16k component of vacuolar H-ATPases. *Nature*, **352**, 347-349.

Grassmann, K., Rapp, B.,Maschek, H., Petry, K. U., Iftner, T. (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**, 2239-2349.

Green, H. (1977) Terminal differentiation of cultured human epidermal cells. *Cell*, **11**, 405-416.

Green, M.R. (1991) Biochemical mechanisms of constitutive and regulated pre-mRNA splicing. *Annu. Rev. Cell Biol.*, **7**, 559-599.

Griffiths, T.H. (1953) *J. Exp. Med*, **97**, 659.

Gunderson, S.I., Polycarpou-Schwarz, Mattaj, I. W. (1998) U1 snRNP inhibits pre-mRNA polyadenylation through a direct interaction between U1 70K and poly(A) polymerase. *Molecular Cell*, **1**, 255-264.

Halpert, R., Fruchter, R. G., Sedlis, A., Butt, K., Boyce, J. G., Sillman, F. H. (1986) Human papillomavirus and lower genital tract neoplasia in renal transplant patients. *Obstet. Gynecol.*, **68**, 251-258.

Hanamura, A., Caceres, J. F., Mayeda, A., Franza, B. R., Krainer, A. R. (1998) Regulated tissue-specific expression of antagonistic pre-mRNA splicing factors. *RNA*, **4**, 430-444.

Harper, D., Philips, Z., Jenkins, D. (2001) HPV testing: Psychosocial and cost-effectiveness studies of screening and HPV disease. *Papillomavirus Report*, **12**, 1-5.

Harro, C.D., Pang, Y-YS., Roden, R. B. S., Hildesheim, A., Wang, Z., Reynolds, M. J., Lowy, D. R. (2001) Safety and immunogenicity trial in adult volunteers of a human papillomavirus 16 L1 virus-like particle vaccine. *J. Natl. Cancer Inst.*, **93**, 284-292.

Harwood, C.A., Surentheran, T., McGregor, J. M., Spink, P. J., Leigh, I. M., Breuer, J., Proby, C. M. (2000) Human papillomavirus infection and non-melanoma skin cancer in immunosuppressed and immunocompetent individuals. *J. Med. Virol.*, **61**, 289-297.

Hennings, H., Michael, D., Cheng, C., Steinert, P., Holbrook, K., Yuspa, S. H. (1980) Calcium regulation of growth and differentiation of mouse epidermal cells in culture. *Cell*, **19**, 245-254.

Higgins, G.D., Uzelin, D. M., Phillips, G. E., McEvoy, P., Burrel, C. J. (1992) Transcription patterns of human papillomavirus type 16 in genital intraepithelial neoplasia: Evidence for promoter usage within the E7 open reading frame during epithelial differentiation. *J. Gen. Virol.*, **73**, 2047-2057.

Hirose, Y., Manley, J. L. (1998) RNA polymerase II is an essential mRNA polyadenylation factor. *Nature*, **395**, 93-96.

Hirose, Y., Manley, J. L. (2000) RNA polymerase II and the integration of nuclear events. *Genes Dev.*, **14**, 1415-1429.

Howley, P.M., Law, M. F., Heilman, C., Engel, L., Alonso, M. C., Israel, M. A., Lowy, D. R., Lancaster, W. D. (1980) Molecular characterisation of papillomavirus genomes. *Cold Spring Harbour Conf. Cell Prolif.*, **7**, 233-247.

Howley, P.M. (1996) Papillomavirinae: The viruses and their replication. In Fields, B.N. (ed.) *Fields Virology.* Lippincott - Raveb, Philadelphia, Vol. 2, pp. 2045-2076.

Huang, J., Yen, T. S. B. (1994) Hepatitis B virus RNA element that facilitates accumulation of surface gene transcripts. *J. Virol.*, **68**, 3193-3199.

Hughes, F.J., Romanos, M. A. (1993) E1 protein of human papillomavirus is a DNA helicase/ATPase. *Nucleic Acid Res.*, **21**, 5817-5823.

Hummel, M., Frattini, M. G., Laimins, L. A. (1992) Differentiation induced and constitutive transcription of human papillomavirus type 31b in cell lines containing viral episomes. *J. Virol.*, **66**, 6070-6080.

Hummel, M., Lim, H. B., Laimins, L. A. (1995) Human papillomavirus type 31b late gene expression is regulated through protein kinase C mediated changes in RNA processing. *J. Virol.*, **69**, 3381-3388.

Jeon, S., Allen-Hoffmann, B. L., Lambert, P. F. (1995a) Integration of human papillomavirus type 16 into the human genome correlates with a selective growth advantage of cells. *J. Virol.*, **69**, 2989-2997.

Jeon, S., Lambert, P. F. (1995b) 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. USA*, **92**, 1654-1658.

Johnson, P.F., McKnight, S. L. (1989) Eukaryotic transcriptional regulatory proteins. *Annu. Rev. Biochem.*, **58**, 799-839.

Kastner, B., Kornstadt, U., Bach, M., Luhrmann, R. (1992) Structure of the small nuclear RNP particle -U1 identification of the 2 structural perturbances with RNP-antigens and RNP-antigens-70k. *J. Cell Biol.*, **116**, 839-849.

Kaufman, R.H., Ervin, A., Vladimir, V. (2000) Human papillomavirus infection and cervical carcinoma. *Clin. Obstet. Gynecol.*, **43**, 363-380.

Kawana, Y., Kawana, K., Yoshikawa, H., Taketani, Y., Yoshike, K., Kanda, T. (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**, 2331-2336.

Kennedy, I.M., Haddow, J. K., Clements, J. B. (1990) Analysis of human papillomavirus type 16 late mRNA 3' processing signals *in vitro* and *in vivo.* *J. Virol.*, **64**, 1825-1829.

Kennedy, I.M., Haddow, J. K., Clements, J. B. (1991) A negative regulatory element in the human papillomavirus type 16 genome acts at the level of late mRNA stability. *J. Virol.*, **65**, 2093-2097.

Kirnbauer, R., Booy, F., Cheng, N., Lowy, D. R., Schiller, J. T. (1992) Papillomavirus L1 major capsid protein self-assembles into virus-like particles that are highly immunogenic.
Proc. Natl. Acad. Sci. USA, **89**, 12180-12184.

Kirnbauer, R., Taub, J., Greenstone, H., Roden, R., Durst, M., Gissmann, L., Lowy, D. R., Schiller, J. T. (1993) Efficient self-assembly of human papillomavirus type 16 L1 and L1-L2 into virus-like particles. *J. Virol.*, **67**, 6929-6936.

Kiviat, N.B. (1999) Papillomaviruses in non-melanoma skin cancer: Epidemiological aspects. *Sem. Cancer Biol.*, **9**, 397-403.

Kiyono, T., Hiraiwa, A., Fujita, M., Hayashi, Y., Akiyama, T., Ishibashi, M. (1997) Binding of high-risk papillomavirus E6 oncoproteins to the human homologue of the Drosophila discs large tumour suppressor protein. *Proc. Natl. Acad. Sci. USA*, **94**, 11612-11616.

Klingelhutz, A.J., Hedrick, L., Cho, K. R., McDougall, J. K. (1995) The DCC gene suppresses the malignant phenotype of transformed human epithelial cells. *Oncogene*, **10**, 1581-1586.

Klingelhutz, A.J., Foster, S. A., McDougall, J. K. (1996) Telomerase activation by the E6 gene product of HPV-16. *Nature*, **380**, 79-82.

Klumpp, D.J., Laimins, L.A. (1999) Differentiation-induced changes in promoter usage for transcripts encoding the human papillomavirus type 31 replication protein E1. *Virology*, **257**, 239-246.

Koffa, M.D., Graham, S. V., Takagaki, Y., Manley, J. B., Clements, J. B. (2000) The human papillomavirus type 16 negative regulatory element interacts with three proteins that act at different posttranscriptional levels. *Proc. Natl. Acad. Sci. USA*, **97**, 4677-4682.

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

Kreider, J.W., Howett, M. K., Wolfe, S. A., Bartlett, S. A., Zaino, R. J., Sedlacek, T. V., Mortel, R. (1985) Morphological transformation in vivo of human uterine cervix with papillomavirus from condylomata acuminata. *Nature*, **317**, 639-641.

Kreider, J.W., Howett, M. K., Lill, N. L., Bartlett, S. A., Zaino, R. J., Sedlacek, T. V., Mortel, R. (1986) In vivo transformation of human skin with HPV 11 from condyloma acuminata. *Virology*, **59**, 369-376.

Kukimoto, I., Kanda, T. (2001) Displacement of YY1 by differentiation-specific transcription factor hSkn-1a activates the P670 promoter of human papillomavirus type 16. *J. Virol.*, **75**, 9302-9311.

Laemmli, U. (1970) Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature*, **277**, 680-685.

Lambert, P.F. (1991) Papillomavirus DNA replication. *J. Virol.*, **65**, 3417-3420.

Lamond, A.I. (1991) ASF/SF2: A splice site selector. *Trends Biochem. Sci.*, **16**, 452-453.

Lerner, E.A., Lerner, M. R., Janeway, C. A., Steitz, J. A. (1981) Monoclonal antibodies to nucleic acid-containing cellular constituents: probes for molecular biology autoimmune disease. *Proc. Natl. Acad. Sci. USA*, **78**, 2737-2741.

Levy, N.S., Chung, S., Furneaux, H., Levy P. A. (1998) Hypoxic stabilisation of vascular endothelial growth factor mRNA by the RNA-binding protein HuR. *J. Biol. Chem.*, **273**, 6417-6423.

Lewis, R.M., Morrill, J. C., Jahrling, P. B., Cosgriff, T. M. (1989) Replication of hemorrhagic fever viruses in monocytic cells. *Rev. Infect. Dis.*, **11**, S736-S742.

Li, Q.X., Yuong, L. S., Niedobitek, G., Dawson, C. W., Birkenbach, M., Wang, F., Rickinson, A. B. (1992) Epstein-Barr virus infection and replication in a human epithelial cell system. *Nature*, **356**, 347-350.

Li, R., Botchan, M. R. (1993) The acidic transcriptional activation domains of VP16 and p53 bind the cellular replication protein A and stimulate in vitro BPV-1 DNA replication. *Cell*, **73**, 1207-1221.

Liu, D.-W., Tsao, Y-P., Hsieh, C-H., Hsieh, J-T., Kung, J. T., Chiang, C-L., Chen, S-L. (2000) Induction of CD8 T cells by vaccination with recombinant adenovirus expressing human papillomavirus type 16 E5 gene reduces tumour growth. *J. Virol.*, **74**, 9083-9089.

Lorincz, A., Anthony, J. (2001) Advances in HPV detection by Hybrid Capture. *Papillomavirus Report*, **12**, 145-154.

Lutz, C.S., Alwine, J. C. (1994) Direct interaction of the U1 snRNP-A protein with the upstream efficiency element of the SV40 polyadenylation signal. *Genes Dev.*, **8**, 576-586.

Lutz, C.S., Murthy, K. G. K., Schek, N., O'Connor, P., Manley, J. L., Alwine, J. C. (1996) Interaction between the U1 snRNP-A protein and the 160kD subunit of cleavage-polyadenylation specificity factor increases polyadenylation efficiency in vitro. *Genes Dev.*, **10**, 325-337.

Maeda, T., Hiranuma, H., Jikko, A. (1999) Differential expression of the splicing regulatory factor genes during two-step chemical transformation in a BALB/3T3-derived cell line, MT-5. *Carcinogenesis*, **20**, 2341-2344.

Maldarelli, F., Martin, M. A., Strebel, K. (1991) Identification of post-transcriptionally active inhibitory sequences in HIV-1 RNA: Novel level of regulation. *J. Virol.*, **65**, 5732-5743.

Mann, K.P., Weiss, E. A., Nevins, J. R. (1993) Alternative poly(A) site utilization during adenovirus infection coincides with a decrease in the activity of a poly(A) site processing factor. *Mol. Cell Biol.*, **13**, 2411-2419.

Mantovani, F., Banks, L. (2001) The human papillomavirus E6 protein and its contribution to malignant progression. *Oncogene*, **20**, 7874-7887.

Maran, A., Amella, C. A., Di Lorenzo, T. P., Auborn, K. J., Taichman, L. B., Steinberg, B. M. (1995) Human papillomavirus type 11 transcripts are present at low abundance in latently infected respiratory tissue. *Virology*, **212**, 285-294.

Matthews, K.R., Tschudi, C., Ullu, E. (1994) A common pyrimidine-rich motif governs trans-splicing and polyadenylation of tubulin polycistronic pre-mRNA in trypanosomes. *Genes Dev.*, **8**, 491-501.

McBride, A.A., Byrne, J. C., Howley, P. M. (1989) E2 polypeptides encoded by BPV-1 from dimers through the carboxyl-terminal DNA binding domain: Transactivation is mediated through the conserved amino-terminal domain. *Proc. Natl. Acad. Sci. USA*, **86**, 510-514.

McBride, A.A., Romanczuk, H., Howley, P. M. (1991) The papillomavirus E2 regulatory proteins. *J. Biol. Chem.*, **266**, 18411-18414.

McCance, D.J., Kopan, R., Fuchs, E., Laimins, L. A. (1988) Human papillomavirus type-16 alters human epithelial cell differentiation in vitro. *Proc. Natl. Acad. Sci. USA*, **85**, 7169-7173.

McClean, C.S., Churcher, M. J., Meinke, J., Smith, G. L., Higgins, G., Stanley, M. A., Minson, A. C. (1990) Production and characterisation of a monoclonal antibody to human papillomavirus type 16 using a recombinant vaccinia virus. *J. Clin. Pathol.*, **43**, 488-492.

McGuire, K., McPhillips, M. G., Kullmann, H., Graham, S. V. (2002) The human papillomavirus type 16 negative regulatory element is a regulator of polyadenylation and a transcription pause site. *Submitted.*

McLauchlan, J., Gaffney, J., Whitton, L., Clements, J. B. (1985) A consensus sequence YGTGTTYY located downstream from the AATAAA signal is required for efficient formation of 3' end termini.
Nucleic Acid Res., **13**, 1347-1367.

Meissner, J.D. (1999) Nucleotide sequences and further characterisation of human papillomavirus DNA present in the CaSki, SiHa, and Hela cervical cell lines. *J. Gen. Virol.*, **80**, 1725-1733.

Melkert, P.W., Hopman, E., van den Burle, A.J. (1993) Prevalence of HPV in cytomorphologically normal cervical smears, as determined by PCR, is age-dependent. *Int. J. Cancer*, **53**, 919-923.

Merrick, D.T., Blanton, R. A., Gown, A. M., McDougall, J. K. (1992) Altered expression of proliferation and differentiation markers in human papillomavirus 16 and 18 immortalized cells grown in organotypic culture. *Am. J. Pathol.*, **140**, 167-177.

Meyers, C., Laimins, L.A. (1994a) *In vitro* systems for the study and propagation of human papillomaviruses.
In zur Hausen, H. (ed.) *Human Pathogenic Papillomaviruses*, pp. 199-215.

Meyers, C., Frattini, M. G., Laimins, L. A. (1994b) Tissue culture techniques for the study of human papillomaviruses in stratified epithelia. In Celis, J.E. (ed.) *Cell Biology: A Laboratory Handbook*. Academic Press, Inc, Orlando, FL.

Meyers, C. (1996) Organotypic (raft) epithelial tissue culture system for the differentiation-dependent replication of papillomavirus.
Methods in Cell Science, **18**, 201-210.

Meyers, C.M., Frattini, M. G., Hudson, J. B., Laimins, L. A. (1992) Biosynthesis of human papillomavirus from a continuous cell line upon epithelial differentiation. *Science*, **257**, 971-973.

Meyers, C.M., Frattini, M. G., Laimins, L. A. (1994) Induction of the protein kinase C signal transduction pathway is required for the biosynthesis of papillomavirus in vitro. *J. Cell. Biochem.*, 94.

Meyers, C.M., Mayer, T. J., Ozbun, M. A. (1997) Synthesis of infectious human papillomavirus type 18 in differentiating epithelium transfected with viral DNA. *J. Virol.*, **71**, 7381-7386.

Molin, M., Akusjarvi, G. (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**, 9002-9009.

Moreira, A., Takagaki, Y., Brackenridge, S., Wollerton, M., Manley, J. L., Proudfoot, N. J. (1998) The upstream sequence element of the C2 complement poly(A) signal activates mRNA 3' end formation by two distinct mechanisms. *Genes Dev.*, **12**, 2522-2534.

Morgan, I.M., Campo, S. M. (2000) Recent developments in bovine papillomavirus. *Papillomavirus Report*, **11**, 127-132.

Mork, J., Lie, K., Glattre, E., Hallmans, G., Jellum, E., Koskela, P., Moller, B., Pukkala, E., Schiller, J. T., Youngman, L., Lehtinen, M., Dillner, J. (2001) Human papillomavirus infection as a risk factor for squamous-cell carcinoma of the head and neck. *N. Engl. J. Med.*, **344**, 1125-1131.

Mounts, P., Shah K. (1984) Respiratory papillomatosis: Etiological relation to genital tract papillomviruses. *Proc. Med. Virol.*, **29**, 90-114.

Muderspach, L., Wilczynski, S., Roman, L., Bade, L., Felix, J., Small, L. A., Weber, J. (2000) A phase 1 trial of a human papillomavirus peptide vaccine for women with high-grade cervical and vulvar intraepithelial neoplasia who are HPV-16 positive. *Clin. Cancer Res.*, **6**, 3406-3416.

Munger, K., Basile, J. R., Duensing, S., Eichten, A., Gonzalez, S. L., Grace, M., Zacny, V. L. (2001) Biological properties and molecular targets of the human papillomavirus E7 oncoprotein. *Oncogene*, **20**, 7888-7898.

Munoz, N. (2000) Human papillomavirus and cancer: The epidemiological evidence. *J. Clin. Virol.*, **19**, 1-5.

Nasseri, M., Hirochika, R., Broker, T. R., Chow, L. T. (1987) A human papillomavirus type 11 transcript encoding an E1^E4 protein. *Genome Res.*, **9**, 27-43.

Negrini, B.P., Schffman, M. H., Kurman, R. J. (1990) Oral contraeptive use, human papillomavirus infection, and risk of early cytological abnormalities of the cervix. *Cancer Res.*, **50**, 4670-4675.

Nicholls, P.K., Stanley, M. A. (2000) The immunology of animal papillomaviruses.
Vet. Immunol. Immunopathol., **73**, 101-127.

Nindl, I. (2002) HPV variants and risk of cervical cancer.
Papillomavirus Report, **13**, 1-6.

Niwa, M., MacDonald, C. C., Berget, S. M. (1992) Are vertebrate exons scanned during splice-site selection? *Nature*, **360**, 277-280.

Nobbenhuis, M.A.E., Walboomers, J. M. M., Helmerhorst, T. J. M. (1999) Relation of human papillomavirus status to cervical lesions and consequences for cervical-cancer screening: A prospective study.
Lancet, **354**, 20-25.

O'Connor, D.P., Kay, E.W., Leader, M., Atkins, G. J., Murphy, G. M., Mabruk, M. J. (2001) p53 codon 72 polymorphism and human papillomavirus associated cancer. *J. Clin. Pathol.*, **5**, 539-542.

Olson, C., Cook, R. H. (1951) Cutaneous sarcoma-like lesions of the horse caused by the agent of bovine papilloma.
Proc. Soc. Exp. Biol. Med., **77**, 281-284.

Ozbun, M.A., Meyers, C. (1997) Characterization of late gene transcripts expressed during vegetative replication of human papillomavirus type 31b.
J. Virol., **71**, 5161-5172.

Ozbun, M.A., Meyers, C. (1998a) Temporal usage of multiple promoters during the life cycle of human papillomavirus type 31b. *J. Virol.*, **72**, 2715-2722.

Ozbun, M.A., Meyers, C. (1998b) Human papillomavirus type 31b E1 and E2 transcript expression correlates with vegetative viral genome amplification. *Virology*, **248**, 218-230.

Ozbun, M.A., Meyers, C. (1999) Two novel promoters in the upstream regulatory region of human papillomavirus type 31b are negatively regulated by epithelial differentiation. *J. Virol.*, **248**, 218-230.

Parazzini, F., La Vecchia, C., Negri, E., Fedele, L., Franceschi, S., Gallotta, L. (1992) Risk factors for cervical intraepithelial neoplasia. *Cancer*, **69**, 2276-2282.

Park, P., Copeland, W., Yang, L., Wang, T., Botchan, M. R., Mohr, I. J. (1994) The cellular DNA polymerase alpha-primase is required for papillomavirus DNA replication and associates with the viral E1 helicase. *Proc. Natl. Acad. Sci. USA*, **91**, 8700-8704.

Parkinson, K., Yeudall, L. (1992) The Epidermis. In Freshney, R.I. (ed.) *Culture of epithelial cells.*, pp. 60-80.

Pastrana, D.V., Vass, W. C., Lowy, D. R., Schiller, J. T. (2001) HPV16 VLP vaccine induces human antibodies that neutralize divergent variants of HPV16. *Virology*, **279**, 361-369.

Pattillo, R.A., Hussa, R. O., Story, M. T., Ruckert, A. C. F., Shalaby, M. R., Mattingly, R. F. (1976) Tumor antigen and human chrionic gonadotrophin in CaSki cells: A new epidermoid cervical cancer cell line.
Science, **196**, 1456-1458.

Petti, L., Nilson, L., DiMaio, D. (1991) Activation of the platelet-derived growth factor receptor by the bovine papillomavirus E5 protein.
EMBO J., **10**, 845-855.

Pirisi, L., Yasumoto, S., Feller, M., Doniger, J., DiPaolo, J. A. (1987) Transformation of human fibroblasts and keratinocytes with human papillomavirus type 16 DNA. *J. Virol.*, **61**, 1061-1066.

Powell, D.M., Amaral, M. C., Wu, J. Y., Maniatis, T., Greene, W. C. (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. USA, **94**, 973-978.

Pray, T.D., Laimins, L. A. (1995) Differentiation-dependent expression of E1^E4 proteins in cell lines maintaining episomes of human papillomavirus type 31b. *Virology*, **206**, 679-685.

Pugh, B.F. (2000) Control of gene expression through regulation of the TATA-binding protein. *Gene*, **255**, 1-14.

Rader, J.S., Golub, T. R., Hudson, J. B., Patel, D., Bedell, M. A., Laimins, L. A. (1990) *In vitro* differentiation of epithelial cells from cervical neoplasias resembles *in vivo* lesions. *Oncogene*, **5**, 571-576.

Rheinwald, J.G., Green, H. (1975) Serial cultivation of strains of human epidermal keratinocytes: The formation of keratinizing colonies from single cells. *Cell*, **6**, 331-343.

Roden, R.B.S., Kirnbauer, R., Jenson, A. B. (1994) Interaction of papillomaviruses with the cell surface. *J. Virol.*, **68**, 7260-7266.

Roden, R.B.S., Greenstone, H. L., Kirnbauer, R., Booy, F. P., Jessie, J., Lowy, D. R., Schiller, J. T. (1996) *In vitro* generation and type-specific neutralization of a human papillomavirus type 16 virion pseudotype. *J. Virol.*, **70**, 5433-5441.

Rohlfs, M., Winkenbach, S., Meyer, S., Rupp, T., Durst, M. (1991) Viral transcription in human keratinocyte cell lines immortalized by human papillomavirus type 16. *Virology*, **183**, 331-342.

Romanczuk, H., Thierry, F., Howley, P. M. (1990) Mutational analysis of cis-elements involved in E2 modulation of human papillomavirus type 16 P97 and type 18 P105 promoters. *J. Virol.*, **64**, 2849-2859.

Ronco, L.V., Karpova, A. Y., Vidal, M., Howley, P. M. (1998) Human papillomavirus 16 E6 oncoprotein binds to interferon regulatory factor-3 and inhibits its transcriptional activity. *Genes Dev.*, **12**, 2061-2072.

Ross, J. (1995) mRNA stability in mammalian cells. *Microbiol. Rev.*, **59**, 15-95.

Ruesch, M.N., Laimins, L. A. (1998a) Human papillomavirus oncoproteins alter differentiation-dependent cell cycle exit on suspension in semisolid medium. *Virology*, **250**, 19-29.

Ruesch, M.N., Stubenrauch, F., Laimins, L. A. (1998b) Activation of papillomavirus late gene transcription and genome amplification upon differentiation in semisolid medium is coincident with expression of involucrin and transglutamine but not keratin-10. *J. Virol.*, **72**, 5016-5024.

Saiga, A., Orita, S., Minoura-Tada, N., Maeda, M., Aono, Y., Asakawa, K., Nakahara, R., Kubota, M., Osame, M., Igarashi, H. (1997) Cis-acting inhibitory elements within the pol-env region of human T-cell leukemia virus type 1 possibly involved in viral persistence. *J. Virol.*, **71**, 4485-4494.

Sang, B-C., Barbosa, M. S. (1992) Single amino acid substituitions in low-risk HPV-6 E7 protein enhances features characteristic of the high-risk E7 oncoproteins. *Proc. Natl. Acad. Sci. USA*, **89**, 8063-8067

Sarnow, P., Shih, H. Y., Williams, J., Levine, A. Y. (1982) Adenovirus E1B-58kD tumour antigen and SV40 large tumour antigen are physically associated with the same 54kDa cellular protein in transformed cells. *Cell,* **38**, 387-394

Schafer, A., Friedmann, W., Mielke, M., Schwartlander, B., Koch, M. A. (1991) The increased frequency of cervical-neoplasia in women infected with the human immunodeficiency virus is related to the degree of immunosuppression. *Am. J. Obstet. Gynecol.*, **164**, 593-599.

Scheffner, M., Werness, B. A., Hubregtse, J. M., Levine, A. J., Howley, P. M. (1990) The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell*, **63**, 1129-1136.

Schek, N., Cooke, C., Alwine, J. C. (1992) Definition of the upstream efficiency element of the simian virus 40 late polyadenylation signal by using in vitro analyses. *Mol. Cell Biol.*, **12**, 5386-5393.

Schmitt, A., Rochat, A., Zeltner, R., Borenstein, L., Barrandon, Y., Wettstein, F. O., Iftner, T. (1996) The primary cells of the high-risk cottontail rabbit papillomavirus colocalize with the hair follicle stem cells.
J. Virol., **70**, 1912-1922.

Schneider, A., Hotz, M., Gissman, L. (1990) Increased prevalence of human papillomavirus in the lower genital tract of pregnant women.
Int. J. Cancer, **40**, 198-201.

Schneider-Gadicke, A., Schwarz, E. (1986) Different human cervical carcinoma cell lines show similar transcription patterns of HPV-18 early genes. *EMBO J.*, **5**, 2285-2292.

Schneider-Schaulies, S., Schneider-Schaulies, J., Bayer, M., Ladler, S., ter Meulen, V. J. (1993) Spontaneous and differentiation-dependent regulation of measles virus gene expression in human glial cells.
Virology, **67**, 3375-3383.

Schoop, V.M., Mirancea, N., Fusenig, N. E. (1999) Epidermal organization and differentiation of HaCaT keratinocytes in organotypic coculture with human dermal fibroblasts. *J. Invest. Dermatol.*, **112**, 343-353.

Schwartz, S., Felber, B. K., Pavlakis, G. N. (1992) Distinct RNA sequences in the gag region of HIV-1 decrease mRNA stability and inhibit expression in the absence of Rev protein. *J. Virol.*, **66**, 150-159.

Schwartz, S. (1998) Cis-acting negative RNA elements on papillomavirus late mRNAs. *Sem. Virol.*, **8**, 291-300.

Schwartz, S., Sokolowski, M., Collier, B., Carlsson, A., Goobar-Larsson, L. (1999) Cis-acting regulatory sequenes on human papillomavirus late mRNAs. *Recent Res. Devel. Virol.*, **1**, 53-74.

Schwarz, E., Freese, U. K., Gissmann, L., Mayer, W., Roggenbuck, A., Stremlau, A., zur Hausen, H. (1985) Structure and trascription of human papillomavirus sequences in cervical carcinoma cells. *Nature*, **314**, 111-114.

Sedman, S.A., Barbosa, M. S., Vass, W. C., Hubbert, N. L., Haas, J. A., Lowy, D. R., Schiller, J. T. (1991) The full-length E6 protein of human papillomavirus type 16 has transforming and transactivating activities and cooperates with E7 to immortalise keratinocytes in culture. *J. Virol.*, **65**, 4860-4866.

Seedorf, K., Krammer, G., Durst, M., Suhai, S., Rowekamp, W. G. (1985) Human papillomavirus type-16 DNA sequence. *Virology*, **145**, 181-185.

Seiki, M., Hikikikoshi, A., Yoshida, M. (1990) The U5 sequence is a cis-acting repressive element for genomic RNA expression of human T cell leukemia virus type 1. *Virology*, **186**, 81-86.

Selvakamur, R., Borenstein, L. A., Lin, Y-L., Ahmed, R. (1995) Immunization with nonstructural proteins E1 and E2 of CRPV stimulates the regression of virus-induced papillomas. *J. Virol.*, **69**, 602-605.

Shah, K.V., Howley, P. M. (1996) Papillomaviruses. In Fields, B.N. (ed.) *Fields Virology.* Lippincott - Raven, Philadelphia, Vol. 2, pp. 2077-2109.

Sherman, L., Alloul, N., Golan, I., Durst, M., Baram, A. (1992) Expression and splicing patterns of human papillomavirus type 16 messenger RNAs in precancerous lesions and carcinomas of the cervix, in human keratinocytes immortalized by HPV-16 and in cell lines established from cervical cancers. *Int. J. Cancer*, **50**, 356-364.

Shope, R. (1933) Infectious papillomatosis of rabbits. *J. Exp. Med.,* **58**, 607-624

Shinozaki, A., Arahata, K., Tsukahara, T. (1999) Changes in pre-mRNA splicing factors during neural differentiation in P19 embryonal carcinoma cells. *Int. J. Biochem. Cell Biol.*, **31**, 1279-1287.

Sibbet, G., Romero-Graillet, C., Meneguzzi, G., Campo, M. S. (2000) Alpha6 integrin is not the obligatory cell receptor for bovine papillomavirus type 4. *J. Gen. Virol.*, **81**, 327-334.

Sillman, F., Stanek, A., Sedlis, A., Rosenthal, J., Lanks, K. W., Buchhagen, D., Nicastri, A., Boyce, J. (1984) The relationship between human papillomavirus and lower genital intraepithelial neoplasia in immunosuppressed women. *Am. J. Obstet. Gynecol.*, **150**, 300-308.

Sirover, M.A. (1997) Role of the glycolytic protein, glyceraldehyde-3-phosphate dehydrogenase, in normal cell function and pathology. *J. Cell. Biochem.*, **66**, 133-140.

Smale, S.T. (1997) Transcription initiation from TATA-less promoters within eukaryotic protein-coding regions. *Biochem. Biophys. Acta*, **1351**, 73-88.

Smotkin, D., Wettstein, F. O. (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. USA*, **83**, 4680-4684.

Sokolowski, M., Zhao, C., Tan, W., Schwartz, S. (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**, 2303-2319.

Sokolowski, M., Tan, W., Jellne, M., Schwartz, S. (1998) mRNA instability elements in the human papillomavirus type 16 L2 coding region. *J. Virol.*, **72**, 1504-1515.

Sokolowski, M., Furneaux, F., Schwartz, S. (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**, 1080-1091.

Spalholz, B.A., Yang, Y-C, Howley, P. M. (1985) Transactivation of a bovine papillomavirus transcriptional regulatory element by the E2 gene product. *Cell*, **42**, 183-191.

Stanley, M.A., Browne, H. M., Appleby, M., Minson, A. C. (1989) Properties of a non-tumourigenic human cervical keratinocyte cell line. *Int. J. Cancer*, **43**, 672-676.

Stanley, M.A., Greenfield, I., M. (1992) Culture of human cervical epithelial cells. In Freshney, R.I. (ed.) *Culture of Epithelial Cells*, 135-158.

Stanley, M.A. (1994) Replication of human papillomaviruses in cell culture. *Antiviral Res.*, **24**, 1-15.

Stanley, M.A., Masterson, P. J., Nicholls, P. K. (1997) *In vitro* and animal models for antiviral therapy in papillomavirus infections.
Antiviral Chem. Chemo., **8**, 381-400.

Stauffer, Y., Raj, K., Masternak, K., Beard, P. (1998) Infectious human papillomavirus type 18 pseudovirions.
J. Mol. Biol., **283**, 529-536.

Sterling, J.C., Stanley, M. A., Gatward, G., Minson, T. (1990) Production of human papillomavirus type 16 virions in a keratinocyte cell line.
J. Virol., **64**, 6305-6307.

Sterling, J.C., Minson, A. C., Stanley, M. A., Gatward, G., Skepper, J. (1991) Culture of human papillomavirus type-16 containing keratinocytes *in vivo* resulting in the development of a differentiated epithelium with completion of the virus life-cycle. *J. Pathol.*, **163**, A156-A156.

Sterling, J.C., Skepper, J. N., Stanley, M. A. (1993) Immunoelectron microscopical localization of human papillomavirus type 16 L1 and E4 proteins in cervical keratinocytes cultured *in vivo*.
J. Invest. Dermatol., **100**, 154-158.

Stoler, M.H., Wolinsky, S. M., Whitbeck, A., Broker, T. R., Chow, L. T. (1989) Differentiation-linked human papillomavirus types 6 and 11 transcription in genital condylomata revealed by *in situ* hybridisation with message-specific RNA probes. *Virology*, **172**, 331-340.

Storey, A., Thomas, M., Kalita, A., Harwood, C., Gardiol, D., Mantovani, F., Breuer, J., Leigh, I. M., Matlashewski, G., Banks, L. (1998) Role of a p53 polymorphism in the development of human papillomavirus-associated cancer. *Nature*, **393**, 229-234.

Straight, S.W., Hinkle, P. M., Jewers, R. J., McCance, D. J. (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**, 4521-4532.

Stubenrauch, F., Laimins, L. A. (1999) Human papillomavirus life cycle: active and latent phases. *Sem. Cancer Biol.*, **9**, 379-386.

Sun, X.W., Kuhn, L., Ellerbrock, T. V., Chiasson, M. A., Bush, T. J., Wright, T. C. (1997) Human papillomavirus infection in women infected with the human immunodeficiency virus. *N. Engl. J. Med.*, **337**, 1343-1349.

Taichman, L.B., LaPorta, R. F. (1986) The expression of papillomaviruses in epithelial cells. In Salzman, N.P., Howley, P. M. (ed.) *The papovaviridae: The papillomaviruses*. Plenum, New York, Vol. 2, pp. 109-139.

Takagaki, Y., Manley, J. L., MacDonald, C. C., Wilusz, J., Shenk, T. (1990) A multisubunit factor, CstF, is required for polyadenylation of mammalian pre-mRNAs. *Genes Dev.*, **4**, 2112-2120.

Takagaki, Y., Manley, J. L. (1997) RNA recognition by the human polyadenylation factor CstF. *Mol. Cell Biol.*, **17**, 3907-3914.

Takagaki, Y., Manley, J. L. (1998) Levels of polyadenylation factor CstF-64 control IgM heavy chain mRNA accumulation and other events associated with B cell differentiation. *Mol. Cell*, **2**, 761-771.

Tan, W., Schwartz, S. (1995a) 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**, 2932-2945.

Tan, W., Felber, B. K., Zoloturkin, A. S., Pavlakis, G. N., Schwartz, S. (1995b) Efficient expression of human papillomavirus type16 L1 protein in epithelial cells by using Rev and the Rev-responsive element of human immunodeficiency virus or the cis-acting transctivation element of simian retrovirus type 1. *Virology*, **69**, 5607-5620.

Terhune, S.S., Milcarek, C., Laimins, L. A. (1999) Regulation of human papillomavirus type 31 polyadenylation during the differentiation-dependent life cycle. *J. Virol.*, **73**, 7185-7192.

Terhune, S.S., Hubert, W. G., Thomas, J. T., Laimins, L. A. (2001) Early polyadenylation signals of human papillomavirus type 31 negatively regulate capsid gene expression. *J. Virol.*, **75**, 8147-8157.

Thomas, J.T., Hubert, W. G., Ruesch, M. N., Laimins, L. A. (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. USA*, **96**, 8449-8454.

Thomas, J.T., Oh, S. T., Terhune, S. S., Laimins, L. A. (2001) Cellular changes induced by low-risk human papillomavirus type 11 keratinocytes that stably maintain viral episomes. *J. Virol.*, **75**, 7564-7571.

Thomas, K.K., Hughes, J. P., Kuypers, J. M., Kiviat, N. B., Lee, S-K., Adam, D. E., Koutsky, L. A. (2000) Concurrent and sequential acquisition of different genital human papillomavirus types. *J. Infect. Dis.*, **182**, 1097-1102.

Thomas, M., Banks, L. (1998) Inhibition of Bak-induced apoptosis by HPV-18 E6. *Oncogene*, **10**, 2943-2954.

Thomas, M., Banks, L. (1999) HPV E6 interactions with Bak are conserved amongst E6 proteins from high and low-risk HPV types.
J. Gen. Virol., **80**, 1513-1517.

Tijong, M.Y., Out, T. A., Ter Schegget, J., Burger, M. P. M., Van der Vang, N. (2001) Epidemiologic and mucosal immunologic aspects of HPV infection and HPV-related cervical neoplasia in the lower female genital tract: A review. *Int. J. Gynecol. Cancer*, **2001**, 9-17.

Tong, X., Howley, P. M. (1997) The bovine papillomavirus E6 oncoprotein interacts with paxillin and disrupts the actin cytoskeleton.
Proc. Natl. Acad. Sci. USA, **94**, 4412-4417.

Towbin, H., Staehelin, T., Gorden, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA*, **76**, 4350-4354.

Trofatter, K.F. (1997) Diagnosis of human papillomavirus genital tract infection. *Am. J. Med.*, **102**, 21-27.

Trus, B.L., Roden, R. B., Greenstone, H. L., Vrhel, M., Schiller, J. T., Booy, F. P. (1997) Novel structural features of bovine papillomavirus capsid revealed by a three-dimensional reconstruction to 9 A resolution.
Nature Struct. Biol., **4**, 413-420.

Umek, R.M., Friedman, A. D., McKnight, S. L. (1991) CCAAT-enhancer binding protein: A component of a differentiation switch.
Science, **251**, 288-292.

Unger, E.R., Vernon, S. D., Lee, D. R., Miller, D. L., Sharma, S., Clancy, K. A., Hart, C. E., Reeves, W. C. (1997) Human papillomavirus type in anal epithelial lesions is influenced by human immunodeficiency virus. *Arch. Pathol. Lab. Med.*, **121**, 820-824.

Ustav, M., Stenlund, A. (1991) Transient replication of BPV-1 requires two viral polypeptides encoded by the E1 and E2 open reading frames. *EMBO J.*, **10**, 449-457.

Vagner, S., Vagner, C., Mattaj, I. M. (2000) The carboxyl terminus of vertebrate poly(A) polymerase interacts with U2AF^{65} to couple 3'-end processing and splicing. *Genes Dev.*, **14**, 403-413.

Venuti, A., Salani, D., Poggiali, F., Manni, V., Bagnato, A. (1998) The E5 oncoprotein of HPV-16 enhances endothelin-1-induced keratinocyte growth. *Virology*, **24**, 1-5.

Vormwald-Dogan, V., Fischer, B., Bludau, H., Freese, U, K., Gissmann, L., Glitz, D., Schwarz, E., Durst, M. (1992) Sense and antisense transcripts of human papillomavirus type 16 in cervical cancers. *J. Gen. Virol.*, **73**, 1833-1838.

Wahle, E., Keller, W. (1994) 3' end processing of mRNA. In Higgins, S.J., Hames, B. D. (ed.) *RNA processing - a practical approach.* Oxford University Press, New York, pp. 1-34.

Wahle, E., Ruegsegger, U. (1999) 3'end processing of pre-mRNA in eukaryotes. *FEMS Microbiol. Reviews*, **23**, 277-295.

Wang, Y.-C., Meenakshi, S., Helfman, D. M. (1997) Alternative pre-mRNA splicing. In Krainer, A.R. (ed.) *Eukaryotic mRNA Processing.* IRL Press, pp. 242-279.

Wank, R., Thomssen, C. (1991) High risk of squamous cell carcinoma of the cervix for women with HLA-DQw3. *Nature*, **352**, 723-725.

Wassarman, K.M., Steitz, J. A. (1993) Association with terminal exons in pre-mRNAs: A new role for the U1 snRNP?
Genes Dev., **7**, 647-659.

Weinshenker, B.G., Wilton, S., Rice, G. P. A. (1988) Phorbol ester-induced differentiation permits productive human cytomegalovirus infection in a monocytic cell line. *J. Immunol.*, **140**, 1625-1631.

Werness, B.A., Levine, A. J., Howley, P. M. (1990) Association of human papillomavirus types 16 and 18 E6 proteins with p53. *Science*, **248**, 76-79.

Will, C.L., Luhrmann, R. (1997) snRNP structure and function. In Krainer, A.R. (ed.) *Eukaryotic mRNA Processing.* IRL Press, pp. 130-173.

Wright, T.C., Sun, X. W., Kuolos, J. (1995) Comparison of management algorithms for the evaluation of women with low-grade cytologic abnormalities. *Obstet. Gynecol.*, **85**, 202-210.

Yonaha, M., Proudfoot, N. J. (1999) Specific transcriptional pausing activates polyadenylation in a coupled in vitro system. *Mol. Cell*, **3**, 593-600.

Yoon, C.-S., Kim, D-D., Park, S-N., Cheong, S-W. (2001) Alpha6 integrin is the main receptor of human papillomavirus type 16 VLP.
Biochem. Biophys. Res. Commun., **283**, 668-673.

Zahler, A.M., Lane, W. S., Stolk, J. A., Roth, M. B. (1992) SR proteins: A conserved family of pre-mRNA splicing factors.
Genes Dev., **6**, 837-847.

Zerfass, K., Schulze, A., Spitkovsky, D., Friedman, V., Henglein, B., Jansen-Durr, P. (1995) Sequential activation of cyclin E and cyclin A gene expression by HPV-16 E7 through sequences necessary for transformation. *J. Virol.*, **69**, 6389-6399.

Zhang, B., Spandau, D. F., Roman, A. (2002) E5 protein of human papillomavirus type 16 protects human foreskin keratinocytes from UV B-irradiation-induced apoptosis. *J. Virol.*, **76**, 220-231.

Zhao, C., Tan, W., Sokolowski, M., Schwartz, S. (1996) Identification of nuclear and cytoplasmic proteins that interact specifically with an AU-rich, cis-acting inhibitory sequence in the 3' untranslated region of human papillomavirus type 1 late mRNAs. *J. Virol.*, **70**, 3659-3667.

Zhao, J., Hyman, L., Moore, C. (1999) Formation of mRNA 3' ends in eukaryotes: Mechanism, regulation and interrelationships with other steps in mRNA synthesis. *Microbiol. Mol. Biol. Rev.*, **63**, 405-445.

Zhao, W., Chow, L. T., Broker, T. R. (1999) A distal element in the HPV-11 upstream regulatory region contributes to promoter repression in basal keratinocytes in squamous epithelium. *Virology*, **253**, 219-229.

Zhou, J., Sun, X. Y., Stenzel, D. J., Frazer, I. H. (1991) Expression of vaccinia recombinant HPV 16 L1 and L2 ORF proteins in epithelial cells is sufficient for assembly of HPV virion-like particles. *Virology*, **185**, 251-257.

zur Hausen, H., de Villiers, E. M. (1994) Human papillomaviruses.
Annu. Rev. Microbiol., **48**, 427-447.

zur Hausen, H. (1999) Immortalization of human cells and their malignant conversion by high risk human papillomavirus genotypes.
Sem. Cancer Biol., **9**, 405-411.

zur Hausen, H. (2000) Papillomavirus causing cancer: Evasion from host-cell control in early events in carcinogenesis.
J. Nat. Cancer Inst., **92**, 690-698.

GLASGOW UNIVERSITY LIBRARY