University of Alberta

The roles of HSV-1 VP16 and ICP0 in modulating cellular innate antiviral responses

by

Meaghan H Hancock

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Virology

Medical Microbiology and Immunology

©Meaghan H Hancock

Spring 2010
Edmonton, Alberta

Permission is hereby granted to the University of Alberta Libraries to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly or scientific research purposes only. Where the thesis is converted to, or otherwise made available in digital form, the University of Alberta will advise potential users of the thesis of these terms.

The author reserves all other publication and other rights in association with the copyright in the thesis and, except as herein before provided, neither the thesis nor any substantial portion thereof may be printed or otherwise reproduced in any material form whatsoever without the author's prior written permission.

Examining Committee

James Smiley, Medical Microbiology and Immunology

Luis Schang, Biochemistry and Medical Microbiology and Immunology

Edan Foley, Medical Microbiology and Immunology

Bart Hazes, Medical Microbiology and Immunology

Vikram Misra, Veterinary Microbiology, University of Saskatchewan

Abstract

Infection of most cell types with herpes simplex virus (HSV) mutants lacking the activation functions of VP16 and/or ICP0 results in repression of viral gene expression. However, the human osteosarcoma cell line U2OS supports the replication of VP16 and ICP0 mutants to nearly wild type levels. Prior to the studies presented in this thesis, the basis for the permissivity of U2OS cells to VP16 and ICP0 mutants had not been explored. Here, somatic cell fusion assays were used to determine that U2OS cells support the replication of VP16 and ICP0 mutants due to a defect in an innate gene silencing mechanism. The artificial induction of interferon stimulated genes that occurs during the somatic cell fusion assays is not the basis for the observed repression of viral gene expression. As one means of identifying components of the antiviral pathway defective in U2OS cells, restrictive cell types were treated with kinase inhibitors and infected with VP16 and/or ICP0 mutants. Although several compounds were identified which compensate for the defect in gene expression of VP16 mutants, these drugs also stimulate mutant virus gene expression in U2OS. Thus, U2OS are most likely not defective in the cellular signalling pathway(s) targeted by these compound(s). Finally, the importance of VP16 and ICP0 in modulating chromatin structure on the viral genome in both restrictive and permissive cells was examined, uncovering an essential role for both proteins in altering histone occupancy and acetylation levels. Importantly, U2OS cells have a defect in the chromatin-based pathway targeted by ICP0. However, evidence suggests that the ability of VP16

and ICP0 to affect histone occupancy and acetylation levels is not required for viral gene expression. Taken together, the results of this thesis demonstrate that U2OS cells support the replication of VP16 and ICP0 mutants due to a defect in an innate antiviral mechanism which does not involve the targets of several well characterized kinase inhibitors. The significance of the defect in a chromatin-based pathway targeted by ICP0 in U2OS cells remains to be elucidated.

Acknowledgements

First and foremost I would like to thank Jim for being an excellent mentor and providing me with the opportunity to succeed in his lab. I appreciate his passion for science and for teaching me to think like a scientist. I am also indebted to members of the Smiley lab for their encouragement and support. Specifically I would like to thank Melany Wagner for sharing the trials and triumphs of the last five years as well as Ervin Hou and Brett Duguay for their encouragement. Drs Jenn Corcoran, Steve Barr and Bianca Dauber all provided their expertise and experience. I would like to thank Rob Maranchuk for his role in keeping the lab organized and running smoothly and Holly Saffran, both for her technical expertise as well as providing me with a place to stay while Chris was in Japan!

I would like to thank my family for their understanding and for making each time we were able to go home very special. My mother Johanna and sister Erin helped to organize our wedding while I was working on this degree and have been a constant support for me.

Finally, I would like to thank my husband, Chris, without whom this thesis would not have been possible. Thank you for being there when I needed you, gone when I needed you to be and for being a perfect foil to my personality.

I would like to dedicate this thesis to the memory of my father, Paul Albert Hancock

Table of Contents

Abstract i

Acknowledgements iii

Table of Contents iv

List of figures x

List of abbreviations xii

Chapter 1: Thesis introduction

1.1 The *Herpesviridae* family 2

1.2 The herpes simplex virus lytic life cycle 3

1.2.1 The HSV virion 3

1.2.1.1 The envelope 3

1.2.1.2 The tegument 4

1.2.1.3 The capsid 4

1.2.1.4 The viral genome 5

1.2.2 Viral attachment and entry 5

1.2.3 Delivery of the HSV genome to the nucleus 6

1.2.4 Initiation of the HSV lytic replication cycle 7

1.2.4.1 Alterations in nuclear architecture mediated by HSV 8

1.2.4.1.1 Alteration of ND10 structures 8

1.2.4.1.2 Viral replication compartment formation 9

1.2.5 Viral gene expression and DNA replication 10

1.2.5.1 Function of VP16 11

1.2.5.2 Phenotype of VP16 mutants 12
1.2.5.3 IE gene expression 14
1.2.5.3.1 ICP47 15
1.2.5.3.2 ICP22 15
1.2.5.3.3 ICP27 17
1.2.5.3.4 ICP4 18
1.2.5.3.5 ICP0 20
1.2.5.3.5.1 Function of ICP0 20
1.2.5.3.5.2 Phenotype of ICP0 mutants 24
1.2.5.4 E gene expression and DNA replication 26
1.2.5.5 L gene expression 28
1.2.6 Viral capsid assembly and egress 29
1.3 Herpes Simplex virus latency 30
1.3.1 HSV primary and latent infections 30
1.3.2 Models of latency 31
1.3.3 Latency-associated transcripts 32
1.3.4 Establishment and maintenance of latency 33
1.3.5 Reactivation from latency 34
1.4 HSV quiescence 35
1.5 Chromatinization of the viral genome 39
1.5.1 Chromatinization of the viral genome during Latency 40
1.5.2 Chromatinization of the viral genome during lytic infection 42
1.5.2.1 Roles of VP16 and ICP0 in regulating chromatin structure 44
1.6 Intracellular antiviral responses 44

1.6.1 The interferon response 44

1.6.2 Interferon-stimulated genes 44

1.6.3 The interferon-independent response to HSV infection 47

Chapter 2: Materials and Methods

2.1 Cell culture and growth of HSV 52

2.1.1 Maintenance of mammalian cell culture 52

2.1.2 Preparation and storage of HSV stocks 53

2.1.3 Titration of HSV stocks 55

2.1.4 Infection of mammalian cells with HSV 56

2.1.5 Acid washing mammalian cells 56

2.1.6 UV-inactivating HSV 57

2.2 Production and manipulation of plasmid DNA 57

2.2.1 Transformation of competent bacteria 57

2.2.2 Growth and maintenance of bacterial cultures 58

2.2.3 Large-scale preparation of plasmid DNA 58

2.2.4 Quantitation of DNA and RNA 58

2.2.5 Restriction enzyme digestion 59

2.2.6 Polymerase Chain Reaction (PCR) 59

2.2.7 DNA agarose gel electrophoresis and gel extraction 60

2.2.8 Random prime labelling of DNA fragments 60

2.2.9 Transfection of plasmid DNA into mammalian cells 61

2.3 Isolation and manipulation of cellular RNA 62

2.3.1 Harvesting total cellular RNA 62

2.3.2 Northern blot analysis 62

2.4 Western blot analysis 64

2.5 Indirect immunofluorescence and microscopy 62
2.5.1 Cell-permeable staining 62
2.5.2 Indirect immunofluorescence 62
2.5.3 Microscopy 66
2.6 DNA fluorescent in situ hybridization 67
2.6.1 Preparation of DNA probe 67
2.6.2 DNA fluorescent in situ hybridization 68
2.7 Chromatin Immunoprecipitation 69
2.7.1 Chromatin Immunoprecipitation 69
2.7.2 Isolation of immunoprecipitated DNA 71
2.8 Real-time PCR 71
2.8.1 Design of Real-time PCR experiments 72

Chapter 3: Herpes Simplex virus regulatory proteins VP16 and ICP0 counteract an innate intranuclear barrier to viral gene expression

Preface 75
Abstract 76
Introduction 77
Results 84
Discussion 94
References 116
Appendix 1: Data not shown 124

Chapter 4: **Cell fusion-induced activation of interferon-stimulated genes in not required for restriction of a herpes simplex virus VP16/ICP0 mutant in heterokarya formed between permissive and restrictive cells**

Preface 127

Abstract 128

Note 129

References 141

Chapter 5: **Herpes Simplex Virus VP16, but not ICP0, is Required to Reduce Histone Occupancy and Enhance Histone Acetylation on Viral Genomes in U2OS Osteosarcoma Cells.**

Preface 146

Abstract 147

Introduction 148

Results 155

Discussion 163

References 183

Appendix 1: Data not shown 189

Appendix 2: Additional data 190

Chapter 6: **Identification of Kinase Inhibitors that Stimulate Herpes Simplex Virus Immediate-Early Gene Expression in the Absence of VP16 Activation Function**

Preface 196

Abstract 197

Introduction 198

Results and Discussion 203

References 233

Appendix 1: Data not shown 243

Chapter 7: Thesis discussion 246

Chapter 8: Thesis references 263

List of Figures

Figure 3.1	Host range phenotype of KM110	100
Figure 3.2	Fusion does not affect the phenotype of U2OS or HEL cells	102
Figure 3.3	Experimental design	104
Figure 3.4	The restrictive phenotype of HEL cells is dominant in heterokarya	106
Figure 3.5	The KM110 genome responds to VP16 delivered *in trans*	108
Figure 3.6	Nuclear delivery of viral genomes is complete within 3 Hours	110
Figure 3.7	Reactivation of the KM110 genome by ICP0 provided *in trans* 48 hours postinfection	112
Figure 3.8	VP16 is unable to reactivate KM110 at late times postinfection	114
Figure 3.A1	The effects of membrane permeable dyes on viral infection	124
Figure 4.1	Effects of p14 expression on ISG mRNA accumulation	136
Figure 4.2	Expression of ICP4 in heterokarya	138
Table 4.1	ICP4 protein expression in p14-induced heterokarya	140
Table 5.1	PCR primers	172
Figure 5.1	Gene expression profiles of KOS, n212 and V422 in HeLa and U2OS cells	173
Figure 5.2	Histone H3 occupancy on KOS, n212 and V422 genomes in HeLa and U2OS cells	175
Figure 5.3	Acetylated histone H3 on KOS, n212 and V422 genomes in HeLa and U2OS cells	177
Figure 5.4	The effects of cycloheximide on histone H3 occupancy on the viral genome in HeLa and U2OS cells	179

Figure 5.5	Effects of HSV infection on histone H3 occupancy at selected regions of the cellular genome	181
Figure 5.A1	H3 occupancy on the cellular genome decreases with KOS and n212 infection	189
Figure 5.A2.1	RNA polymerase II occupancy at the ICP27 transcriptional start site in HeLa and U2OS cells	191
Figure 5A2.2	Input DNA levels in HeLa and U2OS cells	192
Figure 5A2.3	ICP0 mRNA expression in U2OS cells	194
Table 6.1	Activation of IE gene expression with protein kinase inhibitors	218
Figure 6.1	LY294002 compensates for the VP16 lesion of KM110	219
Figure 6.2	V422 gene expression is stimulated in a PI3K-independent manner	221
Figure 6.3	NU7441 activates V422 in a DNA PK-independent manner	223
Figure 6.4	Drugs stimulate V422 gene expression in the absence of new protein synthesis	225
Figure 6.5	Drugs do not block ISG56K induction in response to incoming virus	227
Figure 6.6	U2OS cells express the target(s) of the LY drugs and NU7441	229
Figure 6.7	Chemical structures of LY294002, LY303511 and NU7441	231
Figure 6.A1	The effects of various chemical compounds on viral infection	243

List of abbreviations

ActD	Actinomycin D
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
BHK	Baby hamster kidney
^{o}C	Celsius
cAMP	cyclic adenosine monophosphate
CBP	CREB-binding protein
cDNA	Complementary DNA
CENP	Centromeric protein
ChIP	Chromatin Immunoprecipitation
CKII	Casein Kinase II
CREB	cAMP response element binding
CSK	Cytoskeletal buffer
CTCF	CCCTC-binding factor
CTD	Carboxy-terminal domain
CTL	Cytotoxic T lymphocyte
CTP	Cytidine triphosphate
DAI	DNA activator of IRFs
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic Acid
DNA PK	DNA-dependent protein kinase
dTTP	Deoxythymidine triphosphate
dUTP	Deoxyuridine triphosphate
E	Early
EBV	Epstein Barr Virus
EDTA	Ethylenediaminetetraacetic acid
eIF	Eukaryotic initiation factor
ER	Endoplasmic Reticulum
FAST	Fusion-associated small transmembrane
FBS	Fetal bovine serum
Fig	Figure
FISH	Fluorescent *in situ* hybridization
GFP	Green fluorescent protein
GTP	Guanosine triphosphate
HCMV	Human cytomegalovirus
HAT	Histone acetyltransferase
HAUSP	Herpesvirus-associated ubiquitin specific protease

HCF	Host cell factor
HDAC	Histone deacetylase
HEL	Human embryonic lung
HEXIM	Hexamethylene *bis*-acetimide inducible
HHV	Human herpesvirus
HIV	Human immunodeficiency virus
HMBA	Hexamethylene *bis*-acetimide
HMG	High mobility group
HP	Heterochromatin protein
hpi	Hours post-infection
HSV	Herpes Simplex virus
HVEM	Herpesvirus entry mediator
ICP	Infected cell protein
IE	Immediate early
IF	Immunofluorescence
IFN	Interferon
IPS-1	Interferon β promoter stimulator 1
IRF	Interferon regulatory factor
ISG	Interferon stimulated gene
ISH	*In situ* hybridization
ISRE	Interferon stimulated response element
JAK	Janus activated kinae
JNK	Jun kinase
kDa	kiloDalton
KSHV	Kaposi's sarcoma-associated herpesvirus
L	Late
LAT	Latency-associated transcript
LB	Luria Broth
M	Molar
mL	Millilitre
mM	Millimolar
MDA5	Melanoma differentiation-associated gene 5
MEF	Murine embryonic fibroblast
Mg	Milligram
μg	Microgram
MHC	Major Histocompatability Complex
min	Minute
miRNA	MicroRNA
MOI	Multiplicity of infection
mRNA	Messanger RNA
ng	Nanogram
nt	Nucleotide
OAS	Oligoadenylate Synthetase
Oct-1	Octamer transcription factor-1
ORF	Open reading frame

Ori	Origin of DNA replication
PAA	Phosphonoacetic Acid
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFU	Plaque forming units
PI3K	Phosphoinositol-3 kinase
PIPES	Piperazine-1,4-bis(2-ethane sulfonic acid)
PKR	Protein kinase R
PML	Promyelocytic leukemia
PRR	Pattern recognition receptor
Rb	Retinoblastoma
REST	RE1 silencing transcription factor
RIG-I	Retinoic acid-inducible gene
RING	Really interesting new gene
RIPA	Radioimmunoprecipitation assay
RLH	RIG-I-like helicase
RNA	Ribonucleic Acid
RNase	Ribonuclease
RPA	Replication protein A
rpm	Revolutions per minute
RT PCR	Real-time PCR
SDS	Sodium dodecyl sulphate
snRNP	Small nuclear ribonuclear protein
STAT	Signal transducer and activator of transcription
SUMO	Small ubiquitin-like modifier
TAF	Template activating factor
TAP	Transporter associates with antigen processing
TBP	TATA-binding protein
TE	Tris-EDTA
TF	Transcription factor
TK	Thymidine kinase
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRIM	Tripartite motif
tRNA	Transfer RNA
ts	Temperature sensitive
TSA	Trichostatin A
TTP	Thymidine triphosphate
UL	Unique long
US	Unique short
USP	Ubiquitin specific protease
UTP	Uridine triphosphate
UV	Ultraviolet
vhs	Virion host shutoff

VP	Virion protein
VZV	Varicella zoster virus
w/v	Weight/volume

Chapter One: Thesis Introduction

1.1 The Herpesviridae family

Herpesviridae is a family of large, enveloped, double-stranded DNA viruses which replicate in the nucleus of mammalian cells. This family of viruses is characterized by their distinctive pattern of gene expression during lytic infection, their ability to establish latent infections in their host and a variety of structural features. All members of the *Herpesviridae* have an envelope with glycoprotein spikes, a proteinaceous tegument layer and an icosahedral capsid surrounding the linear dsDNA genome. Numerous different herpesviruses have been identified to date, with eight known to cause disease in humans which can involve skin lesions, tumour formation, encephalitis and several other outcomes (110).

The *Herpesviridae* family is subdivided into *Alphaherpesvirinae, Betaherpesvirinae,* and *Gammaherpesvirinae* on the basis of factors such as host range, growth rate and cell type in which latency is established. The *Alphaherpesvirinae* subfamily includes herpes simplex viruses (HSV) 1 and 2 which predominantly cause oral and genital herpetic lesions respectively, and varicella zoster virus (VZV), the causative agent of chickenpox and shingles. These viruses typically have a broad host range, quickly destroy infected cells, spread rapidly in culture, and establish latency in sensory ganglia. The *Betaherpesvirinae* include human cytomegalovirus (HCMV), human herpesvirus-6 (HHV-6), and HHV-7. They generally have a restricted host range, grow slowly and establish latency in cell types including kidneys and secretory glands. Finally, the *Gammaherpesvirinae* are exemplified by Epstein-Barr virus (EBV)

and Kaposi's sarcoma-associated herpesvirus (KSHV) and often infect and establish latency in lymphoid tissue (110).

1.2 The Herpes Simplex virus lytic lifecycle

1.2.1 The HSV-1 virion

Using cryo-electron tomography, the HSV virion was determined to be a spherical particle with an average diameter of 186 nm (225 nm when the glycoprotein spikes are included) (131).

1.2.1.2 The envelope

The lipid membrane of HSV is derived from a host cytoplasmic membrane, although the exact cellular compartment is still controversial (350, 398). Embedded within this membrane are nine viral glycoproteins (gB, gC, gD, gE, gG, gH, gI, gL and gM). Additionally, at least two nonglycosylated intrinsic membrane proteins (U_L20, U_S9) and potentially more (U_L24, U_L43 and U_L34) are also found in the lipid membrane (110). The viral glycoproteins are non-randomly arranged in spikes projecting from the viral envelope and play roles in mediating attachment and entry into target cells (360).

1.2.1.2 The tegument

The tegument, defined as the region between the lipid membrane and the icosahedral capsid, is composed of over 20 viral proteins (368). Within the tegument there are interactions between tegument proteins, as well as between tegument proteins and the capsid and envelope proteins (390). Proteins packaged in the tegument are involved in stimulating viral gene expression (VP16; (39)), releasing viral DNA from the capsid (VP1-2; (16)) and making the host cell more conducive to viral replication (ICP0; described in more detail below). The tegument also contains vhs, a protein that induces degradation of cellular and viral mRNAs (206, 286).

1.2.1.3 The capsid

The HSV capsid is composed of 162 capsomeres in a T = 16 icosahedral symmetry. There are 12 pentons on the vertices and 150 hexons on the edges and faces (110). VP5 is the major capsid protein, found at both the pentons and the hexons (418). VP26 is also found at each hexon (419), while a trimer formed from one copy of VP19C and two copies of VP23 interact with two capsomeres each (418). The U_L6 protein forms a dodecamer through which the viral DNA is packaged (381). VP24 is a capsid protease and plays a role, along with U_L16, in DNA encapsidation (273, 307).

1.2.1.4 The viral genome

The HSV-1 genome packaged in the capsid is a linear, double-stranded DNA molecule (181). The polyamine spermine is thought to interact with the viral DNA (122). The genome is approximately 152 kbp in length with a G + C content of 68%. It encodes at least 80 proteins (110).

The HSV genome is composed of covalently linked unique long (U_L, 108kb) and unique short (U_S, 13kb) segments flanked by inverted repeats (391). The long and short segments can invert relative to one another, resulting in 4 possible conformations of the viral genome found in equimolar ratios in a virus population (71, 145).

1.2.2 Viral attachment and entry

Initial binding of the virus to the cell surface is mediated by gC and/or gB, which interact with glycosaminoglycans, namely the heparan sulphate moieties, on cell surface proteoglycans (149, 345, 404). In the absence of gC, gB or heparan sulphate, infectivity of the virions is greatly reduced but not abolished, indicating that other glycoproteins and cell surface moieties can mediate the initial attachment step (13, 130, 148, 149, 345).

Following attachment, gD interacts with its cognate receptor. There are three different families of receptors with which gD can interact (56, 120, 267, 347, 394, 396). Herpesvirus entry mediator (HVEM) is a member of the tumour necrosis factor (TNF) receptor family and is expressed in T and B lymphocytes, epithelial

and fibroblast cells but not neurons (156, 205, 242, 267). Nectin-1 is a member of the immunoglobulin superfamily and is expressed on epithelial cells, fibroblasts and neurons and plays important roles in cell adhesion (322). Finally, gD can also interact with specific sites in heparan sulphate which are generated by 3-*O*-sulfotransferases (347).

Binding of gD to one of the above receptors results in conformational changes which trigger recruitment of gB as well as the gH/gL heterodimer and results in membrane fusion (111, 333). gD, gB, gH/gL and the gD receptor are sufficient for entry. It is currently unknown whether the fusion peptide required for membrane fusion resides in the gH or gB glycoproteins (147). The role of gL in this process is also unclear. This glycoprotein does not encode a transmembrane domain and may be required for the correct folding and trafficking of gH to the cell surface (159).

Viral entry can also occur through endocytosis. The viral particles escape the endocytic vesicle through fusion mediated by gD, gB and gH/gL (280). Entry of the virus through endocytic vesicles in the absence of gD results in vesicles fusion with the lysosomal compartment, viral particle degradation and apoptosis (417).

1.2.3 Delivery of the HSV genome to the nucleus

The process of attachment, binding and penetration is fast, taking only several minutes (157). From the site of de-envelopment, the viral capsid is delivered to the nuclear pore, where the DNA is injected into the nucleus. Tegument proteins

are also released. Empty capsids are detected in the cytoplasm shortly after infection, suggesting that release of the viral DNA also occurs rapidly (15).

Movement of capsids from the membrane to the nuclear pore relies on the microtubule minus-end directed motors dynein and dynactin (77, 355). The component(s) of the capsid which interact with the microtubule motors is unknown; VP26 interacts with components of the dynein light chain, but is not necessary for transport (76). At the nuclear pore, importin-β, along with the Ran GTPase cycle is necessary for docking of the capsid and injection of viral DNA *in vitro* (285). The large tegument protein VP1-2 has been implicated in release of DNA from the capsid. VP1-2 may require cleavage by a serine or cysteine protease to release the viral DNA (174).

1.2.4 Initiation of the HSV lytic replication cycle

The tegument proteins released into the cytoplasm by fusion play important roles in making the cellular environment conducive to viral replication. Tegument proteins include VP16, which is required for efficient IE gene expression (Reviewed in (405)) and vhs, which nonspecifically targets cytoplasmic mRNA, resulting in a general cessation of host gene expression (Reviewed in (354)). Other viral proteins are required to alter nuclear morphology early in infection to initiate lytic replication.

1.2.4.1 Alterations in nuclear architecture mediated by HSV

Viral transcription, DNA replication and encapsidation occur in the nucleus. HSV subverts several nuclear processes in order to carry out these processes. Remodeling of the nuclear architecture aids in replication compartment formation, egress and blocks cellular antiviral responses.

1.2.4.1.1 Alteration of ND10 structures

One of the first detectable changes in the infected cell nucleus following injection of the viral DNA is the accumulation of ND10 domain components adjacent to the incoming viral genomes (96). ND10 domains (also known as Promyelocytic Leukemia (PML) nuclear bodies) are dynamic structures which require the PML protein for their formation and harbour many other cellular proteins, either permanently or transiently (Reviewed in (371)). These nuclear structures have been implicated in a wide range of cellular processes, including oncogenesis (324), the DNA damage response (72), apoptosis (18), senescence (25), the ubiquitin pathway (278), the IFN response and viral infection (91).

Transcription of the ND10 constituent proteins PML and Sp100 is directly induced by IFN through the IFN stimulated response elements (ISREs) found within their promoters (128, 359). IFN induction results in both an increase in size and number of ND10 domains (129), directly implicating these structures in the antiviral response.

Studies using both fixed and live cells indicate that ND10 components are non-randomly distributed at the nuclear rim following directional infection with

HSV (96, 104). This is due to *de novo* accumulation of ND10 components at the incoming viral genome rather than migration of pre-formed ND10 domains (96). The rapid recruitment of ND10 components to incoming viral genomes could be the result of chromatinization or a DNA damage response elicited by the viral genome (88). DNA repair proteins, such as ATM, RPA, γH2A.X, are found, at least transiently, in ND10 domains (14, 41). In addition, ND10 domains also harbour proteins involved in chromatin modifications, including the ATP-dependent chromatin remodeling Swi/Snf family member ATRX (164), hDaxx, which interact with histone deacetylases (HDACs) (164), the heterochromatin-associated protein HP1 (341) and the histone chaperone HIRA (414). Interestingly, knockdown of PML results in dispersal of other ND10 component proteins such as Sp100 and hDaxx, however these components still migrate to and nucleate at incoming viral genomes in the absence of PML (103).

HSV encodes a regulatory protein which interacts with ND10 domain components. The immediate early (IE) protein ICP0 precisely colocalizes with PML and Sp100 at very early times after infection and mediates their proteasome-dependent degradation (94, 95, 244, 245). How ICP0 interacts with ND10 domain components and the functional significance will be outlined below.

1.2.4.1.2 Viral replication compartment formation

The viral genomes that initially associate with ND10 domains have a high probability of forming viral replication compartments (104). These replication compartments contain viral regulatory proteins, such as ICP4, as well as the

components of the DNA replication machinery (110). DNA replication, late (L) gene expression and encapsidation all take place in replication compartments, which eventually expands to fill almost the entire nucleus (110). Along with the expansion of the replication compartments, cellular chromatin is marginalized and condensed around the nuclear periphery, while the nuclear volume increases up to two times (266).

The marginalization of cellular chromatin to the nuclear periphery provides a significant impediment to nuclear egress. In addition, the chromatin is further surrounded by the nuclear lamina, a mesh-like network of lamin A/C and lamin B which interacts with the inner nuclear membrane as well as chromatin to provide stability and shape to the nucleus. During infection with HSV, the nuclear lamina and chromatin domains are disrupted so that viral replication compartments can then reach the nuclear membrane (348, 349). This disruption involves the redistribution of lamin A/C and lamin-associated protein B and requires the viral proteins UL31 and UL34 (348, 349).

1.2.5 Viral gene expression and DNA replication

HSV gene expression follows a temporally regulated program. Firstly, IE genes are stimulated by the tegument-associated protein VP16. IE genes encode regulatory proteins which play roles in stimulating viral gene expression and counteracting antiviral responses. Next, IE proteins stimulate early (E) gene

expression, some of which are involved in DNA replication. Finally, late (L) gene expression occurs, and proteins involved in capsid assembly and egress are made.

1.2.5.1 Function of VP16

VP16, encoded by the U_L48 gene, is a 490 amino acid, 65 kilodalton (kDa) phosphoprotein and an essential structural component of the tegument (110). There are 500 – 1000 molecules of VP16 in the tegument that are released into the cytoplasm upon fusion of the host and viral membranes (405). Once in the cytoplasm, VP16 interacts with the cellular protein HCF and is transported to the nucleus (408).

Viral IE promoters contain a specific regulatory sequence, TAATGARAT (where R represents purine), in one or several copies upstream of the cap site, which is recognized by the cellular POU-domain containing transcription factor Oct-1 (116, 194). The VP16-HCF complex recognizes both Oct-1 and sequences in the IE promoter to form the specific activator complex (121, 176, 192, 194, 225, 281, 363). VP16 contains a C-terminal acidic activation domain that is critical for stimulating viral gene expression in the absence of cellular or viral protein synthesis (380).

In heterologous systems, the VP16 activation domain interacts with components of the Mediator complex (160, 265), general transcription factors including TFIIA (186), TFIID (161, 183), TFIIB (125, 222), TFIIH (407), TATA-binding protein (TBP) and TBP-associated factors (33), histone acetyltransferases

(HAT) (190, 199, 259, 384) and ATP-dependent chromatin remodeling complexes (137, 259, 274). These interactions result in the recruitment of RNA polymerase II, stimulation of open complex formation and subsequent gene expression.

In contrast, much less is known about the complexes recruited to IE promoters by the VP16 activation domain during lytic HSV infection. Recent work using chromatin immunoprecipitation (ChIP) has demonstrated that TBP and RNA polymerase II recruitment depends on the presence of the VP16 activation domain, while recruitment of Oct-1 and VP16 itself does not (150). The HATs CBP and p300 also depend on the VP16 activation domain for their recruitment, while the ATP-dependent chromatin remodeling complexes Brg-1 and Brm have reduced, but not abrogated recruitment in the absence of the VP16 activation domain (150). Thus, VP16 is thought to stimulate viral gene expression in a manner similar to that observed in heterologous systems.

The activation domain of VP16 also plays an important role in maintaining low levels of histone proteins on the viral genome during lytic infection (150, 202), which will be discussed in detail below.

1.2.5.2 Phenotype of VP16 mutants

VP16 is an essential component of the tegument (2, 395) and as such, viruses with complete deletions of the U_L48 open reading frame cannot be propagated in culture. VP16 deletion mutants can be grown on complementing cell lines, which provide VP16 *in trans* and form a normal tegument within the viral particles

(395). These mutants display normal IE gene expression, but show a severe defect in viral particle assembly on non-complementing cell lines (395).

VP16 also interacts with the tegument protein vhs both *in vitro* and within infected cells (184, 351). Infection with VP16 mutants incapable of interacting with vhs results in an almost complete inhibition of host and viral mRNA synthesis (209), suggesting the interaction with VP16 may be important in tempering the nuclease function of vhs.

Several different approaches have been taken to mutagenize the VP16 protein. These include truncation (209, 352, 370), linker insertion (3) and point mutations (64, 369) which affect the binding of VP16 to DNA and to its associated protein complexes.

The viral mutant in1814 has a 12 base pair insertion within the U_L48 open reading frame which results in a protein that cannot interact with Oct-1 and HCF (3). Accumulation of IE RNAs is decreased up to 5-fold with this mutant, which displays a high particle-to-plaque forming unit (PFU) ratio (3).

Several mutants have also been constructed with truncations of the activation domain (V422, RP5 (352, 370)). Similar to in1814, IE RNA accumulation is greatly decreased with these mutants, and reduced titres on Vero cells are observed (352). The defect in gene expression with the V422 mutant is more severe than the in1814 mutant (270). Recruitment of TBP, RNA polymerase II, CBP and p300 is diminished in the absence of the VP16 activation domain, correlating with decreased gene expression (150). Thus, it is thought that an

absence of VP16 activation function decreases the probability that a viral particle will initiate the lytic cycle of infection.

The defect in gene expression with VP16 mutants can be complemented by two distinct mechanisms. Firstly, the cytodifferentiating agent hexamethylene *bis*-acetimide (HMBA) aids in reactivation of latent genomes in explant culture (20, 213, 397) and stimulates gene expression from VP16 mutant genomes (248, 304). HMBA directly stimulates IE gene expression to levels approaching that of wild type virus (248) but is only effective when added to culture media within a limited time frame before or immediately after infection (248). The mechanism by which HMBA stimulates HSV IE gene expression has not been elucidated. Recent evidence suggests that the compound activates the phosphoinositol 3-kinase (PI3K)/Akt pathway, which in turn phosphorylates HEXIM1 and releases the transcriptional elongation factor P-TEFb from an inhibitory complex resulting in increased transcription (62).

Infection of the human osteosarcoma cell line U2OS also results in IE gene expression from a VP16 mutant genome (352). Plaque assays reveal that VP16 mutants can replicate to nearly wild type levels in U2OS cells (270, 352), which also complement the defects of ICP0 mutants (409).

1.2.5.3 IE gene expression

Five IE genes have their expression stimulated by VP16. Four of these genes (ICP0, ICP4, ICP22 and ICP27) encode proteins which have important regulatory roles in viral gene expression at the transcriptional and post-transcriptional levels

(110). The fifth, ICP47 is dispensable for replication in cell culture (231), but plays an important role in immune evasion in the host.

1.2.5.3.1 ICP47

ICP47 is a 12 kDa protein encoded by the α47 gene located in the unique short segment of the viral genome (110). ICP47 is a cytoplasmic protein which physically interacts with TAP (transporter associated with antigen processing) (115, 151). This protein is necessary and sufficient for the inhibition of TAP-mediated translocation of antigen-derived peptides across the endoplasmic reticulum (ER) membrane (9, 378). This prevents the assembly of peptides with Class I major histocompatability complex (MHC) and subsequent expression on the cell surface (9, 378), rendering cells resistant to lysis by HSV-specific CD8+ CTL (152, 411).

1.2.5.3.2 ICP22

ICP22 is a 68 kDa protein encoded by the α22 gene in the unique short region of the viral genome (110). ICP22 is serine, threonine and tyrosine phosphorylated (308, 310, 311), nucleotydylated by casein kinase II (CKII) (22, 264) and is detected in at least seven different isoforms on denaturing polyacrylamide gels. It has two nuclear localization signals (362) and is detected in discrete nuclear bodies along with ICP4 and RNA polymerase II after initiation of viral DNA replication and phosphorylation by the viral protein UL13 (166, 216). A UL13 mutant displays the same phenotype as an ICP22 mutant, highlighting the role of

virus-mediated phosphorylation in ICP22 function (308, 310). Additionally, ICP22 may be phosphorylated by the viral kinase US3 (308).

The role of ICP22 in viral infection remains enigmatic. ICP22 mutants can replicate efficiently in Vero and HEp-2 cells (300), but are restricted for growth in some rodent cell lines and human fibroblasts (339). This growth restriction is not MOI-dependent and is the result of decreased late gene expression (339). Additionally, ICP22 has been implicated in regulating the expression of ICP0 but may also play a role in repressing IE gene expression (310, 339).

ICP22 and UL13 are required for the activation and post-translational modification of cdc2, as well as the degradation of cyclins A and B (4, 5, 7). The viral DNA processivity factor UL42 replaces cyclin B as the binding partner for cdc2 (5) and recruits a modified form of topoisomerase IIα (6). Topoisomerase IIα may be required for untangling the newly synthesized viral DNA, allowing for efficient late gene expression (6).

ICP22 also mediates the modification of the carboxy-terminal domain (CTD) of RNA polymerase II (316). While the CTD is normally phosphorylated on both Serine 2 and Serine 5 during active transcription, a modified form of the CTD, phosphorylated only on Serine 5 is detected during HSV infection (113). There is a specific loss of the Serine 2-phosphorylated form of RNA polymerase II mediated by the proteasome and dependent on ICP22 (67, 112, 113). Additionally, both ICP22 and UL13 are required to induce the Serine 5 modified form of RNA polymerase II (113, 230).

1.2.5.3 ICP27

ICP27 is a multifunctional 63 kDa protein encoded by the U_L54 gene (110). Several different protein motifs are found in ICP27 including an RGG-box RNA binding domain (256, 330), a nuclear localization signal (254), and C-terminal protein-protein interaction motifs (389, 416). At early times post-infection, ICP27 is found diffusely throughout the nucleus, whereas at later times it is found within replication compartments and in the cytoplasm (255, 292, 330, 356).

ICP27 plays roles in both activation and repression of viral gene expression (247, 320). Deletion mutants of ICP27 overexpress some E genes, make greatly decreased amounts of viral DNA, make only low levels of leaky-late proteins and do not express true late proteins (247, 252, 315). Some E genes, including the DNA replication proteins, require ICP27 for efficient expression (249, 326, 383), suggesting the decrease in viral DNA replication is due to insufficient expression of these proteins (383).

ICP27 interacts with the CTD of RNA polymerase and relocalizes it to viral transcription centres (67). It also interacts with the chaperone Hsc70, which plays a role in the degradation of Serine 2 phosphorylated RNA polymerase CTD (218).

ICP27 may stimulate expression of some L genes with weak polyadenylation signals by altering the specificity of the polyadenylation machinery in order to stimulate 3’-end mRNA processing (249-251, 332). In addition, ICP27 plays a critical role in inhibiting host cell mRNA splicing at early times post-infection, which aids in the shut off of host protein synthesis (140, 141). ICP27 acts by preventing spliceosome assembly (223). It interacts with spliceosome

components such as snRNPs (243), SR proteins (331), p32 (34), the essential spliceosome assembly factor SAP145 (35) and the kinase SRPK1 (338). Its interaction with SRPK1 diverts this protein to the nucleus, resulting in hypophosphorylation of SR proteins and stalling of spliceosome assembly (338).

ICP27 also plays an essential role in exporting intronless HSV transcripts to the cytoplasm (330), which normally occurs very inefficiently in mammalian cells (236). ICP27 interacts with two different RNA export receptors: Aly/REF (49, 188) and TAP/NXF (48, 170, 171). Recent evidence suggests that only the interaction with TAP/NXF is essential in viral mRNA export (170).

As well as preventing splicing of cellular mRNAs, ICP27 stabilizes some cellular RNAs containing AU-rich instability elements in a process involving the p38 stress-activated protein kinase pathway (63). Additionally, ICP27 interacts with translation initiation factors and has been implicated in regulating translation of a subset of viral mRNAs in vivo (82). Finally, ICP27 has been implicated in altering several cellular signaling pathways. ICP27 activates the p38 and JNK stress activated protein kinase pathways (142). In addition, it mediates NFκB activation through the loss of IκBα (182). It can also regulate the IFN response by downregulating Stat1 phosphorylation and preventing Stat1 translocation to the nucleus (169).

1.2.5.3.4 ICP4

ICP4 is a 175 kDa protein encoded by two copies of the α4 gene located in the inverted repeat region flanking the unique short segment of the viral genome

(110). Early in infection, ICP4 is found diffuse within the nucleus (185). At later times it is recruited into globular viral replication compartments (69) dependent on viral DNA replication (185). Experiments following ICP4 dynamics in live infected cells indicate that the protein also localizes to small punctuate structures adjacent to ND10 domains shortly after infection (104).

ICP4 acts both as a transcriptional activator and repressor. In the absence of ICP4, the only gene products made during infection are ICP0, ICP6, ICP22 and ICP27 (73), implicating this protein in the induction of E and L genes. ICP4 binds DNA as a dimer and recognizes both consensus and non-consensus sequences (191, 261, 262). For ICP4 to activate transcription it must bind 3’ to a transcriptional start site which contains a TATA box and initiator element (42, 132). ICP4 facilitates the binding of TBP (as a component of TFIID) to the TATA box (127) mediated through TAF250 and the C-terminal region of ICP4 (43). At some viral promoters, ICP4 also facilitates binding of TFIIA (412). High mobility group protein 1 (HMG1) associates with this complex and may bend the DNA (42). The binding of these proteins facilitates formation of the transcription pre-initiation complex.

ICP4 also represses transcription from its own promoter, as well as those of the ICP0, ORF P and ORF O genes (198, 272). ICP4 binds across the transcriptional start sites of these genes and blocks the formation of pre-initiation complexes (132, 272).

1.2.5.3.5 ICP0

ICP0 is an 100 kDa protein encoded by the α0 gene. This gene is located within the inverted repeat region flanking the unique long segment of the viral genome (110).

1.2.5.3.5.1 Function of ICP0

ICP0 localizes to the nucleus at early times after infection. Initially, ICP0 can be detected colocalizing precisely with ND10 domain components such as PML and Sp100 (95, 244, 245). At later times ICP0 accumulates and fills the nucleus. When DNA replication is initiated ICP0 then shuttles to the cytoplasm (177, 233, 387), which may be due to an interaction between ICP0 and cyclin D3 (177, 387). Treatment of cells with the proteasome inhibitor MG132 results in ICP0 remaining in the nucleus (233, 386).

ICP0 is one of the few HSV transcripts that are spliced. The N-terminus contains a C_3HC_4 RING (Really Interesting New Gene) zinc finger motif that functions as an E3 ubiquitin ligase (83, 89, 114). It also contains a nuclear localization signal (84), an USP7 interaction domain (84, 85) and a multimerization domain (52, 99). ICP0 is nucleotydylated by CKII (22) and contains at least seven different phosphorylation signals, some of which are targets of UL13 (282) and the cellular protein cdc2 (7). Mutations in these phosphorylation sites do not affect the E3 ligase activity of the enzyme *in vitro*, but the protein no longer colocalizes with conjugated ubiquitin or disrupts ND10 *in vivo* (27, 68).

One of the first activities ascribed to ICP0 was its ability to act as a strong transactivator of viral and cellular genes in both transfection and infection assays (Reviewed in (87)). ICP0 does not bind DNA, but has been shown to interact, and work synergistically, with ICP4 to stimulate gene expression (84, 410). ICP0 expression results in enhanced accumulation of viral mRNAs, and acts at or before initiation of transcription (or both) (173). The RING domain of ICP0 is critical for its ability to stimulate viral gene expression (83).

ICP0 colocalizes with conjugated ubiquitin and interacts with the proteasome (86, 287, 386) as well as the E2 conjugating enzyme cdc34 (386) in infected cells. Mutations in the RING domain abrogate the association with ubiquitin and proteasomes, as well as the transactivating activity of ICP0 (30, 85, 94). ICP0 also interacts with the ubiquitin specific protease USP7 or HAUSP (herpesvirus-associated ubiquitin specific protease) which protects it from autoubiquitination and increases its stability (26, 40).

ICP0 associates with centromeres in both mitotic and interphase cells which is dependent on an intact RING domain (92). Cycling cells are blocked at an unusual stage of mitosis called pseudo-prometaphase in the presence of ICP0 (92, 226) resulting from the proteasome-dependent degradation of the centromeric protein CENP-C (92, 226). Aditionally, both CENP-B (227) and the histone variant CENP-A (228) are destabilized by ICP0. This degradation causes significant ultrastructual changes to the kinetochore, although the relevance of this in regards to HSV infection is unclear (228).

Perhaps the most well studied function of ICP0 is its ability to interact with and cause the destruction of ND10 domains. Viral genomes are associated with ND10 at early times post-infection while ICP0 precisely colocalizes with several ND10 component proteins (95, 244, 245). This results in the proteasome-dependent loss of several isoforms of PML which are conjugated with the small ubiquitin-like modifier SUMO (47, 94). Mutant forms of PML which cannot be modified with SUMO are not degraded by ICP0 (29). ICP0 does not directly ubiquitinate PML *in vitro*, indicating other viral and/or cellular factors are required for this effect (29). In the presence of exogenously overexpressed PML, ND10 domains remain intact but are highly reorganized in the presence of ICP0 (106). In addition, high MOI infection with ICP0 mutants causes the reorganization of ND10 (103), suggesting other viral proteins may play a role in this phenomenon.

The functional significance of the dispersal of ND10 domains is still unknown. Two possibilities, not mutually exclusive, can be envisioned. First, ND10 domains may contain factors which are inhibitory to viral gene expression and hence must be dispersed or degraded. The second possibility is that ND10 domains sequester factors needed to enhance viral gene expression which are released upon dispersal. Everett *et al* used shRNAs targeting PML in human fibroblasts and showed that knock-down of this protein increased gene expression and plaque formation of an ICP0 mutant virus (103). This complementation was not to wild type levels however, indicating that targeting PML for destruction is not the only essential function of ICP0. In the absence of PML, both Sp100 and

hDaxx continue to accumulate at viral genomes, suggesting that other components of ND10 domains also need to be degraded by ICP0 (103). However, knockdown of both PML and Sp100 still does not fully complement an ICP0 mutant (101).

ICP0 mutants are hypersensitive to the effects of IFN; a decrease in mRNA accumulation and plaque formation are seen in the presence of this cytokine (269). ICP0 counteracts the IFN response by preventing translocation of the essential transcription factor IRF3 into the nucleus and increasing the turnover of this protein (221). However, ICP0 mutants are still restricted for growth in human fibroblasts where either IRF3 or Stat1 protein levels have been reduced using shRNAs (105), indicating other essential roles for this protein. At least some of the antiviral effects of IFN are mediated through ND10. IFN greatly inhibits the growth of ICP0 mutants in PML $^{+/+}$ but not PML$^{-/-}$ murine embryonic fibroblasts (MEFs) (46).

In addition to these well-studied facets of ICP0 biology, several other effects have been noted. ICP0 interacts with and may stabilize the transcription-regulatory factor BMAL1 (178). It also interacts with and ubiquitinates the tumour suppressor p53 (28), although this protein is stabilized during HSV infection through an ICP0-independent mechanism (153). In addition, ICP0 interacts with elongation factor EF-1δ, which may alter translation efficiency of viral mRNAs (177). Finally, ICP0 causes the proteasome-dependent degradation of the catalytic subunit of the protein kinase DNA PK (212). The functional relevance of this finding is currently unknown. The interaction of ICP0 with

histone deacetylases (HDACs) and its ability to reactivate quiescent genomes are the subject of a later section.

1.2.5.3.5.2 Phenotype of ICP0 mutants

ICP0 mutant virus replication is greatly attenuated compared to wild type virus in a cell type- and MOI-dependent manner (366). Infection of baby hamster kidney (BHK) cells with an ICP0 null virus results in a reduced yield compared to wild type virus (366). In this cell type, a similar number of particles were produced upon high MOI infection, indicating the ICP0 null virus has a higher particle-to-PFU ratio (366). This difference in viral yields was further increased in Vero and human foetal lung cells (366).

Although ICP0 mutant viruses are highly restricted for growth on some cell types, pioneering work by Yao and Schaffer determined that the U2OS osteosarcoma cell line is permissive to these mutants (409). Single step growth curves indicate that ICP0 null viruses produce 100-fold more virus on U2OS cells than Vero cells and plaquing efficiency of ICP0 null viruses is greatly enhanced (to levels comparable with wild type virus) (409).

Studies have been designed to alter cell physiology in an attempt to induce gene expression from ICP0 mutant genomes. It was originally noted in Vero cells that the plaquing efficiency of an ICP0 mutant was greatest at eight hours post-release from growth arrest mediated by isoleucine deprivation (36). Glutamine deprivation, and to a lesser extent arginine and methionine deprivation can also enhance plaquing efficiency of an ICP0 mutant (31). These effects have been

attributed to causing cellular stress, as heat shock and UV-C irradiation can also enhance the plaquing efficiency of an ICP0 null mutant (32). What common cellular functions are altered by these various cellular stressors is unknown.

A detailed analysis of the fate of ICP0 null mutants after low MOI infection in human fibroblasts was undertaken by Everett *et al* (90). The possible outcomes included quiescence, stalled infection, cell death or plaque formation. In many cells, IE proteins were expressed to low levels. In fewer non-productively infected cells, E proteins were also expressed. Many more cells express IE proteins than go on to produce plaques, highlighting the stalled nature of the infection (90). These results are in accord with those of Cai and Schaffer, who demonstrated that Vero cells infected with an ICP0 null virus expressed IE proteins (at levels lower than wild type virus) but expressed little to no E or L proteins (37). This suggests that IE gene expression is activated mostly by VP16, whereas E and L gene expression may require ICP0. Addition of an VP16 mutation into an ICP0 mutant genome completely abrogates viral gene expression (270). ICP0 provided *in trans* can partially complement a VP16 mutant (3), suggesting that ICP0 can functionally compensate for VP16 activation function.

Everett describes the initial events after infection as a competition between cellular repression mechanisms and viral transactivators (87). ICP0 gives an advantage to the virus through degradation of components of this repression mechanism. The defect of an ICP0 null virus can be overcome by increasing MOI, which may be explained in several ways. Firstly, it is possible that the cellular repression mechanism is titrated out with increasing genome load.

Alternatively, if low MOI infection results in several possible outcomes (90), repression may be random and in some cases incomplete. If several genomes infect the same cell and produce a different complement of viral proteins, a productive infection could ensue.

1.2.5.4 E gene expression and DNA replication

Early genes contain no specific sequence or common promoter element that identifies them as being expressed with early kinetics. Temporally, they are expressed after IE proteins and are made in two general phases. Some E genes are expressed early after IE protein synthesis, such as ICP8, while others are expressed at later times (ie, Thymidine Kinase (TK)) (155). Expression of E genes requires ICP4 and its interactions with cellular transcription factors as well as the IE proteins ICP0 and ICP27. Some proteins made with early kinetics are involved in DNA replication and nucleotide metabolism.

The HSV genome contains three potential origins of DNA replication. OriL is a 144bp palindrome sequence found in the unique long region. OriS is a 45bp palindrome sequence is found in the repeat regions flanking the unique short region (372).

Seven E proteins are required for viral DNA replication (45). Along with several cellular components, these proteins form the complexes which bend, unwind and replicate the viral DNA. Firstly, the UL9 protein binds to the origin of replication and bends the DNA, causing the formation of a single-stranded stem-loop structure (81). ICP8, the single-stranded DNA binding protein (60),

binds to this region and stimulates the helicase activity of UL9, which unwinds a region of the DNA (23). The helicase/primase complex consisting of UL5/UL8/UL52 is recruited by ICP8 and makes the primers for leading strand synthesis (65), which is carried out by the DNA polymerase comprised of UL30 and UL42 (309). This complex also contains 3'-5' exonuclease and proofreading ability (309).

Replication compartments are formed through the recruitment of both viral and cellular proteins to the replicating viral DNA, which eventually increases in size to fill the entire nucleus (193, 296, 313). Cellular proteins, such as p53, Rb, DNA polymerase δ, DNA ligase, topoisomerase IIα, DNA polymerase α-primase, and the DNA damage response proteins Mre11 and ATM are all found in replication compartments (219, 400).

The mechanism used to replicate the viral DNA is complex and not fully understood. The linear genome circularizes by an unknown mechanism upon injection into the nucleus and is thought to initially undergo theta form replication. At some point, replication switches to a rolling circle mechanism, analogous to bacteriophage lambda DNA replication (110). The detection of head-to-tail concatamers late in infection supports the rolling circle mechanism (365). However, analysis of newly-replicated HSV genomes by partial digestion, pulse field gel elecrophoresis and two dimensional gel electrophoresis suggest that these concatemeric DNA molecules are highly branched (343, 344, 415). These highly branched molecules cannot be formed by a rolling circle mechanism only and may require a combination of DNA replication and recombination.

Analysis of the concatameric DNA molecules reveals varying orientations of the unique long and short regions, suggesting replication and recombination may be functionally linked (343, 415). Several cellular recombination proteins, such as RPA, RAD51, p95 and Ku86 are found in replication compartments (401, 402). Additionally, HSV encodes a 5'-3' exonuclease, UL12, which may act in conjunction with ICP8 to form a viral recombinase resembling the lambda Red recombination system (374).

Other E gene products are not essential for growth in cell culture, but may be important in non-dividing cells. These include TK, ribonucleotide reductase, uracil N-glycosylase and the viral dUTPase (110).

1.2.5.5 L gene expression

The products of L gene expression are structural proteins and those involved in assembly of virions and alterations of cellular membranes. L gene expression occurs in replication compartments (292) and requires ICP4, ICP27 and ICP22 (for some). ICP8 is also required for L gene expression (117), either by making L promoters more accessible through viral genome rearrangement or by promoting transcription from progeny genomes after viral DNA replication (253).

Two classes of L genes exist, those that are expressed prior to DNA replication (such as U_L27 and U_S6) as those that are expressed after DNA replication (such as U_L44 and U_S11). Although some late genes do not require DNA replication to be expressed (termed 'leaky-late'), they are greatly enhanced

upon DNA synthesis. The sequence components which result in a gene being expressed with leaky-late or true late kinetics are unknown (110).

1.2.6 Viral capsid assembly and egress

Capsid assembly occurs within replication compartments and does not involve any cellular proteins (69, 276). Capsids are assembled around a scaffolding structure made of three viral proteins: VP21, preVP22a and VP24 (154). These proteins are formed from the complex cleavage of the proteins from the U_L26 and U_L26.5 genes (224). The main component of the capsid, VP5, interacts with preVP22a and self-assembles (276). Hexons and pentons are included, and the portal proteins are added to the structure (277). Newly synthesized viral DNA is packaged into the capsid during this process, and VP22a and VP21 proteins are removed (110).

The mechanism of viral DNA cleavage and packaging is still under investigation. Cleavage of the concatameric DNA requires two specific sequences, termed pac1 and pac2 (70, 388). Two site-specific DNA breaks occur downstream of these sequences (70, 353, 388). Encapsidation requires 7 viral proteins: UL6, UL15, UL17, UL25, UL28, UL32 and UL33. UL15, UL28 and UL33 act as the terminase which cleaves viral DNA into monomers (110).

Once the capsid is properly assembled and filled with viral DNA it exits the nucleus and eventually the cell. The viral capsid is too large to exit via the nuclear pore, and so is thought to bud through the nuclear membrane. The viral

proteins UL31 and UL34, along with the US3 protein kinase play important roles in altering the nuclear lamina to allow access to the nuclear envelope (348, 349). Budding into the perinuclear space occurs through a mechanism not fully defined (260). Evidence suggests that US3, as a component of the perinuclear virion, phosphorylates gB to mediate fusion with the outer nuclear membrane (403). Unenveloped capsids in the cytoplasm then acquire a tegument layer (260) and bind to post-ER cytoplasmic membranes enriched in viral glycoproteins where the final envelope is acquired and the virus is transported to the cell surface (107, 168, 350, 398).

1.3 Herpes Simplex virus latency

1.3.1 HSV primary and latent infections

In humans, primary infection occurs through direct contact with the mucosal epithelium. Lytic replication occurs for several days until the host adaptive immune response limits and eventually clears the infection. During lytic infection, virus spreads to and infects sensory neurons. The viral nucleocapsid travels to the cell body of the neuron in a retrograde fashion. An initial infection occurs in the neurons and is eventually contained by infiltrating CD8+ T cells. At least some viral genomes persist as extrachromosomal, transcriptionally silent, endless or circular DNA molecules (75, 258, 317, 318). In response to a variety of stimuli, the virus can reactivate, replicate the genome and package viral DNA

into capsids. These capsids are thought to travel via an anterograde mechanism to the nerve endings, where new virus is released to infect the surrounding epithelial cells.

1.3.2 Models of latency

Several different model systems have been developed to study latency, including those involving rabbits and mice. The rabbit and mouse eye model involves corneal scarification, lytic replication and establishment of latency in dorsal root ganglia. The rabbit eye model results in spontaneous reactivation which can be measured in tear secretions (17). The mouse model of latency allows for study of latent infection in knockout mice where the immune response is compromised (232). In addition, the rate of spontaneous reactivation is extremely low in mice, yet can be induced through treatments such as UV-light exposure, hyperthermia and some drug treatments (336). In many cases, reactivation is studied by explanting infected trigeminal ganglia tissues and using *in situ* hybridization (ISH) or RT-PCR to detect viral transcripts or DNA.

Although there are several systems to study HSV latency, no encompassing model has been derived by comparing data from different animal models.

1.3.3 Latency-associated transcripts

Only transcripts from the LAT (latency-associated transcript) regions, which are located in the repeats flanking the unique long segment of the viral genome, can be detected during latency (110). LATs are a series of co-linear, nuclear poly A^- transcripts of 2.0 and 1.5kbp (major LATs). These are derived from a much less abundant 8.3kbp transcript, termed the minor LAT, which is transcribed partially antisense to the ICP0 gene (172, 392).

The 2.0kbp LAT is an unusually stable intron that persists in a lariat structure (108, 373). In neuronal cells, the 2.0kbp LAT is able to produce a second RNA of 1.5kbp (239, 413), both of which are found diffusely in the nucleus (12). During lytic infection, the 2.0kbp LAT can be detected at late times, while the 1.5kbp LAT cannot (124).

As assessed by *in situ* PCR, the number of neurons harbouring latent genomes far exceeds those expressing LATs (126, 257, 312). In fact, LATs have not been firmly implicated in latency establishment, maintenance or reactivation.

One potential role of LATs is to protect the infected cells from death. LAT^- viruses induce apoptosis in rabbit neurons (290), however only some groups have been able to reproduce this in mice (8, 376, 377). In addition, transfection of a plasmid expressing LAT protects cells from caspase-3 induced apoptosis (290, 377).

An alternative method of protecting cells from death could be through preventing viral gene expression. LAT^- viruses show higher levels of viral gene

expression both during the initial lytic infection stage in neurons as well as during latency (51, 118, 335). LAT$^-$ viruses cause more severe pathology in infected mice (376, 377). In tissue culture, LATs also reduce viral gene expression and replication (238).

Transient expression of LATs inhibits the function of ICP0 (108). Recent studies have implicated micro RNAs (miRNAs) in this phenomenon. miRNAs have been detected in latently infected neurons, at biologically relevant levels, with specificity for both ICP0 and ICP4 (382). The miRNA targeting ICP0 is derived from the second exon of the spliced ~6.3kbp minor LAT, while the miRNA targeting ICP4 is derived from an unknown portion of the viral genome (382). These miRNAs can reduce ICP0 and ICP4 protein expression in transfected cells, but have no effect on viral mRNA levels (382).

1.3.4 Establishment and maintenance of latency

After lytic replication at the primary site of infection, nucleocapsids (potentially with some tegument) travel to the cell body of the neuron. For several days thereafter, viral gene products of all classes are detected in the infected neurons, and lytic infection within the neuron is controlled by the adaptive immune system (110). Two populations of neurons exist in the infected trigeminal ganglia at this time: those that express lytic gene products and those that express LATs (208, 241, 335, 357, 358).

Latency is generally thought to be the default after a failure to launch the lytic cycle. In neurons, this may be due to a lack of VP16 function. POU domain containing proteins may compete with Oct-1 for binding to IE promoters in neurons (210, 220, 302). Additionally, HCF may be sequestered in the cytoplasm in neurons and hence unable to transport VP16 to the nucleus (195, 207). Inducible expression of VP16 has no effect on the establishment of latency, although this remains controversial (340) (see below).

No one viral gene product is essential in the establishment or maintenance of latency. Many times the low reactivation phenotype of a viral mutant is due to inefficient lytic replication at the primary site of infection (110). Infected neurons can harbour anywhere from one to thousands of copies of viral DNA (334) which could be due to multiple viruses infecting each neuron from the primary site of infection, or through replication of viral DNA before the establishment of latency (79).

1.3.5 Reactivation from latency

Reactivation of latent HSV in humans can occur from injury to the infected neurons as well as physical or emotional stress, exposure to UV light, hyperthermia, or hormonal imbalances (110). At the molecular level, the details surrounding reactivation are unclear. It is thought that only a small proportion of neurons containing latent genomes are stimulated to reactivate, which is thought to be due to changes in neuronal gene expression resulting in induction of cellular

factors that act on the viral genome (110). Gene expression most likely follows the IE, E and L temporal pattern; provision of ICP0, ICP4 or VP16 can reactivate latent genomes (138).

A recent report implicates VP16 in both the acute phase of infection in neurons and reactivation (375). The authors argue that in neurons, VP16 is expressed with different kinetics than in most conventional cell types. They present a model whereby *de novo* synthesis of VP16 is required to induce the lytic replication cycle within the neuron in order to increase the latent reservoir. After establishment of latency, cellular stresses result in changes to the neuronal transcription patterns which uniquely affect the VP16 promoter, allowing the VP16 protein to be expressed in rare reactivating neurons (375). Once enough VP16 is produced, IE gene expression can be stimulated and the temporal cascade of gene expression can initiate.

Gene expression and DNA replication result in the production of nucleocapsids, tegument and envelope proteins. These may travel together or separately along the axon shaft to the nerve endings (211, 284, 323, 379). Whether viral replication results in destruction of the infected neuron, or it survives to be reactivated multiple times is still unknown (110).

1.4 HSV quiescence

Attempts have been made to mimic the latent state in more amenable tissue culture cell lines. Primary human fibroblasts can be infected with wild type virus

at low MOI and higher than physiological temperatures resulting in a lack of gene expression. Compounds such as IFN, cytosine arabinoside and acyclovir are used to aid in establishing '*in vitro* latency'. Reactivation is induced through removal of the chemical compound and decreasing temperature (for example: (319, 337, 346, 399)). These manipulations resulted in this system being labeled too artificial to mimic the molecular events of latency (110).

Quiescence is the term used to define an infection whereby the viral genome can enter a non-neuronal cell, yet is maintained in a transcriptionally silent state. This model system is used to study the cellular mechanisms which prevent viral gene expression at the earliest times post-infection.

Viruses commonly used in the study of quiescence lack the activation functions of proteins such as VP16 and ICP0 (KM110; (270)) or additionally ICP4 (in1820K; (306)) or all IE proteins (d109; (325)). No viral gene expression is detected, including from the LAT region (143), even with high MOI (up to 10 PFU/cell) infection (270, 306, 325). Quiescent genomes are actively repressed, as heterologous promoters embedded within them are also silenced (167, 263, 303, 306, 325). For example, the ICP0 promoter is silenced during quiescent infection, while the same promoter within the cellular genome in cells harbouring the quiescent virus is active (306). This suggests that the silencing of the viral genome is not DNA sequence-dependent and implies that the viral genome may be functionally sequestered from transcriptional machinery.

The repression of gene expression can be broken up into at least two stages. In the initial stages, at early times post-infection (< 24hrs), the viral genome is

responsive to transacting factors, such as ICP0, VP16 or chemicals such as HMBA. At later times (>24 hours), the viral genome is capable of being reactivated by the provision of ICP0 *in trans* (143, 306, 325, 366) or some newly identified chemical compounds (see below) but not by VP16 or HMBA (248, 306).

Quiescent genomes are found in the nucleus surrounded by a shell of the IFN-inducible PML protein (97), suggesting that the viral genome may be physically sequestered. Additionally, the quiescent genome may be functionally inaccessible through its association with histones bearing marks of transcriptional repression as well as the heterochromatin-associated protein HP1 (58). HP1 is found in ND10 domains (93, 235, 341), suggesting a potential link between sequestering the quiescent genome at ND10 and assembling the viral genome into repressive chromatin.

ICP0 is both necessary and sufficient for reactivation of quiescent viral genomes. The ability of ICP0 to act as an ubiquitin E3 ligase, along with a functional proteasome system is absolutely required for reactivation (100, 143, 366), suggesting ICP0 functions by degrading host inhibitory factors. When quiescently infected cells are infected at high MOI with ICP0 mutants, no gene expression is detectable from the quiescent genome, highlighting the functional inaccessibility of the viral genome in the absence of ICP0 (263). However, when quiescently infected cells are superinfected with an ICP0 mutant at low MOI, viral gene expression from a subset of quiescent genomes can be observed (301). Gene expression from the superinfecting ICP0 mutant and the quiescent genome is

extremely sensitive to MOI; a high enough MOI is required to overcome the defect in IE gene expression of an ICP0 mutant, but cannot be so high as to cause competition with the reactivating quiescent genome. Only approximately 10% of the quiescent genomes are reactivated by low MOI infection of an ICP0 mutant (301). This would suggest that the quiescent population, much like the latent population in neurons, is heterogeneous or stochastic, with some genomes more responsive to trans-acting factors than others.

Unlike HMBA and DMSO, which cannot reactivate quiescent genomes (248), sodium arsenate and gramicidin D both stimulate IE gene expression and reactivate ICP0 mutant genomes (305). Sodium arsenate is an inducer of heat shock and oxidative stress, while gramicidin D specifically permeablizes the cellular membranes. The mechanism used by these compounds to reactivate the genome is unknown, although they both induce rearrangements of ND10 domains. Similar to the results described above, only about 10% of the quiescent genomes are activated by these compounds (305), again suggesting a heterogeneous population or a stochastic process. The discovery that chemical compounds can reverse the repression of the quiescent genome may provide insight into the function of ICP0.

1.5 Chromatinization of the viral genome

The HSV genome is associated with histone proteins during both the lytic and latent stages of its lifecycle. Gathering evidence suggests that histone occupancy and modifications may play an important role in regulating viral gene expression.

Nucleosomes are comprised of four core histones (H2A, H2B, H3 and H4) containing N-terminal tails which can be modified in a variety of different manners. Histones are commonly acetylated, methylated, phosphorylated and ubiquitinated (189). These histone modifications create binding sites for multi-protein complexes. For example, bromodomain-containing proteins recognize and bind to lysine acetylated histone tails, while chromodomain-containing proteins recognize methylated lysine residues (342). Acetylation of histones is generally correlated to transcriptional activation, as acetylation may mask the overall positive charge on the nucleosome (119). Histone acetylation also recruits chromatin remodeling complexes which can slide or remove nucleosomes from the chromatin template (144, 175). Some histone modifications are markers of transcription initiation, such as H3K9/14 acetylation and H3K4 methylation (19, 364). Other modifications occur concomitantly with RNA polymerase II-mediated transcription and are markers of transcription elongation, such as H3K36 methylation (217).

1.5.1 Chromatinization of the viral genome during latency

The global repression of gene expression during HSV latency suggests an epigenetic silencing mechanism. Early studies ruled out the possibility of DNA methylation, and instead suggested histone modifications are involved in maintaining a transcriptionally silent genome (197). The latent genome is complexed with nucleosomes in a regular repeating pattern as assessed by micrococcal nuclease (MNase) assays (75) and histone modifications appear to play an important role in regulating gene expression during latency, as described below.

The LAT promoter and 5' exon enhancer regions are hyperacetylated compared to the transcriptionally silent ICP0, ICP4, ICP27 and DNA polymerase genes (196, 197). Part of LAT overlaps the ICP0 coding region, but histone hyperacetylation does not extend into the ICP0 promoter (196). The LAT enhancer and 5' exon are bounded by CTCF binding motifs (11, 50), suggesting this region is insulated as its own chromatin domain within the viral genome.

Histone hyperacetylation can still be detected within the 5' exon enhancer region of a mutant with a deletion of the LAT promoter (196). LAT transcripts are not detectable in the absence of the LAT promoter, suggesting that acetylation is not due to RNA polymerase II-mediated transcription (196).

Expression of LAT results in an increase in H3K9 and H3K27 methylation (54) (both correlated with repressed transcription and facultative heterochromatin) and a decrease in H3K4 methylation (associated with active transcription) on

several lytic genes (393). The polycomb protein Bmi1, which methylates H3K27 is also detectable on the latent viral genome (204). Therefore, one role of LATs may be to recruit histone modification machinery to assemble repressive chromatin over the lytic regions of the viral genome. However, contradictory evidence has been presented which suggests that H3K27 methylation and the association of the histone variant macroH2A (associated with transcriptional silencing) increases with a LAT deletion mutant (204). The reason for this discrepancy has yet to be determined.

In contrast, in the rabbit eye model LATs may be required for low levels of lytic gene expression during latency (123). Higher levels of H3K4 methylation at the LAT enhancer as well as the ICP0 and ICP27 promoters was detected on LAT^+ compared to LAT^- genomes. Levels of H3K9 methylation were similar between the LAT^+ and LAT^- viruses, suggesting the LATs are not required in this model to assemble the lytic regions of the genome into heterochromatin (123). The authors argue that contrary to the mouse model of latency, in rabbits LATs are required to maintain the viral genome in a more transcriptionally permissive state poised for reactivation.

In the mouse explant model, reactivation results in a decrease in LAT acetylation, followed by a decrease in LAT abundance and an increase in acetylation at the ICP0 promoter (10). Similar results were noted with an *in vivo* reactivation model using the HDAC inhibitor sodium butyrate (275). However, no increase in lytic gene transcripts could be detected at 12 hours in the explant model (10). Whether this is a sensitivity issue or the increase in acetylation

detected at the ICP0 promoter does not correlate with transcription has not been addressed.

It is important to note that not all latent genomes express LATs, therefore they may not be absolutely essential for chromatinization of the viral genome. However, the current ChIP evidence strongly suggests that histone modifications regulate gene expression from the latent genome.

1.5.2 Chromatinization of the viral genome during lytic infection

The viral genome within the nucleocapsid is devoid of histone proteins (57, 283, 294). Instead, it is complexed with the highly basic protein spermine (122). For many years it was thought that the lytic HSV genome was transcribed as a naked DNA template. In support of this, GFP-tagged histone H2A is specifically excluded from viral replication compartments (266). However, during lytic infection, viral DNA is protected from MNase (109, 215, 271; J Lacasse, L Schang pers comm). As assessed by ChIP, the viral genome becomes complexed with histones very early after infection, although the levels appear to be much lower than several regions of the cellular genome used as references (150, 179). This may depend on the MOI used, as the viral and cellular genomes are associated with similar amounts of histone H3 at low MOI (55). Assessment of total cellular histone content after HSV infection using western blot analysis indicates no significant changes in the overall amount of total or modified histone H3 (179). Study of the linker histone H1 indicates that it is mobilized upon HSV

infection in a manner partially dependent on IE or E protein expression (61). Therefore, histones may be mobilized away from cellular chromatin and onto viral genomes at early times after infection, although this has not been directly tested.

The histone chaperone HIRA is required to load the histone variant H3.3 onto the viral genome prior to DNA replication (295). Knock-down of HIRA expression results in decreased association of H3.3 with the viral genome as well as decreased viral mRNA accumulation and DNA replication (295). This suggests that at least some histone association with the viral genome is important for lytic infection, although the effects of knocking down this protein on other cellular processes are unclear.

During lytic infection, viral promoters are associated with histones bearing marks of active transcription: H3K9/K14 acetylated histones and H3K4 methylated histones (150, 158, 179). The association of these marks with specific promoters occurs in a temporal manner which mimics viral gene expression.

HCF, which interacts with VP16 at the viral IE promoters, also associates with the Set1/Ash2 H3K4 histone methyltransferase complex (406). Knockdown of Set1 expression results in decreased detection of this histone modification on the viral genome as well as decreased viral gene expression and DNA replication, although these effects are most prominent at late times in infection (158). This indicates that histone modifications may play an important role in regulating viral gene expression.

In addition, the abundant tegument protein VP22 interacts with the histone chaperone TAF-1 and prevents TAF-1 deposition of nucleosomes onto a DNA template *in vitro* (385). In addition, overexpression of TAF-1 is inhibitory to viral infection (385). Whether this interaction also occurs in the context of lytic infection is unknown.

1.5.2.1 The roles of VP16 and ICP0 in regulating viral chromatin structure

While performing the experiments outlined in Chapters three and four, evidence was presented in the literature suggesting that both VP16 and ICP0 are involved in modulating the chromatin structure on the viral genome during lytic infection (55, 109, 150, 201). Chapter five summarizes the information available in the field to date, thus I will defer the discussion of how these proteins function to affect the chromatin structure on the viral genome until Chapter five.

1.6 Intracellular antiviral responses

One of the major pathways activated in response to viral components is the IFN response. IFN stimulates the expression of a wide range of cellular factors, called interferon stimulated genes (ISGs), which act to make the cell inhospitable to viral infection. ISGs can be induced through both IFN-dependent and IFN-independent pathways. HSV is well-adapted to its host and hence encodes a wide

variety of proteins which act to interfere with IFN signaling or the functions of ISGs.

1.6.1 The Interferon Response

IFNs were first discovered by Isaacs and Lindenmann in 1957 (162). IFNs protect cells against pathogens and have immunomodulatory and antiproliferative effects (361). There are three main types of IFNs (Type I, II and III) which are activated via different receptors, but use many of the same signaling molecules and regulate some of the same genes (293).

Viral infection can be detected through a variety of means by proteins known as pattern recognition receptors (PRRs). The most well-characterized to date include Toll-like receptors (TLRs) and retinoic acid-inducible gene I (RIG-I)-like helicases (RLHs). These proteins recognize pathogen-associated molecular patterns (PAMPs) and signal the production of IFN and pro-inflammatory cytokines (293). IFN is secreted from the cell and acts in both an autocrine and paracrine manner to stimulate the production of ISGs (361).

1.6.2 Interferon-stimulated genes

There are over 300 ISGs stimulated by IFN (74), however only a small subset are required to induce an antiviral state. HSV encodes proteins which affect the functions of several known ISGs.

The ISG protein kinase R (PKR) is activated by dsRNA. PKR phosphorylates eukaryotic initiation factor (eIF) 2-α which impairs the guanine nucleotide exchange reaction catalyzed by eIF-2B thereby inhibiting the translation of both viral and cellular mRNA (53, 327, 328). HSV encodes two proteins known to interfere with this pathway: the neurovirulence protein ICP34.5 and US11. ICP34.5 binds protein phosphatase 1α and directs it to de-phosphorylate eIF-2α (44, 146). US11 is a true late RNA binding protein which physically interacts with PKR and prevents its activation (291, 299).

A second set of well-characterized ISGs are 2'5' oligoadenylate synthetase (OAS) and RNAse L which function together to degrade RNA (321). OAS acts as a PRR, which recognizes dsRNA and catalyzes the formation of oligoadenylates with a 2'5'-phosphodiester bond linkage called 2'5'-Oligo Adenylic acid (2-5A) (180). These oligomers bind to latent RNAse L, causing homodimerization, activation and subsequent ssRNA degradation (78). RNA degraded by RNAse L can act as triggers for other cytosolic PRRs like RIG-I and MDA5 which results in type I IFN production (240). US11 has also been implicated in preventing OAS-mediated RNA degradation either through sequestering dsRNA away from the protein or interacting in a high molecular weight complex with OAS and dsRNA (329).

The Mx family of proteins, including MxA and MxB in humans, are cytoplasmic GTPases that are expressed upon IFN treatment (321). These proteins are found near the smooth ER and hence are thought to survey exocytotic events and mediate vesicle trafficking to trap essential viral components (1, 187).

The functions of several other ISGs have been explored in detail, while many remain to be characterized. ISG56K binds to the p48 subunit of eIF-3, inhibiting protein translation (136). IP-10 (CXCL10) is an IFN-inducible chemokine which attracts cells of the adaptive immune system (237). ND10 component proteins PML, Sp100, Sp110, Sp140, ISG20 and PA28 are all IFN-inducible (314).

1.6.3 The IFN-independent response to HSV infection

In some instances, the induction of ISGs can occur in the absence of IFN production. Infection with several enveloped RNA and DNA viruses can induce ISGs, whereas infection with non-enveloped viruses does not, implicating membrane fusion as a potential recognition mechanism used by the cell (59). HSV-1 induces the accumulation of ISGs, but only in the absence of viral gene expression.

Infection of human embryonic lung fibroblasts with the VP16/ICP0 double mutant virus KM110 or UV-inactivated wild type virus results in induction of a small subset of ISGs as assessed by microarray analysis (268), such as ISG56, MxA, OAS, PML, IP-10 and ISG15. These ISGs act to inhibit viral replication and are also induced by IFN treatment (268). In addition, Nicholl *et al* described the induction of ISG54, IFI56, ISG15, 9-27 and MxA in response to infection with wild type virus in the presence of the protein synthesis inhibitor cycloheximide, the VP16 mutant in1814 or the VP16/ICP0/ICP4 mutant in1320 (279). Similarly, infection with a mutant virus which does not express any IE

genes (d109) also induced the accumulation of a subset of ISGs (80). Thus, one or more viral proteins prevent this phenomenon.

In human fibroblasts, entry is required for the induction of ISGs. Viral mutants in gB and gD which cannot fuse and enter cells do not induce ISGs (268, 279). ISGs are also not induced in response to enveloped viruses in the absence of IRF3, while IRF1, 7 and 9 are dispensable (59). Preston *et al* demonstrated that HSV infection in the presence of cycloheximide induces a DNA binding complex, including IRF3 and CBP, which recognizes ISREs.

The mechanism by which HSV prevents the induction of ISGs remains to be determined. Eidson et al determined that ICP0 is involved in preventing the accumulation of ISGs. Inhibiting proteasome function with MG132, as well as infections with viruses harbouring mutations in the RING domain of ICP0, results in ISG accumulation (80). ICP0 expression does not cause the degradation of the signaling molecules TBK-1 or IKKε or the transcription factors IRF3 or CBP (221).

Using a cell line expressing ICP0 in an inducible manner, Everett and Orr demonstrated that ICP0 does not prevent the induction of ISG15 in response to IFN or dsRNA (98). The reason for the discrepancy between these observations and those previously reported is unclear. Pre-expression of ICP0 increased HSV yields at low MOI in the presence of IFN, but yields were still reduced compared to no IFN treatment. At high MOIs, pre-expression of ICP0 relieves IFN-mediated repression to a greater extent (98). It would appear that the effects of ICP0 and IFN is different in tissue culture than intact animals, as in mice the IFN

pathway plays a key role in limiting HSV infection, particularly with ICP0 mutant viruses (139, 214, 288, 289).

When I began this project, it was understood that U2OS cells support the replication of both ICP0 and VP16 mutants to nearly wild-type levels, but the basis for this permissivity was unknown. In order to better understand how this cell type could compensate for two viral proteins which function by distinct mechanisms, I first determined whether the permissive phenotype of U2OS cells was dominant or recessive in heterokaryon formed between restrictive HEL fibroblasts and U2OS. In Chapter three I determined that the permissive phenotype of U2OS cells is recessive in somatic cell fusion assays, indicating this cell line lacks an antiviral response found in other restrictive cell types. In Chapter four I determined that the artificial induction of ISGs that occurs via p14-mediated fusion is not the mechanism by which viral gene expression is inhibited.

I then tested a specific hypothesis, as described in Chapter five: Do U2OS cells have a defect in a chromatin-based repression system? ChIP experiments indicate that both ICP0 and VP16 play roles in modulating histone occupancy and acetylation levels on the viral genome, suggesting this function may be important for stimulating viral gene expression. Knowing that U2OS cells support the replication of VP16 and ICP0 mutants and have a defect in an unidentified antiviral pathway, I hypothesized that this cell type is defective in loading high levels of under-acetylated histones on the viral genome. Using ChIP, I determined that U2OS cells are lacking a pathway that assembles high levels of

under-acetylated histones on the viral genome that is normally targeted by ICP0. However, as described in Chapter five, the role this chromatin-based pathway plays in limiting viral gene expression is unclear.

Finally, I tested a second specific hypothesis, as described in Chapter six: Do U2OS cells have a defect in a signalling pathway that is required to activate the antiviral mechanism? I identified several kinase inhibitors which stimulate viral gene expression in otherwise restrictive cell types in the absence of VP16 activation function, but have not identified the target of these compounds. Additionally, I determined that these kinase inhibitors also function in U2OS cells to enhance viral gene expression. Thus, U2OS cells express the target of these kinase inhibitors and must then support the replication of VP16 and ICP0 mutants through an alternative mechanism.

The data presented in this thesis strongly supports the hypothesis that U2OS cells are defective in an antiviral mechanism normally targeted by ICP0 and VP16. Although U2OS have a defect in a pathway involved in loading high levels of under-acetylated histones on the viral genome, the relevance of this to viral genome repression is currently unclear. Studies into a potential defect in the ND10-based repression system in U2OS cells are clearly warranted, as outlined in the thesis discussion.

Chapter Two: Thesis Materials & Methods

2.1 Cell culture and growth of HSV

2.1.1 Maintenance of mammalian cell culture

Human U2OS osteosarcoma, HeLa cervical carcinoma, human embryonic lung (HEL) fibroblasts and African green monkey kidney epithelial cells (Vero) were obtained from the American Type Culture collection. Cells were maintained in 150cm^2 tissue culture flasks (Corning) using 25mL Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 50 units/mL penicillin and 5µg/mL streptomycin. HEL cells were further supplemented with 1mM sodium pyruvate. MO59J (DNA PKcs- negative) and MO59K (DNA PKcs-wild type) cells, derived from a human malignant glioma, were obtained from Dr S. Lees-Miller. These cells were grown in 10cm^2 dishes in 50% Dulbecco's modified Eagle medium/50% F12 medium supplemented with 10% FBS and antibiotics as above. Flasks were incubated at 37°C in a humidified atmosphere in the presence of 5% CO_2.

Cells were passaged when they reached confluency by washing once with phosphate buffered saline (PBS; 10mg/mL NaCl, 0.25mg/mL KCl, 1.8mg/mL Na_2HPO_4, 0.3mg/mL KH_2PO_4, pH7.5), trypsinizing the cells until no longer adherent to the flask (Trypsin, Gibco), resuspending in DMEM with additives, and reseeding to approximately 25% confluency using pre-warmed growth medium.

HEL cells were passaged 10-18 times, while U2OS, HeLa and Vero were passaged up to 50 times. MO59J and MO59K were passaged a total of 4 times. To prepare for experiments the following day, U2OS, HeLa and Vero cells were seeded at 50% confluency. HEL cells were seeded at 80% confluency, while MO59J/K were seeded at 95% confluency. The concentration of cells was determined using a haemocytometer.

To prepare stocks for freezing in liquid nitrogen, cells from subconfluent, actively dividing cultures were harvested and diluted to approximately 2 x 10^6 cells in a mixture of 50% FBS and 50% DMSO in cryovials (Nalgene). Cells were left in an isopropanol cooler at -80°C overnight, and then transferred to liquid nitrogen for long term storage.

To start cell cultures from frozen stocks, cryovials were thawed briefly at 37°C and the cells are resuspended in 10mL growth medium. Diluted cells were pelleted at low speed, and then resuspended into appropriate growth medium and added to 75cm^2 flasks.

2.1.2 Preparation and storage of HSV stocks

The following viruses were used in this study:

KOS – wild type virus

n212 – an ICP0 mutant where both copies of the ICP0 gene bear an *Spe1* linker. This linker contains an amber chain termination codon that results in truncation of the ICP0 protein after amino acid residue 212 (38).

V422 – a VP16 mutant which includes an *Nhe1* linker containing amber stop codons in all three frames which results in truncation of the protein after amino acid residue 422 (209).

KM110 – is a VP16/ICP0 double mutant including the mutations described for the n212 and V422 mutants (270).

in1814 – is a VP16 mutant with a 12bp insertion after amino acid residue 397 which abolishes interactions with HCF and Oct-1 (3).

in1814R – the marker rescue of in1814 (wild type strain 17+) (3).

All HSV strains used here were propagated on U2OS cells, which complement both ICP0 and VP16 mutants. Titres of wild type virus obtained from this cell line are approximately 2-fold lower than those obtained on Vero cells.

To harvest cell-associated viral stocks, U2OS cells were grown to approximately 85% confluency in 150cm^2 flasks. For n212, V422, in1814 and KM110 stocks, cells were infected using a multiplicity of infection (MOI) of 0.05 in serum-free medium. KOS and in1814R infections were performed at an MOI of 0.03. After one hour of infection at 37°C, rocking every 15 minutes, 20mL growth medium was added back to the flasks. In the case of V422, in1814 and KM110 viral stocks, cells were additionally supplemented with 3mM hexamethylene-*bis*-acetimide (HMBA) during and after the one hour incubation time. When most cells exhibited extensive cytopathic effect (approximately 3 days with KOS, in1814R and n212 stocks, 5 days with V422, in1814 and KM110 stocks), infected cells were collected and pelleted at 1200rpm for 5 minutes on a bench top centrifuge at 4°C. Pelleted cells were resuspended in 1mL serum-free

medium per 150cm^2 flask. Cells were frozen at -80°C for 15 minutes and thawed at 37°C for 5 minutes, repeated three times. Cells were then sonicated (Model 550 Fisher Scientific Sonic Dismembrator) at level 7 using 3 20 second pulses each followed by 20 seconds on ice. Cellular debris was separated using centrifugation at 2000rpm for 15 minutes on a bench top centrifuge at 4°C. Supernatants were then aliquoted as viral stocks and frozen at -80°C.

To harvest cell-free virus, cells were infected as described above. Once CPE had been achieved, 25mL of supernatant was carefully removed from each flask into a 50mL Falcon tube. The supernatant was centrifuged at 2000rpm for 15 minutes at 4°C to remove any large cellular debris. The supernatant was carefully removed and added to Oak Ridge tubes (Nalgene). These were centrifuged at 12 000 rpm using the Avanti J-25 centrifuge and the JS 13.1 rotor (Beckman). After spinning, the supernatant was carefully removed and the viral pellet was resuspended in 100µL of serum-free medium per 150cm^2 flask. These stocks were then aliquoted and frozen at -80°C.

2.1.3 Titration of HSV stocks

10µL of virus stock was diluted into 990µL of serum-free medium to create a 1/100 dilution. 10-fold serial dilutions were made ranging from 10^{-2} to 10^{-7}. 250µL of each dilution were used to infect a confluent monolayer of U2OS cells in a 12-well plate. For V422, in1814 and KM110 titrations, 3mM HMBA was added to each well. After one hour of adsorption, the viral inoculum was removed and 1mL of DMEM containing 1% human serum was added to each

well. 2-3 days later, plaques were counted under a light microscope. Wells were counted which contained at least 20 and no more than 200 plaques.

2.1.4 Infection of mammalian cells with HSV

Cells were plated approximately 24 hours prior to infection. Cells were counted immediately prior to infection using a haemocytometer and virus was diluted in the appropriate amount of serum-free medium. Adsorption was carried out at 37°C in a humidified incubator in the presence of 5% CO_2. Cells were rocked every 15 minutes for one hour. Infecting media was replaced with fresh DMEM containing 10% FBS after acid washing (see below). Cells were then left in a humidified incubator at 37°C for the appropriate length of time for the experiment.

Kinase inhibitors used in Chapter six include LY294002 (Sigma), LY303511 (Calbiochem), wortmannin (Sigma), NU7441 (KuDOS Pharmaceuticals), TBB (Sigma), TBCA (Calbiochem), rapamycin (Sigma), Torin-1 (Dr D. Sabatini, MIT), caffeine (Sigma), DNA PK I and II (Calbiochem) and cycloheximide (Sigma).

2.1.5 Acid Washing mammalian cells

After the one hour adsorption time, infecting medium was aspirated and cells were washed once with PBS. Acid wash (40mM citric acid, 10mM KCl, 135mM NaCl, pH 3.0) was added to the cells for 30 seconds, removed and added again for

another 30 seconds. Growth medium was added to neutralize the acid, removed and the cells were washed one more time with growth medium before DMEM containing 10% FBS was added to the cells for incubation.

2.1.6 UV-inactivating HSV

Viral stocks were diluted in serum-free medium to the appropriate concentrations for each experiment. 500µL of diluted virus was added to each well of a 6-well dish. Dishes were placed in the Stratalinker 2400 (Stratagene) and the lids were removed. Each dish was exposed to 2000 x 10^2 µJ of UV irradiation. This is the amount of irradiation determined to prevent IE gene expression by KOS at an MOI of 10 in HEL cells.

2.2 Production and manipulation of plasmid DNA

2.2.1 Transformation of competent bacteria

Plasmids were transformed into *Escherichia coli* (*E.coli*) strain DH5α. 40µL of competent *E. coli* were mixed with 50ng of plasmid DNA and kept on ice. This mixture was transferred to a pre-chilled electrocuvette and samples were pulsed using a BIORAD Gene Pulser II with the resistance set at 200 ohms, the capacitance at 25 µfaraday and the voltage at 1.8 volts. Immediately after pulsing, bacteria was added to 1mL of SOC medium (0.02g/mL Bacto-tyrptone, 5µg/mL Yeast extract, 5mM NaCl, 25mM KCl, 10mM $MgCl_2$, 20mM glucose) and allowed to recover for 45 minutes at 37°C shaking at 225rpm. 200µL of these

cells were spread-plated onto luria broth (LB; 1% w/v bactotryptone, 0.5% w/v yeast extract, 1% w/v NaCl) agarose with the appropriate antibiotic marker and grown overnight at 37°C.

2.2.2 Growth and maintenance of bacterial cultures

All plasmids were maintained in the DH5α strain of *E. coli*. Selection was performed by growing bacteria in LB in the presence of the appropriate antibiotic at 37°C, shaking at 225rpm. For long term storage, sterile glycerol was added to freshly grown cultures to a final concentration of 30% in LB, mixed and 1mL aliquots were frozen at -80°C.

2.2.3 Large scale preparation of plasmid DNA

Bacteria from a single isolated colony was grown overnight (14-16 hours) in 100mL LB plus the appropriate antibiotic at 37°C. Plasmid DNA was isolated using the Qiagen Maxi Prep DNA isolation kit according to the manufacturer's instructions.

2.2.4 Quantitation of DNA and RNA

DNA and RNA samples were quantified by measuring their absorbance at 260nm with an Ultraspec 3000 (Pharmacia) spectrophotometer. 4-5µL of DNA or RNA was diluted in 1mL of water, transferred to a quartz cuvette and its absorbance was measured. The concentration was calculated automatically by the spectrophotometer using the conversion of 1 absorbance unit being equal to

50mg/mL of dsDNA or 40mg/ml of RNA. Purity of DNA and RNA was assessed by comparing the ratios of absorbance at 260 and 280nm. Generally, a ratio of 1.7 to 2.0 was considered acceptable.

2.2.5 Restriction enzyme digestion

Restriction enzyme digestions were used to generate probe fragments for Northern analysis. Restriction enzymes were purchased from New England Biolabs or Invitrogen. Digests were prepared using 10μg of plasmid DNA, 10 units of the appropriate enzyme and the appropriate buffer diluted to 1X. Double digestions used a total of 10 units of enzymes and a buffer which offered maximal activity for both enzymes. Digested DNA was immediately separated using agarose gel electrophoresis.

To make probes for Northern blot analysis, plasmids were cut with the following enzymes:

pICP22: *SacI, XhoI*, React 4 Buffer
p27 (ICP27): *BstXI, EcoNI*, React 2 Buffer
pTK173: *PvuII, SacI*, NEB#1 Buffer
IP-10 (Image Clone 491243): *HindIII, XhoI*, React 2 Buffer
MxB (Image Clone 471638): *EcoRI, NotI*, React 3 Buffer

2.2.6 Polymerase Chain Reaction (PCR)

PCR was used to amplify DNA sequences for use as probes in Northern blotting. Generally, 50μL reactions were set up as follows:

5μL 10X PCR buffer, 2.5μL $MgCl_2$, 5μL each 10μM primer, 1μL 10mM dNTPs, 0.5μL *Taq* polymerase, 31μL H2O

Generally, PCR programs were set up as follows:

Step 1: 94°C for 2 min, Step 2: 94°C for 30 sec, Step 3: Annealing temp for 30 sec, Step 4: 72°C for 1 min, Step 5: 72°C for 5 min, Repeat Steps 2-4 24 times

PCR primers used in this study:

To amplify the ISG56K probe fragment:
JRS 139: CCCAGTCACGACGACGTTGTAAAACG
JRS140: AGCGGATAACAATTTCACACAGG Annealing temperature: 55°C

To amplify the VP16 probe fragment:
JRS87: CTCGACGACTTGGGCTTTAG
JRS88: AGGGCATCGGTAAACATCTG Annealing temperature: 55°C

PCR reactions were immediately separated using agarose gel electrophoresis.

2.2.7 DNA agarose gel electrophoresis and gel purification

DNA fragments were separated by electrophoresis on 1% agarose gels in 1X TAE (40mM Tris-acetate, 2mM EDTA). Ethidium bromide was added to gels to allow for visualization using an UV transilluminator. Samples were run for 1 – 2 hours in 1X TAE buffer at 100V.

DNA fragments were gel purified by cutting the appropriate bands out of the agarose gel and following the manufacturer’s instructions in the Qiagen Gel Extraction Kit.

2.2.8 Random primer labelling of DNA fragments

2µg of random hexamer DNA primers were mixed with 2.5µg of template DNA and water to bring to reaction to 35µL. This mixture was heated at 95°C for

5 minutes and placed immediately on ice for 2 minutes. Random priming was initiated by adding 7µL of oligo labelling buffer (30mM Tris pH 8, 3mM $MgCl_2$, 0.5% β-mercaptoethanol, 1.25mM dGTP, 1.25mM dATP, 1.25mM dTTP, 1.285M HEPES, pH 6.6), 2µL bovine serum albumin (BSA; New England Biolabs), 5µL α-^{32}P dCTP, and 1µL Klenow (Invitrogen). This mixture was left at 37°C for 30 minutes and then 50µL Tris-EDTA (10mM Tris pH 7.0, 1mM EDTA) was added. The DNA was extracted using an equal volume of phenol/chloroform (Fluka). After spinning in a benchtop centrifuge (Eppendorf centrifuge 5417C) at 14 000rpm for 5 minutes at room temperature, the aqueous layer was removed and passed through a NICK column (Amersham Pharmacia BioTech) to remove unincorporated nucleotides. 400µL of TE was also passed through the column. 500µL of TE was added to the column and the eluate collected. Radioactive counts were determined by measuring 2µL of eluate diluted in 5mL scintillation fluid (Cytoscint, MP Biomedicals) using a Beckman LS6500 scintillation detector. 1 x 10^7 counts were used for each blot.

2.2.9 Transfection of plasmid DNA into mammalian cells

Transient transfections were done using two methods: Lipofectamine 2000 (Invitrogen) was used to transfect U2OS cells and Exgen 500 (Fermentas) was used to transfect HEL cells. All transfections were carried out using the amounts of DNA and reagent according to the manufacturer's instructions. Extremely high transfection efficiency can be obtained using U2OS cells (~80%), while very low transfection efficiency is obtained using HEL cells (~5%).

2.3 Isolation and manipulation of cellular RNA

2.3.1 Harvesting total cellular RNA

Monolayers were grown in 60mm dishes, washed with 5mL of PBS and incubated with 1mL of Trizol (Invitrogen) at room temperature. Cells were scraped into 1.5mL eppendorf tubes and either frozen at -80°C or immediately extracted.

For extractions, 200µL of chloroform was added to each sample, mixed for 3 minutes and centrifuged at 14 000rpm for 15 minutes at 4°C. The aqueous phase was removed and re-extracted with 200µL of chloroform and spun as above. The aqueous phase was again removed, mixed with an equal volume of isopropanol and left for 10 minutes at room temperature. The mixture was then spun at 14 000rpm for 15 minutes at 4°C and the supernatant aspirated. The RNA pellet was washed once with 70% ethanol and centrifuged at 14 000rpm for 5 minutes. The ethanol was aspirated and the RNA pellet was allowed to dry for 9 minutes. After this time, 40µL of DEPC-treated water was added and the dissolved RNA pellets were frozen at -80°C for 10 minutes, and then thawed at 65°C prior to quantitation.

2.3.2 Northern blot analysis

For each 10µg RNA sample, 3µL 10X MOPS (0.2M MOPS, 1mM EDTA, 5mM NaAcetate), 5µL of formaldehyde and 15µL formamide was added.

Samples were denatured at 65°C for 15 minutes, and then left on ice for 2 minutes prior to loading onto a 1% agarose gel containing 2% formaldehyde, 1X MOPS and ethidium bromide. Gels were run for approximately 2 hours at 100V in 1X MOPS running buffer. RNA was transferred to a Genescreen Plus nylon membrane (NEN Life Sciences Products) in 10X SSC (1.5M NaCl, 150mM sodium citrate). After an overnight transfer, membranes were UV-crosslinked using the Stratalinker 2400 before being hybridized with the α-^{32}P-labeled probe. Firstly, blots were preinclubated with 5mL Express Hybridization Solution (BD Biosciences) at 68°C for 30 minutes while being rotated using an H1 16000 hybridization incubator (Tyler Research Instruments). The α-^{32}P-labelled probe was denatured at 95°C for at least 5 minutes and added to the ExpressHyb solution. Hybridization occurred for one hour at 68°C, followed by washes with Express Hybridization wash 1 (0.5% 20X SSC, 0.1% SDS) with several changes over 40 minutes at room temperature. Express Hybridization wash 2 (10% 20X SSC, 0.05% SDS) was then used to wash 2 times for 20 minutes each while being rotated at 50°C. Following the washes, blots were wrapped in saran wrap and exposed. The α-^{32}P signal was detected by exposure to Kodak Biomax MS film at -80°C.

To remove bound probe from the membrane for the purpose of re-probing, blots were washed at 95°C for 15 minutes with stripping buffer (0.5% SDS, 10mM Tris pH 8.0). Blots were allowed to cool and could then be pre-hybridized with Express Hybridization solution again.

2.4 Western blot analysis

Monolayers of various cells were grown in 6-well plates. After the appropriate treatment and time frame, cells were harvested by washing once with PBS, then scraped into 100µL of 1X RIPA (50mM Tris pH 7.4, 150mM NaCl, 1mM EDTA, 1X Complete Protease Inhibitor (Roche), 1% Triton X-100, 0.1% SDS). Samples could be quantitated at this point using the BCA Protein Assay Kit (Thermo Scientific) according to the manufacturer's instructions. 6X SDS loading buffer (0.5M Tris pH 6.8, 30% glycerol, 1% SDS, Bromphenol blue and 0.6% β-mercaptoethanol) was added to a final concentration of 1X and the samples were heated at 95°C for 10 minutes. 10µg of protein was loaded onto 8% acrylamide SDS PAGE gels along with a prestained protein marker (BIORAD). The gels were electrophoresed for approximately 2 hours at 100V in 1X SDS PAGE buffer. Samples were transferred to a nitrocellulose membrane (Hybond ECL, Amersham Pharmacia) using a wet transfer apparatus (BIORAD Trans-blot cell) at 100V for 45 minutes. After transferring, blots were incubated for at least one hour in 5% skim milk/TBS Tween (25mM Tris pH 8.0, 150mM NaCl, 0.1% Tween-20) at room temperature, or overnight at 4°C.

Primary antibodies were diluted in 5% skim milk/TBS Tween for at least one hour at room temperature. Appropriate dilutions of antibody were determined empirically. Three washes were then performed using TBS-Tween for 5 minutes each. Secondary antibodies were diluted 1:10 000 in 5% skim milk/TBS Tween and incubated with the blots for at least one hour at room temperature. Another

three washes were performed as above and the membrane was developed using the ECL+ system (Amersham Biosciences) according to the manufacturer's instructions. Signal was detected by exposure to Fuji Super RX X-Ray film.

To remove bound antibody for the purpose of re-probing, the membranes were washed with stripping buffer (0.02g/L SDS, 6.25mM Tris, pH 6.7, 7% β-mercaptoethanol) at 55°C for 15 minutes. Membranes were rinsed several times with TBS-Tween and 5% skim milk/TBS-Tween was added to the blots again for one hour prior to antibody re-exposure.

2.5 Indirect immunofluoresence and microscopy

2.5.1 Cell-permeable staining

Cells were stained with one of: 15mM blue fluorescent 7-aminocoumarin (CMAC), 15mM far red fluorescent BODIPY methylbromide, 10mM orange fluorescent tetramethylrhodamine (CMTMR) or 10mM green fluorescent fluorescein diacetate (CFMDA), all from Molecular Probes, according to the manufacturer's instructions.

2.5.2 Indirect immunofluorescence

Monolayers on cells were grown on 18mm coverslips (Fisher Scientific) within 12-well dishes. These cells were fixed by washing once in PBS, then adding 5% formaldehyde in PBS for 10 minutes. Cells were again washed with PBS and PBS containing 0.5% Nonidet P-40 was added for 10 minutes. Cells were washed

three times with PBS containing 1% FBS and left in PBS/1% FBS for at least one hour at room temperature or overnight at $4^{o}C$. Cells were then incubated with primary antibody diluted in PBS/1% FBS for 45 minutes, washed three times for 5 minutes each with PBS/1% FBS then incubated with secondary antibody (anti-rabbit or anti-mouse horseradish peroxidise) diluted in PBS/1% FBS (1:10000) for another 45 minutes. After three more washes in PBS/1% FBS, coverslips were dipped into distilled water and inverted onto slides containing 25μL Vectashield (Vector Laboratories) with or without DAPI, depending on the experiment. Finally, coverslips were secured using clear nail polish.

2.5.3 Microscopy

The light microscope used in our laboratory is an Olympus CK40. The fluorescent microscope used was a Zeiss Axiovert 200. The light source was an X-cite 120 Fluorescence Illumination System and a 40X oil immersion objective lens was used for all experiments. Samples were analyzed using the Axiovert Rel. 4.6 software. The confocal microscope used was a Zeiss LSM 510 confocal microscope. Lasers used included those giving excitation lines at 488nm, 546nm and 350nm. A 40X oil immersion objective lens was used for all experiments. Samples were analyzed using the LSM software.

2.6 DNA fluorescent *in situ* hybridization

2.6.1 Preparation of DNA probe

The HSV DNA probe was made by removing the HSV-1 sequence with coordinates (79442–115152) from cosmid 56 (66) using *Pac1* restriction enzyme digestion and purification of the ca. 35-kb fragment with an agarose gel DNA extraction kit (Roche). The probe was biotin labelled using the BioPrime DNA Labeling System (Invitrogen) according to the manufacturer's instructions, purified on a Chroma Spin 100 column (Clonetech) and stored in 1mL 100% ethanol. Before use 50µL of probe was combined with 15µL unlabeled human COT-1 DNA (Invitrogen), 15µL salmon sperm DNA (Invitrogen), 30µL yeast tRNA (Invitrogen), 10µL 3M sodium acetate and 25µL H_2O and precipitated with 250µL 100% ethanol. Samples were spun at 14 000 rpm at 4^oC and washed twice with 70% ethanol (spinning as above), air dried for 5-10 minutes and resuspended in 12.5µL formamide. After incubation for 10 min at 75 °C and quick cooling on ice, the probe was mixed with 12.5µL 2× hybridization buffer (1 part 20× SSC, 2 parts bovine serum albumin (10 mg/ml), 2 parts 50% dextran sulphate), boiled for 5 min, cooled on ice and incubated at 37 °C for 30 min before use. This probe can be stored at -20^oC. The prehybridization mix was made as described above, but in the place of HSV DNA, unlabeled cosmid 56 backbone derived from the initial *Pac1* digestion was added.

2.6.2 DNA fluorescent in situ hybridization

Cells grown in 4-well chamber slides (NUNC) and fixed by washing once with 50 ml of PBS in a Coplin Jar, then once with 50mL Cytoskeletal Buffer (CSK: 100 mM NaCl, 300 mM sucrose, 3 mM MgCl2, 10 mM PIPES pH 6.8) for 30 s, 50 mL CSK + 0.2% Tween-20 for 30 s, then again for 30 s with 50mL CSK. 50mL 4% paraformaldehyde was added to the Coplin Jar for 10 min. Slides were stored in 50mL 70% ethanol at 4 °C until further use. Cells were dehydrated in a 50mL ethanol series (70%, 85%, 95%, and 100%) for 2 min each, then incubated for 2 hours at 37 °C with 50 µg/ml RNase A (Invitrogen) in a humid chamber. After this incubation, the slides were washed 3 times for 5 min each at 39 °C with 50mL 2× SSC (20× SSC: 3 M NaCl, 300 mM sodium citrate), then underwent another ethanol dehydration series and were incubated for 10 min at 70 °C in a 50mL 70% formamide/2× SSC solution. The slides again underwent a dehydration series using ice-cold ethanol and were completely dried before the prehybridization step. 3 µl of prehybridization probe was added to the slides and spread under parafilm. The slides were incubated for 1 hour at 37 °C in a humid chamber, after which time the parafilm was removed and 3 µl of biotin-labeled probe was added. The slides were then incubated in the humid chamber at 37 °C overnight. The next day, slides were washed twice at 39 °C in 50mL 1× SSC/50% formamide for 5 min each, once for 5 min at 39 °C in 50mL 2× SSC and twice for 5 min at 39 °C in 50mL 1× SSC. 30 µl of Fluorescein DCS (Vector Laboratories) diluted 1:50 in detection buffer (1 part 20× SSC, 1part bovine serum albumin (10

mg/ml), 3 parts H_2O) was then added to slides and spread under parafilm. The slides were incubated at 37 °C for 30 min in a humid chamber, after which they were washed once in 50mL 4× SCC at 39 °C for 5 min, once in 50mL 4× SSC/0.2% Tween-20 at 39 °C for 5 min, again in 50mL 4× SSC at 39 °C for 5 min and finally once in 50mL distilled water at room temperature for 5 min. 70 µl of Vectashield was placed on slides, and coverslips were added and sealed with clear nail polish.

2.7 Chromatin immunoprecipitation

2.7.1 Chromatin immunoprecipitation

In all ChIP experiments, the input doses of n212 and V422 were adjusted to give rise to the same levels of viral DNA as 1 PFU of KOS at 3 hours post-infection (in the presence of PAA). The appropriate MOIs (which ranged from 0.875 to 1.1) were determined by infecting cells with 1 PFU/cell of each virus for three hours and harvesting the total cellular DNA as per the ChIP protocol (up to and including the sonication step; see below). The amount of viral DNA in each sample was then assessed by real-time PCR using primers specific for the ICP27 gene.

This protocol is adapted from Cliffe and Knipe (55). $3x10^6$ HeLa or U2OS cells were grown in 100mm dishes and infected as appropriate. Cells were fixed by the addition of 270µL of formaldehyde to a final concentration of 1% for 10 minutes. Cross-linking was halted by the addition of 125mM (1.15 mL)

glycine for 5 minutes. Cells were washed with PBS and scraped into 1mL cold PBS and centrifuged at 4000 rpm for 5 minutes at 4°C. The pellet was resuspended in 200μL 1% SDS lysis buffer (1% SDS, 10mM EDTA, 50mM Tris pH 8.1), left for 10 minutes on ice and diluted with 400μL with RIPA buffer (0.1% SDS. 1% sodium deoxycholate, 150mM NaCl, 10mM Na_2PO_4, 2mM EDTA, 1% NP40). Samples were sonicated with a Model 550 Fisher Scientific Sonic Dismembrator at level 3.8 using 20 sec pulses to obtain DNA fragmented to 200-500bp. Samples were spun at 14 000 rpm for 10 minutes at 4°C. Supernatants were removed (600μL) and 400μL RIPA buffer was added. Samples were pre-cleared with 40μL protein A agarose/salmon sperm DNA (Millipore) for one hour rotating at 4°C. After this time, samples were spun at 3000 rpm at 4°C for 1 minute and the supernatant was removed to a new tube. 10μL of sample was removed at this point as 'Input' DNA and stored at -20°C. Protein-DNA complexes were immunoprecipitated with 5μg anti-histone H3 antibody (Abcam, ab1791) or 10μg anti-acetyl-histone H3 antibody (Millipore 06-599) overnight, rotating at 4°C. Immune complexes were collected by incubation with 30μL protein A-agarose/salmon sperm DNA for 30 minutes at 4°C with rotating, followed by centrifugation at 3000 rpm for one minute at 4°C. Supernatant was removed and washes were performed at 4°C for 5 minutes with 1mL low salt (150mM NaCl, 20mM Tris pH 8.1, 2mM EDTA, 1% Triton X-100, 0.1% SDS), high salt (500mM NaCl, 20mM Tris pH 8.1, 2mM EDTA, 1% Triton X-100, 0.1% SDS) and LiCl buffers (0.25M LiCl, 1% NP40, 1% sodium deoxycholate, 1mM EDTA, 10mM Tris pH 8.1), and then two washes with TE

(10mM Tris pH 7.5, 1mM EDTA). Beads were pelleted at low speed between washes and supernatants removed as described above. Protein-DNA complexes were eluted in 200µL elution buffer (1% SDS, 0.1M $NaHCO_3$) warmed to 65°C, rotated for 15 minutes at room temperature, incubated for 15 minutes at 65°C and rotated for another 15 minutes at room temperature. Samples were centrifuged at room temperature at 3000 rpm. Eluates were incubated at 65°C for at least 4 hours in the presence of 200mM NaCl and 1µg RNase A.

2.7.2 Isolation of immunoprecipitated DNA

DNA was isolated using the Qiagen PCR Purification Kit according to the manufacturer's instructions. DNA was eluted in 100µL H_20 warmed to 65°C and the column was incubated at 65°C for 5 minutes.

2.8 Real-time PCR

10uL PCR reactions were prepared using 2µL of DNA (from above immunoprecipitation), 1.6µM of each primer and 2X Sybr Green Mix (20mM Tris pH 8.3, 100mM KCl, 6mM $MgCl_2$, 1.6% Glycerol, 0.02% Tween-20. 4% DMSO, 0.4mM dNTPs (each), 0.06U/µL Platinum *Taq*, 1X Sybr Green). The PCR primers used are listed in Chapter 5. Real-time PCR was performed using an Eppendorf Mastercycler Realplex^2 PCR machine and the standard 2-step PCR program.

2.8.1 Design of Real-time PCR experiments

Primers for Real-time PCR were designed using the PrimerQuest program from Integrated DNA Technologies (http://www.idtdna.com/Scitools/Applications/Primerquest/), using standard settings for Real-time PCR primer design. All primers were designed with an annealing temperature of 60°C. Efficiency of primer amplification was tested using DNA harvested from HeLa cells infected with KOS virus at an MOI of 10 for 3 hours. 10-fold serial dilutions were performed and the RealPlex PCR program (Eppendorf) was used to calculate the efficiency of each primer set. The specificity of each primer pair was analyzed using melting curves of each PCR product.

Samples were analyzed in duplicate and relative copy numbers were estimated by comparison with a standard curve generated by a 10-fold dilution series of pooled input samples. The C_t values for the samples in the dilution series was plotted against log[relative DNA copy] (10000 – 10) and the slope of the curve and R^2 value were determined. Only experiments where the slope of the curve was close to 3.3 ($2^{\sim 3.3}$ = 10) and R^2 values were no less than 0.98 were the samples further analyzed. The relative DNA copy number for each experimental sample was then calculated by using each C_t value in the equation obtained from the dilution series curve.

Relative DNA copy numbers were obtained both for the Input DNA and the immunoprecipitated DNA for each experimental sample, and were compared:

IP DNA/Input DNA. In the case of comparing multiple IPs: [IP (1) DNA/Input (1) DNA] / [IP DNA (2)/Input DNA (2)].

Chapter Three: Herpes Simplex virus regulatory proteins VP16 and ICP0 counteract an innate intranuclear barrier to viral gene expression

A version of this chapter has been published:

Hancock MH, Corcoran JA, and Smiley JR. 2006. Virology **352**(1):237-52.

Preface to Chapter 3

The work in this chapter was performed as a means to examine the basis for the permissivity of U2OS cells to VP16 and ICP0 mutants. It was originally suggested By Yao and Schaffer that U2OS cells encoded an 'ICP0-like' activator of viral gene expression; however, this hypothesis had never been tested. I felt it was equally possible that this cell type was lacking a component of an innate antiviral response that prevents viral gene expression in the absence of these transcriptional activators and set out to distinguish between these possibilities.

The publication entitled "Herpes Simplex virus regulatory proteins VP16 and ICP0 counteract an innate intranuclear barrier to viral gene expression" is the content of Chapter three of this thesis. This work was published online June 5th 2006 and available in print August 2006 in the journal Virology. Jennifer A. Corcoran suggested the use of the reovirus p14 fusion protein as a means of forming heterokarya and hence was added as an author on this publication. All experiments were performed by me, while writing and editing was shared between Dr Smiley and myself.

ABSTRACT

HSV regulatory proteins VP16 and ICP0 play key roles in launching the lytic program of viral gene expression in most cell types. However, these activation functions are dispensable in U2OS osteosarcoma cells, suggesting that this cell line either expresses an endogenous activator of HSV gene expression or lacks inhibitory mechanisms that are inactivated by VP16 and ICP0 in other cells. To distinguish between these possibilities, we examined the phenotypes of somatic cell hybrids formed between U2OS cells and highly restrictive HEL fibroblasts. The U2OS-HEL heterokarya were as non-permissive as HEL cells, a phenotype that could be overcome by providing either VP16 or ICP0 *in trans*. Our data indicate that human fibroblasts contain one or more inhibitory factors that act within the nucleus to limit HSV gene expression, and argue that VP16 and ICP0 stimulate viral gene expression at least in part by counteracting this innate antiviral defence mechanism.

INTRODUCTION

Innate immune defences protect eukaryotic organisms from harmful invaders by coupling sensor-mediated recognition of common pathogen-associated molecular patterns to potent and specialized anti-pathogen responses (43, 66). In the case of antiviral defence in vertebrates, these responses include a variety of mechanisms that act within infected cells to inhibit virus replication. Some of these antiviral systems are broadly active, being able to inhibit many viruses; examples include the type I interferon (IFN) response and host apoptotic and RNA silencing pathways (2, 30, 91). Others, such as the retrovirus restriction factors APOBEC3G and TRIM5α are more selective and target only a subset of viruses (28). Given the effectiveness of these varied intracellular antiviral defences, it is not surprising that most if not all viruses encode gene products that inactivate one or more of these host systems (2, 30, 91). Indeed, mounting evidence documents that the host-range of many viruses is determined by the balance between host innate defences and viral countermeasures (56, 88, 93).

An effective viral suppressor can mask the effects of an otherwise potent cellular defence mechanism, hindering the initial discovery of the antiviral mechanism. However, mutations that inactivate the viral suppressor often give rise to a host-range phenotype such that the mutant virus is able to replicate efficiently only in cells that lack the cognate host defence mechanism (8, 56, 88, 93). Such viral mutants and permissive cell variants provide powerful tools for the discovery and characterization of novel innate antiviral defence mechanisms.

In particular, somatic cell fusion experiments that probe the basis for host-range phenotypes have, in several cases, provided the first evidence that the viral protein in question acts by overcoming a cellular barrier to virus replication in restrictive cells (10, 55, 65, 82). For example, HIV mutants lacking the viral vif protein can be propagated in only a subset of T cell lines in culture (26, 87, 92). Studies of somatic cell hybrids formed between permissive and non-permissive cell lines revealed that the non-permissive phenotype is dominant, implying that such cells contain a trans-acting antiviral factor that is inactivated by vif (10, 82). These findings set the stage for the subtractive hybridization experiments that identified the cellular cytosine deaminase APOBEC3G as the antiviral factor targeted by vif (81).

In this report we present the results of somatic cell fusion experiments that probe the basis for the host-range phenotype of a herpes simplex virus type 1 (HSV-1) mutant that lacks functional ICP0 and VP16, two proteins that stimulate viral gene expression during the earliest stages of infection. As elaborated below, our data argue that VP16 and ICP0 act at least in part by overcoming one or more cellular barriers to viral gene expression.

HSV-1 is a significant human pathogen and the prototypical member of the herpesviridae, a family of large enveloped nuclear DNA viruses. Depending on the nature of the host cell, the virus either undergoes productive replication leading to cell death or establishes a latent infection (reviewed in (77)). The HSV

lytic cycle involves the temporally regulated expression of three sets of viral genes: immediate-early (IE), early (E), and late (L). This genetic program is initiated by the viral tegument protein VP16, which is released into the cell following fusion of the viral envelope with the host plasma membrane. VP16 binds the cellular factors HCF and Oct-1 and the resulting tripartite complex then associates with specific target sequences within the viral IE promoters (68, 73, 85, 96). The C-terminus of VP16 serves as a strong acidic transcriptional activation domain (89) that recruits factors involved in transcription initiation to the viral IE promoters (37, 45, 47, 58, 60, 90, 95). VP16 stimulates the expression of five IE genes, four of which encode proteins that collaborate to drive progression of the lytic cycle into the E and L stages. One of these, ICP0, appears to act upstream of the other IE proteins, as it is required along with VP16 for efficient IE gene expression (4, 5, 20, 86). Thus, the functions of VP16 and ICP0 seem to be interrelated or overlapping. Consistent with this view, ICP0 expressed *in trans* can partially complement the transcriptional defect of VP16 mutants (1, 34).

Viral mutants lacking the activation functions of ICP0 or VP16 display a greatly increased particle-to-plaque forming unit (PFU) ratio and substantially lower levels of IE gene expression upon low multiplicity infection of many cell types (1, 5, 16, 83, 86). The severity of the mutant phenotype varies between cell types, with primary human fibroblasts being the most restrictive (20). In the absence of either or both of these proteins, the viral genome often fails to engage the lytic program of gene expression in restrictive cells, and instead persists in a

transcriptionally silent, extrachromosomal state (1, 20, 35, 36, 41, 72, 74, 78, 79, 86). Such quiescent genomes appear to be actively repressed, as otherwise constitutively active heterologous promoters embedded within them are also silenced (41, 59, 74, 79). Thus, VP16 and ICP0 appear to modulate the fate of newly delivered viral genomes, diverting them away from one or more cellular gene silencing systems and towards a lytic infection.

ICP0 behaves as a promiscuous transactivator of viral and cellular genes in transient cotransfection assays (15, 27, 69, 75), reviewed in (18), and it stimulates expression of HSV genes belonging to all three temporal classes during lytic infection (4, 5, 16, 20, 42, 80, 86). A plausible hypothesis is that it stimulates viral gene expression at least in part by counteracting the cellular silencing mechanisms alluded to above (reviewed in (18, 19, 72)). Consistent with this hypothesis, ICP0 suffices to block entry into quiescence and is able to reactivate silenced genomes (35, 36, 41, 72, 74, 79, 86). Little is known about the mechanisms underlying silencing and reactivation of quiescent HSV genomes; however data obtained by Preston and colleagues indicate that the newly delivered viral genome is gradually assembled into a functionally inaccessible configuration that precludes the action of many trans-acting factors, including VP16 and the viral DNA replication machinery (74), reviewed in (72). ICP0 is unique among HSV proteins in its ability to reactivate these globally repressed genomes, and such genomes are neither expressed nor replicated following productive superinfection with ICP0-deficient HSV (35, 36, 38, 59, 78). It is tempting to

speculate that these anti-repression activities of ICP0 also underlie its ability to promote efficient IE gene expression during the earliest stages of a normal HSV infection. However, the HSV IE promoters residing within genomes destined for quiescence remain responsive to transacting factors for at least one hour after genome delivery, and repression has been documented only after 24 hours exposure to the restrictive cellular environment in the absence of ICP0 (74). Thus, it is currently unknown whether ICP0 stimulates gene expression at early times postinfection by counteracting host inhibitory mechanisms.

ICP0 is an ubiquitin E3 ligase that interacts with a growing list of cellular proteins, causing the degradation of some (reviewed in (18)). Its E3 ligase function is essential for transactivation and reactivation of quiescent viral genomes, and these processes are blocked by proteasome inhibitors (3, 14, 24, 33). These observations suggest that ICP0 may act by targeting cellular inhibitory proteins for ubiquitination and degradation. ICP0 is known to interact with type II histone deacetylases (52) and components of the CoREST/REST transcriptional repression complex (32), suggesting a possible link with cellular repression mechanisms and chromatin modifications. In addition, ICP0 causes the degradation of the histone variant CENP-A (51), the centromeric protein CENP-C (21) and the catalytic subunit of DNA-PK (48, 70), although the functional significance of these findings remains unknown. ICP0 also causes the degradation of several ND10 component proteins, including the main structural protein PML (7, 22, 23). ND10 domains are distinct nuclear substructures that

appear to play roles in senescence (71), apoptosis (39), DNA repair (12, 19), signal transduction (50) and viral replication (57, 67). Interestingly, incoming HSV genomes induce *de novo* formation of adjacent nuclear ND10 bodies (25), and destruction of these domains has been correlated with ICP0 activation function (22, 23). ICP0 is also critical for the resistance of HSV-1 to type I interferon (IFN) (13, 61, 62). Several ND10 components are IFN-inducible (29, 31, 84) and the antiviral effect of IFN is abrogated in $PML^{-/-}$ mouse embryo fibroblasts (6). However, the precise roles of ND10 domains in modulating HSV gene expression and ICP0 function are not fully understood.

HSV-1 mutants lacking the activation functions of either ICP0 or VP16 display a host-range phenotype: they are impaired for growth in primary human fibroblasts and many established cell lines, but replicate efficiently in U2OS osteosarcoma cells (83, 97). A viral mutant (KM110) that lacks both functions displays a similar but greatly exaggerated phenotype, being incapable of lytic growth or viral gene expression in normal fibroblasts even after high multiplicity infection (63). One possibility, originally proposed by Yao and Schaffer (97), is that U2OS cells express a cellular factor that activates HSV-1 gene expression, thus obviating the requirement for ICP0 and VP16 function; another is that the permissive U2OS cells lack one or more cellular antiviral mechanisms that are targeted by ICP0 and VP16. In order to distinguish between these two possibilities we have performed somatic cell fusion assays similar to those used to document the dominant blocks enforced by APOBEC3G and TRIM5α to

retroviral replication (10, 55, 65, 82). We found that heterokarya formed between permissive and restrictive cells are completely non-permissive for KM110 IE gene expression, indicating that restrictive cells contain one or more rapidly acting dominant inhibitory factors that are absent from permissive U2OS cells. VP16 and ICP0 were each able to override the non-permissive phenotype; in contrast, as previously documented (74), only ICP0 was able to reactivate the viral genome following extended exposure to the restrictive cellular environment. Our results therefore document a dominant, innate, multi-step viral gene silencing pathway which can be countermanded by either VP16 or ICP0 at early times postinfection.

RESULTS

KM110 displays a strict host-range phenotype.

As reviewed in the Introduction, HSV-1 mutants lacking the activation functions of VP16 or ICP0 often enter a transcriptionally silent state following infection of restrictive cells such as human embryonic lung (HEL) fibroblasts. The HSV-1 strain KOS isolate KM110 lacks the activation functions of both VP16 and ICP0, and consequently displays a greatly exaggerated phenotype relative to either singly mutant virus. KM110 does not express its IE genes or replicate following high MOI infection (10 PFU/cell) of HEL cells; the cells survive infection and the viral genome persists in a quiescent state for up to ten days (63). In contrast, KM110 is able to replicate to close to wild-type levels in permissive U2OS osteosarcoma cells (63). To determine if this striking host-range phenotype can be readily visualized at the level of individual cells in mixed cultures, the experiment depicted in Figure 3.1 was performed. HEL and U2OS cells were stained red and blue respectively with the CellTracker probes CMTMR and CMAC, then mixed together in a culture dish. (Control experiments confirmed that these and other dyes used in this study had no detectable effect on viral gene expression or plaquing efficiency; Figure 3A.1). The mixed culture was then infected with wild type HSV-1 strain KOS (Figure 3.1A and 3.1B) or KM110 (Figure 3.1C and 3.1D) at 10 PFU/cell. Nine hours later the cells were fixed and scored for expression of the viral IE protein ICP4 by indirect immunofluorescence (IF) or viral DNA replication by fluorescent *in situ* hybridization (DNA FISH) using a probe spanning a large portion of the viral

genome. Note that the FISH assay was conducted under conditions that do not detect unreplicated input viral genomes. As shown in Figure 3.1A and 3.1B, essentially every cell in the mixed culture was infected by wild-type HSV-1, as evidenced by ICP4 protein production and the formation of large intranuclear compartments containing replicated viral DNA (replication compartments). As expected given the late time point analyzed, in most cells the ICP4 staining localized to large intranuclear structures shown previously to correspond to replication compartments (46). In contrast, only the U2OS cells (blue) displayed evidence of ICP4 protein production and viral genome replication in the cultures infected with KM110: the HEL cells (red) present in the same culture were uniformly negative (Figure 3.1C and 3.1D). These results confirm the strict host-range phenotype of KM110 and suggest that the single cell assays used in this experiment are suitable for assessing the permissivity of somatic cell hybrids formed between U2OS and HEL cells.

Fusion *per se* does not affect the phenotype of U2OS or HEL cells

In order to determine whether hybrids of permissive and non-permissive cells support or block the replication of KM110, we developed a transient heterokaryon assay (outlined in Figure 3.3 below). We elected to use the fusogenic reovirus protein p14, a member of the fusion-associated small transmembrane (FAST) protein family, as the fusion agent in these experiments (9). p14 is a non-structural protein encoded by a reptilian reovirus that promotes cell-cell fusion, aiding rapid reovirus dissemination (9). Cells transfected with a

p14 expression vector rapidly express p14 on their surface and efficiently fuse with neighbouring non-expressing cells, offering a simple and readily manipulated means of efficiently creating somatic cell hybrids. However, as neither the mechanism of p14-induced fusion nor its downstream effects on other cellular processes are fully understood, we first asked if p14-mediated cell-cell fusion alters the phenotypes of U2OS or HEL cells with respect to the host-range of KM110.

To assess the effects of fusion on U2OS cells, the cells were stained red with methyl bromide and one culture was transfected with a p14 expression plasmid; six hours later the transfected cells were mixed with those of a parallel culture of U2OS cells that had been infected with 10 PFU/cell KM110 one hour previously. The mixed culture was then incubated for nine hours, the last seven of which were in the presence of a polyclonal rabbit anti-p14 serum to slow the fusion process and prevent large homokarya from forming. The cells were then fixed and scored for ICP4 production by IF. ICP4 protein was observed in large replication compartment structures in 14 of 14 homokarya examined (for example, Figure 3.2A) and in most unfused cells. These data indicate that U2OS cells remain permissive to KM110 following homotypic fusion.

A similar experiment was performed using HEL cells stained green with CFMDA in order to determine whether fusion alters their restrictive phenotype. Although the transfection efficiency was much lower in these primary cells (ca.

5%), homokarya could nevertheless be identified. KM110 failed to produce ICP4 in any of the five homokarya examined, suggesting that the restrictive phenotype was maintained (Figure 3.2B). To ensure that these restrictive HEL homokarya contained KM110 genomes that were potentially capable of gene expression, we co-transfected a plasmid bearing an ICP0 cDNA driven from the HMCV IE promoter along with the p14-expression plasmid into HEL cells. When these cells were fused with HEL cells previously infected with KM110, ICP4 replication compartments were evident in ten of fourteen homokarya examined (eg., Figure 3.2C). These results indicate that the p14-mediated cell-cell fusion process does not alter the restrictive phenotype of HEL cells with respect to KM110 replication and demonstrate that ICP0 provided *in trans* is able to render HEL cells permissive for KM110 IE gene expression.

Heterokarya formed between U2OS and HEL cells restrict KM110 replication in the absence of viral activators

It has been suggested that U2OS cells express an ‘ICP0-like’ function that activates viral gene expression in the absence of the transactivators VP16 and ICP0 (97); alternatively, it is possible that these cells lack a cellular gene silencing mechanism or other barrier to virus replication present in restrictive HEL cells. Under the first scenario, heterokarya of permissive U2OS and restrictive HEL cells are predicted to be permissive for the replication of KM110, as the activating factor contributed by the U2OS cells should be able to act upon the KM110 genome in the hybrid cells. In contrast, the second scenario predicts that the

heterokarya would not support viral replication, as the incoming genome would be silenced by the repressive mechanism contributed by the HEL cells.

To distinguish between these possibilities we used the p14-mediated somatic cell fusion assay. A timeline of these experiments is outlined in Figure 3.3. U2OS cells were stained blue with CMAC, then transfected with the p14 expression plasmid. Five hours later the cells were mixed with HEL cells stained red with CMTMR that had been infected with 10 PFU/cell KM110 one hour previously. (The HEL cells were washed with an acid glycine solution prior to the addition of the U2OS cells to remove any residual bound virus that had not yet penetrated the plasma membrane). The mixed cultures were then incubated for a total of nine hours in the presence of neutralizing human anti-HSV serum. Anti-p14 serum was present during the last seven hours of this incubation period, to slow the cell fusion process. The cells were then fixed and scored for ICP4 expression and viral DNA replication by IF and DNA FISH, as above. Note that in this experimental design the parental KM110 genomes were delivered into the restrictive HEL cells prior to the introduction of the U2OS cells.

Heterokarya were identified under the confocal microscope as cells with multiple nuclei that fluoresced in both the red and blue channels, indicating fusion between red HEL and blue U2OS cells. In most experiments both types of nuclei retained their characteristic staining during the sample work-up, allowing straightforward discrimination between the U2OS and HEL nuclei present within

individual heterokarya (see for example Figure 3.4). However, the blue CMAC stain was occasionally lost from the U2OS nuclei during the DNA-FISH procedure (for example, Figure 3.6 A and 3.6 B); in such cases the HEL nuclei were readily identified on the basis of their red staining and the U2OS nuclei appeared as unstained areas in both the blue and red channels. Using this procedure, we found that the heterokarya formed between U2OS and HEL cells failed to support viral IE protein production or KM110 genome replication (Figure 3.4A and 3.4C). All of the several hundred heterokarya examined over the course of six independent experiments were uniformly negative; twenty-six and fifteen examples of these were photographed after staining for ICP4 or viral DNA, respectively. These observations suggest that U2OS cells do not harbour a transacting factor capable of substituting for VP16 and/or ICP0; rather the data imply that HEL cells contain one or more dominantly acting negative factors that are able to extinguish the permissive phenotype exhibited by U2OS cells. In order to confirm that the KM110 genomes delivered to the HEL cells prior to fusion with the U2OS cells were potentially capable of undergoing replication in the presence of transactivating proteins, in each of the six experiments described above some of the U2OS cells were co-transfected with an ICP0 expression vector and the p14 expression plasmid. When ICP0 was thus delivered into the infected HEL cells via cell fusion approximately 75% of the heterokarya examined supported IE protein production and the infecting virus underwent genome replication (twenty-eight and seventeen examples of the heterokarya positive for ICP4 and viral DNA respectively were photographed; Figure 3.4B

and 3.4D). Therefore, ICP0 provided *in trans* is capable of activating a viral genome residing in an otherwise restrictive heterokaryon.

Previous work by Preston and Nicholl has shown that the IE promoters of HSV-1 genomes destined for quiescence are initially delivered into cells in a form that is susceptible to trans-activation by VP16 (74). In order to determine if this is also the case in our system, we also tested the ability of a VP16 expression plasmid to transactivate the viral genome in three of the experiments described above (Figure 3.5). The results indicated that VP16 was also capable of activating the newly delivered viral genome as evidenced by IE protein production and viral genome replication in ca. 75% of the heterokarya (eighteen and seven examples of these positive heterokarya respectively were photographed; Figure 3.5B and 3.5D). Hence, VP16, like ICP0, can overcome the rapid, dominant silencing mechanism that prevents IE gene expression in HEL cells shortly after infection.

Evidence for rapid establishment of a restrictive nuclear environment in heterokarya

An interesting observation made throughout the foregoing experiments was that a viral DNA FISH signal was detected in both the U2OS and HEL nuclei within heterokarya when KM110 replication was triggered by ICP0 or VP16 (see Figure 3.4D and 3.5D). The finding that the replicated DNA signal was present in both types of nuclei was at first glance surprising, because the virus was initially

delivered to the HEL cells prior to fusion with the U2OS cells. The observation therefore raised the possibility that not all of the input KM110 virions had productively docked with or delivered their genomes into the HEL cell nuclei prior to fusion with the U2OS cells, leaving some virions free to deliver their genomes to the newly recruited U2OS nuclei. Alternatively, and in our view much less likely, the U2OS nuclei might have been infected by transfer of un-encapsidated viral DNA between nuclei within the heterokaryon, or via progeny virions being misdirected to another nucleus within the heterokaryon during viral egress.

To distinguish between these possibilities we asked if the incoming KM110 genomes became more strictly committed to the HEL nuclei if the period between the initial infection and the onset of fusion with the U2OS cells was increased from one hour (as in the preceding experiments) to three, twelve, and forty-eight hours. As expected according to the nuclear delivery model, when fusion with U2OS cells expressing ICP0 was initiated three or more hours after infection, the DNA FISH signal was detected in only the HEL nuclei of the heterokarya (Figure 3.6 B and D, Figure 3.7 D, and data not shown; note that the CMAC staining of the U2OS nuclei was lost in the experiment depicted in Figure 3.6 A and B but retained in the experiment shown in Figure 3.6 C and D). These results strongly argue that nuclear genome delivery is complete within three hours of infection and imply that the DNA FISH signals within U2OS nuclei observed in the one-hour experiments depicted in Figure 3.4D, 3.5D, 3.6A and 3.6C arise

from U2OS nuclei that were infected with input KM1110 genomes shortly after the onset of cell fusion. If so, then the finding that such infected U2OS nuclei fail to the launch the viral lytic program in the absence of ICP0 or VP16 indicates that the restrictive factors present in HEL cells rapidly render newly recruited U2OS nuclei non-permissive for KM110 replication.

Loss of VP16 responsiveness at late times

The preceding data suggest that ICP0 and VP16 are both capable of antagonizing the action of one or more dominant inhibitory mechanisms that otherwise block viral IE gene expression in restrictive HEL cells. The cellular inhibitory mechanism(s) operate during the earliest stages of infection, and rapidly render U2OS nuclei non-permissive following cell fusion. Preston and colleagues have shown that the viral IE promoters gradually lose their ability to respond to VP16 provided *in trans* over the course of several days as HSV-1 genomes enter quiescence in restrictive cells (74). Such genomes, however, continue to respond to ICP0 even after responsiveness to VP16 has been lost (74). The repression documented by Preston and colleagues differs from the negative regulation that we have detected in that repression was manifest only after 24 hours, while the dominant inhibition that we describe acts very rapidly. To determine if the repressed state characteristic of genome quiescence can be detected using our heterokaryon system, we infected HEL cells with 10 PFU/cell of KM110 two or four days prior to fusion with p14-expressing U2OS cells. As expected based on the preceding experiments, the quiescent viral genome was not

activated following fusion of the HEL cells with U2OS cells in three independent experiments (Figure 3.7, Figure 3.8 and data not shown). When ICP0 or VP16 were provided *in trans*, only ICP0 was capable of reactivating the genome as evidenced by ICP4 protein production and viral genome replication (compare Figure 3.7B and 3.7D to Figure 3.8B and D). We note that reactivation mediated by ICP0 occurred in a lower proportion of the heterokarya (approximately 25%) than was observed when cell fusion was initiated one hour of infection (approximately 75%). The basis for this difference in efficiency remains unclear. These data confirm the observations of Preston and colleagues (74), and suggest that HSV genome silencing is a multi-step process which includes one or more rapidly acting innate barriers to viral gene expression that can be overridden by either VP16 or ICP0, followed by the slower establishment of a globally repressed state that can be counteracted only by ICP0.

DISCUSSION

Our data provide strong evidence that human fibroblasts express one or more inhibitory factors that serve as an innate barrier to the activity of incoming HSV genomes. We further show that the HSV regulatory proteins VP16 and ICP0, long known to play key roles in launching the HSV lytic cycle, stimulate viral gene expression at least in part by counteracting this host inhibitory system. These studies were made possible by the availability of three key biological reagents: a permissive cell line, U2OS, in which HSV mutants lacking the activation functions of ICP0 and/or VP16 replicate freely (83, 97); a viral VP16/ICP0 mutant, KM110, that is unable to replicate in other cell types (63); and reovirus p14, a fusogenic protein which allows rapid and efficient formation of heterokarya (9). The exceptionally tight host-range phenotype of KM110 was an essential feature of our experimental design, as it allowed us to load essentially every cell in the culture with viral genomes via high MOI infection without provoking detectable viral gene expression in the restrictive HEL cells. Using these reagents, we show that the permissive phenotype of U2OS cells is extinguished in somatic cell hybrids formed with restrictive HEL fibroblasts, indicating that U2OS cells lack the inhibitory mechanism that precludes replication of KM110 in other cell types. The inhibitory mechanism acts rapidly and *in trans* to prevent expression of newly delivered viral genomes, as documented by the finding that U2OS nuclei within heterokarya fail to support KM110 gene expression or replication, even when these nuclei are seeded with viral genomes (Figures 3.4-3.6). Inasmuch as previous studies have shown that

viral genomes enter the nucleus in the absence of VP16 or ICP0 function (36, 40, 41) the simplest interpretation is that the inhibitory system presents an intranuclear barrier to viral gene expression. Our data therefore indicate that one or more factors contributed by HEL cells are able to reconstitute an effective nuclear barrier in U2OS nuclei. However, it is important to stress that this result does not necessarily imply that the defect in U2OS cells resides in the nuclear repression machinery *per se*. Indeed, the very rapid induction of a restrictive intranuclear state following cell fusion is consistent with the possibility that the U2OS cells contain an intact nuclear repression machinery but lack a signal transduction pathway required for its activity. Also consistent with a signalling defect, reconstitution of the repression system following cell fusion appears remarkably robust, as it is insensitive to the relative contributions of HEL and U2OS cells to individual heterokarya: heterokarya containing many more U2OS than HEL nuclei remained completely non-permissive (Figure 3A.1). Thus, the potential artefact of 'diluting out' a potential trans-acting factor from the U2OS cell is most likely not occurring.

Previous genetic analysis has indicated that VP16 and ICP0 make largely independent contributions to triggering the onset of the HSV lytic cycle, as the phenotype of the VP16/ICP0 mutant KM110 is much more severe than those of its singly mutant parents in restrictive cells (63). However, the finding that KM110 and its parents replicate freely in U2OS cells suggests that the functions of VP16 and ICP0 are somehow related. Consistent with this hypothesis, we show

here that the inhibitory mechanism present in HEL cells imposes requirements for both viral proteins in heterokarya formed with U2OS cells. Moreover, this barrier can be at least partially overcome at early times by over expressing either VP16 or ICP0. These findings lead us to propose that gene expression is inhibited via a multi-step antiviral gene silencing pathway, and that VP16 and ICP0 each serve as (incompletely effective) inhibitors of separate steps in this pathway. We further suggest that this gene silencing pathway is completely inactive in U2OS cells, accounting for the ability of these cells to "complement" defects in either or both viral functions. Although this model is plausible and consistent with all of the available data, we cannot at present exclude the alternative possibility that U2OS cells are defective in two or more separate anti-viral pathways, accounting for their ability to "complement" lesions in both VP16 and ICP0.

Our finding that newly delivered HSV genomes are rapidly subjected to negative regulation by the host cell is novel, as is our evidence supporting roles for both VP16 and ICP0 in countermanding this inhibition. As reviewed above, ICP0 is able to reactivate repressed quiescent HSV genomes (35, 36, 38, 59, 72, 74, 78, 79). We show here that ICP0 also acts at very early times post-infection to antagonize negative regulation by the host cell, long before the stable repression characteristic of quiescence has been established. These data indicate that the early transactivation function of ICP0 is accomplished, at least in part, by counteracting a constitutive innate barrier to viral gene expression. Such an activity for ICP0 has long been suggested (17, 18, 24, 32, 52), but never directly

demonstrated. It will be interesting to learn whether and how this activity relates to known biochemical activities of ICP0, such as its ability to target host proteins such as PML for degradation. A key question is whether early transactivation and reactivation of quiescent viral genomes are accomplished by the same mechanisms.

VP16 is often regarded as a direct activator of the HSV IE genes, a role that is consistent with its ability to recruit components of the transcription initiation complex such as RNA polymerase II and TF IIB to viral IE promoters (37). However studies of the activity of the VP16 activation domain in heterologous systems have revealed that it also targets chromatin-remodelling coactivators and adaptor proteins, including histone acetyltransferases (HATs) and ATP-dependant chromatin-remodelling complexes (45, 47, 58, 60, 90, 95). The interaction of VP16 with chromatin- remodelling proteins was initially puzzling as the HSV genome was long thought to be transcribed as naked DNA during lytic infection (49, 64). However recent evidence indicates that the newly delivered viral genome rapidly associates with a limited number of nucleosomes and that the histones associated with specific viral genes display activating modifications that change over time in response to viral regulators in a fashion that reflects the sequential activation of the IE, E and L genes during the lytic cascade (37, 44). These data suggest that chromatin modifications might be a method of cellular control of HSV gene expression. Consistent with this view, the C-terminal activation domain of VP16 has recently been shown to recruit HATs

and ATP-dependant remodelling complexes to the IE promoters during lytic infection of mammalian cells (37). In addition, the activation domain induced a specific deficit of histones over the IE promoters (37). Thus, in addition to directly activating gene expression, VP16 may also specifically prevent repression mediated by inhibitory chromatin.

The foregoing considerations raise the possibility that the host innate antiviral defence mechanism that we have documented acts by detecting the incoming HSV genome and then assembling it into repressive chromatin. Indeed, the inhibitory pathway leads to the global repression characteristic of genome quiescence, a state that is reminiscent of cellular heterochromatin. This attractive and readily testable hypothesis is consistent with many of the activities of ICP0 and VP16 described above. If chromatin structure does in fact underlie HSV genome repression, then it seems likely that a specialized DNA replication-independent chromatin assembly pathway (54) such as that mediated by the histone chaperone HIRA (76) is involved. It is interesting to note in this context that HIRA associates with ND10 and has been linked to gene silencing during cellular senescence (98); moreover it functions in *Drosophila* to load histones onto the newly delivered paternal genome immediately after fertilization (53), a situation perhaps analogous to chromatinization of the incoming HSV genome. In view of the foregoing, we speculate that U2OS cells may be defective in a signal transduction pathway that activates the nuclear repression system in response to

incoming HSV virions or genomes, or lack a specialized replication-independent chromatin assembly pathway and/or chromatin-dependent repression system.

Figure 3.1. Host range phenotype of KM110

HEL cells (stained red with CMTMR) and U2OS cells (stained blue with CMAC) were mixed and infected with 10 PFU/cell of wild type HSV-1 KOS (A and B) or KM110 (C and D) for nine hours. Cells were then fixed and scored for ICP4 (A and C) or replicated viral genomes (B and D) via IF and DNA-FISH respectively (green signals). Representative fields of view are shown. Scale bar = 10µm.

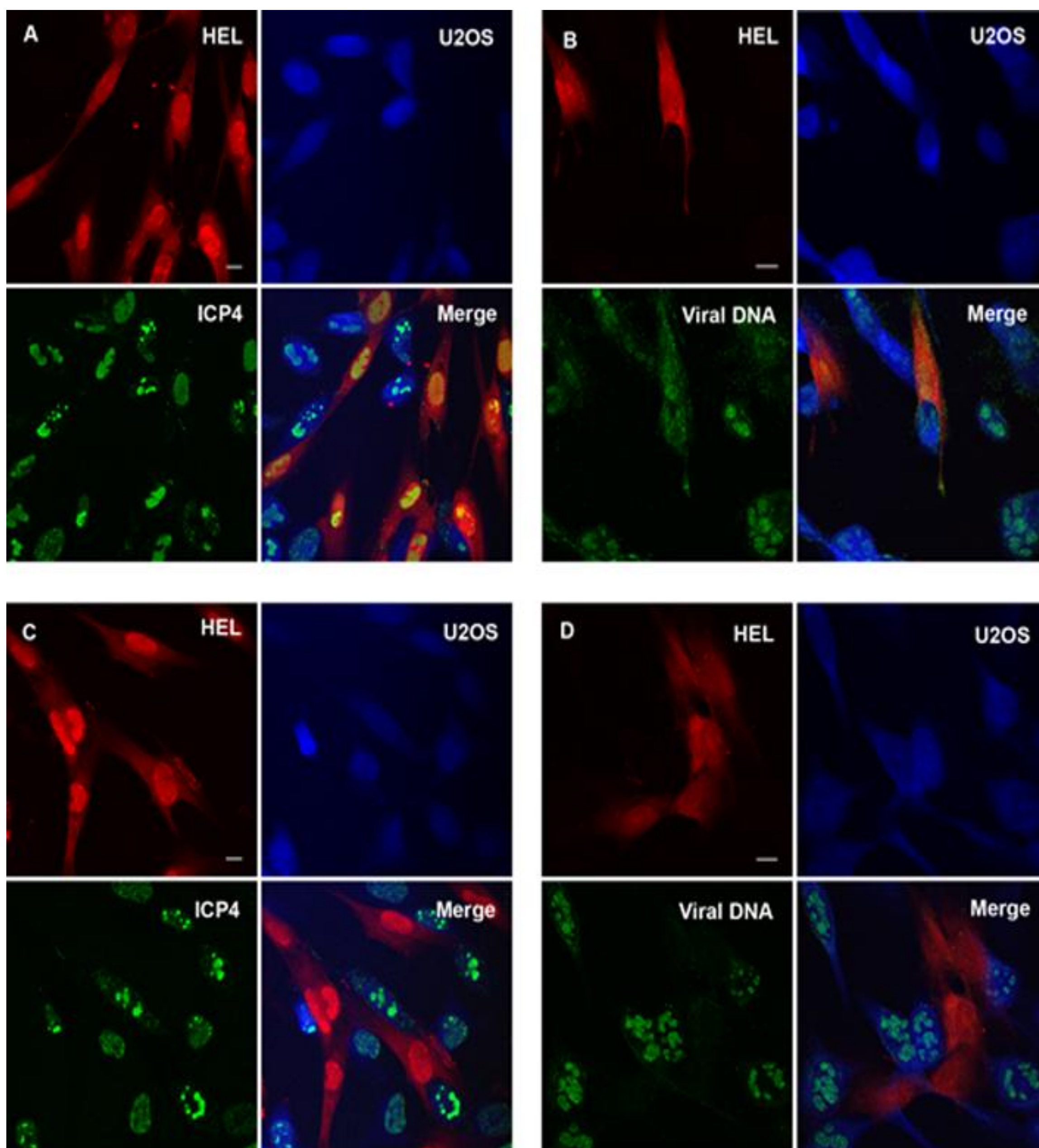

A
HEL
U2OS
ICP4
Merge
B
HEL
U2OS
Viral DNA
Merge
C
HEL
U2OS
ICP4
Merge
D
HEL
U2OS
Viral DNA
Merge

Figure 3.2. Fusion does not affect the phenotype of U2OS or HEL cells

Homokarya formed between U2OS cells (A, stained red with methyl bromide) or HEL cells (B and C, stained green with CFMDA) were scored for their permissivity to KM110. Cells transfected with pcDNA3-p14 (A and B) or pcDNA3-p14 and the ICP0 expression vector pDR27 (C) were mixed six hours post-transfection with a parallel culture that had been infected with KM110 (10 PFU/cell) one hour previously. Anti-p14 antiserum was added after two hours, and cells were fixed and scored for ICP0 via IF (blue) seven hours later. Representative homokarya are shown. Scale bar = 10μm.

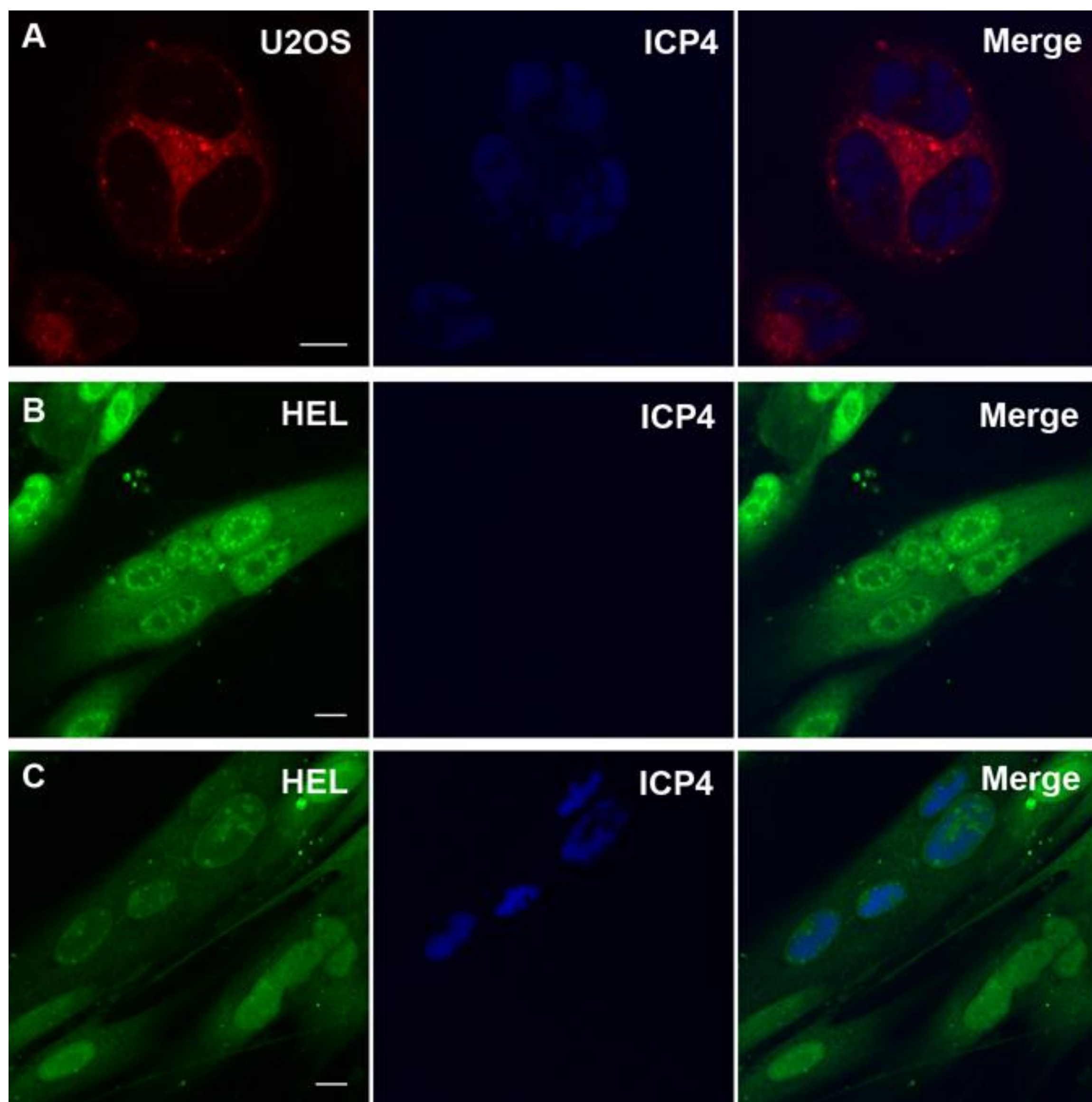

A
U2OS
ICP4
Merge
B
HEL
ICP4
Merge
C
HEL
ICP4
Merge

Figure 3.3. Experimental design

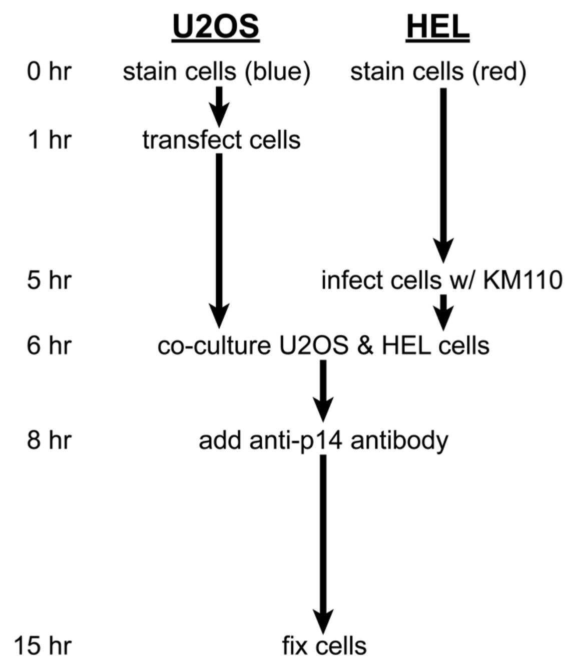

U2OS
HEL
0 hr
stain cells (blue)
stain cells (red)
1 hr
transfect cells
5 hr
infect cells w/ KM110
6 hr
co-culture U2OS & HEL cells
8 hr
add anti-p14 antibody
15 hr
fix cells

Figure 3.4. The restrictive phenotype of HEL cells is dominant in heterokarya.

HEL cells (red) were infected with 10 PFU/cell of KM110, then fused with p14-expressing U2OS cells (blue) one hour later as outlined in Figure 3.3. In panels B and D, the U2OS cells were transfected with the ICP0 expression vector pDR27 in addition to the p14 expression plasmid. Cells were fixed 9 hours later and scored for ICP4 expression (A and B) and replicated viral DNA (C and D) by IF and DNA-FISH respectively (green signals). Representative heterokarya are shown. Scale bar = 10μm.

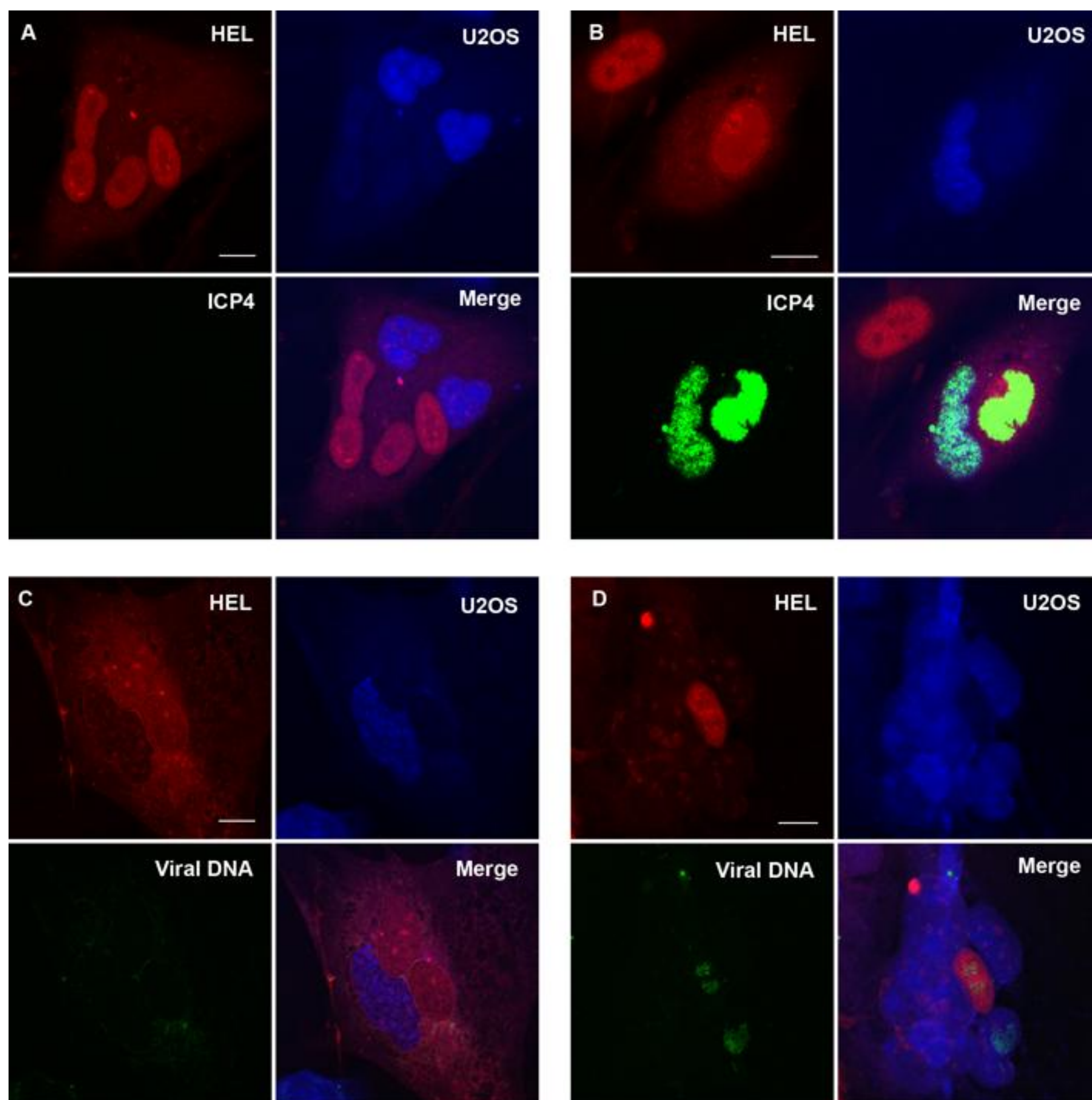

A
HEL
U2OS
ICP4
Merge
B
HEL
U2OS
ICP4
Merge
C
HEL
U2OS
Viral DNA
Merge
D
HEL
U2OS
Viral DNA
Merge

Figure 3.5. The KM110 genome responds to VP16 delivered *in trans* one hour post-infection.

HEL cells (red) were fused with U2OS cells (blue) one hour after infection with KM110, as in Figure 4. In panels B and D, the U2OS cells were transfected with the VP16 expression vector pKOS-VP16 in addition to the p14 expression plasmid. Cells were fixed 9 hours later and scored for ICP4 expression (A and B) and replicated viral DNA (C and D) by IF and DNA-FISH respectively (green signals). Representative heterokarya are shown. Scale bar = 10μm.

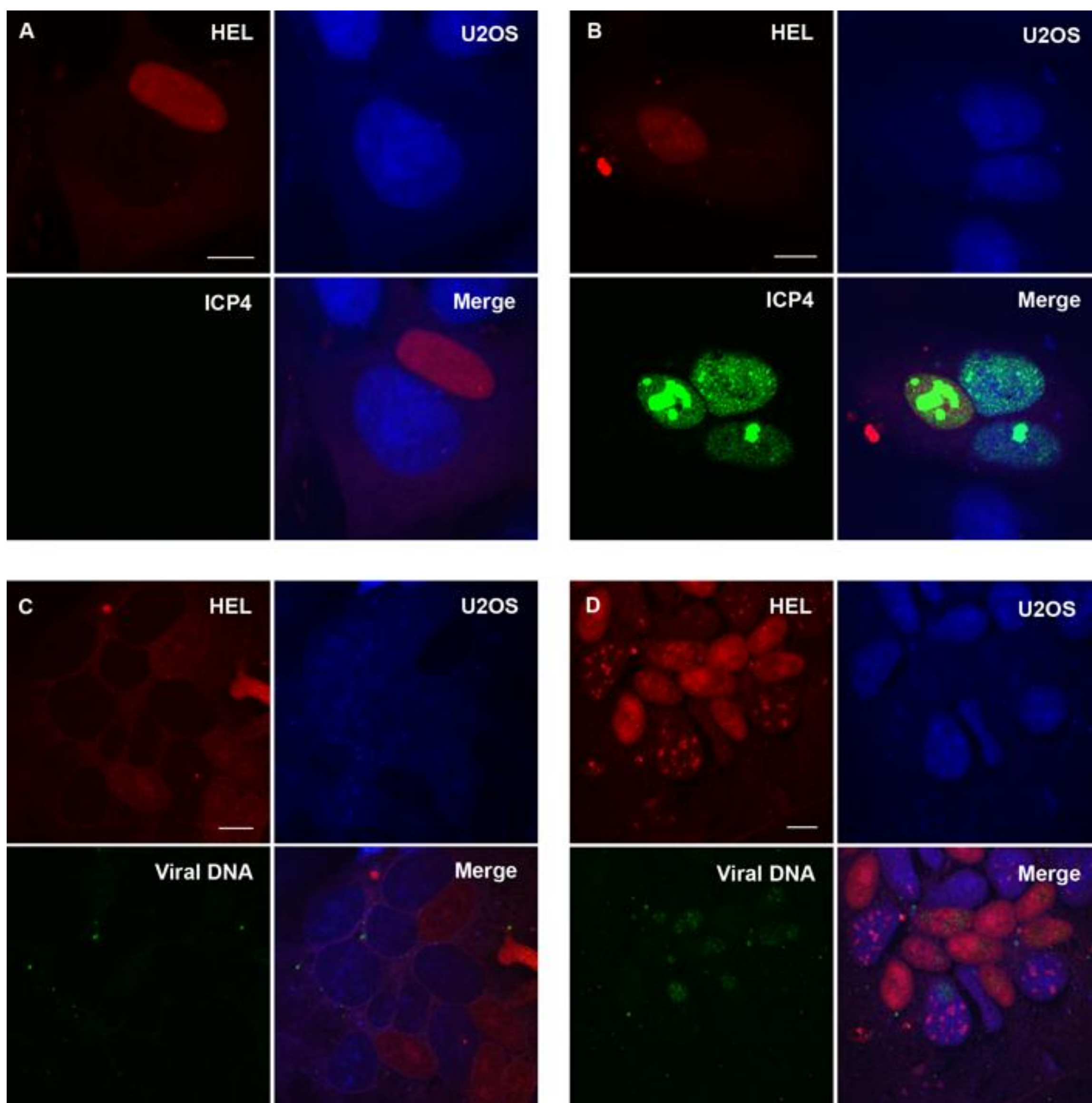

A
HEL
U2OS
ICP4
Merge
B
HEL
U2OS
ICP4
Merge
C
HEL
U2OS
Viral DNA
Merge
D
HEL
U2OS
Viral DNA
Merge

Figure 3.6. Nuclear delivery of viral genomes is complete within three hours. HEL cells (red) were mixed with U2OS cells (blue) expressing ICP0 and p14 at one (A) or three (B) hours after infection with KM110, essentially as outlined in Figure 3. Cells were fixed 9 hours after fusion and scored for ICP4 expression (green signal). The images shown in panels A and B were obtained in an experiment where the CMAC stain was lost from the U2OS nuclei. Representative heterokarya are shown. Scale bar = 10μm.

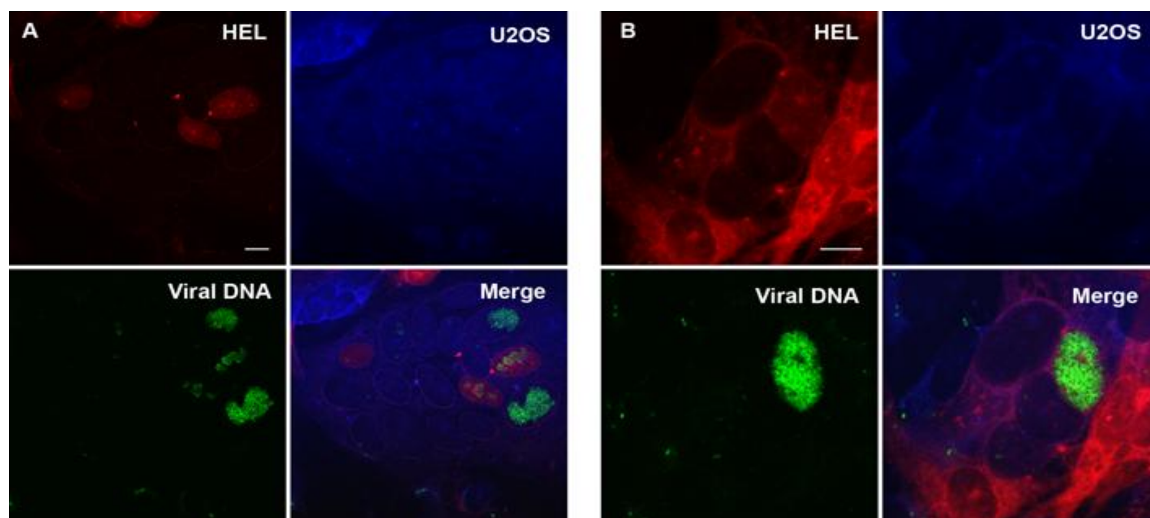

A
HEL
U2OS
Viral DNA
Merge
B
HEL
U2OS
Viral DNA
Merge

Figure 3.7. Reactivation of the KM110 genome by ICP0 provided *in trans* 48 hours post-infection

HEL cells (red) were infected with 10 PFU/cell of KM110, incubated 48 hours, then fused with p14-expressing U2OS cells (blue) as outlined in Figure 3.3. In panels B and D, the U2OS cells were cotransfected with the ICP0 expression vector pDR27. Cells were fixed 9 hours later and scored for ICP4 expression (A and B) and replicated viral DNA (C and D) by IF and DNA-FISH respectively (green signals). Representative heterokarya are shown. Scale bar = 10μm.

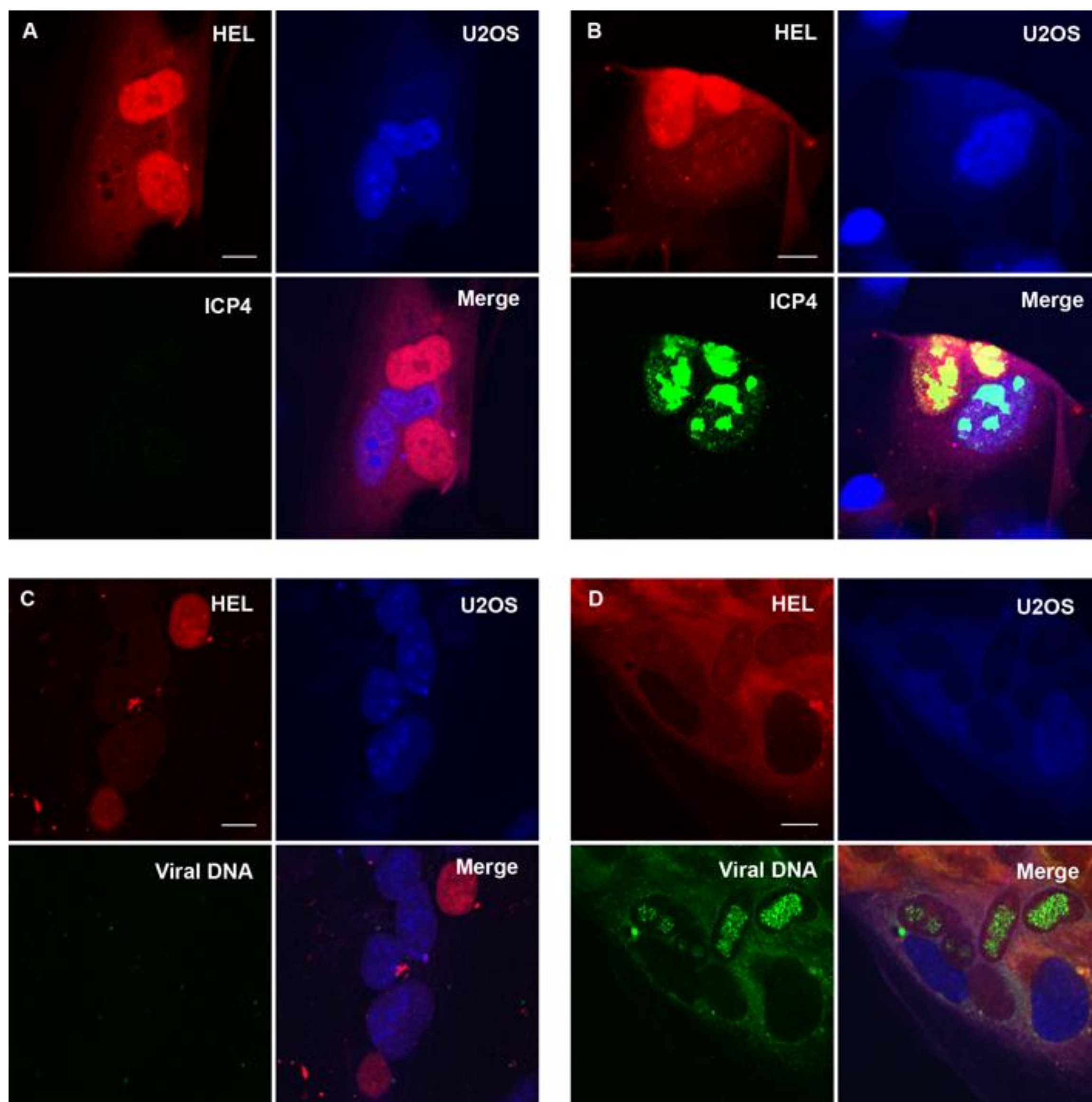
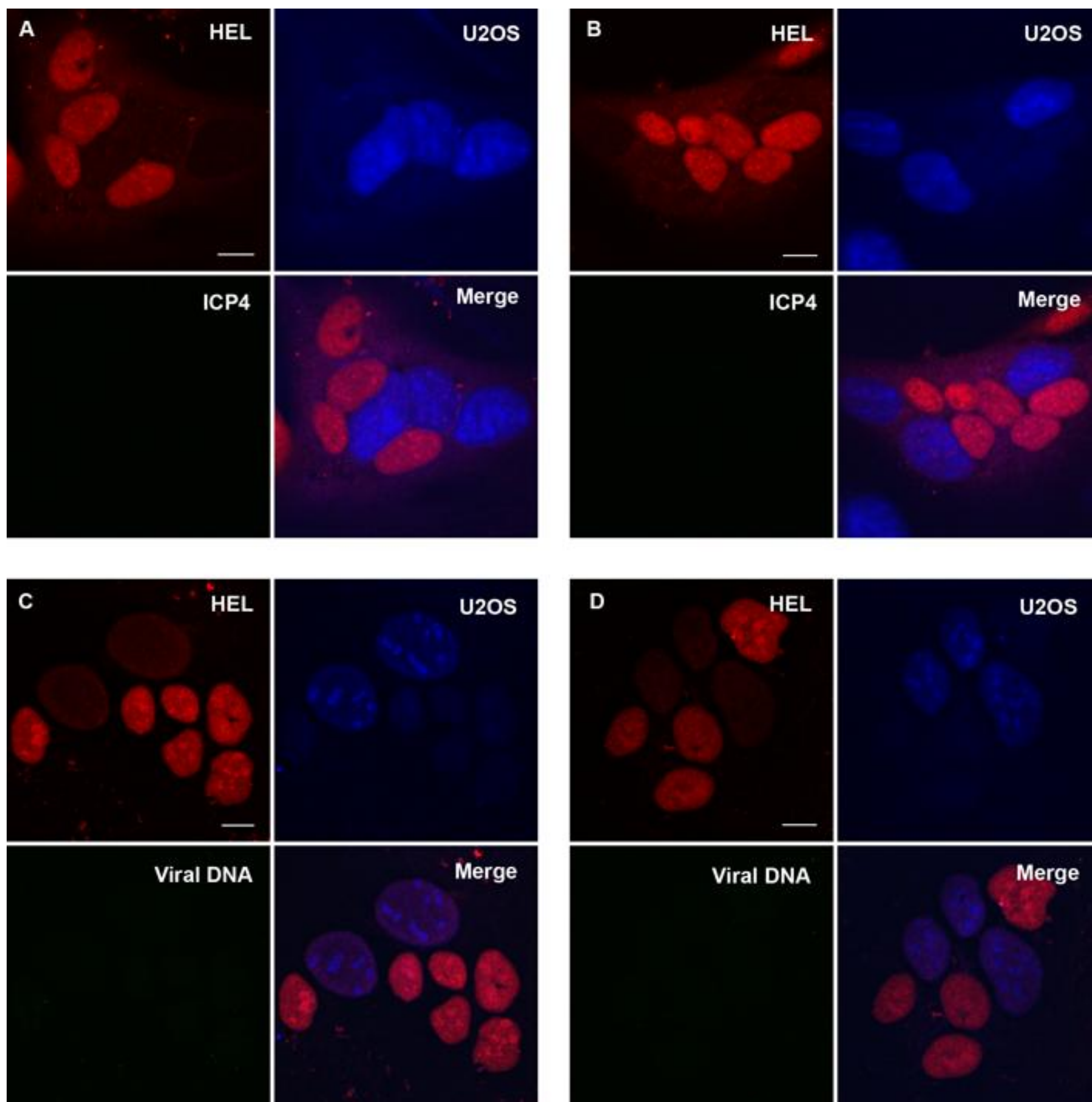

A
HEL
U2OS
ICP4
Merge
B
HEL
U2OS
ICP4
Merge
C
HEL
U2OS
Viral DNA
Merge
D
HEL
U2OS
Viral DNA
Merge

Figure 3.8. VP16 is unable to reactivate KM110 at late times post-infection. HEL cells (red) were infected with 10 PFU/cell of KM110, incubated 48 hours, then fused with p14-expressing U2OS cells (blue) as in figure 7 and as outlined in Figure 3. In panels B and D, the U2OS cells were cotransfected with the VP16 expression plasmid pKOS-VP16. Cells were fixed 9 hours later and scored for ICP4 expression (A and B) and replicated viral DNA (C and D) by IF and DNA-FISH respectively (green signals). Representative heterokarya are shown. Scale bar = 10μm.


A
HEL
U2OS
ICP4
Merge
B
HEL
U2OS
ICP4
Merge
C
HEL
U2OS
Viral DNA
Merge
D
HEL
U2OS
Viral DNA
Merge

REFERENCES

1. **Ace, C. I., T. A. McKee, J. M. Ryan, J. M. Cameron, and C. M. Preston.** 1989. Construction and characterization of a herpes simplex virus type 1 mutant unable to transinduce immediate-early gene expression. J Virol **63:**2260-9.
2. **Benedict, C. A., P. S. Norris, and C. F. Ware.** 2002. To kill or be killed: viral evasion of apoptosis. Nat Immunol **3:**1013-8.
3. **Boutell, C., S. Sadis, and R. D. Everett.** 2002. Herpes simplex virus type 1 immediate-early protein ICP0 and is isolated RING finger domain act as ubiquitin E3 ligases in vitro. J Virol **76:**841-50.
4. **Cai, W., T. L. Astor, L. M. Liptak, C. Cho, D. M. Coen, and P. A. Schaffer.** 1993. The herpes simplex virus type 1 regulatory protein ICP0 enhances virus replication during acute infection and reactivation from latency. J Virol **67:**7501-12.
5. **Cai, W., and P. A. Schaffer.** 1992. Herpes simplex virus type 1 ICP0 regulates expression of immediate-early, early, and late genes in productively infected cells. J Virol **66:**2904-15.
6. **Chee, A. V., P. Lopez, P. P. Pandolfi, and B. Roizman.** 2003. Promyelocytic leukemia protein mediates interferon-based anti-herpes simplex virus 1 effects. J Virol **77:**7101-5.
7. **Chelbi-Alix, M. K., and H. de The.** 1999. Herpes virus induced proteasome-dependent degradation of the nuclear bodies-associated PML and Sp100 proteins. Oncogene **18:**935-41.
8. **Chou, J., J. J. Chen, M. Gross, and B. Roizman.** 1995. Association of a M(r) 90,000 phosphoprotein with protein kinase PKR in cells exhibiting enhanced phosphorylation of translation initiation factor eIF-2 alpha and premature shutoff of protein synthesis after infection with gamma 134.5- mutants of herpes simplex virus 1. Proc Natl Acad Sci U S A **92:**10516-20.
9. **Corcoran, J. A., and R. Duncan.** 2004. Reptilian reovirus utilizes a small type III protein with an external myristylated amino terminus to mediate cell-cell fusion. J Virol **78:**4342-51.
10. **Cowan, S., T. Hatziioannou, T. Cunningham, M. A. Muesing, H. G. Gottlinger, and P. D. Bieniasz.** 2002. Cellular inhibitors with Fv1-like activity restrict human and simian immunodeficiency virus tropism. Proc Natl Acad Sci U S A **99:**11914-9.
11. **Cunningham, C., and A. J. Davison.** 1993. A cosmid-based system for constructing mutants of herpes simplex virus type 1. Virology **197:**116-24.
12. **Dellaire, G., and D. P. Bazett-Jones.** 2004. PML nuclear bodies: dynamic sensors of DNA damage and cellular stress. Bioessays **26:**963-77.
13. **Eidson, K. M., W. E. Hobbs, B. J. Manning, P. Carlson, and N. A. DeLuca.** 2002. Expression of herpes simplex virus ICP0 inhibits the induction of interferon-stimulated genes by viral infection. J Virol **76:**2180-91.

14. **Everett, R., P. O'Hare, D. O'Rourke, P. Barlow, and A. Orr.** 1995. Point mutations in the herpes simplex virus type 1 Vmw110 RING finger helix affect activation of gene expression, viral growth, and interaction with PML-containing nuclear structures. J Virol **69:**7339-44.
15. **Everett, R. D.** 1985. Activation of cellular promoters during herpes simplex virus infection of biochemically transformed cells. EMBO J. **4:**1973-1980.
16. **Everett, R. D.** 1989. Construction and characterization of herpes simplex virus type 1 mutants with defined lesions in immediate early gene 1. J Gen Virol **70 (Pt 5):**1185-202.
17. **Everett, R. D.** 2001. DNA viruses and viral proteins that interact with PML nuclear bodies. Oncogene **20:**7266-73.
18. **Everett, R. D.** 2000. ICP0, a regulator of herpes simplex virus during lytic and latent infection. Bioessays **22:**761-70.
19. **Everett, R. D.** 2006. Interactions between DNA viruses, ND10 and the DNA damage response. Cell Microbiol **8:**365-74.
20. **Everett, R. D., C. Boutell, and A. Orr.** 2004. Phenotype of a herpes simplex virus type 1 mutant that fails to express immediate-early regulatory protein ICP0. J Virol **78:**1763-74.
21. **Everett, R. D., W. C. Earnshaw, J. Findlay, and P. Lomonte.** 1999. Specific destruction of kinetochore protein CENP-C and disruption of cell division by herpes simplex virus immediate-early protein Vmw110. Embo J **18:**1526-38.
22. **Everett, R. D., P. Freemont, H. Saitoh, M. Dasso, A. Orr, M. Kathoria, and J. Parkinson.** 1998. The disruption of ND10 during herpes simplex virus infection correlates with the Vmw110- and proteasome-dependent loss of several PML isoforms. J Virol **72:**6581-91.
23. **Everett, R. D., and G. G. Maul.** 1994. HSV-1 IE protein Vmw110 causes redistribution of PML. Embo J **13:**5062-9.
24. **Everett, R. D., A. Orr, and C. M. Preston.** 1998. A viral activator of gene expression functions via the ubiquitin-proteasome pathway. Embo J **17:**7161-9.
25. **Everett, R. D., G. Sourvinos, C. Leiper, J. B. Clements, and A. Orr.** 2004. Formation of nuclear foci of the herpes simplex virus type 1 regulatory protein ICP4 at early times of infection: localization, dynamics, recruitment of ICP27, and evidence for the de novo induction of ND10-like complexes. J Virol. **78:**1903-17.
26. **Gabuzda, D. H., K. Lawrence, E. Langhoff, E. Terwilliger, T. Dorfman, W. A. Haseltine, and J. Sodroski.** 1992. Role of vif in replication of human immunodeficiency virus type 1 in CD4+ T lymphocytes. J Virol **66:**6489-95.
27. **Gelman, I. H., and S. Silverstein.** 1985. Identification of immediate early genes from herpes simplex virus that transactivate the virus thymidine kinase gene. Proc Natl Acad Sci U S A **82:**5265-9.
28. **Goff, S. P.** 2004. Retrovirus restriction factors. Mol Cell **16:**849-59.

29. **Gongora, C., G. David, L. Pintard, C. Tissot, T. D. Hua, A. Dejean, and N. Mechti.** 1997. Molecular cloning of a new interferon-induced PML nuclear body-associated protein. J Biol Chem **272:**19457-63.
30. **Goodbourn, S., L. Didcock, and R. E. Randall.** 2000. Interferons: cell signalling, immune modulation, antiviral response and virus countermeasures. J Gen Virol **81:**2341-64.
31. **Grotzinger, T., T. Sternsdorf, K. Jensen, and H. Will.** 1996. Interferon-modulated expression of genes encoding the nuclear-dot-associated proteins Sp100 and promyelocytic leukemia protein (PML). Eur J Biochem **238:**554-60.
32. **Gu, H., Y. Liang, G. Mandel, and B. Roizman.** 2005. Components of the REST/CoREST/histone deacetylase repressor complex are disrupted, modified, and translocated in HSV-1-infected cells. Proc Natl Acad Sci U S A **102:**7571-6.
33. **Hagglund, R., C. Van Sant, P. Lopez, and B. Roizman.** 2002. Herpes simplex virus 1-infected cell protein 0 contains two E3 ubiquitin ligase sites specific for different E2 ubiquitin-conjugating enzymes. Proc Natl Acad Sci U S A **99:**631-6.
34. **Halford, W. P., C. D. Kemp, J. A. Isler, D. J. Davido, and P. A. Schaffer.** 2001. ICP0, ICP4, or VP16 expressed from adenovirus vectors induces reactivation of latent herpes simplex virus type 1 in primary cultures of latently infected trigeminal ganglion cells. J Virol **75:**6143-53.
35. **Harris, R. A., R. D. Everett, X. X. Zhu, S. Silverstein, and C. M. Preston.** 1989. Herpes simplex virus type 1 immediate-early protein Vmw110 reactivates latent herpes simplex virus type 2 in an in vitro latency system. J Virol **63:**3513-5.
36. **Harris, R. A., and C. M. Preston.** 1991. Establishment of latency in vitro by the herpes simplex virus type 1 mutant in1814. J Gen Virol **72 (Pt 4):**907-13.
37. **Herrera, F. J., and S. J. Triezenberg.** 2004. VP16-dependent association of chromatin-modifying coactivators and underrepresentation of histones at immediate-early gene promoters during herpes simplex virus infection. J Virol **78:**9689-96.
38. **Hobbs, W. E., D. E. Brough, I. Kovesdi, and N. A. DeLuca.** 2001. Efficient activation of viral genomes by levels of herpes simplex virus ICP0 insufficient to affect cellular gene expression or cell survival. J Virol **75:**3391-403.
39. **Hofmann, T. G., and H. Will.** 2003. Body language: the function of PML nuclear bodies in apoptosis regulation. Cell Death Differ **10:**1290-9.
40. **Jackson, S. A., and N. A. DeLuca.** 2003. Relationship of herpes simplex virus genome configuration to productive and persistent infections. Proc Natl Acad Sci U S A **100:**7871-6.
41. **Jamieson, D. R., L. H. Robinson, J. I. Daksis, M. J. Nicholl, and C. M. Preston.** 1995. Quiescent viral genomes in human fibroblasts after infection with herpes simplex virus type 1 Vmw65 mutants. J Gen Virol **76 (Pt 6):**1417-31.

42. **Jordan, R., and P. Schaffer.** 1997. Activation of gene expression by herpes simplex virus type 1 ICP0 occurs at the level of mRNA synthesis. J. Virol. **71:**6850-6862.
43. **Kawai, T., and S. Akira.** 2005. Pathogen recognition with Toll-like receptors. Curr Opin Immunol **17:**338-44.
44. **Kent, J. R., P. Y. Zeng, D. Atanasiu, J. Gardner, N. W. Fraser, and S. L. Berger.** 2004. During lytic infection herpes simplex virus type 1 is associated with histones bearing modifications that correlate with active transcription. J Virol **78:**10178-86.
45. **Klemm, R. D., J. A. Goodrich, S. Zhou, and R. Tjian.** 1995. Molecular cloning and expression of the 32-kDa subunit of human TFIID reveals interactions with VP16 and TFIIB that mediate transcriptional activation. Proc Natl Acad Sci U S A **92:**5788-92.
46. **Knipe, D. M., D. Senechek, S. A. Rice, and J. L. Smith.** 1987. Stages in the nuclear association of the herpes simplex virus transcriptional activator protein ICP4. J Virol **61:**276-84.
47. **Kobayashi, N., P. J. Horn, S. M. Sullivan, S. J. Triezenberg, T. G. Boyer, and A. J. Berk.** 1998. DA-complex assembly activity required for VP16C transcriptional activation. Mol Cell Biol **18:**4023-31.
48. **Lees-Miller, S. P., M. C. Long, M. A. Kilvert, V. Lam, S. A. Rice, and C. A. Spencer.** 1996. Attenuation of DNA-dependent protein kinase activity and its catalytic subunit by the herpes simplex virus type 1 transactivator ICP0. J Virol **70:**7471-7.
49. **Leinbach, S. S., and W. C. Summers.** 1980. The structure of herpes simplex virus type 1 DNA as probed by microccal nuclease digestion. J. Gen. Virol. **51:**45-59.
50. **Lin, H. K., S. Bergmann, and P. P. Pandolfi.** 2004. Cytoplasmic PML function in TGF-beta signalling. Nature **431:**205-11.
51. **Lomonte, P., K. F. Sullivan, and R. D. Everett.** 2001. Degradation of nucleosome-associated centromeric histone H3-like protein CENP-A induced by herpes simplex virus type 1 protein ICP0. J Biol Chem **276:**5829-35.
52. **Lomonte, P., J. Thomas, P. Texier, C. Caron, S. Khochbin, and A. L. Epstein.** 2004. Functional interaction between class II histone deacetylases and ICP0 of herpes simplex virus type 1. J Virol **78:**6744-57.
53. **Loppin, B., E. Bonnefoy, C. Anselme, A. Laurencon, T. L. Karr, and P. Couble.** 2005. The histone H3.3 chaperone HIRA is essential for chromatin assembly in the male pronucleus. Nature **437:**1386-90.
54. **Loyola, A., and G. Almouzni.** 2004. Histone chaperones, a supporting role in the limelight. Biochim Biophys Acta **1677:**3-11.
55. **Madani, N., and D. Kabat.** 1998. An endogenous inhibitor of human immunodeficiency virus in human lymphocytes is overcome by the viral Vif protein. J Virol **72:**10251-5.
56. **Mariani, R., D. Chen, B. Schrofelbauer, F. Navarro, R. Konig, B. Bollman, C. Munk, H. Nymark-McMahon, and N. R. Landau.** 2003.

Species-specific exclusion of APOBEC3G from HIV-1 virions by Vif. Cell **114:**21-31.

57. **Maul, G. G.** 1998. Nuclear domain 10, the site of DNA virus transcription and replication. BioEssays **20:**660-667.
58. **Memedula, S., and A. S. Belmont.** 2003. Sequential recruitment of HAT and SWI/SNF components to condensed chromatin by VP16. Curr Biol **13:**241-6.
59. **Minaker, R. L., K. L. Mossman, and J. R. Smiley.** 2005. Functional inaccessibility of quiescent herpes simplex virus genomes. Virol J **2:**85.
60. **Mittler, G., T. Stuhler, L. Santolin, T. Uhlmann, E. Kremmer, F. Lottspeich, L. Berti, and M. Meisterernst.** 2003. A novel docking site on Mediator is critical for activation by VP16 in mammalian cells. Embo J **22:**6494-504.
61. **Mossman, K. L., H. A. Saffran, and J. R. Smiley.** 2000. Herpes simplex virus ICP0 mutants are hypersensitive to interferon. J Virol **74:**2052-6.
62. **Mossman, K. L., and J. R. Smiley.** 2002. Herpes simplex virus ICP0 and ICP34.5 counteract distinct interferon-induced barriers to virus replication. J Virol **76:**1995-8.
63. **Mossman, K. L., and J. R. Smiley.** 1999. Truncation of the C-terminal acidic transcriptional activation domain of herpes simplex virus VP16 renders expression of the immediate-early genes almost entirely dependent on ICP0. J Virol **73:**9726-33.
64. **Muggeridge, M. I., and N. W. Fraser.** 1986. Chromosomal organization of the herpes simplex virus genome during acute infection of the mouse central nervous system. J. Virol. **59:**764-767.
65. **Munk, C., S. M. Brandt, G. Lucero, and N. R. Landau.** 2002. A dominant block to HIV-1 replication at reverse transcription in simian cells. Proc Natl Acad Sci U S A **99:**13843-8.
66. **Netea, M. G., C. van der Graaf, J. W. Van der Meer, and B. J. Kullberg.** 2004. Toll-like receptors and the host defense against microbial pathogens: bringing specificity to the innate-immune system. J Leukoc Biol **75:**749-55.
67. **Nisole, S., J. P. Stoye, and A. Saib.** 2005. TRIM family proteins: retroviral restriction and antiviral defence. Nat Rev Microbiol **3:**799-808.
68. **O'Hare, P., and C. R. Gooding.** 1988. Herpes simplex virus regulatory elements and the immunoglobulin octamer domain bind a common factor and are both targets for virion transactivation. Cell **52:**435-445.
69. **O'Hare, P., and G. S. Hayward.** 1985. Three trans-acting regulatory proteins of herpes simplex virus modulate immediate-early gene expression in a pathway involving positive and negative feedback regulation. J Virol **56:**723-33.
70. **Parkinson, J., S. P. Lees-Miller, and R. D. Everett.** 1999. Herpes simplex virus type 1 immediate-early protein vmw110 induces the proteasome-dependent degradation of the catalytic subunit of DNA-dependent protein kinase. J Virol **73:**650-7.

71. **Pearson, M., R. Carbone, C. Sebastiani, M. Cioce, M. Fagioli, S. Saito, Y. Higashimoto, E. Appella, S. Minucci, P. P. Pandolfi, and P. G. Pelicci.** 2000. PML regulates p53 acetylation and premature senescence induced by oncogenic Ras. Nature **406:**207-10.
72. **Preston, C. M.** 2000. Repression of viral transcription during herpes simplex virus latency. J Gen Virol **81:**1-19.
73. **Preston, C. M., M. C. Frame, and M. E. Campbell.** 1988. A complex formed between cell components and an HSV structural polypeptide binds to a viral immediate early gene regulatory DNA sequence. Cell **52:**425-34.
74. **Preston, C. M., and M. J. Nicholl.** 1997. Repression of gene expression upon infection of cells with herpes simplex virus type 1 mutants impaired for immediate-early protein synthesis. J Virol **71:**7807-13.
75. **Quinlan, M. P., and D. M. Knipe.** 1985. Stimulation of expression of a herpes simplex virus DNA-binding protein by two viral functions. Mol. Cell. Biol. **5:**957-963.
76. **Ray-Gallet, D., J. P. Quivy, C. Scamps, E. M. Martini, M. Lipinski, and G. Almouzni.** 2002. HIRA is critical for a nucleosome assembly pathway independent of DNA synthesis. Mol Cell **9:**1091-100.
77. **Roizman, B., and D. M. Knipe.** 2001. Herpes simplex viruses and their replication., p. 2381-2398. *In* D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Strauss (ed.), Field's Virology, 4th ed. Lippincott, Williams & Wilkins, Philadelphia.
78. **Russell, J., N. D. Stow, E. C. Stow, and C. M. Preston.** 1987. Herpes simplex virus genes involved in latency in vitro. J Gen Virol **68 (Pt 12):**3009-18.
79. **Samaniego, L. A., L. Neiderhiser, and N. A. DeLuca.** 1998. Persistence and expression of the herpes simplex virus genome in the absence of immediate-early proteins. J Virol **72:**3307-20.
80. **Samaniego, L. A., N. Wu, and N. A. DeLuca.** 1997. The herpes simplex virus immediate-early protein ICP0 affects transcription from the viral genome and infected-cell survival in the absence of ICP4 and ICP27. Journal of Virology **71:**4614-4625.
81. **Sheehy, A. M., N. C. Gaddis, J. D. Choi, and M. H. Malim.** 2002. Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. Nature **418:**646-50.
82. **Simon, J. H., N. C. Gaddis, R. A. Fouchier, and M. H. Malim.** 1998. Evidence for a newly discovered cellular anti-HIV-1 phenotype. Nat Med **4:**1397-400.
83. **Smiley, J. R., and J. Duncan.** 1997. Truncation of the C-terminal acidic transcriptional activation domain of herpes simplex virus VP16 produces a phenotype similar to that of the in1814 linker insertion mutation. J Virol **71:**6191-3.
84. **Stadler, M., M. K. Chelbi-Alix, M. H. Koken, L. Venturini, C. Lee, A. Saib, F. Quignon, L. Pelicano, M. C. Guillemin, C. Schindler, and et al.** 1995. Transcriptional induction of the PML growth suppressor gene by

interferons is mediated through an ISRE and a GAS element. Oncogene **11:**2565-73.
85. **Stern, S., M. Tanaka, and W. Herr.** 1989. The Oct-1 homoeodomain directs formation of a multiprotein-DNA complex with the HSV transactivator VP16. Nature **341:**624-30.
86. **Stow, N. D., and E. C. Stow.** 1986. Isolation and characterization of a herpes simplex virus type 1 mutant containing a deletion within the gene encoding the immediate early polypeptide Vmw110. J Gen Virol **67 (Pt 12):**2571-85.
87. **Strebel, K., D. Daugherty, K. Clouse, D. Cohen, T. Folks, and M. A. Martin.** 1987. The HIV 'A' (sor) gene product is essential for virus infectivity. Nature **328:**728-30.
88. **Stremlau, M., C. M. Owens, M. J. Perron, M. Kiessling, P. Autissier, and J. Sodroski.** 2004. The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys. Nature **427:**848-53.
89. **Triezenberg, S. J., R. C. Kingsbury, and S. L. McKnight.** 1988. Functional dissection of VP16, the trans-activator of herpes simplex virus immediate early gene expression. Genes Dev. **2:**718-729.
90. **Uesugi, M., O. Nyanguile, H. Lu, A. J. Levine, and G. L. Verdine.** 1997. Induced alpha helix in the VP16 activation domain upon binding to a human TAF. Science **277:**1310-3.
91. **Voinnet, O.** 2005. Induction and suppression of RNA silencing: insights from viral infections. Nat Rev Genet **6:**206-20.
92. **von Schwedler, U., J. Song, C. Aiken, and D. Trono.** 1993. Vif is crucial for human immunodeficiency virus type 1 proviral DNA synthesis in infected cells. J Virol **67:**4945-55.
93. **Wang, F., Y. Ma, J. W. Barrett, X. Gao, J. Loh, E. Barton, H. W. Virgin, and G. McFadden.** 2004. Disruption of Erk-dependent type I interferon induction breaks the myxoma virus species barrier. Nat Immunol **5:**1266-74.
94. **Weinheimer, S. P., B. A. Boyd, S. K. Durham, J. L. Resnick, and D. R. O'Boyle, 2nd.** 1992. Deletion of the VP16 open reading frame of herpes simplex virus type 1. J Virol **66:**258-69.
95. **Xiao, H., A. Pearson, B. Coulombe, R. Truant, S. Zhang, J. L. Regier, S. J. Triezenberg, D. Reinberg, O. Flores, C. J. Ingles, and et al.** 1994. Binding of basal transcription factor TFIIH to the acidic activation domains of VP16 and p53. Mol Cell Biol **14:**7013-24.
96. **Xiao, P., and J. P. Capone.** 1990. A cellular factor binds to the herpes simplex virus type 1 transactivator Vmw65 and is required for Vmw65-dependent protein-DNA complex assembly with Oct-1. Mol Cell Biol **10:**4974-7.
97. **Yao, F., and P. A. Schaffer.** 1995. An activity specified by the osteosarcoma line U2OS can substitute functionally for ICP0, a major regulatory protein of herpes simplex virus type 1. J Virol **69:**6249-58.
98. **Zhang, R., M. V. Poustovoitov, X. Ye, H. A. Santos, W. Chen, S. M. Daganzo, J. P. Erzberger, I. G. Serebriiskii, A. A. Canutescu, R. L.**

Dunbrack, J. R. Pehrson, J. M. Berger, P. D. Kaufman, and P. D. Adams. 2005. Formation of MacroH2A-containing senescence-associated heterochromatin foci and senescence driven by ASF1a and HIRA. Dev Cell **8:**19-30.

Appendix 1. Data Not Shown

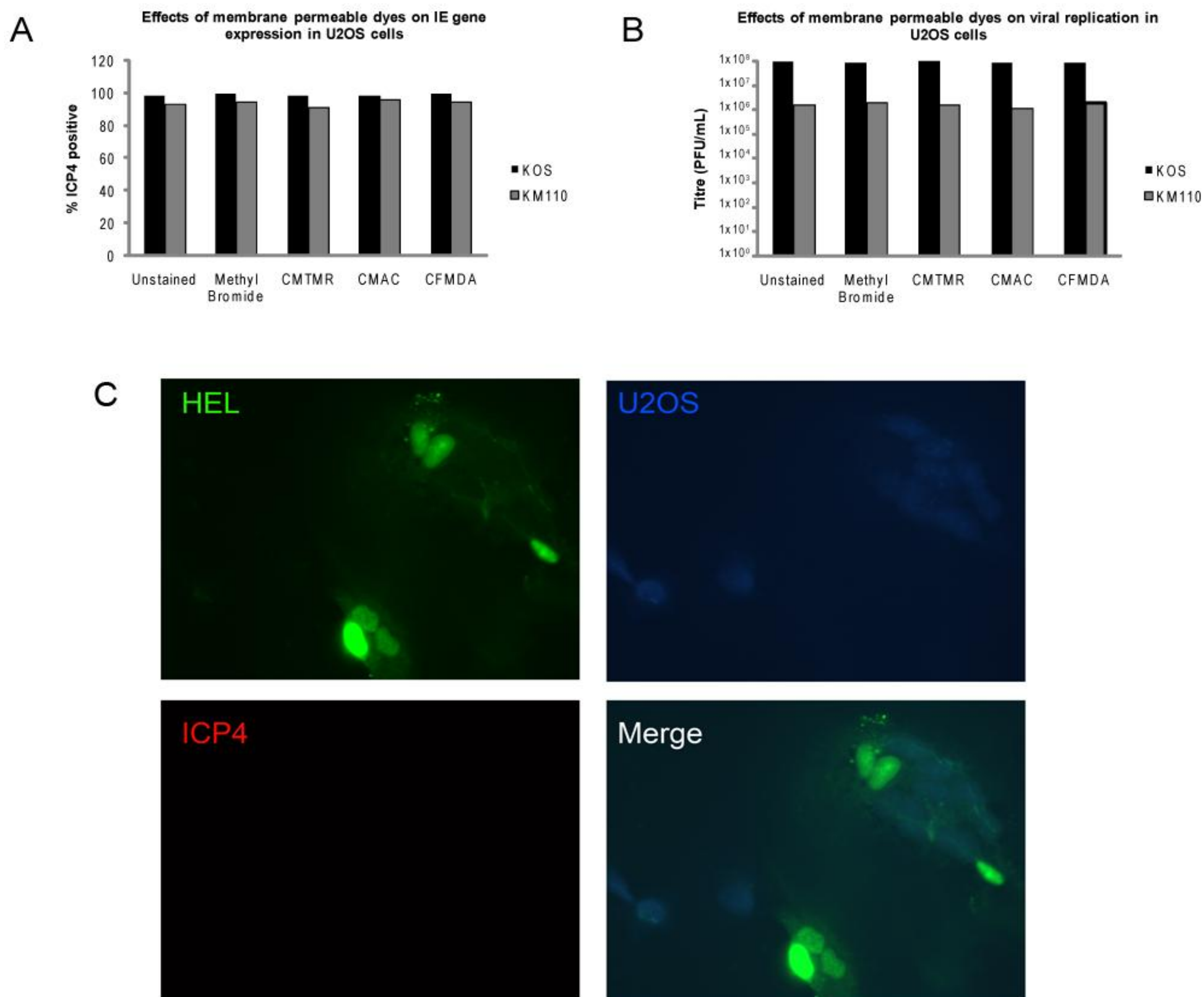


Figure 3A.1. The effects of membrane permeable dyes on viral infection (A) The effects of membrane permeable dyes on IE gene expression. U2OS cells were stained with membrane permeable dyes according to the manufacturer's instructions. Cells were then infected with 10 PFU/cell of KOS or KM110 for 9 hours, fixed and processed for indirect immunofluorescence for ICP4. A minimum of 100 cells were counted and the proportion of cells expressing ICP4 was calculated. (B) The effects of membrane permeable dyes on viral

replication. U2OS cells were stained with membrane permeable dyes according to the manufacturer's instructions. Serial dilutions of KOS and KM110 were titrated on these cells and the number of plaques was counted after three days.

(C) IE gene expression is restricted, even when more U2OS than HEL nuclei are present in a heterokaryon. HEL cells (stained green with CFMDA) were infected with 10 PFU/cell of KM110 then mixed with p14-expressing U2OS cells (stained blue with CMAC) 1 h later. Cells were fixed 9 h later and scored for ICP4 expression.

Chapter Four: Cell fusion-induced activation of interferon-stimulated genes is not required for restriction of a herpes simplex virus VP16/ICP0 mutant in heterokarya formed between permissive and restrictive cells

A version of this chapter has been published:

Hancock MH, Mossman KL, and Smiley JR. 2009. J Virol **83**(17):8976-9.

Preface to Chapter 4

Although I determined in the preceding chapter that p14-induced fusion did not alter the permissive or restrictive phenotype of U2OS and HEL cells respectively, it is unknown what cellular responses are triggered by this unnatural event. I became aware that Dr Karen Mossman and Dr Ryan Noyce had determined that p14-mediated fusion results in the induction of ISGs and an antiviral state in some cell types. It was therefore possible that the lack of viral gene expression detected in our somatic cell hybrids was due to fusion-induced expression of ISGs, rather than a consitutive defense mechanism counteracted by ICP0 and VP16. In this chapter I outline the identification of cell lines which do not induce ISGs in response to p14-mediated fusion and test these cell types in our somatic cell fusion assays.

The publication entitled "Cell fusion-induced activation of interferon-stimulated genes is not required for restriction of a herpes simplex virus VP16/ICP0 mutant in heterokarya formed between permissive and restrictive cells" is the content of Chapter four of this thesis. This work was published online June 17th 2009 and available in print September 2009 in the Journal of Virology. Dr Karen Mossman is an author on this publication for providing us with the information regarding the induction of ISGs by p14. All experiments were performed by me, while writing and editing was shared by Dr Smiley and myself.

ABSTRACT

Herpes simplex virus VP16 and ICP0 mutants replicate efficiently in U2OS osteosarcoma cells but are restricted in other cell types. We previously showed that the restrictive phenotype is dominant in a transient cell fusion assay, suggesting that U2OS cells lack an antiviral mechanism present in other cells. Recent data indicate that unscheduled membrane fusion events can activate expression of interferon-stimulated genes (ISGs) in fibroblasts, raising the possibility that our earlier results were due to a fusion-induced antiviral state. However, we show here that the permissive phenotype is also extinguished following fusion with Vero cells in the absence of ISG induction.

Two herpes simplex virus (HSV) proteins play key roles in launching the lytic program of viral gene expression: the tegument protein VP16, and the immediate-early (IE) protein ICP0. VP16 contains a strong C-terminal acidic activation domain (38) and acts with the cellular factors HCF and Oct-1 to recruit factors involved in transcription initiation to the IE promoters (22-24, 26, 27, 34, 37, 41, 45, 47, 48). ICP0 is also required for efficient IE gene expression (4, 5, 12, 16, 36), and can complement the defects of VP16 mutant viruses (1, 21). ICP0 is capable of activating expression of viral and cellular genes in transient cotransfection assays and stimulates expression of all classes of viral genes during HSV-1 infection (Reviewed in (11)). ICP0 is an ubiquitin E3 ligase that interacts with many cellular proteins, although the significance of these interactions is not yet understood. Its E3 ligase activity is critical for transactivation and reactivation of quiescent genomes and these functions are blocked by proteasome inhibition (2, 15, 20), suggesting that ICP0 acts by targeting cellular inhibitory proteins for ubiquitination and degradation. ICP0 also blocks the action of interferon (IFN) during HSV-1 infection (9, 27, 28) and causes the degradation of the IFN-inducible promyelocytic leukemia (PML) protein and dispersal of ND10 domains (13, 14, 18).

HSV mutants lacking the activation functions of VP16 or ICP0 display a greatly increased particle-to-plaque forming unit (PFU) ratio and substantially lower levels of IE gene expression upon low multiplicity infection of primary human fibroblasts (1, 4, 12, 36, 38), but replicate efficiently in the human

osteosarcoma cell line U2OS (35, 41). In a previous study (22) we fused U2OS cells with HEL fibroblasts to determine if the permissive phenotype is dominant, as would be predicted if U2OS cells express a VP16 and/or ICP0-like activator (41) , or recessive, a result that would suggest these cells lack an antiviral repression mechanism present in restrictive cells. The HEL-U2OS heterokarya strongly restricted the growth of the VP16/ICP0 mutant KM110 (29), supporting the latter conclusion. VP16 or ICP0 provided *in trans* activated viral replication in the hetrokarya, indicating that VP16 and ICP0 are each able to overcome this repression mechanism (22).

One innate cellular response to virus infection is the induction of the type I interferons (IFNα and IFNβ), a family of cytokines that induce a cellular antiviral state (Reviewed in (34)). IFN production can be induced in response to viral infection by signaling through Toll-like receptors (TLRs) or the cytoplasmic nucleic acid detectors RIG-I, MDA5, and DAI (34), leading to activation of the latent transcription factor IRF-3. Activated IRF-3 translocates to the nucleus and binds to the interferon stimulated response element (ISRE) found in the promoter region of the IFNβ gene, and, along with other cellular transcription factors, recruits complexes required to remodel the promoter and initiate transcription. Secreted IFNβ then signals through the JAK/STAT pathway to induce the expression of the full subset of interferon-stimulated genes (ISGs). Virus infection can also induce an antiviral state in the absence of IFN signaling (3, 26, 30, 32, 33, 37) via direct IRF-3 dependent activation of a small subset of ISGs (7, 31, 33).

Such induction requires viral entry (3, 26, 30) occurs in the absence of *de novo* protein synthesis (26, 32), but in the case of HSV-1, the receptor which senses infection is currently unknown (31). HSV-1 is capable of counteracting this response through the E3 ligase activity of ICP0 (24), suggesting that ICP0 causes the degradation of a critical component of this pathway.

Our earlier cell fusion studies employed the fusogenic reptilian reovirus p14 protein to efficiently generate heterokarya (22). p14 is a fusion-associated small transmembrane (FAST) protein which aids in reovirus dissemination through cell-cell fusion (8). Although p14-mediated homotypic fusion did not alter the restrictive or permissive phenotypes of HEL fibroblasts and U2OS cells respectively (22), the cellular events that are triggered by p14-induced fusion have not been investigated. Recent data indicate that p14 activates expression of interferon stimulated gene 56 (ISG56K) in fibroblasts, most likely through its fusogenic activity (K. L. Mossman, unpublished). ISG56K inhibits protein translation through interaction with eukaryotic initiation factor 3 (eIF-3) (19) and is amongst the small subset of ISGs which are activated directly by IRF-3 in the absence of IFN production. The finding that p14-mediated fusion induces ISG56K in at least some cell types raised the possibility that our previous results were due to ISG induction by the cell fusion protocol, rather than a pre-existing repression mechanism.

As one approach to testing this possibility, we sought to identify conditions allowing fusion of U2OS cells with a restrictive cell type without triggering ISG expression. Vero cells restrict the replication of KM110 (although not as effectively as HEL cells (29)), but display impaired IRF3-dependent signaling (6) and fail to express ISG56K in response to p14 (K.L. Mossman, unpublished). These observations suggested that this cell line might be an informative fusion partner. We therefore monitored ISG induction following p14-induced homo- and heterotypic fusion of U2OS, Vero and HEL cells. Homotypic fusion was induced by transfecting cells with pcDNA3-p14 as described in the Materials and Methods. Cell monolayers were then incubated for 19 hr (U2OS and Vero) or 3 days (HEL), at which time large syncitia encompassing almost all of the cells in the monolayer were detected by light microscopy. U2OS/Vero and U2OS/HEL heterokarya were formed by transfecting U2OS cells with pcDNA3-p14 for 6 hrs, then overlaying the transfected cells onto naïve monolayers of Vero or HEL cells for 19 hrs. As a positive control for ISG56 induction, 1000U of IFN α was added to a naïve monolayer of each cell type 6 hrs prior to harvest. RNA harvested for each culture was then analyzed for mRNAs derived from the ISGs ISG56K, MxB and IP-10 by northern blot (Figure 4.1) as previously described (26).

As expected, the p14 expression plasmid efficiently triggered accumulation of ISG mRNAs in HEL cells while empty pcDNA3 was much less active. No such ISG response was observed in fused Vero cells or U2OS cells.

The basis for the reproducible but weak response of HEL cells to the empty vector remains unclear: the response might be triggered by membrane perturbations induced by the liposomal transfection reagent (23), or by intracellular detection of the transfected DNA (reviewed in (40)). Significantly, ISGs were induced during formation of U2OS/HEL heterokarya (although only the U2OS cells were transfected), indicating the ISG response of HEL fibroblasts is dominant. These results were consistent with the hypothesis that a fusion-induced antiviral state contributes to the non-permissive phenotype of U2OS/HEL heterokarya observed in our previous study (22). In contrast, ISGs were not induced following fusion of U2OS with Vero cells.

We therefore asked if the permissive phenotype of U2OS cells is extinguished following fusion with Vero cells (Figure 4.2). Vero cells (stained green with CFMDA, Molecular Probes) were infected with KM110 at a multiplicity of infection (MOI) of 1, then fused with U2OS cells transfected with pcDNA3-p14 (stained blue with CMAC, Molecular Probes), as previously described (22). Heterokarya were identified and expression of the IE ICP4 protein was assessed via indirect immunofluorescence using Alexa Fluor-555 labeled secondary antibody, shown in red. Control experiments revealed that only 2.5% of unfused Vero cells expressed ICP4; in contrast 37.4% of U2OS cells infected at the same MOI scored positive. The majority of the U2OS-Vero heterokarya lacked detectable ICP4 expression (Figure 4.2A, Table 1), similar to our previous observations using U2OS/HEL heterokarya. Because ISGs are not induced with

this experimental design (see Figure 4.1), this result argues that a pre-existing dominant repression mechanism hinders expression of the KM110 genome in Vero cells. As observed previously, ICP4 expression was activated when plasmids encoding either VP16 or ICP0 were cotransfected with pcDNA3-p14 (Figure 2B and 2C, Table 1), indicating that both proteins are capable of overcoming this repression, although the ICP0 plasmid was more active (Table 4.1). Similar results were obtained in parallel experiments using HEL cells as the restrictive partner (Table 4.1), as previously described, although in this case we used an infecting MOI of 10.

The results presented here document that heterokarya formed between U2OS and Vero cells restrict the replication of KM110 in the absence of ISG induction. Although we assessed the induction of only three ISGs, these observations are in accord with those recently published by Everett *et al.* (17), who demonstrated that STAT1 and IRF3, both required for the broad induction of ISGs, are not essential for the ability of human fibroblasts to restrict the growth of an ICP0 mutant. The nature of the antiviral repression mechanisms that limits replication of VP16 and ICP0 mutants in HEL and Vero cells remains to be defined.

Interestingly, both Vero and U2OS cells appear to be defective in the signaling pathway that activates ISG expression in response to p14-mediated fusion; moreover these cells fail to complement each other following fusion

suggesting they may share a common defect. However, both cell types can respond to exogenous IFN-α to induce the transcript, indicating that the pathways activated by p14-mediated fusion and IFN are separate but converge on ISG mRNA induction. The U2OS cell line has previously been suggested to lack components of the IFN pathway (28) while Vero cells can respond to exogenous IFN but cannot produce it (10, 25, 39).

Figure 4.1. Effects of p14 expression on ISG mRNA accumulation. Total cellular RNA extracted from U2OS, Vero and HEL cells following transfection with the indicated plasmids was scored for ISG56K, MxB and IP-10 mRNA by northern blot hybridization, as described in the main text. U2OS-p14/Vero, U2OS-p14/HEL: Transfected U2OS cells were overlayed onto monolayers of Vero or HEL cells, as described in the main text. IFN: 1000U IFN α was added 6 hrs prior to harvest.

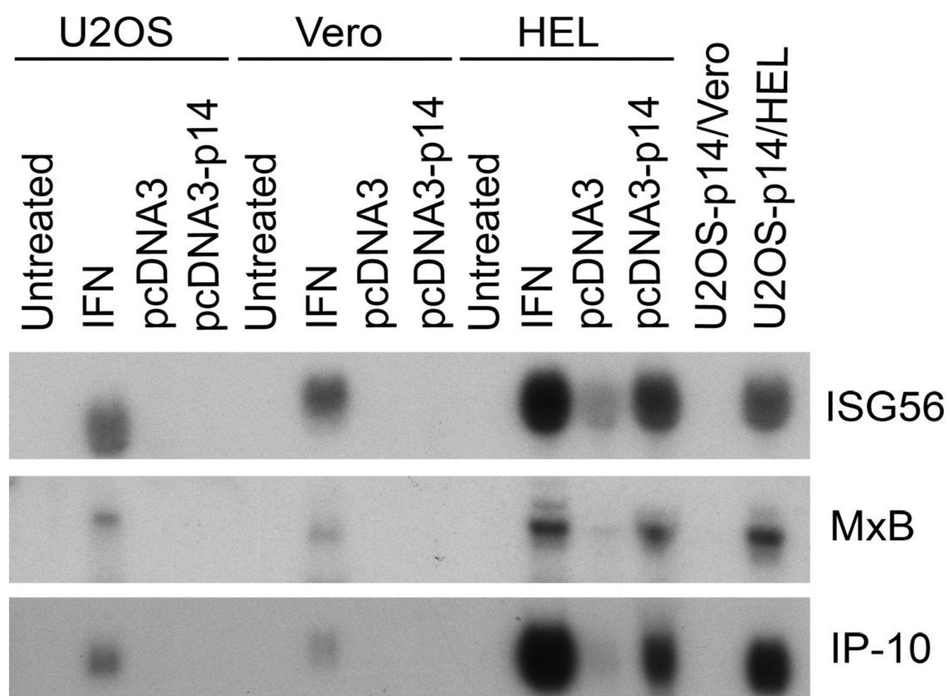

U2OS
Vero
HEL
Untreated
IFN
pcDNA3
pcDNA3-p14
Untreated
IFN
pcDNA3
pcDNA3-p14
Untreated
IFN
pcDNA3
pcDNA3-p14
U2OS-p14/Vero
U2OS-p14/HEL
ISG56
MxB
IP-10

Figure 4.2. Expression of ICP4 in heterokarya. Vero cells (green) were infected with KM110 at 1 PFU/cell then fused with p14-expressing U2OS cells (blue) 1hr later. In panel B, the U2OS cells were also transfected with the ICP0 expression vector pDR27 in addition to the p14 expression vector. In panel C, the U2OS cells were transfected with the VP16 expression vector pKOS-VP16 in addition to the p14 expression vector. Cells were fixed 12hrs later and scored for ICP4 protein expression by IF. Representative heterokarya are shown.

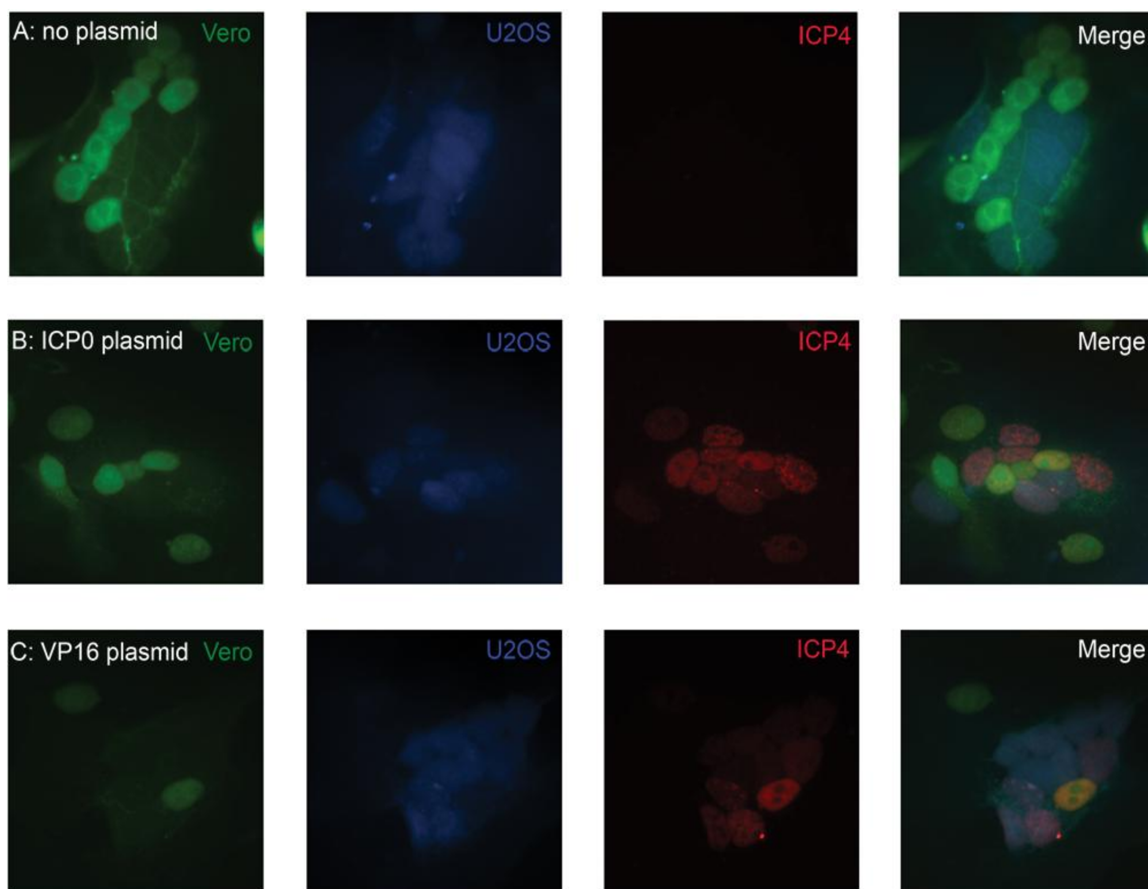

A: no plasmid
Vero
U2OS
ICP4
Merge
B: ICP0 plasmid
Vero
U2OS
ICP4
Merge
C: VP16 plasmid
Vero
U2OS
ICP4
Merge

Table 4.1. ICP4 expression in p14-induced heterokarya

	Percentage of ICP4-positive heterokarya	
Transfected DNA	HEL x U2OS	Vero x U2OS
p14	6.9	8.6
p14 + ICP0	84.0	70.2
p14 + VP16	32.0	42.4

REFERENCES

1. **Ace, C. I., T. A. McKee, J. M. Ryan, J. M. Cameron, and C. M. Preston.** 1989. Construction and characterization of a herpes simplex virus type 1 mutant unable to transinduce immediate-early gene expression. J Virol **63:**2260-9.
2. **Boutell, C., S. Sadis, and R. D. Everett.** 2002. Herpes simplex virus type 1 immediate-early protein ICP0 and is isolated RING finger domain act as ubiquitin E3 ligases in vitro. J Virol **76:**841-50.
3. **Boyle, K. A., R. L. Pietropaolo, and T. Compton.** 1999. Engagement of the cellular receptor for glycoprotein B of human cytomegalovirus activates the interferon-responsive pathway. Mol Cell Biol **19:**3607-13.
4. **Cai, W., T. L. Astor, L. M. Liptak, C. Cho, D. M. Coen, and P. A. Schaffer.** 1993. The herpes simplex virus type 1 regulatory protein ICP0 enhances virus replication during acute infection and reactivation from latency. J Virol **67:**7501-12.
5. **Cai, W., and P. A. Schaffer.** 1992. Herpes simplex virus type 1 ICP0 regulates expression of immediate-early, early, and late genes in productively infected cells. J Virol **66:**2904-15.
6. **Chew, T., R. Noyce, S. E. Collins, M. H. Hancock, and K. L. Mossman.** 2009. Characterization of the interferon regulatory factor 3-mediated antiviral response in a cell line deficient for IFN production. Mol Immunol **46:**393-9.
7. **Collins, S. E., R. S. Noyce, and K. L. Mossman.** 2004. Innate cellular response to virus particle entry requires IRF3 but not virus replication. J Virol **78:**1706-17.
8. **Corcoran, J. A., and R. Duncan.** 2004. Reptilian reovirus utilizes a small type III protein with an external myristylated amino terminus to mediate cell-cell fusion. J Virol **78:**4342-51.
9. **Eidson, K. M., W. E. Hobbs, B. J. Manning, P. Carlson, and N. A. DeLuca.** 2002. Expression of herpes simplex virus ICP0 inhibits the induction of interferon-stimulated genes by viral infection. J Virol **76:**2180-91.
10. **Emeny, J. M., and M. J. Morgan.** 1979. Regulation of the interferon system: evidence that Vero cells have a genetic defect in interferon production. J Gen Virol **43:**247-52.
11. **Everett, R. D.** 2000. ICP0, a regulator of herpes simplex virus during lytic and latent infection. Bioessays **22:**761-70.
12. **Everett, R. D., C. Boutell, and A. Orr.** 2004. Phenotype of a herpes simplex virus type 1 mutant that fails to express immediate-early regulatory protein ICP0. J Virol **78:**1763-74.
13. **Everett, R. D., P. Freemont, H. Saitoh, M. Dasso, A. Orr, M. Kathoria, and J. Parkinson.** 1998. The disruption of ND10 during herpes simplex virus infection correlates with the Vmw110- and proteasome-dependent loss of several PML isoforms. J Virol **72:**6581-91.

14. **Everett, R. D., and G. G. Maul.** 1994. HSV-1 IE protein Vmw110 causes redistribution of PML. Embo J **13:**5062-9.
15. **Everett, R. D., A. Orr, and C. M. Preston.** 1998. A viral activator of gene expression functions via the ubiquitin-proteasome pathway. Embo J **17:**7161-9.
16. **Everett, R. D., G. Sourvinos, C. Leiper, J. B. Clements, and A. Orr.** 2004. Formation of nuclear foci of the herpes simplex virus type 1 regulatory protein ICP4 at early times of infection: localization, dynamics, recruitment of ICP27, and evidence for the de novo induction of ND10-like complexes. J Virol **78:**1903-17.
17. **Everett, R. D., D. F. Young, R. E. Randall, and A. Orr.** 2008. STAT-1- and IRF-3-dependent pathways are not essential for repression of ICP0-null mutant herpes simplex virus type 1 in human fibroblasts. J Virol **82:**8871-81.
18. **Everett, R. D., and A. Zafiropoulos.** 2004. Visualization by live-cell microscopy of disruption of ND10 during herpes simplex virus type 1 infection. J Virol **78:**11411-5.
19. **Guo, J., D. J. Hui, W. C. Merrick, and G. C. Sen.** 2000. A new pathway of translational regulation mediated by eukaryotic initiation factor 3. Embo J **19:**6891-9.
20. **Hagglund, R., C. Van Sant, P. Lopez, and B. Roizman.** 2002. Herpes simplex virus 1-infected cell protein 0 contains two E3 ubiquitin ligase sites specific for different E2 ubiquitin-conjugating enzymes. Proc Natl Acad Sci U S A **99:**631-6.
21. **Halford, W. P., C. D. Kemp, J. A. Isler, D. J. Davido, and P. A. Schaffer.** 2001. ICP0, ICP4, or VP16 expressed from adenovirus vectors induces reactivation of latent herpes simplex virus type 1 in primary cultures of latently infected trigeminal ganglion cells. J Virol **75:**6143-53.
22. **Hancock, M. H., J. A. Corcoran, and J. R. Smiley.** 2006. Herpes simplex virus regulatory proteins VP16 and ICP0 counteract an innate intranuclear barrier to viral gene expression. Virology **352:**237-52.
23. **Li, X. L., M. Boyanapalli, X. Weihua, D. V. Kalvakolanu, and B. A. Hassel.** 1998. Induction of interferon synthesis and activation of interferon-stimulated genes by liposomal transfection reagents. J Interferon Cytokine Res **18:**947-52.
24. **Lin, R., R. S. Noyce, S. E. Collins, R. D. Everett, and K. L. Mossman.** 2004. The herpes simplex virus ICP0 RING finger domain inhibits IRF3- and IRF7-mediated activation of interferon-stimulated genes. J Virol **78:**1675-84.
25. **Mosca, J. D., and P. M. Pitha.** 1986. Transcriptional and posttranscriptional regulation of exogenous human beta interferon gene in simian cells defective in interferon synthesis. Mol Cell Biol **6:**2279-83.
26. **Mossman, K. L., P. F. Macgregor, J. J. Rozmus, A. B. Goryachev, A. M. Edwards, and J. R. Smiley.** 2001. Herpes simplex virus triggers and then disarms a host antiviral response. J Virol **75:**750-8.

27. **Mossman, K. L., H. A. Saffran, and J. R. Smiley.** 2000. Herpes simplex virus ICP0 mutants are hypersensitive to interferon. J Virol **74:**2052-6.
28. **Mossman, K. L., and J. R. Smiley.** 2002. Herpes simplex virus ICP0 and ICP34.5 counteract distinct interferon-induced barriers to virus replication. J Virol **76:**1995-8.
29. **Mossman, K. L., and J. R. Smiley.** 1999. Truncation of the C-terminal acidic transcriptional activation domain of herpes simplex virus VP16 renders expression of the immediate-early genes almost entirely dependent on ICP0. J Virol **73:**9726-33.
30. **Netterwald, J. R., T. R. Jones, W. J. Britt, S. J. Yang, I. P. McCrone, and H. Zhu.** 2004. Postattachment events associated with viral entry are necessary for induction of interferon-stimulated genes by human cytomegalovirus. J Virol **78:**6688-91.
31. **Paladino, P., D. T. Cummings, R. S. Noyce, and K. L. Mossman.** 2006. The IFN-independent response to virus particle entry provides a first line of antiviral defense that is independent of TLRs and retinoic acid-inducible gene I. J Immunol **177:**8008-16.
32. **Prescott, J., C. Ye, G. Sen, and B. Hjelle.** 2005. Induction of innate immune response genes by Sin Nombre hantavirus does not require viral replication. J Virol **79:**15007-15.
33. **Preston, C. M., A. N. Harman, and M. J. Nicholl.** 2001. Activation of interferon response factor-3 in human cells infected with herpes simplex virus type 1 or human cytomegalovirus. J Virol **75:**8909-16.
34. **Randall, R. E., and S. Goodbourn.** 2008. Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. J Gen Virol **89:**1-47.
35. **Smiley, J. R., and J. Duncan.** 1997. Truncation of the C-terminal acidic transcriptional activation domain of herpes simplex virus VP16 produces a phenotype similar to that of the in1814 linker insertion mutation. J Virol **71:**6191-3.
36. **Stow, N. D., and E. C. Stow.** 1986. Isolation and characterization of a herpes simplex virus type 1 mutant containing a deletion within the gene encoding the immediate early polypeptide Vmw110. J Gen Virol **67 (Pt 12):**2571-85.
37. **tenOever, B. R., M. J. Servant, N. Grandvaux, R. Lin, and J. Hiscott.** 2002. Recognition of the measles virus nucleocapsid as a mechanism of IRF-3 activation. J Virol **76:**3659-69.
38. **Triezenberg, S. J., R. C. Kingsbury, and S. L. McKnight.** 1988. Functional dissection of VP16, the trans-activator of herpes simplex virus immediate early gene expression. Genes Dev **2:**718-29.
39. **Wathelet, M. G., P. M. Berr, and G. A. Huez.** 1992. Regulation of gene expression by cytokines and virus in human cells lacking the type-I interferon locus. Eur J Biochem **206:**901-10.
40. **Yanai, H., D. Savitsky, T. Tamura, and T. Taniguchi.** 2009. Regulation of the cytosolic DNA-sensing system in innate immunity: a current view. Curr Opin Immunol **21:**17-22.

41. **Yao, F., and P. A. Schaffer.** 1995. An activity specified by the osteosarcoma line U2OS can substitute functionally for ICP0, a major regulatory protein of herpes simplex virus type 1. J Virol **69:**6249-58.

Chapter Five: Herpes Simplex Virus VP16, but not ICP0, is Required to Reduce Histone Occupancy and Enhance Histone Acetylation on Viral Genomes in U2OS Osteosarcoma Cells.

A version of this chapter has been submitted for publication in the Journal of Virology

Preface to Chapter 5

The role of ICP0 in stimulating viral gene expression through overcoming an innate antiviral response has long been postulated in the literature. The data I have presented in Chapters three and four suggest that VP16 may also stimulate gene expression by counteracting an innate antiviral defense mechanism, potentially by targeting a similar pathway as ICP0. While the previous experiments were being performed, evidence accumulated in the literature that both VP16 and ICP0 play roles in modulating the chromatin structure on the viral genome and that this may be involved in stimulating viral gene expression. Therefore, I hypothesized that U2OS cells have a defect in the chromatin-based pathways targeted by these proteins which allows for viral gene expression in the absence of either ICP0 or VP16. The content of this chapter addresses this hypothesis.

The publication entitled "Herpes Simplex Virus VP16, but not ICP0, is Required to Reduce Histone Occupancy and Enhance Acetylation on Viral Genomes in U2OS Osteosarcoma Cells." is the content of Chapter six of this thesis. This work is in revision with the Journal of Virology. All experiments were performed by me, while writing and editing was shared between Dr Smiley and myself. Drs Knipe and Cliffe provided me with the opportunity to learn chromatin immunoprecipitation and were involved in the editing process.

ABSTRACT

The HSV genome rapidly becomes associated with histones following injection into the host cell nucleus. The viral proteins ICP0 and VP16 are required for efficient viral gene expression and have been implicated in reducing the levels of under-acetylated histones on the viral genome, raising the possibility that high levels of under-acetylated histones inhibit viral gene expression. The U2OS osteosarcoma cell line is permissive for replication of ICP0 and VP16 mutants, and appears to lack an innate antiviral repression mechanism present in other cells types. We therefore used chromatin immunoprecipitation to determine if U2OS cells are competent to load histones onto HSV DNA, and if so, whether ICP0 and/or VP16 are required to reduce histone occupancy and enhance acetylation in this cell type. High levels of under-acetylated histone H3 accumulated at several locations on the viral genome in the absence of VP16 activation function; in contrast, an ICP0 mutant displayed markedly reduced histone levels and enhanced acetylation, similar to wild-type HSV. These results demonstrate that U2OS cells are competent to load under-acetylated histones onto HSV DNA and uncover an unexpected role for VP16 in modulating chromatin structure at viral early and late loci. One interpretation of these findings is that ICP0 and VP16 affect viral chromatin structure through separate pathways, and the pathway targeted by ICP0 is defective in U2OS cells. We also show that HSV infection results in decreased histone levels on some actively transcribed genes within the cellular genome, demonstrating that viral infection alters cellular chromatin structure.

INTRODUCTION

Herpes Simplex virus (HSV) is a double-stranded DNA virus that undergoes productive replication in the nucleus of infected cells. The linear genome is packaged into a nucleocapsid and is released into the cytoplasm upon fusion of the viral and host cell membranes. Also released are the pre-formed tegument proteins, which play important roles in counteracting host defenses and stimulating viral gene expression. The tegument protein VP16 acts to stimulate immediate early (IE) gene expression through the recruitment of general transcription factors and RNA polymerase II to the IE promoters (30, 73), launching the temporal cascade of gene expression.

The HSV genome is thought to be complexed with the polyamine spermine within virions (9, 25, 58). Upon injection into the nucleus, the genome associates with host histones (32, 33, 38, 46), most likely in a form involving the four core histones (22, 46) and at a density significantly less than that of cellular chromatin (22, 32, 46). Nucleosomes are the basic repeating units of chromatin comprised of approximately 146 bp of DNA wrapped around a histone octamer composed of two copies of each of the four core histone proteins (H2A, H2B, H3 and H4). The structure of chromatin can be altered both by post-translational modifications of histones and through ATP-dependent remodeling of the nucleosomes (42).

Chromatin remodeling involves eviction or sliding of nucleosomes along the DNA template, increasing the accessibility of the DNA to other interacting proteins. These processes require specific chromatin remodeling complexes which hydrolyze ATP and are recruited to the DNA through covalent modifications of histones (64). Covalent modifications of histones include acetylation, methylation, phosphorylation and ubiquitination. How each histone modification influences gene expression is not yet fully understood (35), but some marks have been generally linked to transcriptional outcomes. For example, histone acetylation correlates with transcriptional activation, and histone methylation correlates with either activation or silencing depending on which residue within the histone is methylated (42). Acetylation is thought to relax the interactions between histones and DNA by altering the net charge of the nucleosome (23) and additionally enhances transcription by recruiting chromatin remodeling complexes (31, 37). Modified histones provide docking sites for proteins that contain specific interaction motifs. For example, bromodomain-containing proteins bind to acetylated histones, while chromodomain-containing proteins, such as the heterochromatin-associated protein HP1, bind to histone H3 trimethylated at lysine 9 (42).

During latent HSV infection the nucleosomes on the viral genome are arranged in a regular repeating pattern similar to cellular chromatin (12). The histones bound to most regions of the genome display features characteristic of transcriptionally silent chromatin, such as reduced acetylation, increased levels of

H3K9 di- and trimethylation (73) and H3K27 trimethylation (7, 48), and the presence of the histone variant macroH2A (48). The exceptions are the histones found within the promoter and 5' region of the latency-associated transcript (LAT), which bear activating marks such as acetylation of histone H3 lysine 9 and 14 (H3K9/K14Ac) (43, 44, 73). LATs are the only transcripts expressed in latently infected neurons (68, 69) and hence, these observations suggest that covalent histone modifications play an important role in regulating HSV gene expression during latency.

In contrast to latency, during productive infection the histones are arranged on the viral genome in an irregular, non-repeating pattern (49, 56). As assessed by chromatin immunoprecipitation (ChIP), histone occupancy is often significantly lower than on the cellular genome (46), although the levels depend on the multiplicity of infection (MOI) used (8). Accumulating evidence suggests that histone modifications may also play an important role in regulating HSV gene expression during productive infection (reviewed in (40, 47)). For example, marks correlating with active transcription, such as H3K9/K14Ac and H3K4 trimethylation, are found within promoters of actively transcribed viral genes (32, 33, 44). The histone methyltransferase Set1 is important in maintaining the H3K4 trimethylation modification on the viral genome and viral transcription and replication are inhibited when Set1 levels are knocked down by siRNAs. (33). Additionally, knocking down expression of HIRA, a chaperone for the histone H3 variant H3.3, results in decreased association of histone H3.3 with the viral

genome and decreased viral transcription and replication (59). These results are consistent with the idea that HIRA actively loads H3.3 onto the viral genome and that viral gene expression is enhanced by this histone variant.

Two HSV proteins, VP16 and ICP0, have been implicated in regulating the levels and covalent modifications of the histones on the HSV genome during productive infection. Extensive studies into the mechanism of action of the acidic activation domain (AD) of VP16 in heterologous systems have revealed that it recruits general transcription factors, RNA polymerase II, histone acetyltransferases (HATs), and ATP-dependent chromatin remodeling complexes to promoters (32, 39, 41, 52, 54, 72, 74). ChIP experiments indicate that the VP16 AD also recruits these proteins to viral IE promoters during productive HSV infection (32). Interestingly, knocking down expression of these HATs and chromatin remodeling complexes via siRNAs (either singly or in combination) has no detectable effect on viral IE gene expression in cultured cells, arguing that these chromatin modulators do not play a major role during productive infection (45). It therefore remains unclear why the VP16 AD recruits these modulators to IE promoters during productive infection.

The AD of VP16 has also been implicated in preventing the deposition, or enhancing the removal, of histones from viral IE promoters (32, 46): histone occupancy is higher on a VP16 AD mutant genome than on the wild type genome (46). This increase may be due to reduced transcription of the VP16 AD mutant

genome, as treatment with the transcription inhibitor actinomycin D (ActD) increases histone occupancy on a wild type genome. In addition, newly synthesized viral proteins likely play a role in regulating histone occupancy at many regions of the viral genome, as the protein synthesis inhibitor cycloheximide increased histone levels on the ICP27, thymidine kinase (TK) and gC promoters (46). Interestingly, HSV-2 superinfection of cells harbouring a silent HSV-1 VP16 AD mutant virus activated transcription from the mutant genome without reducing histone levels or increasing histone acetylation (46). These observations raise the possibility that once histones are loaded onto the VP16 AD mutant genome they are refractory to subsequent removal or acetylation, and suggest that they do not preclude viral gene expression.

The IE protein ICP0 has also been implicated in regulating chromatinization of the viral genome. Cliffe and Knipe demonstrated that the genome of an ICP0 null mutant displays higher levels of bound histone H3 and reduced H3 acetylation compared to wild type virus during low MOI infection of HeLa cells (8). Consistent with these results, Ferenczy et al demonstrated that a viral mutant that expresses only ICP0 displays lower histone occupancy and higher histone acetylation levels than a mutant expressing no viral genes (22). ICP0 has been shown to dissociate histone deacetylases (HDAC) 1 and 2 from the CoREST/REST/HDAC repressor complex (26, 27) and interacts with HDACs 5,6 and 7 (50), suggesting a direct role in modulating the acetylation of histones bound to the viral genome. Whether these activities contribute to the ability of

ICP0 to stimulate transcription of all classes of viral genes (3, 4, 13-15, 24, 36, 57, 62, 65, 70) remains to be determined.

HSV mutants lacking ICP0 or the activation function of VP16 share several phenotypic similarities, including a much higher than normal particle-to-plaque forming unit (PFU) ratio and lower levels of IE gene expression upon low multiplicity infection of many cell types (1, 4, 14, 66, 70). The phenotype of such mutants varies between cell types, with the most restrictive being primary human fibroblasts (15). Following infection of non-permissive cells, the viral genome is retained in a transcriptionally silent, extrachromosomal state (1, 15, 29, 30, 34, 60, 61, 63, 65, 70). Such quiescent genomes are actively repressed, as shown by the finding that heterologous promoters embedded within them are also silenced (34, 53, 61, 65). However, quiescent genomes can be efficiently reactivated by ICP0 provided in *trans* (29, 30, 34, 60, 61, 65, 70). The histones bound to quiescent genomes are under-acetylated, enriched in H3K9 methylation, and complexed with heterochromatin-associated protein HP1, all classical marks of transcriptional repression (10, 22). Following ICP0-mediated derepression, histone acetylation increases markedly (10), suggesting one role of ICP0 in reactivation of quiescent genomes may be to alter histone modifications.

Although VP16 and ICP0 mutants display severely restricted gene expression profiles in many cell types, the activation functions of these proteins are largely dispensable in the human osteosarcoma cell line U2OS (66, 75). We

previously provided evidence that the permissive phenotype of U2OS cells stems from defects in an innate antiviral repression mechanism that is present in other cells types (28). We further suggested that the defect might inactivate a chromatin assembly pathway that incorporates newly delivered viral genomes into repressive chromatin. We therefore used ChIP assays to determine if U2OS cells are competent to load histones onto HSV DNA, and if so, whether ICP0 and/or VP16 are required to reduce histone H3 occupancy in this cell type. Our results clearly indicate that U2OS cells are able to load high levels of under-acetylated histones onto viral DNA in the absence of VP16 activation function. However in contrast to the situation in HeLa cells, ICP0 does not appear to regulate histone H3 levels or acetylation on the viral genome in this cell line.

RESULTS

Gene expression profiles of n212 and V422 infection in HeLa and U2OS cells

Previous studies documenting the roles of VP16 and ICP0 in the regulation of HSV genome chromatinization were conducted in HeLa cells (8, 32, 46). We sought to examine the effects of VP16 and ICP0 on genome chromatinization in U2OS cells, which "complement" the growth defect of HSV ICP0 and VP16 mutants (55, 66, 75). In order to provide a basis for assessing the functional significance of any such effects, we first compared the consequences of inactivating ICP0 and VP16 on viral gene expression in U2OS and HeLa cells. To this end, we monitored the accumulation of mRNAs derived from the viral ICP27 (IE), TK (early) and VP16 (leaky-late) genes following infection of U2OS and HeLa cells with 1 PFU/cell of wild type KOS, the ICP0 mutant n212 and the VP16 AD mutant V422 (Figure 5.1). Viral DNA replication was blocked by the addition of the viral DNA polymerase inhibitor phosphonoacetic acid (PAA) at the time of infection, in order to restrict the analysis to expression from input viral genomes.

In HeLa cells, ICP27 mRNA accumulated between 3 and 6hpi with both KOS and n212, while virtually no ICP27 mRNA was detected over the 12h time course with V422 (Figure 5.1a). n212 displayed wild-type levels of ICP27 mRNA, but TK and VP16 transcripts accumulated with delayed kinetics and to lower levels. The results with TK mRNA are similar to the previous findings of Jordan and Schaffer (36), who examined accumulation of viral mRNAs during

n212 infection of Vero cells (ICP27 and VP16 mRNA were not examined in that study). In contrast, TK and VP16 mRNA could not be detected during infection with V422. Thus, both mutants exhibit a restricted gene expression profile in HeLa cells, with V422 displaying a much more severe defect.

As expected, U2OS cells supported much higher levels of viral gene expression than HeLa cells following infection with n212 and V422 (Figure 5.1b). However, significant defects relative to wild-type KOS were nevertheless observed. For example, abundant ICP27 mRNA was present by 3hpi with KOS, but accumulation of the transcript was delayed with n212 and V422, and the levels were significantly reduced at all time points with V422 (Figure 5.1b). Similarly, accumulation of TK and VP16 mRNAs was delayed and reduced with both mutants. Overall, these data indicate that V422 is somewhat more impaired than n212 in this cell type, as assayed by gene expression in the presence of PAA.

Histone H3 occupancy on the n212 and V422 genomes in HeLa and U2OS cells

We previously suggested that the defect that renders U2OS cells permissive to VP16 and ICP0 mutants might inactivate a chromatin assembly pathway that represses incoming viral genomes in other cell types (28). To test this hypothesis, we used ChIP assays to follow loading and acetylation of histone H3 on viral genomes in HeLa and U2OS cells infected with wild type KOS, n212 and V422. All experiments were performed in the presence of PAA to restrict the

analysis to input viral genomes. In addition, the n212 and V422 input multiplicities were adjusted to deliver the same amount of input viral DNA (measured at 3hpi) as is achieved with 1 PFU/cell of wild-type KOS (Material and Methods). In no case did this adjustment alter the MOI by more than 25%.

We first monitored histone H3 levels at the transcriptional start site (TSS) of the IE ICP27 gene (Figure 5.2a). In HeLa cells infected with wild-type KOS, histone H3 levels rose between 1 and 3 hpi, then declined by 6 hpi; in contrast, H3 continued to accumulate on the n212 and V422 genomes, leading to significantly higher levels than on the KOS genome by 6hpi, consistent with previous reports (8, 32, 46). U2OS cells displayed a very different pattern: H3 levels remained low on the KOS and n212 genomes over the entire time course, while H3 accumulated on the V422 genome (Figure 5.2a). These results indicate that the AD of VP16 is required to reduce histone occupancy on the ICP27 TSS in both HeLa and U2OS cells, while ICP0 is dispensable in U2OS cells. Interestingly, at 3 hpi in U2OS cells, the level of H3 on the ICP27 region of the n212 genome was similar to wild type virus, whereas at the TK and VP16 promoters it was higher, and more similar to V422. This suggests that ICP0 is not required at IE regions of the genome at 3 hpi, but may be required at E and L regions at that time in U2OS cells. The reasons for these observations are currently unclear. In addition, the accumulation of high levels of histone H3 on the V422 genome in U2OS cells documents that these cells are competent to add histones onto incoming viral genomes.

We next tested whether the increase in histone occupancy at the ICP27 TSS observed with n212 and V422 in HeLa cells, and with V422 in U2OS cells, also occurs at other regions of the viral genome. In Hela cells, enhanced histone H3 levels were observed at the TK and VP16 promoters with both mutants (Figure 5.2b and c), data that are in accord with recent reports documenting that H3 levels are increased at multiple gene loci, including early and late genes, when either VP16 or ICP0 is inactivated (8, 46). Similarly, in U2OS cells, n212 and V422 displayed low and high histone H3 levels respectively at all three loci. Thus, the effects of VP16 and ICP0 on histone occupancy are not restricted to IE genes in either cell type.

VP16 is required for enhanced acetylation of histone H3 bound to the viral genome in U2OS cells, while ICP0 is dispensable

Cliffe and Knipe demonstrated that the histone H3 bound to the genome of an HSV-1 ICP0 null mutant displays lower levels of acetylation than that bound to the corresponding marker rescue virus during low MOI infection of HeLa cells, suggesting that ICP0 plays an important role in stimulating histone acetylation in this cell type (8). In addition, Herrera and Triezenberg documented that VP16 stimulates acetylation of histones bound to viral DNA in HeLa cells (32). We therefore asked if ICP0 and/or VP16 are required for enhanced histone acetylation in permissive U2OS cells.

HeLa and U2OS cells were infected with KOS, n212 or V422 for 6 and 9 hours, and then harvested for ChIP. Sonicated lysates were divided, and immunoprecipitations were performed using the total histone H3 and acetyl-H3 antibodies in parallel. The quantity of acetylated histones immunoprecipitated from each sample was then normalized to the amount of immunoprecipated total histone H3. In HeLa cells the levels of acetylated histone H3 were significantly higher on the KOS genome than on n212 or V422 at 6 and 9 hpi at all regions of the viral genome tested, confirming previous reports (Figure 5.3a) (8, 32). In contrast, in U2OS cells histone H3 acetylation levels were high for both KOS and n212, but acetylation remained low on the V422 genome (Figure 5.3b). These trends were observed at all regions of the viral genome tested (Figure 5.3). Interestingly, n212 displayed somewhat higher levels of histone acetylation than KOS, but this difference was not statistically significant except for the VP16 promoter at 6 hpi. The biological relevance of this observation remains unclear. These observations indicate that in U2OS cells, histone H3 acetylation requires the activation function of VP16, but does not require the action of ICP0.

The effect of cycloheximide on histone H3 occupancy in HeLa and U2OS cells

Kutluay and Triezenberg suggest that the AD of VP16 acts indirectly to reduce histone levels at non-IE promoters through its ability to stimulate transcription of the viral IE genes (46). According to this hypothesis, one or more of the IE proteins acts to reduce histone levels on E and L regions of the viral genome. Histone occupancy at the E and L region of the wild type genome

increases in the presence of the protein synthesis inhibitor cycloheximide in HeLa cells, supporting this hypothesis (46). Given the differences that are observed in the viral factors required to reduce chromatinization of the viral genome in U2OS and HeLa cells, we determined if cycloheximide increases histone occupancy at E and L regions in U2OS cells.

Figure 5.4 shows the results of cycloheximide treatment on histone H3 occupancy at 6 hpi in HeLa cells. At the ICP27, TK and VP16 regions, histone H3 occupancy on the KOS genome increased in the presence of cycloheximide, suggesting that protein synthesis is required to maintain low levels of histones at all regions of the viral genome, as previously reported (46). Kutluay and Triezenberg report that the ICP27 gene is treated differently from other IE genes in that its histone occupancy requires newly synthesized proteins (46). Cycloheximide treatment had little effect on histone H3 levels on the n212 and V422 genomes, which remained high.

The experiment gave strikingly different results in U2OS cells (Figure 5.4). Histone H3 occupancy at the ICP27, TK and VP16 regions of the KOS and n212 genomes remained low in the presence of cycloheximide, while histone H3 levels on the V422 genome dropped significantly in the presence of the drug. These results cannot easily be reconciled with the hypothesis that newly synthesized viral proteins are required to reduce histone levels on the viral genome, as appears to be the case in HeLa cells. Rather, these results imply that

histone loading in U2OS cells requires ongoing protein synthesis. One possibility is that the machinery required to load histones onto viral DNA is not expressed constitutively in U2OS cells, but is induced by infection. According to this scenario, KOS and n212 inactivate this cellular response, while V422 does not, possibly because viral gene expression is significantly delayed or because the AD of VP16 is directly required. These possibilities require further investigation. Given the results of these experiments we were unable to assess whether a newly synthesized viral protein is required to mediate low histone occupancy at non-IE regions of the viral genome in U2OS cells.

Effects of viral infection on histone H3 levels on the cellular genome

During the course of the experiments described above, we also measured histone H3 levels at the TSS of the cellular GAPDH gene, with the intention of using this value as an internal control to correct for differences in cross-linking and immunoprecipitation efficiencies between experiments. However, the results demonstrated that the histone H3 levels on the GAPDH TSS significantly decrease during wild-type HSV infection, in both HeLa and U2OS cells (the data obtained in U2OS cells are shown in Figure 5.5a). A similar decrease in histone occupancy was noted following infection with n212, but not with V422 (Figure 5.5a). To test whether this effect is specific to GAPDH, we also assessed histone occupancy at the promoter for the cellular U3 snoRNA gene (Figure 5.5b) and the pericentric satellite sequence Sat3 (Figure 5.5c). A significant decrease in histone

occupancy was observed at the U3 promoter during KOS and n212 infection, while no change was detected at the Sat3 region with any virus.

To determine whether protein synthesis is required for the observed decreases in histone occupancy, ChIP experiments were performed in U2OS cells infected with KOS, n212 and V422 for 6 hours in the presence or absence of cycloheximide. Cycloheximide treatment increased histone occupancy on the GAPDH TSS in cell infected with KOS and n212 infection, while histone H3 levels remained high with V422 (Figure 5.5d). Similar results were obtained in HeLa cells (Figure 5A.1).

These results indicate that HSV infection depletes histone H3 from specific regions of the cellular genome, and that this process does not require ICP0. Whether this effect represents a cellular response to infection or a specific effect of one or more viral proteins has not yet been determined.

DISCUSSION

Previous studies have shown that HSV-1 mutants lacking the activation functions of VP16 or ICP0 accumulate high levels of under-acetylated histones at multiple regions of the viral genome in HeLa cells (8, 22, 32, 46). One interpretation of the data presented in those reports is that VP16 reduces histone occupancy and enhances acetylation at IE regions, while ICP0 subsequently acts to produce the same effect over the rest of the viral genome. The effects of VP16 and ICP0 on histone occupancy and acetylation are consistent with the hypothesis that these proteins stimulate viral gene expression at least in part by countering the formation of a repressive chromatin structure. However, evidence contrary to this hypothesis has recently been presented by Kutluay and Treizenberg (45, 46). Firstly, a highly chromatinized and silent HSV-1 VP16 mutant genome was efficiently activated by superinfecting HSV-2, without any decrease in histone occupancy or increase in acetylation (46). Secondly, knocking down expression of the various HATs and chromatin remodeling complexes that are recruited to IE promoters by the VP16 AD had no effect on IE gene expression (45). Given these observations, the role of chromatin structure in regulating viral gene expression during productive infections clearly requires further investigation.

As one means of exploring the role of histone loading and acetylation in regulating viral gene expression, we examined these processes in U2OS cells, which “complement” the growth defects of both ICP0 and VP16 AD mutants (66, 75). This permissive phenotype is recessive in somatic cell hybrids, indicating

that U2OS cells lack an antiviral mechanism that is critical for limiting viral gene expression in restrictive cell types (28). If ICP0 and/or VP16 function to stimulate viral gene expression primarily by preventing the assembly of repressive chromatin on the viral genome, then U2OS cells would likely display a defect in this process. Seemingly consistent with this hypothesis, we found that the ICP0 mutant n212 displays a chromatin phenotype similar to that of wild type virus in U2OS cells: multiple regions of the genome were associated with low levels of highly acetylated histone H3. Thus, ICP0 is not required to reduce histone occupancy and enhance histone acetylation on the viral genome in this cell line, a finding that is in keeping with the idea that these cells lack a chromatin-based repression mechanism that is targeted by ICP0. However, the VP16 AD mutant V422 behaved very differently: its genome was loaded with high levels of under-acetylated histones, just as it is in HeLa cells. This result indicates that U2OS cells are competent to load under-acetylated histones onto viral DNA, and leads to the surprising conclusion that the VP16 AD acts, either directly or indirectly, to reduce histone occupancy and enhance histone acetylation at multiple regions of the viral genome, in a process that does not require ICP0.

How does the VP16 AD reduce histone occupancy and enhance acetylation on early and late genes?

Kutluay and Triezenberg (46) suggested that the accumulation of high levels of underacetylated histones on VP16 mutant genomes is a consequence of reduced transcription, rather than loss of VP16 function *per se*. However, V422

and n212 accumulate TK and VP16 mRNAs with roughly similar kinetics in U2OS cells (Figure 5.1) yet display very different patterns of histone loading and acetylation (Figures 5.2 and 5.3), arguing that reduced transcription is not the cause of the increased histone occupancy at the TK and VP16 loci of V422.

A second possibility is that the VP16 AD acts indirectly, by stimulating the expression of one or more viral proteins (other than ICP0) that regulate chromatinization of the viral genome. Arguing against this scenario, V422 replicates quite efficiently in U2OS cells (55, 66), indicating that most or all viral genes are eventually expressed in these cells. Thus, the hypothetical downstream viral factor almost certainly accumulates, but the viral genome nevertheless remains bound to high-levels of under-acetylated histones, just as in restrictive HeLa cells. Therefore, we consider this scenario unlikely.

In our view, the most likely explanation for the effect of VP16 on viral chromatin structure is that VP16 acts directly (either on its own or in concert with other viral proteins) to reduce histone loading and enhance histone acetylation at multiple regions of the genome. For example, the VP16 AD might bind and sequester one or more key proteins required for efficient accumulation of under-acetylated histones on the viral genome (such as an HDAC or a specialized histone chaperone), or actively recruit HATs to multiple regions of the viral genome, in addition to acting specifically at IE promoters. Further studies are required to test this hypothesis.

What is the primary defect in U2OS cells?

Our data argue that ICP0 and VP16 are both required to reduce histone occupancy and enhance acetylation at multiple loci on the viral genome in restrictive HeLa cells. The requirement for ICP0 is relieved in permissive U2OS cells, but the requirement for VP16 is maintained. These findings provide strong evidence that ICP0 and VP16 achieve their effects on viral chromatin structure though separate pathways. One model to account for these findings is that two independent chromatin assembly pathways are capable of loading high levels of under-acetylated histone onto the viral genome. One of these is inactivated by ICP0, while other is modulated by VP16. Under this scenario, U2OS cells lack the pathway targeted by ICP0, but retain the pathway that is targeted by VP16.

It is tempting to speculate that the chromatin assembly pathway targeted by ICP0 is linked to the well-documented ND10-based antiviral repression system. One of the earliest cellular responses to HSV infection is the mobilization of ND10 components to the incoming viral genome (17, 21, 51, 67). A critical role of ICP0 is to block the recruitment of ND10 components to the incoming viral genome through the proteasome-dependent degradation of SUMO-modified forms of PML and Sp100 (2, 6, 16, 17, 19, 20). The ability of ICP0 to cause degradation of ND10 components closely correlates with its ability to stimulate viral gene expression and reactivate quiescent genomes (20). Consistent with a link between ND10 components and chromatin assembly, the HIRA histone

chaperone localizes to ND10 (76) and quiescent HSV genomes bearing high levels of under-acetylated histones (10, 22) are surrounded by a shell of PML and other ND10 components (18). However, it is important to note that HDAC inhibitors such as Trichostatin A are unable to fully replace ICP0 in stimulating gene expression in restrictive human fibroblasts (19), and the domain of ICP0 that interacts with CoREST and causes the translocation of HDACs 1 and 2 to the cytoplasm is not required for ICP0-induced activation of viral gene expression (20). Thus, the under-acetylated histones that accumulate on the viral genome in the absence of ICP0 may not be directly responsible for primary genome repression. Perhaps loading of underacetylated histones is linked to, but downstream of, the ND10-based genome detection and primary repression system. Thus, it is possible that U2OS are defective in the ND10-based detection system, rather than in histone acetylation and removal *per se*.

Alternatively, it could be argued that the ability of ICP0 to reduce histone occupancy and enhance acetylation stems solely from its ability to stimulate expression of a downstream viral modulator of chromatin structure, such as VP16. According to this scenario, ICP0 is not required to modulate chromatin structure in U2OS cells because it is dispensable for viral gene expression in this cell type. However, this scenario is inconsistent with evidence indicating that ICP0 can modulate viral chromatin structure in the absence of significant expression of viral genes other than ICP6 (22).

If, as argued above, two pathways exist to load high levels of under-acetylated histone onto the viral genome, then two key questions arise. First, are the two pathways linked to functionally distinct outcomes? Second, what factors control which pathway predominates in cells where both are potentially operative? Further studies are required to address these questions.

Increased levels of under-acetylated histone H3 on the viral genome do not preclude viral gene expression

Our data demonstrate that viral gene expression profiles do not always strictly correlate with the load and acetylation status of histone H3 on the viral genome. For example, in HeLa cells both n212 and V422 display higher levels of under-acetylated histone H3 than wild-type virus at all loci tested (Figures 5.2, 5.3), but n212 expresses high levels of ICP27 mRNA, while V422 is essentially silent (Figure 5.1). Similarly, V422 displays an increased load of under-acetylated histone H3 in U2OS cells, where significant viral gene expression is observed. These data indicate that the increased levels of under-acetylated histones that accumulate on the viral genome in the absence of ICP0 and/or the VP16 AD do not present an insuperable barrier to viral gene expression. Kutluay and Triezenberg recently reached the same conclusion (46). Although it might be argued that only a small pool of un-chromatinized viral genomes are actively transcribed, Kutluay and Triezenberg's data demonstrate that RNA polymerase II and histones H3 and H2A co-occupy the same DNA templates, suggesting that at

least some of the transcribed RNA comes from highly chromatinized and under-acetylated genomes (46).

It is important to stress that the increased levels of histone H3 found on the viral genome in U2OS cells in the absence of VP16 activation function are similar to, or lower than, the histone density at two actively transcribed cellular genes (compare Figures 5.2 and 5.4) and thus clearly do not by themselves preclude transcription. What is surprising is that histone acetylation does not appear to be required for viral gene expression. On the cellular genome, histone acetylation levels are generally high at promoters of actively transcribed cellular genes and are thought to recruit chromatin remodelers which stimulate transcription (23, 31, 37). In contrast, although the VP16 AD recruits HATs and chromatin remodeling complexes to viral IE promoters (32), these factors are not required for efficient IE transcription during productive infection (45). It is currently unknown why VP16 recruits HATs and remodeling complexes to IE promoters; perhaps these factors are important during VP16-mediated reactivation from latency (71).

Effects of HSV infection on cellular chromatin structure

Our observation that HSV infection significantly decreases histone occupancy at two actively transcribed cellular genes is interesting and requires further investigation. Although ICP0 is not required, newly synthesized viral (or cellular) proteins are needed. It has previously been demonstrated that the linker histone H1 is mobilized upon HSV infection in a manner dependent on IE or E

gene expression (11). One possible interpretation of these results is that histones are mobilized away from cellular chromatin and onto the incoming viral genomes, although this has not been directly demonstrated. The histone variant H3.3 becomes associated with the viral genome at early times post-infection using a mechanism involving the DNA replication-independent histone chaperone HIRA (59). It is not clear if HIRA draws from H3.3 bound to the cellular genome, but cycloheximide treatment increases histone occupancy on the viral genome in HeLa cells (46), suggesting that new synthesis of histone H3.3 is likely not required.

Researchers studying histone occupancy on the HSV genome often normalize their ChIP data to an endogenous cellular gene as a means of controlling for variations in cross-linking and immunoprecipitation efficiencies between experiments. However, the data presented in this report suggest those normalizations may be misleading, especially if infections are performed for longer than 6 hours. These observations may be the result of the relatively high MOI used in these studies (MOI = 1) as no changes in histone occupancy are observed at lower MOIs (ARC and DMK, unpublished observations). Our results indicate that non-transcribed DNA sequences such as the Sat3 pericentric satellite could serve as a more reliable control than an actively transcribed gene in future experiments, as histone occupancy did not change significantly throughout infection on this sequence. It will be interesting to determine whether changes in

cellular histone occupancy are mediated by specific viral proteins or are a response of the infected cell.

TABLE 5.1: REAL TIME PCR PRIMERS

Gene Region	Primer sequence (5’-3’)
GAPDH TSS	ACTAGGCGCTCACTGTTCTCTCCCT
	AACTCACCCGTTGACTCCGACCTT
U3 Promoter	GCGCACCACACCAGGAGCAAACA
	AGCATAATACTGAATGACAGCCAATCA
	CAAACT
hsSat3	CAATCATCCAACGGAAGCTAATGGAA
	TCAACA
	TCCATTCGATGACGAGTCCATCCATTTCAA
ICP27 TSS	CACCACCAGAGGCCATATCCGACA
	AGCATATCAATGTCAGTCGCCATGACCG
ICP27 ORF	TGTGCGGCCTGGACGAACTGTGTT
	TGGCCAGAATGACAAACACGAAGGATGC
TK Promoter	CCACACGCGTCACCTTAATATGCGAA
	ATTGGCGAATTCGAACACGCAGATGCAG
VP16 Promoter	TCGGATTGGGAAACAAAGGCACGCAA
	TCCGTACCCAGACAATAAAGCACCAACAGG

Figure 5.1. Gene expression profiles of KOS, n212 and V422 in HeLa and U2OS cells. HeLa and U2OS cells were infected with the indicated viruses in the presence of PAA as described in the Materials and Methods. Total RNA was harvested after the indicated lengths of time and subjected to northern blot analysis using probes for ICP27, TK and VP16.

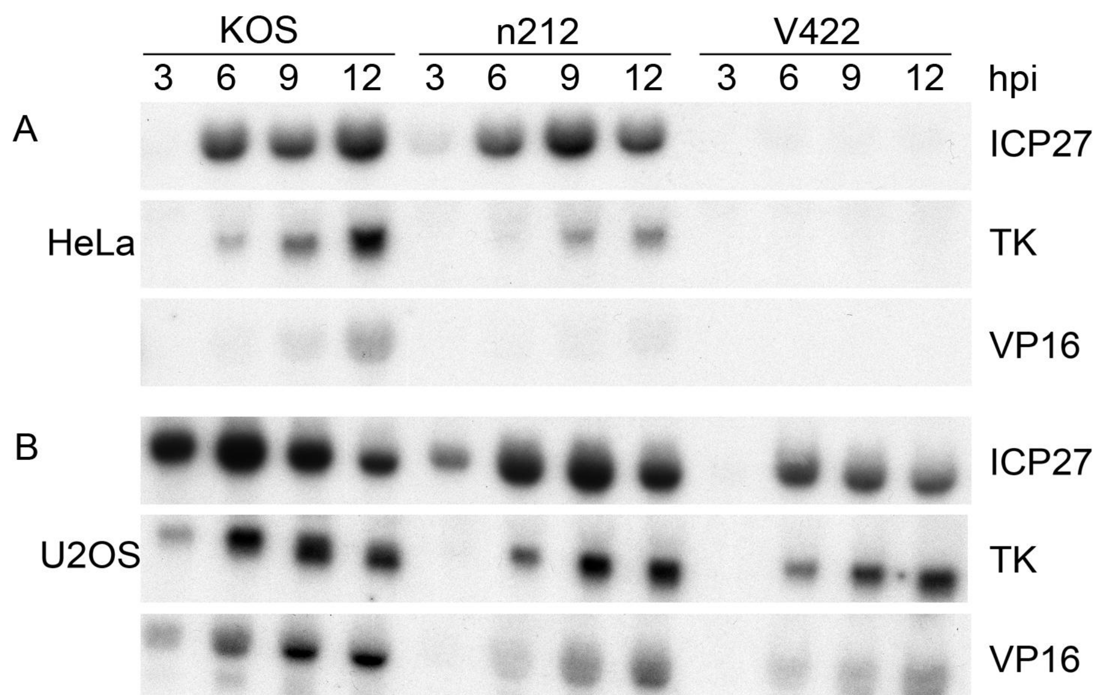

KOS
n212
V422
3
6
9
12
3
6
9
12
3
6
9
12
hpi
A
HeLa
ICP27
TK
VP16
B
U2OS
ICP27
TK
VP16

Figure 5.2. Histone H3 occupancy on KOS, n212 and V422 genomes in HeLa and U2OS cells. HeLa and U2OS cells were infected with the indicated viruses in the presence of PAA as described in the Materials and Methods. Cell lysates were prepared at 1, 3 and 6 hpi. The amount of immunoprecipitated DNA from the (A) ICP27 TSS, (B) TK promoter and (C) VP16 promoter was determined. Pooled data from at least three independent experiments are presented. Samples with mean values that differed significantly ($P < 0.05$, Student's *t* test) are indicated (*).

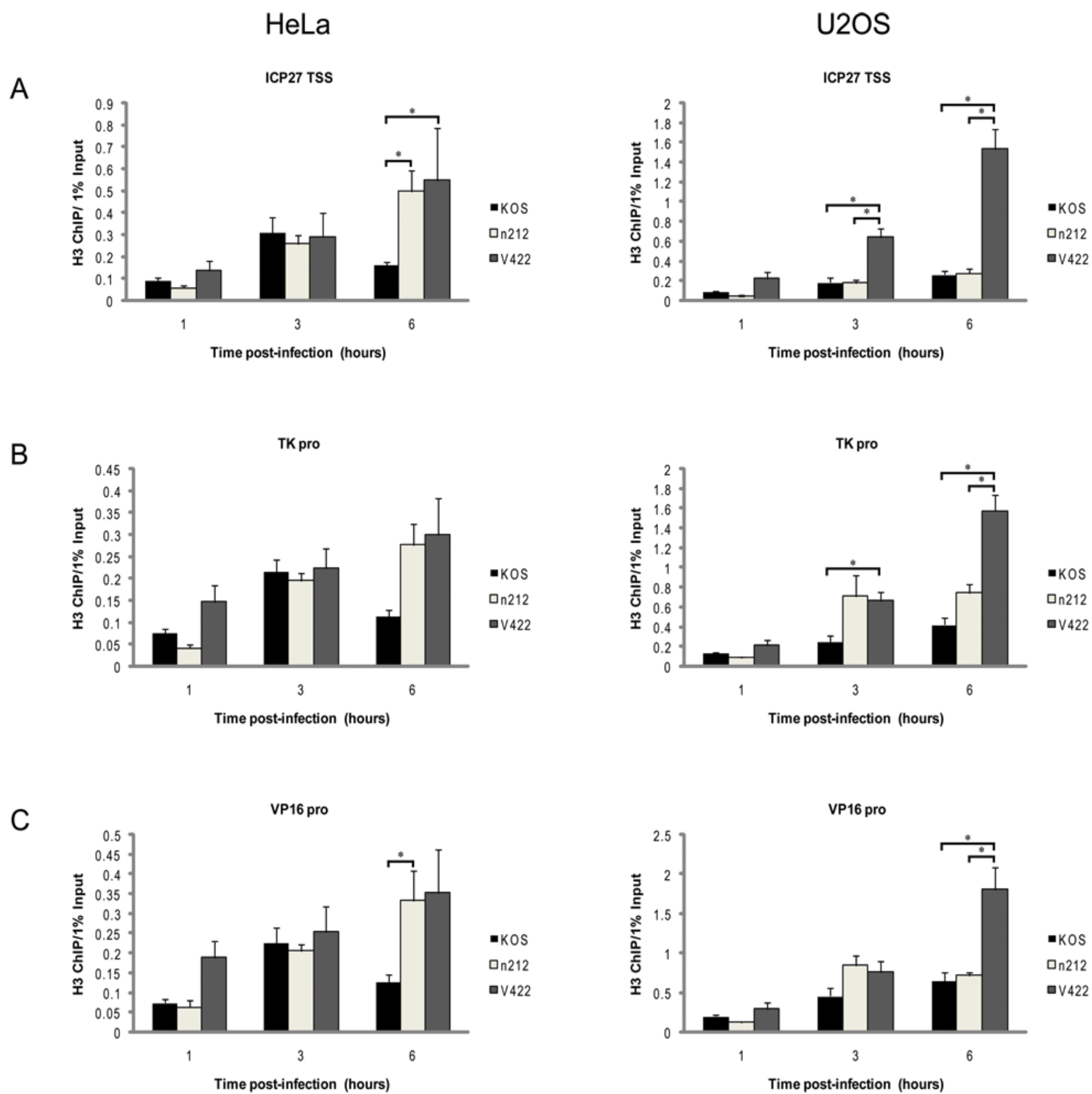

HeLa
U2OS
A
ICP27 TSS
H3 ChIP/ 1% Input
H3 ChIP/1% Input
Time post-infection (hours)
KOS
n212
V422
B
TK pro
C
VP16 pro

Figure 5.3. Acetylated histone H3 on KOS, n212 and V422 genomes in HeLa and U2OS cells. HeLa and U2OS cells were infected with the viruses in the presence of PAA as described in the Materials and Methods. Cell lysates were prepared at 6 and 9 hpi. The proportion of acetylated histone H3 associated with (A) ICP27 TSS, (B) TK promoter and (C) VP16 promoter viral DNA was determined as the fraction of DNA associated with acetylated H3 normalized to the fraction of DNA associated with total histone H3. Pooled data from at least three independent experiments are presented. Samples with mean values that differed significantly ($P < 0.05$, Student's *t* test) are indicated (*).

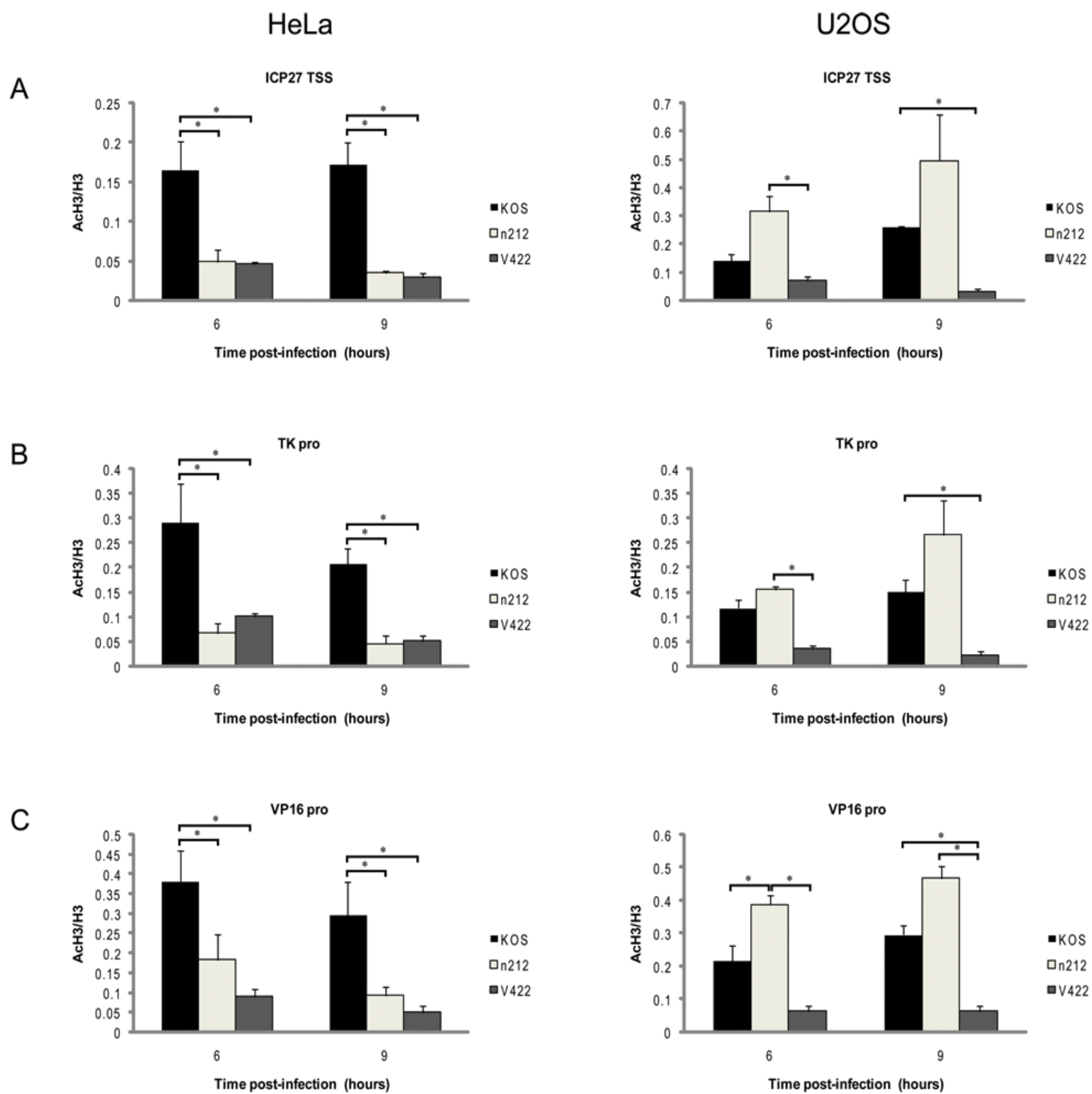
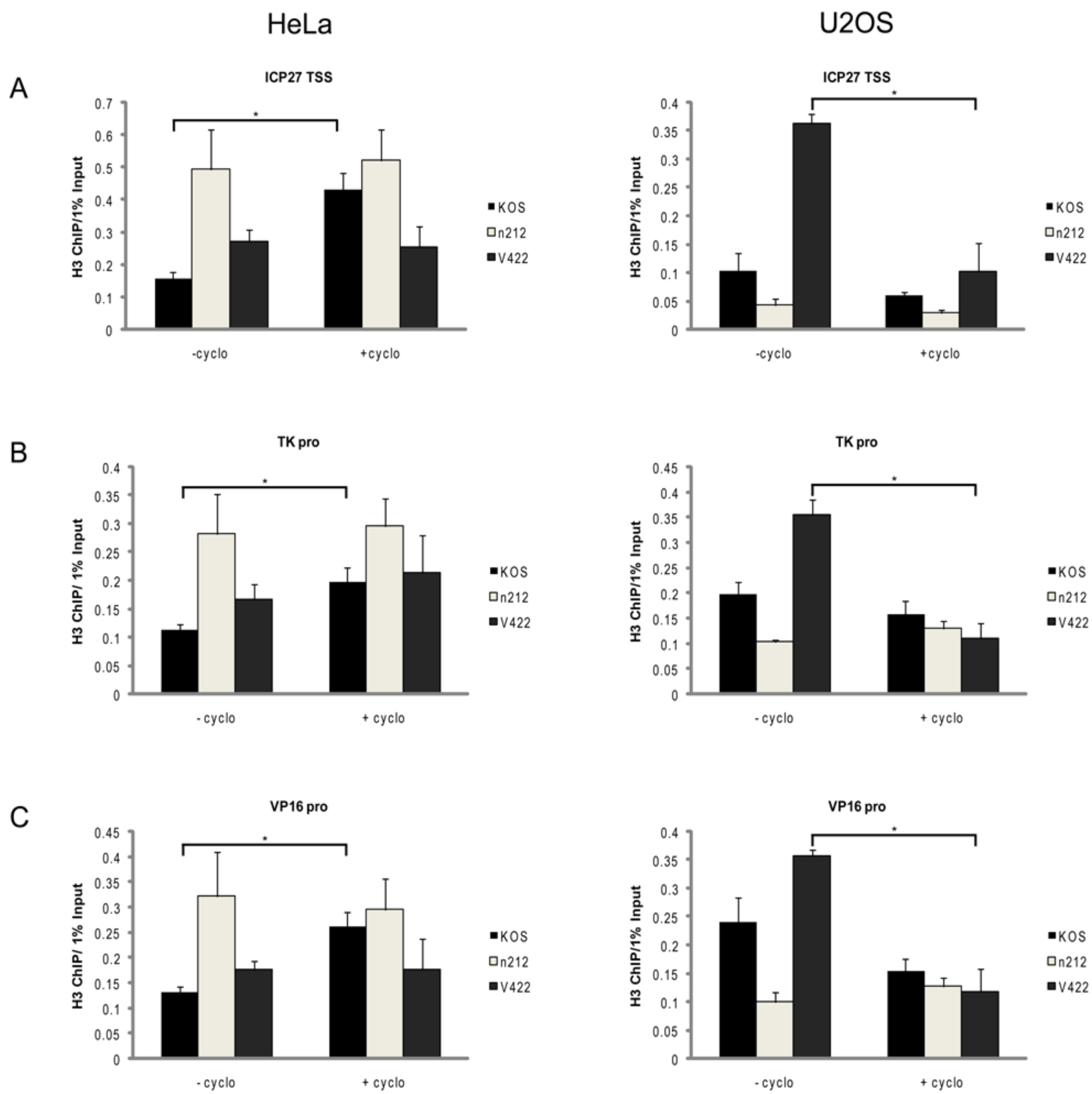
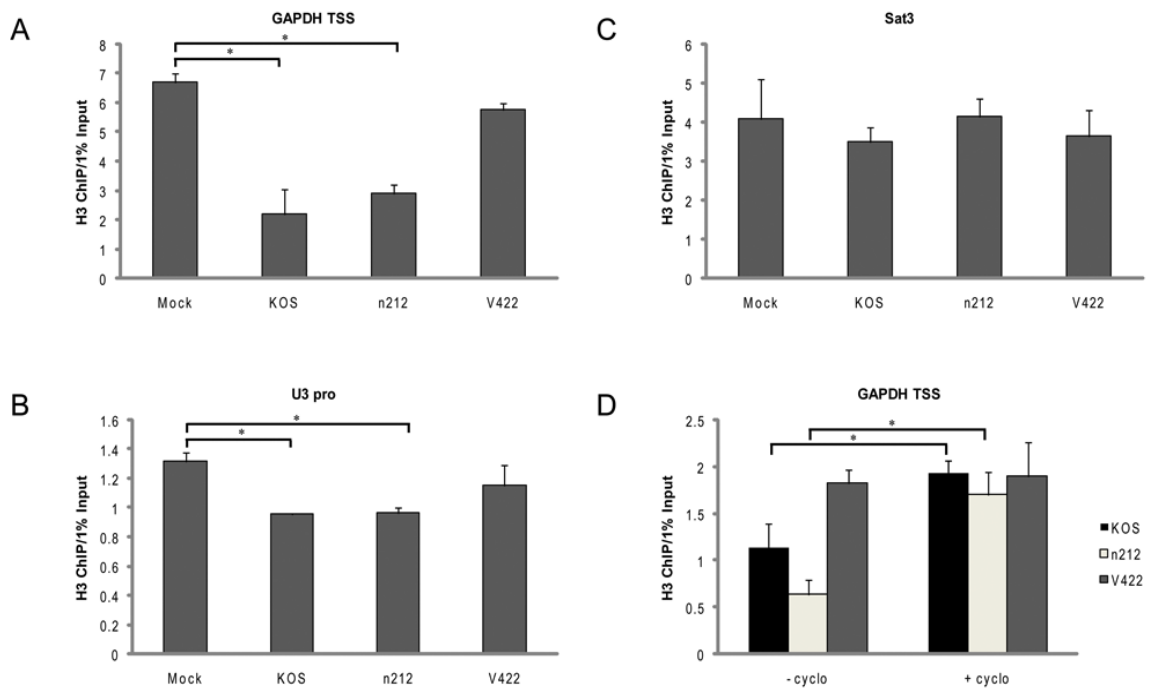

HeLa
U2OS
A
ICP27 TSS
AcH3/H3
Time post-infection (hours)
KOS
n212
V422
B
TK pro
C
VP16 pro

Figure 5.4. The effects of cycloheximide on histone H3 occupancy on the viral genome in HeLa and U2OS cells. Cells were infected with 1 PFU/cell of KOS, n212 or V422 in the presence or absence of cycloheximide for 6 hours and then samples were harvested for ChIP using a total H3 antibody. PCR primers amplified: (A) ICP27 TSS (B) TK promoter (C) VP16 promoter. All experiments were performed in triplicate. Samples with mean values that differed significantly ($P < 0.05$, Student's *t* test) are indicated (*).



HeLa
U2OS
A
ICP27 TSS
H3 ChIP/1% Input
-cyclo
+cyclo
KOS
n212
V422
B
TK pro
H3 ChIP/ 1% Input
H3 ChIP/1% Input
- cyclo
+ cyclo
C
VP16 pro

Figure 5.5. Effects of HSV infection on histone H3 occupancy at selected regions of the cellular genome. U2OS cells were infected with KOS, n212 and V422 or mock infected in the presence of PAA as described in the Materials and Methods. Cell lysates were prepared at 6 hpi. The amount of immunoprecipitated DNA associated with the (A) GAPDH TSS, (B) U3 promoter and (C) Sat3 sequence was determined. 6 hour infections were also performed in the presence of cycloheximide (D) and the amount of GAPDH TSS DNA immunoprecipitated was determined. Pooled data from at least three independent experiments are presented. Samples with mean values that differed significantly ($P < 0.05$, Student's *t* test) are indicated (*).


A
GAPDH TSS
H3 ChIP/1% Input
Mock
KOS
n212
V422
C
Sat3
H3 ChIP/1% Input
Mock
KOS
n212
V422
B
U3 pro
H3 ChIP/1% Input
Mock
KOS
n212
V422
D
GAPDH TSS
H3 ChIP/1% Input
- cyclo
+ cyclo
KOS
n212
V422

REFERENCES

1. **Ace, C. I., T. A. McKee, J. M. Ryan, J. M. Cameron, and C. M. Preston.** 1989. Construction and characterization of a herpes simplex virus type 1 mutant unable to transinduce immediate-early gene expression. J Virol **63:**2260-9.
2. **Boutell, C., A. Orr, and R. D. Everett.** 2003. PML residue lysine 160 is required for the degradation of PML induced by herpes simplex virus type 1 regulatory protein ICP0. J Virol **77:**8686-94.
3. **Cai, W., T. L. Astor, L. M. Liptak, C. Cho, D. M. Coen, and P. A. Schaffer.** 1993. The herpes simplex virus type 1 regulatory protein ICP0 enhances virus replication during acute infection and reactivation from latency. J Virol **67:**7501-12.
4. **Cai, W., and P. A. Schaffer.** 1992. Herpes simplex virus type 1 ICP0 regulates expression of immediate-early, early, and late genes in productively infected cells. J Virol **66:**2904-15.
5. **Cai, W. Z., and P. A. Schaffer.** 1989. Herpes simplex virus type 1 ICP0 plays a critical role in the de novo synthesis of infectious virus following transfection of viral DNA. J Virol **63:**4579-89.
6. **Chelbi-Alix, M. K., and H. de The.** 1999. Herpes virus induced proteasome-dependent degradation of the nuclear bodies-associated PML and Sp100 proteins. Oncogene **18:**935-41.
7. **Cliffe, A. R., D. A. Garber, and D. M. Knipe.** 2009. Transcription of the Herpes Simplex Virus Latency-Associated Transcript Promotes the Formation of Facultative Heterochromatin on Lytic Promoters. J Virol.
8. **Cliffe, A. R., and D. M. Knipe.** 2008. Herpes simplex virus ICP0 promotes both histone removal and acetylation on viral DNA during lytic infection. J Virol **82:**12030-8.
9. **Cohen, G. H., M. Ponce de Leon, H. Diggelmann, W. C. Lawrence, S. K. Vernon, and R. J. Eisenberg.** 1980. Structural analysis of the capsid polypeptides of herpes simplex virus types 1 and 2. J Virol **34:**521-31.
10. **Coleman, H. M., V. Connor, Z. S. Cheng, F. Grey, C. M. Preston, and S. Efstathiou.** 2008. Histone modifications associated with herpes simplex virus type 1 genomes during quiescence and following ICP0-mediated de-repression. J Gen Virol **89:**68-77.
11. **Conn, K. L., M. J. Hendzel, and L. M. Schang.** 2008. Linker histones are mobilized during infection with herpes simplex virus type 1. J Virol **82:**8629-46.
12. **Deshmane, S. L., and N. W. Fraser.** 1989. During latency, herpes simplex virus type 1 DNA is associated with nucleosomes in a chromatin structure. J Virol **63:**943-7.
13. **Everett, R. D.** 1985. Activation of cellular promoters during herpes virus infection of biochemically transformed cells. Embo J **4:**1973-80.
14. **Everett, R. D.** 1989. Construction and characterization of herpes simplex virus type 1 mutants with defined lesions in immediate early gene 1. J Gen Virol **70 (Pt 5):**1185-202.

15. **Everett, R. D., C. Boutell, and A. Orr.** 2004. Phenotype of a herpes simplex virus type 1 mutant that fails to express immediate-early regulatory protein ICP0. J Virol **78:**1763-74.
16. **Everett, R. D., P. Freemont, H. Saitoh, M. Dasso, A. Orr, M. Kathoria, and J. Parkinson.** 1998. The disruption of ND10 during herpes simplex virus infection correlates with the Vmw110- and proteasome-dependent loss of several PML isoforms. J Virol **72:**6581-91.
17. **Everett, R. D., and J. Murray.** 2005. ND10 components relocate to sites associated with herpes simplex virus type 1 nucleoprotein complexes during virus infection. J Virol **79:**5078-89.
18. **Everett, R. D., J. Murray, A. Orr, and C. M. Preston.** 2007. Herpes simplex virus type 1 genomes are associated with ND10 nuclear substructures in quiescently infected human fibroblasts. J Virol **81:**10991-1004.
19. **Everett, R. D., C. Parada, P. Gripon, H. Sirma, and A. Orr.** 2008. Replication of ICP0-null mutant herpes simplex virus type 1 is restricted by both PML and Sp100. J Virol **82:**2661-72.
20. **Everett, R. D., M. L. Parsy, and A. Orr.** 2009. Analysis of the functions of herpes simplex virus type 1 regulatory protein ICP0 that are critical for lytic infection and derepression of quiescent viral genomes. J Virol **83:**4963-77.
21. **Everett, R. D., G. Sourvinos, C. Leiper, J. B. Clements, and A. Orr.** 2004. Formation of nuclear foci of the herpes simplex virus type 1 regulatory protein ICP4 at early times of infection: localization, dynamics, recruitment of ICP27, and evidence for the de novo induction of ND10-like complexes. J Virol **78:**1903-17.
22. **Ferenczy, M. W., and N. A. Deluca.** 2009. Epigenetic Modulation of Gene Expression from Quiescent Hsv Genomes. J Virol.
23. **Garcia-Ramirez, M., C. Rocchini, and J. Ausio.** 1995. Modulation of chromatin folding by histone acetylation. J Biol Chem **270:**17923-8.
24. **Gelman, I. H., and S. Silverstein.** 1985. Identification of immediate early genes from herpes simplex virus that transactivate the virus thymidine kinase gene. Proc Natl Acad Sci U S A **82:**5265-9.
25. **Gibson, W., and B. Roizman.** 1971. Compartmentalization of spermine and spermidine in the herpes simplex virion. Proc Natl Acad Sci U S A **68:**2818-21.
26. **Gu, H., Y. Liang, G. Mandel, and B. Roizman.** 2005. Components of the REST/CoREST/histone deacetylase repressor complex are disrupted, modified, and translocated in HSV-1-infected cells. Proc Natl Acad Sci U S A **102:**7571-6.
27. **Gu, H., and B. Roizman.** 2007. Herpes simplex virus-infected cell protein 0 blocks the silencing of viral DNA by dissociating histone deacetylases from the CoREST-REST complex. Proc Natl Acad Sci U S A **104:**17134-9.

28. **Hancock, M. H., J. A. Corcoran, and J. R. Smiley.** 2006. Herpes simplex virus regulatory proteins VP16 and ICP0 counteract an innate intranuclear barrier to viral gene expression. Virology **352:**237-52.
29. **Harris, R. A., R. D. Everett, X. X. Zhu, S. Silverstein, and C. M. Preston.** 1989. Herpes simplex virus type 1 immediate-early protein Vmw110 reactivates latent herpes simplex virus type 2 in an in vitro latency system. J Virol **63:**3513-5.
30. **Harris, R. A., and C. M. Preston.** 1991. Establishment of latency in vitro by the herpes simplex virus type 1 mutant in1814. J Gen Virol **72 (Pt 4):**907-13.
31. **Hassan, A. H., P. Prochasson, K. E. Neely, S. C. Galasinski, M. Chandy, M. J. Carrozza, and J. L. Workman.** 2002. Function and selectivity of bromodomains in anchoring chromatin-modifying complexes to promoter nucleosomes. Cell **111:**369-79.
32. **Herrera, F. J., and S. J. Triezenberg.** 2004. VP16-dependent association of chromatin-modifying coactivators and underrepresentation of histones at immediate-early gene promoters during herpes simplex virus infection. J Virol **78:**9689-96.
33. **Huang, J., J. R. Kent, B. Placek, K. A. Whelan, C. M. Hollow, P. Y. Zeng, N. W. Fraser, and S. L. Berger.** 2006. Trimethylation of histone H3 lysine 4 by Set1 in the lytic infection of human herpes simplex virus 1. J Virol **80:**5740-6.
34. **Jamieson, D. R., L. H. Robinson, J. I. Daksis, M. J. Nicholl, and C. M. Preston.** 1995. Quiescent viral genomes in human fibroblasts after infection with herpes simplex virus type 1 Vmw65 mutants. J Gen Virol **76 (Pt 6):**1417-31.
35. **Jenuwein, T., and C. D. Allis.** 2001. Translating the histone code. Science **293:**1074-80.
36. **Jordan, R., and P. A. Schaffer.** 1997. Activation of gene expression by herpes simplex virus type 1 ICP0 occurs at the level of mRNA synthesis. J Virol **71:**6850-62.
37. **Kasten, M., H. Szerlong, H. Erdjument-Bromage, P. Tempst, M. Werner, and B. R. Cairns.** 2004. Tandem bromodomains in the chromatin remodeler RSC recognize acetylated histone H3 Lys14. Embo J **23:**1348-59.
38. **Kent, J. R., P. Y. Zeng, D. Atanasiu, J. Gardner, N. W. Fraser, and S. L. Berger.** 2004. During lytic infection herpes simplex virus type 1 is associated with histones bearing modifications that correlate with active transcription. J Virol **78:**10178-86.
39. **Klemm, R. D., J. A. Goodrich, S. Zhou, and R. Tjian.** 1995. Molecular cloning and expression of the 32-kDa subunit of human TFIID reveals interactions with VP16 and TFIIB that mediate transcriptional activation. Proc Natl Acad Sci U S A **92:**5788-92.
40. **Knipe, D. M., and A. Cliffe.** 2008. Chromatin control of herpes simplex virus lytic and latent infection. Nat Rev Microbiol **6:**211-21.

41. **Kobayashi, N., P. J. Horn, S. M. Sullivan, S. J. Triezenberg, T. G. Boyer, and A. J. Berk.** 1998. DA-complex assembly activity required for VP16C transcriptional activation. Mol Cell Biol **18:**4023-31.
42. **Kouzarides, T.** 2007. Chromatin modifications and their function. Cell **128:**693-705.
43. **Kubat, N. J., A. L. Amelio, N. V. Giordani, and D. C. Bloom.** 2004. The herpes simplex virus type 1 latency-associated transcript (LAT) enhancer/rcr is hyperacetylated during latency independently of LAT transcription. J Virol **78:**12508-18.
44. **Kubat, N. J., R. K. Tran, P. McAnany, and D. C. Bloom.** 2004. Specific histone tail modification and not DNA methylation is a determinant of herpes simplex virus type 1 latent gene expression. J Virol **78:**1139-49.
45. **Kutluay, S. B., S. L. DeVos, J. E. Klomp, and S. J. Triezenberg.** 2009. Transcriptional coactivators are not required for herpes simplex virus type 1 immediate-early gene expression in vitro. J Virol **83:**3436-49.
46. **Kutluay, S. B., and S. J. Triezenberg.** 2009. Regulation of Histone Deposition on the Herpes Simplex Virus Type 1 Genome During Lytic Infection. J Virol.
47. **Kutluay, S. B., and S. J. Triezenberg.** 2009. Role of chromatin during herpesvirus infections. Biochim Biophys Acta **1790:**456-66.
48. **Kwiatkowski, D. L., H. W. Thompson, and D. C. Bloom.** 2009. Polycomb group protein Bmi1 binds to the HSV-1 latent genome and maintains repressive histone marks during latency. J Virol.
49. **Leinbach, S. S., and W. C. Summers.** 1980. The structure of herpes simplex virus type 1 DNA as probed by micrococcal nuclease digestion. J Gen Virol **51:**45-59.
50. **Lomonte, P., J. Thomas, P. Texier, C. Caron, S. Khochbin, and A. L. Epstein.** 2004. Functional interaction between class II histone deacetylases and ICP0 of herpes simplex virus type 1. J Virol **78:**6744-57.
51. **Maul, G. G., A. M. Ishov, and R. D. Everett.** 1996. Nuclear domain 10 as preexisting potential replication start sites of herpes simplex virus type-1. Virology **217:**67-75.
52. **Memedula, S., and A. S. Belmont.** 2003. Sequential recruitment of HAT and SWI/SNF components to condensed chromatin by VP16. Curr Biol **13:**241-6.
53. **Minaker, R. L., K. L. Mossman, and J. R. Smiley.** 2005. Functional inaccessibility of quiescent herpes simplex virus genomes. Virol J **2:**85.
54. **Mittler, G., T. Stuhler, L. Santolin, T. Uhlmann, E. Kremmer, F. Lottspeich, L. Berti, and M. Meisterernst.** 2003. A novel docking site on Mediator is critical for activation by VP16 in mammalian cells. Embo J **22:**6494-504.
55. **Mossman, K. L., and J. R. Smiley.** 1999. Truncation of the C-terminal acidic transcriptional activation domain of herpes simplex virus VP16 renders expression of the immediate-early genes almost entirely dependent on ICP0. J Virol **73:**9726-33.

56. **Muggeridge, M. I., and N. W. Fraser.** 1986. Chromosomal organization of the herpes simplex virus genome during acute infection of the mouse central nervous system. J Virol **59:**764-7.
57. **O'Hare, P., and G. S. Hayward.** 1985. Three trans-acting regulatory proteins of herpes simplex virus modulate immediate-early gene expression in a pathway involving positive and negative feedback regulation. J Virol **56:**723-33.
58. **Pignatti, P. F., and E. Cassai.** 1980. Analysis of herpes simplex virus nucleoprotein complexes extracted from infected cells. J Virol **36:**816-28.
59. **Placek, B. J., J. Huang, J. R. Kent, J. Dorsey, L. Rice, N. W. Fraser, and S. L. Berger.** 2009. The histone variant H3.3 regulates gene expression during lytic infection with herpes simplex virus type 1. J Virol **83:**1416-21.
60. **Preston, C. M.** 2000. Repression of viral transcription during herpes simplex virus latency. J Gen Virol **81:**1-19.
61. **Preston, C. M., and M. J. Nicholl.** 1997. Repression of gene expression upon infection of cells with herpes simplex virus type 1 mutants impaired for immediate-early protein synthesis. J Virol **71:**7807-13.
62. **Quinlan, M. P., and D. M. Knipe.** 1985. Stimulation of expression of a herpes simplex virus DNA-binding protein by two viral functions. Mol Cell Biol **5:**957-63.
63. **Russell, J., N. D. Stow, E. C. Stow, and C. M. Preston.** 1987. Herpes simplex virus genes involved in latency in vitro. J Gen Virol **68 (Pt 12):**3009-18.
64. **Saha, A., J. Wittmeyer, and B. R. Cairns.** 2006. Chromatin remodelling: the industrial revolution of DNA around histones. Nat Rev Mol Cell Biol **7:**437-47.
65. **Samaniego, L. A., N. Wu, and N. A. DeLuca.** 1997. The herpes simplex virus immediate-early protein ICP0 affects transcription from the viral genome and infected-cell survival in the absence of ICP4 and ICP27. J Virol **71:**4614-25.
66. **Smiley, J. R., and J. Duncan.** 1997. Truncation of the C-terminal acidic transcriptional activation domain of herpes simplex virus VP16 produces a phenotype similar to that of the in1814 linker insertion mutation. J Virol **71:**6191-3.
67. **Sourvinos, G., and R. D. Everett.** 2002. Visualization of parental HSV-1 genomes and replication compartments in association with ND10 in live infected cells. Embo J **21:**4989-97.
68. **Spivack, J. G., and N. W. Fraser.** 1987. Detection of herpes simplex virus type 1 transcripts during latent infection in mice. J Virol **61:**3841-7.
69. **Stevens, J. G., E. K. Wagner, G. B. Devi-Rao, M. L. Cook, and L. T. Feldman.** 1987. RNA complementary to a herpesvirus alpha gene mRNA is prominent in latently infected neurons. Science **235:**1056-9.
70. **Stow, N. D., and E. C. Stow.** 1986. Isolation and characterization of a herpes simplex virus type 1 mutant containing a deletion within the gene

encoding the immediate early polypeptide Vmw110. J Gen Virol **67 (Pt 12):**2571-85.

71. **Thompson, R. L., C. M. Preston, and N. M. Sawtell.** 2009. De novo synthesis of VP16 coordinates the exit from HSV latency in vivo. PLoS Pathog **5:**e1000352.
72. **Uesugi, M., O. Nyanguile, H. Lu, A. J. Levine, and G. L. Verdine.** 1997. Induced alpha helix in the VP16 activation domain upon binding to a human TAF. Science **277:**1310-3.
73. **Wang, Q. Y., C. Zhou, K. E. Johnson, R. C. Colgrove, D. M. Coen, and D. M. Knipe.** 2005. Herpesviral latency-associated transcript gene promotes assembly of heterochromatin on viral lytic-gene promoters in latent infection. Proc Natl Acad Sci U S A **102:**16055-9.
74. **Xiao, H., A. Pearson, B. Coulombe, R. Truant, S. Zhang, J. L. Regier, S. J. Triezenberg, D. Reinberg, O. Flores, C. J. Ingles, and et al.** 1994. Binding of basal transcription factor TFIIH to the acidic activation domains of VP16 and p53. Mol Cell Biol **14:**7013-24.
75. **Yao, F., and P. A. Schaffer.** 1995. An activity specified by the osteosarcoma line U2OS can substitute functionally for ICP0, a major regulatory protein of herpes simplex virus type 1. J Virol **69:**6249-58.
76. **Zhang, R., M. V. Poustovoitov, X. Ye, H. A. Santos, W. Chen, S. M. Daganzo, J. P. Erzberger, I. G. Serebriiskii, A. A. Canutescu, R. L. Dunbrack, J. R. Pehrson, J. M. Berger, P. D. Kaufman, and P. D. Adams.** 2005. Formation of MacroH2A-containing senescence-associated heterochromatin foci and senescence driven by ASF1a and HIRA. Dev Cell **8:**19-30.

Appendix 1. Data Not Shown

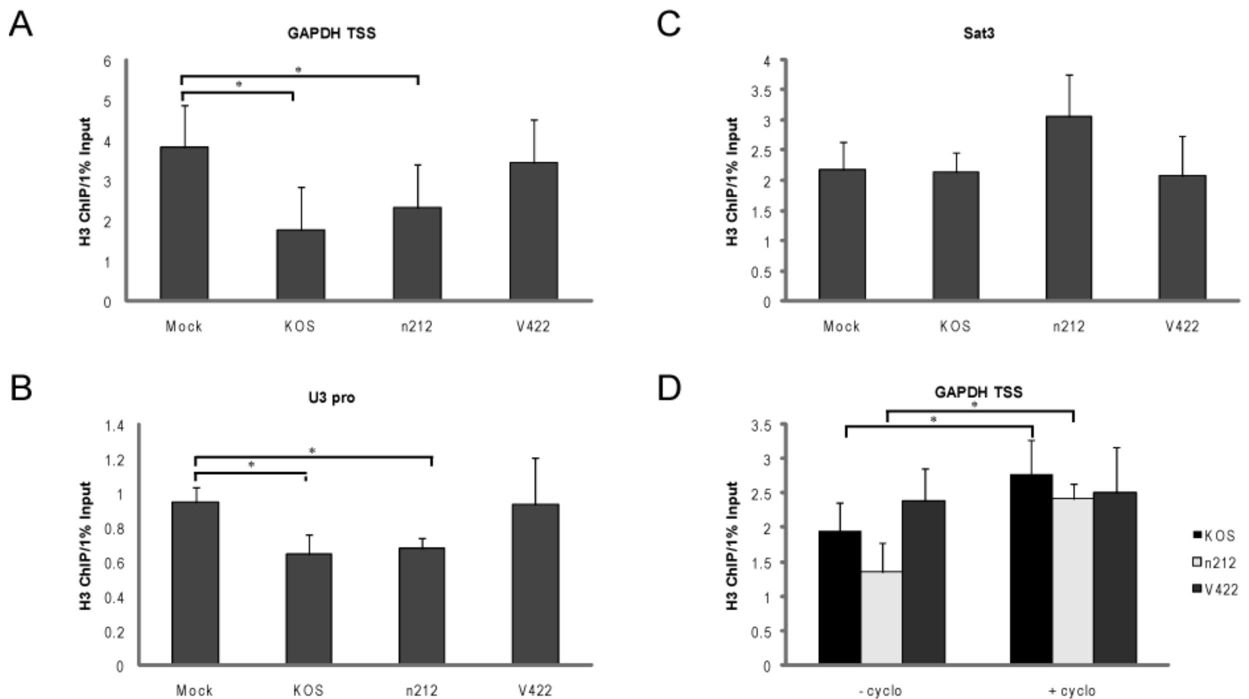


Figure 5A.1. H3 occupancy on the HeLa genome decreases with KOS and n212 infection. HeLa cells were infected with 1 PFU/cell of KOS, n212 or V422 for 6 hours and then samples were harvested for ChIP using a total H3 antibody. PCR primers amplified: (A) GAPDH TSS (B) U3 promoter (C) Sat3. (D) H3 occupancy on the cellular genome is unchanged when infections are carried out in the presence of cycloheximide. HeLa cells were infected with 1 PFU/cell of KOS, n212 or V422 in the presence or absence of cycloheximide for 6 hours. Samples were harvested for ChIP using a total H3 antibody. PCR primers amplified the GAPDH TSS. All experiments were performed in triplicate. Samples with mean values that differed significantly ($P < 0.05$, Student's *t* test) are indicated (*).

Appendix 2. Additional data

RNA polymerase occupancy on the viral genomes in HeLa and U2OS cells

In Chapter five, I demonstrated that U2OS cells support IE gene expression from VP16 mutant genomes, while HeLa cells do not (Figure 5.1). Whether the accumulation of mRNAs from the V422 genome observed in U2OS cells is due to a mechanism involving the stimulation of transcription, or rather the stabilization of the viral mRNAs via a post-transcriptional mechanism is unknown. To test whether U2OS cells enhance V422 gene expression by stimulating recruitment of RNA polymerase II to the mutant viral promoters, I used ChIP to test the occupancy of RNA polymerase II at the ICP27 transcriptional start site with wild type and V422 infection of HeLa and U2OS cells. As shown in Figure A2.1, there is a statistically significant difference in RNA polymerase II occupancy between KOS and V422 genomes in HeLa cells, indicating a barrier to loading RNA polymerase onto the viral genome in the absence of the C-terminal acidic activation domain of VP16. There is no statistical difference in RNA polymerase II loading between the two viruses in U2OS cells, indicating that this barrier is not present in this cell type. Thus, the increased accumulation of viral mRNAs observed in U2OS cells infected with V422 is most likely due to an increase in transcription from the mutant virus genome.

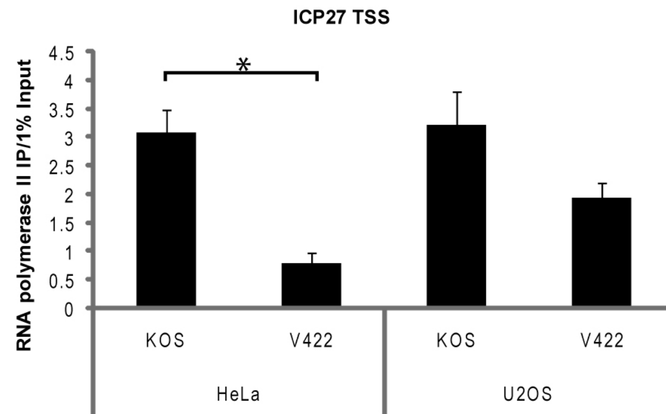


Figure 5A.2.1. RNA polymerase II occupancy at the ICP27 transcriptional start site in HeLa and U2OS cells. Cells were infected with 10 PFU/cell of KOS or V422 for 2 hours and then samples were harvested for ChIP using an antibody detecting the Serine 5-phosphorylated form of RNA polymerase II (8WG16). All experiments were performed in triplicate. Samples with mean values that differed significantly ($P < 0.05$, Student's *t* test) are indicated (*).

Changes in input DNA levels over time

In the ChIP experiments described in Chapter five, I adjusted the MOI used for each mutant virus to reflect an equivalent amount of viral DNA (compared to wild-type virus) isolated from the infected cells at 3 hours post-infection. This was to ensure that any differences in the IP efficiencies did not merely reflect differences in the amount of viral DNA in the infected cells. In Figures 5.2 and 5.3 I assessed total histone H3 and acetylated histone H3 IP efficiences at 1, 3 and 6 hours post-infection, thus I also assayed the amounts of total DNA in the infected cells at all three timepoints. As shown below, input DNA levels (in the presence of PAA) were roughly equivalent between all three

viruses at 3 and 6 hpi in HeLa cells as determined by Real-time PCR (Fig 5A.2.2A). Additionally, input DNA levels were equivalent between all three viruses in U2OS cells at 3 hpi, although a small (but not statistically significant) increase in DNA levels was observed with KOS and n212 infections at 6hpi (Fig 5A.2.2B) which could be due to breakthrough DNA replication in the presence of PAA. Thus, there are significant differences in IP efficiencies between the three viruses at 6 hpi in both cell types (See Fig 5.2), when input DNA levels are still roughly equivalent.

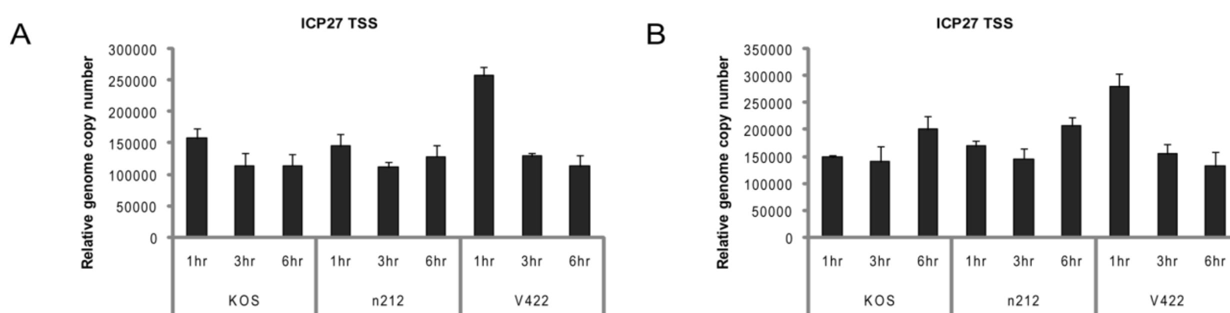


Figure 5A.2.2. Input DNA levels in HeLa and U2OS cells. HeLa (A) and U2OS (B) were infected with KOS, n212 and V422 for 1, 3 or 6 hours. DNA was harvested as per the ChIP protocol and total amounts of Input DNA were assessed using Real-time PCR and primers amplifying the ICP27 transcriptional start site. In each case, three independent experiments were performed.

V422 input DNA levels in both HeLa and U2OS cells are much higher at 1 hpi compared to KOS and n212 virus, and drop over time (Fig A2.2). Given the fact that cells are washed in acid to remove unbound viral particles immediately

after the one hour incubation period, this drop in input DNA levels most likely represents viral DNA which is degraded in the cytoplasm and/or nucleus. The DNA levels stay relatively stable in both cell types between 3 and 6 hpi, suggesting that the viral DNA that remains after this time is not subject to degradation. It is unclear as to why this drop in DNA levels is observed only with the VP16 mutant virus in both restrictive and permissive cell types, but suggests that the VP16 activation domain may play a role in preventing the degradation of viral DNA.

ICP0 expression in U2OS cells infected with V422

Kutluay and Triezenberg suggested that VP16 modulates chromatin structure at non-IE regions of the viral genome through stimulating the expression of another viral gene product (202). Several reports in the literature suggest ICP0 is this protein. Cliffe and Knipe demonstrated that an ICP0 mutant has a high level of under-acetylated histones on its genome, implicating this protein in directly modulating chromatin structure (55). Additionally, work by Ferenczy and DeLuca implicate ICP0 in modulating histone occupancy and acetylation levels on the viral genome (109). In Chapter five I show that the VP16 mutant V422 has a high level of underacetylated histones on the viral genome. However, this mutant can replicate with nearly wild type efficiency in this cell type, suggesting that ICP0 is made during the infection but cannot alone affect the chromatin structure on the viral genome. Northern blot analysis of ICP0 mRNA expression in U2OS cells infected with V422 indicates that the mRNA

accumulates within 4-6 hpi (Figure 5A.2.3), suggesting the protein is most likely produced within the timeframe of the experiments presented in Chapter five. This suggests that although ICP0 is produced during V422 infection of U2OS cells, it is not sufficient to alter the chromatin structure on the viral genome. This reinforces the hypothesis put forth in Chapter five that both VP16 and ICP0 are required to maintain a low level of highly acetylated histones on the viral genome.

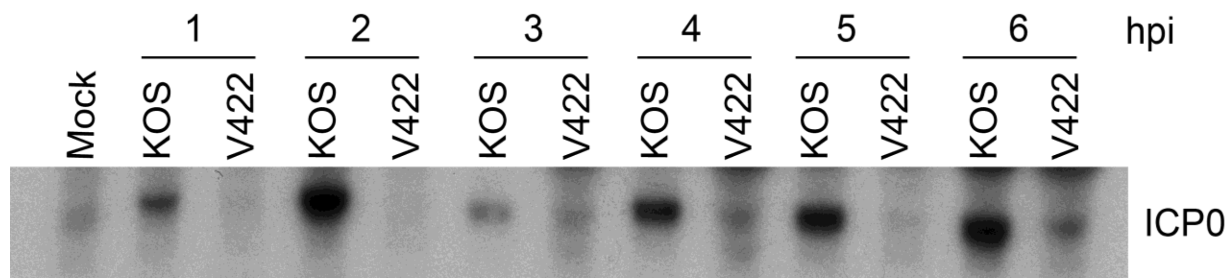


Figure 5A.2.3. ICP0 mRNA expression in U2OS cells. U2OS cells were infected with 1 PFU/cell of KOS or V422 for 1 to 6 hours in the presence of PAA. RNA was harvested and separated on a denaturing agarose gel. RNA was hybridized and blotted for ICP0.

Chapter Six: Identification of Kinase Inhibitors that Stimulate Herpes Simplex Virus Immediate-Early Gene Expression in the Absence of VP16 Activation Function

Preface to Chapter 6

The somatic cell fusion assays described in Chapters three and four indicate that U2OS cells lack a component of an innate antiviral response found in other restrictive cell types. As suggested in Chapter three, one possibility is that this cell line is lacking components of a signalling pathway which activates the nuclear repression mechanism. As one strategy to test this hypothesis, we attempted to convert a restrictive cell type into a cell type permissive to VP16 and ICP0 mutants using kinase inhibitors. I hypothesized that the target of the kinase inhibitors may be lacking in U2OS cells, accounting for their permissive phenotype. The results of these experiments are outlined in this chapter.

The publication entitled "Identification of Kinase Inhibitors that Stimulate Herpes Simplex Virus Immediate-Early Gene Expression in the Absence of VP16 Activation Function" is the content of Chapter six of this thesis. This work is formatted for submission to Journal of Virology. All experiments were performed by me, while writing and editing was shared between Dr Smiley and myself.

ABSTRACT

Herpes Simplex virus VP16 and ICP0 mutants are highly attenuated for growth in many cells types, including primary human fibroblasts, at least in part because they are unable to overcome an innate gene silencing mechanism that represses newly delivered viral genomes. We and others have suggested that genome silencing involves one or more signaling pathways that detect incoming viral genomes. We therefore surveyed inhibitors targeting several major cellular signaling pathways for their ability to activate gene expression from the VP16/ICP0 mutant KM110, in an attempt to identify relevant pathways. We identified two structurally related inhibitors, LY294002 and NU7441, which render human embryonic lung fibroblasts permissive to KM110. Further analysis revealed that these compounds specifically compensate for the VP16 defect of KM110 by stimulating the accumulation of immediate-early mRNA. Although LY294002 and NU7441 are well known as inhibitors of phosphoinositol-3 kinase (PI3K) and DNA protein kinase (DNA PK) respectively, their effects on HSV gene expression do not stem from inhibition of these canonical targets. The potent PI3K inhibitor wortmannin is inactive, while an analog of LY294002 that does not target PI3K (LY303511) is highly effective; in addition, NU7441 activates HSV gene expression in cells that lack DNA PK. The compounds do not act by blocking induction of interferon-stimulated genes in response to incoming viral particles. Moreover, they accelerate viral gene expression in U2OS cells, which are semi-permissive to VP16 mutants, indicating that the target(s) of these inhibitors are also present in this cell line.

INTRODUCTION

Herpes Simplex virus type-1 (HSV-1) lytic infection involves the temporal regulation of viral gene expression. Immediate early (IE) genes are stimulated by the tegument protein VP16, which contains a strong C-terminal acidic activation domain and interacts with the cellular proteins HCF and Oct-1 in order to bind specific target sequences within the IE promoters (78, 83, 96, 102, 113). The activation domain of VP16 is involved in recruiting general transcription factors and RNA polymerase to the IE promoters (48, 53, 57, 69, 71, 103, 112). IE proteins play important roles in driving early (E) and late (L) gene expression, as well as overcoming the innate and adaptive immune response. ICP0 appears to act upstream of the other IE proteins, as it is required along with VP16 for efficient IE gene expression (11, 12, 30, 35, 97). The functions of ICP0 and VP16 appear to be interrelated as ICP0 provided *in trans* can partially alleviate the growth defect of VP16 mutants (1, 43).

ICP0 is an ubiquitin E3 ligase that interacts with several cellular proteins, although the significance of these interactions in many cases is still unknown (Reviewed in (29)). ICP0 has roles in subverting the interferon (IFN) response (24, 73, 74) and causes the degradation of the IFN-inducible protein PML, a major constituent of ND10 domains (31, 32). ICP0 is also critical for preventing entry of viral genomes into quiescence as well as reactivation from this silenced state (11, 12, 28, 30, 33, 46, 70, 90). In addition, ICP0 interacts with histone deacetylases (HDACs) and may play a role in modulating the chromatin structure

of the viral genome (17, 18, 36, 40, 64). Proteasome inhibition or disruption of the RING domain eliminates transactivation and reactivation from quiescence (5, 27, 33, 42). These observations suggest that ICP0 targets cellular inhibitory proteins for proteasome-dependent degradation. Knockdown of PML and Sp100 (another major ND10 component) enhances replication of an ICP0-null virus but the complementation is incomplete (34), suggesting that ICP0 must target additional proteins to fully stimulate viral gene expression.

VP16 and ICP0 mutants show significantly decreased gene expression and greatly increased particle-to-plaque forming unit (PFU) ratios during low multiplicity of infection (MOI) in restrictive cell types such as primary human fibroblasts (1, 12, 28, 95, 97). Instead of undergoing lytic infection, these viral genomes remain in a transcriptionally silent state termed quiescence (1, 30, 46, 47, 50, 82, 86, 90, 91, 97). Quiescent genomes are actively repressed, as heterologous promoters embedded within them are also silenced (50, 70, 86, 91). These observations indicate that VP16 and ICP0 play important roles in preventing global silencing of the viral genome.

In contrast to the defective phenotypes exhibited in most cell lines, HSV VP16 and ICP0 mutants replicate with close to wild type efficiency in the U2OS osteosarcoma cell line (44, 95, 115). The permissive phenotype of U2OS cells was originally hypothesized to be due to expression of an ICP0-like positively acting factor that stimulates viral gene expression (115). However, using

heterotypic cell fusion assays we determined that the permissive phenotype of U2OS cells is recessive, indicating they lack an innate antiviral mechanism found in restrictive cell types (44). Either VP16 or ICP0 provided *in trans* could overcome this antiviral mechanism at early times post-infection. The permissive phenotype of U2OS cells was extinguished within an hour or so after fusion with non-permissive human embryonic lung fibroblasts, a finding that led us to propose that U2OS cells may lack a signaling pathway which normally activates intranuclear antiviral defense, rather than being defective in the repression machinery *per se* (44).

The requirement for ICP0 and VP16 activation function can be at least partially alleviated by agents that alter the physiological state of otherwise restrictive cells. For example, hexamethylene *bis*-acetamide (HMBA), a compound that induces terminal differentiation of several solid tumour and leukemic cell lines (61, 66, 88, 89), specifically compensates for VP16 mutations by stimulating IE gene expression (67, 84). HMBA can enhance reactivation of latent HSV from infected ganglia (108), but it is unable to reactivate quiescent genomes in restrictive primary fibroblasts (67). *N*'-Methylnicotinamide, a compound which induces the differentiation of murine erythroleukemic cells, similarly compensates for VP16 mutations (84). The cellular target(s) of these compounds relevant to activation of HSV IE gene expression have not been identified.

Recently, Preston and Nicholl reported the identification of compounds which similarly stimulate viral gene expression in the absence of ICP0 and are capable of reactivating quiescent viral genomes (85). Sodium arsenite, which induces heat shock and oxidative stress, and gramicidin D, which permeabilizes cell membranes, cause the reorganization of ND10 domains and stimulate viral gene expression (85), although the mechanism of action is unclear. In addition, cellular stressors such as heat shock, UV-C irradiation and glutamine deprivation can enhance infections with ICP0 mutants (7, 8).

Many viruses alter cellular signaling cascades to enhance viral gene expression and replication (6, 10, 104, 116). Well-studied pathways modulated by viruses include the phosphoinositol 3-kinase (PI3K), extracellular-regulated kinase (ERK) 1/2 mitogen activated protein kinase (MAPK), and p38 kinase and Jun N-terminal kinase (JNK) stress-activated protein kinase (SAPK) pathways. Both the p38 and JNK pathways are activated during HSV-1 infection by the IE protein ICP27 and are thought to increase the efficiency of viral replication and prevent apoptosis (38, 45, 51, 68, 117, 118). ICP0 may also activate the JNK pathway (22). The PI3K pathway has been implicated in HSV-1 entry (15), and the downstream effector protein Akt is transiently activated early after infection (4). Interestingly, PI3K has also been implicated in the innate immune response to dsRNA through the phosphorylation of IRF3 and subsequent induction of an antiviral state (92).

In this study, we sought to determine if any of the canonical signaling pathways discussed above play roles in limiting the replication of VP16 and/or ICP0 mutants in restrictive cell types. To this end, we asked if HEL fibroblasts, which are highly restrictive to the VP16/ICP0 double mutant virus KM110 (75), could be rendered permissive by treatment with kinase inhibitors. We report here that the well-studied PI3K inhibitor LY294002 and the DNA PK inhibitor NU7441 (59) render HEL fibroblasts permissive to KM110 infection, and that these compounds specifically compensate for the VP16 lesion in the virus. However, in both cases activation of gene expression does not stem from inhibition of the canonical targets of these agents. The compounds stimulate IE gene transcription in the absence of new viral and protein synthesis; however, the cellular target(s) of these kinase inhibitors remains elusive.

RESULTS AND DISCUSSION

LY294002 compensates for a lack of VP16 activation function

KM110, an HSV-1 mutant which lacks the activation functions of ICP0 and VP16, is highly restricted for growth in human embryonic lung (HEL) fibroblasts (75). These cells can be infected with up to 10 PFU/cell of KM110 without detectable gene expression or cellular cytotoxicity (75). Using heterotypic cell fusion assays we previously determined that the restrictive phenotype of HEL fibroblasts stems from an innate antiviral mechanism which prevents KM110 gene expression (44). Additionally, either VP16 or ICP0, provided *in trans*, can overcome this silencing mechanism. Given the previous demonstrations that certain compounds are able to compensate for the absence of VP16 or ICP0 (67, 85), we asked if selected kinase inhibitors targeting well characterized signaling pathways are able to activate viral gene expression in HEL cells infected with KM110. Specifically, we tested inhibitors that target the ERK (U0126), p38 (SB203580), JNK (SP600125) and PI3K (LY294002) pathways. HMBA, which compensates for VP16 activation function, was included as a positive control.

HEL cells were pretreated with the kinase inhibitors for 1 hr, and then infected with 10 PFU/cell KM110. After 9hrs of infection in the presence of drugs, cells were fixed and processed for immunofluorescence (IF) for the IE protein ICP4 (Table 6.1, Experiment 1). The concentrations of drugs used in this and all other experiments described in this report did not cause obvious visible cytotoxicity during the time-course of the experiments. As expected, only a small

fraction (ca. 5%) of the cells infected with KM110 expressed detectable levels of ICP4 in control cultures lacking any drug, and this value was not altered by the solvent DMSO at the concentrations used. Also as expected, HMBA induced ICP4 expression in ca 90% of the cells. Strikingly, the PI3K inhibitor LY294002 was almost as effective, inducing ICP4 expression in ca. 70% of the treated cells. In contrast, SP600125, U0126 and SB203580 had no significant effect (Table 6.1). Notably, ICP4 localized predominantly to large intranuclear domains characteristic of viral replication compartments in a large fraction of the LY294402-induced positive cells (Fig. 6.1a), suggesting that the infection had proceeded at least to the stage of viral DNA replication (56). These results indicate that LY294002 efficiently triggers the onset of the KM110 lytic cycle in an otherwise highly restrictive cell type.

KM110 bears mutations that eliminate the activation functions of both ICP0 and VP16 (75). To determine if LY294002 specifically compensates for one or both of these mutations, we examined the effects of the drug on the parental isolates n212 (13) and V422 (95), which bear the ICP0 and VP16 mutations present in KM110 respectively (Fig. 6.1b). Cells were pretreated for one hour with the indicated kinase inhibitors and then infected with 1 PFU/cell of each virus. Infections were carried out for a total of 9 hours in the presence of the kinase inhibitors and the proportion of cells expressing ICP4 was determined by IF. LY294002 greatly increased the number of ICP4-positive cells during V422 infection while little or no effect was observed with n212, a pattern similar to that

produced by HMBA However, unlike HMBA, LY294002 consistently reduced the fraction of ICP4-postive cells in cultures infected with wild-type KOS. Whether this inhibitory effect stems from generalized toxicity or inhibition of one or more signaling pathways that enhance virus infection has yet to determined (see below). These results suggest that, like HMBA, LY294002 acts to compensate specifically for the VP16 mutation present in KM110.

As noted above, KM110 forms viral replication compartments in LY294002-treated HEL cells (Fig. 6.1a). To determine if infectious progeny virions are also produced, viral burst assays were performed. HEL cells were pretreated for one hour with the indicated kinase inhibitors and then infected with 0.1 PFU/cell of each virus. After 24 hours in the presence of the inhibitors, virus was harvested and titred on U2OS cells. As shown in Fig. 6.1c, both LY294002 and HMBA markedly increased the yields of infectious progeny KM110 and V422 virions, but had little effect on wild type KOS and n212. These results indicate that LY294002 allows viral replication and production of progeny virions in the absence of VP16 activation function.

The mutant form of VP16 specified by V422 is truncated after amino acid residue 422 within the C-terminal activation domain (58). Similarly truncated versions of VP16 retain the ability to associate with viral IE promoters, but fail to activate transcription (48, 99). To determine if the stimulatory effect of LY294002 requires mutant VP16 bound to IE promoters, we examined the HSV-1 strain 17-

based VP16 mutant, in1814. This mutant contains a 12 base pair insertion in the VP16 gene that eliminates the ability of VP16 to interact with IE promoters (1). LY294002 strongly enhanced in1814 viral yields following low MOI infection of HEL cells (Figure 6A.1). Thus, the effect of LY294002 is not mediated through VP16 bound to IE promoters.

LY294002 acts through a PI3K-independent mechanism

LY294002 is often used as an experimental tool to inhibit PI3K and as such can affect a wide range of cellular functions. PI3K is a member of a conserved family of lipid kinases that phosphorylate phosphoinositides on the 3'-hydroxyl group. The enzyme is recruited to the plasma membrane following stimulation of receptor tyrosine kinases. This brings PI3K into proximity with its membrane phosphoinositide substrate, leading to production of phosphoinositol-3,4,5-triphosphosphate (PIP_3). PIP_3 is a critical second messenger that recruits PDK1 to the membrane, which in turn directly phosphorylates Akt/PKB. Akt is a key signaling molecule involved in survival and proliferation (Reviewed in (26, 106)). During HSV infection Akt is transiently activated and may function to inhibit apoptosis early in infection (4).

To determine whether LY294002 compensates for VP16 mutations through its ability to inhibit PI3K signaling, we tested the activity of two additional compounds: wortmannin, an unrelated irreversible inhibitor of PI3K, and LY303511, an inactive structural analog of LY294002 which contains an

amine substitution for the morpholino oxygen (107). We first verified that LY294002 and wortmannin prevent the phosphorylation of Akt in response to platelet-derived growth factor (PDGF) at the concentrations used (Fig. 6.2a). In contrast, and as expected, LY303511 had no effect on Akt phosphorylation. Despite the similar ability of LY294002 and wortmannin to block Akt phosphorylation, wortmannin did not detectably stimulate ICP4 expression in KM110-infected HEL cells (Table 6.1, Experiment 2), even when it was tested at a wide range of concentrations (1nM – 10μM) or replenished each hour throughout the course of the experiment (Figure 6A.1). These data argue that inhibition of PI3K is not the mechanism by which LY294002 acts to stimulate viral gene expression and replication. Supporting this conclusion, LY303511 was at least as effective as LY294002 at activating ICP4 expression from the KM110 genome (Table 6.1, Experiments 2 and 3), and specifically compensated for the VP16 mutation (Fig. 6.2b,c) at concentrations which do not prevent Akt phosphorylation in response to PDGF. The simplest interpretation of these data is that LY294002 and LY303511 activate viral gene expression through a common target, which is not PI3K.

There is increasing evidence in the literature for PI3K-independent effects of LY294002 and LY303511. For example, LY294002 and LY303511 both inhibit nitric oxide production in response to LPS in macrophages (52). Both compounds block K(V) currents in pancreatic β cells (25) and inhibit monocyte chemoattractant protein-1 (MCP-1) expression in HUVEC cells (16). Also,

LY294002 and LY303511 suppress corticosterone-induced COX-2 expression in cardiomyocytes (98). Finally, both compounds cause an increase in intracellular H_2O_2 in some tumour cell lines (81, 93). In all of these cases, the direct targets of the LY drugs are unknown.

In addition to blocking PI3K, LY294002 also inhibits other members of the PI3K-related kinase (PIKK) family (54, 105). Members of this family include DNA Protein kinase (DNA PK), Ataxia-telangiectasia mutated (ATM), ATM Rad3-related (ATR), mammalian target of rapamycin (mTOR), ATX/SMG1 and the non-catalytic member TRRAP (65). This family of proteins is defined by their large size and the presence of a shared PI3 kinase-like catalytic domain (PIK). PIKK family members have diverse and important biological functions. mTOR is a key player in the regulation of protein synthesis and cell growth, while ATX/SMG1 is involved in nonsense-mediated mRNA decay. Although TRRAP lacks key residues within the catalytic domain, it plays a scaffolding role in chromatin assembly and is involved in recruiting histone acetyltransferases to the genome. DNA PK, ATM and ATR are recruited to sites of DNA damage and signal to other cellular proteins involved in maintaining genomic integrity (65).

Some cellular dsDNA repair proteins are required for efficient HSV DNA replication and localize to viral replication compartments (62, 94, 100, 110). PIKK family member ATM is activated during HSV infection (49, 62, 94); however the downstream effector protein Mre11 is lost at late times post-infection

(39). In contrast, ATR signaling is disrupted in HSV infection, and cellular repair proteins involved in recognizing ssDNA are sequestered from viral replication compartments in a manner dependent on ICP0 (109). Additionally, the DNA PK catalytic subunit, an essential component of the non-homologous end joining (NHEJ) pathway, is targeted for proteasome-dependent degradation by ICP0 (60, 80) and viral replication is enhanced in cells lacking components of the NHEJ pathway (80, 100). Thus, DNA damage signaling pathways are altered during HSV infection, with some pathways being important for viral replication while others are detrimental.

Given that LY294002 targets some PIKK family members which may be inhibitory to HSV infection, we tested the ability of additional inhibitors that target certain PIKK family members to overcome the restrictive phenotype of HEL cells. Caffeine, an inhibitor of ATM and ATR, had no effect on KM110 gene expression in HEL cells, while rapamycin and Torin-1 (101) (mTOR inhibitors) and DNA PK inhibitors I and II from Calbiochem also showed no stimulatory effects (Figure 6A.1). LY294002 also has documented effects on casein kinase II activity (20). We tested the ability of several inhibitors of casein kinase II (TBB, TBCA (79, 121)) to activate gene expression from the KM110 genome in restrictive HEL cells, and found no stimulatory effects (Figure 6A.1). A recent report suggested that LY294002 and LY303511increase levels of intracellular H_2O_2 in some tumour cell lines (81). However, in our hands, the

addition of exogenous H_2O_2 did not affect gene expression from the KM110 genome in HEL cells (Figure 6A.1).

In summary, our studies to date suggest that PI3K-independent activities of LY294002 and LY303511 are able to compensate for a lack of VP16 activation function, and appear to exclude the PIKK family members ATM, ATR, DNA-PK and mTOR, casein kinase II and H_2O_2 as mediators of this effect. The role of ATX/SMG1 in stimulating gene expression in the absence of VP16 activation function has not been assessed.

NU7441 also activates V422 gene expression, through a DNA-PK-independent mechanism

As described above, the DNA PK inhibitors I and II from Calbiochem did not alter the restrictive phenotype of HEL cells for KM110. However, another DNA PK inhibitor, NU7441, was capable of activating gene expression from KM110 in these cells. (Table 6.1, Experiment 3). In addition, NU7441 compensated for the VP16 defect of KM110 in a plaque enhancement assay in Vero cells (Fig 6.3a). Similar results were obtained with the in1814 VP16 mutant (Figure 6A.1). NU7441 is structurally related to LY294002 (Fig. 6.7), and has been described as a potent and highly selective inhibitor of DNA PK (IC_{50} 14nM) (59). It currently is being tested in preclinical trials as a cancer therapeutic (111, 119).

To determine whether NU7441 relieves the requirement for VP16 activation function by inhibiting DNA PK, we tested the effectiveness of the drug in DNA PKcs-deficient (MO59J) and DNA PKcs-wild type (MO59K) human glioma cell lines. Both cell types were pretreated for one hour with the indicated kinase inhibitors and then infected with 5 PFU/cell of V422 for a total of 9 hours in the presence of the drugs. Protein was harvested and assayed for ICP4 by western blot analysis. Both MO59J and MO59K were restrictive to V422 IE protein production in the absence of drug treatments (Fig 6.3b), indicating that loss of DNA PK does not compensate for the absence of functional VP16. Each drug was capable of stimulating ICP4 protein production in both MO59J and MO59K cell lines (Fig. 6.3b). These results indicate that the target of NU7441 (as well as LY303511 and HMBA) which compensates for VP16 activation function is not DNA PK. These data demonstrate that NU7441 activates gene expression from the V422 genome in a DNA PK-independent manner.

The kinase inhibitors stimulate accumulation of HSV IE mRNA in the absence of viral protein synthesis

The ability of LY294002, LY303511 and NU7441 to enhance in1814 replication indicates that VP16 does not need to be present at the IE promoters for these drugs to stimulate viral gene expression. We next asked whether these drugs require newly synthesized viral or cellular proteins in order to exert their effects.

Northern blot analysis of ICP22 transcript accumulation in the presence of the inhibitors and the protein synthesis inhibitor cycloheximide was performed. HEL cells were pretreated with the indicated kinase inhibitors in the presence or absence of cycloheximide for one hour. Infection was carried out with 10 PFU/cell of KOS or V422 for a total of 6hr in the presence of the inhibitors, after which time RNA was harvested for northern blot analysis (Fig. 6.4). The kinase inhibitors appear to have little effect on wild type mRNA accumulation. However, all three drugs stimulated ICP22 transcript accumulation during V422 infection in the absence or presence of cycloheximide. Cycloheximide itself is known to stimulate IE gene expression (87) (see untreated and DMSO treated lanes), yet also seems to block the full induction of IE genes from the V422 genome in the presence of the drugs. The reason behind this is unclear; however, the drugs enhance ICP22 transcript accumulation above mock- and DMSO-treated levels despite this reduction in accumulation. The ability of these compounds to directly stimulate IE gene expression in the presence of cycloheximide indicates that new viral or cellular protein synthesis is not required for their action.

The kinase inhibitors do not act by preventing the activation of interferon-stimulated genes in response to incoming viral particles

Entry of enveloped viruses elicits an IFN-independent antiviral state in human fibroblasts (9, 19, 72, 76, 120). This IFN-independent response induces the expression of a small subset of IFN-stimulated genes (ISGs), the protein

products of which act to limit viral replication. For example, ISG56K inhibits translation through interaction with eukaryotic initiation factor 3 (eIF-3) (41). In the case of HSV infection, expression of ICP0 inhibits the accumulation of ISGs (24). However, during infections with KM110, V422 or UV-inactivated virus, an antiviral response is induced (72) and may be a factor in inhibiting viral gene expression.

Treatment of cells with LY294002 blocks the induction of ISG56K in response to UV-inactivated HSV-1 (77). Both PI3K and DNA PK are dispensable for ISG induction, suggesting that viral entry triggers an antiviral response that involves a novel target of LY294002 (77). We hypothesized that the LY drugs and/or NU7441 may enhance viral gene expression by blocking the antiviral signaling pathway used to activate ISG expression.

We tested the abilities of the drugs to prevent ISG56K induction in HEL cells in response to UV-inactivated KOS using northern blot analysis. Cells were pretreated with the kinase inhibitors for one hour and infected with 10 PFU/cell of UV-inactivated KOS for a total of 6hrs in the presence of the drugs. IFNα was used as a positive control for ISG56K induction. LY294002 (25μM) decreased ISG56K mRNA transcript accumulation however the inhibition was incomplete (Fig. 6.5). Noyce *et al* used a higher concentration of LY294002 (50μM (77)) which may account for the discrepancy in ISG56K RNA levels upon drug treatment. The increased accumulation of ISG56K with NU7441 treatment was

not reproducible; DMSO, HMBA and LY303511 treatment had no effect ISG56K levels in response to UV-inactivated KOS. These data argue that ISG induction is not the cellular response inhibited by the LY drugs and NU7441.

U2OS cells express the target(s) of the LY drugs and NU7441

HSV-1 VP16 and ICP0 mutants form plaques with close to wild-type efficiency in U2OS osteosarcoma cells (44, 95, 115) and these cells are often used to grow such mutants to high titres in culture. We previously determined that U2OS cells are lacking a cellular antiviral mechanism found in restrictive cell types (44) and hypothesized that this cell line may be missing or defective in a signaling pathway used to activate a nuclear antiviral mechanism. It therefore seemed possible that the pathway targeted by the kinase inhibitors might be defective in these cells. If so, then one would predict that the drugs would not stimulate viral gene expression in this cell line. Although V422 plaques with greatly enhanced efficiency on U2OS cells, viral gene expression is delayed by approximately 4-5 hours relative to wild-type HSV-1 (Figure 5.1), allowing us to determine whether the kinase inhibitors enhance IE gene expression at early times post-infection in these cells.

Northern blot analysis of ICP27 transcript accumulation in the presence of the inhibitors and cycloheximide was performed (Fig. 6.6). U2OS cells were pretreated with kinase inhibitors with or without cycloheximide for one hour. Cells were infected with 10 PFU/cell of KOS or V422 for 4hr in the presence of

the drugs and then RNA was harvested for northern blot analysis. Similar to what was observed in HEL cells (Fig. 6.4), drug treatments had little effect on IE mRNA accumulation in wild type infected cells. However, the drugs stimulated ICP27 mRNA accumulation with V422 infection in both the absence and presence of cycloheximide, indicating that U2OS cells express the target(s) of LY303511 and NU7441. Again, we see a decrease in IE mRNA accumulation in the presence of cycloheximide, yet the mRNA levels with drug treatments are still higher than mock- or DMSO-treated cells. This suggests that the defect in U2OS cells that allows for viral replication in the absence of VP16 activation function is separate from the pathway involving the targets of the LY drugs and NU7441.

Potential mechanisms and concluding remarks

The compounds examined in this report compensate for VP16 activation function by stimulating IE gene expression in the absence of VP16 activation function, in this sense resembling HMBA. They differ from Gramicidin D and sodium arsenite, which also stimulate IE gene expression (85), in that the kinase inhibitors are unable to reactivate quiescent HSV genomes. Gramicidin D and sodium arsenite were proposed to activate HSV gene expression via induction of cellular stress responses (85), and cellular stress can also reactivate latent genomes in infected neurons (21, 23). The inability of the kinase inhibitors to reactivate quiescent HSV argues that their effects on HSV IE gene expression do not stem from induction of a general cellular stress response. Indeed, the

compounds were used at low doses that did not cause obvious morphological changes indicative of cytotoxicity.

Our data strongly suggest that LY294002 and NU7441 activate HSV gene expression in the absence of VP16 activation function through ‘off-target’ effects on one or more proteins other than their intended targets. LY294002 is much less potent than the natural PI3K inhibitor wortmannin, and as such has many documented ‘off-target’ effects (16, 20, 25, 52, 55, 81, 93, 98, 105). Both LY294002 and NU7441 are ATP-competitive kinase inhibitors and bind in the active site of their intended targets (55). ATP-competitive inhibitors may also have effects on structurally related kinases, as documented by the ability of LY294002 to inhibit some members of the PIKK family (54, 105). There is extensive structural similarity between LY294002, LY303511 and NU7441 (Figure 6.7), suggesting they may all target a similar or related kinase.

The kinase inhibitors studied in this report could activate HSV IE gene expression by inactivating a repression mechanism or by stimulating an activator of viral gene expression; in either case, the direct target of the drug might be several steps removed from repressor or activator. Thus, many possible models could be envisioned for how these compounds function. In this context it is interesting to note that both LY294002 and LY303511 cause the phosphorylation and activation of Egr-1 in some cell types (63, 114). HSV infection induces Egr-1 expression (14) and this protein is thought to regulate ICP4 and ICP22 expression

(3, 14). However, the relevance of Egr-1 to the effects that we have described here remain to be determined.

The relevant direct target of the kinase inhibitors is likely a currently unidentified lipid or protein kinase. However, we cannot rule out a non-kinase ATP binding protein such as an ATPase, or even a protein that does not bind ATP. Indeed, the cellular transcriptional cofactor Brd4 can interact with an LY294002 derivative (37). A genome-wide siRNA screen for proteins that repress V422 gene expression in restrictive cell types is currently underway in our laboratory. Identifying these target(s) will provide valuable insight into the regulation of HSV gene expression.

Table 6.1: Activation of IE gene expression with protein kinase inhibitors

compound	Percent ICP4 positive HEL cells		
	Expt 1 (MOI = 10)	Expt 2 (MOI = 10)	Expt 3 (MOI = 5)
Mock	4.7	7.3	1
DMSO	6.4	7.2	1
HMBA (3mM)	91.2	90	24.9
SP600125 (25µM)	1	-	-
U0126 (10µM)	4.3	-	-
SB203580 (25µM)	4.5	-	-
LY294002 (25µM)	71.2	59.8	20.5
Wortmannin (1µM)	-	2.5	-
LY303511 (10µM)	-	71.6	25.5
NU7441 (10µM)	-	-	30.3

Figure 6.1. LY294002 compensates for the VP16 lesion of KM110. (A) HEL cells were left untreated or pretreated with LY294002 (25µM) for one hour. Cells were infected with 10 PFU/cell of KM110 and left for 9 hours in the presence of the drug. Cells were then processed for IF. Green staining represents ICP4 and the nuclei are stained blue with DAPI. (B) HEL cells were pretreated for one hour with DMSO, HMBA (3mM), LY294002 (25µM) or left untreated and infected with KOS, KM110, n212 or V422 at 1 PFU/cell for 9 hours. A minimum of 100 cells were assessed for ICP4 expression. (C) HEL cells were pretreated for one hour with the indicated drugs and infected with KOS, KM110, n212 or V422 at 0.1 PFU/cell for 24 hours. Total virus was harvested and titred on U2OS cells. Samples in which no plaques were detected on U2OS cells are indicated with (*).

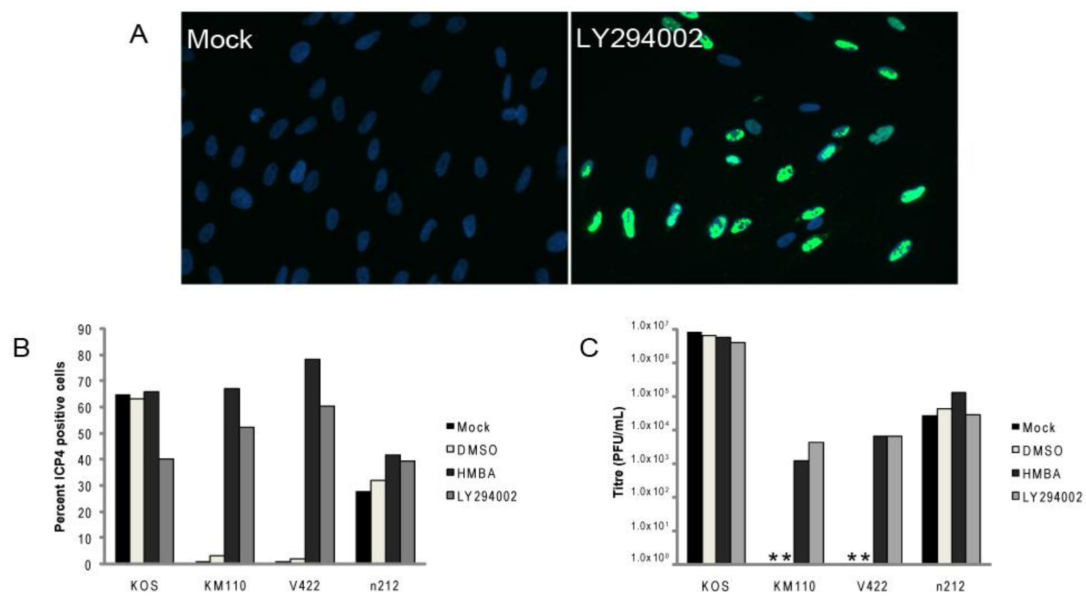

A
Mock
LY294002
B
Percent ICP4 positive cells
90
80
70
60
50
40
30
20
10
0
KOS
KM110
V422
n212
Mock
DMSO
HMBA
LY294002
C
Titre (PFU/mL)
1.0x10^7
1.0x10^6
1.0x10^5
1.0x10^4
1.0x10^3
1.0x10^2
1.0x10^1
1.0x10^0
* *
* *
KOS
KM110
V422
n212
Mock
DMSO
HMBA
LY294002

Figure 6.2. V422 gene expression is stimulated in a PI3K-independent manner. (A) HEL cells were serum-starved, then treated with LY294002 (25µM), LY303511 (10µM) or wortmannin (1µM) for 30 minutes. PDGF was added for 30 minutes and protein was harvested and blotted for total and phospho-AKT. (B and C) Infections were carried out as in Fig 6.1B and 6.1C (MOI = 10) also using 10µM LY303511. Samples in which no plaques were detected on U2OS cells are indicated with (*).

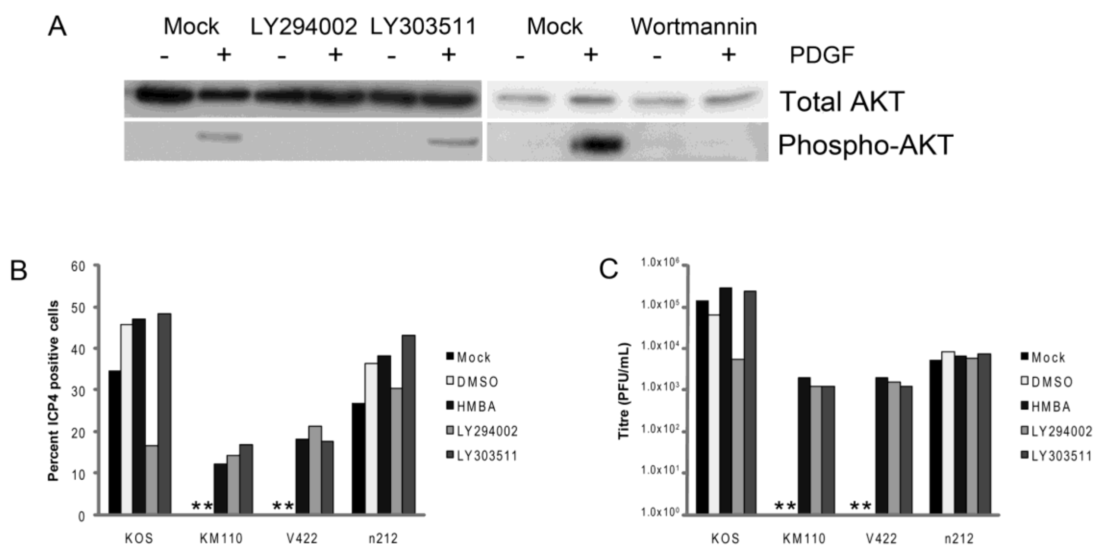
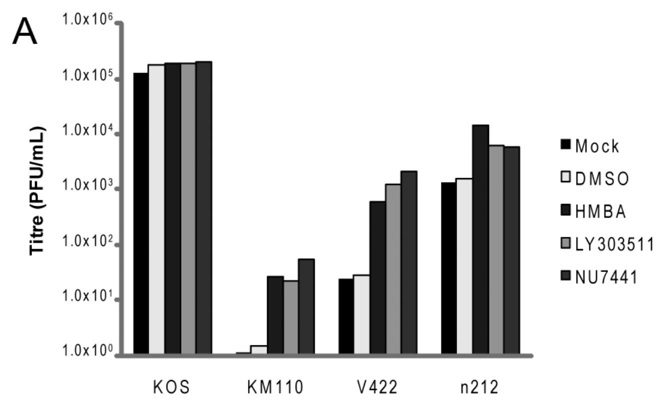

A
Mock
LY294002
LY303511
Mock
Wortmannin
PDGF
Total AKT
Phospho-AKT
B
Percent ICP4 positive cells
KOS
KM110
V422
n212
Mock
DMSO
HMBA
LY294002
LY303511
C
Titre (PFU/mL)
KOS
KM110
V422
n212
Mock
DMSO
HMBA
LY294002
LY303511

Figure 6.3. NU7441 activates V422 in a DNA PK-independent manner. (A) The indicated viruses were titred on Vero cells in the presence of DMSO, HMBA (3mM), LY303511 (10μM) or NU7441 (10μM). (B) MO59J and MO59K cells were treated with the indicated drugs and infected with 5 PFU/cell of V422 for 9 hours. Protein was harvested and blotted for ICP4.


A
1.0x10^6
1.0x10^5
1.0x10^4
1.0x10^3
1.0x10^2
1.0x10^1
1.0x10^0
Titre (PFU/mL)
Mock
DMSO
HMBA
LY303511
NU7441
KOS
KM110
V422
n212

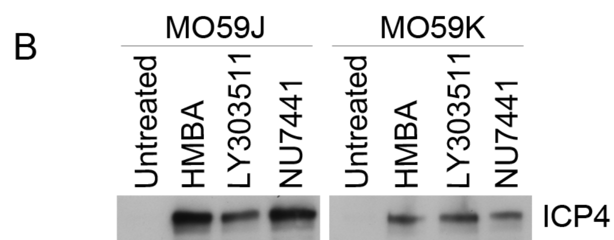
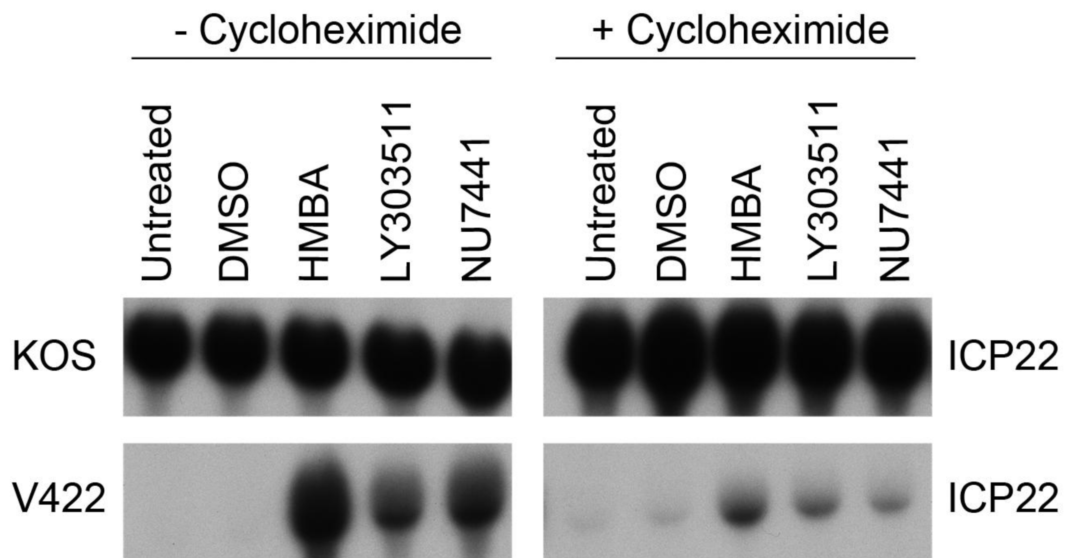
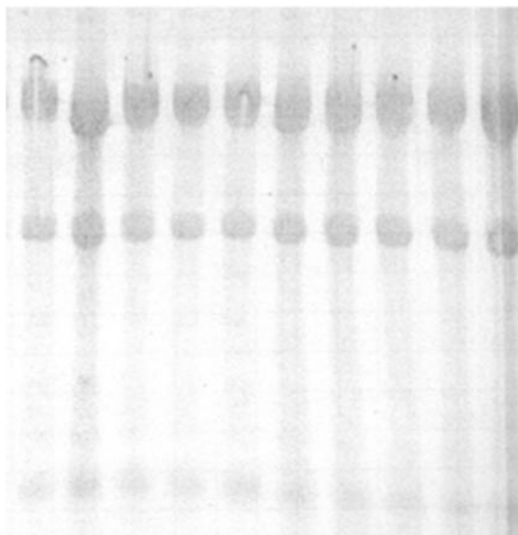

B
MO59J
MO59K
Untreated
HMBA
LY303511
NU7441
Untreated
HMBA
LY303511
NU7441
ICP4

Figure 6.4. Drugs stimulate V422 gene expression in the absence of new protein synthesis. Total cellular RNA was extracted from HEL cells following treatment with the indicated drugs and infection with 10 PFU/cell of KOS or V422 for 6 hours. 100μg/mL of cycloheximide was added where appropriate. ICP22 mRNA accumulation was monitored by northern blot hybridization. Below are Sybr Gold stained RNA gels (KOS, left; V422, right).


- Cycloheximide
+ Cycloheximide
Untreated
DMSO
HMBA
LY303511
NU7441
Untreated
DMSO
HMBA
LY303511
NU7441
KOS
V422
ICP22
ICP22



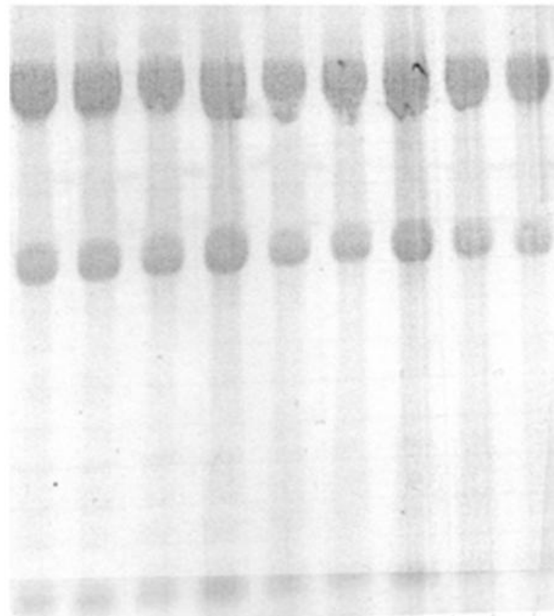


Figure 6.5. Drugs do not block ISG56K induction in response to incoming virus. Total cellular RNA extracted from HEL cells was scored for ISG56K mRNA by northern blot hybridization following drug treatments and infection with 10 PFU/cell of UV-inactivated KOS for 6 hours. 1000U of IFNα was added to one well as a positive control. 1/10th the amount of RNA was run in the IFN lane. Below is the ethidium bromide stained RNA gel.

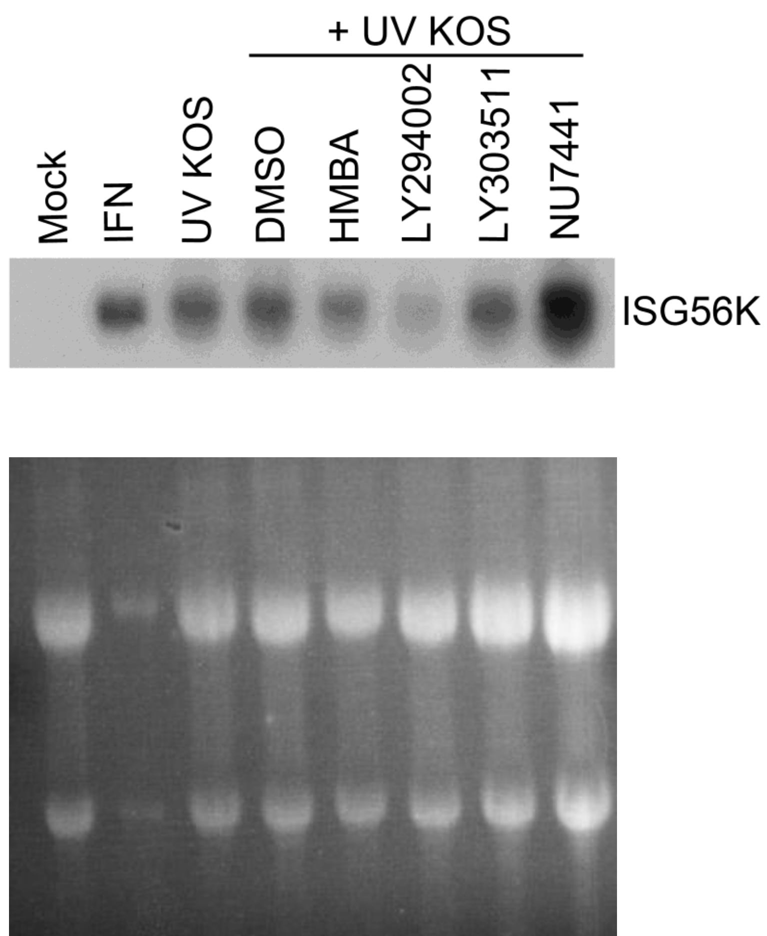

+ UV KOS
Mock
IFN
UV KOS
DMSO
HMBA
LY294002
LY303511
NU7441
ISG56K

Figure 6.6. U2OS cells express the target(s) of the LY drugs and NU7441. Total cellular RNA was extracted from U2OS cells following treatment with the indicated drugs and infection with 10 PFU/cell of KOS or V422 for 4 hours. ICP27 mRNA accumulation was monitored by northern blot hybridization. Below is the ethidium bromide stained RNA gels (KOS, left; V422, right).

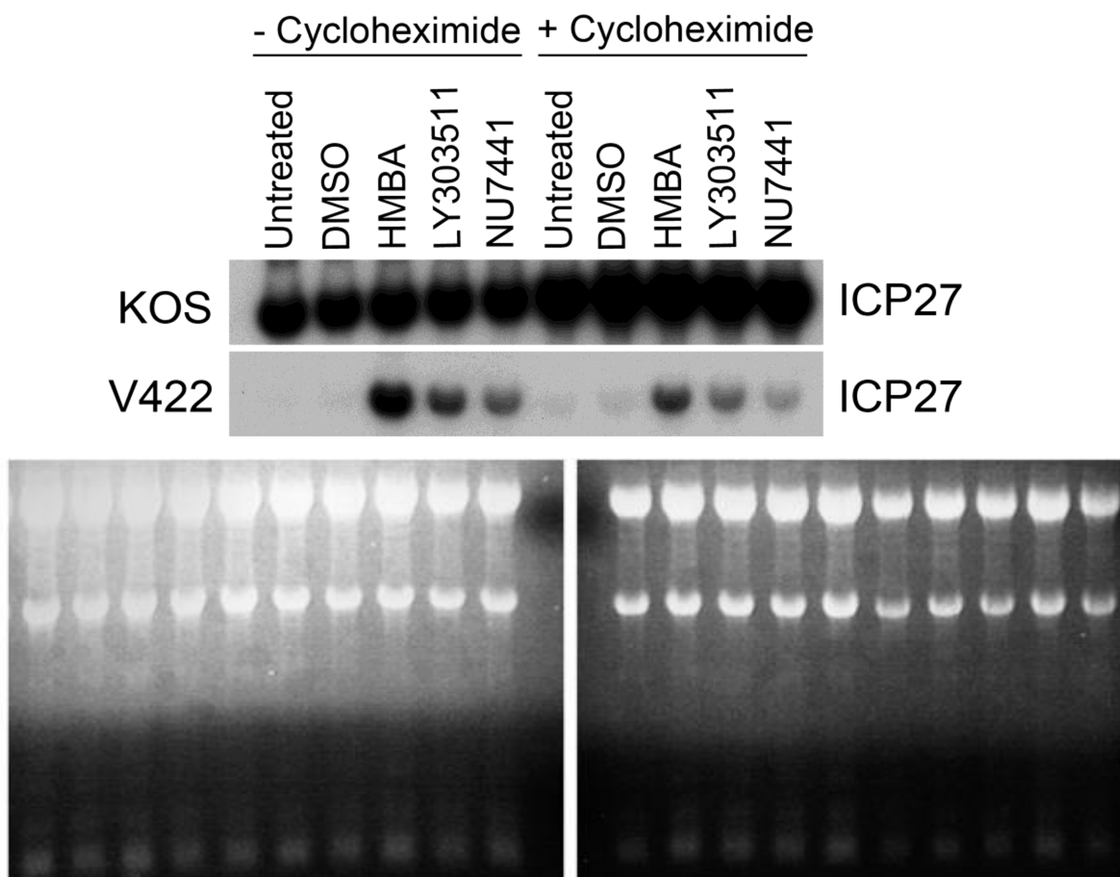
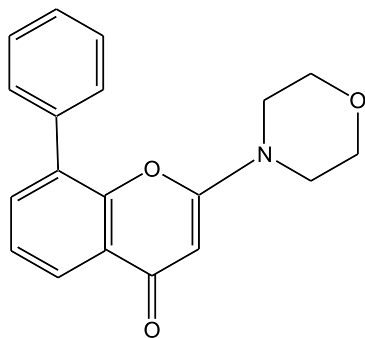
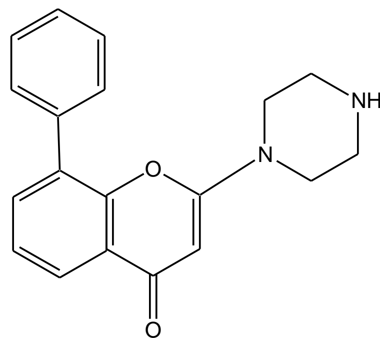

- Cycloheximide
+ Cycloheximide
Untreated
DMSO
HMBA
LY303511
NU7441
Untreated
DMSO
HMBA
LY303511
NU7441
KOS
ICP27
V422
ICP27

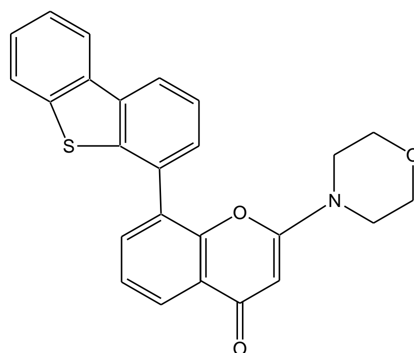
Figure 6.7. Chemical structures of LY294002, LY303511 and NU7441



Ly294002



Ly303511



NU7441

REFERENCES

1. **Ace, C. I., T. A. McKee, J. M. Ryan, J. M. Cameron, and C. M. Preston.** 1989. Construction and characterization of a herpes simplex virus type 1 mutant unable to transinduce immediate-early gene expression. J Virol **63:**2260-9.
2. **Allalunis-Turner, M. J., G. M. Barron, R. S. Day, 3rd, K. D. Dobler, and R. Mirzayans.** 1993. Isolation of two cell lines from a human malignant glioma specimen differing in sensitivity to radiation and chemotherapeutic drugs. Radiat Res **134:**349-54.
3. **Bedadala, G. R., R. C. Pinnoji, and S. C. Hsia.** 2007. Early growth response gene 1 (Egr-1) regulates HSV-1 ICP4 and ICP22 gene expression. Cell Res **17:**546-55.
4. **Benetti, L., and B. Roizman.** 2006. Protein kinase B/Akt is present in activated form throughout the entire replicative cycle of deltaU(S)3 mutant virus but only at early times after infection with wild-type herpes simplex virus 1. J Virol **80:**3341-8.
5. **Boutell, C., S. Sadis, and R. D. Everett.** 2002. Herpes simplex virus type 1 immediate-early protein ICP0 and is isolated RING finger domain act as ubiquitin E3 ligases in vitro. J Virol **76:**841-50.
6. **Bowie, A. G., and L. Unterholzner.** 2008. Viral evasion and subversion of pattern-recognition receptor signalling. Nat Rev Immunol **8:**911-22.
7. **Bringhurst, R. M., A. A. Dominguez, and P. A. Schaffer.** 2008. Glutamine deprivation causes enhanced plating efficiency of a herpes simplex virus type 1 ICP0-null mutant. J Virol **82:**11472-5.
8. **Bringhurst, R. M., and P. A. Schaffer.** 2006. Cellular stress rather than stage of the cell cycle enhances the replication and plating efficiencies of herpes simplex virus type 1 ICP0- viruses. J Virol **80:**4528-37.
9. **Browne, E. P., B. Wing, D. Coleman, and T. Shenk.** 2001. Altered cellular mRNA levels in human cytomegalovirus-infected fibroblasts: viral block to the accumulation of antiviral mRNAs. J Virol **75:**12319-30.
10. **Buchkovich, N. J., Y. Yu, C. A. Zampieri, and J. C. Alwine.** 2008. The TORrid affairs of viruses: effects of mammalian DNA viruses on the PI3K-Akt-mTOR signalling pathway. Nat Rev Microbiol **6:**266-75.
11. **Cai, W., T. L. Astor, L. M. Liptak, C. Cho, D. M. Coen, and P. A. Schaffer.** 1993. The herpes simplex virus type 1 regulatory protein ICP0 enhances virus replication during acute infection and reactivation from latency. J Virol **67:**7501-12.
12. **Cai, W., and P. A. Schaffer.** 1992. Herpes simplex virus type 1 ICP0 regulates expression of immediate-early, early, and late genes in productively infected cells. J Virol **66:**2904-15.
13. **Cai, W. Z., and P. A. Schaffer.** 1989. Herpes simplex virus type 1 ICP0 plays a critical role in the de novo synthesis of infectious virus following transfection of viral DNA. J Virol **63:**4579-89.

14. **Chen, S. H., H. W. Yao, I. T. Chen, B. Shieh, and C. Li.** 2008. Suppression of transcription factor early growth response 1 reduces herpes simplex virus lethality in mice. J Clin Invest **118:**3470-7.
15. **Cheshenko, N., B. Del Rosario, C. Woda, D. Marcellino, L. M. Satlin, and B. C. Herold.** 2003. Herpes simplex virus triggers activation of calcium-signaling pathways. J Cell Biol **163:**283-93.
16. **Choi, E. K., H. J. Park, J. S. Ma, H. C. Lee, H. C. Kang, B. G. Kim, and I. C. Kang.** 2004. LY294002 inhibits monocyte chemoattractant protein-1 expression through a phosphatidylinositol 3-kinase-independent mechanism. FEBS Lett **559:**141-4.
17. **Cliffe, A. R., and D. M. Knipe.** 2008. Herpes simplex virus ICP0 promotes both histone removal and acetylation on viral DNA during lytic infection. J Virol **82:**12030-8.
18. **Coleman, H. M., V. Connor, Z. S. Cheng, F. Grey, C. M. Preston, and S. Efstathiou.** 2008. Histone modifications associated with herpes simplex virus type 1 genomes during quiescence and following ICP0-mediated de-repression. J Gen Virol **89:**68-77.
19. **Collins, S. E., R. S. Noyce, and K. L. Mossman.** 2004. Innate cellular response to virus particle entry requires IRF3 but not virus replication. J Virol **78:**1706-17.
20. **Davies, S. P., H. Reddy, M. Caivano, and P. Cohen.** 2000. Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem J **351:**95-105.
21. **Devi-Rao, G. B., J. S. Aguilar, M. K. Rice, H. H. Garza, Jr., D. C. Bloom, J. M. Hill, and E. K. Wagner.** 1997. Herpes simplex virus genome replication and transcription during induced reactivation in the rabbit eye. J Virol **71:**7039-47.
22. **Diao, L., B. Zhang, C. Xuan, S. Sun, K. Yang, Y. Tang, W. Qiao, Q. Chen, Y. Geng, and C. Wang.** 2005. Activation of c-Jun N-terminal kinase (JNK) pathway by HSV-1 immediate early protein ICP0. Exp Cell Res **308:**196-210.
23. **Ecob-Prince, M., and K. Hassan.** 1994. Reactivation of latent herpes simplex virus from explanted dorsal root ganglia. J Gen Virol **75 (Pt 8):**2017-28.
24. **Eidson, K. M., W. E. Hobbs, B. J. Manning, P. Carlson, and N. A. DeLuca.** 2002. Expression of herpes simplex virus ICP0 inhibits the induction of interferon-stimulated genes by viral infection. J Virol **76:**2180-91.
25. **El-Kholy, W., P. E. Macdonald, J. H. Lin, J. Wang, J. M. Fox, P. E. Light, Q. Wang, R. G. Tsushima, and M. B. Wheeler.** 2003. The phosphatidylinositol 3-kinase inhibitor LY294002 potently blocks K(V) currents via a direct mechanism. Faseb J **17:**720-2.
26. **Engelman, J. A., J. Luo, and L. C. Cantley.** 2006. The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. Nat Rev Genet **7:**606-19.

27. **Everett, R., P. O'Hare, D. O'Rourke, P. Barlow, and A. Orr.** 1995. Point mutations in the herpes simplex virus type 1 Vmw110 RING finger helix affect activation of gene expression, viral growth, and interaction with PML-containing nuclear structures. J Virol **69:**7339-44.
28. **Everett, R. D.** 1989. Construction and characterization of herpes simplex virus type 1 mutants with defined lesions in immediate early gene 1. J Gen Virol **70 (Pt 5):**1185-202.
29. **Everett, R. D.** 2000. ICP0, a regulator of herpes simplex virus during lytic and latent infection. Bioessays **22:**761-70.
30. **Everett, R. D., C. Boutell, and A. Orr.** 2004. Phenotype of a herpes simplex virus type 1 mutant that fails to express immediate-early regulatory protein ICP0. J Virol **78:**1763-74.
31. **Everett, R. D., P. Freemont, H. Saitoh, M. Dasso, A. Orr, M. Kathoria, and J. Parkinson.** 1998. The disruption of ND10 during herpes simplex virus infection correlates with the Vmw110- and proteasome-dependent loss of several PML isoforms. J Virol **72:**6581-91.
32. **Everett, R. D., and G. G. Maul.** 1994. HSV-1 IE protein Vmw110 causes redistribution of PML. Embo J **13:**5062-9.
33. **Everett, R. D., A. Orr, and C. M. Preston.** 1998. A viral activator of gene expression functions via the ubiquitin-proteasome pathway. Embo J **17:**7161-9.
34. **Everett, R. D., C. Parada, P. Gripon, H. Sirma, and A. Orr.** 2008. Replication of ICP0-null mutant herpes simplex virus type 1 is restricted by both PML and Sp100. J Virol **82:**2661-72.
35. **Everett, R. D., G. Sourvinos, C. Leiper, J. B. Clements, and A. Orr.** 2004. Formation of nuclear foci of the herpes simplex virus type 1 regulatory protein ICP4 at early times of infection: localization, dynamics, recruitment of ICP27, and evidence for the de novo induction of ND10-like complexes. J Virol **78:**1903-17.
36. **Ferenczy, M. W., and N. A. Deluca.** 2009. Epigenetic Modulation of Gene Expression from Quiescent Hsv Genomes. J Virol.
37. **Gharbi, S. I., M. J. Zvelebil, S. J. Shuttleworth, T. Hancox, N. Saghir, J. F. Timms, and M. D. Waterfield.** 2007. Exploring the specificity of the PI3K family inhibitor LY294002. Biochem J **404:**15-21.
38. **Gillis, P. A., L. H. Okagaki, and S. A. Rice.** 2008. Herpes Simplex Virus Type 1 ICP27 Induces p38 Mitogen-activated Protein Kinase Signaling and Apoptosis in HeLa Cells. J Virol.
39. **Gregory, D. A., and S. L. Bachenheimer.** 2008. Characterization of mre11 loss following HSV-1 infection. Virology **373:**124-36.
40. **Gu, H., Y. Liang, G. Mandel, and B. Roizman.** 2005. Components of the REST/CoREST/histone deacetylase repressor complex are disrupted, modified, and translocated in HSV-1-infected cells. Proc Natl Acad Sci U S A **102:**7571-6.
41. **Guo, J., D. J. Hui, W. C. Merrick, and G. C. Sen.** 2000. A new pathway of translational regulation mediated by eukaryotic initiation factor 3. Embo J **19:**6891-9.

42. **Hagglund, R., C. Van Sant, P. Lopez, and B. Roizman.** 2002. Herpes simplex virus 1-infected cell protein 0 contains two E3 ubiquitin ligase sites specific for different E2 ubiquitin-conjugating enzymes. Proc Natl Acad Sci U S A **99:**631-6.
43. **Halford, W. P., C. D. Kemp, J. A. Isler, D. J. Davido, and P. A. Schaffer.** 2001. ICP0, ICP4, or VP16 expressed from adenovirus vectors induces reactivation of latent herpes simplex virus type 1 in primary cultures of latently infected trigeminal ganglion cells. J Virol **75:**6143-53.
44. **Hancock, M. H., J. A. Corcoran, and J. R. Smiley.** 2006. Herpes simplex virus regulatory proteins VP16 and ICP0 counteract an innate intranuclear barrier to viral gene expression. Virology **352:**237-52.
45. **Hargett, D., T. McLean, and S. L. Bachenheimer.** 2005. Herpes simplex virus ICP27 activation of stress kinases JNK and p38. J Virol **79:**8348-60.
46. **Harris, R. A., R. D. Everett, X. X. Zhu, S. Silverstein, and C. M. Preston.** 1989. Herpes simplex virus type 1 immediate-early protein Vmw110 reactivates latent herpes simplex virus type 2 in an in vitro latency system. J Virol **63:**3513-5.
47. **Harris, R. A., and C. M. Preston.** 1991. Establishment of latency in vitro by the herpes simplex virus type 1 mutant in1814. J Gen Virol **72 (Pt 4):**907-13.
48. **Herrera, F. J., and S. J. Triezenberg.** 2004. VP16-dependent association of chromatin-modifying coactivators and underrepresentation of histones at immediate-early gene promoters during herpes simplex virus infection. J Virol **78:**9689-96.
49. **Iwahori, S., N. Shirata, Y. Kawaguchi, S. K. Weller, Y. Sato, A. Kudoh, S. Nakayama, H. Isomura, and T. Tsurumi.** 2007. Enhanced phosphorylation of transcription factor sp1 in response to herpes simplex virus type 1 infection is dependent on the ataxia telangiectasia-mutated protein. J Virol **81:**9653-64.
50. **Jamieson, D. R., L. H. Robinson, J. I. Daksis, M. J. Nicholl, and C. M. Preston.** 1995. Quiescent viral genomes in human fibroblasts after infection with herpes simplex virus type 1 Vmw65 mutants. J Gen Virol **76 (Pt 6):**1417-31.
51. **Karaca, G., D. Hargett, T. I. McLean, J. S. Aguilar, P. Ghazal, E. K. Wagner, and S. L. Bachenheimer.** 2004. Inhibition of the stress-activated kinase, p38, does not affect the virus transcriptional program of herpes simplex virus type 1. Virology **329:**142-56.
52. **Kim, Y. H., K. H. Choi, J. W. Park, and T. K. Kwon.** 2005. LY294002 inhibits LPS-induced NO production through a inhibition of NF-kappaB activation: independent mechanism of phosphatidylinositol 3-kinase. Immunol Lett **99:**45-50.
53. **Klemm, R. D., J. A. Goodrich, S. Zhou, and R. Tjian.** 1995. Molecular cloning and expression of the 32-kDa subunit of human TFIID reveals interactions with VP16 and TFIIB that mediate transcriptional activation. Proc Natl Acad Sci U S A **92:**5788-92.

54. **Knight, Z. A., G. G. Chiang, P. J. Alaimo, D. M. Kenski, C. B. Ho, K. Coan, R. T. Abraham, and K. M. Shokat.** 2004. Isoform-specific phosphoinositide 3-kinase inhibitors from an arylmorpholine scaffold. Bioorg Med Chem **12:**4749-59.
55. **Knight, Z. A., and K. M. Shokat.** 2005. Features of selective kinase inhibitors. Chem Biol **12:**621-37.
56. **Knipe, D. M., D. Senechek, S. A. Rice, and J. L. Smith.** 1987. Stages in the nuclear association of the herpes simplex virus transcriptional activator protein ICP4. J Virol **61:**276-84.
57. **Kobayashi, N., P. J. Horn, S. M. Sullivan, S. J. Triezenberg, T. G. Boyer, and A. J. Berk.** 1998. DA-complex assembly activity required for VP16C transcriptional activation. Mol Cell Biol **18:**4023-31.
58. **Lam, Q., C. A. Smibert, K. E. Koop, C. Lavery, J. P. Capone, S. P. Weinheimer, and J. R. Smiley.** 1996. Herpes simplex virus VP16 rescues viral mRNA from destruction by the virion host shutoff function. Embo J **15:**2575-81.
59. **Leahy, J. J., B. T. Golding, R. J. Griffin, I. R. Hardcastle, C. Richardson, L. Rigoreau, and G. C. Smith.** 2004. Identification of a highly potent and selective DNA-dependent protein kinase (DNA-PK) inhibitor (NU7441) by screening of chromenone libraries. Bioorg Med Chem Lett **14:**6083-7.
60. **Lees-Miller, S. P., M. C. Long, M. A. Kilvert, V. Lam, S. A. Rice, and C. A. Spencer.** 1996. Attenuation of DNA-dependent protein kinase activity and its catalytic subunit by the herpes simplex virus type 1 transactivator ICP0. J Virol **70:**7471-7.
61. **Li, X. N., Z. W. Du, and Q. Huang.** 1996. Modulation effects of hexamethylene bisacetamide on growth and differentiation of cultured human malignant glioma cells. J Neurosurg **84:**831-8.
62. **Lilley, C. E., C. T. Carson, A. R. Muotri, F. H. Gage, and M. D. Weitzman.** 2005. DNA repair proteins affect the lifecycle of herpes simplex virus 1. Proc Natl Acad Sci U S A **102:**5844-9.
63. **Liu, P., B. Xu, J. Li, and H. Lu.** 2008. LY294002 inhibits leukemia cell invasion and migration through early growth response gene 1 induction independent of phosphatidylinositol 3-kinase-Akt pathway. Biochem Biophys Res Commun **377:**187-90.
64. **Lomonte, P., J. Thomas, P. Texier, C. Caron, S. Khochbin, and A. L. Epstein.** 2004. Functional interaction between class II histone deacetylases and ICP0 of herpes simplex virus type 1. J Virol **78:**6744-57.
65. **Lovejoy, C. A., and D. Cortez.** 2009. Common mechanisms of PIKK regulation. DNA Repair (Amst).
66. **Marks, P. A., V. M. Richon, and R. A. Rifkind.** 1996. Induced differentiation of cancer cells: second generation potent hybrid polar compounds target cell cycle regulators. Eur J Cancer Prev **5 Suppl 2:**75-7.
67. **McFarlane, M., J. I. Daksis, and C. M. Preston.** 1992. Hexamethylene bisacetamide stimulates herpes simplex virus immediate early gene

expression in the absence of trans-induction by Vmw65. J Gen Virol **73 (Pt 2):**285-92.

68. **McLean, T. I., and S. L. Bachenheimer.** 1999. Activation of cJUN N-terminal kinase by herpes simplex virus type 1 enhances viral replication. J Virol **73:**8415-26.
69. **Memedula, S., and A. S. Belmont.** 2003. Sequential recruitment of HAT and SWI/SNF components to condensed chromatin by VP16. Curr Biol **13:**241-6.
70. **Minaker, R. L., K. L. Mossman, and J. R. Smiley.** 2005. Functional inaccessibility of quiescent herpes simplex virus genomes. Virol J **2:**85.
71. **Mittler, G., T. Stuhler, L. Santolin, T. Uhlmann, E. Kremmer, F. Lottspeich, L. Berti, and M. Meisterernst.** 2003. A novel docking site on Mediator is critical for activation by VP16 in mammalian cells. Embo J **22:**6494-504.
72. **Mossman, K. L., P. F. Macgregor, J. J. Rozmus, A. B. Goryachev, A. M. Edwards, and J. R. Smiley.** 2001. Herpes simplex virus triggers and then disarms a host antiviral response. J Virol **75:**750-8.
73. **Mossman, K. L., H. A. Saffran, and J. R. Smiley.** 2000. Herpes simplex virus ICP0 mutants are hypersensitive to interferon. J Virol **74:**2052-6.
74. **Mossman, K. L., and J. R. Smiley.** 2002. Herpes simplex virus ICP0 and ICP34.5 counteract distinct interferon-induced barriers to virus replication. J Virol **76:**1995-8.
75. **Mossman, K. L., and J. R. Smiley.** 1999. Truncation of the C-terminal acidic transcriptional activation domain of herpes simplex virus VP16 renders expression of the immediate-early genes almost entirely dependent on ICP0. J Virol **73:**9726-33.
76. **Nicholl, M. J., L. H. Robinson, and C. M. Preston.** 2000. Activation of cellular interferon-responsive genes after infection of human cells with herpes simplex virus type 1. J Gen Virol **81:**2215-8.
77. **Noyce, R. S., S. E. Collins, and K. L. Mossman.** 2006. Identification of a novel pathway essential for the immediate-early, interferon-independent antiviral response to enveloped virions. J Virol **80:**226-35.
78. **O'Hare, P., and C. R. Goding.** 1988. Herpes simplex virus regulatory elements and the immunoglobulin octamer domain bind a common factor and are both targets for virion transactivation. Cell **52:**435-45.
79. **Pagano, M. A., G. Poletto, G. Di Maira, G. Cozza, M. Ruzzene, S. Sarno, J. Bain, M. Elliott, S. Moro, G. Zagotto, F. Meggio, and L. A. Pinna.** 2007. Tetrabromocinnamic acid (TBCA) and related compounds represent a new class of specific protein kinase CK2 inhibitors. Chembiochem **8:**129-39.
80. **Parkinson, J., S. P. Lees-Miller, and R. D. Everett.** 1999. Herpes simplex virus type 1 immediate-early protein vmw110 induces the proteasome-dependent degradation of the catalytic subunit of DNA-dependent protein kinase. J Virol **73:**650-7.
81. **Poh, T. W., and S. Pervaiz.** 2005. LY294002 and LY303511 sensitize tumor cells to drug-induced apoptosis via intracellular hydrogen peroxide

production independent of the phosphoinositide 3-kinase-Akt pathway. Cancer Res **65:**6264-74.

82. **Preston, C. M.** 2000. Repression of viral transcription during herpes simplex virus latency. J Gen Virol **81:**1-19.
83. **Preston, C. M., M. C. Frame, and M. E. Campbell.** 1988. A complex formed between cell components and an HSV structural polypeptide binds to a viral immediate early gene regulatory DNA sequence. Cell **52:**425-34.
84. **Preston, C. M., and M. McFarlane.** 1998. Cytodifferentiating agents affect the replication of herpes simplex virus type 1 in the absence of functional VP16. Virology **249:**418-26.
85. **Preston, C. M., and M. J. Nicholl.** 2008. Induction of cellular stress overcomes the requirement of herpes simplex virus type 1 for immediate-early protein ICP0 and reactivates expression from quiescent viral genomes. J Virol **82:**11775-83.
86. **Preston, C. M., and M. J. Nicholl.** 1997. Repression of gene expression upon infection of cells with herpes simplex virus type 1 mutants impaired for immediate-early protein synthesis. J Virol **71:**7807-13.
87. **Preston, C. M., A. Rinaldi, and M. J. Nicholl.** 1998. Herpes simplex virus type 1 immediate early gene expression is stimulated by inhibition of protein synthesis. J Gen Virol **79 (Pt 1):**117-24.
88. **Reuben, R. C., R. A. Rifkind, and P. A. Marks.** 1980. Chemically induced murine erythroleukemic differentiation. Biochim Biophys Acta **605:**325-46.
89. **Rifkind, R. A., V. M. Richon, and P. A. Marks.** 1996. Induced differentiation, the cell cycle, and the treatment of cancer. Pharmacol Ther **69:**97-102.
90. **Russell, J., N. D. Stow, E. C. Stow, and C. M. Preston.** 1987. Herpes simplex virus genes involved in latency in vitro. J Gen Virol **68 (Pt 12):**3009-18.
91. **Samaniego, L. A., L. Neiderhiser, and N. A. DeLuca.** 1998. Persistence and expression of the herpes simplex virus genome in the absence of immediate-early proteins. J Virol **72:**3307-20.
92. **Sarkar, S. N., K. L. Peters, C. P. Elco, S. Sakamoto, S. Pal, and G. C. Sen.** 2004. Novel roles of TLR3 tyrosine phosphorylation and PI3 kinase in double-stranded RNA signaling. Nat Struct Mol Biol **11:**1060-7.
93. **Shenoy, K., Y. Wu, and S. Pervaiz.** 2009. LY303511 enhances TRAIL sensitivity of SHEP-1 neuroblastoma cells via hydrogen peroxide-mediated mitogen-activated protein kinase activation and up-regulation of death receptors. Cancer Res **69:**1941-50.
94. **Shirata, N., A. Kudoh, T. Daikoku, Y. Tatsumi, M. Fujita, T. Kiyono, Y. Sugaya, H. Isomura, K. Ishizaki, and T. Tsurumi.** 2005. Activation of ataxia telangiectasia-mutated DNA damage checkpoint signal transduction elicited by herpes simplex virus infection. J Biol Chem **280:**30336-41.
95. **Smiley, J. R., and J. Duncan.** 1997. Truncation of the C-terminal acidic transcriptional activation domain of herpes simplex virus VP16 produces a

phenotype similar to that of the in1814 linker insertion mutation. J Virol **71:**6191-3.

96. **Stern, S., M. Tanaka, and W. Herr.** 1989. The Oct-1 homoeodomain directs formation of a multiprotein-DNA complex with the HSV transactivator VP16. Nature **341:**624-30.
97. **Stow, N. D., and E. C. Stow.** 1986. Isolation and characterization of a herpes simplex virus type 1 mutant containing a deletion within the gene encoding the immediate early polypeptide Vmw110. J Gen Virol **67 (Pt 12):**2571-85.
98. **Sun, H., B. Xu, E. Sheveleva, and Q. M. Chen.** 2008. LY294002 inhibits glucocorticoid-induced COX-2 gene expression in cardiomyocytes through a phosphatidylinositol 3 kinase-independent mechanism. Toxicol Appl Pharmacol **232:**25-32.
99. **Tal-Singer, R., R. Pichyangkura, E. Chung, T. M. Lasner, B. P. Randazzo, J. Q. Trojanowski, N. W. Fraser, and S. J. Triezenberg.** 1999. The transcriptional activation domain of VP16 is required for efficient infection and establishment of latency by HSV-1 in the murine peripheral and central nervous systems. Virology **259:**20-33.
100. **Taylor, T. J., and D. M. Knipe.** 2004. Proteomics of herpes simplex virus replication compartments: association of cellular DNA replication, repair, recombination, and chromatin remodeling proteins with ICP8. J Virol **78:**5856-66.
101. **Thoreen, C. C., S. A. Kang, J. W. Chang, Q. Liu, J. Zhang, Y. Gao, L. J. Reichling, T. Sim, D. M. Sabatini, and N. S. Gray.** 2009. An ATP-competitive mammalian target of rapamycin inhibitor reveals rapamycin-resistant functions of mTORC1. J Biol Chem **284:**8023-32.
102. **Triezenberg, S. J., R. C. Kingsbury, and S. L. McKnight.** 1988. Functional dissection of VP16, the trans-activator of herpes simplex virus immediate early gene expression. Genes Dev **2:**718-29.
103. **Uesugi, M., O. Nyanguile, H. Lu, A. J. Levine, and G. L. Verdine.** 1997. Induced alpha helix in the VP16 activation domain upon binding to a human TAF. Science **277:**1310-3.
104. **Unterholzner, L., and A. G. Bowie.** 2008. The interplay between viruses and innate immune signaling: recent insights and therapeutic opportunities. Biochem Pharmacol **75:**589-602.
105. **Vanhaesebroeck, B., S. J. Leevers, K. Ahmadi, J. Timms, R. Katso, P. C. Driscoll, R. Woscholski, P. J. Parker, and M. D. Waterfield.** 2001. Synthesis and function of 3-phosphorylated inositol lipids. Annu Rev Biochem **70:**535-602.
106. **Vivanco, I., and C. L. Sawyers.** 2002. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nat Rev Cancer **2:**489-501.
107. **Vlahos, C. J., W. F. Matter, K. Y. Hui, and R. F. Brown.** 1994. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). J Biol Chem **269:**5241-8.
108. **Whitby, A. J., W. A. Blyth, and T. J. Hill.** 1987. The effect of DNA hypomethylating agents on the reactivation of herpes simplex virus from

latently infected mouse ganglia in vitro. Brief report. Arch Virol **97:**137-44.

109. **Wilkinson, D. E., and S. K. Weller.** 2006. Herpes simplex virus type I disrupts the ATR-dependent DNA-damage response during lytic infection. J Cell Sci **119:**2695-703.
110. **Wilkinson, D. E., and S. K. Weller.** 2004. Recruitment of cellular recombination and repair proteins to sites of herpes simplex virus type 1 DNA replication is dependent on the composition of viral proteins within prereplicative sites and correlates with the induction of the DNA damage response. J Virol **78:**4783-96.
111. **Willmore, E., S. L. Elliott, T. Mainou-Fowler, G. P. Summerfield, G. H. Jackson, F. O'Neill, C. Lowe, A. Carter, R. Harris, A. R. Pettitt, C. Cano-Soumillac, R. J. Griffin, I. G. Cowell, C. A. Austin, and B. W. Durkacz.** 2008. DNA-dependent protein kinase is a therapeutic target and an indicator of poor prognosis in B-cell chronic lymphocytic leukemia. Clin Cancer Res **14:**3984-92.
112. **Xiao, H., A. Pearson, B. Coulombe, R. Truant, S. Zhang, J. L. Regier, S. J. Triezenberg, D. Reinberg, O. Flores, C. J. Ingles, and et al.** 1994. Binding of basal transcription factor TFIIH to the acidic activation domains of VP16 and p53. Mol Cell Biol **14:**7013-24.
113. **Xiao, P., and J. P. Capone.** 1990. A cellular factor binds to the herpes simplex virus type 1 transactivator Vmw65 and is required for Vmw65-dependent protein-DNA complex assembly with Oct-1. Mol Cell Biol **10:**4974-7.
114. **Yamaguchi, K., S. H. Lee, J. S. Kim, J. Wimalasena, S. Kitajima, and S. J. Baek.** 2006. Activating transcription factor 3 and early growth response 1 are the novel targets of LY294002 in a phosphatidylinositol 3-kinase-independent pathway. Cancer Res **66:**2376-84.
115. **Yao, F., and P. A. Schaffer.** 1995. An activity specified by the osteosarcoma line U2OS can substitute functionally for ICP0, a major regulatory protein of herpes simplex virus type 1. J Virol **69:**6249-58.
116. **Yurochko, A. D.** 2008. Human cytomegalovirus modulation of signal transduction. Curr Top Microbiol Immunol **325:**205-20.
117. **Zachos, G., B. Clements, and J. Conner.** 1999. Herpes simplex virus type 1 infection stimulates p38/c-Jun N-terminal mitogen-activated protein kinase pathways and activates transcription factor AP-1. J Biol Chem **274:**5097-103.
118. **Zachos, G., M. Koffa, C. M. Preston, J. B. Clements, and J. Conner.** 2001. Herpes simplex virus type 1 blocks the apoptotic host cell defense mechanisms that target Bcl-2 and manipulates activation of p38 mitogen-activated protein kinase to improve viral replication. J Virol **75:**2710-28.
119. **Zhao, Y., H. D. Thomas, M. A. Batey, I. G. Cowell, C. J. Richardson, R. J. Griffin, A. H. Calvert, D. R. Newell, G. C. Smith, and N. J. Curtin.** 2006. Preclinical evaluation of a potent novel DNA-dependent protein kinase inhibitor NU7441. Cancer Res **66:**5354-62.

120. **Zhu, H., J. P. Cong, and T. Shenk.** 1997. Use of differential display analysis to assess the effect of human cytomegalovirus infection on the accumulation of cellular RNAs: induction of interferon-responsive RNAs. Proc Natl Acad Sci U S A **94:**13985-90.
121. **Zien, P., J. S. Duncan, J. Skierski, M. Bretner, D. W. Litchfield, and D. Shugar.** 2005. Tetrabromobenzotriazole (TBBt) and tetrabromobenzimidazole (TBBz) as selective inhibitors of protein kinase CK2: evaluation of their effects on cells and different molecular forms of human CK2. Biochim Biophys Acta **1754:**271-80.

Appendix 1. Data not shown

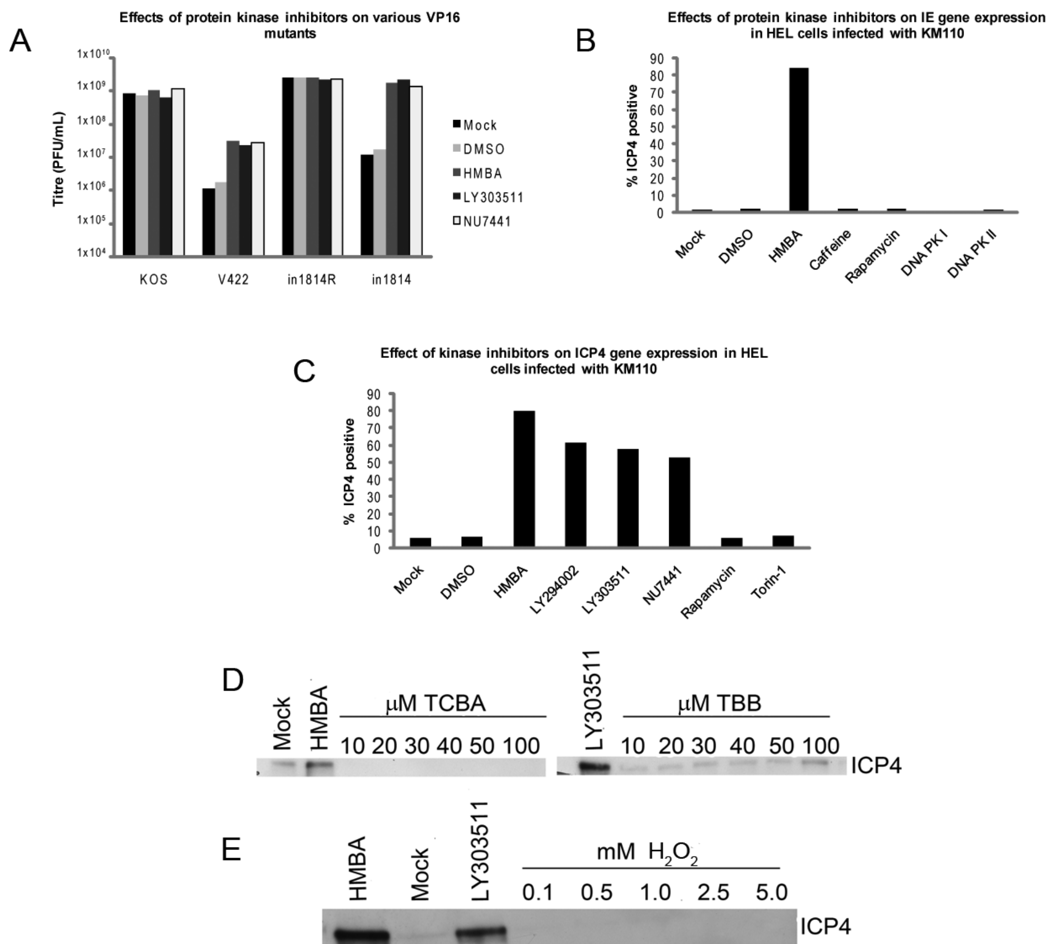


Figure 6A.1. The effects of various chemical compounds on viral infection (A) Protein kinase inhibitors stimulate in1814 replication. HEL cells were pretreated with the indicated kinase inhibitors and infected with 0.1 PFU/cell of KOS, V422, in1814R and in1814. After 24 hours virus was harvested and titred on U2OS cells. (B and C) PIKK inhibitors do not activate KM110 gene expression. HEL cells were pretreated with the indicated drugs and infected with 10 PFU/cell of KM110 for 9 hours. The proportion of cells expressing ICP4 was assessed using

indirect immunofluorescence. (D) Casein kinase II inhibitors do not activate KM110 gene expression. HEL cells were treated with the indicated drugs and infected with 10 PFU/cell of KM110 for 9 hours. Protein was harvested and ICP4 protein expression was assessed by western blot analysis. (E) H_2O_2 does not activate KM110 gene expression. HEL cells were treated with various concentrations of H_2O_2 and infected with 10 PFU/cell of KM110 for 9 hours. Protein was harvested and ICP4 protein expression was assessed by western blot analysis.

Chapter Seven: Thesis Discussion

The work presented in this thesis is directed towards understanding how the human osteosarcoma cell line U2OS support the replication of HSV-1 VP16 and ICP0 mutants. Before I began this project, Yao and Schaffer had proposed that U2OS cells express an 'ICP0-like' protein which can stimulate viral gene expression in the absence of ICP0 (409). U2OS cells also support the replication of VP16 mutants (352), which would argue either that the activation functions of VP16 and ICP0 are related, or that U2OS cells also express a second protein which can compensate for VP16 activation function. I felt it was alternatively possible that U2OS cells lack a component of an antiviral pathway targeted by VP16 and ICP0, and set out to distinguish between these possibilities. In Chapter three, I approached this problem by employing heterotypic somatic cell fusion assays using permissive U2OS and restrictive human fibroblasts. From these experiments it was evident that the permissive phenotype of U2OS cells was recessive in the somatic cell hybrids, strongly suggesting that U2OS are lacking a component of an innate antiviral gene silencing mechanism found in other restrictive cell types. Thus, VP16 and ICP0 may stimulate gene expression, at least in part, by overcoming this antiviral mechanism. In Chapter four, I excluded the possibility that the artificial induction of interferon stimulated genes caused by p14-mediated fusion was the basis for the repression of viral gene expression observed in the somatic cell fusion assays. These observations provided the basis for interpreting the data presented in subsequent chapters of this thesis.

While I was performing these somatic cell fusion experiments, evidence was presented in the literature indicating that VP16 and ICP0 play roles in

modulating the chromatin structure on the viral genome (Reviewed in Chapter five; (55, 109, 150, 202)). Knowing that U2OS cells lack a component of an innate antiviral mechanism normally counteracted by VP16 and ICP0, I hypothesized that this cell type has a defect in a process involved in loading or modifying histones on the viral genome. In Chapter five, I performed ChIP experiments assaying histone occupancy and acetylation levels on wild type and VP16 and ICP0 mutant genomes in both restrictive and permissive cell types. Several surprising observations arose from these studies. Firstly, VP16 is required in both restrictive and permissive cell types in order to maintain a low density of highly acetylated histones at multiple loci on the viral genome. In fact, evidence presented in Chapter five strongly argues that VP16 and ICP0 modulate chromatin structure on the viral genome by each counteracting an independent pathways. Additionally, I determined that the chromatin phenotype of an ICP0 mutant is strikingly different in restrictive and permissive cell types. While ICP0 is required in order to maintain a low density of highly acetylated histones on the viral genome in HeLa cells, ICP0 is dispensable for this effect in U2OS. Thus, the pathway targeted by ICP0 appears to be missing or defective in U2OS cells.

In this chapter, I will discuss possible mechanisms by which VP16 and ICP0 could modulate the histone occupancy and acetylation levels on the viral genome in the light of my findings and those of others. I will also present evidence to support the hypothesis that U2OS cells allow for the replication of VP16 and ICP0 mutants due to a defect in the ND10-based repression system. Finally, I will outline several possible mechanisms for how ND10 domains could

be recruited to viral genomes and present the interesting questions that have arisen from this research.

Mechanisms by which VP16 and ICP0 could affect histone occupancy and acetylation levels on the viral genome

VP16 and ICP0 play critical roles in maintaining low histone occupancy on the viral genome as well as being required to maintain the viral chromatin in a highly acetylated state (55, 109, 150, 202). Evidence presented in Chapter five suggests that VP16 and ICP0 accomplish these functions by each counteracting separate chromatin-based pathways. The increased acetylation of histones on the viral genome mediated by VP16 and ICP0 may be linked to the decrease in histone occupancy. Acetylation masks the overall positive charge of the nucleosome and is thought to relax the interactions between histones and DNA (119). In addition, acetylated lysine residues provide a binding motif for chromatin remodelling complexes which can slide or remove histones from chromatinized DNA (144, 175). The simplest interpretation would suggest that the ability of VP16 and ICP0 to increase acetylation levels on the viral genome would also affect histone density. However, it remains possible that histone occupancy and acetylation levels on the viral genome are regulated by VP16 and ICP0 independently of one another.

One possible mechanism by which VP16 and ICP0 could affect viral chromatin structure is by counteracting the effects of HDACs or repressive chromatin remodelling complexes which specifically target the viral genome.

ND10 proteins associate with the viral genome immediately after their injection into the nucleus (96, 104) and many transcriptional repressors are found within these structures, including type I and II HDACs and repressive chromatin remodelling complexes such as that formed between ATRX and hDaxx (371). The close proximity of these transcriptional repressors to the viral genome may result in a high density of under-acetylated histones on the viral DNA. Therefore, ICP0 could affect chromatin structure on the viral genome via its ability to disperse ND10 domains and prevent the association of these factors with the viral genome. In fact, knockdown of several transcriptional repressors which are components of ND10 domains, including PML, Sp100, ATRX and hDaxx can partially alleviate the repression of ICP0 mutant genomes in human fibroblasts ((101), R. Everett, personal comm.). Thus, ICP0 may affect histone occupancy and acetylation levels on the viral genome by preventing the association of ND10 components involved in mediating chromatin assembly or histone modifications.

Evidence presented in Chapter five indicates that VP16 counteracts a pathway that is separate from that modulated by ICP0. VP16 may function by binding and sequestering HDACs or chromatin remodelling factors which are not found in ND10. VP16 binds only IE promoter sequences, yet can affect histone occupancy and acetylation levels at multiple regions of the viral genome (Figure 5.2-3). This suggests that VP16 acts in a promoter-independent manner to modulate chromatin structure and supports a model whereby VP16 globally prevents the association of repressive chromatin factors with the viral genome.

As one means to test whether VP16 is required to bind to viral DNA in order to exert its effects on chromatinization of the viral genome, histone occupancy and acetylation levels could be assayed on the genome of the VP16 mutant in1814. This mutant retains the C-terminal acidic activation domain but contains a linker insertion mutation which results in a protein unable to interact with IE promoters (3). Thus, if this mutant virus can still modulate histone occupancy and acetylation levels on the viral genome, this would suggest that VP16 acts in a manner not requiring association with DNA and supports the hypothesis that VP16 functions, at least in part, by interacting with histone modifying or assembly factors away from the viral genome.

What is the defect in U2OS cells which allows for the replication of ICP0 mutants?

In Chapter five, I show that an ICP0 mutant has histone H3 levels similar to wild type virus in U2OS cells. Based on the premise that U2OS cells are lacking a component of an innate antiviral mechanism normally targeted by ICP0, these results would suggest that U2OS cells have a defect in a chromatin assembly and/or acetylation pathway normally targeted by ICP0. Although I cannot fully exclude the possibility that the ability of U2OS cells to support the replication of ICP0 mutants stems, at least in part, from a defect in chromatin assembly or acetylation, evidence presented in Chapter five and elsewhere in the literature suggests that the ability of ICP0 to modulate the viral chromatin structure is not directly required to stimulate viral gene expression.

Perhaps the strongest evidence that chromatinization of the viral genome is not the antiviral response that limits viral gene expression at the earliest time post-infection is the timing of histone loading and modifications. Histones are detected on the viral genome within 30 minutes after infection (158), yet Figure 5.2 indicates that mutant genomes do not begin to accumulate histones at levels significantly higher than wild type virus until between 3 and 6 hours post-infection. Viral gene expression is thought to be repressed very soon after infection, much earlier than this increased loading of histones on the viral genome. This suggests that the process of histone loading and modification occurs downstream of the innate mechanism silencing viral gene expression. In addition, Figures 5.1 demonstrates that ICP27 mRNA accumulates after infection of HeLa cells with an ICP0 mutant, yet the genome is associated with high levels of under-acetylated histones at the ICP27 promoter (Figures 5.2-3), arguing that the chromatin-based pathway targeted by ICP0 which involves loading high levels of under-acetylated histones on the viral genome does not prevent IE gene expression. Finally, although evidence has been put forth to suggest that the ability of ICP0 to counteract the effects of HDACs is important in regulating viral gene expression (133-135), the Everett group has presented data which indicates that HDAC inhibitors are unable to fully complement ICP0 mutants in restrictive human fibroblasts (101). In addition, the ability of ICP0 to interact with the CoREST repressor complex and dissociate HDACs 1 and 2 to the cytoplasm is not essential for the transactivation activity of ICP0 (102). Therefore, the ability of ICP0 to affect HDAC activity may not be related to its ability to stimulate viral

gene expression. Thus, the hypothesis put forth in Chapter three, suggesting that U2OS cells support the replication of ICP0 mutants due to a defect in a chromatin assembly or modification pathway may be incorrect, at least with respect to histone acetylation.

The defect in U2OS cells which allows for replication of ICP0 mutants is thus still unknown. Observations from the Everett group suggest that ND10 components do not properly localize to incoming viral genomes in U2OS cells (personal comm.). Thus, U2OS cells may be missing, or defective in, an essential component of the ND10-based repression system and therefore do not properly target the incoming viral genomes for repression. The near-complete complementation of IE gene expression in U2OS cells infected with the ICP0 mutant n212 (Figure 5.1) supports the hypothesis that dispersal of ND10 domains by ICP0 is important for stimulating viral gene expression at the earliest times of infection. In the absence of repression mediated by ND10, the viral genome may also be free from the effects of HDACs and/or chromatin assembly factors, accounting for the chromatin structure of ICP0 mutants in this cell type. Thus, U2OS cells complement both the repression of viral gene expression mediated by ND10 and the chromatin phenotype which results from the association of these domains with the viral genome.

One interesting question which arises from these observations is whether the ability to localize ND10 to the viral genome can be restored in U2OS cells by fusion with restrictive human fibroblasts. To test whether this is the case, the localization of ND10 with viral genomes in U2OS cells can be assayed pre- and

post-fusion. Using the protocol of Everett et al (97, 104), viral DNA can be detected using DNA FISH and ND10 components with IF shortly after infection. According to the Everett group, unfused U2OS cells would have poor localization of ND10 components to the viral DNA. If, after fusion with human fibroblasts, the ability of ND10 to localize to the viral genomes found in the U2OS nuclei (see Figure 3.6) is restored, this would suggest that human fibroblasts can provide the necessary component(s) of the ND10-based repression system that are lacking in U2OS cells. This would provide evidence that U2OS cells support ICP0 mutant virus gene expression due to a defect in the ND10-based repression system.

Complementation of VP16 mutants in U2OS cells

In Chapter three, I hypothesized that both VP16 and ICP0 serve as inhibitors of separate steps in the same antiviral pathway. This hypothesis was based on two observations: firstly, the VP16/ICP0 double mutant virus KM110 can replicate in U2OS cells (270), suggesting the functions of these two proteins are related. Secondly, overexpression of either protein can overcome the barrier to viral gene expression in heterokarya, suggesting they both target the same pathway (Figure 3.4-5). However, this hypothesis must be re-evaluated based on the evidence presented in Chapter five. Figure 5.1 indicates that IE gene expression from the VP16 mutant V422 is significantly delayed in U2OS cells as compared to either wild type virus or the ICP0 mutant n212. This suggests that U2OS cells complement IE gene expression from ICP0 mutants to a greater extent

that VP16 mutants and suggests that U2OS do not directly complement VP16 mutants.

The phenotype of VP16 mutant gene expression in U2OS cells can be accommodated in a model whereby U2OS have a defect in the ND10-based repression system. If ND10 domains do not properly localize to incoming genomes in U2OS cells, the VP16 mutant genome may be free from the actions of the transcriptional repressors found at ND10. In the absence of these transcriptional repressors, the VP16 mutant genome may be accessible to other cellular transcription factors that can act in place of the VP16 activation domain, although evidence suggests this occurs in an inefficient manner (Figure 5.1). Once IE gene expression is initiated by cellular factors, the viral replication cycle can then proceed as in a wild-type infection. Thus, U2OS cells are most likely not defective in an antiviral pathway targeted by VP16, but instead VP16 mutants can have their gene expressed by cellular factors because they are not in the proximity of ND10-based transcriptional repressors. It is important to note that the data presented in Chapter five demonstrates that the chromatin-based pathway targeted by VP16 is intact in U2OS cells, yet does not prevent viral gene expression. Thus, U2OS cells do not complement VP16 mutants due to a defect in a chromatin assembly or acetylation pathway, as hypothesized in Chapter three.

Therefore, the question remains as to why expression of VP16 was capable of stimulating gene expression from the KM110 genome in restrictive heterokarya (Figure 3.5). The high levels of VP16 expression obtained from the transfection assays may have overwhelmed the restrictive mechanism and/or

accelerated IE gene expression so that there was insufficient time to repress the viral genomes. Alternatively, VP16 may play a role in modulating the ND10-based repression mechanism which has yet to be defined.

The hypothesis that cellular proteins can stimulate viral gene expression is supported by evidence from Chapter six, which demonstrates that several chemical compounds can stimulate gene expression in the absence of the VP16 activation domain (Figure 6.1-2). In fact, experiments using the VP16 mutant in1814 indicate that VP16 does not need to be bound to the viral genome for these compounds to exert their effects (Figure 6A.1). Unfortunately, the protein(s) targeted by these compounds have not been identified. These drugs can stimulate IE gene expression from a VP16 mutant genome in U2OS cells (Figure 6.6), indicating that this cell type does express protein(s) which can act on the viral genome in the absence of VP16.

How is the incoming viral genome detected by the ND10-based repression system?

The aspect of viral infection that is detected by the cell soon after infection and results in mobilizing ND10 components to the viral genome has not been elucidated. Everett and others have identified several steps in the viral life cycle that could be recognized and result in ND10 mobilization including the circularization of the incoming genome, the association of chromatin binding factors and histones with the viral DNA, or viral transcription itself (88, 97, 244).

Although viral transcription was originally hypothesized to be required for mobilization of ND10, infection in the presence of transcription inhibitors, such as actinomycin D, does not prevent the association of ND10 with the incoming viral genome (97, 163, 246). However, Everett et al argue that VP16, as well as cellular chromatin and DNA binding proteins may still associate with the viral genome even when transcription is inhibited and thus could trigger ND10 recruitment (97). Therefore, the role of binding of viral or cellular proteins to the viral genome in the recruitment of ND10 requires further examination.

The circularization of the viral genome and the association of histones with the viral DNA most likely involve cellular proteins and occur immediately after injection of the DNA into the nucleus. DNA repair and chromatin-associated proteins are found within ND10 domains (14, 41, 164, 341, 414), suggesting these processes could be involved in ND10 mobilization.

Double stranded and single stranded DNA breaks in the cellular genome are rapidly detected and repaired in order to maintain genomic integrity (21). The viral DNA injected into the nucleus is in a linear form with free dsDNA ends which may be quickly recognized as dsDNA breaks by cellular DNA repair proteins. Such proteins initiate cellular signalling cascades which could result in the repair of the ends of the HSV genome into a circular DNA molecule. Some DNA repair proteins are found in ND10 (14, 164, 341), and hence this may be one mechanism used to recruit these domains to the incoming viral genome.

Jackson and DeLuca presented evidence to suggest that circularization of the viral genome, as outlined above, leads to the repression of viral gene

expression (165). Using Gardella gel electrophoresis, Jackson and DeLuca demonstrated that linear genomes are detected during lytic infection, while circular genomes are only found in the absence of viral gene expression. They demonstrate that expression of ICP0 prevents the circularization of the viral genome and argue that the key role of ICP0 in stimulating viral gene expression is through counteracting a DNA repair pathway (165). Perhaps the ability of ICP0 to disperse ND10 domains prevents the association of DNA repair proteins with the viral genome, thus preventing circularization and allowing for viral gene expression.

Despite the observations outlined above, the model presented by Jackson and DeLuca remains controversial. It has long been thought that viral genomes circularize soon after injection into the nucleus and provides the template for transcription and DNA replication. Strang and Stow performed elegant genetic experiments which demonstrated that the lytic viral genome circularizes shortly after infection using a mechanism most likely involving direct end-joining (367). Strang and Stow made genetic lesions in the viral packaging sequences which resulted in viruses with unique genomic termini. Using these viruses, linear and circularized DNA molecules could be distinguished by Southern blot hybridization. They demonstrate that viral genomes circularize in the presence or absence of ICP0 (367). The discrepancies between these findings and those of Jackson and DeLuca may be due to the different approaches used in these studies, but this controversy remains unresolved. No new information has been presented since these findings were reported, thus the role, if any, of DNA repair proteins

found in ND10 in recruiting these domains to the incoming viral genome remains to be determined.

The second possible mechanism for recruiting ND10 domains to the viral genome could involve direct recognition of the 'naked' viral DNA. HSV-1 DNA in the virion is complexed with the protamine spermine, but not histone proteins (57, 122, 283, 294). The cell may recognize the spermine and spermidine associated with the viral DNA and recruit chromatin assembly factors to exchange these factors for histones, as occurs with paternal DNA during fertilization (24, 234). The chromatin assembly factor HIRA transfers the histone variant H3.3 onto the paternal DNA during fertilization (24, 234) and also onto the HSV-1 genome prior to viral DNA replication (295). HIRA has been detected as a component of ND10 domains (414) and thus may be involved in targeting these domains to the incoming viral genome. To test the importance of HIRA in ND10 domain recruitment to incoming viral genomes, IF and DNA FISH can be used to examine the localization of ND10 components and viral DNA during infection of fibroblast cells stably expressing a HIRA-targeted shRNA. If proper localization of ND10 components does not occur in the absence of HIRA, this argues that the chromatin assembly factor is essential for targeting ND10 to the incoming viral genome, as well as chromatinizing the viral genome.

It is also possible that the trigger(s) involved in recruiting ND10 domains to the viral genome resides outside the nucleus and may require recognition of infection prior to the injection of the viral DNA. Events that occur at very early times, such as engagement of the viral gylcoproteins with the cell surface

receptors or the process of membrane perturbation/fusion itself could alert ND10 components in the nucleus to an imminent infection, similar to the TLR and RLH signalling pathways. For example, a signalling cascade that begins outside the nucleus may culminate in post-translational modifications of ND10 component proteins that could increase their mobilization.

In Chapter 4, I determined that p14-mediated fusion in U2OS cells does not result in the expression of ISGs, as is observed in restrictive human fibroblasts (Figure 4.1). I hypothesize that this defect in U2OS cells may be related to a defect in the ND10-based repression mechanism. Perhaps membrane fusion triggers multiple signalling pathways that activate different arms of the antiviral response, one of which is the activation of ISGs and another is the mobilization of ND10 components. However, any link between membrane fusion events and ND10 component mobilization remains to be addressed. Elucidating the mechanism used to recognize viral infection at the earliest times is essential for understanding the ND10-based repression system.

Future Directions

By attempting to understand the basis for the permissivity of U2OS cells to VP16 and ICP0 mutants, this work has led to many interesting observations that warrant further investigation and are outlined in this section. Firstly, in Chapter four I demonstrated that membrane fusion results in the induction of ISGs in human fibroblasts, but not in U2OS or Vero cells. Fusion of U2OS and Vero cells does not result in restoration of this antiviral pathway, suggesting they share

a common defect. The defect in these cell lines which prevents the induction of ISGs in response to membrane fusion is unknown, but both cell types can respond to endogenous IFNα to induce ISGs, suggesting the problem may lie upstream of IRF3 activation, potentially in signalling the recognition of membrane fusion events.

In Chapter six I identified several chemical compounds which can stimulate gene expression from a VP16 mutant genome. Identifying the target(s) of these compounds will provide a greater understanding of how viral gene expression is regulated. A large-scale siRNA-based screen for proteins which limit VP16 mutant virus gene expression in restrictive cell types is currently under construction. As one example of a mechanism by which these drugs could function, the chemical compound HMBA also enhances VP16 mutant virus yields in many cell types, including U2OS (248, 304, 352). Recent evidence suggests that HMBA acts by releasing the positive transcription elongation factor P-TEFb from the repressive HEXIM1-containing complex in order to stimulate gene expression (62). Interestingly, in heterologous systems the activation domain of VP16 interacts with P-TEFb (200). However, a link between HMBA, P-TEFb and VP16 has not been made in the context of HSV infection.

Another very interesting observation made from the work presented in Chapter five indicates that VP16 globally affects the chromatin structure on the viral genome, while only directly interacting with IE promoters. Several possible mechanisms can account for these observations, and are outlined in detail in the discussion of Chapter five.

Finally, as discussed above, identifying the aspects of the viral lifecycle which are recognized by the cell and results in mobilization of ND10 domains to the viral genome is an important area of future research. Much remains to be understood about the cellular and viral processes which could result in ND10 mobilization. Further examination into any possible link between chromatin assembly, circularization of the viral genome and ND10 mobilization awaits further understanding of the proteins involved in these processes.

Chapter eight: Thesis References

1. **Accola, M. A., B. Huang, A. Al Masri, and M. A. McNiven.** 2002. The antiviral dynamin family member, MxA, tubulates lipids and localizes to the smooth endoplasmic reticulum. J Biol Chem **277:**21829-35.
2. **Ace, C. I., M. A. Dalrymple, F. H. Ramsay, V. G. Preston, and C. M. Preston.** 1988. Mutational analysis of the herpes simplex virus type 1 trans-inducing factor Vmw65. J Gen Virol **69 (Pt 10):**2595-605.
3. **Ace, C. I., T. A. McKee, J. M. Ryan, J. M. Cameron, and C. M. Preston.** 1989. Construction and characterization of a herpes simplex virus type 1 mutant unable to transinduce immediate-early gene expression. J Virol **63:**2260-9.
4. **Advani, S. J., R. Brandimarti, R. R. Weichselbaum, and B. Roizman.** 2000. The disappearance of cyclins A and B and the increase in activity of the G(2)/M-phase cellular kinase cdc2 in herpes simplex virus 1-infected cells require expression of the alpha22/U(S)1.5 and U(L)13 viral genes. J Virol **74:**8-15.
5. **Advani, S. J., R. R. Weichselbaum, and B. Roizman.** 2001. cdc2 cyclin-dependent kinase binds and phosphorylates herpes simplex virus 1 U(L)42 DNA synthesis processivity factor. J Virol **75:**10326-33.
6. **Advani, S. J., R. R. Weichselbaum, and B. Roizman.** 2003. Herpes simplex virus 1 activates cdc2 to recruit topoisomerase II alpha for post-DNA synthesis expression of late genes. Proc Natl Acad Sci U S A **100:**4825-30.
7. **Advani, S. J., R. R. Weichselbaum, and B. Roizman.** 2000. The role of cdc2 in the expression of herpes simplex virus genes. Proc Natl Acad Sci U S A **97:**10996-1001.
8. **Ahmed, M., M. Lock, C. G. Miller, and N. W. Fraser.** 2002. Regions of the herpes simplex virus type 1 latency-associated transcript that protect cells from apoptosis in vitro and protect neuronal cells in vivo. J Virol **76:**717-29.
9. **Ahn, K., T. H. Meyer, S. Uebel, P. Sempe, H. Djaballah, Y. Yang, P. A. Peterson, K. Fruh, and R. Tampe.** 1996. Molecular mechanism and species specificity of TAP inhibition by herpes simplex virus ICP47. EMBO J **15:**3247-55.
10. **Amelio, A. L., N. V. Giordani, N. J. Kubat, E. O'Neil J, and D. C. Bloom.** 2006. Deacetylation of the herpes simplex virus type 1 latency-associated transcript (LAT) enhancer and a decrease in LAT abundance precede an increase in ICP0 transcriptional permissiveness at early times postexplant. J Virol **80:**2063-8.
11. **Amelio, A. L., P. K. McAnany, and D. C. Bloom.** 2006. A chromatin insulator-like element in the herpes simplex virus type 1 latency-associated transcript region binds CCCTC-binding factor and displays enhancer-blocking and silencing activities. J Virol **80:**2358-68.
12. **Arthur, J. L., R. Everett, I. Brierley, and S. Efstathiou.** 1998. Disruption of the 5' and 3' splice sites flanking the major latency-associated transcripts of herpes simplex virus type 1: evidence for

alternate splicing in lytic and latent infections. J Gen Virol **79 (Pt 1):**107-16.

13. **Banfield, B. W., Y. Leduc, L. Esford, K. Schubert, and F. Tufaro.** 1995. Sequential isolation of proteoglycan synthesis mutants by using herpes simplex virus as a selective agent: evidence for a proteoglycan-independent virus entry pathway. J Virol **69:**3290-8.
14. **Barr, S. M., C. G. Leung, E. E. Chang, and K. A. Cimprich.** 2003. ATR kinase activity regulates the intranuclear translocation of ATR and RPA following ionizing radiation. Curr Biol **13:**1047-51.
15. **Batterson, W., D. Furlong, and B. Roizman.** 1983. Molecular genetics of herpes simplex virus. VIII. further characterization of a temperature-sensitive mutant defective in release of viral DNA and in other stages of the viral reproductive cycle. J Virol **45:**397-407.
16. **Batterson, W., and B. Roizman.** 1983. Characterization of the herpes simplex virion-associated factor responsible for the induction of alpha genes. J Virol **46:**371-7.
17. **Berman, E. J., and J. M. Hill.** 1985. Spontaneous ocular shedding of HSV-1 in latently infected rabbits. Invest Ophthalmol Vis Sci **26:**587-90.
18. **Bernardi, R., A. Papa, and P. P. Pandolfi.** 2008. Regulation of apoptosis by PML and the PML-NBs. Oncogene **27:**6299-312.
19. **Bernstein, B. E., E. L. Humphrey, R. L. Erlich, R. Schneider, P. Bouman, J. S. Liu, T. Kouzarides, and S. L. Schreiber.** 2002. Methylation of histone H3 Lys 4 in coding regions of active genes. Proc Natl Acad Sci U S A **99:**8695-700.
20. **Bernstein, D. I., and J. C. Kappes.** 1988. Enhanced in vitro reactivation of latent herpes simplex virus from neural and peripheral tissues with hexamethylenebisacetamide. Arch Virol **99:**57-65.
21. **Bernstein, K. A., and R. Rothstein.** 2009. At loose ends: resecting a double-strand break. Cell **137:**807-10.
22. **Blaho, J. A., C. Mitchell, and B. Roizman.** 1993. Guanylylation and adenylylation of the alpha regulatory proteins of herpes simplex virus require a viral beta or gamma function. J Virol **67:**3891-900.
23. **Boehmer, P. E., M. S. Dodson, and I. R. Lehman.** 1993. The herpes simplex virus type-1 origin binding protein. DNA helicase activity. J Biol Chem **268:**1220-5.
24. **Bonnefoy, E., G. A. Orsi, P. Couble, and B. Loppin.** 2007. The essential role of Drosophila HIRA for de novo assembly of paternal chromatin at fertilization. PLoS Genet **3:**1991-2006.
25. **Bourdeau, V., D. Baudry, and G. Ferbeyre.** 2009. PML links aberrant cytokine signaling and oncogenic stress to cellular senescence. Front Biosci **14:**475-85.
26. **Boutell, C., M. Canning, A. Orr, and R. D. Everett.** 2005. Reciprocal activities between herpes simplex virus type 1 regulatory protein ICP0, a ubiquitin E3 ligase, and ubiquitin-specific protease USP7. J Virol **79:**12342-54.

27. **Boutell, C., R. Everett, J. Hilliard, P. Schaffer, A. Orr, and D. Davido.** 2008. Herpes simplex virus type 1 ICP0 phosphorylation mutants impair the E3 ubiquitin ligase activity of ICP0 in a cell type-dependent manner. J Virol **82:**10647-56.
28. **Boutell, C., and R. D. Everett.** 2003. The herpes simplex virus type 1 (HSV-1) regulatory protein ICP0 interacts with and Ubiquitinates p53. J Biol Chem **278:**36596-602.
29. **Boutell, C., A. Orr, and R. D. Everett.** 2003. PML residue lysine 160 is required for the degradation of PML induced by herpes simplex virus type 1 regulatory protein ICP0. J Virol **77:**8686-94.
30. **Boutell, C., S. Sadis, and R. D. Everett.** 2002. Herpes simplex virus type 1 immediate-early protein ICP0 and is isolated RING finger domain act as ubiquitin E3 ligases in vitro. J Virol **76:**841-50.
31. **Bringhurst, R. M., A. A. Dominguez, and P. A. Schaffer.** 2008. Glutamine deprivation causes enhanced plating efficiency of a herpes simplex virus type 1 ICP0-null mutant. J Virol **82:**11472-5.
32. **Bringhurst, R. M., and P. A. Schaffer.** 2006. Cellular stress rather than stage of the cell cycle enhances the replication and plating efficiencies of herpes simplex virus type 1 ICP0- viruses. J Virol **80:**4528-37.
33. **Brou, C., J. Wu, S. Ali, E. Scheer, C. Lang, I. Davidson, P. Chambon, and L. Tora.** 1993. Different TBP-associated factors are required for mediating the stimulation of transcription in vitro by the acidic transactivator GAL-VP16 and the two nonacidic activation functions of the estrogen receptor. Nucleic Acids Res **21:**5-12.
34. **Bryant, H. E., D. A. Matthews, S. Wadd, J. E. Scott, J. Kean, S. Graham, W. C. Russell, and J. B. Clements.** 2000. Interaction between herpes simplex virus type 1 IE63 protein and cellular protein p32. J Virol **74:**11322-8.
35. **Bryant, H. E., S. E. Wadd, A. I. Lamond, S. J. Silverstein, and J. B. Clements.** 2001. Herpes simplex virus IE63 (ICP27) protein interacts with spliceosome-associated protein 145 and inhibits splicing prior to the first catalytic step. J Virol **75:**4376-85.
36. **Cai, W., and P. A. Schaffer.** 1991. A cellular function can enhance gene expression and plating efficiency of a mutant defective in the gene for ICP0, a transactivating protein of herpes simplex virus type 1. J Virol **65:**4078-90.
37. **Cai, W., and P. A. Schaffer.** 1992. Herpes simplex virus type 1 ICP0 regulates expression of immediate-early, early, and late genes in productively infected cells. J Virol **66:**2904-15.
38. **Cai, W. Z., and P. A. Schaffer.** 1989. Herpes simplex virus type 1 ICP0 plays a critical role in the de novo synthesis of infectious virus following transfection of viral DNA. J Virol **63:**4579-89.
39. **Campbell, M. E., J. W. Palfreyman, and C. M. Preston.** 1984. Identification of herpes simplex virus DNA sequences which encode a trans-acting polypeptide responsible for stimulation of immediate early transcription. J Mol Biol **180:**1-19.

40. **Canning, M., C. Boutell, J. Parkinson, and R. D. Everett.** 2004. A RING finger ubiquitin ligase is protected from autocatalyzed ubiquitination and degradation by binding to ubiquitin-specific protease USP7. J Biol Chem **279:**38160-8.
41. **Carbone, R., M. Pearson, S. Minucci, and P. G. Pelicci.** 2002. PML NBs associate with the hMre11 complex and p53 at sites of irradiation induced DNA damage. Oncogene **21:**1633-40.
42. **Carrozza, M. J., and N. DeLuca.** 1998. The high mobility group protein 1 is a coactivator of herpes simplex virus ICP4 in vitro. J Virol **72:**6752-7.
43. **Carrozza, M. J., and N. A. DeLuca.** 1996. Interaction of the viral activator protein ICP4 with TFIID through TAF250. Mol Cell Biol **16:**3085-93.
44. **Cassady, K. A., M. Gross, and B. Roizman.** 1998. The second-site mutation in the herpes simplex virus recombinants lacking the gamma134.5 genes precludes shutoff of protein synthesis by blocking the phosphorylation of eIF-2alpha. J Virol **72:**7005-11.
45. **Challberg, M. D.** 1986. A method for identifying the viral genes required for herpesvirus DNA replication. Proc Natl Acad Sci U S A **83:**9094-8.
46. **Chee, A. V., P. Lopez, P. P. Pandolfi, and B. Roizman.** 2003. Promyelocytic leukemia protein mediates interferon-based anti-herpes simplex virus 1 effects. J Virol **77:**7101-5.
47. **Chelbi-Alix, M. K., and H. de The.** 1999. Herpes virus induced proteasome-dependent degradation of the nuclear bodies-associated PML and Sp100 proteins. Oncogene **18:**935-41.
48. **Chen, I. H., L. Li, L. Silva, and R. M. Sandri-Goldin.** 2005. ICP27 recruits Aly/REF but not TAP/NXF1 to herpes simplex virus type 1 transcription sites although TAP/NXF1 is required for ICP27 export. J Virol **79:**3949-61.
49. **Chen, I. H., K. S. Sciabica, and R. M. Sandri-Goldin.** 2002. ICP27 interacts with the RNA export factor Aly/REF to direct herpes simplex virus type 1 intronless mRNAs to the TAP export pathway. J Virol **76:**12877-89.
50. **Chen, Q., L. Lin, S. Smith, J. Huang, S. L. Berger, and J. Zhou.** 2007. CTCF-dependent chromatin boundary element between the latency-associated transcript and ICP0 promoters in the herpes simplex virus type 1 genome. J Virol **81:**5192-201.
51. **Chen, X., M. C. Schmidt, W. F. Goins, and J. C. Glorioso.** 1995. Two herpes simplex virus type 1 latency-active promoters differ in their contributions to latency-associated transcript expression during lytic and latent infections. J Virol **69:**7899-908.
52. **Ciufo, D. M., M. A. Mullen, and G. S. Hayward.** 1994. Identification of a dimerization domain in the C-terminal segment of the IE110 transactivator protein from herpes simplex virus. J Virol **68:**3267-82.
53. **Clemens, M. J., and A. Elia.** 1997. The double-stranded RNA-dependent protein kinase PKR: structure and function. J Interferon Cytokine Res **17:**503-24.

54. **Cliffe, A. R., D. A. Garber, and D. M. Knipe.** 2009. Transcription of the Herpes Simplex Virus Latency-Associated Transcript Promotes the Formation of Facultative Heterochromatin on Lytic Promoters. J Virol.

55. **Cliffe, A. R., and D. M. Knipe.** 2008. Herpes simplex virus ICP0 promotes both histone removal and acetylation on viral DNA during lytic infection. J Virol **82:**12030-8.

56. **Cocchi, F., L. Menotti, P. Mirandola, M. Lopez, and G. Campadelli-Fiume.** 1998. The ectodomain of a novel member of the immunoglobulin subfamily related to the poliovirus receptor has the attributes of a bona fide receptor for herpes simplex virus types 1 and 2 in human cells. J Virol **72:**9992-10002.

57. **Cohen, G. H., M. Ponce de Leon, H. Diggelmann, W. C. Lawrence, S. K. Vernon, and R. J. Eisenberg.** 1980. Structural analysis of the capsid polypeptides of herpes simplex virus types 1 and 2. J Virol **34:**521-31.

58. **Coleman, H. M., V. Connor, Z. S. Cheng, F. Grey, C. M. Preston, and S. Efstathiou.** 2008. Histone modifications associated with herpes simplex virus type 1 genomes during quiescence and following ICP0-mediated de-repression. J Gen Virol **89:**68-77.

59. **Collins, S. E., R. S. Noyce, and K. L. Mossman.** 2004. Innate cellular response to virus particle entry requires IRF3 but not virus replication. J Virol **78:**1706-17.

60. **Conley, A. J., D. M. Knipe, P. C. Jones, and B. Roizman.** 1981. Molecular genetics of herpes simplex virus. VII. Characterization of a temperature-sensitive mutant produced by in vitro mutagenesis and defective in DNA synthesis and accumulation of gamma polypeptides. J Virol **37:**191-206.

61. **Conn, K. L., M. J. Hendzel, and L. M. Schang.** 2008. Linker histones are mobilized during infection with herpes simplex virus type 1. J Virol **82:**8629-46.

62. **Contreras, X., M. Barboric, T. Lenasi, and B. M. Peterlin.** 2007. HMBA releases P-TEFb from HEXIM1 and 7SK snRNA via PI3K/Akt and activates HIV transcription. PLoS Pathog **3:**1459-69.

63. **Corcoran, J. A., W. L. Hsu, and J. R. Smiley.** 2006. Herpes simplex virus ICP27 is required for virus-induced stabilization of the ARE-containing IEX-1 mRNA encoded by the human IER3 gene. J Virol **80:**9720-9.

64. **Cress, W. D., and S. J. Triezenberg.** 1991. Critical structural elements of the VP16 transcriptional activation domain. Science **251:**87-90.

65. **Crute, J. J., E. S. Mocarski, and I. R. Lehman.** 1988. A DNA helicase induced by herpes simplex virus type 1. Nucleic Acids Res **16:**6585-96.

66. **Cunningham, C., and A. J. Davison.** 1993. A cosmid-based system for constructing mutants of herpes simplex virus type 1. Virology **197:**116-24.

67. **Dai-Ju, J. Q., L. Li, L. A. Johnson, and R. M. Sandri-Goldin.** 2006. ICP27 interacts with the C-terminal domain of RNA polymerase II and facilitates its recruitment to herpes simplex virus 1 transcription sites,

where it undergoes proteasomal degradation during infection. J Virol **80:**3567-81.

68. **Davido, D. J., W. F. von Zagorski, W. S. Lane, and P. A. Schaffer.** 2005. Phosphorylation site mutations affect herpes simplex virus type 1 ICP0 function. J Virol **79:**1232-43.
69. **de Bruyn Kops, A., S. L. Uprichard, M. Chen, and D. M. Knipe.** 1998. Comparison of the intranuclear distributions of herpes simplex virus proteins involved in various viral functions. Virology **252:**162-78.
70. **Deiss, L. P., J. Chou, and N. Frenkel.** 1986. Functional domains within the a sequence involved in the cleavage-packaging of herpes simplex virus DNA. J Virol **59:**605-18.
71. **Delius, H., and J. B. Clements.** 1976. A partial denaturation map of herpes simplex virus type 1 DNA: evidence for inversions of the unique DNA regions. J Gen Virol **33:**125-33.
72. **Dellaire, G., and D. P. Bazett-Jones.** 2007. Beyond repair foci: subnuclear domains and the cellular response to DNA damage. Cell Cycle **6:**1864-72.
73. **DeLuca, N. A., A. M. McCarthy, and P. A. Schaffer.** 1985. Isolation and characterization of deletion mutants of herpes simplex virus type 1 in the gene encoding immediate-early regulatory protein ICP4. J Virol **56:**558-70.
74. **Der, S. D., A. Zhou, B. R. Williams, and R. H. Silverman.** 1998. Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays. Proc Natl Acad Sci U S A **95:**15623-8.
75. **Deshmane, S. L., and N. W. Fraser.** 1989. During latency, herpes simplex virus type 1 DNA is associated with nucleosomes in a chromatin structure. J Virol **63:**943-7.
76. **Dohner, K., K. Radtke, S. Schmidt, and B. Sodeik.** 2006. Eclipse phase of herpes simplex virus type 1 infection: Efficient dynein-mediated capsid transport without the small capsid protein VP26. J Virol **80:**8211-24.
77. **Dohner, K., A. Wolfstein, U. Prank, C. Echeverri, D. Dujardin, R. Vallee, and B. Sodeik.** 2002. Function of dynein and dynactin in herpes simplex virus capsid transport. Mol Biol Cell **13:**2795-809.
78. **Dong, B., and R. H. Silverman.** 1995. 2-5A-dependent RNase molecules dimerize during activation by 2-5A. J Biol Chem **270:**4133-7.
79. **Efstathiou, S., and C. M. Preston.** 2005. Towards an understanding of the molecular basis of herpes simplex virus latency. Virus Res **111:**108-19.
80. **Eidson, K. M., W. E. Hobbs, B. J. Manning, P. Carlson, and N. A. DeLuca.** 2002. Expression of herpes simplex virus ICP0 inhibits the induction of interferon-stimulated genes by viral infection. J Virol **76:**2180-91.
81. **Elias, P., M. E. O'Donnell, E. S. Mocarski, and I. R. Lehman.** 1986. A DNA binding protein specific for an origin of replication of herpes simplex virus type 1. Proc Natl Acad Sci U S A **83:**6322-6.

82. **Ellison, K. S., R. A. Maranchuk, K. L. Mottet, and J. R. Smiley.** 2005. Control of VP16 translation by the herpes simplex virus type 1 immediate-early protein ICP27. J Virol **79:**4120-31.
83. **Everett, R., P. O'Hare, D. O'Rourke, P. Barlow, and A. Orr.** 1995. Point mutations in the herpes simplex virus type 1 Vmw110 RING finger helix affect activation of gene expression, viral growth, and interaction with PML-containing nuclear structures. J Virol **69:**7339-44.
84. **Everett, R. D.** 1988. Analysis of the functional domains of herpes simplex virus type 1 immediate-early polypeptide Vmw110. J Mol Biol **202:**87-96.
85. **Everett, R. D.** 1987. A detailed mutational analysis of Vmw110, a trans-acting transcriptional activator encoded by herpes simplex virus type 1. EMBO J **6:**2069-76.
86. **Everett, R. D.** 2000. ICP0 induces the accumulation of colocalizing conjugated ubiquitin. J Virol **74:**9994-10005.
87. **Everett, R. D.** 2000. ICP0, a regulator of herpes simplex virus during lytic and latent infection. Bioessays **22:**761-70.
88. **Everett, R. D.** 2006. Interactions between DNA viruses, ND10 and the DNA damage response. Cell Microbiol **8:**365-74.
89. **Everett, R. D., P. Barlow, A. Milner, B. Luisi, A. Orr, G. Hope, and D. Lyon.** 1993. A novel arrangement of zinc-binding residues and secondary structure in the C3HC4 motif of an alpha herpes virus protein family. J Mol Biol **234:**1038-47.
90. **Everett, R. D., C. Boutell, and A. Orr.** 2004. Phenotype of a herpes simplex virus type 1 mutant that fails to express immediate-early regulatory protein ICP0. J Virol **78:**1763-74.
91. **Everett, R. D., and M. K. Chelbi-Alix.** 2007. PML and PML nuclear bodies: implications in antiviral defence. Biochimie **89:**819-30.
92. **Everett, R. D., W. C. Earnshaw, J. Findlay, and P. Lomonte.** 1999. Specific destruction of kinetochore protein CENP-C and disruption of cell division by herpes simplex virus immediate-early protein Vmw110. EMBO J **18:**1526-38.
93. **Everett, R. D., W. C. Earnshaw, A. F. Pluta, T. Sternsdorf, A. M. Ainsztein, M. Carmena, S. Ruchaud, W. L. Hsu, and A. Orr.** 1999. A dynamic connection between centromeres and ND10 proteins. J Cell Sci **112 (Pt 20):**3443-54.
94. **Everett, R. D., P. Freemont, H. Saitoh, M. Dasso, A. Orr, M. Kathoria, and J. Parkinson.** 1998. The disruption of ND10 during herpes simplex virus infection correlates with the Vmw110- and proteasome-dependent loss of several PML isoforms. J Virol **72:**6581-91.
95. **Everett, R. D., and G. G. Maul.** 1994. HSV-1 IE protein Vmw110 causes redistribution of PML. EMBO J **13:**5062-9.
96. **Everett, R. D., and J. Murray.** 2005. ND10 components relocate to sites associated with herpes simplex virus type 1 nucleoprotein complexes during virus infection. J Virol **79:**5078-89.
97. **Everett, R. D., J. Murray, A. Orr, and C. M. Preston.** 2007. Herpes simplex virus type 1 genomes are associated with ND10 nuclear

substructures in quiescently infected human fibroblasts. J Virol **81:**10991-1004.

98. **Everett, R. D., and A. Orr.** 2009. Herpes simplex virus type 1 regulatory protein ICP0 aids infection in cells with a preinduced interferon response but does not impede interferon-induced gene induction. J Virol **83:**4978-83.
99. **Everett, R. D., A. Orr, and M. Elliott.** 1991. High level expression and purification of herpes simplex virus type 1 immediate early polypeptide Vmw110. Nucleic Acids Res **19:**6155-61.
100. **Everett, R. D., A. Orr, and C. M. Preston.** 1998. A viral activator of gene expression functions via the ubiquitin-proteasome pathway. EMBO J **17:**7161-9.
101. **Everett, R. D., C. Parada, P. Gripon, H. Sirma, and A. Orr.** 2008. Replication of ICP0-null mutant herpes simplex virus type 1 is restricted by both PML and Sp100. J Virol **82:**2661-72.
102. **Everett, R. D., M. L. Parsy, and A. Orr.** 2009. Analysis of the functions of herpes simplex virus type 1 regulatory protein ICP0 that are critical for lytic infection and derepression of quiescent viral genomes. J Virol **83:**4963-77.
103. **Everett, R. D., S. Rechter, P. Papior, N. Tavalai, T. Stamminger, and A. Orr.** 2006. PML contributes to a cellular mechanism of repression of herpes simplex virus type 1 infection that is inactivated by ICP0. J Virol **80:**7995-8005.
104. **Everett, R. D., G. Sourvinos, C. Leiper, J. B. Clements, and A. Orr.** 2004. Formation of nuclear foci of the herpes simplex virus type 1 regulatory protein ICP4 at early times of infection: localization, dynamics, recruitment of ICP27, and evidence for the de novo induction of ND10-like complexes. J Virol **78:**1903-17.
105. **Everett, R. D., D. F. Young, R. E. Randall, and A. Orr.** 2008. STAT-1- and IRF-3-dependent pathways are not essential for repression of ICP0-null mutant herpes simplex virus type 1 in human fibroblasts. J Virol **82:**8871-81.
106. **Everett, R. D., and A. Zafiropoulos.** 2004. Visualization by live-cell microscopy of disruption of ND10 during herpes simplex virus type 1 infection. J Virol **78:**11411-5.
107. **Farnsworth, A., K. Goldsmith, and D. C. Johnson.** 2003. Herpes simplex virus glycoproteins gD and gE/gI serve essential but redundant functions during acquisition of the virion envelope in the cytoplasm. J Virol **77:**8481-94.
108. **Farrell, M. J., A. T. Dobson, and L. T. Feldman.** 1991. Herpes simplex virus latency-associated transcript is a stable intron. Proc Natl Acad Sci U S A **88:**790-4.
109. **Ferenczy, M. W., and N. A. Deluca.** 2009. Epigenetic Modulation of Gene Expression from Quiescent Hsv Genomes. J Virol.

110. **Fields, B. N., D. M. Knipe, and P. M. Howley.** 2007. Fields' virology, 5th ed. Wolters Kluwer Health/Lippincott Williams & Wilkins, Philadelphia.
111. **Forrester, A., H. Farrell, G. Wilkinson, J. Kaye, N. Davis-Poynter, and T. Minson.** 1992. Construction and properties of a mutant of herpes simplex virus type 1 with glycoprotein H coding sequences deleted. J Virol **66:**341-8.
112. **Fraser, K. A., and S. A. Rice.** 2007. Herpes simplex virus immediate-early protein ICP22 triggers loss of serine 2-phosphorylated RNA polymerase II. J Virol **81:**5091-101.
113. **Fraser, K. A., and S. A. Rice.** 2005. Herpes simplex virus type 1 infection leads to loss of serine-2 phosphorylation on the carboxyl-terminal domain of RNA polymerase II. J Virol **79:**11323-34.
114. **Freemont, P. S., I. M. Hanson, and J. Trowsdale.** 1991. A novel cysteine-rich sequence motif. Cell **64:**483-4.
115. **Fruh, K., K. Ahn, H. Djaballah, P. Sempe, P. M. van Endert, R. Tampe, P. A. Peterson, and Y. Yang.** 1995. A viral inhibitor of peptide transporters for antigen presentation. Nature **375:**415-8.
116. **Gaffney, D. F., J. McLauchlan, J. L. Whitton, and J. B. Clements.** 1985. A modular system for the assay of transcription regulatory signals: the sequence TAATGARAT is required for herpes simplex virus immediate early gene activation. Nucleic Acids Res **13:**7847-63.
117. **Gao, M., and D. M. Knipe.** 1991. Potential role for herpes simplex virus ICP8 DNA replication protein in stimulation of late gene expression. J Virol **65:**2666-75.
118. **Garber, D. A., P. A. Schaffer, and D. M. Knipe.** 1997. A LAT-associated function reduces productive-cycle gene expression during acute infection of murine sensory neurons with herpes simplex virus type 1. J Virol **71:**5885-93.
119. **Garcia-Ramirez, M., C. Rocchini, and J. Ausio.** 1995. Modulation of chromatin folding by histone acetylation. J Biol Chem **270:**17923-8.
120. **Geraghty, R. J., C. Krummenacher, G. H. Cohen, R. J. Eisenberg, and P. G. Spear.** 1998. Entry of alphaherpesviruses mediated by poliovirus receptor-related protein 1 and poliovirus receptor. Science **280:**1618-20.
121. **Gerster, T., and R. G. Roeder.** 1988. A herpesvirus trans-activating protein interacts with transcription factor OTF-1 and other cellular proteins. Proc Natl Acad Sci U S A **85:**6347-51.
122. **Gibson, W., and B. Roizman.** 1971. Compartmentalization of spermine and spermidine in the herpes simplex virion. Proc Natl Acad Sci U S A **68:**2818-21.
123. **Giordani, N. V., D. M. Neumann, D. L. Kwiatkowski, P. S. Bhattacharjee, P. K. McAnany, J. M. Hill, and D. C. Bloom.** 2008. During herpes simplex virus type 1 infection of rabbits, the ability to express the latency-associated transcript increases latent-phase transcription of lytic genes. J Virol **82:**6056-60.

124. **Godowski, P. J., and D. M. Knipe.** 1986. Transcriptional control of herpesvirus gene expression: gene functions required for positive and negative regulation. Proc Natl Acad Sci U S A **83:**256-60.
125. **Goodrich, J. A., T. Hoey, C. J. Thut, A. Admon, and R. Tjian.** 1993. Drosophila TAFII40 interacts with both a VP16 activation domain and the basal transcription factor TFIIB. Cell **75:**519-30.
126. **Gressens, P., and J. R. Martin.** 1994. In situ polymerase chain reaction: localization of HSV-2 DNA sequences in infections of the nervous system. J Virol Methods **46:**61-83.
127. **Grondin, B., and N. DeLuca.** 2000. Herpes simplex virus type 1 ICP4 promotes transcription preinitiation complex formation by enhancing the binding of TFIID to DNA. J Virol **74:**11504-10.
128. **Grotzinger, T., K. Jensen, and H. Will.** 1996. The interferon (IFN)-stimulated gene Sp100 promoter contains an IFN-gamma activation site and an imperfect IFN-stimulated response element which mediate type I IFN inducibility. J Biol Chem **271:**25253-60.
129. **Grotzinger, T., T. Sternsdorf, K. Jensen, and H. Will.** 1996. Interferon-modulated expression of genes encoding the nuclear-dot-associated proteins Sp100 and promyelocytic leukemia protein (PML). Eur J Biochem **238:**554-60.
130. **Gruenheid, S., L. Gatzke, H. Meadows, and F. Tufaro.** 1993. Herpes simplex virus infection and propagation in a mouse L cell mutant lacking heparan sulfate proteoglycans. J Virol **67:**93-100.
131. **Grunewald, K., P. Desai, D. C. Winkler, J. B. Heymann, D. M. Belnap, W. Baumeister, and A. C. Steven.** 2003. Three-dimensional structure of herpes simplex virus from cryo-electron tomography. Science **302:**1396-8.
132. **Gu, B., and N. DeLuca.** 1994. Requirements for activation of the herpes simplex virus glycoprotein C promoter in vitro by the viral regulatory protein ICP4. J Virol **68:**7953-65.
133. **Gu, H., Y. Liang, G. Mandel, and B. Roizman.** 2005. Components of the REST/CoREST/histone deacetylase repressor complex are disrupted, modified, and translocated in HSV-1-infected cells. Proc Natl Acad Sci U S A **102:**7571-6.
134. **Gu, H., and B. Roizman.** 2009. Engagement of the lysine-specific demethylase/HDAC1/CoREST/REST complex by herpes simplex virus 1. J Virol **83:**4376-85.
135. **Gu, H., and B. Roizman.** 2007. Herpes simplex virus-infected cell protein 0 blocks the silencing of viral DNA by dissociating histone deacetylases from the CoREST-REST complex. Proc Natl Acad Sci U S A **104:**17134-9.
136. **Guo, J., D. J. Hui, W. C. Merrick, and G. C. Sen.** 2000. A new pathway of translational regulation mediated by eukaryotic initiation factor 3. EMBO J **19:**6891-9.

137. **Gutierrez, J. L., M. Chandy, M. J. Carrozza, and J. L. Workman.** 2007. Activation domains drive nucleosome eviction by SWI/SNF. EMBO J **26:**730-40.
138. **Halford, W. P., C. D. Kemp, J. A. Isler, D. J. Davido, and P. A. Schaffer.** 2001. ICP0, ICP4, or VP16 expressed from adenovirus vectors induces reactivation of latent herpes simplex virus type 1 in primary cultures of latently infected trigeminal ganglion cells. J Virol **75:**6143-53.
139. **Halford, W. P., C. Weisend, J. Grace, M. Soboleski, D. J. Carr, J. W. Balliet, Y. Imai, T. P. Margolis, and B. M. Gebhardt.** 2006. ICP0 antagonizes Stat 1-dependent repression of herpes simplex virus: implications for the regulation of viral latency. Virol J **3:**44.
140. **Hardwicke, M. A., and R. M. Sandri-Goldin.** 1994. The herpes simplex virus regulatory protein ICP27 contributes to the decrease in cellular mRNA levels during infection. J Virol **68:**4797-810.
141. **Hardy, W. R., and R. M. Sandri-Goldin.** 1994. Herpes simplex virus inhibits host cell splicing, and regulatory protein ICP27 is required for this effect. J Virol **68:**7790-9.
142. **Hargett, D., T. McLean, and S. L. Bachenheimer.** 2005. Herpes simplex virus ICP27 activation of stress kinases JNK and p38. J Virol **79:**8348-60.
143. **Harris, R. A., and C. M. Preston.** 1991. Establishment of latency in vitro by the herpes simplex virus type 1 mutant in1814. J Gen Virol **72 (Pt 4):**907-13.
144. **Hassan, A. H., P. Prochasson, K. E. Neely, S. C. Galasinski, M. Chandy, M. J. Carrozza, and J. L. Workman.** 2002. Function and selectivity of bromodomains in anchoring chromatin-modifying complexes to promoter nucleosomes. Cell **111:**369-79.
145. **Hayward, G. S., R. J. Jacob, S. C. Wadsworth, and B. Roizman.** 1975. Anatomy of herpes simplex virus DNA: evidence for four populations of molecules that differ in the relative orientations of their long and short components. Proc Natl Acad Sci U S A **72:**4243-7.
146. **He, B., M. Gross, and B. Roizman.** 1997. The gamma(1)34.5 protein of herpes simplex virus 1 complexes with protein phosphatase 1alpha to dephosphorylate the alpha subunit of the eukaryotic translation initiation factor 2 and preclude the shutoff of protein synthesis by double-stranded RNA-activated protein kinase. Proc Natl Acad Sci U S A **94:**843-8.
147. **Heldwein, E. E., and C. Krummenacher.** 2008. Entry of herpesviruses into mammalian cells. Cell Mol Life Sci **65:**1653-68.
148. **Herold, B. C., R. J. Visalli, N. Susmarski, C. R. Brandt, and P. G. Spear.** 1994. Glycoprotein C-independent binding of herpes simplex virus to cells requires cell surface heparan sulphate and glycoprotein B. J Gen Virol **75 (Pt 6):**1211-22.
149. **Herold, B. C., D. WuDunn, N. Soltys, and P. G. Spear.** 1991. Glycoprotein C of herpes simplex virus type 1 plays a principal role in the adsorption of virus to cells and in infectivity. J Virol **65:**1090-8.
150. **Herrera, F. J., and S. J. Triezenberg.** 2004. VP16-dependent association of chromatin-modifying coactivators and underrepresentation of histones

at immediate-early gene promoters during herpes simplex virus infection. J Virol **78:**9689-96.

151. **Hill, A., P. Jugovic, I. York, G. Russ, J. Bennink, J. Yewdell, H. Ploegh, and D. Johnson.** 1995. Herpes simplex virus turns off the TAP to evade host immunity. Nature **375:**411-5.
152. **Hill, A. B., B. C. Barnett, A. J. McMichael, and D. J. McGeoch.** 1994. HLA class I molecules are not transported to the cell surface in cells infected with herpes simplex virus types 1 and 2. J Immunol **152:**2736-41.
153. **Hobbs, W. E., 2nd, and N. A. DeLuca.** 1999. Perturbation of cell cycle progression and cellular gene expression as a function of herpes simplex virus ICP0. J Virol **73:**8245-55.
154. **Homa, F. L., and J. C. Brown.** 1997. Capsid assembly and DNA packaging in herpes simplex virus. Rev Med Virol **7:**107-122.
155. **Honess, R. W., and B. Roizman.** 1974. Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. J Virol **14:**8-19.
156. **Hsu, H., I. Solovyev, A. Colombero, R. Elliott, M. Kelley, and W. J. Boyle.** 1997. ATAR, a novel tumor necrosis factor receptor family member, signals through TRAF2 and TRAF5. J Biol Chem **272:**13471-4.
157. **Huang, A. S., and R. R. Wagner.** 1964. Penetration of Herpes Simplex Virus into Human Epidermoid Cells. Proc Soc Exp Biol Med **116:**863-9.
158. **Huang, J., J. R. Kent, B. Placek, K. A. Whelan, C. M. Hollow, P. Y. Zeng, N. W. Fraser, and S. L. Berger.** 2006. Trimethylation of histone H3 lysine 4 by Set1 in the lytic infection of human herpes simplex virus 1. J Virol **80:**5740-6.
159. **Hutchinson, L., H. Browne, V. Wargent, N. Davis-Poynter, S. Primorac, K. Goldsmith, A. C. Minson, and D. C. Johnson.** 1992. A novel herpes simplex virus glycoprotein, gL, forms a complex with glycoprotein H (gH) and affects normal folding and surface expression of gH. J Virol **66:**2240-50.
160. **Ikeda, K., T. Stuehler, and M. Meisterernst.** 2002. The H1 and H2 regions of the activation domain of herpes simplex virion protein 16 stimulate transcription through distinct molecular mechanisms. Genes Cells **7:**49-58.
161. **Ingles, C. J., M. Shales, W. D. Cress, S. J. Triezenberg, and J. Greenblatt.** 1991. Reduced binding of TFIID to transcriptionally compromised mutants of VP16. Nature **351:**588-90.
162. **Isaacs, A., and J. Lindenmann.** 1957. Virus interference. I. The interferon. Proc R Soc Lond B Biol Sci **147:**258-67.
163. **Ishov, A. M., and G. G. Maul.** 1996. The periphery of nuclear domain 10 (ND10) as site of DNA virus deposition. J Cell Biol **134:**815-26.
164. **Ishov, A. M., O. V. Vladimirova, and G. G. Maul.** 2004. Heterochromatin and ND10 are cell-cycle regulated and phosphorylation-dependent alternate nuclear sites of the transcription repressor Daxx and SWI/SNF protein ATRX. J Cell Sci **117:**3807-20.

165. **Jackson, S. A., and N. A. DeLuca.** 2003. Relationship of herpes simplex virus genome configuration to productive and persistent infections. Proc Natl Acad Sci U S A **100:**7871-6.
166. **Jahedi, S., N. S. Markovitz, F. Filatov, and B. Roizman.** 1999. Colocalization of the herpes simplex virus 1 UL4 protein with infected cell protein 22 in small, dense nuclear structures formed prior to onset of DNA synthesis. J Virol **73:**5132-8.
167. **Jamieson, D. R., L. H. Robinson, J. I. Daksis, M. J. Nicholl, and C. M. Preston.** 1995. Quiescent viral genomes in human fibroblasts after infection with herpes simplex virus type 1 Vmw65 mutants. J Gen Virol **76 (Pt 6):**1417-31.
168. **Johnson, D. C., and M. T. Huber.** 2002. Directed egress of animal viruses promotes cell-to-cell spread. J Virol **76:**1-8.
169. **Johnson, K. E., B. Song, and D. M. Knipe.** 2008. Role for herpes simplex virus 1 ICP27 in the inhibition of type I interferon signaling. Virology **374:**487-94.
170. **Johnson, L. A., L. Li, and R. M. Sandri-Goldin.** 2009. The cellular RNA export receptor TAP/NXF1 is required for ICP27- mediated export of herpes simplex virus 1 RNA, whereas, the TREX-complex adaptor protein Aly/REF appears to be dispensable. J Virol.
171. **Johnson, L. A., and R. M. Sandri-Goldin.** 2009. Efficient nuclear export of herpes simplex virus 1 transcripts requires both RNA binding by ICP27 and ICP27 interaction with TAP/NXF1. J Virol **83:**1184-92.
172. **Jones, C.** 2003. Herpes simplex virus type 1 and bovine herpesvirus 1 latency. Clin Microbiol Rev **16:**79-95.
173. **Jordan, R., and P. A. Schaffer.** 1997. Activation of gene expression by herpes simplex virus type 1 ICP0 occurs at the level of mRNA synthesis. J Virol **71:**6850-62.
174. **Jovasevic, V., L. Liang, and B. Roizman.** 2008. Proteolytic cleavage of VP1-2 is required for release of herpes simplex virus 1 DNA into the nucleus. J Virol **82:**3311-9.
175. **Kasten, M., H. Szerlong, H. Erdjument-Bromage, P. Tempst, M. Werner, and B. R. Cairns.** 2004. Tandem bromodomains in the chromatin remodeler RSC recognize acetylated histone H3 Lys14. EMBO J **23:**1348-59.
176. **Katan, M., A. Haigh, C. P. Verrijzer, P. C. van der Vliet, and P. O'Hare.** 1990. Characterization of a cellular factor which interacts functionally with Oct-1 in the assembly of a multicomponent transcription complex. Nucleic Acids Res **18:**6871-80.
177. **Kawaguchi, Y., R. Bruni, and B. Roizman.** 1997. Interaction of herpes simplex virus 1 alpha regulatory protein ICP0 with elongation factor 1delta: ICP0 affects translational machinery. J Virol **71:**1019-24.
178. **Kawaguchi, Y., M. Tanaka, A. Yokoymama, G. Matsuda, K. Kato, H. Kagawa, K. Hirai, and B. Roizman.** 2001. Herpes simplex virus 1 alpha regulatory protein ICP0 functionally interacts with cellular transcription factor BMAL1. Proc Natl Acad Sci U S A **98:**1877-82.

179. **Kent, J. R., P. Y. Zeng, D. Atanasiu, J. Gardner, N. W. Fraser, and S. L. Berger.** 2004. During lytic infection herpes simplex virus type 1 is associated with histones bearing modifications that correlate with active transcription. J Virol **78:**10178-86.
180. **Kerr, I. M., and R. E. Brown.** 1978. pppA2'p5'A2'p5'A: an inhibitor of protein synthesis synthesized with an enzyme fraction from interferon-treated cells. Proc Natl Acad Sci U S A **75:**256-60.
181. **Kieff, E. D., S. L. Bachenheimer, and B. Roizman.** 1971. Size, composition, and structure of the deoxyribonucleic acid of herpes simplex virus subtypes 1 and 2. J Virol **8:**125-32.
182. **Kim, J. C., S. Y. Lee, S. Y. Kim, J. K. Kim, H. J. Kim, H. M. Lee, M. S. Choi, J. S. Min, M. J. Kim, H. S. Choi, and J. K. Ahn.** 2008. HSV-1 ICP27 suppresses NF-kappaB activity by stabilizing IkappaBalpha. FEBS Lett **582:**2371-6.
183. **Klemm, R. D., J. A. Goodrich, S. Zhou, and R. Tjian.** 1995. Molecular cloning and expression of the 32-kDa subunit of human TFIID reveals interactions with VP16 and TFIIB that mediate transcriptional activation. Proc Natl Acad Sci U S A **92:**5788-92.
184. **Knez, J., P. T. Bilan, and J. P. Capone.** 2003. A single amino acid substitution in herpes simplex virus type 1 VP16 inhibits binding to the virion host shutoff protein and is incompatible with virus growth. J Virol **77:**2892-902.
185. **Knipe, D. M., D. Senechek, S. A. Rice, and J. L. Smith.** 1987. Stages in the nuclear association of the herpes simplex virus transcriptional activator protein ICP4. J Virol **61:**276-84.
186. **Kobayashi, N., T. G. Boyer, and A. J. Berk.** 1995. A class of activation domains interacts directly with TFIIA and stimulates TFIIA-TFIID-promoter complex assembly. Mol Cell Biol **15:**6465-73.
187. **Kochs, G., and O. Haller.** 1999. Interferon-induced human MxA GTPase blocks nuclear import of Thogoto virus nucleocapsids. Proc Natl Acad Sci U S A **96:**2082-6.
188. **Koffa, M. D., J. B. Clements, E. Izaurralde, S. Wadd, S. A. Wilson, I. W. Mattaj, and S. Kuersten.** 2001. Herpes simplex virus ICP27 protein provides viral mRNAs with access to the cellular mRNA export pathway. EMBO J **20:**5769-78.
189. **Kouzarides, T.** 2007. Chromatin modifications and their function. Cell **128:**693-705.
190. **Kraus, W. L., E. T. Manning, and J. T. Kadonaga.** 1999. Biochemical analysis of distinct activation functions in p300 that enhance transcription initiation with chromatin templates. Mol Cell Biol **19:**8123-35.
191. **Kristie, T. M., and B. Roizman.** 1986. DNA-binding site of major regulatory protein alpha 4 specifically associated with promoter-regulatory domains of alpha genes of herpes simplex virus type 1. Proc Natl Acad Sci U S A **83:**4700-4.

192. **Kristie, T. M., and B. Roizman.** 1987. Host cell proteins bind to the cis-acting site required for virion-mediated induction of herpes simplex virus 1 alpha genes. Proc Natl Acad Sci U S A **84:**71-5.
193. **Kristie, T. M., and B. Roizman.** 1984. Separation of sequences defining basal expression from those conferring alpha gene recognition within the regulatory domains of herpes simplex virus 1 alpha genes. Proc Natl Acad Sci U S A **81:**4065-9.
194. **Kristie, T. M., and P. A. Sharp.** 1990. Interactions of the Oct-1 POU subdomains with specific DNA sequences and with the HSV alpha-trans-activator protein. Genes Dev **4:**2383-96.
195. **Kristie, T. M., J. L. Vogel, and A. E. Sears.** 1999. Nuclear localization of the C1 factor (host cell factor) in sensory neurons correlates with reactivation of herpes simplex virus from latency. Proc Natl Acad Sci U S A **96:**1229-33.
196. **Kubat, N. J., A. L. Amelio, N. V. Giordani, and D. C. Bloom.** 2004. The herpes simplex virus type 1 latency-associated transcript (LAT) enhancer/rcr is hyperacetylated during latency independently of LAT transcription. J Virol **78:**12508-18.
197. **Kubat, N. J., R. K. Tran, P. McAnany, and D. C. Bloom.** 2004. Specific histone tail modification and not DNA methylation is a determinant of herpes simplex virus type 1 latent gene expression. J Virol **78:**1139-49.
198. **Kuddus, R., B. Gu, and N. A. DeLuca.** 1995. Relationship between TATA-binding protein and herpes simplex virus type 1 ICP4 DNA-binding sites in complex formation and repression of transcription. J Virol **69:**5568-75.
199. **Kundu, T. K., V. B. Palhan, Z. Wang, W. An, P. A. Cole, and R. G. Roeder.** 2000. Activator-dependent transcription from chromatin in vitro involving targeted histone acetylation by p300. Mol Cell **6:**551-61.
200. **Kurosu, T., and B. M. Peterlin.** 2004. VP16 and ubiquitin; binding of P-TEFb via its activation domain and ubiquitin facilitates elongation of transcription of target genes. Curr Biol **14:**1112-6.
201. **Kutluay, S. B., S. L. DeVos, J. E. Klomp, and S. J. Triezenberg.** 2009. Transcriptional coactivators are not required for herpes simplex virus type 1 immediate-early gene expression in vitro. J Virol **83:**3436-49.
202. **Kutluay, S. B., and S. J. Triezenberg.** 2009. Regulation of histone deposition on the herpes simplex virus type 1 genome during lytic infection. J Virol **83:**5835-45.
203. **Kutluay, S. B., and S. J. Triezenberg.** 2009. Role of chromatin during herpesvirus infections. Biochim Biophys Acta.
204. **Kwiatkowski, D. L., H. W. Thompson, and D. C. Bloom.** 2009. Polycomb group protein Bmi1 binds to the HSV-1 latent genome and maintains repressive histone marks during latency. J Virol.
205. **Kwon, B. S., K. B. Tan, J. Ni, K. O. Oh, Z. H. Lee, K. K. Kim, Y. J. Kim, S. Wang, R. Gentz, G. L. Yu, J. Harrop, S. D. Lyn, C. Silverman, T. G. Porter, A. Truneh, and P. R. Young.** 1997. A newly

identified member of the tumor necrosis factor receptor superfamily with a wide tissue distribution and involvement in lymphocyte activation. J Biol Chem **272:**14272-6.

206. **Kwong, A. D., and N. Frenkel.** 1987. Herpes simplex virus-infected cells contain a function(s) that destabilizes both host and viral mRNAs. Proc Natl Acad Sci U S A **84:**1926-30.
207. **La Boissiere, S., T. Hughes, and P. O'Hare.** 1999. HCF-dependent nuclear import of VP16. EMBO J **18:**480-9.
208. **Lachmann, R. H., M. Sadarangani, H. R. Atkinson, and S. Efstathiou.** 1999. An analysis of herpes simplex virus gene expression during latency establishment and reactivation. J Gen Virol **80 (Pt 5):**1271-82.
209. **Lam, Q., C. A. Smibert, K. E. Koop, C. Lavery, J. P. Capone, S. P. Weinheimer, and J. R. Smiley.** 1996. Herpes simplex virus VP16 rescues viral mRNA from destruction by the virion host shutoff function. EMBO J **15:**2575-81.
210. **Latchman, D. S.** 1999. POU family transcription factors in the nervous system. J Cell Physiol **179:**126-33.
211. **LaVail, J. H., A. N. Tauscher, J. W. Hicks, O. Harrabi, G. T. Melroe, and D. M. Knipe.** 2005. Genetic and molecular in vivo analysis of herpes simplex virus assembly in murine visual system neurons. J Virol **79:**11142-50.
212. **Lees-Miller, S. P., M. C. Long, M. A. Kilvert, V. Lam, S. A. Rice, and C. A. Spencer.** 1996. Attenuation of DNA-dependent protein kinase activity and its catalytic subunit by the herpes simplex virus type 1 transactivator ICP0. J Virol **70:**7471-7.
213. **Leib, D. A., D. M. Coen, C. L. Bogard, K. A. Hicks, D. R. Yager, D. M. Knipe, K. L. Tyler, and P. A. Schaffer.** 1989. Immediate-early regulatory gene mutants define different stages in the establishment and reactivation of herpes simplex virus latency. J Virol **63:**759-68.
214. **Leib, D. A., T. E. Harrison, K. M. Laslo, M. A. Machalek, N. J. Moorman, and H. W. Virgin.** 1999. Interferons regulate the phenotype of wild-type and mutant herpes simplex viruses in vivo. J Exp Med **189:**663-72.
215. **Leinbach, S. S., and W. C. Summers.** 1980. The structure of herpes simplex virus type 1 DNA as probed by micrococcal nuclease digestion. J Gen Virol **51:**45-59.
216. **Leopardi, R., P. L. Ward, W. O. Ogle, and B. Roizman.** 1997. Association of herpes simplex virus regulatory protein ICP22 with transcriptional complexes containing EAP, ICP4, RNA polymerase II, and viral DNA requires posttranslational modification by the U(L)13 proteinkinase. J Virol **71:**1133-9.
217. **Li, J., D. Moazed, and S. P. Gygi.** 2002. Association of the histone methyltransferase Set2 with RNA polymerase II plays a role in transcription elongation. J Biol Chem **277:**49383-8.

218. **Li, L., L. A. Johnson, J. Q. Dai-Ju, and R. M. Sandri-Goldin.** 2008. Hsc70 focus formation at the periphery of HSV-1 transcription sites requires ICP27. PLoS ONE **3:**e1491.
219. **Lilley, C. E., C. T. Carson, A. R. Muotri, F. H. Gage, and M. D. Weitzman.** 2005. DNA repair proteins affect the lifecycle of herpes simplex virus 1. Proc Natl Acad Sci U S A **102:**5844-9.
220. **Lillycrop, K. A., M. K. Howard, J. K. Estridge, and D. S. Latchman.** 1994. Inhibition of herpes simplex virus infection by ectopic expression of neuronal splice variants of the Oct-2 transcription factor. Nucleic Acids Res **22:**815-20.
221. **Lin, R., R. S. Noyce, S. E. Collins, R. D. Everett, and K. L. Mossman.** 2004. The herpes simplex virus ICP0 RING finger domain inhibits IRF3- and IRF7-mediated activation of interferon-stimulated genes. J Virol **78:**1675-84.
222. **Lin, Y. S., I. Ha, E. Maldonado, D. Reinberg, and M. R. Green.** 1991. Binding of general transcription factor TFIIB to an acidic activating region. Nature **353:**569-71.
223. **Lindberg, A., and J. P. Kreivi.** 2002. Splicing inhibition at the level of spliceosome assembly in the presence of herpes simplex virus protein ICP27. Virology **294:**189-98.
224. **Liu, F., and B. Roizman.** 1992. Differentiation of multiple domains in the herpes simplex virus 1 protease encoded by the UL26 gene. Proc Natl Acad Sci U S A **89:**2076-80.
225. **Liu, Y., W. Gong, C. C. Huang, W. Herr, and X. Cheng.** 1999. Crystal structure of the conserved core of the herpes simplex virus transcriptional regulatory protein VP16. Genes Dev **13:**1692-703.
226. **Lomonte, P., and R. D. Everett.** 1999. Herpes simplex virus type 1 immediate-early protein Vmw110 inhibits progression of cells through mitosis and from G(1) into S phase of the cell cycle. J Virol **73:**9456-67.
227. **Lomonte, P., and E. Morency.** 2007. Centromeric protein CENP-B proteasomal degradation induced by the viral protein ICP0. FEBS Lett **581:**658-62.
228. **Lomonte, P., K. F. Sullivan, and R. D. Everett.** 2001. Degradation of nucleosome-associated centromeric histone H3-like protein CENP-A induced by herpes simplex virus type 1 protein ICP0. J Biol Chem **276:**5829-35.
229. **Lomonte, P., J. Thomas, P. Texier, C. Caron, S. Khochbin, and A. L. Epstein.** 2004. Functional interaction between class II histone deacetylases and ICP0 of herpes simplex virus type 1. J Virol **78:**6744-57.
230. **Long, M. C., V. Leong, P. A. Schaffer, C. A. Spencer, and S. A. Rice.** 1999. ICP22 and the UL13 protein kinase are both required for herpes simplex virus-induced modification of the large subunit of RNA polymerase II. J Virol **73:**5593-604.
231. **Longnecker, R., and B. Roizman.** 1986. Generation of an inverting herpes simplex virus 1 mutant lacking the L-S junction a sequences, an

origin of DNA synthesis, and several genes including those specifying glycoprotein E and the alpha 47 gene. J Virol **58:**583-91.
232. **Lopez, C.** 1975. Genetics of natural resistance to herpesvirus infections in mice. Nature **258:**152-3.
233. **Lopez, P., C. Van Sant, and B. Roizman.** 2001. Requirements for the nuclear-cytoplasmic translocation of infected-cell protein 0 of herpes simplex virus 1. J Virol **75:**3832-40.
234. **Loppin, B., E. Bonnefoy, C. Anselme, A. Laurencon, T. L. Karr, and P. Couble.** 2005. The histone H3.3 chaperone HIRA is essential for chromatin assembly in the male pronucleus. Nature **437:**1386-90.
235. **Luciani, J. J., D. Depetris, Y. Usson, C. Metzler-Guillemain, C. Mignon-Ravix, M. J. Mitchell, A. Megarbane, P. Sarda, H. Sirma, A. Moncla, J. Feunteun, and M. G. Mattei.** 2006. PML nuclear bodies are highly organised DNA-protein structures with a function in heterochromatin remodelling at the G2 phase. J Cell Sci **119:**2518-31.
236. **Luo, M. J., and R. Reed.** 1999. Splicing is required for rapid and efficient mRNA export in metazoans. Proc Natl Acad Sci U S A **96:**14937-42.
237. **Luster, A. D., and J. V. Ravetch.** 1987. Biochemical characterization of a gamma interferon-inducible cytokine (IP-10). J Exp Med **166:**1084-97.
238. **Mador, N., D. Goldenberg, O. Cohen, A. Panet, and I. Steiner.** 1998. Herpes simplex virus type 1 latency-associated transcripts suppress viral replication and reduce immediate-early gene mRNA levels in a neuronal cell line. J Virol **72:**5067-75.
239. **Mador, N., A. Panet, D. Latchman, and I. Steiner.** 1995. Expression and splicing of the latency-associated transcripts of herpes simplex virus type 1 in neuronal and non-neuronal cell lines. J Biochem **117:**1288-97.
240. **Malathi, K., B. Dong, M. Gale, Jr., and R. H. Silverman.** 2007. Small self-RNA generated by RNase L amplifies antiviral innate immunity. Nature **448:**816-9.
241. **Margolis, T. P., F. Sedarati, A. T. Dobson, L. T. Feldman, and J. G. Stevens.** 1992. Pathways of viral gene expression during acute neuronal infection with HSV-1. Virology **189:**150-60.
242. **Marsters, S. A., T. M. Ayres, M. Skubatch, C. L. Gray, M. Rothe, and A. Ashkenazi.** 1997. Herpesvirus entry mediator, a member of the tumor necrosis factor receptor (TNFR) family, interacts with members of the TNFR-associated factor family and activates the transcription factors NF-kappaB and AP-1. J Biol Chem **272:**14029-32.
243. **Martin, T. E., S. C. Barghusen, G. P. Leser, and P. G. Spear.** 1987. Redistribution of nuclear ribonucleoprotein antigens during herpes simplex virus infection. J Cell Biol **105:**2069-82.
244. **Maul, G. G., and R. D. Everett.** 1994. The nuclear location of PML, a cellular member of the C3HC4 zinc-binding domain protein family, is rearranged during herpes simplex virus infection by the C3HC4 viral protein ICP0. J Gen Virol **75 (Pt 6):**1223-33.

245. **Maul, G. G., H. H. Guldner, and J. G. Spivack.** 1993. Modification of discrete nuclear domains induced by herpes simplex virus type 1 immediate early gene 1 product (ICP0). J Gen Virol **74 (Pt 12):**2679-90.
246. **Maul, G. G., A. M. Ishov, and R. D. Everett.** 1996. Nuclear domain 10 as preexisting potential replication start sites of herpes simplex virus type-1. Virology **217:**67-75.
247. **McCarthy, A. M., L. McMahan, and P. A. Schaffer.** 1989. Herpes simplex virus type 1 ICP27 deletion mutants exhibit altered patterns of transcription and are DNA deficient. J Virol **63:**18-27.
248. **McFarlane, M., J. I. Daksis, and C. M. Preston.** 1992. Hexamethylene bisacetamide stimulates herpes simplex virus immediate early gene expression in the absence of trans-induction by Vmw65. J Gen Virol **73 (Pt 2):**285-92.
249. **McGregor, F., A. Phelan, J. Dunlop, and J. B. Clements.** 1996. Regulation of herpes simplex virus poly (A) site usage and the action of immediate-early protein IE63 in the early-late switch. J Virol **70:**1931-40.
250. **McLauchlan, J., A. Phelan, C. Loney, R. M. Sandri-Goldin, and J. B. Clements.** 1992. Herpes simplex virus IE63 acts at the posttranscriptional level to stimulate viral mRNA 3' processing. J Virol **66:**6939-45.
251. **McLauchlan, J., S. Simpson, and J. B. Clements.** 1989. Herpes simplex virus induces a processing factor that stimulates poly(A) site usage. Cell **59:**1093-105.
252. **McMahan, L., and P. A. Schaffer.** 1990. The repressing and enhancing functions of the herpes simplex virus regulatory protein ICP27 map to C-terminal regions and are required to modulate viral gene expression very early in infection. J Virol **64:**3471-85.
253. **McNamee, E. E., T. J. Taylor, and D. M. Knipe.** 2000. A dominant-negative herpesvirus protein inhibits intranuclear targeting of viral proteins: effects on DNA replication and late gene expression. J Virol **74:**10122-31.
254. **Mears, W. E., V. Lam, and S. A. Rice.** 1995. Identification of nuclear and nucleolar localization signals in the herpes simplex virus regulatory protein ICP27. J Virol **69:**935-47.
255. **Mears, W. E., and S. A. Rice.** 1998. The herpes simplex virus immediate-early protein ICP27 shuttles between nucleus and cytoplasm. Virology **242:**128-37.
256. **Mears, W. E., and S. A. Rice.** 1996. The RGG box motif of the herpes simplex virus ICP27 protein mediates an RNA-binding activity and determines in vivo methylation. J Virol **70:**7445-53.
257. **Mehta, A., J. Maggioncalda, O. Bagasra, S. Thikkavarapu, P. Saikumari, T. Valyi-Nagy, N. W. Fraser, and T. M. Block.** 1995. In situ DNA PCR and RNA hybridization detection of herpes simplex virus sequences in trigeminal ganglia of latently infected mice. Virology **206:**633-40.

258. **Mellerick, D. M., and N. W. Fraser.** 1987. Physical state of the latent herpes simplex virus genome in a mouse model system: evidence suggesting an episomal state. Virology **158:**265-75.
259. **Memedula, S., and A. S. Belmont.** 2003. Sequential recruitment of HAT and SWI/SNF components to condensed chromatin by VP16. Curr Biol **13:**241-6.
260. **Mettenleiter, T. C.** 2002. Herpesvirus assembly and egress. J Virol **76:**1537-47.
261. **Michael, N., and B. Roizman.** 1989. Binding of the herpes simplex virus major regulatory protein to viral DNA. Proc Natl Acad Sci U S A **86:**9808-12.
262. **Michael, N., D. Spector, P. Mavromara-Nazos, T. M. Kristie, and B. Roizman.** 1988. The DNA-binding properties of the major regulatory protein alpha 4 of herpes simplex viruses. Science **239:**1531-4.
263. **Minaker, R. L., K. L. Mossman, and J. R. Smiley.** 2005. Functional inaccessibility of quiescent herpes simplex virus genomes. Virol J **2:**85.
264. **Mitchell, C., J. A. Blaho, and B. Roizman.** 1994. Casein kinase II specifically nucleotidylylates in vitro the amino acid sequence of the protein encoded by the alpha 22 gene of herpes simplex virus 1. Proc Natl Acad Sci U S A **91:**11864-8.
265. **Mittler, G., T. Stuhler, L. Santolin, T. Uhlmann, E. Kremmer, F. Lottspeich, L. Berti, and M. Meisterernst.** 2003. A novel docking site on Mediator is critical for activation by VP16 in mammalian cells. EMBO J **22:**6494-504.
266. **Monier, K., J. C. Armas, S. Etteldorf, P. Ghazal, and K. F. Sullivan.** 2000. Annexation of the interchromosomal space during viral infection. Nat Cell Biol **2:**661-5.
267. **Montgomery, R. I., M. S. Warner, B. J. Lum, and P. G. Spear.** 1996. Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. Cell **87:**427-36.
268. **Mossman, K. L., P. F. Macgregor, J. J. Rozmus, A. B. Goryachev, A. M. Edwards, and J. R. Smiley.** 2001. Herpes simplex virus triggers and then disarms a host antiviral response. J Virol **75:**750-8.
269. **Mossman, K. L., H. A. Saffran, and J. R. Smiley.** 2000. Herpes simplex virus ICP0 mutants are hypersensitive to interferon. J Virol **74:**2052-6.
270. **Mossman, K. L., and J. R. Smiley.** 1999. Truncation of the C-terminal acidic transcriptional activation domain of herpes simplex virus VP16 renders expression of the immediate-early genes almost entirely dependent on ICP0. J Virol **73:**9726-33.
271. **Muggeridge, M. I., and N. W. Fraser.** 1986. Chromosomal organization of the herpes simplex virus genome during acute infection of the mouse central nervous system. J Virol **59:**764-7.
272. **Muller, M. T.** 1987. Binding of the herpes simplex virus immediate-early gene product ICP4 to its own transcription start site. J Virol **61:**858-65.

273. **Nalwanga, D., S. Rempel, B. Roizman, and J. D. Baines.** 1996. The UL 16 gene product of herpes simplex virus 1 is a virion protein that colocalizes with intranuclear capsid proteins. Virology **226:**236-42.
274. **Neely, K. E., A. H. Hassan, A. E. Wallberg, D. J. Steger, B. R. Cairns, A. P. Wright, and J. L. Workman.** 1999. Activation domain-mediated targeting of the SWI/SNF complex to promoters stimulates transcription from nucleosome arrays. Mol Cell **4:**649-55.
275. **Neumann, D. M., P. S. Bhattacharjee, N. V. Giordani, D. C. Bloom, and J. M. Hill.** 2007. In vivo changes in the patterns of chromatin structure associated with the latent herpes simplex virus type 1 genome in mouse trigeminal ganglia can be detected at early times after butyrate treatment. J Virol **81:**13248-53.
276. **Newcomb, W. W., F. L. Homa, D. R. Thomsen, Z. Ye, and J. C. Brown.** 1994. Cell-free assembly of the herpes simplex virus capsid. J Virol **68:**6059-63.
277. **Newcomb, W. W., B. L. Trus, F. P. Booy, A. C. Steven, J. S. Wall, and J. C. Brown.** 1993. Structure of the herpes simplex virus capsid. Molecular composition of the pentons and the triplexes. J Mol Biol **232:**499-511.
278. **Nichol, J. N., L. A. Petruccelli, and W. H. Miller, Jr.** 2009. Expanding PML's functional repertoire through post-translational mechanisms. Front Biosci **14:**2293-306.
279. **Nicholl, M. J., L. H. Robinson, and C. M. Preston.** 2000. Activation of cellular interferon-responsive genes after infection of human cells with herpes simplex virus type 1. J Gen Virol **81:**2215-8.
280. **Nicola, A. V., A. M. McEvoy, and S. E. Straus.** 2003. Roles for endocytosis and low pH in herpes simplex virus entry into HeLa and Chinese hamster ovary cells. J Virol **77:**5324-32.
281. **O'Hare, P., C. R. Goding, and A. Haigh.** 1988. Direct combinatorial interaction between a herpes simplex virus regulatory protein and a cellular octamer-binding factor mediates specific induction of virus immediate-early gene expression. EMBO J **7:**4231-8.
282. **Ogle, W. O., T. I. Ng, K. L. Carter, and B. Roizman.** 1997. The UL13 protein kinase and the infected cell type are determinants of posttranslational modification of ICP0. Virology **235:**406-13.
283. **Oh, J., and N. W. Fraser.** 2008. Temporal association of the herpes simplex virus genome with histone proteins during a lytic infection. J Virol **82:**3530-7.
284. **Ohara, P. T., A. N. Tauscher, and J. H. LaVail.** 2001. Two paths for dissemination of Herpes simplex virus from infected trigeminal ganglion to the murine cornea. Brain Res **899:**260-3.
285. **Ojala, P. M., B. Sodeik, M. W. Ebersold, U. Kutay, and A. Helenius.** 2000. Herpes simplex virus type 1 entry into host cells: reconstitution of capsid binding and uncoating at the nuclear pore complex in vitro. Mol Cell Biol **20:**4922-31.

286. **Oroskar, A. A., and G. S. Read.** 1987. A mutant of herpes simplex virus type 1 exhibits increased stability of immediate-early (alpha) mRNAs. J Virol **61:**604-6.

287. **Parkinson, J., and R. D. Everett.** 2001. Alphaherpesvirus proteins related to herpes simplex virus type 1 ICP0 induce the formation of colocalizing, conjugated ubiquitin. J Virol **75:**5357-62.

288. **Pasieka, T. J., B. Lu, S. D. Crosby, K. M. Wylie, L. A. Morrison, D. E. Alexander, V. D. Menachery, and D. A. Leib.** 2008. Herpes simplex virus virion host shutoff attenuates establishment of the antiviral state. J Virol **82:**5527-35.

289. **Pasieka, T. J., B. Lu, and D. A. Leib.** 2008. Enhanced pathogenesis of an attenuated herpes simplex virus for mice lacking Stat1. J Virol **82:**6052-5.

290. **Perng, G. C., C. Jones, J. Ciacci-Zanella, M. Stone, G. Henderson, A. Yukht, S. M. Slanina, F. M. Hofman, H. Ghiasi, A. B. Nesburn, and S. L. Wechsler.** 2000. Virus-induced neuronal apoptosis blocked by the herpes simplex virus latency-associated transcript. Science **287:**1500-3.

291. **Peters, G. A., D. Khoo, I. Mohr, and G. C. Sen.** 2002. Inhibition of PACT-mediated activation of PKR by the herpes simplex virus type 1 Us11 protein. J Virol **76:**11054-64.

292. **Phelan, A., and J. B. Clements.** 1997. Herpes simplex virus type 1 immediate early protein IE63 shuttles between nuclear compartments and the cytoplasm. J Gen Virol **78 (Pt 12):**3327-31.

293. **Pichlmair, A., and C. Reis e Sousa.** 2007. Innate recognition of viruses. Immunity **27:**370-83.

294. **Pignatti, P. F., and E. Cassai.** 1980. Analysis of herpes simplex virus nucleoprotein complexes extracted from infected cells. J Virol **36:**816-28.

295. **Placek, B. J., J. Huang, J. R. Kent, J. Dorsey, L. Rice, N. W. Fraser, and S. L. Berger.** 2009. The histone variant H3.3 regulates gene expression during lytic infection with herpes simplex virus type 1. J Virol **83:**1416-21.

296. **Plummer, G., C. R. Goodheart, D. Henson, and C. P. Bowling.** 1969. A comparative study of the DNA density and behavior in tissue cultures of fourteen different herpesviruses. Virology **39:**134-7.

297. **Poon, A. P., H. Gu, and B. Roizman.** 2006. ICP0 and the US3 protein kinase of herpes simplex virus 1 independently block histone deacetylation to enable gene expression. Proc Natl Acad Sci U S A **103:**9993-8.

298. **Poon, A. P., Y. Liang, and B. Roizman.** 2003. Herpes simplex virus 1 gene expression is accelerated by inhibitors of histone deacetylases in rabbit skin cells infected with a mutant carrying a cDNA copy of the infected-cell protein no. 0. J Virol **77:**12671-8.

299. **Poppers, J., M. Mulvey, D. Khoo, and I. Mohr.** 2000. Inhibition of PKR activation by the proline-rich RNA binding domain of the herpes simplex virus type 1 Us11 protein. J Virol **74:**11215-21.

300. **Post, L. E., and B. Roizman.** 1981. A generalized technique for deletion of specific genes in large genomes: alpha gene 22 of herpes simplex virus 1 is not essential for growth. Cell **25:**227-32.

301. **Preston, C. M.** 2007. Reactivation of expression from quiescent herpes simplex virus type 1 genomes in the absence of immediate-early protein ICP0. J Virol **81:**11781-9.
302. **Preston, C. M.** 2000. Repression of viral transcription during herpes simplex virus latency. J Gen Virol **81:**1-19.
303. **Preston, C. M., R. Mabbs, and M. J. Nicholl.** 1997. Construction and characterization of herpes simplex virus type 1 mutants with conditional defects in immediate early gene expression. Virology **229:**228-39.
304. **Preston, C. M., and M. McFarlane.** 1998. Cytodifferentiating agents affect the replication of herpes simplex virus type 1 in the absence of functional VP16. Virology **249:**418-26.
305. **Preston, C. M., and M. J. Nicholl.** 2008. Induction of cellular stress overcomes the requirement of herpes simplex virus type 1 for immediate-early protein ICP0 and reactivates expression from quiescent viral genomes. J Virol **82:**11775-83.
306. **Preston, C. M., and M. J. Nicholl.** 1997. Repression of gene expression upon infection of cells with herpes simplex virus type 1 mutants impaired for immediate-early protein synthesis. J Virol **71:**7807-13.
307. **Preston, V. G., M. F. al-Kobaisi, I. M. McDougall, and F. J. Rixon.** 1994. The herpes simplex virus gene UL26 proteinase in the presence of the UL26.5 gene product promotes the formation of scaffold-like structures. J Gen Virol **75 (Pt 9):**2355-66.
308. **Prod'hon, C., I. Machuca, H. Berthomme, A. Epstein, and B. Jacquemont.** 1996. Characterization of regulatory functions of the HSV-1 immediate-early protein ICP22. Virology **226:**393-402.
309. **Purifoy, D. J., R. B. Lewis, and K. L. Powell.** 1977. Identification of the herpes simplex virus DNA polymerase gene. Nature **269:**621-3.
310. **Purves, F. C., W. O. Ogle, and B. Roizman.** 1993. Processing of the herpes simplex virus regulatory protein alpha 22 mediated by the UL13 protein kinase determines the accumulation of a subset of alpha and gamma mRNAs and proteins in infected cells. Proc Natl Acad Sci U S A **90:**6701-5.
311. **Purves, F. C., and B. Roizman.** 1992. The UL13 gene of herpes simplex virus 1 encodes the functions for posttranslational processing associated with phosphorylation of the regulatory protein alpha 22. Proc Natl Acad Sci U S A **89:**7310-4.
312. **Ramakrishnan, R., M. Levine, and D. J. Fink.** 1994. PCR-based analysis of herpes simplex virus type 1 latency in the rat trigeminal ganglion established with a ribonucleotide reductase-deficient mutant. J Virol **68:**7083-91.
313. **Randall, R. E., and N. Dinwoodie.** 1986. Intranuclear localization of herpes simplex virus immediate-early and delayed-early proteins: evidence that ICP 4 is associated with progeny virus DNA. J Gen Virol **67 (Pt 10):**2163-77.

314. **Regad, T., and M. K. Chelbi-Alix.** 2001. Role and fate of PML nuclear bodies in response to interferon and viral infections. Oncogene **20:**7274-86.
315. **Rice, S. A., and D. M. Knipe.** 1990. Genetic evidence for two distinct transactivation functions of the herpes simplex virus alpha protein ICP27. J Virol **64:**1704-15.
316. **Rice, S. A., M. C. Long, V. Lam, and C. A. Spencer.** 1994. RNA polymerase II is aberrantly phosphorylated and localized to viral replication compartments following herpes simplex virus infection. J Virol **68:**988-1001.
317. **Rock, D. L., and N. W. Fraser.** 1983. Detection of HSV-1 genome in central nervous system of latently infected mice. Nature **302:**523-5.
318. **Rock, D. L., and N. W. Fraser.** 1985. Latent herpes simplex virus type 1 DNA contains two copies of the virion DNA joint region. J Virol **55:**849-52.
319. **Russell, J., and C. M. Preston.** 1986. An in vitro latency system for herpes simplex virus type 2. J Gen Virol **67 (Pt 2):**397-403.
320. **Sacks, W. R., C. C. Greene, D. P. Aschman, and P. A. Schaffer.** 1985. Herpes simplex virus type 1 ICP27 is an essential regulatory protein. J Virol **55:**796-805.
321. **Sadler, A. J., and B. R. Williams.** 2008. Interferon-inducible antiviral effectors. Nat Rev Immunol **8:**559-68.
322. **Sakisaka, T., T. Taniguchi, H. Nakanishi, K. Takahashi, M. Miyahara, W. Ikeda, S. Yokoyama, Y. F. Peng, K. Yamanishi, and Y. Takai.** 2001. Requirement of interaction of nectin-1alpha/HveC with afadin for efficient cell-cell spread of herpes simplex virus type 1. J Virol **75:**4734-43.
323. **Saksena, M. M., H. Wakisaka, B. Tijono, R. A. Boadle, F. Rixon, H. Takahashi, and A. L. Cunningham.** 2006. Herpes simplex virus type 1 accumulation, envelopment, and exit in growth cones and varicosities in mid-distal regions of axons. J Virol **80:**3592-606.
324. **Salomoni, P., B. J. Ferguson, A. H. Wyllie, and T. Rich.** 2008. New insights into the role of PML in tumour suppression. Cell Res **18:**622-40.
325. **Samaniego, L. A., L. Neiderhiser, and N. A. DeLuca.** 1998. Persistence and expression of the herpes simplex virus genome in the absence of immediate-early proteins. J Virol **72:**3307-20.
326. **Samaniego, L. A., A. L. Webb, and N. A. DeLuca.** 1995. Functional interactions between herpes simplex virus immediate-early proteins during infection: gene expression as a consequence of ICP27 and different domains of ICP4. J Virol **69:**5705-15.
327. **Samuel, C. E.** 1993. The eIF-2 alpha protein kinases, regulators of translation in eukaryotes from yeasts to humans. J Biol Chem **268:**7603-6.
328. **Samuel, C. E.** 1979. Mechanism of interferon action: phosphorylation of protein synthesis initiation factor eIF-2 in interferon-treated human cells by a ribosome-associated kinase processing site specificity similar to

hemin-regulated rabbit reticulocyte kinase. Proc Natl Acad Sci U S A **76:**600-4.

329. **Sanchez, R., and I. Mohr.** 2007. Inhibition of cellular 2'-5' oligoadenylate synthetase by the herpes simplex virus type 1 Us11 protein. J Virol **81:**3455-64.
330. **Sandri-Goldin, R. M.** 1998. ICP27 mediates HSV RNA export by shuttling through a leucine-rich nuclear export signal and binding viral intronless RNAs through an RGG motif. Genes Dev **12:**868-79.
331. **Sandri-Goldin, R. M., M. K. Hibbard, and M. A. Hardwicke.** 1995. The C-terminal repressor region of herpes simplex virus type 1 ICP27 is required for the redistribution of small nuclear ribonucleoprotein particles and splicing factor SC35; however, these alterations are not sufficient to inhibit host cell splicing. J Virol **69:**6063-76.
332. **Sandri-Goldin, R. M., and G. E. Mendoza.** 1992. A herpesvirus regulatory protein appears to act post-transcriptionally by affecting mRNA processing. Genes Dev **6:**848-63.
333. **Sarmiento, M., M. Haffey, and P. G. Spear.** 1979. Membrane proteins specified by herpes simplex viruses. III. Role of glycoprotein VP7(B2) in virion infectivity. J Virol **29:**1149-58.
334. **Sawtell, N. M.** 1997. Comprehensive quantification of herpes simplex virus latency at the single-cell level. J Virol **71:**5423-31.
335. **Sawtell, N. M., and R. L. Thompson.** 1992. Herpes simplex virus type 1 latency-associated transcription unit promotes anatomical site-dependent establishment and reactivation from latency. J Virol **66:**2157-69.
336. **Sawtell, N. M., and R. L. Thompson.** 1992. Rapid in vivo reactivation of herpes simplex virus in latently infected murine ganglionic neurons after transient hyperthermia. J Virol **66:**2150-6.
337. **Scheck, A. C., B. Wigdahl, E. De Clercq, and F. Rapp.** 1986. Prolonged herpes simplex virus latency in vitro after treatment of infected cells with acyclovir and human leukocyte interferon. Antimicrob Agents Chemother **29:**589-93.
338. **Sciabica, K. S., Q. J. Dai, and R. M. Sandri-Goldin.** 2003. ICP27 interacts with SRPK1 to mediate HSV splicing inhibition by altering SR protein phosphorylation. EMBO J **22:**1608-19.
339. **Sears, A. E., I. W. Halliburton, B. Meignier, S. Silver, and B. Roizman.** 1985. Herpes simplex virus 1 mutant deleted in the alpha 22 gene: growth and gene expression in permissive and restrictive cells and establishment of latency in mice. J Virol **55:**338-46.
340. **Sears, A. E., V. Hukkanen, M. A. Labow, A. J. Levine, and B. Roizman.** 1991. Expression of the herpes simplex virus 1 alpha transinducing factor (VP16) does not induce reactivation of latent virus or prevent the establishment of latency in mice. J Virol **65:**2929-35.
341. **Seeler, J. S., A. Marchio, D. Sitterlin, C. Transy, and A. Dejean.** 1998. Interaction of SP100 with HP1 proteins: a link between the promyelocytic leukemia-associated nuclear bodies and the chromatin compartment. Proc Natl Acad Sci U S A **95:**7316-21.

342. **Seet, B. T., I. Dikic, M. M. Zhou, and T. Pawson.** 2006. Reading protein modifications with interaction domains. Nat Rev Mol Cell Biol **7:**473-83.
343. **Severini, A., A. R. Morgan, D. R. Tovell, and D. L. Tyrrell.** 1994. Study of the structure of replicative intermediates of HSV-1 DNA by pulsed-field gel electrophoresis. Virology **200:**428-35.
344. **Severini, A., D. G. Scraba, and D. L. Tyrrell.** 1996. Branched structures in the intracellular DNA of herpes simplex virus type 1. J Virol **70:**3169-75.
345. **Shieh, M. T., D. WuDunn, R. I. Montgomery, J. D. Esko, and P. G. Spear.** 1992. Cell surface receptors for herpes simplex virus are heparan sulfate proteoglycans. J Cell Biol **116:**1273-81.
346. **Shiraki, K., and F. Rapp.** 1986. Establishment of herpes simplex virus latency in vitro with cycloheximide. J Gen Virol **67 (Pt 11):**2497-500.
347. **Shukla, D., J. Liu, P. Blaiklock, N. W. Shworak, X. Bai, J. D. Esko, G. H. Cohen, R. J. Eisenberg, R. D. Rosenberg, and P. G. Spear.** 1999. A novel role for 3-O-sulfated heparan sulfate in herpes simplex virus 1 entry. Cell **99:**13-22.
348. **Simpson-Holley, M., J. Baines, R. Roller, and D. M. Knipe.** 2004. Herpes simplex virus 1 U(L)31 and U(L)34 gene products promote the late maturation of viral replication compartments to the nuclear periphery. J Virol **78:**5591-600.
349. **Simpson-Holley, M., R. C. Colgrove, G. Nalepa, J. W. Harper, and D. M. Knipe.** 2005. Identification and functional evaluation of cellular and viral factors involved in the alteration of nuclear architecture during herpes simplex virus 1 infection. J Virol **79:**12840-51.
350. **Skepper, J. N., A. Whiteley, H. Browne, and A. Minson.** 2001. Herpes simplex virus nucleocapsids mature to progeny virions by an envelopment --> deenvelopment --> reenvelopment pathway. J Virol **75:**5697-702.
351. **Smibert, C. A., B. Popova, P. Xiao, J. P. Capone, and J. R. Smiley.** 1994. Herpes simplex virus VP16 forms a complex with the virion host shutoff protein vhs. J Virol **68:**2339-46.
352. **Smiley, J. R., and J. Duncan.** 1997. Truncation of the C-terminal acidic transcriptional activation domain of herpes simplex virus VP16 produces a phenotype similar to that of the in1814 linker insertion mutation. J Virol **71:**6191-3.
353. **Smiley, J. R., J. Duncan, and M. Howes.** 1990. Sequence requirements for DNA rearrangements induced by the terminal repeat of herpes simplex virus type 1 KOS DNA. J Virol **64:**5036-50.
354. **Smiley, J. R., M. M. Elgadi, and H. A. Saffran.** 2001. Herpes simplex virus vhs protein. Methods Enzymol **342:**440-51.
355. **Sodeik, B., M. W. Ebersold, and A. Helenius.** 1997. Microtubule-mediated transport of incoming herpes simplex virus 1 capsids to the nucleus. J Cell Biol **136:**1007-21.
356. **Soliman, T. M., R. M. Sandri-Goldin, and S. J. Silverstein.** 1997. Shuttling of the herpes simplex virus type 1 regulatory protein ICP27

between the nucleus and cytoplasm mediates the expression of late proteins. J Virol **71:**9188-97.

357. **Speck, P. G., and A. Simmons.** 1991. Divergent molecular pathways of productive and latent infection with a virulent strain of herpes simplex virus type 1. J Virol **65:**4001-5.
358. **Speck, P. G., and A. Simmons.** 1992. Synchronous appearance of antigen-positive and latently infected neurons in spinal ganglia of mice infected with a virulent strain of herpes simplex virus. J Gen Virol **73 (Pt 5):**1281-5.
359. **Stadler, M., M. K. Chelbi-Alix, M. H. Koken, L. Venturini, C. Lee, A. Saib, F. Quignon, L. Pelicano, M. C. Guillemin, C. Schindler, and et al.** 1995. Transcriptional induction of the PML growth suppressor gene by interferons is mediated through an ISRE and a GAS element. Oncogene **11:**2565-73.
360. **Stannard, L. M., A. O. Fuller, and P. G. Spear.** 1987. Herpes simplex virus glycoproteins associated with different morphological entities projecting from the virion envelope. J Gen Virol **68 (Pt 3):**715-25.
361. **Stark, G. R., I. M. Kerr, B. R. Williams, R. H. Silverman, and R. D. Schreiber.** 1998. How cells respond to interferons. Annu Rev Biochem **67:**227-64.
362. **Stelz, G., E. Rucker, O. Rosorius, G. Meyer, R. H. Stauber, M. Spatz, M. M. Eibl, and J. Hauber.** 2002. Identification of two nuclear import signals in the alpha-gene product ICP22 of herpes simplex virus 1. Virology **295:**360-70.
363. **Stern, S., M. Tanaka, and W. Herr.** 1989. The Oct-1 homoeodomain directs formation of a multiprotein-DNA complex with the HSV transactivator VP16. Nature **341:**624-30.
364. **Sterner, D. E., and S. L. Berger.** 2000. Acetylation of histones and transcription-related factors. Microbiol Mol Biol Rev **64:**435-59.
365. **Stow, N. D.** 1982. Localization of an origin of DNA replication within the TRS/IRS repeated region of the herpes simplex virus type 1 genome. EMBO J **1:**863-7.
366. **Stow, N. D., and E. C. Stow.** 1986. Isolation and characterization of a herpes simplex virus type 1 mutant containing a deletion within the gene encoding the immediate early polypeptide Vmw110. J Gen Virol **67 (Pt 12):**2571-85.
367. **Strang, B. L., and N. D. Stow.** 2005. Circularization of the herpes simplex virus type 1 genome upon lytic infection. J Virol **79:**12487-94.
368. **Subak-Sharpe, J. H., and D. J. Dargan.** 1998. HSV molecular biology: general aspects of herpes simplex virus molecular biology. Virus Genes **16:**239-51.
369. **Sullivan, S. M., P. J. Horn, V. A. Olson, A. H. Koop, W. Niu, R. H. Ebright, and S. J. Triezenberg.** 1998. Mutational analysis of a transcriptional activation region of the VP16 protein of herpes simplex virus. Nucleic Acids Res **26:**4487-96.

370. **Tal-Singer, R., R. Pichyangkura, E. Chung, T. M. Lasner, B. P. Randazzo, J. Q. Trojanowski, N. W. Fraser, and S. J. Triezenberg.** 1999. The transcriptional activation domain of VP16 is required for efficient infection and establishment of latency by HSV-1 in the murine peripheral and central nervous systems. Virology **259:**20-33.
371. **Tavalai, N., and T. Stamminger.** 2008. New insights into the role of the subnuclear structure ND10 for viral infection. Biochim Biophys Acta **1783:**2207-21.
372. **Taylor, T. J., M. A. Brockman, E. E. McNamee, and D. M. Knipe.** 2002. Herpes simplex virus. Front Biosci **7:**d752-64.
373. **Thomas, D. L., M. Lock, J. M. Zabolotny, B. R. Mohan, and N. W. Fraser.** 2002. The 2-kilobase intron of the herpes simplex virus type 1 latency-associated transcript has a half-life of approximately 24 hours in SY5Y and COS-1 cells. J Virol **76:**532-40.
374. **Thomas, M. S., M. Gao, D. M. Knipe, and K. L. Powell.** 1992. Association between the herpes simplex virus major DNA-binding protein and alkaline nuclease. J Virol **66:**1152-61.
375. **Thompson, R. L., C. M. Preston, and N. M. Sawtell.** 2009. De novo synthesis of VP16 coordinates the exit from HSV latency in vivo. PLoS Pathog **5:**e1000352.
376. **Thompson, R. L., and N. M. Sawtell.** 2001. Herpes simplex virus type 1 latency-associated transcript gene promotes neuronal survival. J Virol **75:**6660-75.
377. **Thompson, R. L., and N. M. Sawtell.** 2000. HSV latency-associated transcript and neuronal apoptosis. Science **289:**1651.
378. **Tomazin, R., A. B. Hill, P. Jugovic, I. York, P. van Endert, H. L. Ploegh, D. W. Andrews, and D. C. Johnson.** 1996. Stable binding of the herpes simplex virus ICP47 protein to the peptide binding site of TAP. EMBO J **15:**3256-66.
379. **Tomishima, M. J., and L. W. Enquist.** 2002. In vivo egress of an alphaherpesvirus from axons. J Virol **76:**8310-7.
380. **Triezenberg, S. J., R. C. Kingsbury, and S. L. McKnight.** 1988. Functional dissection of VP16, the trans-activator of herpes simplex virus immediate early gene expression. Genes Dev **2:**718-29.
381. **Trus, B. L., N. Cheng, W. W. Newcomb, F. L. Homa, J. C. Brown, and A. C. Steven.** 2004. Structure and polymorphism of the UL6 portal protein of herpes simplex virus type 1. J Virol **78:**12668-71.
382. **Umbach, J. L., M. F. Kramer, I. Jurak, H. W. Karnowski, D. M. Coen, and B. R. Cullen.** 2008. MicroRNAs expressed by herpes simplex virus 1 during latent infection regulate viral mRNAs. Nature **454:**780-3.
383. **Uprichard, S. L., and D. M. Knipe.** 1996. Herpes simplex ICP27 mutant viruses exhibit reduced expression of specific DNA replication genes. J Virol **70:**1969-80.
384. **Utley, R. T., K. Ikeda, P. A. Grant, J. Cote, D. J. Steger, A. Eberharter, S. John, and J. L. Workman.** 1998. Transcriptional

activators direct histone acetyltransferase complexes to nucleosomes. Nature **394:**498-502.

385. **van Leeuwen, H., M. Okuwaki, R. Hong, D. Chakravarti, K. Nagata, and P. O'Hare.** 2003. Herpes simplex virus type 1 tegument protein VP22 interacts with TAF-I proteins and inhibits nucleosome assembly but not regulation of histone acetylation by INHAT. J Gen Virol **84:**2501-10.
386. **Van Sant, C., R. Hagglund, P. Lopez, and B. Roizman.** 2001. The infected cell protein 0 of herpes simplex virus 1 dynamically interacts with proteasomes, binds and activates the cdc34 E2 ubiquitin-conjugating enzyme, and possesses in vitro E3 ubiquitin ligase activity. Proc Natl Acad Sci U S A **98:**8815-20.
387. **Van Sant, C., P. Lopez, S. J. Advani, and B. Roizman.** 2001. Role of cyclin D3 in the biology of herpes simplex virus 1 ICPO. J Virol **75:**1888-98.
388. **Varmuza, S. L., and J. R. Smiley.** 1985. Signals for site-specific cleavage of HSV DNA: maturation involves two separate cleavage events at sites distal to the recognition sequences. Cell **41:**793-802.
389. **Vaughan, P. J., K. J. Thibault, M. A. Hardwicke, and R. M. Sandri-Goldin.** 1992. The herpes simplex virus immediate early protein ICP27 encodes a potential metal binding domain and binds zinc in vitro. Virology **189:**377-84.
390. **Vittone, V., E. Diefenbach, D. Triffett, M. W. Douglas, A. L. Cunningham, and R. J. Diefenbach.** 2005. Determination of interactions between tegument proteins of herpes simplex virus type 1. J Virol **79:**9566-71.
391. **Wadsworth, S., R. J. Jacob, and B. Roizman.** 1975. Anatomy of herpes simplex virus DNA. II. Size, composition, and arrangement of inverted terminal repetitions. J Virol **15:**1487-97.
392. **Wagner, E. K., and D. C. Bloom.** 1997. Experimental investigation of herpes simplex virus latency. Clin Microbiol Rev **10:**419-43.
393. **Wang, Q. Y., C. Zhou, K. E. Johnson, R. C. Colgrove, D. M. Coen, and D. M. Knipe.** 2005. Herpesviral latency-associated transcript gene promotes assembly of heterochromatin on viral lytic-gene promoters in latent infection. Proc Natl Acad Sci U S A **102:**16055-9.
394. **Warner, M. S., R. J. Geraghty, W. M. Martinez, R. I. Montgomery, J. C. Whitbeck, R. Xu, R. J. Eisenberg, G. H. Cohen, and P. G. Spear.** 1998. A cell surface protein with herpesvirus entry activity (HveB) confers susceptibility to infection by mutants of herpes simplex virus type 1, herpes simplex virus type 2, and pseudorabies virus. Virology **246:**179-89.
395. **Weinheimer, S. P., B. A. Boyd, S. K. Durham, J. L. Resnick, and D. R. O'Boyle, 2nd.** 1992. Deletion of the VP16 open reading frame of herpes simplex virus type 1. J Virol **66:**258-69.
396. **Whitbeck, J. C., C. Peng, H. Lou, R. Xu, S. H. Willis, M. Ponce de Leon, T. Peng, A. V. Nicola, R. I. Montgomery, M. S. Warner, A. M. Soulika, L. A. Spruce, W. T. Moore, J. D. Lambris, P. G. Spear, G. H. Cohen, and R. J. Eisenberg.** 1997. Glycoprotein D of herpes simplex

virus (HSV) binds directly to HVEM, a member of the tumor necrosis factor receptor superfamily and a mediator of HSV entry. J Virol **71:**6083-93.

397. **Whitby, A. J., W. A. Blyth, and T. J. Hill.** 1987. The effect of DNA hypomethylating agents on the reactivation of herpes simplex virus from latently infected mouse ganglia in vitro. Brief report. Arch Virol **97:**137-44.
398. **Whiteley, A., B. Bruun, T. Minson, and H. Browne.** 1999. Effects of targeting herpes simplex virus type 1 gD to the endoplasmic reticulum and trans-Golgi network. J Virol **73:**9515-20.
399. **Wigdahl, B., A. C. Scheck, R. J. Ziegler, E. De Clercq, and F. Rapp.** 1984. Analysis of the herpes simplex virus genome during in vitro latency in human diploid fibroblasts and rat sensory neurons. J Virol **49:**205-13.
400. **Wilcock, D., and D. P. Lane.** 1991. Localization of p53, retinoblastoma and host replication proteins at sites of viral replication in herpes-infected cells. Nature **349:**429-31.
401. **Wilkinson, D. E., and S. K. Weller.** 2004. Recruitment of cellular recombination and repair proteins to sites of herpes simplex virus type 1 DNA replication is dependent on the composition of viral proteins within prereplicative sites and correlates with the induction of the DNA damage response. J Virol **78:**4783-96.
402. **Wilkinson, D. E., and S. K. Weller.** 2003. The role of DNA recombination in herpes simplex virus DNA replication. IUBMB Life **55:**451-8.
403. **Wisner, T. W., C. C. Wright, A. Kato, Y. Kawaguchi, F. Mou, J. D. Baines, R. J. Roller, and D. C. Johnson.** 2009. Herpesvirus gB-induced fusion between the virion envelope and outer nuclear membrane during virus egress is regulated by the viral US3 kinase. J Virol **83:**3115-26.
404. **WuDunn, D., and P. G. Spear.** 1989. Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. J Virol **63:**52-8.
405. **Wysocka, J., and W. Herr.** 2003. The herpes simplex virus VP16-induced complex: the makings of a regulatory switch. Trends Biochem Sci **28:**294-304.
406. **Wysocka, J., M. P. Myers, C. D. Laherty, R. N. Eisenman, and W. Herr.** 2003. Human Sin3 deacetylase and trithorax-related Set1/Ash2 histone H3-K4 methyltransferase are tethered together selectively by the cell-proliferation factor HCF-1. Genes Dev **17:**896-911.
407. **Xiao, H., A. Pearson, B. Coulombe, R. Truant, S. Zhang, J. L. Regier, S. J. Triezenberg, D. Reinberg, O. Flores, C. J. Ingles, and et al.** 1994. Binding of basal transcription factor TFIIH to the acidic activation domains of VP16 and p53. Mol Cell Biol **14:**7013-24.
408. **Xiao, P., and J. P. Capone.** 1990. A cellular factor binds to the herpes simplex virus type 1 transactivator Vmw65 and is required for Vmw65-dependent protein-DNA complex assembly with Oct-1. Mol Cell Biol **10:**4974-7.

409. **Yao, F., and P. A. Schaffer.** 1995. An activity specified by the osteosarcoma line U2OS can substitute functionally for ICP0, a major regulatory protein of herpes simplex virus type 1. J Virol **69:**6249-58.
410. **Yao, F., and P. A. Schaffer.** 1994. Physical interaction between the herpes simplex virus type 1 immediate-early regulatory proteins ICP0 and ICP4. J Virol **68:**8158-68.
411. **York, I. A., C. Roop, D. W. Andrews, S. R. Riddell, F. L. Graham, and D. C. Johnson.** 1994. A cytosolic herpes simplex virus protein inhibits antigen presentation to CD8+ T lymphocytes. Cell **77:**525-35.
412. **Zabierowski, S. E., and N. A. Deluca.** 2008. Stabilized binding of TBP to the TATA box of herpes simplex virus type 1 early (tk) and late (gC) promoters by TFIIA and ICP4. J Virol **82:**3546-54.
413. **Zabolotny, J. M., C. Krummenacher, and N. W. Fraser.** 1997. The herpes simplex virus type 1 2.0-kilobase latency-associated transcript is a stable intron which branches at a guanosine. J Virol **71:**4199-208.
414. **Zhang, R., M. V. Poustovoitov, X. Ye, H. A. Santos, W. Chen, S. M. Daganzo, J. P. Erzberger, I. G. Serebriiskii, A. A. Canutescu, R. L. Dunbrack, J. R. Pehrson, J. M. Berger, P. D. Kaufman, and P. D. Adams.** 2005. Formation of MacroH2A-containing senescence-associated heterochromatin foci and senescence driven by ASF1a and HIRA. Dev Cell **8:**19-30.
415. **Zhang, X., S. Efstathiou, and A. Simmons.** 1994. Identification of novel herpes simplex virus replicative intermediates by field inversion gel electrophoresis: implications for viral DNA amplification strategies. Virology **202:**530-9.
416. **Zhi, Y., K. S. Sciabica, and R. M. Sandri-Goldin.** 1999. Self-interaction of the herpes simplex virus type 1 regulatory protein ICP27. Virology **257:**341-51.
417. **Zhou, G., V. Galvan, G. Campadelli-Fiume, and B. Roizman.** 2000. Glycoprotein D or J delivered in trans blocks apoptosis in SK-N-SH cells induced by a herpes simplex virus 1 mutant lacking intact genes expressing both glycoproteins. J Virol **74:**11782-91.
418. **Zhou, Z. H., M. Dougherty, J. Jakana, J. He, F. J. Rixon, and W. Chiu.** 2000. Seeing the herpesvirus capsid at 8.5 A. Science **288:**877-80.
419. **Zhou, Z. H., J. He, J. Jakana, J. D. Tatman, F. J. Rixon, and W. Chiu.** 1995. Assembly of VP26 in herpes simplex virus-1 inferred from structures of wild-type and recombinant capsids. Nat Struct Biol **2:**1026-30.