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The mechanism of inhibition of herpes simplex virus type 1 DNA replication by roscovitine

by

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ABSTRACT

Transcription and DNA replication of herpes simplex virus type 1 (HSV-1) occur in nuclear domains adjacent to structures named ND10. The HSV-1 single-stranded DNA binding protein ICP8 localizes to these nuclear domains to direct the assembly of the pre- and replication compartments.

Inhibition of cyclin dependent kinases with roscovitine inhibits HSV-1 DNA replication, even in the presence of all required HSV-1 proteins, at an unidentified step. Here I show that roscovitine inhibits the localization of pre-expressed ICP8 to new replication sites. Therefore, the inhibition of HSV-1 DNA replication occurs at a step prior to initiation. I next evaluated the mechanisms of inhibition of proper ICP8 localization. ICP8 was extracted at lower salt concentrations from roscovitine-treated than untreated cells, but the affinity of ICP8 for ssDNA *in vitro* was not affected.

I propose that roscovitine inhibits HSV-1 DNA replication by inhibiting DNA accessibility. I also discuss alternative mechanisms.

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LIST OF ABBREVIATIONS

ADNP	Activity-dependent neuroprotective protein
ADP	Adenosine diphosphate
Arg	Arginine
ATP	Adenosine triphosphate
BAF57/60a/155/170	BRG1 or BRM-associated factor 57/60a/155/170
BLM	Bloom helicase
BRCA1	Breast cancer gene 1
BrdU	Bromodeoxyuridine
bp	Base pair
BRG1	BRM related gene 1
BRM	Brahma
BSA	Bovine serum albumin
CAF-1 p48	Chromatin assembly factor 1 p48 subunit
CAK	CDK associated kinase
cAMP	Cyclic adenosine 3'5' monophosphate
CAPS	N-cyclohexyl-3-aminopropanesulfonic acid
CBP	CREB binding protein
CDK	Cyclin dependent kinase
CHD3-IP	Chromo-helicase DNA-binding protein-3-interacting protein
ChIP	Chromatin immunoprecipitation
CHX	Cycloheximide
CK1α/δ	Casein kinase 1α/δ
CKI	CDK inhibitor
CPE	Cytopathic effect
CREB	cAMP response element binding protein
CTD	C-terminal domain
Daxx	Death-domain-association protein
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulfoxide
DNMTAP-1	DNA methyltransferase-associated protein 1
DNA-PKcs	DNA-protein kinase catalytic subunit
ds	Double-stranded
DTT	Dithiothreitol
DYRK1A	Dual-specificity tyrosine phosphorylation-regulated kinase 1A
E	Early
EBV	Epstein-Barr virus
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
EM	Electron microscopy
EPHB2	Ephrin-B2 kinase
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal-regulated kinase 1/2
FACT p140	Facilitates chromain transcription p140

FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FISH	Fluorescent *in situ* hybridization
G0/G1/G2	Gap 0/1/2
gC	Glycoprotein C
γH68	Murine gammaherpesvirus 68
GR	Glucocorticoid receptor
GRY-RBP	Glycine, arginine, tyrosine-rich RNA binding protein
GSK3β	Glycogen synthase kinase 3 beta
GTF II-I	General transcription factor II-I
GTPase	Guanosine triphosphatase
HAT	Histone acetyltransferase
HAUSP	Herpesvirus-associated ubiquitin-specific protease
HCF-1	Host cell factor 1
HCMV	Human cytomegalovirus
HDAC2	Histone deacetylase 2
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HHV-6A/6B/7	Human herpesvirus type-6A/6B/7
HIV-1	Human immunodeficiency virus type-1
hpi	Hours post infection
HPV	Human papilloma virus
HTLV-1	Human T-cell lymphotropic virus type-1
hSNF2H/2L	Human sucrose non-fermenting 2H/2L
HSV-1/-2	Herpes simplex virus type-1/-2
HVEM	Herpesvirus entry mediator ligand
IC_{50}	50% inhibitory concentration
ICP	Infected cell polypeptide
ICTV	International Committee on the Taxonomy of Viruses
IE	Immediate early
IFN	Interferon
IMPDH-2	Inosine-5´-monophosphate dehydrogenase 2
(co-)IP	(co)-immunoprecipitate
IFN	Interferon
IRAK4	Interleukin-1 receptor-associated kinase 4
JCV	JC virus
K	Lysine
KSHV	Kaposi sarcoma-associated herpesvirus
L	Late
L	Long
LAT	Latency-associated transcript
LCMV	Lymphocytic choriomeningitis virus
Lys	Lysine
M	Mitosis
Mat1	Ménage à trois 1
MC	Methyl cellulose
MCM	Mini chromosome maintenance

MCN	Micrococcal nuclease
MMTV	Mouse mammary tumor virus
Mre11	Meiotic recombination 11
MSH2/3/6	MutS homolog 2/3/6
NAP-1-like	Nucleosome-associated protein 1-like
NGS	Normal goat serum
NBS1	Nibrin
ND10	Nuclear dot 10
NMP200/238	Nuclear matrix protein 200/238
n/t	not tested
OBP	Origin binding protein
oct-1	Octamer binding protein 1
ORC	Origin recognition complex
oriL/S	Origin L/S
ORF	Open reading frame
PAA	Phosphonoacetic acid
PAGE	Polyacrylamide gel electrophoresis
PARP-1	Poly (ADP-ribose) polymerase-1
PBS	Phosphate buffered saline
PCAF	p300/CREB-binding protein-associated factor
PCI	Pharmaceutical CDK inhibitor
PCNA	Proliferating nuclear antigen
PFU	Plaque forming unit
PI3K	Phosphoinositide 3-kinase
PLK1	Polo-like kinase 1
PML	Promyelocytic leukemia protein
POD	PML oncogenic domain
PP1A	Protein phosphatase 1A subunit
pRb	phosphorylated Rb
Pre-RC	Pre-replication compartment
PrV	Pseudorabies virus
P-TEFb	Positive transcription elongation factor b
PVDF	Polyvinylidene difluoride
R	Arginine
Rb	Retinoblastoma protein
RBCC	RING-box-coiled coil domain
RC	Replication compartment
RCC1	Regulator of chromosome condensation 1
REST	RE1-silencing transcription corepressor
RING	Really interesting new gene
Rosco	Roscovitine
RPA	Replication protein A
RPM	Revolutions per minute
RSB	Reticulocyte swelling buffer
S	Short
S	Synthesis

SAP130/155	Spiceosome-associated protein 130/155
SBR	Swinging bucket rotor
SDS	Sodium-dodecyl sulfate
Ser	Serine
SFM	Serum-free media
SIN3a	SWI-independent 3a
SMC1	Structural maintenance of chromosomes 1
SNF	Mating-type sucrose non-fermenting
Sp100	Sp100 nuclear antigen
ss	Single-stranded
SUMO-1	Small ubiquitin-like modifier 1
SV40	Simian virus 40
SWI	Mating-type switching
T_{50}	Time to degrade 50% of the DNA
TAg	Large T-Antigen
TAF1	TBP-associated factor 1
TAF172	TAF of 172 kDa
TBP	TATA-binding protein
Thr	Threonine
TK	Thymidine kinase
ts	temperature sensitive
U_L	Unique long
U_S	Unique short
UV	Ultraviolet
vhs	Virion host shut off
VP	Virion protein
VSV	Vesicular stomatitis virus
VSVG	VSV glycoprotein G
VV	Vaccinia virus
VZV	Varicella-zoster virus
WRN	Werner helicase
XRCC4	X-ray repair cross complimenting 4
3-OS HS	3-O-sulfated heparin sulfate

CHAPTER 1: INTRODUCTION

1.1 HERPESVIRIDAE

According to the most current classification by the International Committee on the Taxonomy of Viruses (ICTV), viruses from the *Herpesviridae* family infect mammals, bird, and reptiles (Davison et al., 2009; ICTV, 2009). At least one herpesvirus has been found to infect each of the species investigated in these taxa (Fields et al., 2007). The most recent publication from ICTV identifies 120 species in the *Herpesviridae* family. Nine infect humans beings, herpes simplex virus type-1 (HSV-1), herpes simplex virus type-2 (HSV-2), human cytomegalovirus (HCMV), varicella-zoster virus (VZV), Epstein-Barr virus (EBV), human herpesviruses-6A, -6B, -7, (HHV-6A, HHV-6B, HHV-7, respectively) and Kaposi's sarcoma-associated herpesvirus (KSHV) (Davison et al., 2009).

The first defining characteristic of the *Herpesviridae* family is virion structure. *Herpesviridae* virions consist of a core containing a linear double stranded (ds) DNA inside an icosahedral capsid, which is surrounded by a proteinaceous tegument and an envelope. In addition to structural similarities, members of *Herpesviridae* also share four important biologic properties. They encode for enzymes involved in nucleic acid metabolism, DNA synthesis, and protein processing. Viral DNA synthesis and capsid assembly occur in the nucleus. They kill infected cells and they have the ability to remain latent in

infected animals, although there are no obvious general common mechanisms for establishment, maintenance, or termination of latency.

Twenty-six genes are conserved across all *Herpesviridae*, and most herpes genes belong to one of seven conserved core gene blocks. Within each of these blocks, the orders and orientations (5′ → 3′ or 3′ → 5′) of the genes are conserved. The gene blocks are rearranged in various orders and orientations in different herpesviruses, although gene block arrangements are typically conserved at the subfamily level. Whereas there are recognized exceptions (e.g. EBV), most *Herpesviridae* members do not encode for many spliced genes. In contrast, gene overlaps are common.

Members of the *Herpesviridae* family are classified according to the ICTV on the basis of their biological properties into three subfamilies: *Alphaherpesvirinae, Betaherpesvirinae,* and *Gammaherpesvirinae* (ICTV, 2009). Members of the subfamily *Alphaherpesvirinae* infect a wide variety of hosts, replicate quickly, spread rapidly in culture, destroy infected cells efficiently, and establish latency primarily in sensory ganglia (Fields et al., 2007). This subfamily contains the genera *Simplexvirus* (for example, HSV-1) and *Varicellovirus* (for example, VZV).

1.1.1 Herpes simplex virus type-1

According to ICTV, "*a virus species is defined as a polythetic class of viruses that constitutes a replicating lineage and occupies a particular ecological niche*" (ICTV, 2009). As of 2009, the *Simplexvirus* genus contained 11 species. Two

infect human beings, HSV-1 and HSV-2. HSV-1 and HSV-2 genomes share 83% homology, and each of their genes has a homolog in the same gene block and orientation as in the other species (Dolan et al., 1998; McGeoch et al., 1988). HSV-1 and HSV-2 are considered different species due to historical differences in sites of infection, as well as several differences in biological characteristics. HSV-1 was originally described as infecting oral mucosa and HSV-2 as infecting genital mucosa. However, HSV-1 and HSV-2 are equally capable of productive infection at either site [reviewed in (Malkin, 2004)].

HSV-1 and HSV-2 establish latency and reactivate in different populations of cultured mouse neurons (Imai et al., 2009; Margolis et al., 2007). All neuronal subpopulations are capable of supporting productive infection of HSV-1 and HSV-2. However, HSV-1 preferentially reactivates in neurons expressing Galβ1-4GlcNAc-R epitopes and HSV-2 in neurons expressing Galα1-3Galβ1-4Ac-R epitopes. The neurons expressing these different epitopes are functionally different (Imai et al., 2009; Margolis et al., 2007). The differential reactivation is dependent on the species of the latency-associated transcript (LAT) (Imai et al., 2009), a transcript expressed during HSV latency. LATs improve the efficiency of latency establishment or reactivation, however, they are not required for latency or reactivation. The differential reactivation in different neuronal populations requires more extensive characterization.

HSV-1 is the most extensively studied member of the *Herpesviridae* family, and is consequently considered the archetype of the family. Like all other *Herpesviridae*, HSV-1 is an enveloped, dsDNA virus that replicates in the

nucleus. The HSV-1 genome is approximately 152 kilobases (kb) long, depending on strain. The linear genome is encapsidated into an icosahedral capsid, which is surrounded by a proteinaceous tegument. The tegument is in turn enclosed by a host derived lipid envelope, which contains the virally encoded glycoproteins.

HSV-1 genomes are rich in guanine and cytosine [approximately 68% (McGeoch et al., 1988)]. The genomes consist of two covalently linked components, designated long (L) and short (S), which comprise 82% and 18% of the total genome, respectively. Each component consists of unique sequences (U_L and U_S, respectively) flanked by inverted repeated sequences. Inverted repeats of the L component are designated *ab* and *b′a′* and those of the S component are designated *a′c′* and *ca*. The number of *a* sequence repeats at the L-S junction and at the L terminus is variable. Although the basic structure of the *a* sequence is highly conserved, *a* sequences consist of a variable number of repeat elements. L and S components invert relative to each other. Consequently, there are four subpopulations in any population of HSV-1 virions, differing only in the relative orientation of L and S. However, inversion is not required for virus viability in cell culture or *in vivo* (Poffenberger et al., 1983).

Like most other eukaryotic transcripts, most HSV-1 transcripts also encode for single proteins. There are only three known exceptions, *open reading frame* (*ORF*) *P/ORF O*, U_L26, and U_L3. In contrast, many clusters of transcriptional HSV-1 units are 3′ co-terminal, and some are antisense to others. Few HSV-1 transcripts are spliced, and the transcripts expressed to the highest

levels during latency appear to encode for no protein (LATs). The number of HSV-1 genes is estimated to range from 74 to 100 [(Stingley et al., 2000) and reviewed in (Mcgeoch et al., 2006; Rajcani et al., 2004)].

1.1.1.1 HSV-1 pathology

HSV-1 most commonly causes oral and genital lesions. The vermillion border of the lip, lips, mouth, genitals, and eyes are the most common sites of primary HSV-1 infection. Clinical manifestations vary from asymptomatic to any combination of ulcerative and vesicular lesions, fever, sore throat, gingivostomatitis, edema, localized lymphadenopathy, anorexia, and malaise. The clinical manifestations depend on factors such as the site of infection and the state of the immune system.

HSV-1 replicates at the site of primary infection. Such replication eventually results in infection of sensory nerve endings and nucleocapsid transport to the trigeminal or dorsal root sensory ganglia (depending on the site of the primary infection), where latency is established.

The factors required for the maintenance of latency are yet unclear. A variety of stimuli cause reactivation, although the specific molecular mechanisms mediating reactivation are not well understood either. The reactivation stimuli include fever, physical or emotional stress, tissue damage, immunesuppression, or exposure to ultraviolet (UV) light. Reactivated virus then reinfects, and replicates at, the primary site. The pathological changes induced by reactivation are similar to those in primary infections, although varying in severity. These pathological

changes are the result of the virally-induced cellular death and of the associated inflammatory response.

Although rarely in healthy humans, infection can become systemic and spread beyond the dorsal root ganglia. Such spread can cause disseminated neonatal HSV-1 infection with multi-organ involvement and multi-organ disease during pregnancy, or general dissemination in immunosuppressed patients. Spread can also result in life-threatening infections of the brain (encephalitis).

1.2 REPLICATION OF HERPES SIMPLEX VIRUS TYPE-1

The HSV-1 replication cycle can be described as a sequence of eight events. The first is entry. Entry itself is comprised of attachment and fusion of the virion to the cell membrane. The second and third events consist of the delivery of the nucleocapsid to the nucleus and subsequent delivery of viral DNA through the nuclear pore complex into the nucleus. The fourth event is circularization of the HSV-1 DNA. The fifth and sixth events, which are concomitant and dependent on each other, are the expression of HSV-1 proteins and HSV-1 DNA replication. The last two events of the replication cycle consist of virion assembly and egress.

1.2.1 HSV-1 entry

Entry of HSV-1 into cells occurs in three stages; low affinity attachment, followed by high affinity binding and then fusion. Initial low affinity attachment involves the interaction of the viral glycoprotein embedded in the envelope,

glycoprotein C (gC), and to a lesser extent gB, with glycosaminoglycan moieties of cell surface moiety heparin sulfate [reviewed in (Heldwein and Krummenacher, 2008)]. The high affinity binding occurs by binding of gD to either herpesvirus entry mediator (HVEM), nectin-1, nectin-2, or a modified cell surface carbohydrate, 3-O-sulfated heparin sulfate (3-OS HS), which is attached to the extracellular domain of integral membrane proteins. The high affinity interaction of gD with any of its receptors is required for subsequent fusion of the viral envelope with the host plasma membrane. HVEM is a member of the tumor necrosis factor receptor family, and the nectins are members of the immunoglobulin superfamily.

The actual mechanisms whereby the lipid bilayers of the virion envelope fuse those of the with cell membranes are not entirely understood. The most commonly accepted model is the hemifusion stalk (Maurer et al., 2008). In this model, the outer leaflets of the HSV-1 envelope and cell membranes fuse first, forming what is known as a "hemifusion stalk". At this stage, the outer leaflets form a continuous membrane, whereas the tegument and capsid are still physically separated from the cytoplasm by a bilayer formed by the inner leaflets of the viral envelope and cellular membrane. These two inner leaflets merge next, forming a pore, which is then enlarged to allow the contents of the virion and cell to mix, and the capsid and tegument to enter the cytoplasm.

HSV-1 virions do not fuse to the cell membrane spontaneously. Rather, fusion is dependent on the HSV-1 envelope glycoproteins gD, gB, gH, and gL. gD binding to cellular receptors brings the virion envelope and cell membrane

into close proximity. Conformational changes in gD then induce the recruitment and possible conformational changes of the other HSV-1 envelope glycoproteins (Atanasiu et al., 2007; Carfi et al., 2001; Krummenacher et al., 2005).

gB is most likely the effector of fusion (Heldwein et al., 2006). The domains of gB are highly homologous in three-dimensional shape and orientation (relative to the viral and cellular membranes) to the post-fusion state of vesicular stomatitis virus (VSV) glycoprotein G (VSV G) (Heldwein et al., 2006). Unlike VSV G, however, HSV-1 gB does not induce fusion alone, neither does it depend on pH decreases to induce fusion. Rather, the gH/gL heterodimer is also required, to form a gB/gH/gL complex (Atanasiu et al., 2010). The interaction of gH and gL is considered to be critical for the biological function of these two glycoproteins. gH and gL are always in a stable 1:1 complex and each protein is required for the proper localization of the other. Furthermore, the structure of the gH/gL heterodimer shows extensive interaction between gH and gL suggesting that each protein is required for the proper folding of the other (Chowdary et al., 2010).

The role of gH/gL in fusion has been difficult to interpret. Fusion peptides and heptad repeats, two common features of viral glycoproteins involved in membrane fusion, have been identified in gH (Galdiero et al., 2005; Gianni et al., 2005a; Gianni et al., 2005b). However, the gH/gL heterodimer does not structurally resemble a viral fusion protein (Chowdary et al., 2010). Furthermore, the gH/gL heterodimer is not sufficient to induce full fusion (Subramanian and Geraghty, 2007).

Current models suggest that after the gD conformational changes, gD triggers the physical interaction between gB and gH/gL and activates gH/gL to up-regulate gB into a fusogenic state. The interaction between gB and gH/gL has been proposed to decrease the activation energy required to shift gB into the post fusion conformation (Atanasiu, 2010; Chowdary et al., 2010).

1.2.2 Nucleocapsid transport and HSV-1 genome entry into the nucleus

After the fusion of the inner leaflets of the virion envelope and the cell membrane, the nucleocapsid and tegument enter the cytoplasm. Some tegument proteins then remain in the cytoplasm (e.g. virion host shut off [vhs], U_L41 and U_S11), whereas others are transported to the nucleus (e.g. virion protein -16, VP16). The nuclear transport of tegument proteins occurs as a result of either nuclear localization signals (e.g. VP16), or continuous association with the capsids while in the cytoplasm (e.g. VP1/2) (Granzow et al., 2005; Maurer et al., 2008; Ojala et al., 2000).

The capsid, with the associated tegument proteins, is transported to the nucleus through the cellular microtubular network (Sodeik et al., 1997). This transport is inhibited by microtubule depolymerizing agents such as colchicine and nocodazole (Kristensson et al., 1986; Sodeik et al., 1997). Antibody labeling of dynein showed colocalization with HSV-1 capsids (Sodeik et al., 1997), and likely depends on dynactin. Inhibition of dynein and dynactic association with dynamitin inhibited capsid transport (Dohner et al., 2002). Therefore, the transport of HSV-1 capsids to the nucleus is likely mediated by dynein. However,

the specific capsid-tegument structure loaded onto the microtubules is yet unknown. It is not clear how the nucleocapsids are delivered to the nuclear pore complex either. Dynein motors normally deliver cargo to microtubule-organizing centers.

The nucleocapsid delivery to the nucleus results in their association with nuclear pore complexes. A comprehensive list of all proteins playing important roles in capsid docking is not available. However, docking may depend on importin-β and Ran guanosine triphosphatase (GTPase) (Ojala et al., 2000). The nucleocapsids then release the linear dsDNA into the nucleus, leaving empty capsids at the nuclear pore (Ojala et al., 2000). Proteolysis of the tegument protein VP1/2 may play a role in such DNA release (Batterson et al., 1983; Jovasevic et al., 2008), and VP1/2 proteolysis is required for HSV-1 DNA release into the nucleus. However, little is known about the mechanisms of HSV-1 DNA release and transport through the nuclear pore.

1.2.3 HSV-1 protein expression and DNA replication

Inside the nucleus, the incoming viral genomes circularize (Garber et al., 1993; Strang and Stow, 2005). Circularization is independent of viral protein synthesis (Garber et al., 1993), indicating that it is mediated by cellular or structural virion proteins. However, the precise circularization mechanisms are unclear. Circularization has been suggested to involve the regulator of chromosome condensation (RCC1) (Umene and Nishimoto, 1996), or to result from

recombination between direct repeats in the terminal *a* sequences or require dsDNA breaks (Sarisky and Weber, 1994).

Through also unknown mechanisms, the circularized HSV-1 genomes are then delivered to sites adjacent to specific nuclear structures called "nuclear dot 10" (ND10s) (Phelan et al., 1997). The localization of the HSV-1 genomes to sites adjacent to these domains was discovered when it was observed that HSV-1 transcription and DNA replication occurred at nonrandom nuclear sites. These sites were dictated by preexisting nuclear architecture (de Bruyn Kops and Knipe, 1994). These domains were identified to be adjacent to ND10s, which shall be revisited in section 1.4.2 (ND10 dispersal). Nucleocapsid assembly also occurs at these nuclear domains.

The HSV-1 genome encodes for approximately 100 genes, which are coordinately expressed. Based on their requirement for expression, the HSV-1 genes are classified into three categories, immediate-early (IE or α), early (E or β), and late (L or γ). IE genes are defined as genes the expression of which occurs in the absence of *de novo* protein synthesis. Expression of E proteins requires previous expression of IE proteins, whereas L protein expression requires HSV-1 DNA replication, which itself requires E proteins (**Figure 1.1**). A more comprehensive discussion of IE, E, and L genes and their expression is provided in section 1.2.6 (regulation of HSV-1 gene expression). Capsid assembly occurs after L proteins have been expressed.

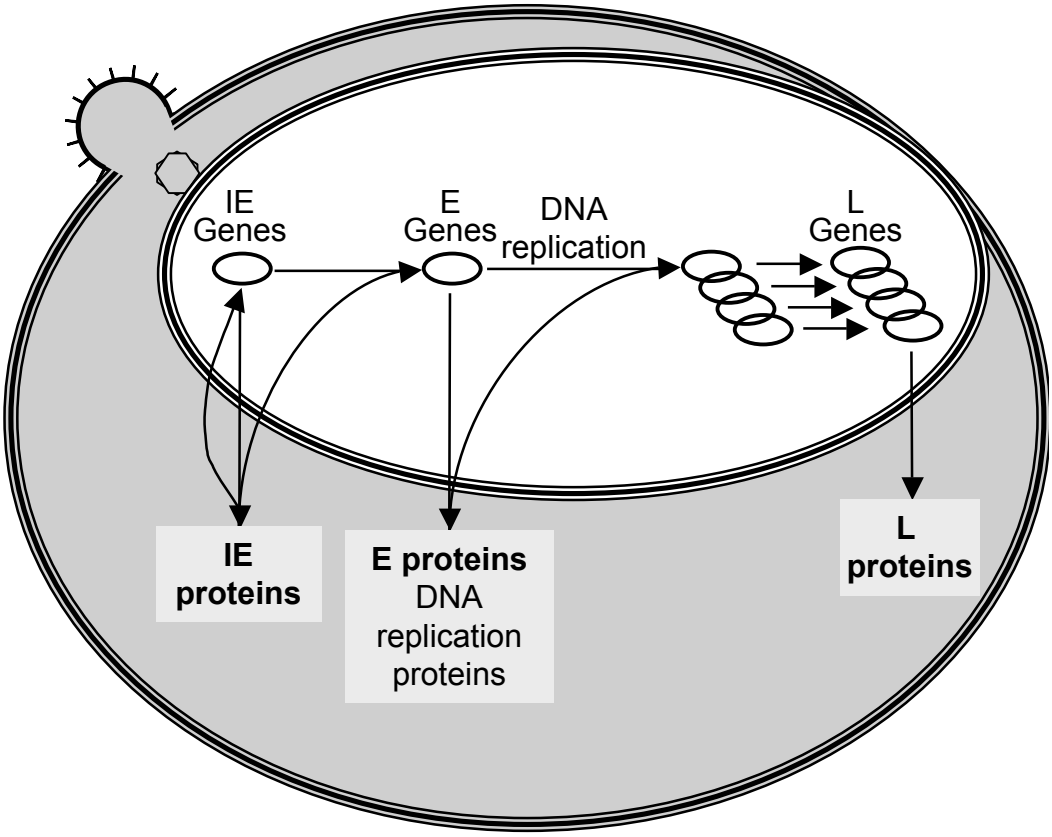


Figure 1.1 HSV-1 protein expression is sequential and coordinated. Cartoon representation of the coordinated expression of the HSV-1 proteins. Immediate early (**IE**) proteins activate the expression of early (**E**) proteins and regulate their own expression. The E genes encode for the DNA replication proteins. Therefore, HSV-1 DNA is replicated after E proteins are expressed. DNA replication then activates the expression of the late (**L**) proteins.

1.2.4 Capsid assembly and DNA encapsidation

The HSV-1 capsid is composed of the products of six HSV-1 genes, which result in seven capsid proteins: VP5, VP19C, VP21, VP22a, VP23, VP24, and VP26. These proteins accumulate within HSV-1 replication compartments, where they assemble into capsids (de Bruyn Kops et al., 1998). The HSV-1 capsids are a proteinaceous shell approximately 15 nm thick and 125 nm in diameter [reviewed in (Homa and Brown, 1997)]. They are icosahedra composed of 162 capsomers with a T = 16 lattice. The capsomers themselves are composed of 150 hexons and 12 pentons, all made up of VP5, the major capsid protein. VP26 is found at the tips of the hexons. Capsomers are connected in groups of three by structures called triplexes, which are composed of one molecule of VP19C and two molecules of VP23.

Capsid assembly requires VP5, VP19C, VP23, and VP21 or VP22a. The cavity of the capsid shell is filled with cleaved forms of the scaffolding protein, VP22a, the minor scaffolding protein VP21, and the viral protease VP24. VP21 and VP22a were discovered to have scaffolding properties in recombinant baculovirus capsid assembly assays. Partial and deformed capsid shells were formed in the absence of VP21 and VP22a in this system (Tatman et al., 1994). While VP22a is the major scaffolding protein, VP21 can partially substitute for VP22a, albeit with reduced efficiency (Tatman et al., 1994).

As capsid proteins accumulate, partial capsids composed of the shell (VP5, VP19C, and VP23) and core (scaffolding) proteins are formed [reviewed in (Homa and Brown, 1997)]. When sufficient molar amounts of the required

proteins have accumulated, the partial capsids convert to closed procapsids. The round procapsids then undergo structural transformations to become mature angular capsids. Capsid cavity proteins are removed upon DNA encapsidation. The protease activity of VP24 is required for the release of scaffolding proteins from the capsid interior and, therefore, for proper capsid assembly (Homa and Brown, 1997; Preston et al., 1983). However, the step at which the cleavage is required is unclear, as is the stage at which the linear HSV-1 DNA is packaged into progeny virions.

Two packaging and cleavage signals have been identified within the HSV-1 genome, within the domains of the *a* sequences (Deiss et al., 1986). HSV-1 DNA encapsidation involves cleavage of HSV-1 progeny DNA concatemers into unit-length monomers. Seven genes that encode for cleavage and packaging proteins have been identified [reviewed in (Nishiyama, 2004)]. Five of their products have known functions, including a portal protein (U_L6), alkaline nuclease (U_L12) and terminase (U_L15, U_L28, and U_L33), whereas the specific functions of U_L32 and U_L25 have yet to be established.

1.2.5 Egress

After encapsidation of HSV-1 DNA, nucleocapsids bud through the inner nuclear membrane. The route for egress of the virion particle from the space between the inner and outer nuclear membranes to the exterior of the cell has been the subject of controversy for over 40 years (Cheung et al., 1991; Darlington and Moss, 1968; Johnson and Spear, 1982; Leuzinger et al., 2005; Schwartz and Roizman, 1969;

van Genderen et al., 1994; Whealy et al., 1991). Three models have been proposed. In the first, particles enveloped at the inner nuclear membrane fuse with the outer nuclear membrane (Skepper et al., 2001). This results in the de-envelopment of nucleocapsids and entry of the naked capsids into the cytoplasm. These nucleocapsids then bud into the trans-Golgi network and the enveloped particles are released through secretory vesicles (Browne et al., 1996; Whiteley et al., 1999). The second model proposes that the enveloped particles in the perinuclear space move into the lumen of the endoplasmic reticulum (ER) and then follow the vesicular exocytic pathway (Whealy et al., 1991). In the third proposed model, nuclear pores are dilated and disassembled such that the capsids exit the nucleus through distorted pores (Leuzinger et al., 2005).

Nowadays, the most generally accepted model is envelopment and de-envelopment at the nuclear membrane followed by Golgi trafficking. Naked nucleocapsids are visible in the cytoplasm by electron microscopy (EM), and inhibition of the secretory pathways blocks viral egress and causes accumulation of virions in cytoplasmic vesicles (Cheung et al., 1991; Johnson and Spear, 1982; Whealy et al., 1991). In contrast, microscopic studies do not typically report capsids in the ER. Furthermore, HSV-1 envelope proteins engineered to be targeted to or retained in the ER are not incorporated into mature virions (Browne et al., 1996; Whiteley et al., 1999). The lipid content of virions show similarities to trans-Golgi membranes rather than nuclear membranes, further supporting the first model (van Genderen et al., 1994). Genetic evidence also supports the first model, in that nucleocapsids accumulate in the perinuclear space in HSV-1 or

pseudorabies virus (PrV; another *Alphaherpesviridae* member) knockouts in U_S3 or gK (Klupp et al., 2001; Reynolds et al., 2002; Wagenaar et al., 1995). The egress of virions completes the replication cycle of HSV-1.

1.2.6 Regulation of HSV-1 gene expression

As already described, the expression of HSV-1 proteins is sequential and coordinated (**Figure 1.1**). This differential regulation results from differences in the general organization of the promoter and regulatory sequences of different HSV-1 gene classes [reviewed in (Rajcani et al., 2004)]. In general, IE promoters contain numerous binding sites for cellular transcription factors upstream of a TATA box. IE promoter sequences also contain enhancer-binding sites. These sites consist of reiterated core enhancer elements (often designated TAATGARAT, for the most conserved consensus sequence). E promoters contain binding sites for only two or three cellular transcription factors upstream of the transcriptional start site. CAAT or CCATT boxes are also common in the promoters of E genes. L gene promoters contain only one or two upstream binding sites of a TATA box, an initiator element, and a downstream activator element. The key feature that distinguishes E and L promoters from each other is the arrangement and composition of the regulatory elements.

IE gene transcription is activated by the assembly of the HSV-1 IE transcription enhancer core complexes onto TAATGARAT sequences. TAATGARAT sequences are bound by a complex of host cell factor 1 (HCF-1), octamer binding protein (oct-1), and VP16. The binding of the trimeric complex

stimulates the transcription of IE genes. The IE proteins infected cell polypeptide (ICP) 0, ICP4, ICP22, and ICP27 coordinate the expression of E and L proteins. E proteins are involved in HSV-1 DNA replication and metabolism. Such E proteins include the viral DNA polymerase and thymidine kinase (TK), among many others. L gene products are mostly structural proteins and are expressed following HSV-1 DNA replication. Although a typical structure of L gene promoters has been described, the mechanism of L gene activation as a result of DNA replication is yet unclear. As infection progresses, expression of IE and E proteins decreases and that of L proteins increases.

1.3 CHROMATIN

Like cellular DNA, the nuclear HSV-1 genomes also associate with histones, albeit to different degrees. Histones are the proteins that compact DNA into a complex called chromatin. Chromatin is basically composed of chains of so-called "nucleosomes", ~146 base pairs (bp) of DNA wrapped 1.75 turns around a histone core octamer. This histone octamer is composed of two copies each of the core histones H2A, H2B, H3, and H4. Linker histone H1 binds at entry and exit points on nucleosomes and to linker DNA in between nucleosomes. H1 induces chromatin folding into higher order structures.

Histones bound to the DNA in the nucleosome present an obstruction for DNA replication, transcription, and repair. To access the DNA in these higher order chromatin structures, chromatin must be altered such that histones can be removed from DNA. Chromatin that is transcriptionally inert and condensed is

called heterochromatin, whereas that which is transcriptionally active is called euchromatin.

Eight different types of posttranslational modifications have been detected on over 60 different residues on histones [reviewed in (Kouzarides, 2007)], acetylation, methylation, phosphorylation, ubiquitination, sumoylation, adenosine diphosphate (ADP) ribosylation, deinimation, and proline isomerization (**Table 1.1**). These modifications do not always occur independently. An additional level of complexity comes from modifications that occur in different forms, such as methylation (mono-, di-, and tri- for lysines and mono- or di- for arginines [Arg, R]). These posttranslational modifications are not uniformly distributed throughout chromatin either. Rather, the specific combinations of the histone modifications are indicative of whether or not chromatin is transcriptionally competent. Certain chromatin modifications are often referred to as "marks" of transcriptional competence.

In yeast, different patterns in the types of modifications on the histones on inert or transcribed genes have been discovered. For example, acetylation is enriched on lysines (Lys, K) 9, 18, and 27 on H3 tails within active promoters and at the 5′ end of the coding regions of their respective genes [reviewed in (Lennartsson and Ekwall, 2009)]. Lysine trimethylation at H3K4, H3K36, and H3K79 is also enriched in the coding region of active genes. Each of these three methylation sites has specific distribution patterns, depending on the location within the actively transcribed gene [(Steger et al., 2008) and reviewed in

Table 1.1 Histone modifications

Histone modification	Core histone residues modified	Histone modifying enzymes and examples*
Acetylation	H2A (K5) H2B (K12, K15) H3 (K9, K14, K18, K23, K56) H4 (K5, K8, K12, K16)	Acetyltranferases (CBP/p300), Deacetylases (SirT2)
Methylation	H3 (R2, K4, R8, K9, R17, R26, K27, K36, K79) H4 (R3, K20)	Lys (SET1) and Arg (CARM1) methylases, Lys demethylases (LSD1/BHC110)
Phosphorylation	H2B (S14) H3 (T3, S28) H4 (S1)	Ser/Thr Kinases (CKII)
Ubiquitylation	H2A (K119) H2B (K112, K120)	Ubiquitilases (Bmi/Ring1A)
Sumoylation	Not known (K?)	SUMO-conjugating E2 (UBC9)
ADP ribosylation	H2A (K13) H2B (E2, K30) H3 (K27, K37) H4 (K16)	poly(ADP-ribose) tranferase (Ap4A)
Deimination	H2B (R3) H3 (R2, R8, R17, R26) H4 (R3)	Deiminase (PADI4)
Proline isomerization	H3 (P30, P38)	Proline isomerases (FPR4)

K, lysine; R, arginine; S, serine; T, Threonine; E, glutamic acid; P, proline

* Examples are in parentheses

(Kouzarides, 2007)]. Evidence from mouse and human tissues indicates that such patterning is conserved in higher eukaryotes.

Replacement of canonical histones with certain histone variants is another indicator of transcriptionally active loci. For example, the H3 variant H3.3 is enriched in active chromatin (Ahmad and Henikoff, 2002). This H3 variant is also enriched in "marks" of transcriptional activation. In both yeast and mammals, the nucleosomes flanking active initiation sites are also enriched in the H2A variant H2A.Z [termed Hzt1 in yeast; (Albert et al., 2007)].

Transcription, DNA repair, replication, and chromosome condensation are all regulated by histone modifications (**Table 1.1**). Acetylation, phosphorylation, and ubiquitination are functionally relevant for transcription activation. Methylation, ubiquitination, sumoylation, deimination, and proline isomerization are functionally relevant for repression. The function of ADP ribosylation is still unclear.

There are two roles for histone modifications in gene regulation. Histone modifications alter chromatin structure and the binding sites for histone-binding proteins. For example, certain histone modifications such as acetylation can disrupt histone-DNA contacts or histone contacts within or between nucleosomes, thus affecting the chromatin structure. Disruptions of histone-DNA or histone-histone contacts cause the chromatin to become less dense (decompact). The replacement of canonical histones with histone variants is also thought to also influence the stability of nucleosomes and the density of chromatin.

Histone modifications also provide, or disrupt, binding sites for non-histone proteins. For example, proteins containing bromodomains recognize particular lysine acetylation patterns. Bromodomains are present in transcription factors such as p300/cyclic adenosine 3'5' monophosphate [cAMP] response element binding protein (CREB)-binding protein-associated factor (PCAF) and proteins required for transcription such as TATA-binding protein (TBP)-associated factor 1 (TAF1). Bromodomains are also present in proteins that modify chromatin such as the histone acetyltransferase (HAT) co-activator CREB binding protein (CBP) and the chromatin remodeler Brahma (BRM)-related gene 1 protein (BRG1; the central catalytic ATPase subunit of numerous chromatin-remodeling complexes [reviewed in (Sanchez and Zhou, 2009)]). Therefore, certain lysine acetylation patterns induce the recruitment of proteins that are required for, or facilitate, transcription.

1.3.1 Chromatin and HSV-1

HSV-1 genomes are devoid of histones within the virions (Cohen et al., 1980; Gibson and Roizman, 1971; Hall et al., 1982; Loret et al., 2008; Oh and Fraser, 2008; Pignatti and Cassai, 1980). However, the chromatinization state of HSV-1 DNA in the nucleus of lytically infected cells has been a subject of debate, and is still an area of active research.

Micrococcal nuclease (MCN) digestion is one of the techniques used to evaluate chromatinization. MCN preferentially cleaves linker DNA in between nucleosomes. In early studies using MCN digestion, HSV-1 genomes were

shown to associate with nucleosomes during latent infection, such that regular repeating nucleosome patterns were observed (Deshmane and Fraser, 1989). In contrast, such regular repeating nucleosome patterns were not detected in lytically infected cells (Leinbach and Summers, 1980). From these observations, it was concluded that HSV-1 genomes were not regularly chromatinized during lytic infections.

More recently, a technique called chromatin immunoprecipitation (ChIP) was used to identify DNA sequences associated with histones in cells lytically infected with HSV-1. In ChIP assays, DNA and its associated proteins are cross-linked and the DNA is then sheared. Histones are immunoprecipitated, and the coimmunoprecipitated DNA sequences are analyzed. ChIP assays suggested that several genes in each of the HSV-1 gene classes were associated with histones at early times during lytic infections (Herrera and Triezenberg, 2004; Huang et al., 2006; Kent et al., 2004). Furthermore, the HSV-1 genomes were associated with histones containing marks of chromatin competent for transcription (Huang et al., 2006; Kent et al., 2004). ChIP analyses also showed a enrichment of the histone variant H3.3, in comparison to the canonical histone H3.1, on HSV-1 genes during lytic infection (Placek et al., 2009). However, a far smaller percentage of HSV-1 DNA than cellular DNA was detected to be associated with histones.

Several other lines of evidence suggest a role for chromatin in the regulation of HSV-1 protein expression. The activation-domain of VP16 associates with, and recruits, a number of transcription factors and chromatin modifiers, such as HATs p300 and CBP, or adenosine triphosphate (ATP)-

dependent remodeling complexes BRG1 (the central catalytic ATPase subunit of numerous chromatin-remodeling complexes) and BRM to IE gene promoters (Herrera and Triezenberg, 2004). ICP0 also alters the chromatin associated with HSV-1 genomes (Cliffe and Knipe, 2008). Furthermore, VP16 forms a complex with HCF-1, which has been identified as a component of several chromatin-modifying complexes, including a Set1 methyltransferase complex (Wysocka et al., 2003). Such evidence suggests that HSV-1 genomes do indeed associate with histones.

Our group developed a modified MCN digestion technique to detect unstable MCN digestion intermediates. In classic MCN digestions, nucleosomes are exposed to MCN for long periods. MCN therefore digests the DNA in any unstable nucleosome. In the modified protocols, infected cells were harvested. Their nuclei were isolated and the so-called "soluble" and "insoluble" chromatin was fractionated by differential centrifugation. The soluble chromatin is the supernatant containing soluble proteins, protein-free DNA, and small complexes containing mono-, di-, and short polynucleosomes. The insoluble chromatin is the pellet containing large complexes, including large polynucleosome chains and nuclear matrix. The insoluble chromatin was then resuspended in fresh MCN buffer and digested during a differential centrifugation. The soluble chromatin released into the supernatant was removed every 5 min. The MCN activity in these supernatants was promptly quenched by chelating calcium with ethylene glycol tetraacetic acid (EGTA). The insoluble chromatin was then again resuspended in fresh MCN buffer, centrifuged, and the MCN activity was

quenched. The entire process was repeated nine times and all the soluble fractions were pooled. The digestion products were then resolved by sucrose gradients and evaluated by Southern blot.

Using this technique, our group showed that lytic HSV-1 genomes are actually in complexes with the properties of unstable nucleosomes (Lacasse and Schang, 2010). The unstable nucleosomes were regularly spaced, much like cellular nucleosomes. The presence of unstable nucleosomes suggests that histones are easily removed from the HSV-1 DNA, which is consistent with the observation that only a small percentage of the viral genome appeared to associate with histones when evaluated by ChIP assays (Oh and Fraser, 2008).

1.4 FATE OF INFECTED CELLS

A series of obvious morphological changes occur in HSV-1 infected cells. For example, HSV-1 infected cells round, swell, and aggregate. The nucleolus becomes enlarged, is then displaced towards nuclear membrane, and eventually disaggregates and fragments. Concurrently, infection induces chromatin marginalization. The nucleus itself becomes distorted and multilobed at later times of infection. Some HSV-1 mutants also cause the plasma membranes of infected cells to fuse with neighboring cells to form multinucleated cells.

1.4.1 ND10 dispersal

In addition to the gross morphological changes described above, HSV-1 also induces several subtler ones. For example, several cellular proteins are dispersed away from ND10s. ND10s, also known as nuclear dots, promyelocytic leukemia protein (PML) nuclear bodies, or PML oncogenic domains (PODs) (herein referred to as ND10s), are spherical proteinaceous substructures ranging from 0.2 – 1 μm in diameter. These structures form distinct foci within the interchromosomal space of the nucleus. ND10s are present in all rat and human cell types tested. However, their frequency and size depends on the cell line. Their frequency and size also change physiologically throughout the cell cycle, and pathologically due to viral infection (Everett et al., 1999). During the cell cycle and viral infection, the changes in ND10 number and size result from both post-translational modification and levels of ND10 proteins (Ascoli and Maul, 1991; Everett et al., 1999). The number of ND10s is also increased by interferon (IFN) or heat shock stimulation, due to the upregulation of a number of ND10 associated proteins (Guldner et al., 1992). ND10s normally occur with a frequency of 2 – 30 per cell, with an average of 10 per cell - hence the original designation nuclear dots 10 [**Figure 1.2**; (Ascoli and Maul, 1991)].

An expanding list of proteins have been reported to be constitutively or transiently present at ND10s (Negorev and Maul, 2001; Tavalai and Stamminger, 2008). However, their functions at these domains remain mostly unclear. ND10s

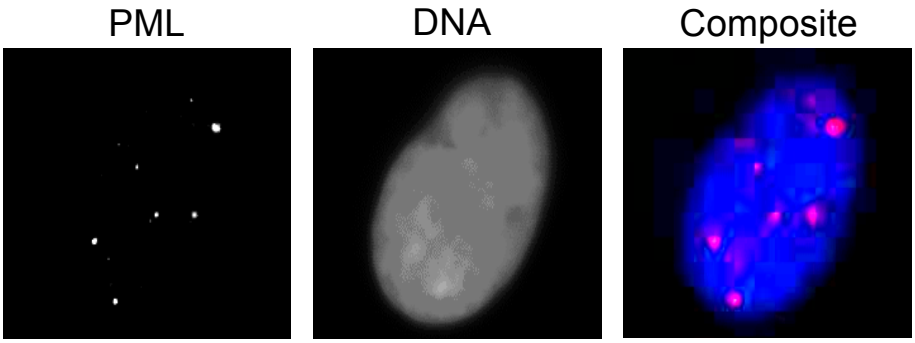


Figure 1.2 ND10s in mock infected U2OS cells. PML immunofluorescence and Hoescht 33258 stained images of fixed uninfected cells. Images were collected using a Zeiss LSM 720 confocal microscope. Uninfected U2OS cells were fixed for 15 min in 4% formaldehyde. ND10s were detected by the visualization of **PML** by indirect immunofluorescence. The **DNA** was counterstained with Hoescht 33258.

contain proteins involved in diverse biological processes such as regulation of chromatin structure, transcription, apoptosis, and DNA damage repair (Dellaire and Bazett-Jones, 2007; Tavalai and Stamminger, 2008).

The PML protein is among the constitutive ND10 proteins. PML belongs to a family of proteins characterized by the presence of the really interesting new gene (RING)-B-box-coiled-coil (RBCC) motifs. PML and its modification by the small ubiquitin-like modifier 1 (SUMO-1) are required for ND10 formation and persistence (Ishov et al., 1999; Zhong et al., 2000). In addition to PML, the other major permanent components of ND10s are Sp100 nuclear antigen (Sp100), death-domain-associated protein (Daxx), SUMO-1, and Bloom helicase (BLM) (Negorev and Maul, 2001; Yankiwski et al., 2000). Some other proteins, such as the phosphorylated retinoblastoma protein (pRb) (Alcalay et al., 1998), CBP (Boisvert et al., 2001), and the DNA repair proteins Nibrin (NBS1) and meiotic recombination 11 (Mre11) (Lombard and Guarente, 2000), localize to ND10s transiently. Neither chromatin nor RNA is found within the central core of ND10s, but newly synthesized RNA appears to associate with their periphery (Boisvert et al., 2000).

The genomes of a variety of DNA viruses are also detected in close association with ND10 [reviewed in (Ishov and Maul, 1996; Maul, 1998)]. Several viruses have also been shown to induce the dispersal of ND10 proteins, or their relocalization into novel structures (Maul et al., 1993; Tavalai et al., 2006). For example, HSV-1 genomes localize to sites adjacent to ND10s, but expression of IE proteins then induces the disruption of the ND10. This disruption is

dependent on the IE protein ICP0 and the proteasome (Everett and Maul, 1994; Maul and Everett, 1994; Maul et al., 1993). ICP0 is a non-specific transcriptional activator with E3 ubiquitin ligase activity. ICP0 is sufficient to induce ND10 disruption (Everett and Maul, 1994; Maul and Everett, 1994; Parkinson and Everett, 2000). ICP0 disruption of ND10s requires ubiquitination (Everett, 2000), and subsequent proteasome-dependent degradation (Chelbi-Alix and de The, 1999), of PML and Sp100 modified or unmodified by SUMO-1 (Everett et al., 1998). The RING finger domain of ICP0 is required for this proteasome-dependent degradation (Boutell et al., 2002; Maul and Everett, 1994). After the dispersal of ND10s, HSV-1 DNA and HSV-1 DNA replication proteins remain in the nuclear domains adjacent to where the ND10s used to be.

1.5 HSV-1 DNA REPLICATION

The current model of HSV-1 DNA replication proposes that it proceeds in two phases. The initial phase consists of origin-dependent theta replication, initiated at one or more origins. The second phase is origin-independent rolling-circle replication in conjunction with recombination. This latter phase produces concatemers, which are subsequently cleaved and packaged into infectious virions. Reconstitution of the rolling-circle phase of HSV-1 DNA replication using cellular extracts and the HSV-1 DNA replication proteins *in vitro* has been performed with only partial success (Skaliter and Lehman, 1994). Theta phase replication has not been reconstituted, nor has any evidence been found of theta

replication intermediates. Furthermore, it is not clear how the switch from Theta replication to rolling-circle would occur, or how would it be triggered. Recombination is likely very important during HSV-1 infection. Evidence of the involvement of recombination during HSV-1 infection includes the high frequency of recombination between coinfecting viral genomes [reviewed in (Fields et al., 2007)], and the genome isomerization (Sarisky and Weber, 1994), which is thought to result from homologous recombination stimulated by dsDNA breaks. This recombination utilizes the replication machinery of HSV-1 [reviewed in (Wilkinson and Weller, 2003)]. Furthermore, replicating HSV-1 DNA has branched structures. These branched structures consist of Y- and X-junctions, which likely represent DNA replication forks and recombination intermediates or merging replication forks, respectively (Dutch et al., 1995; Severini et al., 1996).

1.5.1 A model for HSV-1 genome replication

Nuclear HSV-1 DNA accumulates in the nucleus and adopts an "endless" configuration, consistent with circularization, even in the absence of viral protein synthesis (Garber et al., 1993; Strang and Stow, 2005). The mechanisms of circularization have not been characterized, but may involve recombination. Presumably, the circularized genomes then act as templates for origin-dependent Theta DNA replication. After a period of Theta replication, HSV-1 DNA replication then likely continues via a rolling circle mechanism. Head-to-tail concatemers are then detected. However, pulsed-field gel electrophoresis did not

identify the head-to-tail DNA concatamers as linear molecules when cut only once per genome with sequence-specific endonucleases (Severini et al., 1996). When analyzed by EM, HSV-1 DNA replication intermediates contained DNA replication forks, loops and branched structures (Friedmann et al., 1977). These lines of evidence all suggest that the mechanism of replication is sigma or rolling-circle accompanied by the formation of complex branched intermediates, at least late in infection (Severini et al., 1996). However, other mechanisms of replication should not be discarded, such as the *Saccharomyces cerevisiae* 2μ plasmid replication model.

The 2μ yeast plasmid has evolved a mechanism whereby its copy number is amplified without the initiation of multiple rounds of replication (Nakai, 1993). The plasmid induces site-specific recombination (unique to the plasmid), which induces copy number amplification (Murray et al., 1987). During replication, the intra-molecular recombination reverses one replication fork relative to the other. Both forks then travel in the same direction around a circular monomeric template. This generates large multimers from a single monomeric template and a single initiation of replication (Futcher, 1986). Like the Theta to rolling circle model described above, 2μ replication also uses circular templates and requires only one origin of replication. Furthermore, 2μ replication results in DNA segment inversion, concatemer production, and high frequency recombination. However, the position of the origin is important for the establishment of the 2μ rolling circle and it is not known whether the positions of the HSV-1 origin of

DNA replication allow the intramolecular recombination event necessary for the establishment of the 2μ rolling circle.

1.5.2 HSV-1 proteins required for HSV-1 DNA replication

Seven HSV-1 proteins are required for HSV-1 DNA replication, the ssDNA binding protein ICP8 (U_L29), the heterotrimeric helicase-primase (U_L5, U_L8, and U_L52), the origin binding protein (OBP; U_L9), the DNA polymerase (U_L30), and the DNA polymerase processivity factor (U_L42). The localization of these HSV-1 proteins to the HSV-1 genomes at sites adjacent to ND10s occurs sequentially and is organized by the first protein to localize to these domains, ICP8.

1.5.2.1 ICP8, the ssDNA binding protein

ICP8 is required for HSV-1 replication (Conley et al., 1981) and is highly conserved across all of *Herperviridae* [reviewed in (Fields et al., 2007)]. ICP8 conditional lethal mutants do not generate progeny DNA (Gao and Knipe, 1993; Weller et al., 1983) due to defects in HSV-1 DNA replication. ICP8 is the product of the *U_L29* gene. It consists of 1196 amino acids and has a theoretical molecular mass of 128,342 Daltons (Da). It is a Zinc metalloprotein that binds DNA (Gupte et al., 1991). ICP8 contains a nuclear localization signal, DNA-binding domain, and a Zinc-binding domain (**Figure 1.3**). Each ICP8 protein contains equimolar amounts of chelated Zinc (Gupte et al., 1991), which is

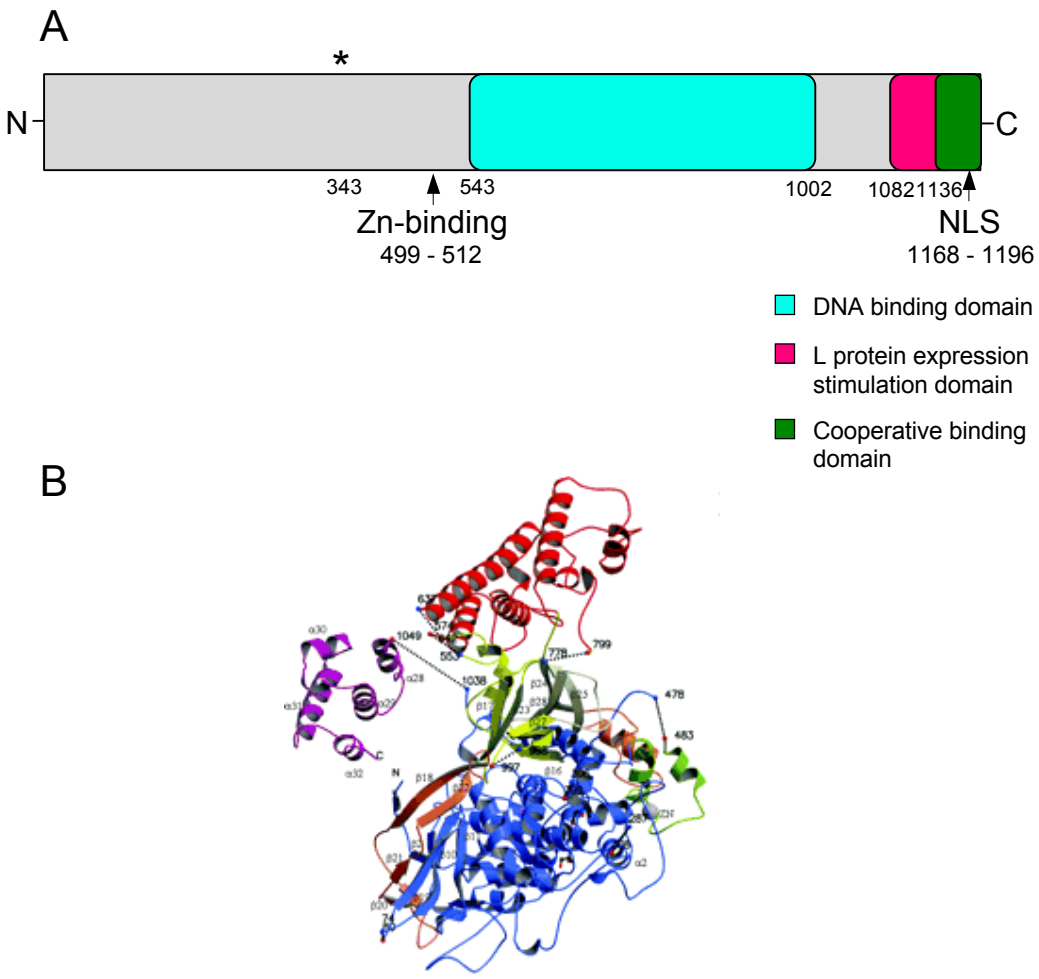


Figure 1.3 The structure of ICP8 and its domains. **A,** schematic representation of the primary structure of ICP8. The DNA binding domain (543 - 1002) is colored light blue, the L protein expression stimulation domain (1082 - 1169) is magenta, and the cooperative binding domain (1136 - 1196) is dark green. ***,** the location of the serine to phenylalanine mutation in the ICP8 *ts* mutant A1. **B**, crystal structure of ICP8 [taken from (Mapelli et al., 2005b)] Dotted lines represent disordered regions. The shoulder region is blue, the neck is yellow (front) and gray (back), and the head is red. The zinc binding region is green and the polypeptide chain linking the neck and shoulders is orange. The helical C-terminal domain is purple.

required for structural integrity. No posttranslational modifications have been reported on ICP8.

ICP8 binds to single-stranded (ss) DNA with fivefold greater affinity than it binds to dsDNA (Lee and Knipe, 1985). ICP8 binding to ssDNA is cooperative (Dudas and Ruyechan, 1998; Powell et al., 1981; Ruyechan et al., 1986). Binding is not sequence specific and occurs optimally at pH 7.6 in 150 mM NaCl (Lee and Knipe, 1985; Ruyechan and Weir, 1984). The frequency of ICP8 binding has been estimated to range from one ICP8 molecule per 12 nucleotides, as estimated by nuclease protection (O'Donnell et al., 1987a), to up to 40 nucleotides, as estimated by EM (Ruyechan, 1983). ICP8 also has the capacity to destabilize DNA helices (Boehmer and Lehman, 1993a), thereby holding the DNA in an extended conformation (Ruyechan, 1983), and lowering the melting temperature of synthetic polynucleotides such as poly(dA)-poly(dT) (Powell et al., 1981; Wang and Hall, 1990) or partially duplexed DNA (Boehmer and Lehman, 1993a).

ICP8 stimulates the activity of three DNA replication enzymes, the HSV-1 DNA polymerase (Hernandez and Lehman, 1990; Ruyechan and Weir, 1984), the helicase-primase, and the OBP. ICP8 stimulates both the helicase and DNA-dependent nucleoside triphophosphatase activities of the OBP (Boehmer et al., 1993; Dodson and Lehman, 1993). ICP8 is consequently required for the unwinding of HSV-1 origins of replication (*oriS* and *oriL*) by OBP (He and Lehman, 2000). ICP8 is also required for the complete unwinding of duplex DNA by the helicase-primase complex (Crute and Lehman, 1991). ICP8 therefore likely interacts with OBP to recognize and destabilize origins of

replication (Boehmer et al., 1993; Dodson and Lehman, 1993) and maintain the origins in extended conformations.

ICP8 may have a role in homologous pairing and strand transfer, suggesting a role in recombination (Bortner et al., 1993; Dutch et al., 1995; Dutch and Lehman, 1993; Reuven et al., 2003). ICP8 has been proposed to aid in the annealing of separated DNA strands. Complexes formed between ICP8 and ssDNA promoted pairing with homologous duplex DNA (Dutch and Lehman, 1993; Makhov et al., 2009; Nimonkar and Boehmer, 2003a). Deproteinization of these complexes releases products typical of a strand transfer reaction, such as ssDNA circles with dsDNA segments (a gapped circle) and an equal number of ssDNA fragments (the displaced strand) (Reuven et al., 2003). Strand transfer is dependent on Mg^{2+}, but not on ATP or other cofactors.

ICP8 also has activities not commonly associated with classical ssDNA binding proteins. ICP8 has been suggested to play a role in gene regulation, for example. ICP8 was reported to induce L protein expression from the progeny genome (Gao and Knipe, 1991). However, it is difficult to separate the role of ICP8 in DNA replication from any role in L gene expression. ICP8 is required for DNA replication, which is itself required for L gene expression. Nonetheless, certain ICP8 mutants inhibit L gene expression to a greater extent than they inhibit viral DNA replication (Chen and Knipe, 1996). Although more evidence supporting this proposed direct function of ICP8 in L gene expression is required, such reports do suggest such a possibility.

In addition to its direct roles in HSV-1 DNA replication, ICP8 is also required for the organization of viral DNA replication proteins into nuclear replication compartments (RCs) before the initiation of DNA replication. ICP8 localizes to sites adjacent to ND10s independently of other HSV-1 DNA replication proteins (Lukonis and Weller, 1997). This localization to ND10s is not a characteristic of other HSV-1 DNA replication proteins, suggesting that ICP8 is the organizational protein that results in the recruitment of all other DNA replication proteins to these sites. Consistent with this model, ICP8 directly interacts with U_L8 (Hamatake et al., 1997), U_L9 (Boehmer and Lehman, 1993b), and U_L42 (Hernandez and Lehman, 1990).

1.5.2.2 Helicase-Primase

The purification of a multisubunit complex required for HSV-1 DNA replication with helicase and primase activities resulted in the identification of three subunits, of 114,416; 98,710; and 79,921 Da (Crute et al., 1989). The subunits were then identified as the products of the *U_L52*, *U_L5*, and *U_L8* genes, respectively. Each of them is essential for viral replication. Amino acid sequence analysis of the U_L5 protein led to the identification of conserved ATP-binding and DNA helicase motifs (Hodgman, 1988). The U_L52 protein contains a proposed divalent metal-binding motif, which is conserved in DNA polymerases and primases. Site-directed mutagenesis of this motif inactivated the *in vitro* primase activity of the holoenzyme (Dracheva et al., 1995). U_L8 lacks detectable enzymatic or DNA-binding activities and a sub-assembly of U_L5 and U_L52 retains DNA-dependent

ATPase, helicase, and primase activities. U_L52 and U_L5 therefore constitute the catalytic core of the enzyme. However, U_L8 stimulates primer synthesis by the U_L5/U_L52 core enzyme by three-fold (Falkenberg et al., 1997). Furthermore, U_L8 is required for the interaction between the helicase-primase enzyme and ICP8 (Hamatake et al., 1997). Therefore, U_L8 was also required for efficient DNA helicase, DNA-dependent nucleoside triphosphatase, and primase activity in the presence of ICP8 (Hamatake et al., 1997).

1.5.2.3 OBP

The HSV-1 genome contains three origins DNA replication, two copies of *oriS*, a sequence located in the *c* sequences flanking the S component of HSV-1 genomes, and one copy of *oriL*, a sequence located between the genes for ICP8 and DNA polymerase [reviewed in (Lehman and Boehmer, 1999)]. The role of the multiple origins is unclear. Deletion of both copies of *oriS* (Igarashi et al., 1993) or the single copy of *oriL* (Polvino-Bodnar et al., 1987) does not have any effect on HSV-1 DNA replication in cultured cells.

The search for a required viral protein that binds to sequences within *oriS* led to the identification of OBP (Elias et al., 1986; Fierer and Challberg, 1992). OBP is the 94,246 Da product of the *U_L9* gene. It consists of 851 amino acids and has conserved ATP-binding and DNA helicase motifs, which are both essential for viral replication (Martinez et al., 1992). The DNA binding activity of U_L9 resides in the C-terminal 317 amino acids. U_L9 exists as a homodimer both in

solution or when bound to its dimeric DNA target sequence. Dimerization is mediated through the U_L9 N-terminal sequence.

The HSV-1 origins are palindromes centered at AT-rich regions (18 or 20 bp). OBP binds to two inverted repeats that flank the stretch of A + T residues within *oriS*, designated Boxes I and II (Elias and Lehman, 1988). In *oriL*, however, there is no Box II. Instead, additional copies of the Box I are located on both sides of the AT rich region. Occasional binding to a third homologous site (Box III) could be detected (Elias et al., 1992). Box III flanks Box I in *oriL* and the 5′ side of Box I in *oriS*. The precise DNA recognition site in Box I was mapped to a 10-bp sequence (5′ CGTTCGCACT) (Elias et al., 1992; Elias and Lehman, 1988; Hazuda et al., 1991; Koff and Tegtmeyer, 1988). Homologous sequences, in inverted orientations, also constitute the binding sites of Boxes II and III.

Binding of OBP to the dimeric origin sequences induces a bend in the DNA and the formation of a stem-loop structure. The loop at the tip of the stem-loop structure is the A-T rich region at the center of the origin. However, OBP is not sufficient for origin unwinding, ICP8 is also required (He and Lehman, 2000). ICP8 stimulates the helicase and DNA-dependent nucleoside triphophosphatase activities of the OBP (Boehmer et al., 1993; Dodson and Lehman, 1993). OBP and ICP8 physically interact with each other (Boehmer and Lehman, 1993b). Deletion of the 16 C-terminal amino acids of the U_L9 protein creates a mutant that retains origin-specific DNA binding, DNA-dependent ATPase, and DNA helicase activities, but has greatly reduced affinity for ICP8. This truncated protein had a

reduced capacity to sustain origin-dependent DNA replication (Boehmer et al., 1994), indicating that the interaction between ICP8 and U_L9 is important to HSV-1 DNA replication.

1.5.2.4 DNA polymerase and processivity factor

The DNA polymerase and processivity factor are the last of the HSV-1 proteins required for HSV-1 DNA replication to localize to the proper nuclear domains. HSV-1 DNA polymerase is the 136,413 Da (1235 amino acid) product of the *U_L30* gene. The HSV-1 DNA polymerase exists as a heterodimer with the 51,154 Da (488 amino acid) HSV-1 DNA polymerase processivity factor encoded by the *U_L42* gene. The processivity factor is a phosphoprotein that stimulates the HSV-1 DNA polymerase (Gallo et al., 1989). It has dsDNA binding activity and tethers the HSV-1 polymerase to DNA (Chow and Coen, 1995). The polymerase catalytic subunit possesses intrinsic $3' \rightarrow 5'$ exonuclease activity on a variety of substrates, including gapped DNA, DNA hairpins, and single-stranded oligodeoxynucleotides. It also removes unpaired deoxynucleotides (O'Donnell et al., 1987b). As expected, the exonuclease activity provides proofreading abilities (O'Donnell et al., 1987b). The structure of the HSV-1 DNA polymerase suggests a putative viral primase-helicase-interacting domain and the location of a putative RNA binding domain of unknown function (Liu et al., 2006).

1.5.3 Formation of the pre-replication and replication compartments

After HSV-1 genomes have localized to sites adjacent to ND10s, and IE and E proteins have been expressed, the HSV-1 DNA replication proteins localize to HSV-1 genomes. The first to do so is the ssDNA binding protein ICP8 (Lukonis and Weller, 1997) (top panels of **Figure 1.4**; **Table 1.2**). The heterotrimeric helicase-primase complex and OBP are subsequently recruited, likely through their interactions with ICP8. The localization of these five proteins is sufficient to recruit the HSV-1 DNA polymerase and processivity factor (U_L30 and U_L42, respectively) (Bush et al., 1991; Liptak et al., 1996). The recruitment of the polymerase and processivity factor is also thought to result from interactions with ICP8 (O'Donnell et al., 1987a). The recruitment of these seven proteins completes the formation of the so-called pre-replication compartments (pre-RCs; **Table 1.2**). HSV-1 DNA replication then starts and the sites become RCs (bottom panels of **Figure 1.4** and **Table 1.2**). It is unclear whether pre-RCs and RCs fuse or expand to become larger RCs (Taylor et al., 2003), which eventually fill the entire nucleus.

1.5.4 Inhibition of HSV-1 DNA replication

HSV-1 DNA replication has classically been inhibited by compounds that inhibit the HSV-1 DNA polymerase [phosphonoacetic acid (PAA) and acyclovir]. PAA is an orthophosphate analog, which does not inhibit the formation of the DNA polymerase-DNA complex, but rather interacts with the DNA polymerase at the pyrophosphate site. PAA, therefore, inhibits DNA replication elongation

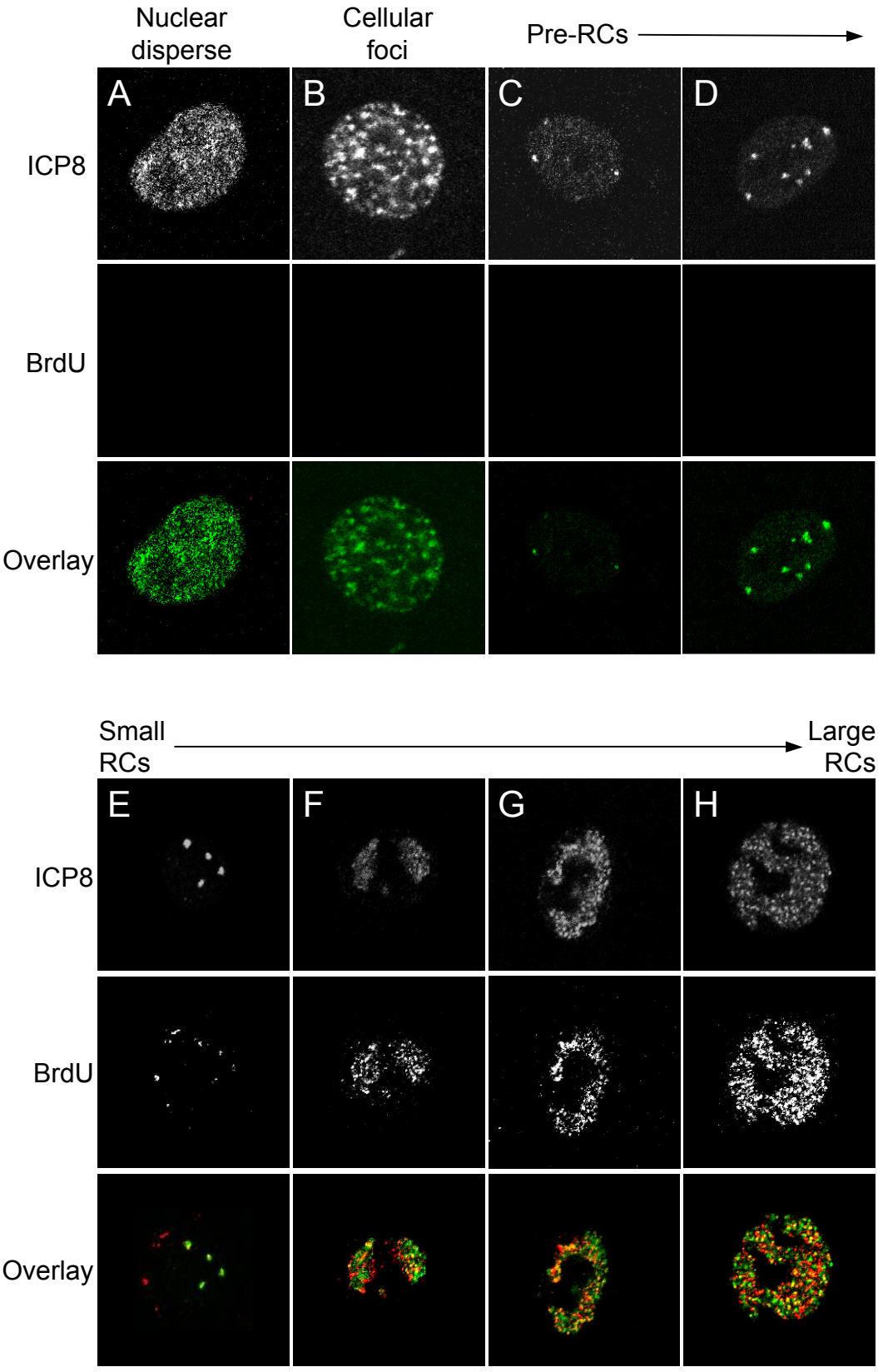

Nuclear disperse
Cellular foci
Pre-RCs
A
B
C
D
ICP8
BrdU
Overlay
Small RCs
Large RCs
E
F
G
H
ICP8
BrdU
Overlay

Figure 1.4 The progression of HSV-1 DNA replication as evaluated by immunofluorescence against ICP8 and BrdU. ICP8 and BrdU immunofluorescence images of fixed HSV infected cells. Images collected using a Zeiss LSM 720 confocal microscope. Vero cells infected with 30 infectious particles of HSV ICP8 *ts* mutant A1 per cell at 38^{o}C for 5 h. Cells were transferred to 33^{o}C for 0 to 6 h. At the end of this incubation, cells were pulsed with the thymidine analog BrdU for 15 min and fixed for 15 min in 4% formaldehyde. **ICP8** or **BrdU** were detected by indirect immunofluorescence. Nuclei were counterstained with Hoescht 33258. At early times after infection, ICP8 is **nuclear disperse** (**A**)**.** As the infection progresses, ICP8 localizes to either cellular ssDNA sites as **cellular foci** (**B**) or sites of HSV genomes as pre-replication compartments (**pre-RCs**; **C** and **D**). When HSV DNA replication starts, ICP8 localizes to replication compartments (**RCs**; **E** to **H**). Pre-RCs can be differentiated from RCs by the localization of ICP8 and BrdU at the same nuclear domain.

Table 1.2 Different localizations of ICP8

	ICP8 localization	Definition	BrdU staining	Examples*	Likely mechanisms
Non-HSV replication structures	Nuclear disperse	ICP8 punctae (<1 pixel) disperse throughout the nucleus	-	A	ICP8 nuclear localization signal[a,b,c]
	Cellular foci	>20 ICP8 foci (>1 pixel), evenly distributed throughout nucleus	-	B	ICP8 at sites of cellular ssDNA. For example, at stalled DNA replication forks[d]
HSV replication structures	Pre-RCs	<15 ICP8 foci (>1 pixel), not evenly distributed throughout nucleus	-	C, D	ICP8 bound to HSV DNA[d,e]
	RCs	Clustered ICP8 punctae (<1 pixel)	+	E - H	ICP8 bound to HSV DNA[f]

***** Figure 1.4

[a](Gao and Knipe, 1992); [b](Mapelli et al., 2005); [c](Taylor and Knipe, 2003); [d](Lukonis et al., 1997); [e](Burkham et al., 1998); [f](Taylor et al., 2003)

(Leinbach et al., 1976). Acyclovir also inhibits HSV-1 DNA replication through inhibition of the DNA polymerase. However, the mechanism of action of acyclovir is different from that of PAA. Acyclovir is phosphorylated first by the HSV-1 TK (Fyfe et al., 1978). A second phosphorylation event on the mono-phosphorylated acyclovir is performed by cellular guanylate kinase (Miller and Miller, 1980). The third and final phosphorylation of acyclovir could be performed by number of kinases such as phosphoglycerate kinase, nucleoside diphosphate kinase, or phosphoenol pyruvate kinase (Miller and Miller, 1982). Triphosphate acyclovir is the active antiviral agent. The triphosphate acyclovir competes with deoxyguanosine triphosphate for the DNA polymerase. Moreover, incorporation of the triphosphate acyclovir into elongating DNA chains results in chain termination. Acyclovir does not have the 3´- hydroxyl group required for the formation of a phosphodiester bond to extend the DNA chain. While the HSV-1 DNA polymerase is the typical target in the development of HSV-1 DNA replication inhibitors, compounds that inhibit the helicase-primase also inhibit HSV-1 DNA replication (Crute et al., 2002).

1.5.5 Cellular proteins required for HSV-1 DNA replication

Even in the presence of all the required HSV-1 proteins, origin-dependent HSV-1 DNA replication cannot be reconstituted without cellular factors (Nimonkar and Boehmer, 2003b). This inability to reconstitute HSV-1 DNA replication in the presence of all required HSV-1 proteins indicates a requirement of cellular proteins for HSV-1 DNA replication. For example, the HSV-1 genome does not

encode for ligases or topoisomerases although they are required for HSV-1 DNA replication. Consistently, knockdown or inhibition of cellular topoisomerases and ligases results in reduced viral titers (Hammarsten et al., 1996; Muylaert and Elias, 2007). Inhibition of a subset of cyclin dependent kinases (CDKs; CDK1, 2, 5, 7, and 9) with the pharmacological CDK inhibitor (PCI) roscovitine also inhibits HSV-1 DNA replication. The latter results suggest that HSV-1 DNA replication also requires a subset of CDKs. However, the mechanisms for such requirement are not entirely elucidated.

1.6 THE CELL CYCLE

The cell cycle is a conserved series of events required for growth, replication of the DNA, and division of a cell. It consists of four phases, Gap 1 (G1), synthesis (S), Gap 2 (G2), and mitosis (M; **Figure 1.5**). G1, S, and G2 phases are known together as interphase. M phase consists of mitosis and cytokinesis. Cells that have temporarily or permanently stopped dividing are said to have entered the Gap 0 (G0) phase, or quiescence. Control of the cell cycle is dependent on the catalytic CDKs and their regulatory subunits, the cyclins.

Classic studies concluded that the cell cycle is governed by families of CDKs, each paired with a specific cyclin (**Figure 1.5**). According to this classic model, specific CDK-cyclin complexes are critical for driving each phase of the cell cycle (van den Heuvel and Harlow, 1993). For example, CDK1 pairing with cyclin B1 is required for the G2/M transition in eukaryotes. This classic model has recently been challenged by transgenic mice, in which CDK loci were

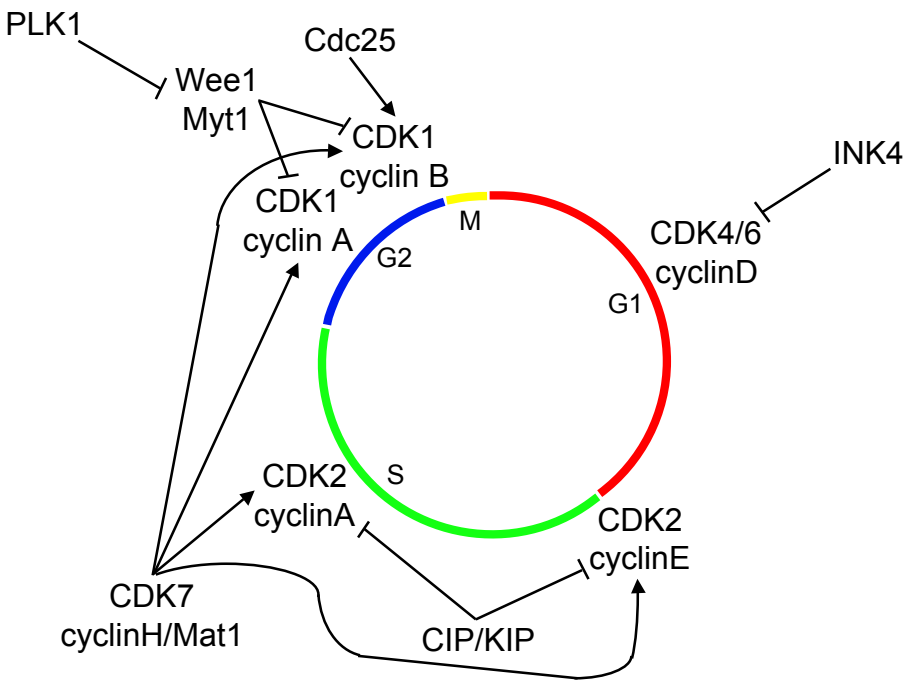


Figure 1.5 The classic cell cycle model. Cartoon representation of the classic model of the cell cycle. According to this model, progression through the different phases requires the activity of five CDKs. **CDK4** and **CDK6** associated with D-type cyclins are active during Gap1 (**G1**). **CDK2/cyclin E** complexes are active mainly during the G1/synthesis (**S**) transition. **CDK2/cyclin A** complexes are active during **S**. **CDK1/cyclin A** and **CDK1/cyclin B** complexes are active during Gap2 (**G2**) and mitosis (**M**). The activity of CDK1 and CDK2 are activated by **CDK7**/**cyclin H**/ménage à trois 1 (**Mat1**). The activity of CDKs is inhibited by two families of CDK inhibitors (CKIs; **INK4** inhibits CDK4 and CDK6 complexes and **CIP/KIP** inhibits CDK2 complexes). CDK1 is also inhibited when phosphorylated by **Wee1** and **Myt1**. Wee1 and Myt1 activity is inhibited by polo-like kinase (**PLK1**). The inhibitory phosphorylation of Wee1 and Myt1 are removed by the phosphatase Cdc25. Therefore, CDK1 is activated by the Cdc25.

systematically knocked out. These mutations resulted in developmental defects only in specialized cell types [reviewed in (Malumbres and Barbacid, 2009)], which suggested that the classic model was incorrect.

1.6.1 Eukaryotic cell cycle regulatory CDKs and cyclins

According to the classic model, five CDKs and their associated cyclins play direct roles in the regulation of the cell cycle: CDK1, 2, 4, 6, and 7 [reviewed in (Fung and Poon, 2005; Golias et al., 2004; Malumbres and Barbacid, 2009; Morgan, 1995)]. CDK3 is also thought to play a role in cell cycle regulation, but its specific role is not understood. Supporting a role for CDK3 in the cell cycle, CDK3 RNA is not detected in organs that contain few cycling cells, such as human heart, brain, skeletal muscle, and pancreas (Meyerson et al., 1992). However, CDK3 is truncated and inactive in the Castle lineage of *Mus musculus*, which includes many common laboratory mice. Therefore, mouse strains such as BALB/C and C57/B6 express no functional CDK3 (Ye et al., 2001). CDK3 will not be discussed any further. CDK5 likely plays no role in cell cycle regulation. CDK5 plays important roles in neuronal maturation and migration. CDK8 and 9 are not considered to be classic cell cycle regulators either. CDK8 negatively regulates transcription (Knuesel et al., 2009). CDK9 (and CDK7) facilitate transcription by phosphorylating the C-terminal domain of the RNA polymerase. Therefore, CDK8 and -9 are indirectly important for cell cycle progression, but are not directly involved in its regulation. CDK 8 or 9 will not be discussed here either.

The activity of CDKs is regulated by five mechanisms (Morgan, 1995). By definition, CDKs must bind to a regulatory cyclin subunit to be active. Cellular levels of CDKs do not fluctuate significantly throughout the cell cycle. Therefore, the first mechanism for CDK activity regulation is the level of their regulatory units, the cyclins. The level of cyclins (and therefore the activity of their associated CDK) is controlled at the level of transcription and degradation. Cyclin degradation is proteasome- and ubiquitin-dependent. CDK4 and CDK6 bind to, and are activated by, D-type cyclins. CDK2 binds to, and is activated by, A- and E-type cyclins. CDK1 binds to, and is activated by, A- and B-type cyclins. CDK7 binds to, and is activated by, H-type cyclins and ménage à trois homolog 1 (Mat1).

The second mechanism for the regulation of CDK activity is the subcellular localization of the CDK/cyclin complex. For example, cyclin B levels increase in S phase and G2, but cyclin B remains in the cytoplasm in a complex with CDK1 [reviewed in (O'Farrell, 2001)]. Just before mitosis, the nuclear export signal on cyclin B (in complexes with CDK1) is phosphorylated and activated (Jackman et al., 2003). The complex then translocates to the nucleus to phosphorylate its nuclear targets.

The third mechanism for regulation of CDK activity is phosphorylation on a conserved residue on the CDK [threonine (Thr) 161 on human CDK1, Thr160 on CDK2, and Thr170 on CDK7). This required phosphorylation is on the activation T-loop, which when unphosphorylated blocks the substrate binding

site [reviewed in (Morgan, 1995; Pines, 1995)]. The T-loop phosphorylation is required for the activation of certain CDKs [-1, -2, and -7; (Larochelle et al., 2001; Russo et al., 1996) and reviewed in (Morgan, 1997)], but not others (-8). CDK7/cyclin H/Mat1 is the CDK activating kinase (CAK) in mammals, and therefore also plays an important role in CDK regulation (**Figure 1.5**).

The fourth mechanism of regulation of CDK activity is the association of CDKs with inhibitory subunits [CKIs; reviewed in (Malumbres et al., 2000; Pei and Xiong, 2005); **Figure 1.5**]. There are two classes of mammalian CKIs, as classified by their structures and CDK targets. The CIP/KIP proteins p21 (CIP1/WAF1/CAP20/SDI1), p27 (KIP1), and p57 (KIP2) have a preference for cyclin E- and cyclin A-dependent kinase complexes (CDK2-cyclin complexes). The INK4 family of CDKIs p16 (INK4), p15 (INK4B), p18 (INK4C), and p19 (INK4D) has a preference for CDK4 and CDK6. The cellular levels of the inhibitory subunits are also controlled by transcription, translation, and degradation.

The fifth mechanism of CDK regulation is inhibitory phosphorylation at a conserved site (Thr14 and Thr15 in human CDK1 and CDK2). This inhibitory phosphorylation induces steric hindrance in the substrate binding site (Welburn et al., 2007). CDK1/cyclin B Thr14 and 15 are phosphorylated by the kinases Wee1 and Myt1, and dephosphorylated by the Cdc25 phosphatases [reviewed in (Doonan and Kitsios, 2009); **Figure 1.5**]. Cdc25 dephosphorylates CDK1/cyclin B at the end of G2, resulting in the activation of CDK1 and mitosis. Further

increases in CDK1/cyclin B activity occur during mitosis, when the activity of the Wee1 and Myt1 decreases due to phosphorylation by polo-like kinase 1 [PLK1; reviewed in (O'Farrell, 2001)].

After cytokinesis, mitogenic signals such as growth factors and hormones stimulate the expression of transcription factors such as Myc. The increased expression of these transcription factors results in increased proliferation [reviewed in (Berthet and Kaldis, 2007)]. D-type cyclins are among the many proteins the expression of which is upregulated by these growth factors. The activity of CDKs in complexes with D-type cyclins is further stimulated by mitogen signaling in early G1, when the phosphoinositide 3-kinase (PI3K) pathway is stimulated by growth factors [reviewed in (Poznic, 2009)]. The stimulated PI3K pathway inhibits glycogen synthase kinase 3-β (GSK3β) phosphorylation and consequent degradation of cyclin D (Huang et al., 2007).

D-type cyclins preferentially bind to, and activate, CDK4 and CDK6. The primary role of these CDK-cyclin complexes is to phosphorylate, and thereby inactivate, the pocket proteins, such as the retinoblastoma protein (Rb), p107, and p130 [reviewed in (Harbour and Dean, 2000)]. Dephosphorylated pocket proteins bind to the E2F transcription factor. This binding results in two mechanisms of inhibition (Poznic, 2009). The Rb-E2F complex recruits histone deactylases to E2F-dependent promoters (Ferreira et al., 2001). In addition, E2F and Rb binding prevents the binding of E2F to certain E2F-dependent promoters and activation of E2F-dependent gene transcription [reviewed in (Poznic, 2009)]. The derepression

of E2F through Rb phosphorylation activates the expression of proteins required for processes such as DNA replication, including cyclin E, cyclin A, and itself, thus providing a positive feedback loop [reviewed in (Doonan and Kitsios, 2009)].

The levels of cyclin E also increase during G1. Cyclin E associates with CDK2. CDK2/cyclin E complexes function mainly at the G1/S transition by further phosphorylating Rb, and as a result further increasing the level of E2F activity. Cyclin D/CDK4 and -6 complexes may also perform a non-catalytic role, sequesteration of the CDK2 inhibitors p21 and p27. During S phase, cyclin A accumulates and binds to CDK2. These complexes phosphorylate E2F inhibiting its DNA-binding activity. Consequently, CDK2 directly, and CDK4 and -6 indirectly, inhibit E2F transcriptional transctivation activities.

CDK1/cyclin A complexes form during late S and early G2 phases (Merrick et al., 2008). CDK1/cyclin B complexes form during S and early G2 phases. They translocate to the nucleus and become active immediately before the G2/M transition. CDK1/cyclin A and CDK1/cyclin B complexes drive the G2/M transition. Following nuclear envelope breakdown, A-type cyclins are degraded by the proteasome. B-type cyclins are degraded shortly after. Approximately two hundred potential human CDK1 substrates have been identified (Ubersax et al., 2003), but only a subset of them have been identified to be relevant *in vivo*.

The cell cycle is also regulated by checkpoints, regulatory pathways which prevent progression of the cell cycle until all previous events have been properly completed. Checkpoints ensure the proper timing and completion of events such as DNA replication, chromosomal condensation, nuclear envelope breakdown, centrosome separation, and assembly of the mitotic spindle. Checkpoints arrest the cell cycle to allow for sufficient time to repair the errors. Checkpoints also stimulate signaling pathways to induce the transcription of certain genes that facilitate repair, such as those required for DNA damage repair.

The classic model described above has been challenged by transgenic mice with disrupted cyclin or CDK genes [reviewed in (Sherr and Roberts, 2004) and (Malumbres and Barbacid, 2009)]. CDK1, cyclin A2, and cyclin B1 are the only CDK or cyclin family members required for embryonic viability and as such cell cycle progression. The deletion of other CDKs and cyclins resulted only in tissue specific phenotypes. This surprising discovery demonstrates that the all the CDKs but CDK1 are dispensable for the cell cycle. It appears that other CDKs can bind to, and be activated by, the cyclin partners of the ablated CDKs (Aleem et al., 2005).

1.7 PHARMACOLOGICAL CDK INHIBITORS (PCIs)

PCIs were discovered during screens to identify selective inhibitors of CDK1/cyclin B complexes [reviewed in (Meijer and Raymond, 2003)]. The selective inhibitors were discovered by screens of compounds from a variety of

sources, such as microbial and plant products, small-molecule libraries, and combinatorial libraries (Hardcastle et al., 2002). During these screens, a number of compounds were identified as specific inhibitors of CDKs. Structure activity relationship studies of 2, 6, 9-trisubstituted purines were performed by the Meijer group in collaboration with Dr. J. Vesely, resulting in the discovery of roscovitine as a potent and selective inhibitor of a subset of CDKs [(De Azevedo et al., 1997) and reviewed in (Meijer, 2006)].

1.7.1 Roscovitine

Roscovitine (also called CYC202 or seliciclib) contains substitutions at positions two, six, and nine of an adenine moiety. Roscovitine contains a 1-ethyl-2-hydroxyethylamino group at position two, a benzylamino group at position six, and an isopropyl group at position nine. Roscovitine contains a chiral carbon in the position two substituent.

The selectivity of roscovitine has been tested experimentally or inferred theoretically (from the sequences of critical residues and crystal structure of the catalytic domain) against a total panel of 491 kinases [94.8% of the kinome; (Bach et al., 2005; Bain et al., 2003; Bain et al., 2007; Caffrey et al., 2008; Chen et al., 2007; Fabian et al., 2005; Fedorov et al., 2007; Graczyk, 2007; Karaman et al., 2008; Subramanian, 2010) and reviewed in (Meijer, 2006; Schang et al., 2006)]. Roscovitine is selective for only a very limited subset of kinases. Below 1 μM, roscovitine only inhibits 50% of activity (IC_{50}) of CDK1/cyclin B (0.45 μM), CDK2/cyclin A (0.25 μM), CDK2/cyclin E (0.10 μM), CDK5/p35 (0.16 μM),

CDK7/cyclin H (0.45 μM), and CDK9/cyclin T1 (positive transcription elongation factor b [P-TEFb]; 0.60 μM) [reviewed in (Schang et al., 2006)]. Another small subset of kinases is at least two-fold less sensitive to inhibition by roscovitine than CDK1, -2, -5, -7, and -9. These kinases are still sensitive to inhibition by 1 to 40 μM roscovitine. They are CDK3/cyclin E, casein kinase 1α (CK1α), casein kinase 1δ (CK1δ), dual-specificity tyrosine phosphorylation-regulated kinase 1A (DYRK1A), ephrin-B2 kinase (EPHB2), extracellular signal-regulated kinase 1 and 2 (ERK1 and ERK2, respectively), focal adhesion kinase (FAK), and interleukin-1 receptor-associated kinase 4 (IRAK4).

Roscovitine competes with ATP for binding at the ATP-binding site of CDKs. Such binding was confirmed by direct cocrystallization of (R)-roscovitine with CDK1/cyclin B and CDK2 (De Azevedo et al., 1997). Later, the crystal structures were also solved for CDK5/p25 (Mapelli et al., 2005) and CDK2/cyclin A (unpublished data from the Meijer group). The binding site for all these kinases is also the ATP-binding site.

Roscovitine has been tested on a wide panel of mammalian cell lines. Two major effects have been described. The first is an arrest in cell cycle progression. Roscovitine arrests the cell cycle of all cell lines. Depending on the cell line, dose, and treatment duration, roscovitine blocked the cell cycle at G0/G1, S, G2/M, or at a combination of these. The inhibition at these stages can be attributed to the inhibition of CDK2/cyclin E (inhibition of G1/S transition), CDK2/cyclin A (inhibition of S phase progression), CDK1/cyclin B (inhibition of prophase-to-metaphase transition). Roscovitine also inhibits the CDK7/cyclin

H/MAT1 complex. Therefore, roscovitine likely also prevents the activation of various CDKs (CDK1 and CDK2). Furthermore, inhibition of CDK2/cyclin E results in decreased p27 phosphorylation and, therefore, in its stabilization, accumulation, enhanced inhibition of its CDK targets (CDK2 and CDK4) and, consequently, arrest in G1.

The second cellular effect of roscovitine is induction of cell death. Roscovitine induces cell death in many cell lines at all phases of the cell cycle (McClue et al., 2002). The induction of cell death is not dependent on functional p53 (Payton et al., 2006; Raynaud et al., 2005) and is not thought to be exclusively a result of cell cycle arrest. Cell death is also induced in non-cycling cells.

The metabolism of roscovitine administered intravenously to mice results in metabolites such as oxidation products, conjugates with glucose, or products lacking the isopropyl group (Meijer, 2006; Nutley et al., 2005). Following oral administration of a single dose to healthy men, roscovitine was distributed throughout all tissues. The main metabolite was a carboxylated derivative (de la Motte and Gianella-Borradori, 2004). All roscovitine metabolites are inactive and cleared renally.

Roscovitine has been tested against a variety of human tumors in nude mice models, including colon, uterine carcinoma, breast, nasopharyngeal carcinoma, and Ewing's sarcoma (Meijer, 2006). In all cases, there was either inhibition of tumor growth or reduction in tumor size (Hui et al., 2009; Maggiorella et al., 2009; Maggiorella et al., 2003; McClue et al., 2002; Payton et

al., 2006; Raynaud et al., 2005; Tirado et al., 2005). The single maximum tolerated dose of roscovitine was 100 mg/kg in mice (intravenous) (Raynaud et al., 2005). Maximum tolerated dose in mice was not attained even at the highest possible doses either intraperitoneally or orally. Roscovitine was well tolerated up to 150 mg/kg intraperitoneally and up to 2,000 mg/kg orally (Raynaud et al., 2005).

Through sponsorship by Cyclacel, roscovitine has been evaluated in sixteen clinical trials including more than four hundred and fifty humans (Cyclacel, 2010). Roscovitine is currently in phase II human clinical trials in the US in patients with advanced solid tumors (NIH, 2010). No results have yet been published. Roscovitine was also tested in US human clinical trials for patients with non-small cell lung carcinoma, but the study was terminated for undisclosed reasons (NIH, 2010). Phase I trials have been completed in the United Kingdom in patients with malignant solid tumors (Benson et al., 2007), in Singapore in patients with undifferentiated nasopharyngeal carcinoma (Hsieh et al., 2009), and in France in patients with advanced solid tumors (Pierga, 2003).

In the United Kingdom clinical trial (Benson et al., 2007), 21 patients were given roscovitine orally at either 100, 200, or 800 mg twice daily for 7 days every 21 days (1 – 6 cycles per patient). Dose-limiting toxicities were observed at 800 mg, grade three (severe) fatigue, grade three skin rash, grade three hyponatraemia, and grade four (potentially life threatening) hypokalaemia. Other toxicities included reversible raised urinal creatinine (grade two; moderate), reversible grade three abnormal liver function and grade two vomiting. Similar

toxicities were seen when 16 patients were given roscovitine orally at 800 mg twice daily on days 1 to 3 and 8 to 12 in the Singapore clinical trial (Hsieh et al., 2009). No significant toxicities were observed when roscovitine was administered orally at 400 mg twice daily.

The French clinical trial (Pierga, 2003) included 49 patients with advanced solid tumors. The patients received roscovitine orally at escalating doses. Dose-limiting toxicities were observed at an oral administration of 800 mg twice daily for three consecutive days every two weeks. Roscovitine was well tolerated at doses up to 2,000 mg/day for five consecutive days every three weeks. The maximum tolerated dose was reached at 3,200 mg/day. The dose limiting toxicity was grade 3-4 (grade 3 – severe and grade 4 – potentially life threatening) vomiting. In a later trial by the same group, the maximum tolerated dose with a biweekly schedule was reached at 3,600 mg/day due to grade 3 hypokalemia.

The most recent French clinical trial included fifty-six patients with metastatic or locally advanced solid tumor or lymphoma (Le Tourneau, 2010). The conclusions reached from this study recommends oral administration of 1250 mg twice daily for 5 days every 3 weeks or 1600 mg twice daily every 2 weeks for phase II clinical trials. These recommendations are based on dose-limiting toxicities at higher doses, which were nausea, vomiting, asthenia, and hypokalaemia. Another toxicity was an increase of creatinine in serum due to abnormal liver function.

1.7.2 Antiviral effects of PCIs

Viruses that replicate in the nucleus do not often encode for all the proteins required for replication. These viruses therefore depend on the infected cell for the proteins that they require but do not encode. Often, the proteins required by the virus are only expressed during certain phases of the cell cycle. Therefore, manipulation of the host cell cycle replication machinery by viruses is a common strategy to optimize their replication. Because viruses often manipulate the cell cycle to their advantage, it is not surprising that drugs targeting CDK activity inhibit the replication of many nuclear viruses.

1.7.2.1 HSV-1 and -2

Experiments aimed at identifying cellular proteins that participate in viral replication led to the discovery of PCIs as inhibitors of HSV-1 replication (Schang et al., 1998). HSV-1 DNA replication (Schang et al., 2000) and transcription (Jordan et al., 1999; Schang et al., 1999) were later identified to be the targets of roscovitine. Roscovitine inhibits a step prior to the initiation of HSV-1, but not cellular, transcription. Therefore, roscovitine acts at a level at which neither CDK7 nor P-TEFb are known to play significant roles. Roscovitine does not inhibit transcription elongation. Roscovitine does not inhibit either the formation of the VP16-dependent transactivating complexes or the affinity of these complexes for the TAATGARAT sequences (Jordan et al., 1999).

Surprisingly, transcription of cellular genes under control of a cellular promoter recombined into the HSV-1 genome was inhibited by roscovitine. In

contrast, transcription of an HSV-1 gene under control of an HSV-1 promoter recombined into the cellular genome was not (Diwan et al., 2004). Furthermore, transcription of an HSV-1 gene under control of an HSV-1 promoter in transiently transfected plasmids was also inhibited (Diwan et al., 2004). Therefore, the inhibition of HSV-1 transcription by roscovitine is independent of promoter-specific factors, but specific for extrachromosomal DNA (Diwan et al., 2004).

Consistently with the specificity for HSV-1 genomes, roscovitine inhibited transcription from HSV-1 mutants resistant to acyclovir and PAA, as well as of HSV-2 (Schang et al., 2002a). HSV-1 DNA replication was inhibited even in the presence of all the required DNA replication proteins (Schang et al., 2000). Roscovitine also inhibits the reactivation of HSV-1 from latently infected neurons (Schang et al., 2002b).

1.7.2.2 VZV

The replication of the *Alphaherpesvirinae* VZV is also inhibited by PCIs such as roscovitine, even *in vivo* (Rowe et al., 2010). The role of CDKs in the replication of VZV is complex and involves several stages. VZV infection induces CDK1, CDK2, cyclin A, cyclin B1, and cyclin D3 expression. However, inhibition of replication by roscovitine appears to be primarily be a result of inhibition of transcription and altered subcellular localization of several VZV proteins, including IE proteins such as the major IE transactivator IE62 (Taylor et al., 2004). Roscovitine also inhibited phosphorylation and resulting essential activity of IE63. Active IE63 represses the transcription of specific VZV genes

(Habran et al., 2005). The inhibition of replication by roscovitine was not a consequence of apoptosis or cell cycle arrest (Taylor et al., 2004). Roscovitine was also shown to inhibit VZV DNA replication. However, VZV DNA replication is dependent on expression levels of IE proteins, which are affected by roscovitine treatment. Therefore, whether roscovitine directly inhibits VZV DNA replication is still unclear.

Roscovitine directly inhibits the CDK2 activity induced by VZV infection (Moffat et al., 2004). Moreover, roscovitine inhibits specific phosphorylations, such as the phosphorylation of the VZV glycoprotein gI by CDK1 and CDK2 (Ye et al., 1999). However, the role of this phosphorylation in the VZV life cycle and the consequence of the inhibition by roscovitine are not clear. CDK1/cyclin B complexes also phosphorylate IE62. CDK1/cyclin B complexes are then incorporated into virions (Leisenfelder et al., 2008). The effect of roscovitine on the CDK1/cyclin B phosphorylation of IE62 and incorporation into virions has not been evaluated.

1.7.2.3 HCMV

The inhibition of viral replication by PCIs such as roscovitine was first published for the *Betaherpesvirinae* member HCMV (Bresnahan et al., 1997; Bresnahan et al., 1996). Much like for HSV-1, roscovitine inhibited HCMV replication even when added at late times of infection (up to 48 h).

There are major differences in regulation of their gene expression between HSV-1 and HCMV. Therefore, it is not surprising that roscovitine had different

effects on the expression of HCMV or HSV-1 genes. Whereas roscovitine inhibits the expression of likely all HSV genes, it has differential effects on the expression of different HCMV genes. For example, roscovitine added at the time of infection resulted in decreased IE72 and increased IE86 expression from 0 – 24 h post infection (hpi) (Sanchez et al., 2004). These two proteins are translated from alternatively spliced transcripts from the same transcriptional unit (U_L122-123). Alternative splicing appears to be the cause of the altered expression levels. The differential splicing of the IE U_L37 RNAs was similarly affected (Sanchez et al., 2004). Differential expression of these transcripts was not dependent on the proteasome, nor did it require de novo protein synthesis. The inhibition of these few IE proteins was sufficient to inhibit the expression of selected E proteins. For example, the levels of two major E proteins (encoded by the U_L112 – 113 loci) were only modestly affected, whereas expression of U_L57, the ssDNA binding protein, was almost completely inhibited. Delaying the addition of roscovitine until 6 hpi still resulted in significantly reduced titers. However, roscovitine had no effects on IE and E protein expression if treatment was delayed until 6 hpi. Viral DNA replication was not directly inhibited by roscovitine (Sanchez et al., 2004).

HCMV infection induced changes in expression and activity of CDK7, CDK9, and their activating cyclins (Kapasi and Spector, 2008). HCMV infection also induces the hyperphosphorylation of the C-terminal domain (CTD) of RNA polymerase II [at serine (Ser) 2 and 5, which are phosphorylated by CDK7 complexes and CDK9 in P-TEFb respectively]. Roscovitine inhibits the

hyperphosphorylation of the CTD of RNA polymerase II (at Ser 2 and 5) in HCMV infected cells (Tamrakar et al., 2005). Furthermore, roscovitine inhibited the localization of CDK7 and P-TEFb to the sites of HCMV replication.

Roscovitine also inhibits late HCMV functions. Roscovitine added 24 h after infection still inhibited the release of infectious HCMV virions, likely by downregulating the levels of the structural proteins pp150 and gB. Roscovitine also induces the mislocalization of pU_L69 and decrease in pU_L69 activity (mRNA export). pU_L69 is phosphorylated *in vitro* by CDK1/cyclin B, CDK7/cyclin H/Mat1 and P-TEFb complexes and perhaps by CDK2/cyclin E (Rechter et al., 2009). This pU_L69 phosphorylation is partly inhibited by roscovitine.

The observations described above suggest that viral transcript processing and E gene expression require CDK activity during early stages of HCMV infection, and virion maturation at later stages.

1.7.2.4 EBV

The lytic replication of the *Gammaherpesvirinae* EBV is also inhibited by PCIs such as roscovitine (Kudoh et al., 2004). As for HSV-1, inhibition occurs even when roscovitine is added up to 9 h post induction of lytic replication. Afterward, there were significantly lower levels of inhibition. These results indicated that a step in EBV lytic replication that occurs up to 9 h post induction was the step primarily inhibited. As for HSV-1, PCIs inhibited the accumulation of IE and E proteins. The inhibition of both IE and E protein expression occurred even in the presence of the IE transactivator BZLF1, which is required for the switch from

latent to lytic replication in latently-infected cell lines. The effect of roscovitine on the expression of BZLF1 itself, however, was not evaluated. PCIs also inhibited the expression of reporter genes driven by EBV promoters introduced to cells by transfection (Kudoh et al., 2004). The specificity of roscovitine for EBV promoter-specific factors has not been evaluated.

1.7.2.5 HIV-1

Roscovitine also inhibits the replication of human immunodeficiency virus type-1 (HIV-1), including primary field isolates [syncytium-inducing or not; (Wang et al., 2001)] and drug resistant mutants (Agbottah et al., 2008; Schang et al., 2002a).

HIV-1 infection induces the expression of cyclins A and E. Roscovitine inhibited this induced expression, and decreased the levels of phosphorylation of substrates of CDK/cyclin A and CDK/cyclin E complexes. Roscovitine also resulted in decreased levels of CDK1 and CDK2, although the mechanisms for such inhibition remain unknown.

Inhibition of HIV-1 replication by roscovitine appears to be primarily a result of inhibition of transcription. P-TEFb bound to HIV-1 Tat plays a critical role during elongation of HIV-1 transcription (Fujinaga et al., 1998; Isel and Karn, 1999; Mancebo et al., 1997). P-TEFb associates with the RNA polymerase II to phosphorylate serine 2 of the RNA polymerase II CTD, thereby enhancing elongation. Roscovitine decreased the levels of CDK9 and CTD phosphorylation. Roscovitine also inhibits CDK2, which has also been implicated in stimulating

Tat-mediated transcription by phosphorylating serine 2 of the RNAPII CTD (Nekhai et al., 2002). The effect of roscovitine on CDK2-mediated Tat transcriptional stimulation has not been evaluated.

As for cellular transcription, CDK7 phosphorylation of serine 5 on the CTD is required for the initiation of basal HIV-1 transcription. It was therefore not surprising that roscovitine inhibited basal and activated HIV-1 transcription (Wang et al., 2001). However, it was surprising that roscovitine had no effects on cellular transcription. The concentration of roscovitine required to inhibit by 50% the replication of HIV-1 infected cells was 0.36 – 1.8 μM. The concentration of roscovitine required to inhibit the replication of uninfected cells by 50% was at least 10-fold higher (22 – 35 μM), indicating that replication of cells infected with HIV-1 is more sensitive to CDK inhibition (Agbottah et al., 2005). The inhibition of CDK7 by roscovitine also explains why Tat is not essential for the inhibition of HIV-1 transcription by roscovitine, as well as why the levels of all genomic, structural, and regulatory HIV-1 RNAs were decreased.

Roscovitine also induced HIV-1 infected cell death (Guendel et al., 2010). Roscovitine selectively induced apoptosis of HIV-1 infected cells (Wang et al., 2001). Up to 70% of HIV-1 infected cells were killed at 10 μM roscovitine (Guendel et al., 2010). In contrast, a maximum of only 25% of uninfected cells were killed at the same concentration of roscovitine. Roscovitine has only moderate selectivity for killing cells infected with another retrovirus, human T-cell lymphotropic virus type-1 (HTLV-1). Another PCI (purvanol A), however,

has high selectivity to induce death of HTLV-1 infected cells (Agbottah et al., 2008). Therefore, purvanol inhibits HTLV-1 replication (Wang et al., 2001).

1.7.2.6 Other oncogenic viruses

Roscovitine also inhibits the replication of oncogenic viruses such as members of the *Gammaherpesvirinae*, *Polyomaviridae*, *Papillomaviridae*, and *Retroviridae* families.

KSHV encodes for its own cyclin, v-cyclin [reviewed in (Verschuren et al., 2004)]. The KSHV v-cyclin activates CDK4 and (preferentially) CDK6 (GoddenKent et al., 1997). These v-cyclin/CDK complexes phosphorylate CDK4 and -6 substrates, as expected, but also phosphorylate CDK2 substrates (Ellis et al., 1999). v-cyclin expressing cells undergo continuous DNA replication and nuclear division, in the absence of cytokinesis (Verschuren et al., 2002). Roscovitine inhibited CDK2 activity in, and progression of, KSHV infected cells into G2/M (Ellis et al., 1999), although it does not bind CDK4 or CDK6. Therefore, the mechanism of KSHV replication inhibition by roscovitine is likely a result of the direct inhibition of CDK2 complexes, or inhibition of phosphorylation and subsequent degradation of p27 (CDK2 and CDK4 inhibitor) by CDK2/cyclin E complexes (Sheaff et al., 1997). The phosphorylation and subsequent degradation of p27 is required for endogenous CDK activation and full S-phase progression.

KSHV v-cyclin also interacts with CDK9 (Chang and Li, 2008). This interaction enhances phosphorylation of the p53 transactivation domain and

induces a p53-dependent growth suppression. The effect of roscovitine on CDK9 substrate phosphorylation has not been evaluated.

Like KSHV, the *Gammaherpervirinae* virus murine γ herpesvirus-68 (γH68) encodes a v-cyclin. The γH68 v-cyclin binds to CDK1 and CDK2 and activates CDK2 (Upton et al., 2005). This v-cyclin promotes cell cycle progression and is a critical regulator of γH68 reactivation from certain cell types (Upton and Speck, 2006). The interaction of v-cyclin with CDKs is not required for *in vitro* replication or *in vivo* replication in the spleen, but is required for *in vivo* replication in the lungs (Upton and Speck, 2006). Roscovitine inhibits the phosphorylation of γH68 v-cyclin/CDK complex substrates (Upton et al., 2005). However, the effect of roscovitine on γH68 replication has not been evaluated.

Roscovitine inhibits the replication of the oncogenic polyomavirus JC virus (JCV), and JCV-induced cytopathic effects (Orba et al., 2008). This inhibition of JCV production is likely the result of the inhibition of DNA replication and transcription of L genes. Levels of the virally encoded large T-antigen (TAg; an E protein) were unchanged by roscovitine, whereas the levels of its phosphorylation on a conserved residue within the CDK recognition motif were lower. Mutation of a conserved threonine (Thr124 in the polyoma virus simian vacuolating virus 40 or simian virus 40 [SV40] and Thr125 in JCV), in the CDK recognition sequence of the TAg, inhibits polyoma virus DNA replication. Phosphorylation of this threonine is required for polyoma virus DNA replication (McVey et al., 1989). Therefore, the inhibition of phosphorylation of the JC TAg resulted in the expected decrease in DNA replication (Orba et al., 2008).

CDKs are also important for human papilloma virus (HPV) replication. The papillomavirus proteins E1 (an origin binding protein) and E2 (a transcriptional activator) are phosphorylated *in vitro* by CDKs complexed with E- and A-type cyclins (Ma et al., 1999). The role of E2 phosphorylation by CDKs has not been evaluated, whereas CDK-mediated phosphorylation of the E1 cyclin-binding motif is required for efficient HPV DNA replication. Therefore, the DNA replication of HPV-16 is inhibited by roscovitine (Atanasova et al., 2007). Furthermore, roscovitine induces cell death in HPV-16 E6- and E7-transformed keratinocytes. It also inhibits E7-induced abnormal centrosome duplication (Duensing et al., 2000).

Roscovitine inhibits the replication of the retrovirus HTLV-1. This inhibition results from inhibition of HTLV-1 activated, but not basal, transcription (Wang et al., 2001; Wang et al., 2002). Unlike HIV-1, however, roscovitine does not induce infected cell death in HTLV-1 infected cells.

The replication of the retrovirus mouse mammary tumor virus (MMTV) is also inhibited by PCIs such as Roscovitine. The long terminal repeat of MMTV contains a glucocorticoid hormone response element. CDK2 phosphorylation of H1 at MMTV promoters leads to loss of H1 from the promoter and a decrease in chromatin density (Bhattacharjee et al., 2001; Horn et al., 2002). The loss of H1 and decrease in chromatin density allows increased access of the glucocorticoid receptor (GR) to the promoter. Roscovitine inhibited the increased phosphorylation levels of H1 and decreased GR mobility at MMTV sites of

replication. Roscovitine also inhibited transcription and chromatin remodeling at the MMTV promoters (Stavreva and McNally, 2006).

1.7.2.7 Other viruses

The E1A protein from nuclear replicating adenovirus is essential for viral replication. E1A is phosphorylated by CDK1, CDK2, and CDK4 *in vitro* (Mal et al., 1996). E1A phosphorylation by CDKs increases its association with Rb. This increased E1A/Rb phosphorylation results in the disruption of more E2F/Rb complexes. Therefore, the functional consequence of roscovitine on the phosphorylation of E1A would be interesting to analyze.

The human influenza A virus multivirulence factor NS1 protein is phosphorylated by CDK1/cyclin B, CDK2/cyclin A, CDK5/p35 and ERK2 *in vitro*. This phosphorylation is important for efficient viral replication in tissue culture. However, the *in vivo* implications of this phosphorylation remain to be determined (Hale et al., 2009). The effect of roscovitine on the phosphorylation of this virulence factor has not been evaluated either. Therefore, the effect of roscovitine on influenza replication and virulence may be of interest to investigate.

Vaccinia virus (VV) or lymphocytic choriomeningitis virus (LCMV) replicate in the cytoplasm, and their replication is not known to require CDKs. It is therefore not surprising that roscovitine does not inhibit the replication of either (Schang et al., 2002a).

1.7.3 Roscovitine as an antiviral compound

Antiviral drugs that target cellular proteins have become accepted as an alternative to drugs that target viral proteins. One obvious potential limitation of drugs that target cellular proteins is toxicity. However, PCIs were fairly well tolerated in small early clinical trials (against cancer).

During the development of PCIs, monospecific inhibitors were initially thought to be the ultimate goal (Fischer and Gianella-Borradori, 2005). However, transgenic mice studies indicate that the inhibition of a single CDK is unlikely to be sufficient to have a major effect on the phosphorylation of CDK substrates. Therefore, a compound like roscovitine that inhibits several related CDKs may actually be more clinically useful.

In subsequent human clinical trials, roscovitine was proven to be orally bioavailable and sufficiently safe. Furthermore, the pharmacokinetics, tissue distribution, and dose limiting profile of roscovitine also suggest that it may be useful clinically as a drug. One of the major limitations of currently approved antiviral drugs is their rapid selection for resistance. The specificity of roscovitine for HSV-1 genomes could minimize selection for resistance. Consistently, attempts to select for HSV-1 resistance to roscovitine have been unsuccessful to date (Schang et al., 1998). Therefore, a compound like roscovitine may be useful clinically as an antiviral.

1.8 RATIONALE AND HYPOTHESIS

1.8.1 Rationale

Our group showed that HSV-1 genomes in lytically infected cells are in complexes with properties of unstable nucleosomes (Lacasse and Schang, 2010). Therefore, HSV-1 DNA replication and transcription are dependent on overcoming the physical barriers posed by chromatin. Transcription and DNA replication must overcome this barrier at a step prior to initiation. Dr. Lacasse discovered that HSV-1 IE and L DNA from infected, roscovitine-treated cells at 7 hpi was digested by MCN slower than the cellular DNA from roscovitine-treated cells and HSV-1 DNA from infected cells not treated with drug at 7 hpi. When infected cells were treated with roscovitine, the time that it took to degrade 50% of the DNA (T_{50}; IE and L DNA) was not reached (more than 60 min). In contrast, the T_{50} of the cellular DNA in infected cells treated with roscovitine was 35.7 min. The T_{50} of infected cells not treated with drug was under 15 min [14.3 min for IE DNA and 12.4 min for L DNA (Lacasse, 2010)]. These results indicate that the HSV-1 DNA from untreated cells is more accessible to MCN digestion than cellular DNA. However, treatment with roscovitine resulted in a decrease in the accessibility of the HSV-1 DNA to MCN digestion (Lacasse, 2010) to levels below HSV-1 DNA from untreated cells and cellular DNA from roscovitine-treated cells.

Roscovitine inhibits both HSV-1 transcription and DNA replication. Roscovitine inhibits a step prior to the initiation of HSV-1 transcription, but does not inhibit ongoing transcription (Diwan et al., 2004; Jordan et al., 1999; Schang

et al., 1999). The inhibition of transcription by roscovitine is independent of promoter-specific factors and specific for extrachromosomal HSV-1 DNA (Diwan et al., 2004). Moreover, roscovitine does not inhibit the formation of the VP16-dependent transactivating complex or decrease its affinity for its cognate sequence either (Jordan et al., 1999).

The molecular mechanisms of inhibition of HSV-1 DNA replication by roscovitine have not been described. Roscovitine inhibits HSV-1 DNA replication even in the presence of all the required HSV-1 DNA replication proteins (Schang et al., 2000). However, little else is known regarding the inhibition of HSV-1 DNA replication by roscovitine.

1.8.2 Hypothesis

Histones bound to DNA in nucleosomes obstruct DNA replication and transcription. Lytic HSV-1 DNA is in complexes with the properties of unstable nucleosomes. However, HSV-1 DNA from roscovitine-treated cells was digested slower by MCN than its cellular counterpart. HSV-1 transcription is inhibited by roscovitine at a step prior to initiation. Therefore, I propose a model whereby roscovitine inhibits HSV-1 DNA replication and transcription by common mechanisms, inhibition of DNA accessibility.

My hypothesis is that the inhibition of HSV-1 DNA replication occurs at a step prior to initiation. In this model, the inhibition of HSV-1 DNA replication by roscovitine is not exclusively a consequence of direct inhibition of HSV-1 DNA

replication. Therefore, my first objective was to identify the steps at which HSV-1 DNA replication is inhibited.

To this end, I evaluated the formation of replication structures in the presence of roscovitine by evaluating ICP8 localization. ICP8 is the first HSV-1 protein to localize to HSV-1 pre-replication or replication structures. As such, ICP8 is required for replication structure formation. However, roscovitine inhibits the expression of HSV-1 proteins. Therefore, I induced the accumulation of ICP8 during a PAA- or temperature-induced HSV-1 DNA replication block. Roscovitine was only added after ICP8 was expressed, and a time course evaluation of the localization of ICP8 was then performed. I discovered that roscovitine inhibited ICP8 formation of new replication structures.

I then evaluated possible mechanisms of inhibition of replication structure formation by ICP8. A decreased affinity of ICP8 for its binding partners would account for the inhibition of replication structure formation in the presence of roscovitine. Therefore, I evaluated how easily ICP8 could be extracted from the nuclei of roscovitine-treated cells, to indirectly test whether ICP8 had altered interactions with its binding partners. ICP8 was more easily extracted from the nuclei of roscovitine-treated cells. I next evaluated whether the increase in extractability was a result of a decreased affinity of ICP8 for ssDNA. The affinity of ICP8 for ssDNA *in vitro* was not affected by roscovitine treatment.

In summary, I have discovered that roscovitine inhibits HSV-1 DNA replication prior to initiation. This inhibition may be the result of decreased

interactions of ICP8 with its binding partners, but is not likely a result of a decreased affinity of ICP8 for ssDNA.

CHAPTER 2: MATERIALS AND METHODS

2.1 CELLS AND VIRUSES

Vero cells (African Green Monkey kidney fibroblasts) were obtained from ATCC (Rockville, MD, USA; catalog no.CCL-81) and maintained in Dulbecco's modified Minimum Eagle's Medium (DMEM; pH 7.4) supplemented with 5% fetal bovine serum (FBS). A low passage (p10) HSV-1 strain KOS, a generous gift from the late Dr. P. Schaffer (University of Pennsylvania, Philadelphia, PA, USA), was used throughout this study.

HSV-1 temperature sensitive (*ts*) strain A1 (also called 343 and HA1, herein referred to as A1) is an ICP8 point mutant (serine to phenylalanine) at residue 343 [**Figure 1.3**; (Gao et al., 1988)]. HSV-1 strain A1 stock seed was also a generous gift from the late Dr. P. Schaffer (University of Pennsylvania).

2.2 HSV-1 KOS PROPAGATION, HARVEST, AND TITRATION

To propagate stocks of HSV-1 KOS, $2.5X10^6$ Vero cells were seeded onto 100 mm^2 dishes. Cells were allowed to settle and adhere for 4 -12 h at 37^oC in 5% CO_2. Adhered cells were infected with 1.5 ml of 4^oC DMEM (serum-free media; SFM) containing 0.05 plaque forming unit (PFU) per cell of HSV-1 KOS. Dishes were incubated at 37^oC in 5% CO_2, rocking and rotating them every 10 min. After 1 h, inocula were vacuumed off and cells were washed twice with 5 ml of 4^oC phosphate-buffered saline (PBS; 1 mM KH_2PO_4, 154 mM NaCl,

3 mM Na_2PO_4, pH 7.4). Cells were then incubated at 33°C in 5% CO_2 in 6 ml of DMEM supplemented with 10% FBS until all cells were rounded and 50% of the cells were floating (3+ cytopathic effect; CPE; 4 – 5 days).

Cells and supernatant were harvested after scraping. Cells were pelleted by centrifugation for 30 min at 3220 × g (4000 revolutions per minute; RPM) in a swinging bucket rotor (SBR; rotor no.A-4-62) at 4°C in an Eppendorf 5810R centrifuge. The supernatants were subsequently centrifuged at 4°C for 2 h at 10,000 × g (8073 RPM) in a Beckman Coulter Avanti J-E centrifuge in a JA-14 rotor to pellet virus. Meanwhile, the cell pellets from the first centrifugation were resuspended in 250 μl of SFM for every 4 dishes harvested. The cell suspensions were then freeze-thawed 3 times in an ethanol-dry ice bath and sonicated with a 550 W sonicator ultrasonic processor XL2020 (Heat Systems Inc.) at 15% (82.5 W) for 3 cycles of 30 s separated by 30 s pauses. Cellular debris was then pelleted by centrifugation for 30 min at 3220 × g in a SBR rotor in an Eppendorf 5810R centrifuge at 4°C. Supernatants from the last centrifugation were used to resuspend the viral pellets from the 2 h 10,000 × g centrifugation.

HSV-1 strain KOS stocks were titrated in 6 well plates on Vero cells seeded at $3X10^5$ cells per well. Cells were allowed to adhere for 4 – 12 h at 37°C in 5% CO_2. Viral stocks were then serially diluted (1:10) in 4°C SFM. Two hundred microliters of each dilution was used to infect the cells. Plates were incubated at 37°C in 5% CO_2, rocking and rotating them every 10 min. Inocula were vacuumed off 1 h after incubation, and the cells were washed with 2 ml of 4°C PBS (pH 7.4). The cells in each well were then overlaid with 3 ml of 37°C

2% w/v methylcellulose (MC) in DMEM supplemented with 5% FBS. Overlaid cells were incubated at 37°C in 5% CO_2 until plaques were well defined (approximately 3 days). Cells were fixed and stained with crystal violet in methanol (1% w/v crystal violet in 17% v/v methanol in ddH_2O). Twenty-four hours later, the plates were washed and dried and the plaques were counted.

2.3 HSV-1 *TS* STRAIN A1 PROPAGATION, HARVEST, AND TITRATION

To propagate stocks of HSV-1 *ts* strain A1, $4X10^6$ Vero cells were seeded onto 100 mm^2 dishes. Cells were allowed to adhere for 4 -12 h at 33°C (permissive temperature for HSV-1 *ts* strain A1). Cells were then infected with 0.05 PFU/cell of HSV-1 *ts* strain A1 in 4°C SFM and incubated at 33°C for 1 h. Dishes were rocked and rotated every 10 min. Inocula were then vacuumed off and cells were washed twice with 5 ml 4°C PBS (pH 7.4). Cells were then incubated at 33°C in 6 ml of DMEM supplemented with 10% FBS until 3+ CPE (4 – 5 days).

Cells and supernatant were harvested after scraping, and the cells were pelleted by centrifugation for 30 min at 3220 × g (4000 RPM) in a SBR rotor in an Eppendorf 5810R centrifuge at 4°C. The supernatants from this spin were subsequently centrifuged for 2 h at 10,000 × g (8073 RPM) in a JA-14 rotor in a Beckman Coulter Avanti J-E centrifuge at 4°C to pellet the virus. Meanwhile, the cell pellets from the first centrifugation were resuspended in 250 μl SFM for every 4 dishes harvested. These cell suspensions were freeze-thawed 3 times in ethanol-dry ice and 37°C water baths and then sonicated with a 550 W sonicator

ultrasonic processor XL2020 (Heat Systems Inc.) at 15% (82.5 W) for 3 cycles of 30 s separated by 30 s pauses. Cellular debris was pelleted from the cell suspensions by centrifugation for 30 min at 3220 × g in a SBR in the Eppendorf 5810R centrifuge at 4°C. Supernatants from the last centrifugation were used to resuspend the viral pellets (from the 2 h 10,000 × g spin).

HSV-1 *ts* strain A1 stocks were titrated at 33°C (permissive temperature) and 39.5°C (non-permissive temperature) on $3X10^5$ Vero cells per well in 6 well plates. Four to twelve hours after seeding the cells, viral stocks were serially diluted (1:10) in 4°C (33°C titrations) or 37°C (39.5°C titrations) SFM, and cells were infected with 200 μl of each dilution. Plates were then incubated at 33°C or 39.5°C for 1 h (without rocking or rotating, to maximize temperature accuracy). Inocula were vacuumed off and the cells were washed with 2 ml of 4°C (33°C titration) or 37°C (39.5°C titration) PBS (pH 7.4). Cells in each well were then overlaid with 3 ml of room temperature (33°C titration) or 37°C (39.5°C titration) 2% MC in DMEM supplemented with 5% FBS. Overlaid cells were incubated at 33°C or 39.5°C in 5% CO_2 until plaques were well defined (approximately 3 days at 39.5°C or 4 days at 33°C). Due to the sensitivity of the virus to changes in temperature, plates at 39.5°C were not removed from the incubator to be checked over the course of infection. The 39.5°C plates were always fixed and stained when small plaques appeared in the 33°C plates. The plates at 33°C were then fixed 24 h later. This 24 h difference compensates for the faster replication at 39.5°C than at 33°C. Cells were fixed and stained with crystal violet in methanol. The plates were washed and dried and the plaques were counted 24 h later. The

titration of the A1 stocks at 39.5°C was performed to ensure temperature sensitivity. HSV-1 strain A1 consistently showed at least a 100-fold reduction in titre at the non-permissive temperature.

2.4 DRUGS

Stocks of 100 mg/ml PAA (Sigma, St. Louis, MO, USA; catalog no.284270) were prepared in SFM. The pH was adjusted to neutrality with NaOH, and the stocks were filter sterilized, aliquoted, and stored at -20°C. PAA was used at concentrations of 200 or 400 μg/ml, as indicated in each experiment. Stocks of 100 mM roscovitine (LC laboratories, Woburn, MA, USA; catalog no.R-1234) were prepared in dimethyl sulfoxide (DMSO), stored at -20°C, and used at a concentration of 100 μM. Stocks of 5 mg/ml cycloheximide (CHX; Sigma; catalog no.C7698) were prepared in SFM, filter sterilized, stored at 4°C, and used at a concentration of 50 μg/ml. Final working solutions of the drugs were prepared immediately prior to their use.

2.5 ANTIBODIES

Two different ICP8 antibodies were used. Polyclonal antibody R8 is directed against recombinant full length ICP8 (a generous gift from Dr. W. Ruyechan, University at Buffalo, NY, USA). Antibody R8 was used at a concentration of 1:20,000 in blocking buffer (2% bovine serum albumin [BSA] and 5% normal goat serum [NGS]) for immunofluorescence. Monoclonal antibody 11E2

(Abcam, Cambridge, MA, USA; catalog no.ab20194) was used at a concentration of 1:1,000 in blocking buffer (2% BSA and 5% NGS) for immunofluorescence, or 1:2,000 in membrane blocking solution (50 % PBS [pH 7.4] and 50% Licor blocking solution; LI-COR Biosciences) for Western blot. ICP4 was detected with a monoclonal ICP4 antibody (Goodwin Institute for Cancer Research Inc., Plantation, FL, USA; catalog no.1101-897) used at a concentration of 1:10,000 in blocking buffer (2% BSA and 5% NGS) for immunofluorescence. BrdU was detected using a polyclonal BrdU antibody (BD Pharmingen; San Diego, CA, USA; catalog no.555627) at a concentration of 1:500 in blocking buffer (2% BSA and 5% NGS for immunofluorescence.

Secondary goat anti-rabbit IgG Alexa-488 (Invitrogen, Carlsbad, CA, USA; catalog no.A-11008) and goat anti-mouse IgG Alexa-594 (Invitrogen; catalog no.A-11005) antibodies were diluted 1:1000 in blocking buffer (2% BSA and 5% NGS).

For Western blots, the secondary antibody was goat anti-mouse labeled with IRDye 800 (Rockland Immunochemicals, Inc., Gilbertsville, PA, USA; catalog no.610-132-121), diluted 1:20,000 in membrane blocking solution (50 % PBS [pH 7.4], 50% Licor blocking solution; LI-COR Biosciences).

2.6 LABELING AND STAINING REAGENTS

Ten millimolar stocks of bromodeoxyuridine (BrdU; Sigma; catalog no.B5002) were prepared in ddH_2O, filter sterilized, stored at -20^{o}C, and used at 10 μM.

Hoechst 33258 (Sigma, catalog no. 861405) was diluted in PBS (pH 7.4) to 0.1 mg/ml, stored at 4°C, and used at a concentration of 1 μg/ml.

2.7 HSV-1 INFECTION FOR ANALYSES OF ICP8 LOCALIZATION

2.7.1 PAA-induced HSV-1 DNA replication block followed by drug treatments

For each treatment, $5x10^5$ Vero cells were seeded onto sterile 12 mm (thickness 0.13 – 0.17 mm; number 1) coverslips (Fisher Scientific, Pittsburgh, PA, USA; catalog no.12-545-82) in 24 well plates at 37°C in 5% CO_2. Seeded cells were incubated for 1 h at 37°C in 5% CO_2 with 0.5 ml of DMEM supplemented with 5% FBS and 200 μg/ml PAA. PAA-treated cells were then infected with 30 PFU per cell of HSV-1, strain KOS, in 0.15 ml of 4°C SFM supplemented with 200 μg/ml PAA. Plates were incubated at 37°C in 5% CO_2 for 1 h, rocking and rotating them every 10 min. Inocula were then vacuumed off and cells were washed twice with 0.5 ml of 4°C PBS (pH 7.4) supplemented with 200 μg/ml PAA. Infected cells were incubated for 4 h with 0.5 ml of DMEM supplemented with 5% FBS and 200 μg/ml PAA. Cells were washed twice with 1 ml of either 37°C PBS (pH 7.4) alone (cells that were afterwards were treated with roscovitine or with no drug) or supplemented with 200 μg/ml PAA (cells that were afterwards treated with PAA) or 50 μg/ml CHX (cells that were afterwards treated with CHX). Cells were then incubated for 2, 4, or 6 h in 0.3 ml of DMEM supplemented with 5% FBS and supplemented or not with 200 μg/ml PAA,

100 μM roscovitine, or 50 μg/ml CHX. Fifteen minutes prior to the end of this incubation, 10 μM BrdU was added to the media (in 0.3 ml of DMEM supplemented with 5% FBS, 20 μM BrdU and the respective drug at the respective concentration). At the end of this 15 min incubation, cells were washed with 0.5 ml of room temperature PBS (pH 7.4) and 0.5 ml of 4°C 10% formalin, and fixed in 0.5 ml of fresh 4°C 10% formalin for 15 min at room temperature. Fixed cells were washed with 0.5 ml of 4°C PBS (pH 7.4), and stored in 0.5 ml of PBS (pH 7.4) at 4°C for future immunofluorescent analyses. Plates were sealed with parafilm to prevent drying.

2.7.2 Temperature-induced HSV-1 DNA replication block followed by drug treatments

For each treatment, $5x10^5$ Vero cells were seeded onto sterile 12 mm (number 1) circular coverslips in 24 well plates at 38°C in 5% CO_2. Four to twelve hours later, cells were infected with 0.15 ml of 37°C SFM containing 30 PFU per cell of the HSV-1 strain A1. Cells were incubated at 38°C in 5% CO_2 for 1 h (without rocking or rotating to minimize temperature changes), before vacuuming off the inocula. Cells were washed once with 0.5 ml of 37°C PBS (pH 7.4) and then incubated in 0.5 ml of 37°C DMEM supplemented with 5% FBS for 3.5 h. Media was then replaced with 0.3 ml of 37°C DMEM supplemented with 5% FBS and no drug, 100 μM roscovitine, 400 μg/ml PAA, or 50 μg/ml CHX. After 30 min, plates were transferred to the permissive temperature (33°C), and further incubated for 2 or 4 h. Fifteen minutes prior to the end of the incubation period,

10 μM BrdU was added in 0.3 ml of DMEM supplemented with 5% FBS, 20 μM BrdU, and the respective drug at the respective concentration. Cells were incubated with BrdU for 15 min before washing with 0.5 ml of room temperature PBS (pH 7.4), and then with 0.5 ml of 4°C 10% formalin. Cells were fixed for 15 min in 0.5 ml of fresh 4°C 10% formalin at room temperature. Fixed cells were washed with 0.5 ml of 4°C PBS (pH 7.4) and stored at 4°C in 0.5 ml of fresh PBS (pH 7.4) for future immunofluorescent analyses. Plates were sealed with parafilm to avoid drying.

2.8 IMMUNOFLUORESCENCE

All reagents were prepared fresh and all procedures were performed at room temperature on a compact rocker (CR300; FINEPCR, Guemjeong-dong, Gunpo-si, Gyenggi-do, Korea) at a speed of 60 RPM unless otherwise indicated. Fixed cells were washed with 0.5 ml of ddH_2O. DNA was denatured with 0.3 ml of 2 N HCl in 0.1 % Triton-X for 25 min. Cells were then washed with 0.3 ml of 0.1 M $Na_2B_4O_7$ (pH 8.5) 4 times for 10 min to neutralize pH. Cell membranes were permeabilized with 2 ml of 0.1 % Triton-X in PBS (pH 7.4) 3 times for 10 min each. Permeabilized cells were blocked with 0.2 ml of blocking buffer (2 % BSA and 5 % NGS in PBS; pH 7.4) for 1 h. Blocked cells were incubated with 0.2 ml of ICP8 (R8) and BrdU antibodies in blocking buffer overnight (16 – 20 h) at 4°C without rocking. The next day, cells were washed 3 times with 0.5 ml of 0.2 % Tween-20 in PBS (pH 7.4) for 15 min each. Cells were then incubated with 0.2 ml of labeled secondary antibody in blocking buffer for 1 h, covered in foil (to

keep them in the dark). All subsequent incubations were performed with samples in the dark (covered in foil). Cells were washed with 0.5 ml of 0.2 % Tween-20 in PBS (pH 7.4) 3 times for 15 min each. Nuclei were counterstained with 0.2 ml of 1 μg/ml Hoescht 33258 for 15 min. Coverslips were then washed with 0.5 ml of PBS (pH 7.4), mounted facedown onto microscope slides with 1 drop (approximately 10 μl) of Vectashield mounting medium (Vector labs, Burlingame, CA, USA; catalog no.H-1000), and sealed with clear nail enamel. Cells were viewed and quantitated using a fluorescence microscope with a UV light source (Leica DM IRB, Wetzlar, Germany) at 1000 magnification. At least 100 cells from each treatment were analyzed. Twenty cells were quantitated from the left-hand, right-hand, top, bottom, and middle regions of each coverslip to ensure that quantitation occurred from all areas of the coverslip.

2.9 CONFOCAL MICROSCOPY

Confocal images were collected using a Zeiss LSM 720 laser scanning confocal microscope on a 40× Plan-apochromat objective (numerical aperture, 1.3; working diameter, 0.12 mm) with 25 mW argon (488 nm) and 1 mW HeNe (543 nm) lasers and band-pass filters of 500 to 550 and 548 to 623 nm, respectively. The images (512 by 512; 8 bit) were analyzed with Zeiss LSM or Zen software. Image contrast and brightness were adjusted for figure preparation using Adobe Photoshop®.

2.10 ICP8 EXTRACTABILITY

For each treatment, $1x10^7$ cells were infected with 2.5 ml of SFM containing 10 PFU per cell of HSV-1, strain A1 prewarmed to 37°C. Inocula were vacuumed off after 1 h at 38°C. Cells were washed with 10 ml of PBS (pH 7.4) at 37°C and then incubated in 15 ml of DMEM supplemented with 5% FBS for 3.5 h. Media was vacuumed off and cells were incubated for 30 min in 10 ml of DMEM supplemented 5% FBS and 50 μg/ml CHX or 100 μM roscovitine (pre-warmed to 37°C). Cells were then transferred to the permissive temperature (33°C) for 4 h.

To harvest, medium was vacuumed off and the cells were washed with 10 ml of 37°C PBS (pH 7.4). Cells were then trypsinized for 2 min at room temperature with 3 ml of 0.05% Trypsin-Ethylene diamine tetraacetic acid (EDTA) at 4°C. Cell suspensions were collected with DMEM supplemented with 5% FBS to a total volume of 20 ml and transferred to 50 ml conical tubes. Samples were kept on ice, all solutions were used at 4°C, and all centrifugations were performed at 4°C in an Eppendorf 5810R centrifuge in a SBR rotor for all subsequent steps. Cells were pelleted for 10 min at 4000 RPM (3220 × g). The cell pellets were resuspended and washed with 20 ml PBS (pH 7.4) and pelleted again for 10 min at 4000 RPM. The cell pellets were resuspended in 20 ml RSB and pelleted again for 10 min at 4000 RPM. Cell membranes were permeabilized with 20 ml of 0.5 % NP40 in RSB. One milliliter of the cell suspension was transferred to a 1.5 ml eppendorf tube and kept on ice for 8 min. Cell lysis was verified by microscopy evaluation at 7.5 min. Nuclei were pelleted by centrifugation for 25 min at 3000 RPM. Pelleted nuclei were resuspended in

25 μl of 0.05 M NaCl extraction buffer (1 mM dithiothreitol [DTT], 0.2 mM EDTA [pH 8.5], 1.5 mM $MgCl_2$, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES; pH 7.6], 25 % Glycerol, and 50 mM NaCl). Nuclear suspensions were incubated on a tube rotator (Wheaton Reax 2) at 30 RPM at 4°C. After 20 min, the nuclei were pelleted by centrifugation for 5 min at 14,000g in a GE 035 rotor. Supernatants were collected as 50 mM NaCl nuclear extracts. The resuspension in extraction buffer, incubation, nuclear pelleting, and supernatant collection was serially repeated with extraction buffers at increasing NaCl concentrations (0.2, 0.3, 0.4, 0.5, 0.6, 0.7, and 0.8 M). The proteins not yet extracted from the nuclei after the final extraction were recovered by incubating the remainder of the pellets in lysis buffer (1% NP-40, 50 mM Tris [pH 8.8], and 150 mM NaCl) for 30 min, followed by 6 rounds of 82.5 W sonication at 10 s each separated by 10 s pauses. The nuclear debris from these lysates was pelleted by centrifugation for 5 min at 14,000g (GE 035 rotor), and the supernatants were collected. All extracts with NaCl concentrations higher than 0.5 M were diluted to 0.5 M NaCl with salt-free extraction buffer. The extracts were then loaded into Amicon ultra-4 concentrators (molecular weight cut-off 30 kDa; Millipore) and concentrated to ~60 (55 – 65) μl by centrifugation (5 to 15 min at 4000 RPM in the SBR).

Proteins were resolved on 10% sodium dodecyl sulfate (SDS) - polyacrylamide gel electrophoresis (PAGE) gels. A voltage of 8 V/cm (6 cm; 50 V) was applied until the dye front had migrated into the resolving gel (approximately 30 min). The voltage was then increased to 16 V/cm (6 cm; 100

V) until the dye front had migrated to the bottom of the resolving gel (approximately 1.5 h). Gels were then removed from the apparatus and equilibrated in cathode buffer (1X Tris-CAPS buffer [60mM Tris and 40 mM N-cyclohexyl-3-aminopropanesulfonic acid (CAPS), pH 9.6] and 0.1% SDS). Meanwhile, the pre-cut polyvinylidene fluoride (PVDF) membranes (6 cm by 8.9 cm) were activated in methanol and then equilibrated with anode buffer (1X Tris-CAPS [pH 9.6] and 15% methanol). After a 20 min equilibration, two sheets of pre-cut thick chromatography paper (Fisherbrand; Fisher Scientific, Pittsburg, PA, USA; catalog no.05-714-4) were added to each anode and cathode equilibration solutions. After soaking for 10 min, the two sheets of filters paper soaked in anode buffer were stacked on the platinum anode of the Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (BIO-RAD, Hercules, CA, USA; catalog no.170-3940). A plastic pipet was rolled over the surface of the top of each stack of filter papers to remove any air bubbles. Each pre-wetted membrane was placed on top of the filter papers and air bubbles were rolled out. Equilibrated gels were then placed on top of the membranes and air bubbles were rolled out again. Two filter papers soaked in cathode buffer were then placed on top of each membrane and any bubbles were rolled out. The cathode was placed onto the stack, and proteins were transferred for 1 h at 2.0 mA/cm^2 (107 mA for 1 gel, 214 mA for 2 gels, etc.). Membranes were then analyzed by Western blot.

2.11 ICP8 AFFINITY FOR ssDNA

For each treatment, $1x10^7$ cells were infected with 2.5 ml of inocula containing 10 PFU per cell of HSV-1, strain A1 prewarmed to 37°C. Inocula were vacuumed off after 1 h at 38°C. Cells were washed with 10 ml of 37°C PBS (pH 7.4) and then incubated in 10 ml of 37°C DMEM supplemented with 5% FBS for 3.5 h. Medium was vacuumed off and cells were incubated in 10 ml of 37°C DMEM supplemented with 5% FBS and 50 μg/ml of CHX or 100 μM roscovitine for 30 min. Flasks were then transferred to the permissive temperature (33°C) for 4 h.

To collect infected cells, the media was vacuumed off and the cells were washed with 10 ml of 37°C PBS (pH 7.4) and then trypsinized with 3 ml of 0.05% Trypsin-EDTA at 4°C for approximately 2 min at 37°C. Cell suspensions were collected with DMEM supplemented with 5% FBS to a total volume of 20 ml and transferred to 50 ml conical tubes. Samples were kept on ice, all solutions were used at 4°C, and centrifugations were performed at 4°C in an Eppendorf 5810R centrifuge in a SBR rotor in all subsequent steps. Cells were pelleted by a 10 min centrifugation at 4000 RPM (3220 × g). Cell pellets were resuspended and washed with 20 ml PBS (pH 7.4) and pelleted again for 10 min at 4000 RPM. Cell pellets were resuspended in 20 ml reticulocyte swelling buffer (RSB; 20 mM HEPES [pH 7.6], 10 mM NaCl, 5 mM $MgCl_2$) and repelleted for 10 min at 4000 RPM. Cells were then lysed with 20 ml of 0.5 % NP40 in RSB for 8 min. Cell lysis was verified by microscopy at 7.5 min. Nuclei were pelleted by a 25 min centrifugation at 3000 RPM (1811 × g). Pelleted nuclei were resuspended

in 500 μl of 4 M NaCl extraction buffer (1 mM DTT, 0.2 mM EDTA [pH 8.5], 1.5 mM $MgCl_2$, 20 mM HEPES [pH 7.6], 25 % Glycerol, and 4 M NaCl) and transferred to 1.5 ml eppendorf tubes. Samples were then sonicated on ice at 82.5 W for 6 cycles of 10 s, separated by rest periods of 10 s. Protein extracts were incubated on a tube rotator (Wheaton Reax 2) for 30 min at 30 RPM at 4^{o}C and cellular debris was then pelleted by centrifugation at 14,000 RPM in a GE 035 rotor. Nuclear extracts were dialyzed in 0.5 – 3.0 ml SLIDE-A-LYZER dialysis cassettes (Thermo Scientific, Waltham, MA, USA; catalog no.PI66330) against 2 L of 1X dialysis buffer (150 mM NaCl, 10 mM HEPES [pH 7.6], 1.5 mM $MgCl_2$, and 0.2 mM EDTA [pH 8.5]; prepared fresh from a 10X stock) for 1 h at 4^{o}C on a stir plate. The dialysis buffer was changed once after 30 min. Protein concentrations were determined using Bradford's assays.

One hundred micrograms of dialyzed nuclear extract was loaded onto 0.4 ml of hydrated ssDNA (calf thymus DNA) cellulose (Sigma; catalog no.D8273) in a 2 ml eppendorf tube and incubated at 4^{o}C on a tube rotator (Wheaton Reax 2) at 30 rpm. After 1 h, beads were pelleted by centrifugation for 7 min at 2100 × g (GE 035 rotor). The supernatant was collected, the beads were resuspended in 1 ml of wash buffer (0.5 mM DTT, 0.5 mM EDTA [pH 8.5], and 20 mM HEPES [pH 7.6]) with 0.15 M NaCl, and then incubated for 5 min on a tube rotator at 4^{o}C. Beads were pelleted by centrifugation for 7 min at 2100 × g (GE 035 rotor), and supernatants were collected. These supernatants were then pooled with the first fractions. Beads were resuspended a third time in 1 ml of wash buffer containing 0.15 M NaCl, incubated for 5 min on the tube rotator at

4°C, and pelleted by centrifugation for 7 min at 2100 × g (GE 035 rotor). The supernatants were collected and pooled with the first fractions. These three pooled fractions were labeled as "unbound" protein. The beads were then resuspended in wash buffer containing 0.3 M NaCl, incubated for 5 min at 4°C on the tube rotator, and pelleted by centrifugation for 7 min at 2100 × g (GE 035 rotor). The supernatants were collected and labeled as 0.3 M NaCl eluates. The process was repeated with increasing NaCl concentrations (0.4, 0.5, 0.6, 0.7, 0.8, and 2 M) in the wash buffer. All extracts with NaCl concentrations above 0.15 M were diluted to 0.15 M NaCl with salt-free elution buffer. The extracts were then loaded into Amicon ultra-4 concentrators (molecular weight cut-off 30 kDa; Millipore) and concentrated to ~50 µl (45 – 55) by centrifugation (3 times for 15 min each at 4000 RPM in the SBR).

The proteins were resolved on 10% SDS-PAGE gels and transferred to PVDF membranes as described in pages 80 and 81. Membranes were then analyzed by Western blot (see below).

2.12 WESTERN BLOT

Membranes were blocked for 1 h at room temperature in 10 ml of membrane blocking solution (50 % PBS [pH 7.4] and 50% Licor blocking solution; LI-COR) in 50 ml conicals placed in a personal hybridization oven (Labnet International Inc., Problot™ Jr.) at 13 RPM. Blocked membranes were subsequently incubated overnight (16 to 22 h) at 4°C on a maxi rotator (Lab-Line Model 4631 Maxi Rotator) at 25 RPM in 4 ml of membrane blocking solution containing primary

monoclonal ICP8 antibody 11E2 diluted 1:2000. Membranes were washed on a compact rocker (CR300; FINEPCR, Guemjeong-dong, Gunpo-si, Gyenggi-do, Korea) at 60 RPM three times for 5 min each with approximately 15 ml of 0.1 % Tween-20 in PBS (pH 7.4) and then once for 5 min with approximately 15 ml of PBS (pH 7.4). All washes were performed at room temperature. Membranes were then incubated with goat anti-mouse IRDye 800 (Rockland) diluted 1:20,000 in 10 ml of membrane blocking solution for 1 h in a 50 ml conical (in the dark) on the rotator. Membranes were washed again three times for 5 min each with approximately 15 ml of 0.1 % Tween-20 in PBS (pH 7.4) at room temperature and once for 5 min with approximately 15 ml of PBS (pH 7.4). Membranes were kept covered during these washes to keep them in the dark.

2.13 ODYSSEY SCANNING AND BLOT QUANTITATION

Blots were scanned and quantitated using in an Odyssey system (LI-COR Biosciences). Blots were scanned at a resolution of 169 μm. The 700 μm channel (red) was scanned at a relative intensity of 4.0 and the 800 μm channel (green) at a relative intensity of 6.0. The integrated intensity (total intensity minus total background) of bands from the blots was quantitated using the Odyssey applications software version 3.0.

CHAPTER 3:

ROSCOVITINE INHIBITS ICP8-FORMED REPLICATION STRUCTURES

3.1 INTRODUCTION

Roscovitine is a relatively non-toxic and orally bioavailable small molecule inhibitor of a subset of CDKs. It also inhibits the replication of unrelated nuclear replicating viruses, such as HSV, HIV, and polyomavirus. Unlike most antivirals, roscovitine does not promptly select for drug-resistant mutants. The lack of major overt toxicities, the selectivity for a subset of CDKs, the inhibition of viral replication, and the lack of selection for drug-resistant mutants suggest that roscovitine has the potential to be developed as an antiviral drug. However, the molecular mechanisms of its inhibition of viral replication are not yet fully understood.

Our group has studied the mechanisms of inhibition of HSV-1 replication by roscovitine. Our group has discovered that roscovitine inhibits both HSV-1 transcription and HSV-1 DNA replication. The molecular mechanisms of the inhibition of transcription by roscovitine have been well described. Roscovitine prevents the initiation of HSV-1 transcription, but it does not inhibit ongoing transcription (Diwan et al., 2004; Jordan et al., 1999; Schang et al., 1999). The transcription of genes driven by HSV-1 promoters recombined into the cellular genome is not inhibited by roscovitine, whereas the transcription of cellular genes

recombined into the HSV-1 genome is. Therefore, the inhibition of transcription by roscovitine is independent of promoter-specific factors (Diwan et al., 2004). Rather, roscovitine inhibits the transcription of extrachromosomal HSV-1 genomes.

The molecular mechanisms of the inhibition of HSV-1 DNA replication by roscovitine have not been so well described. Our group has discovered that roscovitine inhibits HSV-1 DNA replication even in the presence of all the required HSV-1 DNA replication proteins (Schang et al., 2000). However, little else is known regarding the inhibition of HSV-1 DNA replication by roscovitine. It is not yet even clear which HSV-1 DNA replication steps are inhibited by roscovitine. Furthermore, it is not clear whether the observed inhibition of HSV-1 DNA replication is a consequence of the direct inhibition of HSV-1 DNA replication or of steps prior to it. Therefore, my first objective was to identify the specific stages at which roscovitine inhibits HSV-1 DNA replication.

CDKs play several roles in several stages of cellular DNA replication. For example, CDK2/cyclin E phosphorylation of cdc6 and mini chromosome maintenance (MCM) subunits is required for the initiation of cellular DNA replication. Therefore, the inhibition of HSV-1 DNA replication by roscovitine could be a result of inhibition of phosphorylation of such initiation factors. However, MCM subunits or cdc6 have not been described to play any role in the initiation of HSV-1 DNA replication. Alternatively, roscovitine could inhibit HSV-1 DNA replication by inhibiting CDKs required during HSV-1 DNA replication itself. For example, CDKs could be required for phosphorylating a

component of the DNA replication complex such as a primase. Roscovitine could also inhibit HSV-1 DNA replication by inhibiting a later step in HSV-1 DNA replication, such as termination. For example, roscovitine could inhibit the required phosphorylation of a component of the termination complex such as a topoisomerase.

3.2 ROSCOVITINE INHIBITS THE ICP8-INDUCED FORMATION OF NEW REPLICATION STRUCTURES WHEN ICP8 WAS PRE-EXPRESSED DURING A PHOSPHONOACETIC ACID (PAA)-INDUCED DNA REPLICATION BLOCK

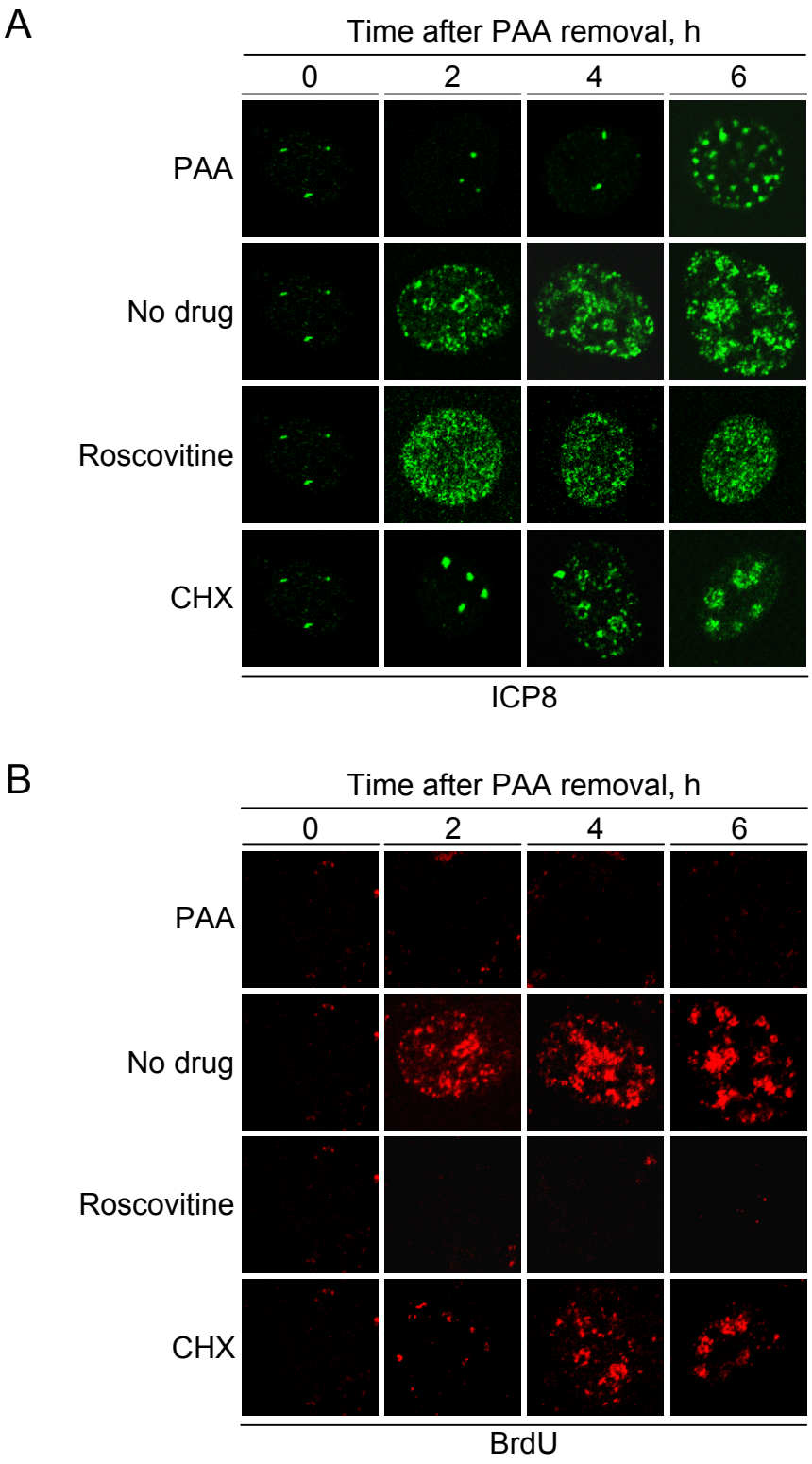
The stages of HSV-1 DNA replication can be identified by the presence of pre-RCs (prior to initiation) or RCs (after initiation, during DNA replication and termination). To identify the steps at which roscovitine inhibits HSV-1 DNA replication, I therefore evaluated the formation of pre-RCs and RCs. To this end, I analyzed the localization of ICP8, the first HSV-1 protein to localize to the sites of HSV-1 DNA replication, and BrdU, a thymidine analog that is incorporated into replicating DNA (**Figure 1.4**). However, roscovitine inhibits transcription as well as DNA replication. Therefore, the required HSV-1 DNA replication proteins would not be expressed in the presence of roscovitine.

ICP8 (along with the rest of the HSV-1 DNA replication proteins) was therefore first expressed, in the absence of HSV-1 DNA replication, using the DNA replication inhibitor PAA. ICP8 was allowed to accumulate for 5 h in the

presence of partially inhibitory concentrations PAA (200 μg/ml). I had to use such partially inhibitory concentrations because HSV-1 DNA replication did not resume after removing fully inhibitory concentrations (400 μg/ml). The formation of HSV-1 replication structures was then evaluated over the course of 6 h after removal of the PAA DNA replication block.

Treatment of cells with partially inhibitory concentrations of PAA for 5 h resulted in two patterns of ICP8 staining, as expected. The staining was either granular and dispersed throughout the nucleus (nuclear disperse), at sites of cellular ssDNA (cellular foci), or at ICP8 formed viral sites (**Figure 1.4**). PAA induces the stalling of DNA polymerases (Leinbach et al., 1976). Although it preferentially inhibits the HSV-1 DNA polymerase, it also inhibits cellular DNA polymerases. Therefore, predominantly sites of cellular ssDNA (most likely stalled DNA replication forks), or ICP8 formed viral pre-RCs or RCs (**Figure 3.1** and **3.2**) were observed. Viral pre-RCs and RCs were differentiated by the absence or presence of BrdU and ICP8 localization at the same nuclear domains, respectively (**Figure 3.1**). The ICP8 localizations can also be described as HSV-1 replication structures (pooled pre-RCs and RCs) or non-replication structures (pooled granular nuclear disperse and cellular ssDNA; **Table 1.2**).

In the presence of partially inhibitory concentrations of PAA, ICP8 formed pre-RCs or at sites of cellular ssDNA in most cells (44.8±12.2% or 24.3±9.1 % of cells; **Figures 3.1** and **3.2**, respectively). Under these conditions, ICP8 was granularly dispersed throughout the nucleus in only 10.3±5.7% of cells.


A
Time after PAA removal, h
0
2
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6
PAA
No drug
Roscovitine
CHX
ICP8
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Time after PAA removal, h
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BrdU

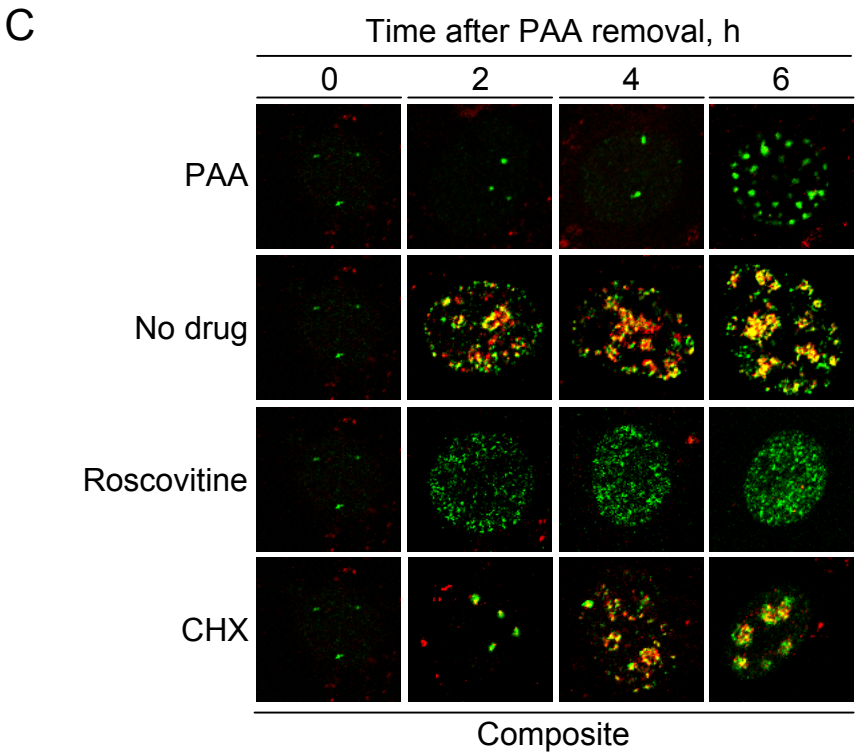


Figure 3.1 ICP8 accumulated during a PAA-induced DNA replication block localizes differentially in the presence of roscovitine. Representative confocal immunofluorescence images showing ICP8 localization in cells treated with different drugs after release from a PAA-induced DNA replication block. Images were collected using a Zeiss LSM 720 confocal microscope. Vero cells were infected with 30 plaque forming units (PFU) of HSV-1 per cell and incubated for 4 h in 200 μg/mL PAA. Infected cells were then transferred to media containing 200 μg/mL **PAA**, **no drug**, 100 μM **roscovitine**, or 50 μg/mL **CHX** for 2, 4, or 6 h, pulsed with 10 μM of the thymidine analog BrdU for 15 min and then fixed with 10% formalin for 15 min. Immunofluorescence was performed using ICP8 (**A**) or BrdU (**B**) primary antibodies; nuclei were counterstained with Hoescht 33258 (data not shown). RCs were differentiated from pre-RCs by ICP8 (green) and BrdU (red) localization at the same nuclear domain (**C**). The images at 0 h (left-hand panels) are all the same picture, which was copied for each drug treatment for the clarity of the presentation.

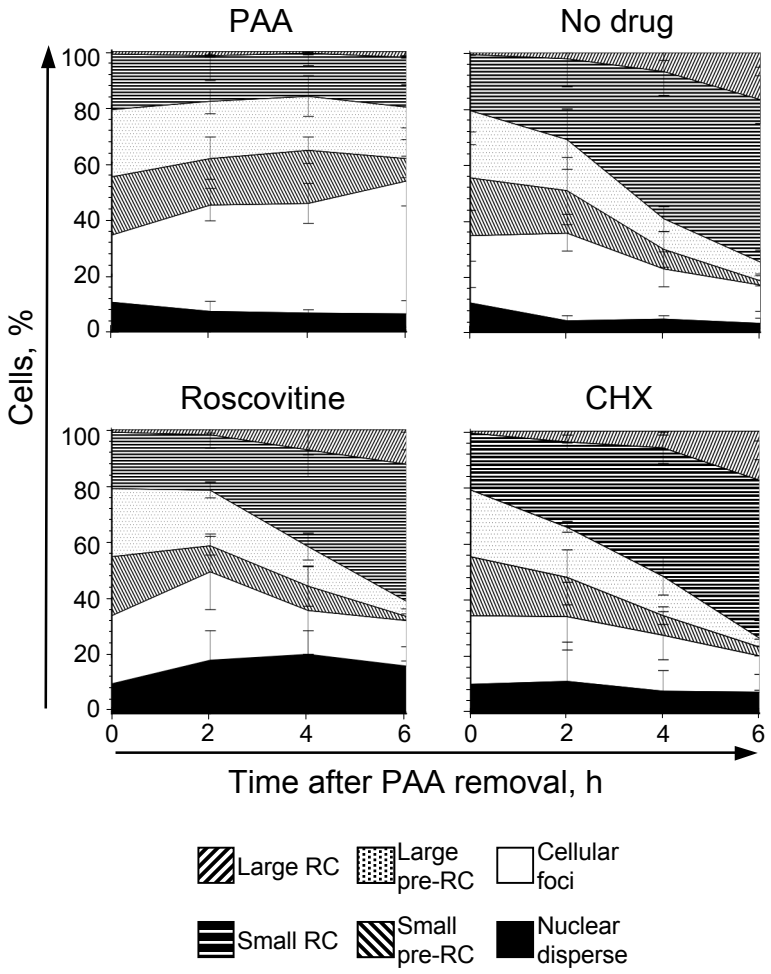


Figure 3.2 Roscovitine inhibits the localization of ICP8 accumulated during a PAA-induced DNA replication block to new replication sites. Area graphs presenting the percentage of cells with ICP8 **nuclear dispersed** or localized to sites of cellular ssDNA (**Cellular foci**), viral **pre-RCs** or viral **RCs** after release from a PAA-induced DNA replication block. Vero cells were infected with 30 PFU of HSV-1 per cell and incubated for 4 h in partially inhibitory concentrations of PAA (200 μg/mL). Cells were then incubated in media with 200 μg/mL **PAA**, **no drug**, 100 μM **roscovitine**, or 50 μg/mL **CHX** for 0, 2, 4, or 6 h. The cells were then pulsed for 15 min with 10 μM of the thymidine analog BrdU and fixed with 10% formalin for 15 min. Immunofluorescence was performed with ICP8 or BrdU primary antibodies and the nuclei were counterstained with the DNA stain Hoescht 33258. RCs were differentiated from pre-RCs by ICP8 and BrdU localization at the same nuclear domain. Error bars, standard deviation of four independent experiments (100 cells were counted for each treatment in each experiment).

As a result of using only partially inhibitory PAA concentrations, ICP8 formed RCs in 20.8±13.7% of cells. In the presence of partially inhibitory concentrations of PAA, therefore, 65.5±2.7% of cells had ICP8 formed HSV-1 replication structures and 34.5% had non-HSV-1 replication structures (**Figure 3.3**).

As expected, there were only slight changes in ICP8 formed replication structures when PAA was added again for 6 h after the 5 h incubation, for a total of 11 h incubation. PAA induces the stalling of DNA polymerases (Leinbach et al., 1976). Although it preferentially inhibits the HSV-1 DNA polymerase (thus inhibiting RC formation and inducing pre-RC accumulation), it also inhibits cellular DNA polymerases. Therefore, cellular replication forks are mostly stalled at late times, and many sites of cellular ssDNA are produced (Burkham et al., 1998; Leinbach et al., 1976; Quinlan et al., 1984). Long-term inhibition of the maturation of pre-RCs into RCs also induces pre-RC dispersal. Therefore, long PAA incubations result in an increase in the percentage of cells with sites of cellular ssDNA, accompanied by a decrease in the percentage of cells with ICP8 formed pre-RCs.

During the further 6 h incubation in the presence of PAA, the percentage of cells with sites of cellular ssDNA did indeed increase. This increase was accompanied by a decrease in the percentage of cells with ICP8 formed pre-RCs, as expected. The percentage of cells with sites of cellular ssDNA increased by 23.2% (to a total of 47.5±8.8%; $P<0.01$; **Figures 3.1** and **3.2**). The percentage of cells with ICP8 formed pre-RCs decreased by 18.5% (to a total of 26.5±10.9%; $P<0.10$). The percentage of cells with ICP8 formed RCs did not change

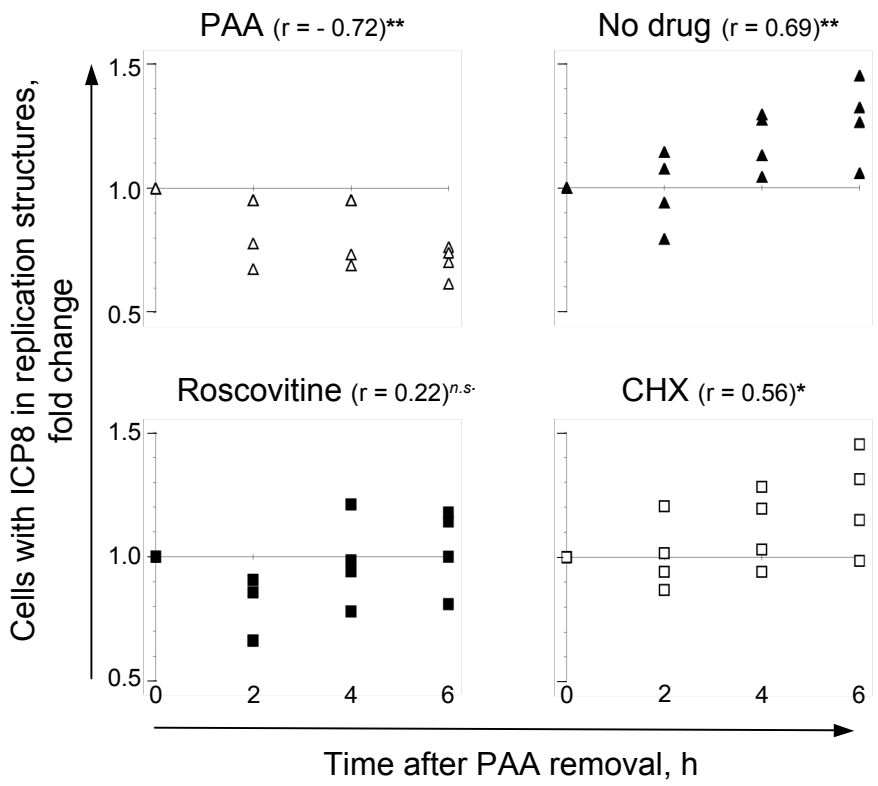


Figure 3.3 Correlation analyses of the formation of new replication structures after removal of a PAA-induced DNA replication block. A scatter plot presenting the relative number of cells with ICP8 in new replication structures, formed after the release from a PAA-induced DNA replication block. Vero cells were infected with 30 PFU of HSV-1 per cell and incubated for 4 h in partially inhibitory concentrations of PAA (200 μg/mL). Cells were incubated in media with 200 μg/mL **PAA**, **no drug**, 100 μM **roscovitine**, or 50 μg/mL **CHX** for 0, 2, 4, or 6 h. Cells were then pulsed for 15 min with 10 μM of the thymidine analog BrdU and fixed with 10% formalin for 15 min. Immunofluorescence was performed with ICP8 or BrdU primary antibodies and the nuclei were counterstained with the DNA stain Hoescht 33258. Data from four independent experiments, in each of which at least 100 cells were counted for each treatment. *, $p<0.05$; **, $p<0.005$; *n.s.*, not significant.

significantly (P>0.10; a non-significant decrease of 1.0% to a total of 19.8±11.7% of cells). The percentage of cells with ICP8 granularly dispersed throughout the nucleus did not change significantly either (P>0.10; a non-significant decrease of 4.0% to a total of 6.3±4.9%).

ICP8 formed HSV-1 replication structures in 19.2% fewer cells (for a total of 46.3±4.2% of cells treated with PAA for another 6 h after the primary PAA block; P<0.0001; **Figure 3.3**), which was accompanied by an equivalent 19.2% increase in cells with non-HSV-1 replication structures (for a total of 53.8±4.2% of cells; P<0.0001).

ICP8 formed RCs in 54.2% more cells when PAA was removed after the primary 5 h DNA replication block and media containing no drug was added (for a total of 75.0±10.7% cells; **Figures 3.1** and **3.2**; P<0.0001). This increase in the percentage of cells with ICP8 formed RCs was accompanied by a decrease in the percentage of cells with ICP8-formed pre-RCs, at sites of cellular ssDNA, or with ICP8 granularly dispersed through the nucleus. The percentage of cells with ICP8-formed pre-RCs decreased by 36.5% (to a total of 8.3±4.6% of cells; P<0.0001). The percentage of cells that had sites of cellular ssDNA did not change significantly (decreased by 10.5% to a total of 13.8±9.5% of cells; P>0.10). The percentage of cells that had ICP8 granularly dispersed throughout the nucleus decreased by 7.3% (to a total of 3.0±1.8% of cells; P<0.0001).

ICP8 formed HSV-1 replication structures in 17.8% more cells (for a total of 83.3±8.6% of cells; **Figure 3.3**; P<0.05), which was accompanied with an equivalent 17.8% decrease in the percentage of cells with ICP8 not localized to

HSV-1 replication structures (for a total of 16.7±8.6% of cells; P<0.05), in cells treated with no drug for 6 h after removal of the PAA-induced DNA replication block.

Fewer cells had ICP8-formed RCs when PAA was removed after the primary 5 h DNA replication block and media containing roscovitine was added for 6 h, as compared to when medium with no drug was added. Only 39.7% more cells (P<0.0001) had ICP8-formed RCs in the presence of roscovitine, whereas 54.2% more cells (P<0.0001) treated with no drug had ICP8-formed RCs (**Figures 3.1** and **3.2**). These increases are not quite statistically different (P=0.17). However, the increase in the percentage of cells with ICP8-formed RCs in the presence of roscovitine was accompanied by a statistically equivalent 38.0% decrease (P>0.10) in the percent of cells with ICP8-formed pre-RCs (for a total of 6.8±3.1% of cells; P<0.0001). When roscovitine was added after the 5 h primary block in DNA replication, therefore, the percentage of cells with ICP8-formed replication structures did not change significantly (65.5±2.7 at the time of PAA removal and 67.3±8.5% at the end of the 6 h; P>0.10; **Figure 3.3**). Under these conditions, the percentage of cells with sites of cellular ssDNA did not change significantly and was not statistically different to time zero (8.3% fewer cells [P>0.10] for a total of 16.0±14.4% [P>0.10]). The percentage of cells that had granular ICP8 dispersed throughout the nucleus did not change statistically (6.5% more cells for a total of 16.8±6.6%; P>0.10).

The percentage of cells with ICP8-formed HSV-1 replication structures increased significantly from 65.5±2.7% to 83.3±8.6% (P<0.0001) in the 6 h after

PAA was removed and no drug was added. At this endpoint, only 16.8±8.6% of cells had non-HSV-1 replication structures. In contrast, there was no significant increase in the percentage of cells with ICP8-formed HSV-1 replication structures when roscovitine was added at the time of PAA removal (from 65.5±2.7 to 67.3±8.5%; $P>0.10$).

Roscovitine inhibits both DNA replication and transcription. Therefore, the results discussed above may have been a consequence of inhibition of ICP8 expression after removing PAA and adding roscovitine. To account for ICP8 expression levels, the localization of ICP8 was tested when the translation inhibitor CHX was added at the time of PAA removal. Under these conditions, CHX inhibits ICP8 translation after removing PAA. ICP8 formed RCs in 52.8% more cells (for a total of 73.6±6.8%; $P<0.0001$) 6 h after adding CHX at the time of PAA removal (**Figures 3.1** and **3.2**). This increase in the number of cells with ICP8-formed RCs was accompanied by a 38.6% decrease in the number of cells with ICP8-formed pre-RCs (for a total of 6.2±5.4% of cells; $P<0.0001$). Conversely, the percentage of cells with sites of cellular ssDNA or with granular ICP8 dispersed throughout the nucleus was not significantly different to zero ($P>0.10$ for both) and did not significantly change in 6 h (11.5% fewer cells [$P>0.10$] for a total of 12.8±12.4%; and 2.9% fewer cells [$P>0.10$] for a total of 7.4±6.1%, respectively).

The percentage of cells with ICP8-formed HSV-1 replication structures increased by 14.3% ($P<0.0001$), to a total of 79.8±9.8% of cells, even when protein synthesis was inhibited with CHX at the time of PAA removal

(**Figure 3.3**). The observed inhibition of the formation of replication structures when roscovitine was added at the time of PAA removal was therefore not exclusively a result of the inhibition of ICP8 expression after adding the drug.

PAA inhibits HSV-1 DNA replication (the formation of RCs), and eventually also induces stalling of cellular replication forks. Therefore, PAA induces the accumulation of ICP8-formed pre-RCs and cellular ICP8 foci. The observed inhibition of replication structure formation may have consequently resulted from residual PAA effects. Alternatively, the inhibition of replication structure formation could have been the result of an incomplete release from the inhibition of DNA replication after PAA removal. Furthermore, the PAA concentrations required for efficient inhibition of HSV-1 DNA replication did not allow for release of inhibition. Consequently, only partially inhibitory PAA concentrations could be used. The observed inhibition of replication structures formation also may have therefore also been the result of an incomplete inhibition of HSV-1 DNA replication during the accumulation of ICP8 prior to roscovitine treatment. The localization of ICP8 in the presence of roscovitine, therefore, was further evaluated without previous use of PAA.

3.3 ROSCOVITINE INHIBITS THE ICP8-INDUCED FORMATION OF NEW REPLICATION STRUCTURES WHEN ICP8 WAS EXPRESSED DURING A TEMPERATURE SENSITIVE MUTANT-INDUCED DNA REPLICATION BLOCK

The results described in **Figures 3.1** to **3.3** may have resulted from an incomplete release from the inhibition of DNA replication after removing PAA, from residual PAA effects, or from an incomplete block of HSV-1 DNA replication during the accumulation of ICP8. Therefore, I next evaluated the formation of replication structures without using PAA. Instead, I used a temperature-induced replication block. More specifically, I used an HSV-1 *ts* ICP8 mutant, which expresses a form of ICP8 that does not support HSV-1 DNA replication at the non-permissive temperature (A1; **Figure 1.3**). However, this *ts* ICP8 fully supports HSV-1 DNA replication at the permissive temperature (**Figure 3.4**).

ICP8 is required for HSV-1 DNA replication. Therefore, cells infected with ICP8 *ts* mutant A1 at non-permissive temperatures express IE and E proteins in the absence of HSV-1 DNA replication. In addition to not supporting significant levels of HSV-1 DNA replication, *ts* A1 ICP8 is unstable at the non-permissive temperature (39.5^{o}C) typically used for such experiments. I discovered a non-permissive temperature (38^{o}C) whereby ICP8 is stable but does not contribute to HSV-1 DNA replication.

ts A1 ICP8 is stable at 38^{o}C for 5 h and localizes to the nucleus. It forms replication structures in approximately half of cells (45.6±14.6%, **Figure 3.5** and **3.6**; 0 h after temperature change). However, only 9.9±8.7% of cells had ICP8-

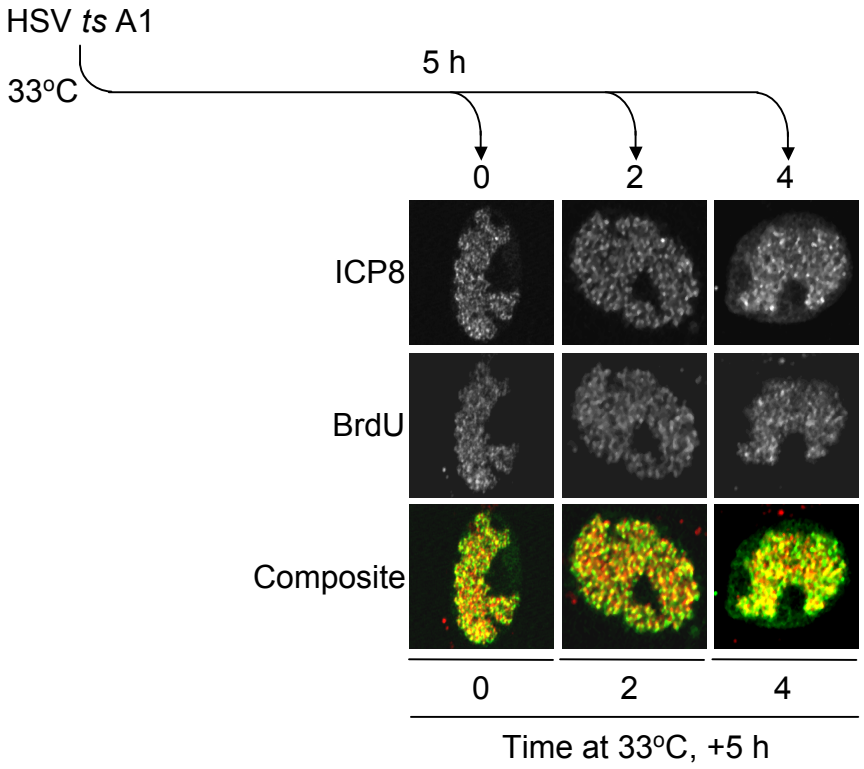
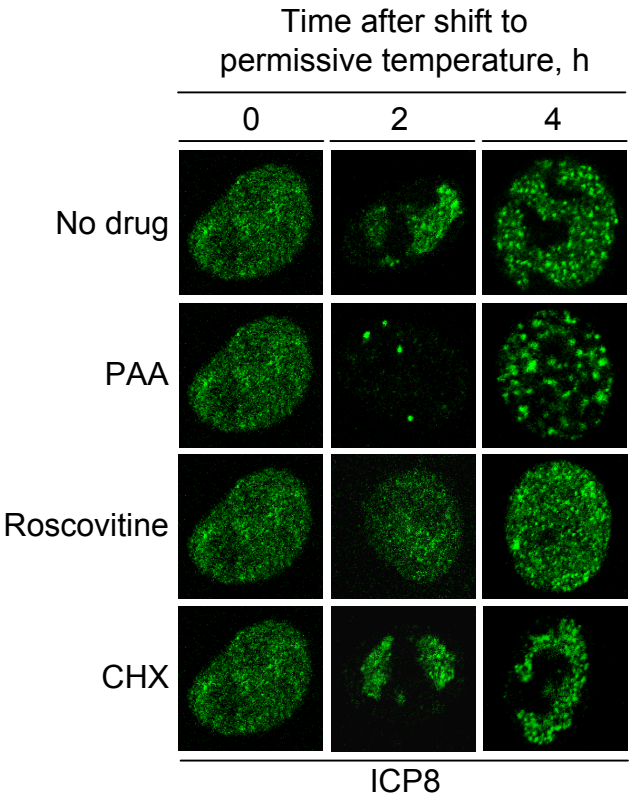


Figure 3.4 *ts* A1 ICP8 localizes to replication compartments at the permissive temperature. Representative confocal immunofluorescence images showing ICP8 localization in cells treated with no drug after infection with the ICP8 *ts* mutant A1 and incubated at the permissive temperature over the entire time course, as described in Figure 3.4. Images were collected using a Zeiss LSM 510 confocal microscope. Vero cells infected with 30 PFU of HSV ICP8 *ts* mutant A1 per cell were incubated at the permissive temperature (33°C) for up to **9** h. Cells were pulsed with 10 μM of the thymidine analog BrdU for 15 min and fixed for 15 min in 10% formalin. **ICP8** or **BrdU** were detected by indirect immunofluorescence, nuclei were counterstained with Hoescht 33258 (not shown). RCs were differentiated from pre-RCs by ICP8 (green) and BrdU (red) localization at the same nuclear domains (**composite**).

A


Time after shift to
permissive temperature, h
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No drug
PAA
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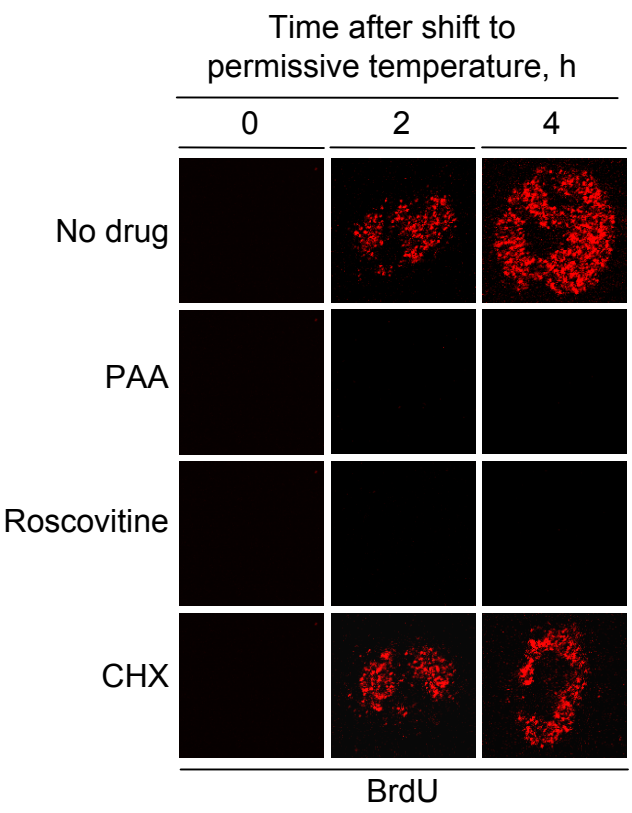
CHX
ICP8

B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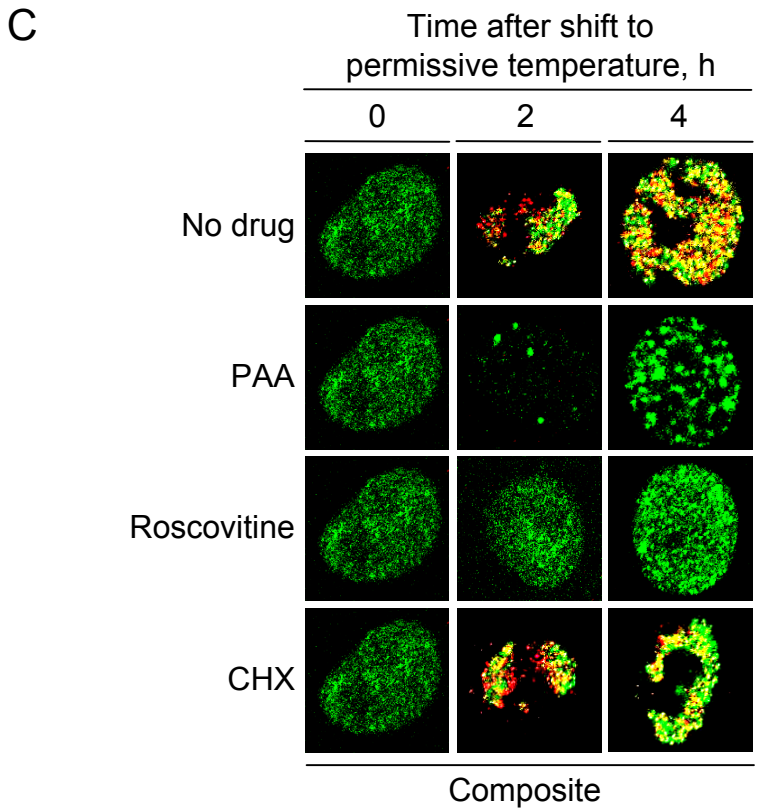

Figure 3.5 ICP8 accumulated during a temperature-induced DNA replication block localizes differentially in the presence of roscovitine. Representative confocal immunofluorescence images showing ICP8 localization in cells treated with no drug, PAA, roscovitine, or CHX after a *ts*-induced DNA replication block. Images were collected using a Zeiss LSM 510 confocal microscope. Vero cells infected with 30 PFU of HSV ICP8 *ts* mutant A1 per cell were incubated at the non-permissive temperature (38°C) for 5 h. Cells were then transferred to the permissive temperature (33°C) in medium containing **no drug**, 200 μg/mL **PAA**, 100 μM **roscovitine**, or 50 μg/mL **CHX** for 2 or 4 h after the shift. Cells were pulsed with 10 μM of the thymidine analog BrdU for 15 min and fixed for 15 min in 10% formalin. ICP8 (**A**) or BrdU (**B**) were detected by indirect immunofluorescence; nuclei were counterstained with Hoescht 33258 (not shown). RCs were differentiated from pre-RCs by ICP8 (green) and BrdU (red) localization at the same nuclear domain (**C**). The images at 0 h (left-hand panels) are all the same picture, which was copied for each drug treatment for clarity of the presentation.

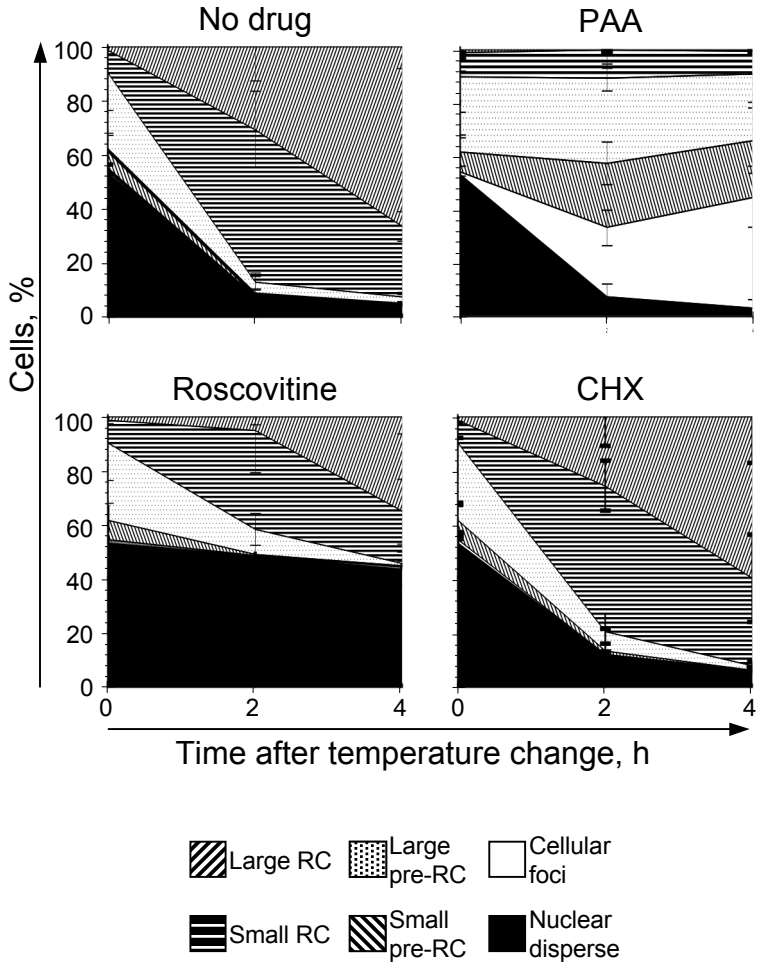

Figure 3.6 The localization of ICP8 accumulated during a temperature-induced DNA replication block to new replication sites is inhibited by roscovitine. Area graphs presenting the percentage of cells with ICP8 as **nuclear disperse** or in sites of cellular ssDNA (**Cellular foci**), viral **pre-RCs**, or viral **RCs** after releases from a *ts*-induced DNA replication block. Vero cells were infected with 30 PFU of HSV-1 ICP8 *ts* mutant strain A1 per cell at the non-permissive temperature (38^{o}C) and then incubated for 4 h. Cells were then transferred to the permissive temperature (33^{o}C) in media with **no drug**, 200 μg/mL **PAA**, 100 μM **roscovitine**, or 50 μg/mL **CHX**. Cells were pulsed 0, 2, or 4 h after the temperature shift-down with 10 μM of the thymidine analog BrdU for 15 min and fixed for 15 min with 10% formalin. Indirect immunofluorescence was then performed with ICP8 or BrdU antibodies and the nuclei were counterstained with Hoescht 33258. RCs were distinguished from pre-RCs by ICP8 and BrdU localization at the same nuclear domain. Error bars, standard deviation of five independent experiments (100 cells were counted for each treatment in each experiment).

formed RCs. This percentage of cells ICP8-formed RCs is not significantly different to zero (P>0.10). Therefore, ICP8 forms new replication structures at non-permissive temperatures, but does not significantly contribute to HSV-1 DNA replication.

Just over half of the cells (53.3±14.5%) infected with *ts* A1 and maintained at the non-permissive temperature of 38^{o}C had granular ICP8 dispersed throughout the nucleus (**Figures 3.5** and **3.6**). The remaining cells had ICP8-formed pre-RCs (35.7±17.6%), RCs (9.9±8.7%), or sites of cellular ssDNA (1.2±1.7%). Therefore, ICP8 formed HSV-1 replication structures in 45.6±14.5% and not in the other 54.5±14.5% of cells (**Figure 3.7**).

The percent of cells with granular ICP8 dispersed throughout the nucleus decreased by 49.1% (to a total of 4.2±4.0% of cells; P<0.0001) 4 h after decreasing to the permissive temperature in media containing no drug (**Figures 3.5** and **3.6**). Reciprocally, ICP8 formed RCs in 39.5% more cells (P<0.0001) than at non-permissive temperatures, for a total of 92.8±4.5% of cells. Only a few cells still had ICP8-formed pre-RCs (3.0±1.9%) after 4 h at the permissive temperature.

The addition of PAA at the time of temperature shift down predominantly resulted in ICP8-formed pre-RCs (46.0±16.5%) or sites of cellular ssDNA (41.4±11.4%; **Figures 3.5** and **3.6**) after 4 h, much like in the PAA release experiment. Occasionally, ICP8 formed RCs (9.2±8.4%) or was granularly dispersed throughout the nucleus (3.4±2.8%). Under these conditions, therefore, ICP8 formed HSV-1 replication structures in 55.2±11.8% of cells, and not in

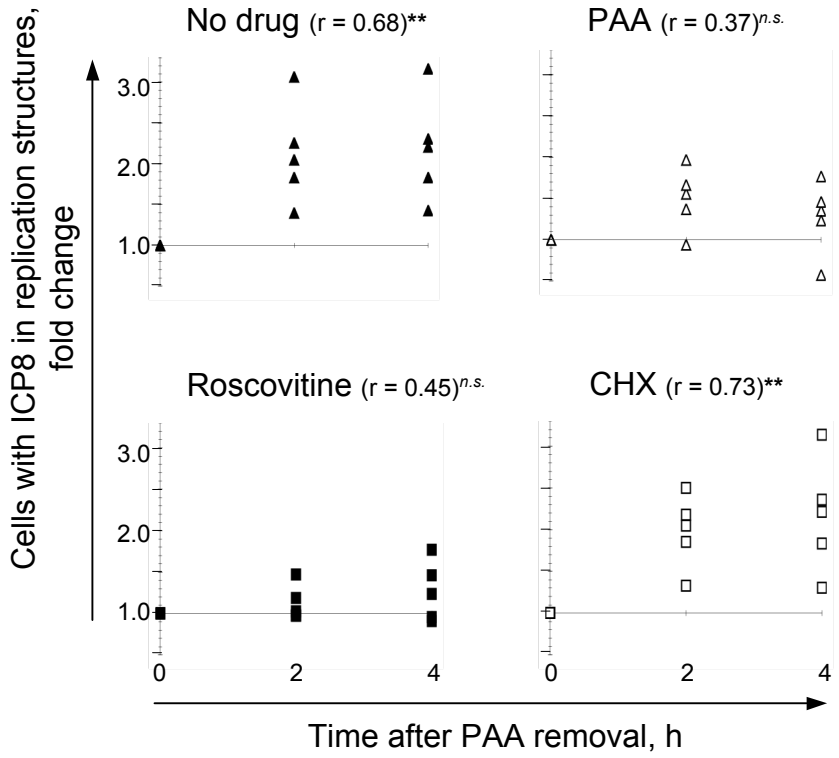

Figure 3.7 Correlation analyses of the formation of new replication structures after removal of temperature sensitive mutant-induced DNA replication block. A scatter plot presenting the relative number of cells with ICP8 in new replication structures formed after release from a PAA-induced DNA replication block. Vero cells infected with 30 PFU of HSV ICP8 *ts* mutant A1 per cell were incubated at the non-permissive temperature (38°C) for 5 h. Cells were then transferred to the permissive temperature (33°C) in media with **no drug**, 200 μg/mL **PAA**, 100 μM **roscovitine**, or 50 μg/mL **CHX**. Cells were pulsed 0, 2, or 4 h after the temperature shift-down with 10 μM of the thymidine analog BrdU for 15 min and fixed for 15 min with 10% formalin. Immunofluorescence was performed with ICP8 or BrdU primary antibodies and the nuclei were counterstained with the DNA stain Hoescht 33258. Data from five independent experiments, in each of which at least 100 cells were counted for each treatment. **, $p<0.005$; *n.s.*, not significant.

44.8±11.8% of cells. The inhibition of ICP8 formation of replication structures was not a consequence of the mutation in A1 ICP8. A1 *ts* ICP8 fully supported the formation of replication structures at the permissive temperature (**Figure 3.4**).

Addition of roscovitine at the time of the shift-down did not result in a statistically significant decrease in the percentage of cells with granular ICP8 dispersed throughout the nucleus after 4 h (only 9.7% fewer cells [P>0.10] as compared to 49.1% when treated with no drug; **Figures 3.5** and **3.6**). These decreases are significantly different (P<0.0001). A total of 43.6±6.2% of cells had granular ICP8 dispersed throughout the nucleus. The percentage of cells with ICP8-formed RCs increased by 44.5% (to a total of 54.4±7.1%; P<0.0001). However, this increase was accompanied by a statistically equivalent 34.3% decrease (P>0.10) in the percentage of cells with ICP8-formed pre-RCs (to a total of 1.4±0.5% of cells; P<0.0001). Therefore, the percentage of cells with ICP8-formed replication structures did not change significantly, slightly increasing from 45.6±14.6% of cells at non-permissive temperatures to 55.8±3.3 after 4 h (P>0.10; **Figure 3.7**). Only 0.6±1.3% of the cells had sites of cellular ssDNA.

As discussed, roscovitine inhibits both DNA replication and transcription. The translational inhibitor CHX was therefore included to account for the inhibition of new ICP8 expression. The addition of CHX at the time of shift down to permissive temperatures resulted in 47.5% fewer cells (P<0.0001; compared to 49.1% fewer cells treated with no drug) with ICP8 granularly dispersed throughout the nucleus 4 h later (for a total of only 5.8±3.1% of cells; **Figures 3.5** and **3.6**). The remaining cells had ICP8-formed RCs (92.6±3.5% of

cells) with occasional ICP8-formed pre-RCs (1.6±0.5% of cells). Therefore, 94.2±3.1% of cells had ICP8-formed HSV-1 replication structures 4 h after shift to the permissive temperature (**Figure 3.6**).

The trend in the ICP8-formed replication structures (pre-RCs or RCs) in cells treated with no drug or CHX at the time of temperature shift-down was different to that in the cells treated with roscovitine. Virtually all cells had ICP8-formed replication structures after 4 h at the permissive temperature in the presence of no drug or CHX (no drug, 95.8±4.0%; CHX, 94.2±3.1%; **Figure 3.6**). Conversely, only 4.2±4.0% or 5.8±3.1% of cells treated with no drug or CHX did not have ICP8-formed HSV-1 replication structures. Therefore, 50.2% more cells (or 48.6% in the presence of CHX) had ICP8-formed replication structures 4 h after the transfer to the permissive temperature than while at the non-permissive temperature ($P<0.0001$). In contrast, the percentage of cells with ICP8-formed replication structures did not increase significantly when roscovitine was added at the time of shift-down ($P>0.10$; 10.2% increase; to a total of 55.8±3.3%). The percentage of cells with non-HSV-1 DNA replication structures, therefore, also did not decrease significantly ($P>0.10$; 10.2% decrease; for a total of 44.2% cells) when the cells were treated with roscovitine at the time of temperature shift-down. Therefore, ICP8 did not form new replication structures in the presence of roscovitine (**Figure 3.7**).

I next evaluated whether the inhibition of replication structure formation was a consequence of the temperature shifts. Cells were infected with wild-type HSV-1, incubated at 38^{o}C, and then shifted to 33^{o}C after 5 h. Zero or three hours

later, the cells were pulsed with BrdU and fixed with folmaldehyde. ICP8 and BrdU were detected by immunofluorescence. Wild-type ICP8 formed replication compartments before or after the temperature shift from 38 to 33^{o}C. Wild-type ICP8 predominantly formed large replication compartments during the 5 h incubation at 38^{o}C (**Figure 3.8**), and the shift to the permissive temperature (33^{o}C) did not induce a decrease in the number of cells with ICP8-formed large replication compartments.

3.4 DISCUSSION

To identify the steps at which roscovitine inhibits HSV-1 DNA replication, I evaluated the formation of pre-RCs and RCs after release from HSV-1 DNA replication blocks, induced by either PAA or non-permissive temperatures. Under either condition, roscovitine inhibited the formation of replication structures (pre-RCs and RCs). The inhibition of new replication structure formation was not a exclusively a result of the inhibition of transcription by roscovitine, as ICP8 still formed new replication structures in the presence of the protein synthesis inhibitor CHX.

ICP8 is the first protein to localize to the sites adjacent to ND10s during the formation of HSV-1 DNA replication compartments. The localization of ICP8 to these new replication structures occurs before, and is required for, the initiation of HSV-1 DNA replication. In the presence of roscovitine, however, ICP8 did not form new replication structures. The inhibition occurred after either PAA- or temperature-induced DNA replication blocks. Therefore, the inhibition

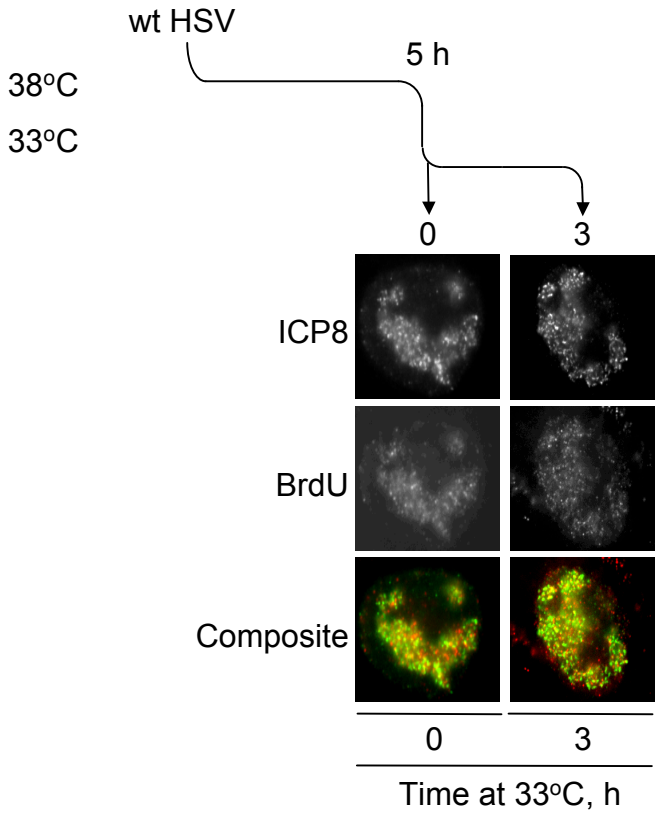

Figure 3.8 Wild-type ICP8 localizes to replication compartments at 38 or 33°C. Representative immunofluorescence images showing wild-type ICP8 localization in nuclei after a temperature shift. Images were collected using a fluorescence microscope with a UV light source (Leica DM, IRB, Wetzlar, Germany) and camera (QIMAGING RETIGA 1300, Burnaby, Canada). Vero cells infected with 30 PFU of wild type HSV-1 per cell were incubated at 38°C for 5 h. Cells were then transferred to 33°C for **0** or **3** h. Cells were pulsed with 10 μM of the thymidine analog BrdU for 15 min and fixed for 15 min in 10% formalin. **ICP8** or **BrdU** were detected by indirect immunofluorescence, and the nuclei were counterstained with Hoescht 33258 (not shown). RCs were differentiated from pre-RCs by ICP8 (green) and BrdU (red) localization at the same nuclear domains (**composite**).

of new replication structure formation was not a result of an incomplete release from inhibition of DNA replication after removing PAA, an incomplete block of HSV-1 DNA replication, or residual PAA effects during the accumulation of ICP8.

Roscovitine, therefore, inhibits an early step in the formation of pre-RCs. The discovery that roscovitine inhibits the formation of replication structures indicates that roscovitine inhibits a step prior to the initiation of HSV-1 DNA replication. Therefore, the previously observed inhibition of HSV-1 DNA replication by roscovitine is not exclusively a result of direct inhibition of HSV-1 DNA replication.

CHAPTER 4

ICP8 IS MORE EASILY EXTRACTED FROM ROSCOVITINE-TREATED CELLS, BUT THE INCREASE IN EXTRACTABILITY IS NOT A RESULT OF A DECREASED AFFINITY FOR SSDNA

4.1 INTRODUCTION

Before I started my thesis work, it was unclear which HSV-1 DNA replication steps were inhibited by roscovitine. It was not even clear whether the observed inhibition of HSV-1 DNA replication was a result of the direct inhibition of HSV-1 DNA replication. I discovered that roscovitine inhibits the formation of replication structures. Therefore, the inhibition of HSV-1 DNA replication by roscovitine after the expression of E proteins occurs at a step prior to initiation. It is still not clear, however, which steps prior to HSV-1 DNA replication are inhibited by roscovitine.

A series of steps are required after the expression of E proteins and prior to the initiation of replication. For example, ICP8 is required to localize to the proper nuclear domains, bind to HSV-1 ssDNA, and recruit the HSV-1 DNA replication enzymes. However, ICP8 does not localize to the proper nuclear domains in the presence of roscovitine. Therefore, ICP8 may not bind to HSV-1 ssDNA in the proper nuclear domains or may not recruit the required HSV-1 DNA replication proteins. ICP8 may fail to localize to the proper nuclear domains

in the presence of roscovitine due to inhibition of required interactions with ssDNA or other binding partners.

ICP8 binds to ssDNA and interacts with both viral and cellular proteins (**Table 4.1**). Therefore, the observed mislocalization of ICP8 could have resulted from altered interactions of ICP8 with ssDNA. A decrease in the affinity of ICP8 for ssDNA in the presence of roscovitine would result in the observed inhibition of HSV-1 DNA replication in the presence of all the required HSV-1 DNA replication proteins. A decrease in ICP8 affinity for ssDNA in the presence of roscovitine would also result in the observed inhibition of ICP8 accumulation at new HSV-1 DNA replication domains. Therefore, I evaluated the ssDNA affinity of ICP8 extracted from roscovitine and CHX treated cells.

A decreased affinity of ICP8 for binding either ssDNA (or other binding proteins) would result in ICP8 being easier to extract from the nuclei. I, therefore, first evaluated whether ICP8 was more easily extracted from the nuclei of cells treated with roscovitine.

4.2 ICP8 IS MORE EASILY EXTRACTED FROM NUCLEI OF CELLS TREATED WITH ROSCOVITINE

To evaluate the so-called "extractability" of ICP8 from the nuclei of roscovitine-treated cells, I performed serial extractions with increasing concentrations of NaCl. Vero cells were infected with the ICP8 *ts* mutant A1. Infected cells were incubated at the non-permissive temperature (38^{o}C) for 5 h to induce the accumulation of ICP8 in the absence of DNA replication. Cells were then treated

Table 4.1 ICP8 binding proteins

Binding protein	Peptides‡	co-IP	Interaction in the absence DNA§	Localization ND10	Localization RCs
Replication/repair/ recombination					
DNA-protein kinase catalytic subunit (DNA-PKcs)	51	+[a]	n/t	-	+[a]
Rad50	29	+[a]	**yes**	+[b]	+[a,c]
Ku86	26	+[a]	**yes**	-	+[a,d]
Ku70	23	n/t	n/t	-	n/t
Poly ADP-ribose polymerase-1(PARP-1)	16	n/t	n/t	-	n/t
MutS homolog 6 (MSH6)	5	n/t	n/t	-	n/t
Meiotic recombination 11 (Mre11)	4	n/t	n/t	+[b,e]	+[f]
Replication protein A (RPA)	4	+[a]	**yes**	+[g,h]	+[d,i]
DNA methyltransferase-associated protein 1 (DNMTAP-1)	3	n/t	n/t	n/t	n/t
Dead-box p68	3	n/t	n/t	n/t	n/t
Dead-box protein	2	n/t	n/t	-	n/t
Minichromosome maintenance protein 2 (MCM2)	2	n/t	n/t	-	n/t
X-ray repair cross complementing protein 4 (XRCC4)	1	n/t	n/t	-	n/t
MSH3	1	n/t	n/t	-	n/t
Proliferating nuclear antigen (PCNA)	1	n/t	n/t	-	+[i]
Werner helicase (WRN)†		+[a]	**yes**	+[j]	+[a]
MSH2†		n/t	n/t	-	+[a]
Breast cancer-associated protein 1 (BRCA1) †		n/t	n/t	+[k,l]	+[a]
Bloom helicase (BLM)†		n/t	n/t	+[m,n]	+[a]

Binding protein	Peptides‡	co-IP	Interaction in the absence DNA§	Localization ND10	Localization RCs
Chromatin remodeling					
Human sucrose non-fermenting 2H (hSNF2H)	18	+[a]	partial	-	+[a]
Facilitates chromatin transcription p140 (FACT p140)	16	n/t	n/t	-	-
Brahma (BRM)	15	n/t	n/t	-	+[a]
BRM-related gene-1 protein (BRG1)	13	+[a]	**yes**	-	+[a]
BRG1 or BRM-associated factor 155 (BAF155)	11	+[a]	partial	-	+[a]
Structural maintenance of chromosomes 1 (SMC1)	9	n/t	n/t	-	n/t
BAF57	9	n/t	n/t	-	n/t
BAF170	8	n/t	n/t	-	n/t
Chromo-helicase DNA-binding protein-3-interacting protein (CHD3-IP)	8	n/t	n/t	n/t	n/t
SWI-independent 3a (SIN3a)	7	+[a]	partial	-	+[a]
hSNF2L	6	n/t	n/t	-	n/t
DEK	4	n/t	n/t	n/t	n/t
BAF 60a	3	n/t	n/t	-	n/t
Histone deacetylase 2 (HDAC2)	3	+[a]	partial	-	+[a]
Nucleosome-associated protein 1-like (NAP-1-like)	3	n/t	n/t	-	n/t
Chromatin assembly factor I p48 subunit (CAF-1 p48)	2	n/t	n/t	-	n/t

ICP8 binding proteins	Peptides[‡]	co-IP	Interaction DNA-dependent[§]	Localization ND10	Localization RCs
RNA binding/ splicing					
Spliceosome-associated protein 130 (SAP130)	7	n/t	n/t	-	n/t
SAP155	5	n/t	n/t	-	n/t
Glycine, arginine, tyrosine-rich RNA-binding protein (GRY-RBP)	3	n/t	n/t	-	n/t
Nuclear matrix protein 200 (NMP200)	3	n/t	n/t	-	n/t
Transcription factors					
General transcription factor II-I (GTF II-I)	7	n/t	n/t	-	n/t
TBP-associated factor of 172 kDa (TAF172)	3	n/t	n/t	-	n/t
Activity-dependent neuroprotective protein (ADNP)	3	n/t	n/t	-	n/t
RE1-silencing transcription (REST) co-repressor	2	n/t	n/t	-	-
Other					
Inosine-5'-monophosphate dehydrogenase 2 (IMPDH-2)	12	n/t	n/t	-	n/t
Herpesvirus-associated ubiquitin-specific protease (HAUSP)	6	n/t	n/t	+[o]	+[o]
Emerin	3	n/t	n/t	-	-
NMP238	2	n/t	n/t	-	n/t
Protein phoshpatase 1A subunit (PP1A subunit)	2	n/t	n/t	-	-
Lamin A/C	2	n/t	n/t	-	-
HSV-1 proteins					
U_L5	25	+[a]	**yes**	-	+[a,p]
ICP4	19	+[a]	no	-	+[a,q]
U_L12	8	n/t	n/t	-	n/t
U_L42	6	n/t	n/t	-	+[p,r]
ICP27	4	+[a]	**yes**	-	+[a,s]
U_L8	3	n/t	n/t	-	+[p]

* Adapted from (Taylor and Knipe, 2004)

‡ Number of peptides detected by mass spectroscopy after trypsin digestion of an excised polyacrylamide gel band

† Protein predicted to associate indirectly with ICP8 or localize to RCs based upon interaction with another protein in the table

§ The interaction between the binding partners is not dependent on the presence of DNA (partial, indicates a more than 50% decreased level of immunoprecitated protein in the presence of ethidium bromide)

co-IP, co-immunoprecipitation; ND10, nuclear dot 10; RC, replication compartment; +, detected; -, not detected; n/t, not tested; ADP, adenosine diphosphate; SWI, mating-type switching; TBP, TATA-binding protein

[a](Taylor and Knipe, 2004); [b](Lombard and Guarente, 2000); [c](Lilley et al., 2005); [d](Wilkinson and Weller, 2004); [e](Mirzoeva and Petrini, 2003); [d](Negorev and Maul, 2001); [f](Gregory and Bachenheimer, 2008); [g](Barr et al., 2003); [h](Yeager et al., 1999); [i](Wilcock and Lane, 1991); [j](Johnson et al., 2001); [k](Maul, 1998); [l](Wu et al., 2003); [m](Ishov et al., 1999); [n](Yankiwski et al., 2000); [o](Everett et al., 1997); [p](Liptak et al., 1996); [q](Knipe et al., 1987); [r](Goodrich et al., 1990); [s](de Bruyn Kops et al., 1998)

with either roscovitine or CHX (to account for the inhibition of ICP8 expression). Cells were collected 4 h later and their nuclei were isolated. Nuclei were then resuspended in buffer containing increasing concentrations of NaCl. The first extraction buffer contained 50 mM NaCl. The second extraction buffer contained 200 mM NaCl. Subsequent extraction buffers contained NaCl concentrations increasing by 100 mM increments (from 300 mM to 800 mM). Nuclei suspensions were then centrifuged to pellet the nuclei, and the supernatants containing the extracted proteins were collected. The proteins that were still in the nuclear pellet after the last wash (800 mM) were released by lysis. Extracted proteins were resolved by SDS-PAGE and transferred to PVDF membranes. ICP8 was then detected by Western blot, and the levels of ICP8 extracted at each NaCl concentration were quantitated using Licor Odyssey software.

The levels of ICP8 extracted at each NaCl concentration during the serial extraction differed depending on whether the infected cells had been treated with roscovitine or not (**Figures 4.1** and **4.2**). Most ICP8 was extracted from the nuclei of CHX-treated cells at 0.4 to 0.6 M NaCl. In contrast, most ICP8 was extracted from the nuclei of roscovitine-treated cells at 0.3 to 0.4 M NaCl. Unfortunately, the standard deviations of the averages of the three repeats are considerable.

To analyze the extractability, the cumulative levels of extracted ICP8 were considered. Then, the concentration of NaCl required to extract 50% ICP8 was calculated. To this end, the cumulative levels of ICP8 extracted were plotted against the NaCl concentration (**Figure 4.3**). Using the resulting sigmoid curves,

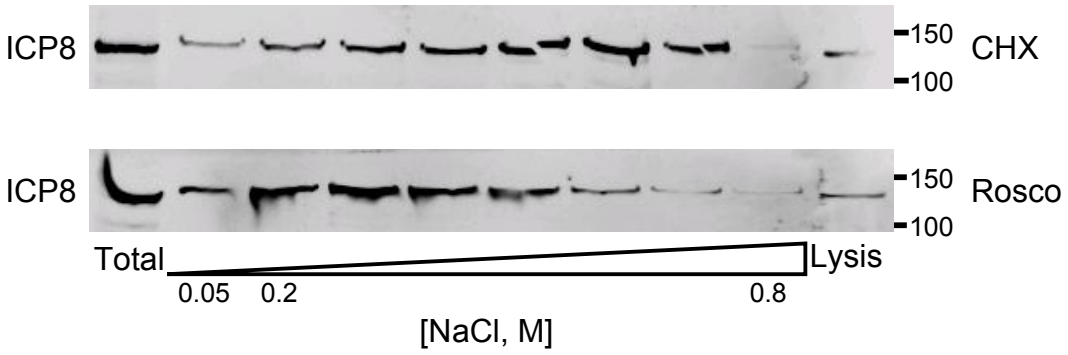

Figure 4.1 ICP8 is more easily extracted from the nuclei of roscovitine-treated cells. Representative Western blots showing the amounts of ICP8 extracted from the nuclei of cells treated with CHX or roscovitine. Vero cells were infected with 10 PFU of HSV-1 ICP8 *ts* mutant strain A1 per cell at the non-permissive temperature (38^{o}C) and incubated for 5 h. Cells were then transferred to the permissive temperature (33^{o}C) for 4 h in media with 100 μM **roscovitine** or 50 μg/mL **CHX**. Nuclei were harvested and the nuclear proteins were extracted at increasing concentrations of NaCl ( ). Proteins remaining in the nuclei after the last extraction were released by lysis (**lysis**). Proteins were separated in 10% SDS-PAGE and transferred to PVDF membranes. ICP8 detected by Western blot was quantitated using LICOR Odyssey software. **Rosco**, roscovitine. Protein levels in each lane were normalized to **Total** (only 10% of the total protein in the extracts was loaded into the gels) during quantitation.

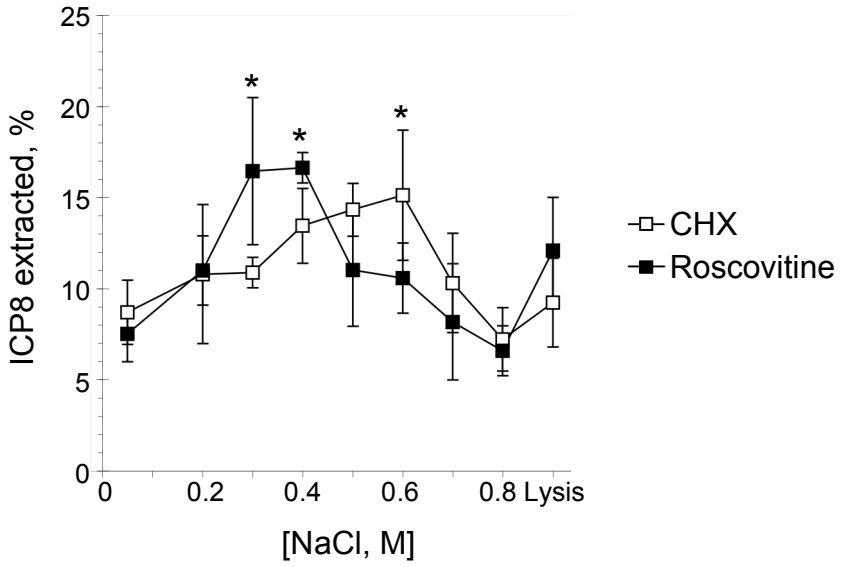

Figure 4.2 ICP8 is more easily extracted from the nuclei of roscovitine-treated cells. Line graphs presenting the percentage of ICP8 extracted from the nuclei of cells treated with CHX or roscovitine. Vero cells were infected with 10 PFU of HSV-1 ICP8 *ts* mutant strain A1 per cell at the non-permissive temperature (38^{o}C) and then incubated for 5 h. Cells were then transferred to the permissive temperature (33^{o}C) in media with 100 μM **roscovitine** or 50 μg/mL **CHX** for 4 h. Nuclei were harvested and the nuclear proteins were extracted at increasing concentrations of NaCl. Proteins remaining in the nuclei after the last extraction were released by lysis (**lysis**). Proteins were separated in 10% SDS-PAGE and transferred to PVDF membranes. ICP8 detected by Western blot was quantitated using LICOR Odyssey software. Error bars, standard deviation of 3 independent experiments. *, $p < 0.10$.

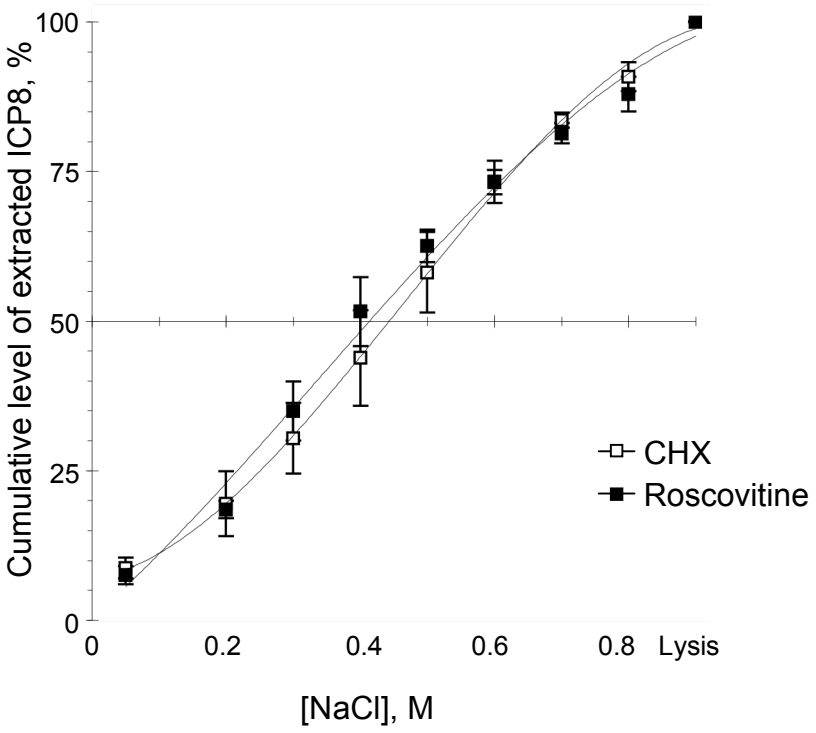

Figure 4.3 The concentration required to extract 50% ICP8 from roscovitine-treated cells is not significantly different than that required to extract it from CHX-treated cells. Line graphs presenting the cumulative percentage of ICP8 extracted at different NaCl concentrations from cells treated with CHX or roscovitine. Vero cells were infected with 10 PFU of HSV-1 ICP8 *ts* mutant strain A1 per cell at the non-permissive temperature (38^{o}C). Cells were transferred to the permissive temperature (33^{o}C) 5 h later in media containing 100 μM **roscovitine** or 50 μg/mL **CHX**. Four hours later, nuclei were harvested and the nuclear proteins were extracted at increasing concentrations of NaCl. Proteins remaining in the nuclei after the last extraction were released by lysis (**lysis**). Proteins were separated in 10% SDS-PAGE and transferred to PVDF membranes. ICP8 detected by Western blot was quantitated using LICOR Odyssey software. Error bars, standard deviation of three independent experiments.

the NaCl concentration required to extract 50% ICP8 was determined for each individual experiment. A concentration of 441.0±49.0 mM (average ± range) NaCl was required to extract 50% ICP8 from the nuclei of CHX-treated cells. In contrast, only 396.0±41.5 mM NaCl was required to extract 50% ICP8 from the nuclei of roscovitine-treated cells. There was high variability between the experiments. The decrease of 45 mM NaCl in the average concentration of NaCl required to extract 50% ICP8 from the nuclei of roscovitine-treated cells in comparison to the CHX-treated cells was consequently not statistically significant ($P>0.10$). The differences in concentration required to extract 50% ICP8 from nuclei of CHX- than roscovitine-treated cells ranged for the three experiments from 25 to 70 mM. The individual experiments had decreases of 25, 40, and 70 mM in the concentration of NaCl required to extract 50% ICP8 from the nuclei of roscovitine-treated cells in comparison to CHX-treated cells.

There were no statistically significant differences between the NaCl concentrations required to extract 50% ICP8 from the nuclei of roscovitine- or CHX-treated cells. However, most ICP8 was extracted at different concentration ranges for each treatment (0.3 to 0.4 M for CHX and 0.4 to 0.6 M for roscovitine). Furthermore, an analysis of the individual points along the extraction curve revealed that a significantly higher percentage of ICP8 was extracted from the nuclei of roscovitine-treated cells at 0.3 and 0.4 M NaCl ($P<0.10$). Conversely, a significantly higher percentage of ICP8 was extracted from the nuclei of CHX-treated cells at 0.6 M NaCl ($P<0.10$). Differences between the percentages of ICP8 extracted from the nuclei were not statistically significant at NaCl

concentrations of 50, 200, 500, 700, and 800 mM, or after the lysis at the end of the serial extraction ($P<0.10$).

Therefore, there was a non-statistically significant trend for ICP8 to be more easily extracted from the nuclei of roscovitine-treated cells than of CHX-treated cells. The increase in ICP8 extractability from the nuclei or roscovitine-treated cells in comparison to CHX-treated cells may have resulted from altered interactions with viral or cellular binding proteins (**Table 4.1**), or with ssDNA. A decrease in the affinity of ICP8 for ssDNA would result in the previously observed inhibition of HSV-1 DNA replication. Furthermore, a decrease in ICP8 affinity for ssDNA would also result in the observed lack of ICP8 localization to new sites of DNA replication, and in the increase in ICP8 extractability. Therefore, I evaluated whether ICP8 had an altered *in vitro* affinity for ssDNA.

4.3 ICP8 FROM CELLS TREATED WITH ROSCOVITINE DOES NOT HAVE A DECREASED AFFINITY FOR ssDNA

I next used affinity chromatography to evaluate the *in vitro* affinity of ICP8 for ssDNA. Vero cells were infected with the ICP8 *ts* mutant A1 at the non-permissive temperature (38^{o}C) to induce the accumulation of ICP8 in the absence of HSV-1 DNA replication. CHX or roscovitine was added after 5 h, and the cells were then incubated at the permissive temperature (33^{o}C) for 4 more hours. The cells were then collected, the nuclei isolated, and the nuclear proteins were extracted in a 4 M NaCl buffer. The nuclear proteins were then incubated with ssDNA coupled to cellulose in a 150 mM NaCl buffer and allowed to bind for 1 h.

The beads and their interacting proteins were then pelleted by centrifugation and the supernatants containing unbound proteins were collected. The beads were resuspended in buffer containing 150 mM NaCl and incubated on a rocker for 5 min before pelleting them again by centrifugation. The supernatants containing the unbound proteins were collected and pooled with the unbound proteins collected during the previous centrifugation. The resuspension, incubation, centrifugation, and supernatant collections were repeated at 300, 400, 500, 600, 700, 800 mM, and 2 M NaCl. Each of these extractions was analyzed individually. The proteins in the supernatants were separated by SDS-PAGE and transferred to a PVDF membrane. ICP8 was detected by Western blot, and its levels were quantitated using Licor Odyssey software.

The percentage of ICP8 that bound to ssDNA did not change significantly when cells were treated with CHX or roscovitine ($P>0.10$). The average percentage of ICP8 bound to ssDNA was 38.4±10.5% (ranging from 31.5 to 51.7%) when ICP8 was extracted from cells were treated with CHX, or 31.3±6.1% (ranging from 24.6 to 40.6%) when ICP8 was extracted from cells treated with roscovitine.

The concentrations of NaCl required to elute most ICP8 from ssDNA were not different when ICP8 had been extracted from cells treated with CHX or roscovitine (**Figures 4.4** and **4.5**). The average NaCl concentration required to elute 50% ICP8 from ssDNA when ICP8 had been extracted from cells treated with CHX was 389.4±37.9 mM (average±standard deviation; n = 5; **Figure 4.6**). The range of concentrations required to elute 50% ICP8 from ssDNA when ICP8

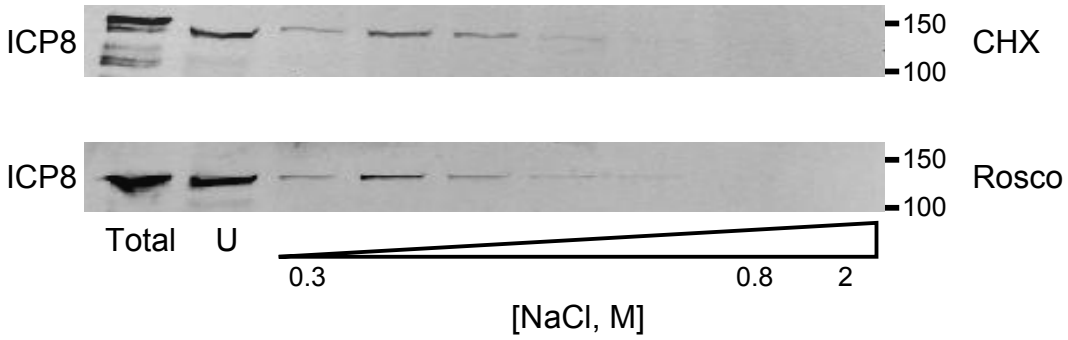

Figure 4.4 ICP8 extracted from roscovitine-treated cells does not have a decreased affinity for ssDNA *in vitro*. Representative Western blots showing the amount of ICP8 eluted from ssDNA at different NaCl concentrations. Vero cells were infected with 10 PFU of HSV-1 ICP8 *ts* mutant strain A1 per cell at the non-permissive temperature (38°C). Cells were transferred to the permissive temperature (33°C) 5 h later in media containing 100 μM **roscovitine** or 50 μg/mL **CHX**. Four hours later, the nuclei were harvested and the nuclear proteins were extracted. Nuclear proteins were incubated with ssDNA coupled to cellulose for 1 h. Bound proteins were eluted from ssDNA at increasing NaCl concentrations ( ). Extracted proteins were separated in 10% SDS-PAGE and transferred to PVDF membranes. ICP8 detected by Western blot was quantitated using LICOR Odyssey software. Protein levels in each lane were normalized to **Total** (only 10% of total protein loaded onto the column); **U**, 50% of unbound fraction collected; **Rosco**, roscovitine.

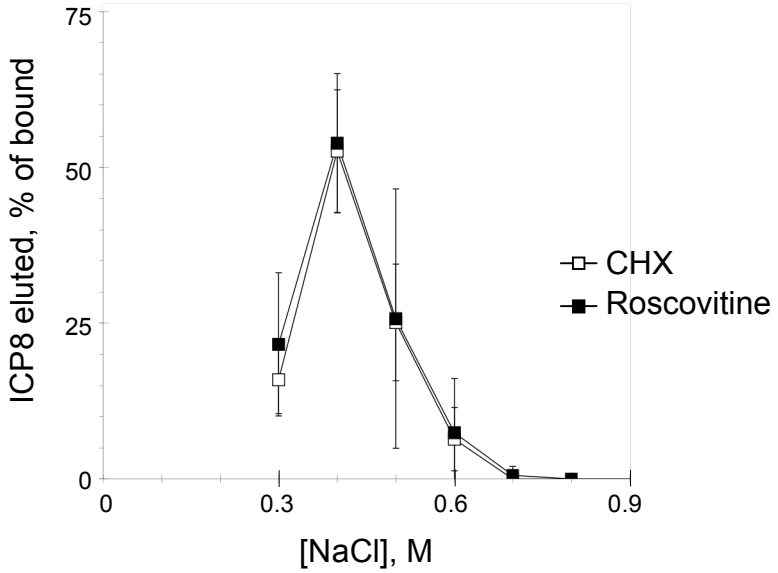

Figure 4.5 ICP8 extracted from roscovitine-treated cells does not have a decreased affinity for ssDNA. Line graph presenting the ssDNA elution profile of ICP8 extracted from cells treated with CHX or roscovitine. Vero cells were infected with 10 PFU of HSV-1 ICP8 *ts* mutant strain A1 per cell at the non-permissive temperature (38^{o}C). Cells were transferred to the permissive temperature (33^{o}C) 5 h later in media containing 100 μM **roscovitine** or 50 μg/mL **CHX**. Four hours later, the nuclei were harvested and the nuclear proteins were extracted. Nuclear proteins were then incubated for 1 h with ssDNA coupled to cellulose. Bound proteins were eluted from the ssDNA at increasing NaCl concentrations. Proteins were separated in 10% SDS-PAGE and transferred to PVDF membranes. ICP8 detected by Western blot was quantitated using LICOR Odyssey software. Error bars, standard deviation of four independent experiments.

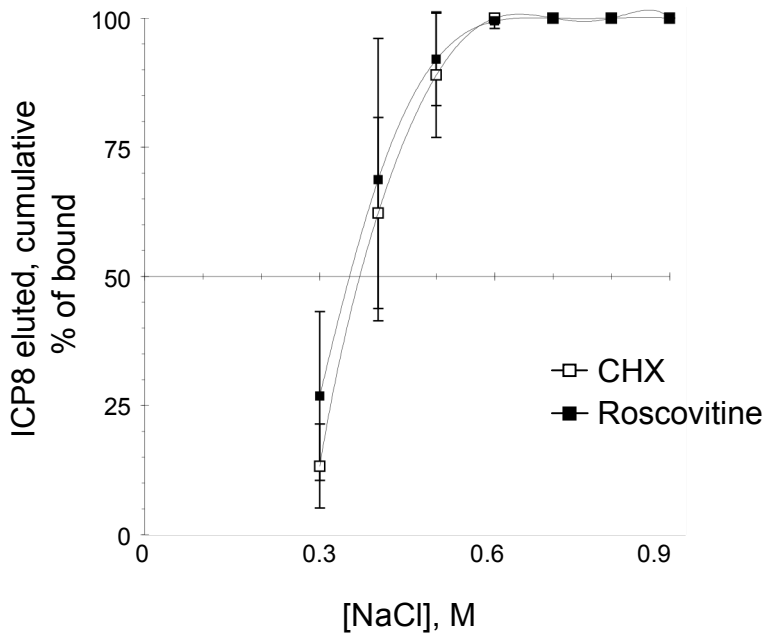

Figure 4.6 The concentration required to elute 50% ICP8 from ssDNA when extracted roscovitine-treated cells is not significantly different than that extracted from CHX-treated cells. Line graph presenting the cumulative percentage of ICP8 extracted from CHX- or roscovitine-treated cells which was eluted from ssDNA with increasing concentrations of NaCl. Vero cells were infected with 10 PFU of HSV-1 ICP8 *ts* mutant strain A1 per cell at the non-permissive temperature (38^{o}C) and then incubated for 5 h. Cells were transferred to the permissive temperature (33^{o}C) in media containing 100 μM **roscovitine** or 50 μg/mL **CHX** for 4 h. Nuclei were harvested and the nuclear proteins were extracted. Nuclear proteins were then incubated with ssDNA coupled to cellulose for 1 h. Bound proteins were eluted from ssDNA at increasing NaCl concentrations. Proteins were separated in 10% SDS-PAGE and transferred to PVDF membranes. ICP8 detected by Western blot was quantitated using LICOR Odyssey software. Error bars, standard deviation of four independent experiments.

had been extracted from cells treated with CHX was 360 to 455 mM (with four of the five experiments ranging from 360 to 385 mM). The average NaCl concentration required to elute 50% ICP8 from ssDNA when ICP8 was extracted from cells treated with roscovitine was 368.6±60.6 mM (average±standard deviation; n = 5). The range of concentrations required to elute 50% ICP8 from ssDNA when ICP8 was extracted from cells treated with roscovitine was 293 to 440 mM. The concentration required to elute 50% ICP8 from ssDNA when cells were treated with CHX or roscovitine was not statistically different. Moreover, no trend was detected among the experiments although there was a wide variation in the NaCl concentrations required to elute ICP8. The concentration of NaCl required to elute ICP8 from ssDNA when extracted from CHX- as compared to roscovitine-treated cells was 54 mM higher, 80 mM lower, 33 mM lower, 92 mM higher, and 75 mM higher in the five different experiments. Therefore, ICP8 extracted from the nuclei of cells treated with roscovitine did not have any obviously altered affinity for ssDNA than ICP8 from cells treated with CHX.

4.4 DISCUSSION

The results described in this chapter show that treatment of cells with roscovitine results in ICP8 being more easily extracted from the nuclei, but does not alter the *in vitro* affinity of ICP8 for ssDNA. Therefore, the increased extractability of ICP8 is not a result of a decreased affinity of ICP8 for ssDNA.

ICP8 interacts with HSV-1 and cellular proteins. The interaction of ICP8 with HSV-1 DNA replication proteins results in the recruitment of HSV-1

proteins to the HSV-1 replication structures. ICP8 also interacts with forty-five cellular proteins (**Table 4.1**), ranging from DNA replication and chromatin remodeling to transcriptional activation proteins. The role of the interaction of ICP8 with cellular proteins is not known. However, of the forty-five ICP8-interacting proteins, five localize to ND10s [reviewed in (Negorev and Maul, 2001)]. ND10s are defined by an accumulation of specific proteins, not by a specific location in the nucleus. ICP8 localizes to nuclear domains adjacent to ND10s even in the absence of other HSV-1 DNA replication proteins or HSV-1 DNA. I speculate that the localization of ICP8 to ND10s is a result of recruitment by cellular proteins, or an interaction with extrachromosomal DNA. However, the mechanism whereby ICP8 localizes to nuclear domains adjacent to ND10s is yet unknown.

The observed inhibition of replication site formation by roscovitine and the increase in ICP8 accessibility after roscovitine treatment may have therefore resulted from decreased ICP8 affinity for proteins or altered interactions with DNA. The affinity of ICP8 for its interacting partners remains to be tested.

CHAPTER 5: DISCUSSION

Our group studies the mechanisms of inhibition of HSV-1 replication by roscovitine. Our group has discovered that roscovitine inhibits both HSV-1 transcription and DNA replication. The molecular mechanisms of the inhibition of transcription have been well described. Roscovitine inhibits the initiation of HSV-1 transcription, but it does not inhibit ongoing transcription (Diwan et al., 2004; Jordan et al., 1999; Schang et al., 1999). The transcription of genes driven by HSV-1 promoters recombined into the cellular genome is not inhibited by roscovitine, whereas the transcription of cellular genes recombined into the HSV-1 genome is. Therefore, the inhibition of transcription by roscovitine is independent of promoter-specific factors (Diwan et al., 2004). In fact, roscovitine inhibits transcription from extrachromosomal HSV-1 genomes.

In contrast to its effects on transcription, the molecular mechanisms of the inhibition of HSV-1 DNA replication by roscovitine have not been well described. Our group has discovered that roscovitine inhibits HSV-1 DNA replication even in the presence of all the required HSV-1 DNA replication proteins (Schang et al., 2000). However, little else is known regarding the inhibition of HSV-1 DNA replication by roscovitine.

HSV-1 genomes are detected in close association with ND10 (Ishov and Maul, 1996; Maul et al., 1996). Therefore, HSV-1 genomes likely localize to these nuclear domains prior to the initiation of HSV-1 transcription and DNA replication. These processes occur at these nuclear domains (Ishov and Maul,

1996; Maul et al., 1996). IE and E proteins are then expressed. ICP8 then localizes to the HSV-1 genomes at these nuclear domains and recruits the rest of the HSV-1 DNA replication complex to initiate HSV-1 DNA replication. My first objective was to identify which steps during the HSV-1 DNA replication cycle are inhibited by roscovitine. To this end, I evaluated the formation of replication structures. As such, I first evaluated the localization of ICP8 and its co-localization with BrdU. ICP8 is the first HSV-1 protein to localize to HSV-1 replication structures. BrdU is a thymidine analog that becomes incorporated into replicating DNA. Localization of ICP8 and BrdU to the same nuclear domain therefore identifies domains of active HSV-1 DNA replication (RCs).

Any evaluation of the localization of ICP8 in the presence of roscovitine requires the expression of ICP8 prior to the addition of roscovitine, as otherwise roscovitine would prevent ICP8 transcription. ICP8 was expressed in the absence of roscovitine (or HSV-1 DNA replication) using PAA- or *ts* mutant-induced HSV-1 DNA replication blocks. Roscovitine was then added after ICP8 was already expressed.

If roscovitine inhibited DNA synthesis, then small RCs would form in its presence, but would not increase in size. If roscovitine inhibited the initiation of HSV-1 DNA replication, then pre-RCs would still form, but would not mature into RCs. Surprisingly, I discovered that ICP8 did not induce the formation of replication structures in the presence of roscovitine (**Section 3.2 and 3.3**). Such ICP8 formation normally occurs prior to initiation of DNA replication. Therefore, roscovitine inhibits HSV-1 DNA replication at a step prior to

initiation, even in the presence of all the E proteins required for HSV-1 DNA replication.

My next objective was therefore to test several possible mechanisms whereby treatment with roscovitine may result in the inhibition of ICP8 localization to the proper nuclear domains.

A decreased affinity of ICP8 for ssDNA or other binding partners could result in inhibition of HSV-1 DNA replication and proper ICP8 localization. Such a decreased affinity would also result in ICP8 being more easily extracted from the nuclei. I therefore evaluated whether ICP8 was extracted more easily from the nuclei of roscovitine-treated cells. I discovered that ICP8 was extracted at lower salt concentrations from roscovitine- than from CHX-treated cells (**Section 4.2**). However, I also discovered that ICP8 extracted from roscovitine-treated cells did not have a decreased affinity for ssDNA *in vitro* (**Section 4.3**). Therefore, the increased extractability is not likely a result of a decreased affinity of ICP8 for ssDNA. Consequently, the mislocalization of ICP8 in the presence of roscovitine is also not likely a result of a decreased affinity of ICP8 for ssDNA.

Roscovitine-sensitive CDKs phosphorylate several proteins that participate in cellular transcription or DNA replication. Therefore, it is not entirely surprising that the inhibition of CDKs by roscovitine results in inhibition of DNA replication and transcription. However, the specific roles of CDKs in HSV-1 and cellular transcription and DNA replication differ, as discussed below.

5.1 ROLE OF CDKS IN CELLULAR AND HSV-1 TRANSCRIPTION AND DNA REPLICATION

CDKs phosphorylate proteins that initiate cellular and HSV-1 transcription and DNA replication. Therefore, CDKs play important roles at steps prior to the initiation of cellular transcription and DNA replication. For example, CDK phosphorylation of the CTD on the cellular RNA polymerase II is required for initiation (and elongation) of cellular DNA replication [reviewed in (Hirose and Ohkuma, 2007)]. Likewise, cdc6 and MCM proteins must be phosphorylated by CDKs for the initiation of cellular DNA replication to occur [(Petersen et al., 1999; Tanaka et al., 1997) and reviewed in (Tanaka et al., 2007; Teer, 2006)]. CDK phosphorylation of the components of the cellular DNA replication complex cdc6 and MCMs is required for the assembly and maintenance of prereplication complexes at replication origins [reviewed in (Bell and Dutta, 2002; Dutta and Bell, 1997)]. However, localization of the first component of the cellular DNA replication complex to the origins (the origin recognition complex; ORC) does not require CDK phosphorylation.

CDKs are important prior to the initiation of HSV-1 transcription (Diwan et al., 2004; Schang et al., 1999). I have discovered that CDKs are also important prior to the initiation of HSV-1 DNA replication (**Chapter 3**). However, the exact roles of CDKs in HSV-1 transcription and DNA replication have not yet been described. Moreover, the steps prior to the initiation of DNA replication that require CDKs have not yet been identified. Differing from cellular DNA replication, the proteins that form the HSV-1 DNA replication preinitiation

complex do not localize to the proper nuclear domains when CDK activity is inhibited (**Chapter 3**). In contrast, CDKs play no known role prior to the localization of the cellular ORC to the proper nuclear domains during the initiation of cellular DNA replication. Therefore, the specific details of the roles of CDKs in HSV-1 and cellular DNA replication are different.

5.2 POSSIBLE MECHANISMS OF INHIBITION OF HSV-1 DNA REPLICATION BY ROSCOVITINE

I have shown that roscovitine inhibits the localization of ICP8 to the nuclear domains adjacent to ND10s. However, the mechanisms whereby ICP8 localizes to these nuclear domains are yet unknown. ICP8 was reported to localize to nuclear domains adjacent to ND10s in the absence of HSV-1 DNA or HSV-1 DNA replication proteins (Lukonis and Weller, 1997). However, HSV-1 plasmid DNA localizes to sites adjacent to ND10s (Tang et al., 2003). ICP8 may therefore localize to nuclear domains adjacent to ND10s as a result of interactions with plasmid DNA. Plasmid DNA localizes to different domains depending on sequences within the plasmid (Gasiorowski and Dean, 2007). For example, SV40 plasmid transcription only occurred at sites adjacent to ND10s when the TAg binding region of the origin and the TAg were present (Tang et al., 2000). Furthermore, infection or transfection of an HSV-1 amplicon sequence was not sufficient for accumulation at ND10s of the amplicon DNA or of the transcript of the reporter, supporting a model whereby no all HSV-1 DNA localizes to sites adjacent to ND10s (Tang et al., 2003). Rather, the expression of ICP4 and ICP27

appeared to be required for the localization of HSV-1 plasmid DNA to the proper nuclear domains. In contrast, infecting HSV-1 DNA may accumulate at sites adjacent to ND10s even when protein and RNA synthesis is inhibited at the time of infection (Ishov and Maul, 1996). ICP8 plasmid DNA therefore may or may not localize to sites adjacent to ND10s during transient transfection. Consequently, the localization of the ICP8-expressing plasmid in transiently transfected cells should be evaluated. If the ICP8 plasmid DNA did localize to sites adjacent to ND10s, then whether the proper localization of ICP8 is dependent on the proper localization of DNA should be evaluated. To evaluate whether the localization of ICP8 to the proper nuclear domains is dependent on extrachromosomal DNA, the localization of ICP8 should be evaluated under conditions whereby ICP8 does not bind to DNA (as discussed in **Section 5.4.3**). If ICP8 localizes to the proper nuclear domains under conditions whereby it does not bind to DNA, then ICP8 localizes to sites adjacent to ND10s independently of DNA binding.

ICP8 may therefore localize to the proper nuclear domains as a result of interactions with either specific proteins or HSV-1 DNA. I propose that ICP8 may not localize to the proper nuclear domains in the presence of roscovitine as a result of decreased affinity with HSV-1 DNA or recruiting proteins.

ICP8 localizes to nuclear domains adjacent to ND10s in the absence of other HSV-1 proteins (Lukonis and Weller, 1997). Therefore, cellular, not viral, proteins are likely to recruit ICP8 to these nuclear domains. ICP8 interacts with forty-five cellular proteins [**Table 4.1**; (Taylor and Knipe, 2004)]. Although the

significance of the interactions of ICP8 with these cellular proteins is yet unknown, seven of them localize to ND10s [reviewed in (Negorev and Maul, 2001)]. It is therefore tempting to envision a model whereby the localization of ICP8 to nuclear domains adjacent to ND10s is a result of recruitment by cellular proteins that interact with ICP8 and localize to ND10s. A decreased affinity of ICP8 for one (or more) of these putative proteins would prevent the recruitment of ICP8 to the proper nuclear domains (**Chapter 3**). Such decreased affinity could be a direct inhibition of required phosphorylations. Such an effect could therefore be the mechanism whereby roscovitine treatment results in ICP8 mislocalization. Decreased affinity for cellular partners would also result in ICP8 being more easily extracted from the nuclei, as it was observed (**Section 4.2**).

The inhibition of recruitment of ICP8 to nuclear domains adjacent to ND10s in the presence of roscovitine is sufficient to explain the inhibition of HSV-1 DNA replication in the presence of all required DNA replication proteins (Schang et al., 2000).

Ten cellular proteins that interact with ICP8 have been reported to localize to RCs (**Table 4.1**). However, none of them is known to localize to ND10s. Therefore, none of these is proteins is likely to recruit ICP8 to sites adjacent to ND10s prior to the formation of RCs. Nevertheless, a potential role for these proteins in the recruitment of ICP8 to the proper nuclear domains should not be ignored.

If the affinity of ICP8 for any binding partners were decreased, then the effect of roscovitine on the phosphorylation of these proteins could be tested to

further characterize the mechanism of inhibition. Roscovitine is a protein kinase inhibitor. However, ICP8 is not phosphorylated. Therefore, the simplest mechanism whereby roscovitine may inhibit ICP8 recruitment to the proper nuclear domains by a cellular protein would be inhibition of phosphorylation of this putative cellular protein. Therefore, the phosphorylation status of the proteins of interest should be evaluated. Such experiments are discussed in **Section 5.4.2**.

Although unexpected, an increased affinity of ICP8 for binding proteins could also result in inhibition of HSV-1 DNA replication. Such increased affinity could sequester ICP8 from the proper nuclear domains (**Chapter 3**), preventing ICP8 from binding to the HSV-1 ssDNA located at these domains. The lack of binding to ssDNA (in the proper nuclear domains) could then result in ICP8 being more accessible for extraction (**Section 4.2**). However, it is not clear whether an increased affinity for binding proteins would allow for the observed increases in extractability (**Figures 4.1** – **4.2**).

As described above, it is not clear whether ICP8 localizes to nuclear domains adjacent to ND10s independently of interactions with extrachromosomal DNA (plasmid or HSV-1). Therefore, the mislocalization of ICP8 in the presence of roscovitine could also result from inhibited interactions with HSV-1 DNA, localized at the proper nuclear domains. However, ICP8 from cells treated with roscovitine does not have a decreased affinity for ssDNA (**Section 4.3**), contrary to what would be expected from such a model. Alternatively, the mislocalization of ICP8 in the presence of roscovitine could also result from HSV-1 DNA mislocalization to other nuclear domains. Therefore, the inhibition of ICP8

localization to the proper nuclear domains could also result from mislocalization of HSV-1 genomes. It could also result from inaccessibility to properly localized HSV-1 genomes. The evaluation of the accessibility and localization of HSV-1 genomes is therefore important to elucidate the mechanism whereby roscovitine inhibits HSV-1 DNA replication.

In the model describing the inhibition of proper ICP8 localization as a result of the mislocalization of HSV-1 genomes, ICP8 would bind to HSV-1 DNA at other nuclear domains, thereby resulting in the mislocalization of ICP8 (**Chapter 3**). Inhibition of HSV-1 genomes localization to the proper nuclear domains would result in the inhibition of both HSV-1 transcription and DNA replication. Therefore, the inhibition of HSV-1 genome localization to the proper nuclear domains is a possible mechanism whereby roscovitine inhibits HSV-1 DNA replication and transcription. Under this model, however, it is not obvious how ICP8 would be more easily extracted (**Section 4.2**).

The transcription of HSV-1 genomes occurs at nuclear domains adjacent to ND10s (Maul et al., 1996). Therefore, the localization of HSV-1 genomes to nuclear domains adjacent to ND10s is likely required for HSV-1 transcription. IE and E proteins are expressed in the presence of PAA (in the PAA-release experiment described in **Section 3.2**) and at non-permissive temperatures (in the *ts* mutant experiments described in **Section 3.3**). Therefore, in addition to inducing the mislocalization of incoming HSV-1 genomes, roscovitine would also have to induce delocalization of the properly localized HSV-1 genomes that were previously permissive for IE and E gene transcription. Therefore, the

mislocalization of HSV-1 genomes would be unexpected to be the cause of ICP8 mislocalization. Nonetheless, the localization of HSV-1 genomes in the presence of roscovitine should be evaluated (as discussed in **Section 5.4.1**).

Any model whereby ICP8 is dependent on HSV-1 DNA binding for proper localization requires HSV-1 DNA to be accessible for ICP8 binding at the nuclear domains adjacent to ND10s. Dr. Lacasse evaluated the accessibility of HSV-1 IE and L DNA from roscovitine-treated cells. To this end, Dr. Lacasse used a modified MCN digestion technique designed to detect unstable HSV-1 nucleoprotein complexes (described in **chapter 1.3.1**). Dr. Lacasse discovered that HSV-1 IE and L DNA from infected, roscovitine-treated cells were resistant to digestion by MCN than the cellular DNA from roscovitine-treated cells and HSV-1 DNA from infected cells not treated with drug. When infected cells were treated with roscovitine, 50% of the digested HSV-1 DNA (T_{50}; IE and L DNA) was not reached in 60 min. In contrast, the T_{50} of the cellular DNA in infected cells treated with roscovitine was 35.7 min. The HSV-1 DNA T_{50} in infected cells treated with no drug was 14.3 min for IE DNA and 12.4 min for L DNA (Lacasse, 2010). The increase in the time required to digest 50% of the DNA indicates that the HSV-1 DNA from roscovitine-treated cells is less accessible to MCN digestion than cellular DNA or the HSV-1 DNA from cells not treated with drug. Therefore, the increased extractability and mislocalization of ICP8 in the presence of roscovitine may have resulted from an inaccessibility of the HSV-1 DNA to ICP8. The mechanisms whereby treatment with roscovitine results in an decreased accessibility to HSV-1 DNA are under investigation.

If HSV-1 DNA were less accessible to ICP8 in the presence of roscovitine, ICP8 would mislocalize and be more easily extracted. However, the mechanisms whereby ICP8 localizes to nuclear domains adjacent to ND10s have not been elucidated. Therefore, alternative mechanisms to identify the cause of the mislocalization and increased extractability of ICP8 observed should not be discarded. ICP8 may not depend on HSV-1 DNA for its proper localization. In this model, ICP8 would localize to the proper nuclear domains even in the absence of accessible HSV-1 DNA.

The models presented above all provide possible mechanisms whereby HSV-1 DNA replication would be inhibited by inhibition of CDKs. I postulate that the mislocalization of ICP8 in the presence of roscovitine is due to the inaccessibility of HSV-1 DNA. Although the recruitment of ICP8 to the proper nuclear domains by a cellular protein is tempting, it is at present entirely speculative. The mislocalization of ICP8 as a result of the mislocalization of HSV-1 genomes appears unlikely, as discussed above. The models described above may also identify roles for CDKs in HSV-1 DNA replication not been previously described.

An evaluation of the localization of HSV-1 genomes and the affinity of ICP8 for its binding partners is required to further characterize the mechanism whereby roscovitine inhibits HSV-1 DNA replication. Such characterization could identify the mechanisms whereby HSV-1 genomes localize to the proper nuclear domains and identify novel roles for ICP8 during HSV-1 DNA replication.

5.3 OTHER POSSIBLE ROLES FOR ICP8 IN HSV-1 DNA REPLICATION

ICP8 recruits the HSV-1 DNA replication machinery to the proper nuclear domains. Even though entirely speculative at present, it is tempting to consider that ICP8 may also recruit cellular factors required for HSV-1 DNA replication. ICP8 interacts with factors required for the initiation of cellular DNA replication (such as replication protein A; RPA) or chromatin remodeling [such as BRG1; the central catalytic ATPase subunit of numerous chromatin-remodeling complexes including mating-types switching/sucrose non-fermenting (SWI/SNF); **Table 4.1** and (Taylor and Knipe, 2004)]. A decreased interaction of ICP8 with such proteins in the presence of roscovitine could prevent ICP8 from recruiting required cellular protein to new replication domains. A role for these proteins in the recruitment of ICP8 to the proper nuclear domains should not be discarded either, even though a decreased interaction of ICP8 with the proteins that it recruits would not likely explain its mislocalization (**Chapter 3**).

ICP8 interacts with two cellular proteins required for the initiation of cellular DNA replication [**Table 4.1** and (Taylor and Knipe, 2004)], RPA and minichromosome maintenance subunit MCM2. RPA is phosphorylated by CDKs (Dutta and Stillman, 1992), relocalizes to RCs during HSV-1 infection (Wilcock and Lane, 1991), and localizes to ND10s in certain cell types (Yeager et al., 1999). Therefore, the affinity of ICP8 for RPA in the presence of roscovitine will be evaluated (as discussed in **Section 5.4.2**).

RPA is required for cellular DNA replication. However, no role for RPA in HSV-1 DNA replication has been reported. It is also not obvious why HSV-1 DNA replication would require the use of two ssDNA binding proteins, RPA and ICP8. Nonetheless, if RPA were required for HSV-1 DNA replication, then RPA is likely required to localize to sites adjacent to ND10s.

The phosphorylation state of RPA is important for its localization to sites of cellular DNA synthesis (Vassin et al., 2004), and roscovitine may inhibit the CDK phosphorylation of RPA. Therefore, roscovitine may induce the mislocalization of RPA as a result of altering its phosphorylation state. The phosphorylation state of RPA may also be important for its interaction with ICP8. Therefore, the localization and phosphorylation state of RPA in the presence of roscovitine would be evaluated (as discussed in **Section 5.4.2**).

MCM2 also interacts with ICP8 and is required for the initiation of cellular DNA replication. In steps prior to the initiation of cellular DNA replication, MCM2 recruits other essential DNA replication proteins (the other MCM proteins). Interestingly, MCM2 is also phosphorylated by CDKs *in vitro*. Therefore, it would be of interest to evaluate whether roscovitine decreases the affinity of ICP8 for MCM2. Furthermore, CDK activity has been implicated in regulating MCM localization [(Liku et al., 2005; Nguyen et al., 2000; Pacek et al., 2006) and reviewed in (Bochman and Schwacha, 2009; Tanaka, 2010)], although the precise role of CDKs in MCM localization is yet incompletely understood. MCM2, however, has not been reported to localize to either HSV-1 replication structures or ND10s. Therefore, the localization of MCM2 during infection

would have to be evaluated prior to the evaluation of the affinity of ICP8 for MCM2, MCM2 phosphorylation, or its localization in the presence of roscovitine.

As discussed, the interaction of ICP8 with cellular proteins could be required for the recruitment of proteins required for the initiation of DNA replication. However, the interaction of ICP8 with certain cellular proteins could also be required for the recruitment of proteins necessary to increase the accessibility to the chromatinized HSV-1 DNA. Consistent with this model, HSV-1 IE and L DNA from infected, roscovitine-treated cells is more resistant to degradation by MCN than the cellular DNA from the same cells (Lacasse, 2010).

The mislocalization of ICP8 in the presence of roscovitine may result in cellular proteins required to increase the accessibility to HSV-1 genomes not being recruited to the proper nuclear domains. Consistent with such models, ICP8 interacts with sixteen proteins that play roles in chromatin remodeling (Taylor and Knipe, 2004). These chromatin-remodelers include BRG1 or BRM-associated factors (BAFs), and members of the SWI/SNF family of proteins (the required helicase subunit from chromatin remodeling complexes).

CHAPTER 6: FUTURE DIRECTIONS

Several potential mechanisms for the inhibition of HSV-1 DNA replication by roscovitine remain to be tested. As described earlier, roscovitine could inhibit the localization of ICP8 to the proper nuclear domains as a result of inhibition of HSV-1 genome localization to the proper nuclear domains, decreased affinity of ICP8 for the cellular proteins that recruit it, or restrictions in the accessibility to HSV-1 DNA.

6.1 THE LOCALIZATION OF HSV-1 GENOMES IN THE PRESENCE OF ROSCOVITINE

HSV-1 genomes localize to nuclear domains adjacent to ND10s even in the absence of HSV-1 protein expression (Maul et al., 1996). However, the HSV-1 genomes may not localize to the proper nuclear domains in the presence of roscovitine. Therefore, ICP8 may mislocalize (**Chapter 3**) as a result of HSV-1 genome mislocalization. If ICP8 does not bind to ssDNA at the proper nuclear domains, then it may also be more accessible for extraction (**Chapter 4**). The limitations of this model are discussed in **Section 5.2**.

To evaluate the localization of HSV-1 genomes in the presence of roscovitine, fluorescence in situ hybridization (FISH) will be used in collaboration with the Lomonte and Catez groups. Two different types of infections have been performed to evaluate the localization of HSV-1 genomes in relation to ND10s. Cells were infected for 1 h with wild type KOS. The cells

were then incubated for 1 or 5 h in the absence of drug or in the presence of PAA, roscovitine, or CHX. After these incubations, the cells were fixed and dried. Alternatively, cells were infected with the HSV-1 *ts* ICP8 mutant A1 and incubated at the non-permissive temperature (38^{o}C) for 5 h. The cells were then transferred to the permissive temperature (33^{o}C) in the absence of drug or in the presence of PAA, roscovitine, or CHX. After the incubation, the cells were fixed and dried. The localization of HSV-1 genomes in these two experiments will be evaluated by FISH by members of the Lomonte and Catez group.

Alternatively, I have developed a technique to evaluate the localization of labeled HSV-1 genomes. Briefly, cells were infected with HSV-1. Cells were then incubated in media supplemented with 10 μg/ml, such that the BrdU is incorporated into the replicating HSV-1 DNA. Twenty-four hours later, the cells were harvested. This virus, the genomes of which are labeled with BrdU, was then used to infect cells. The localization of the labeled infecting HSV-1 genomes was evaluated by immunofluorescence for BrdU.

I was not able to complete these latter experiments due to the anti-PML primary antibodies and the only anti-BrdU primary antibody sensitive enough to detect the labeled genomes being from the same host species. Therefore, the evaluation of the localization of these labeled genomes in relation to ND10s would require the generation of either stable cell lines containing labeled PML or fluorescently-labeled PML antibodies. The localization of HSV-1 genomes could then be evaluated in relation to PML, as described for the FISH experiments (without drying).

6.2 THE AFFINITY OF ICP8 FOR ITS CELLULAR BINDING PARTNERS

Another future direction for this project is the evaluation of the affinity of ICP8 for its cellular binding partners. ICP8 localizes to nuclear domains adjacent to ND10s via unknown mechanisms. ND10s are proteinaceous structures. While it is still not clear whether extrachromosomal DNA plays any role in the localization of ICP8 to the proper nuclear domains, it is tempting to speculate that ICP8 localizes to the nuclear domains adjacent to ND10s as a result of its interactions with cellular ND10s proteins.

Seven proteins localize to ND10s (Negorev and Maul, 2001) and interact with ICP8, herpesvirus-associated ubiquitin-specific protease (HAUSP), Rad50, Mre11, RPA, breast cancer associated gene-1 protein (BRCA1), and the DNA helicases Werner (WRN) and BLM (Taylor and Knipe, 2004). BLM is the only one present at all ND10s when not over-expressed, and in all vertebrate cell lines tested (Ishov et al., 1999; Yankiwski et al., 2000). HAUSP, Rad50, Mre11, and RPA are present in a subset of ND10s (Barr et al., 2003; Everett et al., 1997; Lombard and Guarente, 2000; Mirzoeva and Petrini, 2003). WRN is only present at ND10s in telomerase-negative cells (Yeager et al., 1999). BRCA1 localizes to ND10s predominantly during late S and G2 in telomerase negative cells (Wu et al., 2003). BRCA1 also localizes to sites adjacent to ND10s upon transient overexpression and infection with adenovirus 5 (Maul et al., 1998). Infection with HSV-1 redistributed BRCA1 away from ND10s in an ICP0-dependent manner (Maul et al., 1998).

The affinity of ICP8 for HAUSP, Rad50, Mre11, RPA, BRCA1, WRN, and BLM will be evaluated. None of them have been described as having a role in protein recruitment. As discussed in **Section 5.3**, RPA also plays a role in the initiation of cellular DNA replication, relocalizes to RCs during HSV-1 infection, and is phosphorylated by CDKs (Dutta and Stillman, 1992; Taylor and Knipe, 2004; Wilcock and Lane, 1991). Therefore, the affinity of ICP8 for RPA may be of particular interest.

ICP4 and ICP27 also interact with ICP8. ICP4 and ICP27 have been implicated in the recruitment of HSV-1 genomes to the proper nuclear domains (Tang et al., 2003). However, localization of ICP4 or ICP27 to ND10s independently of other HSV-1 proteins was not detected (Mears et al., 1995; Wang, 2008; Zhu and Schaffer, 1995). Furthermore, a requirement for ICP4 and ICP27 in the recruitment of ICP8 to the proper nuclear domains would not account for the localization of ICP8 independently of other HSV-1 proteins (Lukonis and Weller, 1997). Nonetheless, the affinity of ICP8 for the viral IE proteins ICP4 and ICP27 may also be of interest.

To evaluate the affinity of ICP8 for its binding partners, cells would be infected with the ICP8 *ts* mutant A1 and incubated at the non-permissive temperature (38^{o}C) for 5 h to induce the accumulation of ICP8. CHX or roscovitine would be added before transferring the cells to the permissive temperature. Cells would be harvested after 4 h at 33^{o}C, their nuclei would be isolated, and the nuclear proteins would be extracted. ICP8 would be immunoprecipitated from the nuclear proteins with an ICP8 antibody. ICP8 and

the proteins that interact with ICP8 would be resolved by SDS-PAGE and the amounts of the co-immunoprecipitating ICP8-binding proteins would be evaluated by Western blot.

The percentage change in the amounts of co-immunoprecipitating proteins from roscovitine- as compared to CHX-treated cells would then determined. Increases or decreases of more than 50% in the levels of the co-immunoprecipitating proteins after CHX or roscovitine treatment will identify proteins of interest.

These studies may identify proteins that have decreased affinity for ICP8 in the presence of roscovitine. The interaction of ICP8 with these proteins may be important for the recruitment of ICP8 to the proper nuclear domains if these proteins normally localize to sites adjacent to ND10s, and recruit ICP8 to these domains. The phosphorylation of these proteins may be important for their interaction with ICP8 or its localization to ND10s. Roscovitine being a protein kinase inhibitor, the simplest mechanism whereby it may decrease the affinity of ICP8 for its putative recruiting proteins would be inhibition of phosphorylation of these proteins. Therefore, the effect of roscovitine on the localization and phosphorylation state of the proteins of interest would be evaluated.

To evaluate the localization of the proteins of interest in the presence of roscovitine, cells would be infected with the *ts* ICP8 mutant A1 at the non-permissive temperature (38^{o}C). The cells would then be incubated for 5 h to induce the accumulation of ICP8. CHX or roscovitine would be added and the cells would be transferred to the permissive temperature (33^{o}C). The cells would

be fixed with formaldehyde at 0, 2, and 4 h after transfer to 33^{o}C. The protein of interest and its localization would be detected by immunofluorescence.

The proteins that interact with ICP8 may be directly phosphorylated by roscovitine-sensitive CDKs, or be targets of signal cascades involving roscovitine-sensitive CDKs. The characterization of such pathways would be important for the elucidation of the mechanism (as discussed in **Section 5.4.2**).

To evaluate the phosphorylation status of the proteins of interest, cells would be infected with the *ts* ICP8 mutant A1 at the non-permissive temperature (38^{o}C). Five hours later, CHX or roscovitine would be added and the cells would be transferred to the permissive temperature (33^{o}C). The cells would be harvested 4 h later. The nuclear proteins would be extracted and resolved by SDS-PAGE. The proteins of interest would be identified by Western blot and the phosphorylation status of those proteins would be determined by evaluating their mobility shifts or with a phospho-specific antibody. If a mobility shift of the protein is not detectable, or a phospho-specific antibody is not available, the phosphorylation status of the proteins would be evaluated by supplementing cells with radiolabeled ATP and determining the level of radioactivity of the protein.

These experiments may identify proteins of interest that have altered localization and phosphorylation after treatment with roscovitine, but which are not directly phosphorylated by roscovotine-sensitive kinases. Under these conditions, the signal cascades resulting in the altered phosphorylation state would be elucidated. Alternatively, these experiments may not identify any recruiting proteins, as the localization of ICP8 may not depend on any other

protein. Under this model, an evaluation of the requirement of ICP8 for DNA binding in its proper localization would be more relevant (as discussed below).

6.3 THE REQUIREMENT FOR DNA BINDING FOR THE PROPER LOCALIZATION OF ICP8

HSV-1 IE and L DNA extracted at 7 hpi from infected, roscovitine-treated cells is digested by MCN slower than the cellular DNA from the same cells (Lacasse, 2010). Therefore, it is tempting to conclude that the mislocalization of ICP8 in the presence of roscovitine is a result of this HSV-1 DNA inaccessibility. However, it is not clear whether ICP8 localization to the nuclear domains adjacent to ND10s is dependent on its interactions with extrachromosomal DNA. The localization of ICP8 will therefore be evaluated under conditions whereby ICP8 does not bind to DNA.

To evaluate the localization of ICP8 under conditions whereby ICP8 does not bind to DNA, wild type ICP8 or ICP8 with a mutation in the DNA binding domain will be transfected into cells. The cells would then be fixed, and ICP8 and PML would be detected by immunofluorescence.

If the ICP8 with a point mutation in the DNA binding domains does not localize to sites adjacent to ND10s, and wild type ICP8 does, then DNA-binding would seem to be required for ICP8 to localize to the proper nuclear domains. However, the mutation in the binding domain may result in conformational changes that induce ICP8 mislocalization. Therefore, if ICP8 with a mutation in

the DNA binding domain does localize to the proper nuclear domains, then DNA binding is likely not required for ICP8 localization to sites adjacent to ND10s.

CONCLUSION

During clinical trials against cancer, roscovitine is proving to be relatively safe for humans. *In vitro*, roscovitine inhibits the replication of a range of unrelated viruses. Therefore, roscovitine has the potential to be used as an antiviral. However, the mechanisms whereby roscovitine inhibits the replication of these unrelated viruses has not been fully elucidated. Roscovitine inhibits HSV-1 transcription and DNA replication. The mechanism whereby roscovitine inhibits HSV-1 transcription has been extensively evaluated. Inhibition occurs at a step prior to initiation. However, the mechanism whereby roscovitine inhibits HSV-1 DNA replication had not been so extensively evaluated. Roscovitine inhibits HSV-1 DNA replication in the presence of all required HSV-1 DNA replication proteins. However, it was not even clear until the completion of my Thesis whether the observed inhibition of HSV-1 DNA replication was exclusively a consequence of the inhibition of HSV-1 DNA replication. Alternatively, roscovitine could inhibit a step prior to the initiation of DNA replication.

I have discovered that roscovitine does indeed inhibit a step prior to the initiation of HSV-1 DNA replication. I then discovered that ICP8 is more easily extracted from the nuclei of roscovitine-treated cells. These results indicate that interactions of ICP8 with its binding partners (DNA or proteins) were likely affected by roscovitine treatment. The increased extractability, however, was not a result of a decrease in the affinity of ICP8 for ssDNA. I have therefore discussed several possible mechanisms whereby treatment with roscovitine may lead to the observed inhibition of a step prior to HSV-1 DNA replication. I

postulate that the mislocalization of ICP8 in the presence of roscovitine may be due to the inaccessibility of HSV-1 DNA in the presence of roscovitine. Although the recruitment of ICP8 to the proper nuclear domains by a cellular protein is tempting, it is entirely speculative. The mislocalization of ICP8 as a result of the mislocalization of HSV-1 genomes appears unlikely, as discussed in **Section 5.2**. Further elucidation of the mechanism of inhibition of HSV-1 DNA replication by roscovitine is important for understanding the role of CDKs in HSV-1 DNA replication.

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