Expression of ICP0 from the simian simplexvirus SA8 and a study of its transactivation activity

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“A fact is a simple statement that everyone believes. It is innocent, unless found guilty. A hypothesis is a novel suggestion that no one wants to believe. It is guilty, until found effective.”

Physicist Edward Teller (1908-2003)

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A	Adenine
bp	Base pairs
°C	Degrees Celsius
C	Cytosine
CHX	Cycloheximide
CPE	Cytopathic effect
C-terminal	Carboxy terminal end
DEPC	Diethyl Pyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Mixture of four deoxynucleotides
ds	Double stranded
E	Early
E. coli	Escherichia coli
EDTA	Ethylenediaminetetracacetic acid
eIF2α	Eukaryotic translation initiation factor 2α
FBS	Fetal Bovine Serum
G	Guanine
G+C	Guanine and Cytosine % composition
gDNA	Genomic DNA
HCF	Host Cell Factor
Hpi	Hours post-infection
HSV1	Herpes Simplex Virus Type 1
HSV2	Herpes Simplex Virus Type 2
HVP2	Herpes Virus Papio, Type 2 (Cercopithecine herpesvirus 16)
IE	Immediate Early
IFN	Interferon
kDa	Kilo Daltons
L	Late
LATs	Latency Associated Transcripts
LB	Luria-Bertani
M	Molar Concentration
MEM	Minimal Essential Medium
$MgCl_2$	Magnesium Chloride
MOI	Multiplicity of Infection
mRNA	Messenger ribonucleic acid
MW	Molecular Weight
NaCl	Sodium Chloride
NCBI	National Center for Biotechnology Information
ND10	Nuclear Domain 10
NML	National Microbiology Laboratory
N-terminal	Amino terminal end
Oct-1	Cellular Octamer-binding Factor 1

OD	Optical Density
ORF	Open Reading Frame
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PML	Promyelotic Leukemia Protein
RNA	Ribonucleic acid
RPM	Rotations per minute
SA8	Simian Agent 8 (Cercopithecine herpesvirus 2)
U_L	Unique Long Region
U_S	Unique Short Region
VP16	Virion Protein 16

Abstract

Human Herpes Simplex viruses and Simian Herpes Simplex viruses share a high degree of genome homology, but despite this, important differences arise when the viruses are compared at the level of gene expression and virulence in non-host primates. In Human Herpes viruses (HSV-1 and HSV-2); 5 genes (RL02, US01, RS01, UL54 and US12) are expressed with an immediate early kinetics, i.e. their transcriptional activation does not require *de novo* synthesis of host or viral factors. The five immediate early (IE) genes regulate the cascade of expression of the other early and late HSV genes. Literature indicates that in HSV-1 infections, ICP4, ICP27 and to a lesser extent, ICP0, are mandatory for the full expression of the early and late gene classes. In contrast, our data on the Simian simplexviruses SA8, HVP-2 and B virus indicate that ICP0 (RL2) is the only gene with true IE kinetics. It is possible that in Simian Herpes viruses, ICP0 is necessary for the expression of all other viral genes, and to test this hypothesis I have cloned and expressed in Vero cells the ICP0 protein for the simian simplexvirus SA8 and studied its effect on the SA8 genes that are homologous to the immediate early genes in HSV. Results demonstrate that ICP0 does not appear to be sufficient to activate the transcription of the other IE genes but it is likely that ICP0 functionality is a necessary component in the activation process.

Introduction to Herpesviridae

The Herpes family comprises large (120-250kb) double stranded DNA viruses which are highly disseminated in nature. To date, over 130 different herpesvirus species have been identified, and at least one type of herpesvirus (but often several different types) has been isolated from many mammalian, avian and some fish and invertebrate species [72]. As more refined techniques become available for isolation and detection, it is almost certain that this family of viruses will increase in number. Of the identified viruses, eight are known to cause disease in humans; Herpes simplexviruses 1 (HSV-1), Herpes simplexvirus 2 (HSV-2), Varicella Zoster Virus (VZV), Epstein-Barr Virus (EBV) and Cytomegalovirus (CMV) and lastly, Human herpes viruses 6, 7 and -8 (HHV 6-8). In addition, Cercopithecine herpesvirus 1 (B virus) can be transmitted from macaques to humans causing severe encephalitis [86,87,90]. The herpes viruses are characterized by their unique virion morphology. Herpes viruses are typically 120-220nm in diameter, comprising a viral DNA core wrapped up like a torus within an icosahedral nucleocapsid [72] coated in a glycoprotein-rich envelope. The capsomeres are in a pentameric arrangement at the vertices and hexameric along the longitudinal axis. Interestingly, high resolution images taken of the capsid demonstrate a protein fold suggestive of an evolutionary link to tailed DNA bacteriophages [3]. Surrounding the nucleocapsid is an additional, unique, structural component called the tegument. The tegument houses many important viral proteins that help facilitate development of a productive infection, and, depending on the cellular location of the viral particle, the distribution of the tegument matrix may appear asymmetric. Coating the tegument is an

envelope which the virus acquires from cellular membranes during budding. In general, the herpesvirus envelope is densely packed with short glycoproteins but the type and relative amounts of each glycoprotein are species-specific [72].

In addition to their unique morphology, herpesviruses are further defined by their ability to cause both lytic and latent infections. Once infected, the herpesviruses are able to remain latent for the entire lifetime of their host. In the case of HSV (and potentially of the other simplexviruses) the viral genome circularizes and gene expression is reduced to a few mRNAs, collectively referred to as latency-associated transcripts, or LATs. Although the precise molecular explanation has yet to be defined, when appropriate circumstances are present the latent genomes have the capacity to reactivate, thereby replicating and causing disease. While during latency replication is null, following reactivation, progeny virions are produced and this is accompanied by the destruction of the infected cell [72].

Humans exposed to herpes viruses develop infections which may be divided into three phases; primary infection, latency and reactivation. The transition between lytic and latent phases of infection is poorly understood but of critical importance with respect to herpesvirus candidacy in gene therapy. Primary lytic infection may be asymptomatic, or characterized by high virus titer, and often breaks in skin.

Taxonomy

The *Herpesviridae* were originally classified into three subfamilies; alphaherpesviruses, betaherpesviruses and gammaherpesviruses. The distinction is

primarily based on their host range, length of reproductive cycle, as well as their location during the latent phase of infection. The alphaherpesviruses in general can infect a broad range of hosts (HSV can infect non-human primates rabbits and rodents) and of cell types whereas the beta and gamma herpesviruses can only infect their natural host [72]. The alphaherpesviruses establish latency in sensory ganglia, the betaherpesviruses remain latent in lymphoreticular cells or in secretory glands while the gamma-herpes viruses appear to remain latent in lymphoid tissues. With the introduction of molecular genetic techniques, these subfamilies were maintained and further classification into different genera was made possible. The alphaherpesviruses comprise two genera implicated in human diseases, namely, simplexvirus and Varicellovirus [89].

Simplexviruses

The simplexviruses are characterized by their ability to cause a variety of infections, specifically ocular, orogenital and neuronal. These viruses display an affinity towards infecting mucosal surfaces and they tend to replicate in epithelial cells. Members of this genus include the human simplex virus types 1 and 2 (HSV-1 and HSV-2), Cercopithecine herpesvirus 1 (B virus), Cercopithecine herpesvirus 2 (Simian Agent 8 or SA8) and Cercopithecine herpesvirus 16 (herpes virus papio type 2). The genomes of the aforementioned simplexviruses are between 152-157kb in size, and are marked by an unusually high G+C content ranging between 68 to 76.5% [89]. Simplexviruses establish latency in the sensory ganglia which innervate the primary site of infection. A latent infection may reactivate to cause asymptomatic shedding, or a recurrence of lesions.

Clinical Manifestations

The human simplexviruses, HSV1 and HSV2, primarily infect mucosal tissue. Herpes infections can be asymptomatic (at least 75%) but symptomatic infections are marked by painful, watery blisters that may be recurrent [78]. Primary HSV infection is introduced onto a mucosal surface or through a break in the skin where it replicates productively in epithelial cells at the site of inoculation. Next, via retrograde transport, HSV spreads down sensory axons to neuronal ganglia, the site of secondary infection unless the virus DNA assumes a circularized state and becomes assembled in chromatin in which case the virus enters a state of latency in the ganglia. Following neuronal damage or activation, the virus reactivates and experiences a short productive cycle. This reactivation results in capsids travelling anterograde along the sensory neuron resulting in new virion shedding from mucosal tissue [89]. Symptomatic shedding is accompanied by lesions, however in the absence of lesions, virus can still be shed (asymptomatic shedding). Historically, HSV1 was linked to oral lesions and HSV2 was linked to genital lesions [44]. Recently, there has been an apparent shift in epidemiology and the distinction no longer appears to hold true as HSV2 has been isolated from some oral infections, and, HSV1 has been attributed to around 50% of primary genital infections in North America. Infections of both types are highly contagious, and they spread during primary infection (or during symptomatic reactivation or asymptomatic shedding). The virus is spread through direct contact, commonly sexual contact, or through saliva. In the unfortunate event that a pregnant woman is infected actively producing herpes virus may be transmitted to the newborn during labour. Preventive measures can be taken (suppressive

antiviral therapy or Caesarean section), but otherwise, neonatal herpes is a severe and often lethal perinatal infection, causing encephalitis or disseminated infection. The estimated prevalence of herpesvirus infections is remarkably high. It is estimated that 50-80% of North American adults have been exposed to HSV1 and between 13-40% have been exposed to HSV2 [44].

Genome Organization

Simplexvirus genomes are classified as having a type E organization, as defined by Roizman and Pellet (Fig. 1) [71]. This type of arrangement is characterized by two main components; a unique short (S) and a unique long (L) segment. The U_S and U_L are flanked on either side by inverted repeat segments, R_S and R_L respectively, which can be further classified based on their terminal (TR_S) or internal (IR_S) orientation. In this instance, both components can invert relative to each other by recombination during replication, and DNA extracted from infected cells consists of four equimolar populations of the four predicted isomers. At each of the genome termini, a 400bp direct repeat sequence is located, termed the 'a' repeat region. This 'a' repeat is also present in one or more copies at the junction between the U_S and U_L segments but in the opposite direction. The 'a' repeats contain *cis*-acting signals which are required for the cleavage and packing of the concatermeric genomes. An exception to this genome arrangement is herpesvirus saimiri 2, a more distantly related simplexvirus which shows a genomic organization of type D, like varicella-zoster virus (Tyler et al. 2010. Virologyin press). The genome of prototypic HSV-1 approaches 155kbp which encodes about 90 unique transcriptional

units, accounting for at least 84 proteins. HSV genes can be classified into one of three transcription kinetic classes; immediate-early (α or IE), early (β or E) or late (γ or L).

Figure 1: Schematic of Genome Organization Type E

The IE genes are localized near or on the inverted repeats, whereas the E and L genes are scattered in the U_S and U_L segments There are two immediate-early transcripts, RL02 and RS01, which are transcribed in the same direction on either side of the 'a' sequence which separates the two repeats and the repeat sequences contain a few ORFs which are transcribed in both directions. Some of these are discussed in more detail in subsequent sections.

Based on the size of the genome and the number of proteins it encodes, the herpes virus genome may appear crowded. A closer inspection, however, reveals how remarkably the virus utilizes the space to its advantage. This genome design may be summarized by a few special features. First, a few of HSV-1 genes overlap, or are collinear with other genes, including UL26 and UL26.5. In this instance, the mRNA of UL26.5 is initiated within the UL26 ORF. Additionally, some genes are produced from an overlapping antisense transcript as is the case with RL1 and ORFs O and P [65]. Only two HSV-1 genes are spliced within the coding region, RL02 and UL15. The intron of

UL15 actually contains ORFs UL16 and UL17 in antisense orientation [52]. In HSV-2 there is an additional gene, RL1, which is spliced within the coding region [51]. The majority of HSV-1 ORFs encode a single protein, the only exception being UL03. Most notably however, is that nearly half of all simplexvirus genes produce 3' coterminal transcripts with at least one other ORF. As a result, transcription is initiated at the appropriate promoter for each gene, but it terminates at a shared poly (A) signal. This occasionally results in long mRNA molecules.

Early work using radioimmununoassay (RIA) demonstrated antigenic similarities between human and simian simplexviruses. Comparing antigenic determinants between HSV-1, HSV-2 and four related simian simplexviruses, relative degrees of cross-reactivity between the viruses was established [34]. As expected, the human viruses were the most closely related, however SA8 and B virus also displayed a close relationship to HSV-1. Using recombinant HSV-1 plasmids as probes for Southern blots, homology was observed between the DNA Polymerase, p40 capsid, VP5 capsid, thymidine kinase and the major DNA binding protein of HSV-1 and the simian viruses [34]. The completion of the genome sequences of the simian viruses enabled more extensive comparison (Fig. 2) [84]. Transcriptional groupings of all genes in the U_S regions of the simian simplexviruses were revealed to be identical to groupings in HSV-1 [19]. The only apparent exception was that in the simian viruses, US3/US4/US5 and US6/US7 formed two 3' co-terminal transcript sets but in HSV-1, the transcripts were arranged in a US3/US4 and US5/US6/US7 manner . Notably, simian simplexviruses SA8, B virus and HVP-2 lack the RL1 ORF, coding for the γ34.1 protein, an essential virulence gene in HSV-1 (Fig. 3) [83]. The γ34.5 protein plays an important role in counteracting the host interferon

response which shuts off viral protein synthesis and it is also responsible for neurovirulence in mice.

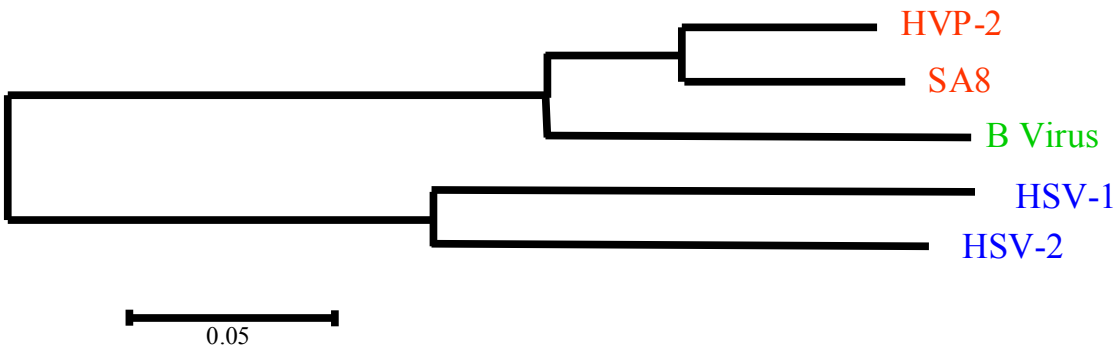


Figure 2: Phylogenic relationship between the simplexviruses. Whole genome alignments of HVP2, SA8, B virus, HSV 1 and HSV 2 were compared and the above dendogram was generated by our laboratory.

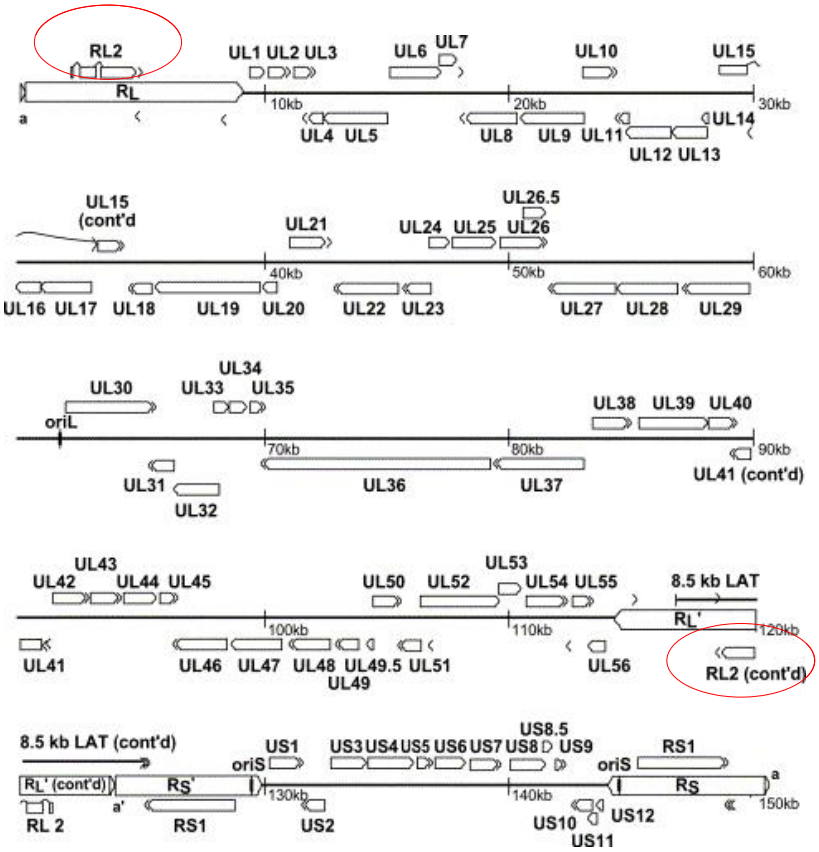


Figure 3: Map of the Open Reading Frames and features of the SA8 genome. Polyadenylation sites are indicated by single chevrons pointing in the predicted direction of transcription. The RL02 gene is highlighted by ovals

Summary of an HSV-1 Infection

The herpes virus must first bind to a suitable cell type which is mostly dictated by available cell surface receptors and coreceptors. Attachment is mediated through viral envelope glycoproteins gB, gC, gD, gH and gL, which enable the virus to fuse with the cell membrane and penetrate the cytoplasm. The success of HSV-1 rests in the virus' ability to establish a cellular environment conducive to viral replication. Using its arsenal of proteins, HSV-1 exploits cellular transcription and translation machinery and it

efficiently counteracts the ability of the cell to mount an antiviral response [53]. Some of the newly released tegument proteins linger in the cytoplasm to help maintain a favourable environment for the infection to transpire, while others are transported to the nucleus. Three notable tegument proteins, VP16, vhs, and US11, serve a multitude of functions at early and late times during infection. Early on, VP16 (UL48) helps to focus RNA Pol II onto the viral α genes and it also plays a role in reducing histone H3 associations with viral α gene promoters. US11 encodes an RNA-binding protein and it is expressed by an early promoter to block protein kinase R (PKR) activation [89]. US11 is found localized to polyribosomes. Virion host shutoff or vhs (UL41) acts as an endoribonuclease to selectively degrade mRNA at early times of infection.

Almost immediately following entry, the nucleocapsid translocates to the nucleus and this is accompanied by the circularization of the viral DNA, probably by the action of the host repair mechanism [71]. Next, the viral DNA localizes to sites near ND10 structures, which are nuclear substructures that ultimately evolve into replication compartments. ICP0, an immediate-early viral protein, executes several functions early during infection whilst residing in the nucleus. Of particular importance is the role ICP0 plays in degrading and dispersing these ND10 structures. HSV utilizes cellular transcriptional machinery in order to progress through a lytic infection. The cellular RNA polymerase (RNA Pol III) is responsible for transcription of viral genes, but many viral and cellular proteins help make transcription possible [71]. HSV gene expression is an elegant cascade of events to which the subsequent section is devoted.

Viral DNA synthesis occurs in the nucleus and seven viral gene products are necessary for this process. The helicase priming complex comprises UL5, UL8 and UL52

as well as the origin-binding protein (UL9). Once these factors have arrived, HSV DNA Polymerase (UL30) and its processivity factor (UL42) are then assembled into the complex. The viral single-stranded DNA (UL29) binding protein is also required for the opening and the progress of the replication fork. As replication is initiated, the prereplicative sites enlarge to form replication compartments that coalesce and eventually fill the nucleus. Viral replication occurs simultaneously to the condensation of the host chromatin. HSV has three origins of replications, oriL and two copies of oriS [91]. DNA synthesis probably occurs through a combination of rolling-circle replication and extensive homologous recombination, resulting in progeny head-to-tail concatemers of the four isomers of the viral genome.

After viral DNA has been synthesized it is cleaved and packaged in the nucleus. The nucleocapsid buds from the inner nuclear membrane, acquiring its tegument and envelope. The newly formed viral particle is then transported to the Golgi apparatus to complete the final stages of the viral glycoprotein maturation process.

HSV Gene Expression and the Immediate-Early Genes

Herpesviruses display a temporal regulation of gene expression that occurs at the level of gene transcription. This regulation is complex and reflects the intricate balance between the virus and the host cell. HSV gene expression during a lytic infection occurs at different stages such that each of the herpes simplex genes falls under one of three general kinetic classes of gene expression; Immediate-Early (IE, α), Early (E, β) or Late (L, γ) [38 ,65, 71]. These categories are distinguished by the requirement for protein synthesis

or viral DNA replication, as revealed by chemical inhibitors of these processes. During an ordinary infection without inhibitors, expression is not confined to temporal boundaries, but rather appears as a seamless continuum.

The first class of genes is designated α (alpha), or immediate-early (IE). These genes function as transcriptional regulators and as such, they are involved in the transcriptional activation of genes in later kinetic classes. These genes distinguish themselves from later genes inasmuch as they do not require *de novo* protein synthesis for their expression. The alpha genes are expressed between 2 and 4 hours postinfection [80, 88]. The β (beta) gene products are proteins required for viral DNA synthesis and nucleotide metabolism. The expression of a few beta genes nearly coincides with the expression of the IE genes (β_1) while the appearance of other transcripts is slightly delayed (β_2). The beta transcripts are detected between 4 and 8 hours postinfection. Cycloheximide (CHX) is a known inhibitor of protein synthesis and it is often used to distinguish between α and β classes [80, 88, 91]. Cycloheximide inhibits the translation of the alpha gene products and therefore blocks the transcription of β genes. Since the IE gene product ICP4 is in turn a repressor of α gene promoters, cycloheximide causes an accumulation of α gene transcripts.

The third class of genes is designated γ (gamma) and these gene products account mostly for structural proteins. Their expression requires viral DNA synthesis. Further distinction is necessary between gamma transcripts that are simply enhanced (γ1) or completely dependent on (γ2) the synthesis of viral DNA. Acyclovir, a selective inhibitor of viral DNA synthesis, provides an elegant means to distinguish between β and γ transcripts.

During a latent infection, the HSV genome is quiescent. Gene expression in this case is limited to a few transcripts, collectively referred to as latency-associated transcripts or LATs.

Virion Protein 16 (VP16) is a viral tegument protein which stimulates the expression of viral immediate early genes. VP16 forms a trihomodimeric complex with two ubiquitous cellular factors, the host cell factor HCF and the octamer transcription factor Oct-1, and, through a direct interaction with viral DNA [2,] activates transcription of the immediate-early genes.

Upon release from the tegument, VP16 associates with the first cellular protein, specifically, host cell factor (HCF). Some articles suggest this binding is responsible for bringing VP16 into the nucleus. In the nucleus, the VP16-HCF complex binds to octamer transcription factor (Oct-1) that is already associated with the viral DNA on the IE gene promoters [42, 64, 71]. The IE promoter region contains many binding sites for cellular and viral transcriptional activators and they are marked by the presence of a consensus sequence, TAATGARAT (where "R" is either purine) in one to several copies upstream from the cap site [27, 49, 65, 79]. Oct-1 binds to the octamer region, and the binding affinity is improved if there is an accompanying ATGC sequence immediately preceeding the TAAT [16, 42]. VP16 also functions to recruit general transcription factors, like TFIIB and TATA-binding protein (TBP) to the IE promoter [92]. Lastly, VP16 serves to reduce histone H3 on viral promoters thereby facilitating the access of co-activators to the pol II initiation complex. VP16 activates the transcription of the immediate-early class of genes and all of the IE genes are involved in the regulation of gene expression to varying degrees.

In HSV-1 there are five IE genes, RL02, RS01, US01, US12, UL54 and a sixth IE transcript, US01.5, which encode for infected cell proteins ICP0, ICP4, ICP22, ICP27, ICP47 and US1.5 respectively [7, 65, 71]. The simian simplexviruses lack US01.5, which is a collinear gene that produces a C-terminal domain of the larger protein encoded by US01. The α gene products collectively exhibit diverse regulatory and immunomodulatory functions. All of the IE gene products, with the exception of ICP47, help to stimulate the expression of β in some cell types. At least four of these proteins are known to be phosphorylated, suggesting that phosphorylation might be an important modulator of these functions [65].

The functions of the IE proteins were initially inferred from the phenotypes of viruses with mutations in IE genes. While ICP4 and ICP27 are the only two absolutely essential for viral replication, each IE contributes to a productive HSV-1 infection. ICP4 is the major transcriptional regulator of viral gene expression, repressing synthesis of its own mRNA and that of other IE genes and activating transcription of E and L viral genes[1]. ICP4 has the ability to bind DNA and interact with various transcription factors providing the means to both activate and repress gene expression [1, 36, 57]. It appears to activate transcription indirectly by recruiting various transcription factors (TFIIB and TFIID) to the preinitiation complex [76], whereas it represses transcription directly by binding with high-affinity to consensus sequences near initiation sites. ICP27 is another multifunctional protein in HSV-1. ICP27 interacts with the C-terminal domain of the large pol II subunit and it also promotes viral DNA replication and late gene expression by a poorly understood manner [60, 73]. It also regulates the processing of viral mRNA [67]. ICP22 is responsible for the activation of the cellular cyclin-dependent kinase 1. ICP22 is

responsible for the activation of cellular-dependent kinase 1 (cdk1). Cdk1 subsequently binds the viral dsDNA binding protein, UL42 and this complex bind topoisomerase IIα. ICP22 is phosphorylated by both viral kinases, US03 and UL13. ICP22 operates with the protein kinases to the phosphorylation of RNA pol II. ICP22 and UL13 are both required for the phosphorylation of RNA Pol II and they are further required for the optimal expression of a subset of late viral genes including US11, UL41 and UL38 [61, 63]. ICP47 has only been linked to the inhibition of the presentation of antigenic peptides to CD8+ cells [65, 71].

ICP0 is a "promiscuous" transactivator of viral and host genes.

ICP0 acts at an unusual position in this regulatory cascade. VP16 and ICP0 seem to play interrelated roles in stimulating the onset of HSV lytic cycle – specifically, ICP0 helps to obtain the full expression of the remaining IE genes. ICP0 displays promiscuous transactivating activity, resulting in the activation of both viral and cellular promoters that exhibit basal activity [6, 65]. ICP0 also plays an important role in creating a permissive environment favourable for viral replication. Unlike ICP4 and ICP27, ICP0 is not absolutely required for viral growth and replication in cell culture but it increases the probability of the virus entering lytic infection. In the absence of ICP0, viral genomes are more likely to become repressed and establish a quiescent infection.

Although the dynamic between VP16 and ICP0 is not fully clear, mutants deficient in either display a striking number of similarities, despite operating via different mechanisms. In-frame linker insertions into VP16 as well as truncations in the C-terminal

end of VP16 both produce a virus with an inflated particle to PFU ratio. The same phenotype is observed in an ICP0 null mutant. The consequence of having an increased particle to PFU ratio is that the mutant viruses have an intrinsically low probability of initiating a productive infection when compared to an infection with wild-type HSV-1. Additionally, both VP16 and ICP0 mutants exhibit a significantly reduced IE gene expression during infection. The phenotypes seen for ICP0 mutants, however, are observed in both a cell-type dependent as well as a multiplicity of infection dependent manner. In most cell types, an infection with a mutant lacking functional ICP0 shows a phenotype, whereby viral yields are reduced 10 to 100-fold lower than cells infected with the wild-type [21, 74]. Similarly, the defective phenotypes become more pronounced at low MOIs yet the requirement for ICP0 seems to be dispelled at MOIs greater than 10. Interestingly, in the osteosarcoma cell line (U2OS) functional ICP0 mutants are recovered in which gene expression and viral yields are restored to reach wild-type levels[21]. Mossman et al. demonstrated that ICP0 provided in *trans* helps to partially complement the effects seen in a VP16 mutant. This finding suggests that the functions of VP16 and ICP0 are interlinked or overlap. It is possible that VP16 primarily functions to activate ICP0 alone, which leaves ICP0 to complete the activation of the other IE genes. To further explore the dynamic between VP16 and ICP0, two different mutants deficient in both of the genes were created[21]. One mutant comprised an ICP0 loss of function mutation (n212) alongside a VP16 linker insertion mutation (in1814) in which the ability of VP16 to form a complex with Oct-1, HCF and DNA is disrupted. The other mutant had the same ICP0 loss of function mutation but in this case, the C-terminal domain of VP16 was deleted (V422). These double mutants infected cells and were investigated on

several criteria; plaquing efficiency, particle to PFU ratio and the degree to which activation of gene expression is possible for representative genes from each kinetic class. The double mutant lacking the C-terminal acidic activation domain (V422) displayed a more severe when compared to the VP16 linker mutation that possessed an intact activation domain [56]. Of note, both phenotypes were less pronounced in U2OS cells, supporting the idea these cells have some inherent factor that promotes transcription activation. Taken together, these findings indicate that once expressed, ICP0 carries the burden of IE transactivation when the C-terminal domain of VP16 is deleted.

After the IE proteins are synthesized, transcription shifts to the β genes, followed by the γ genes. Through a mechanism that is not entirely understood, ICP4 shuts-off the transcription of α genes so that the virus can proceed through the complete expression cycle [71, 76, 77]. ICP4 seems to repress the immediate-early genes through an interaction with a consensus sequence (ATCGTC) in the IE promoter region before it activates transcription of the later genes. ICP4 forms a tripartite complex with TFIIB and TFIID, which binds to the TATA box and to ICP4 binding sites located upstream of the TATA box [76].

Molecular biology of ICP0 in HSV-1

Infected cell protein 0 (ICP0 or Vmw110) is a 110kDa multifunctional nuclear phosphoprotein. The gene coding for ICP0 is called RL2 and is present in two copies of the HSV-1 genome due to its location in the repeat sequence flanking the UL region [50, 71]. It arises from a spliced transcript comprising 3 exons a.a. 1-19, 20-241 and 301-775

respectively. There are two introns present in ICP0, and intron 1 is comparatively large to intron 2. Intron 1 RNA is nonpolyadenylylated and appears to be more stable than authentic ICP0, as it accumulates to high cytoplasmic levels during infection [93]. The second intron is reported to play a role in regulatory events. Intron 2 contains an in-frame stop codon that terminates translation of ICP0 if the intron is not excised. Failure to excise intron 2 results in a truncated protein called ICP0R. ICP0R accumulates to low levels during a normal infection and it appears to function as a dominant negative repressor of the normal role of ICP0 in transactivation. ICP0 interacts with a wide variety of cellular proteins, a testament to the many functions ICP0 has been associated with. To carry out its diverse functions during a lytic infection, ICP0 migrates to different locations within the infected cell. ICP0 may be found in both the nucleus and the cytoplasm, at early (between 2 and 4 hpi) and late (after 4 hpi) times respectively. The activities of ICP0 are generalized as two main functions; major transactivating potential and the ability to degrade intracellular proteins. These functions of ICP0 have been inferred from a battery of insertion, deletion and substitution mutants [23, 74, 85]. The ICP0 coding region is full of stretches of overrepresented amino acids, a unique cysteine-histidine arrangement, a serine-rich tract, two regions that are abundant in acidic amino acid residues, among others. While the relationship between these sequence peculiarities and their function has not been established in all cases, it is clear that the coding region within exon 2 is crucial to ICP0 function.

Originally, the cysteine-histidine arrangement in the N-terminus was thought to be reminiscent of a zinc- finger DNA-binding motif (thereby providing a potential site for DNA interaction); however, further investigation revealed it to be a novel domain, which

was later called a zinc-binding RING finger [8]. ICP0 is extensively modified post-translationally, which is believed to contribute to its diverse functions during infection [4, 13]. Studies mutating the phosphorylation sites of ICP0 have been conducted, revealing some sites to be more crucial than others. Two types of kinases have been shown to affect the phosphorylation site on ICP0; cellular cyclin-dependent kinases (cdks) and the viral kinase (UL13). Davido et al. demonstrated three main phosphorylation sites on ICP0[13], identifying the site closest to the RING finger domain to have the greatest consequence on reducing the activity of ICP0 in viral replication and transactivation.

The strong and broad transactivating activity exhibited by ICP0 on both viral and cellular genes requires the involvement of the cellular ubiquitin-proteosome pathway. Specifically, ICP0 is dependent on the ubiquitin-specific protease 7 (USP7) and the cellular 26S proteasome complex to degrade a variety of cellular proteins in order to create a microenvironment favourable to replication [20, 88]. Ubiquitin and ubiquitin-like modifications act as a major regulatory mechanism in various cellular actitivites including signal transduction, nuclear transport, transcription and membrane protein trafficking. The attachment of a short string of linked ubiquitin molecules (usually four or more) to a protein acts to target them for subsequent proteolytic processing. This process requires the sequential action of three enzymes; E1, E2 and E3 that function as a modifier activator enzyme, a modifier carrier enzyme and a modifier target ligase respectively [47]. The E3 ubiquitin ligase serves to determine the specificity of target substrates, thereby enabling the transfer of ubiquitin from the E2 to the target substrate.

The E3 ubiquitin ligase activity is associated with exon 2 of ICP0, which encodes the RING finger domain [5, 20]. E3 ubiquitin-ligase activity was first suggested in vitro, as

the foci of accumulated ICP0 (in either transfected or infected cells) contain enhanced levels of conjugated ubiquitin. Paradoxically, ICP0 also contains a domain in the C-terminal end that interacts with USP7 or HAUSP (herpesvirus-associated ubiquitin-specific protease) which functions in polyubiquitin chain cleavage [8]. The implications of the ICP0-USP7 interaction have yet to be thoroughly investigated. So far, it appears ICP0 can induce its own ubiquitination, and comparing wild-type ICP0 to an ICP0-mutant lacking the USP7-binding motif, USP7 was shown to inhibit this ICP0 autoubiquintation. The stability of ICP0 was investigated *in vivo*, and USP7 seems to play role in the stabilization and turnover of ICP0 [8]. A number of different isoforms of ICP0 exist and can be isolated from infected cells [4]. These isoforms are believed to be generated by various posttranslational modifications in addition to both proteosome-dependent and proteosome-independent processing pathways [30].

Immediately after ICP0 enters the nucleus, it specifically and temporarily localizes to two cellular protein clusters; the ND10 and the centromeres. The RING finger domain is required for the proteasome-dependent degradation of these two protein clusters [81]. These nuclear substructures are involved in modulating many cellular processes including proliferation, differentiation, apoptosis and innate immunity. The targeted degradation of proteins in these regions is the key to promoting a favourable and unique environment for which viral replication and transactivation may take place. While residing in the nucleus, ICP0 functions to create a more "replication friendly" microenvironment. It accomplishes this through two general mechanisms, it becomes associated with pre-existing nuclear substructures called nuclear domain 10 (ND10) and it counteracts the ICP0 elicited host IFN response. There are approximately 5-20 ND10s

per nucleus and they have been implicated in modulating many cellular processes including proliferation, differentiation innate immunity and DNA repair. ND10s respond to a variety of stimuli and they become modified during the course of the cell cycle. Several cellular proteins are localized in these structures, PML, Daxx, sp100, CPB, p53, Rb, p300 and likely other cellular factors.

Promyelocytic leukemia protein (PML) plays an important role because it is responsible for organizing and assembling the ND10 structures [25, 66]. PML is not a single protein, but rather, a family of related isoforms that arise from extensive splicing and posttranslational modifications. The PML genomic locus is about 35kb, which is divided into 9 exons, and the isoforms range in size from 48kDa to 97kDa. The isoforms share identical N-terminus sequences (exons 1-5) but differ in the C-terminus due to alternative splicing of exons 6-9 [37]. Using a newly developed, virus-sensitive splicing reporter, it was reported that HSV-2 ICP27 might play a role as an alternative-splicing regulator of PML [37]. ICP27 appears to suppress the removal of intron 7a by modulating the 3' splice site, which results in a switch from PML-II to PML-V [37]. Although this has yet to be reproduced, it is not difficult to imagine that the preferential selection of a particular PML isoform might be advantageous to the virus, only adding to the complexity of the virus-host interaction. Analysis of the ND10 structures revealed that PML, Sp100, Daxx (and likely others) are modified by a small, ubiquitin-like protein called SUMO-1. This particular modification appears to play a role in maintaining the integrity of ND10, possibly by stabilizing these individual components. ND10 structures have a repressive effect on viral infection, and, in order for a complete, productive infection to occur, the

virus needs to disrupt these structures. Without PML, these nuclear substructures become dispersed and the components diffuse within the nucleus [66].

A short time later during infection, ICP0 migrates out to the cytoplasm. ICP0 binds cyclin D3 prior to translocating to the cytoplasm and ICP0 mutants that are unable to bind cyclin D3 remain in the nucleus [40]. Yeast two-hybrid studies revealed that in its cytoplasmic phase, ICP0 interacts with the translation elongation factor 1-delta (EF-1δ) [41]. The interaction is mediated through a region in the C-terminal end of ICP0 located in exon 3, which is distinct from the exon 2 region that mediates ubitiquination of ND10-associated proteins. EF-1δ is involved in the elongation of polypeptide chains during the translation of mRNA. EF-1δ is a subunit of a larger complex, comprising EF-1βγδ which is responsible for the hydrolysis of GTP. The conversion of GDP-GTP is required to transport the aminoacyl tRNA to the ribosome in order to begin translation. EF-1δ consists of two isoforms, one that is hypophosphorylated and one that is hyperphosphorylated. EF-1δ becomes hyperphosphorylated by UL13, one of two viral protein kinases encoded by HSV-1 [41]. Together, ICP0, the protein kinase and EF-1δ stimulate protein synthesis.

ICP0 and the HDAC1/2-CoREST-REST complex

The combination of modifications on DNA and its associated histones largely determine whether a region is openly accessible for transcription or alternatively, compacted and transcriptionally repressed. Histones experience an array of modifications including acetylation, methylation, ubiquintination, phosphorylation and ADP

ribosylation [75]. Similarly, multicomponent transcriptional complexes can also become associated with the DNA as an additional means to control and regulate transcription. Both cellular DNA and viral DNA alike make use of these mechanisms to finely tune transcriptional events to coincide/be compatible with the surrounding state of the (infected) cell.

One of the best-known complexes is the mammalian CoREST/histone deacetylase repressor complex. CoREST ([co]repressor for element-1-silencing transcription factor) associates with REST to regulate neuronal gene expression and neuronal stem cell fate and acts as a scaffold on which the complex assembles. This complex comprises six proteins in total, and two are histone deacetylases [45]. HDAC1 and HDAC 2 repress transcription by removing activating acetyl groups from lysine residues on histones. HSV-1 possesses qualities enabling it to block the silencing of viral DNA and this, in large part, is attributed to ICP0. In attempt to better understand the mechanism behind the role of ICP0 in enhancing viral gene expression, lysates of uninfected and wild-type HSV-1 infected cells were compared in co-immunoprecipitation assays. Antibody to CoREST pulled down CoREST, REST and HDAC1 in uninfected cells whereas in wild-type virus infected cells, only CoREST and REST precipitated together and HDAC1 was absent [31].

Extending from the observation that a viral component(s) was responsible for the dissociation of HDAC1 from the complex, and considering the consequences this dissociation would have on viral gene expression, ICP0 was a strong candidate protein. Gu et al investigated whether or not ICP0 acquired a sequence that mimics a host protein involved in gene repression. Interestingly, they discovered 79 homologous residues

between CoREST and the C-terminal of ICP0 [31]. Using HSV-1 mutant viruses, they demonstrate that a region in the C-terminal end of ICP0 mediates the dissociation of HDAC1 from the CoREST/REST complex. Following infection over various time points, HDAC1 was detected in lysates from infections with all viruses yet it was only shown to coprecipitate with CoREST Ab in cells infected with viruses lacking ICP0. Furthermore, results show that CoREST and HDAC1 become phosphorylated in cells infected with HSV-1. To determine the role of viral gene products in these posttranslational modifications, they repeated coprecipitation assays with mutant viruses lacking the two viral kinases, US3 and UL13 [29]. They demonstrate that HDAC1 and CoREST indeed become phosphorylated in an HSV-1 dependent manner, by US3 and UL13 respectively. In a later article they further elaborate on this process, demonstrating that in addition to mediating the dissociation, HSV-1 also results in the translocation of CoREST, REST and HDAC1 to the cytoplasm [32]. Although mostly speculative at this point, this translocation is believed to be a crucial step facilitating the shift of expression to the β and γ genes.

ICP0 counteracts inhibition of viral-gene expression by interferon

The human body comprises a complex system to resist infection by a broad range of pathogens, both bacterial and viral. The host responses to infection often involve several steps, from detecting virus infection to the activation of signal transduction cascades to the transcription of antiviral genes. Each process requires a concerted effort of multiple proteins which provides viruses with many potential targets to inhibit. One of

the major components involved in responding to a pathogen is the interferon (IFN) family of cytokines. IFNs have antiviral, cell regulatory and immunomodulatory functions which are mediated through various interferon stimulated genes (ISGs). Infection with wild-type HSV-1 seems to dampen the IFN response [9, 46, 53, 62].

Interestingly, HSV-1 mutants deficient only in IE gene expression have been shown to induce the expression of ISGs. Using two different mutant viruses, d109 and d106, Mossman et al used microarray analysis to investigate which cellular genes become activated or repressed as a result of an HSV-1 infection [46]. The difference between the two mutants is that d106 only expresses a single IE gene; specifically, it slightly overexpresses ICP0 compared to a wild-type HSV-1 virus. The non-IE expressing mutant, d109, appeared to induce many IFN-stimulated genes whereas the other mutant, d106 did not [46].

IFN-α and -β are classified together as type I IFN. Binding of type I IFN to the IFN α/β receptor modifies the transcriptional and translational environment in cells such that an "antiviral state" is induced. Melroe et al. used a "reverse complementation" approach to demonstrate that HSV-1 ICP0 mutants replicate like wild-type virus in interferon (IFN)-α/β receptor knockout mice [54]. Effects on plaque formation and viral titers were examined following the pre-treatment of Vero cells with IFN-α, β or γ. Wild-type HSV-1 virus (KOS) plaque formation was significantly reduced in the cells that were initially exposed to IFNs.

Many viruses have evolved an efficient way of impeding the host immune response by tampering with a crucial transcription factor, interferon regulatory factor-3 (IRF-3). Functional IRF-3 is absolutely required for the immediate transcription of

interferon resulting from viral infection. In HSV-1, IRF-3 is activated in a cell-type-dependent manner but the explanation for this remains unknown. During an HSV-1 infection, the activation of the immune response is much stronger in the absence of viral IE protein synthesis. By examining the effects of coinfection with Sendai virus and HSV-1, Melroe et al. showed that typical SeV-induced nuclear accumulation of IRF-3 was inhibited and the degradation of IRF-3 was enhanced in the presence of HSV-1 [54]. Using mutant viruses, they were able to determine that ICP0 was necessary and sufficient for this inhibition of IRF-3 nuclear accumulation, as well as the degradation of IRF-3 [54]. ICP0 was not shown to be crucial for the reduction in IFN-B production indicated by infections with HSV-1 ICP0 mutants which continue to display suppressive effects.

In a subsequent study, Melroe et al. went on to explore a more specific role of ICP0 in IRF-3 inhibition. Using d106, the HSV-1 mutant virus which expresses only ICP0 of the IE genes, they observed overt changes in the normal localization patterns of IRF-3 [53]. At early times post infection with Sendai virus alone, IRF-3 was diffuse but localized in the nucleus. In d106 HSV-1 infection alone, no IRF-3 was detected in the nucleus, yet in coinfection with Sendai and d106 HSV-1, IRF-3 was restricted to punctate nuclear structures. Additionally, this accumulation overlapped with the localization of a subset ICP0 indicating that IRF-3, (as well as other proteins required for interferon transcription) became sequestered to nuclear domains containing ICP0 [53]. They also showed that the sequestered IRF-3, in turn, colocalized with p300 and CBP. Levels of IRF-3 localized in the nucleus was minimal at late times during infection and using a proteosomal inhibitor, authors show that proteosomal activity was required for ICP0-mediated inhibition of nuclear IRF-3. The zinc binding RING finger domain of ICP0 has

been shown to be essential for the inhibition of IRF-3-mediated activation of IFN-responsive genes.

In summary ICP0 has pleiotropic effects on important functions of the host cell:

- Transcription regulation via ubiquitination and degradation of the ND10 structures (including PML, Sp100 and Daxx proteins) which occurs at early times during infection to create a favourable and unique environment in which viral replication and transactivation can take place.
- In the cytoplasm, regulation of protein synthesis by interaction with the ribosomal elongation factor EF-1δ. The C-terminal end of ICP0 is associated with the interaction with EF-1δ which is part of a larger complex, EF-1βγδ which is responsible for the conversion of GDP to GTP required to transport the aminoacyl tRNA to the ribosome during protein synthesis.
- Modification of histone acetylation and chromatin structure by interaction with the HDAC1/2-CoREST-REST complex. The C-terminal end of ICP0 mediates the dissociation of HDAC1 from the CoREST-REST complex.
- Inhibition of the interferon response by preventing IFN mediated gene activation. This is achieved through RING finger domain of ICP0 which is responsible for the inhibition of nuclear accumulation as well as the degradation of IRF-3.

HSV Latency

During latency, HSV adopts a transcription regulation strategy much like cellular DNA in that it associates with nucleosomes while residing in neuronal cell bodies.

Specifically, the linear viral genome assumes a circularized "state" and the genome becomes complexed with covalently modified histones [10, 24, 26]. This is accompanied by the silencing of all genes except for the latency associated (RNA) transcripts located in the repeat region flanking the U_L segment. Latency is characterized by cessation of replication and a lack of progeny virus production [26]. Additionally, the LAT promoter and enhancer become associated with acetylated H3 histones, a feature of transcriptionally active chromatin. The LAT gene yields three mRNA species by splicing (collectively referred to as LATs) [80]. LAT functions to repress apoptosis in infected cells, enabling HSV to be maintained discreetly and seemingly unbeknownst to the host immune system.

In some people, this latent state appears to be perpetually maintained and the only indication of HSV-exposure boils down to the detection of HSV antibodies in their serum. Many others are not as lucky, as recurrent outbreaks, often resulting in watery blisters at the site of primary infection are commonplace. The exact molecular mechanism has yet to be worked out but it appears that stress leads, or at least contributes to reactivation. At the clinical level, reactivation is not the same for everyone as the degree and frequency of reactivation episodes experienced is highly variable. From the perspective of the virus, reactivation involves the switch back to high level gene expression, in addition to the completion of a full replicative cycle. During reactivation, LAT transcript levels decrease the H3 histones formerly associated with the promoter and enhancer become deacetylated. There is some evidence to indicate that an HSV-specific immune response might help to minimize the extent of the reactivation, but it does not appear to be capable of outright *preventing* a reactivation. After exposure to HSV, the

adaptive immune system responds by generating antibodies and also activates CD4+ and CD8+ T cells. The HSV-specific antibodies are unsuccessful at neutralizing the virus because two HSV encoded glycoproteins; gE and gI appear to mimic an immunoglobulin G (IgG) Fc receptor. Together, the gE and gI bind the Fc domain of anti-HSV IgG thereby saturating this portion of the immune response and allowing HSV lytic infection to progress [15, 58]. Another HSV glycoprotein, gC, is involved in evasion of the complement system. Not all is lost; however, as there is some redeeming evidence to support a beneficial role of local HSV- CD4+ and CD8+ T cells in minimizing the magnitude of reactivation.

More recently, two small RNAs (sRNA1 and sRNA2), encoded in the first 1.5kb of the HSV genome have been implicated in inhibiting productive infection as well as inhibiting apoptosis. They are detected in cells latently infected with HSV-1. The effect of each of the RNA species was observed in a transfection assay, whereby HSV genomic DNA was co-transfected with either sRNA1 or sRNA2. Results demonstrated that while sRNA1 seemed to contribute more of an effect, both species were capable of reducing the expression of the major transcriptional regulator, ICP4, in addition to substantially reducing the amount of infectious progeny virus that was released. Considering the important consequences of manipulating the chromatin structure of the viral genome, the potentially important role of the small RNA species in addition to the differential outcomes of each reactivation episode, it seems likely that both viral and cellular gene products play a role in this delicate regulation.

ICP0 and Reactivation from Latency

Working to elucidate the necessary conditions and molecular feats required for HSV to reactivate has proven to be no small task, a task which is further complicated by the inherent association between the LATs and ICP0 [80]. Using Northern blots, early work by Stevens et al revealed that LAT ORF almost entirely overlapped with the ICP0 ORF but they are transcribed in the opposite direction[80]. Therein lies the obstacle, due to the overlapping transcriptional nature of some of the important genes involved in reactivation, any type of mutational analysis or deletion affecting one of the genes simultaneously affects the other gene. This makes it particularly difficult, if not impossible, to determine specific effects caused by either LAT mutations or ICP0 mutations on reactivation. Experiments have been conducted using cells that have either been transiently or stably infected with ICP0 [24], and most recently an inducible-ICP0 cell line has been created [25], but creating an in-vivo model of reactivation remains an elusive task. ICP0 has been implicated in reactivation because of its ability to prevent the silencing of viral DNA of all three classes of viral genes.

Using a co-immunoprecipitation approach, Cliffe and Knipe (2008) demonstrate that in HSV-1 infection at low MOI the viral genome becomes associated with histones to a similar extent seen in cellular genes at early times, yet this association decreases later during infection independently of viral DNA replication or transcription [10]. Using an ICP0 null virus in parallel with a rescued virus, they show that the decreased level of H3 histones associated with viral genes is dependent on expression of ICP0. By determining

the proportion of histone H3 that was acetylated in both groups, they also concluded that ICP0 was involved in promoting histone H3 acetylation [11].

Simian simplexviruses

The simplexviruses share a high degree of genome homology, and similar clinical manifestations in their natural hosts. Despite a close phylogenetic relationship between the simian and the human simplexviruses, however, these viruses display differences in clinical presentation when they infect other non-host primates. The simian simplexviruses generally cause similar symptoms to HSV1 and HSV2 in their natural hosts [68]. HVP2 and SA8 are the naturally occurring herpes viruses in baboons (genus papio), and while usually asymptomatic, close to 90% of the baboon population has been exposed HVP-2 [17,84]. The symptomatic SA8 cases are predominantly seen in females and severe infection in newborns has been observed, similar to neonatal infection with HSV-2, causing high degree of morbidity within baboon colonies [83]. B virus is endemic to rhesus macaques, and infection is usually acquired at sexual maturity. Most infected macaques are asymptomatic, however, animals experiencing stress or immunosuppresion present oral lesions and ulcers, in addition to conjunctivitis [87]. Interestingly, despite detection of B virus in genital mucosa, genital lesions have not been observed. Similar to HSV1 in the human population, the rates of B virus seropositivity increases with the age of the population so between 40-50% of captive adult macaques are infected [90]. In addition to the sexual contact, and saliva the virus may also be passed via a bite or a scratch [86]. This presents an occupational risk for individuals who come in close contact, either during

caretaking or research using these animals. B virus seems to be far more pathogenic in humans as infection results in encephalitis. Human infection with B virus has a mortality rate approaching 80% if infection is left untreated [86, 90].

Even more variation is observed in infections with these primate alphaherpesviruses in mice. With the exception of SA8, all of them cause severe encephalitis, and the severity of disease caused by B virus and HVP-2 varies by strain [69, 70]. Moreover, there appears to be two distinct phenotypes for HVP-2 such that strains are either highly neurovirulent or entirely non-pathogenic [69]. The varied clinical presentations in humans, other primates and mice are summarized in Table 1.

Table 1: Simplexvirus Characteristics and Clinical Manifestation

Designation	Natural Host	Presentation in Natural Host	Disease in Other Primates	Virulence in Mice
HSV-1	Humans	oral herpes	SEVERE	DEADLY for most clinical isolates
HSV-2	Humans	genital herpes	SEVERE	DEADLY for most clinical isolates
B virus	Asian Macaques	oro/genital herpes	DEADLY	DEADLY or nonpathogenic depending on isolate
HVP-2	African Baboons	genital herpes	NONE	DEADLY or variably pathogenic depending on isolate
SA8	African Baboons	genital herpes	NONE	NONE

The inherent genetic similarities between the human and simian simplexviruses led our lab to perform a comparative analysis of gene transcription (IE genes) and protein expression (US11) of representative viruses in this genus. By identifying subtle differences that may be responsible for the dramatically different clinical manifestations observed in xenogenic hosts we could gain a better understanding of pathogenic mechanisms of simplex virus infection in humans. Together, this research could bring us closer to creating an attenuated HSV strain for the purpose of vaccines toward HSV and potentially as a backbone for vaccines against other more pathogenic viruses.

In HSV-1, there are five IE genes; RL02, RS01, US01, US12 and UL54 (which encode for six proteins ICP0, ICP4, ICP22/US1.5, ICP47 and ICP27 respectively), the expression of which is independent of cellular protein synthesis. As described in detail in an earlier section, the expression of these genes is activated by the tegument protein VP16 and it does not require *de novo* protein synthesis and therefore their transcription is not prevented by inhibitors of protein synthesis such as cycloheximide. By inhibiting the production of the IE proteins by cycloheximide, the activation of the E genes is prevented, while IE transcripts accumulate due to lack of feedback suppression by the IE protein ICP27. This coordinated pattern of gene expression in HSV-1 is well described in the literature and our lab wanted to determine if this pattern of gene expression was consistent across related simplexviruses. The kinetics of gene expression was investigated during a productive infection with several human and simian herpes simplexviruses using Northern blot and quantitative PCR. Results confirmed that HSV-1 produced five IE transcripts which is consistent with previous studies; however, differences were observed in the case of simian simplexviruses. SA8, HVP-2 and B virus

produced only one IE transcript, RL02, while the other 4 transcripts were expressed as E genes. Lanes 4A,4B,4C,4D and 2E of figure 4 represent gene expression of US12, UL54, RL02, US01 and RS01 respectively, in the absence of cycloheximide. Lanes 5A,5B,5C,5D and 3E represent gene expression of the same genes in the presence of cycloheximide. In the case of US12, UL54, US01 and RS01 gene expression is reduced, albeit to varying degrees. Surprisingly, the expression of RL02 (lane 5C) became enhanced in the presence of the drug. Since this pattern was observed in all the simian viruses investigated, it suggested a potentially important role of RL02 as the only, or key, transcriptional regulator of the β class of genes in simian herpes simplexviruses.

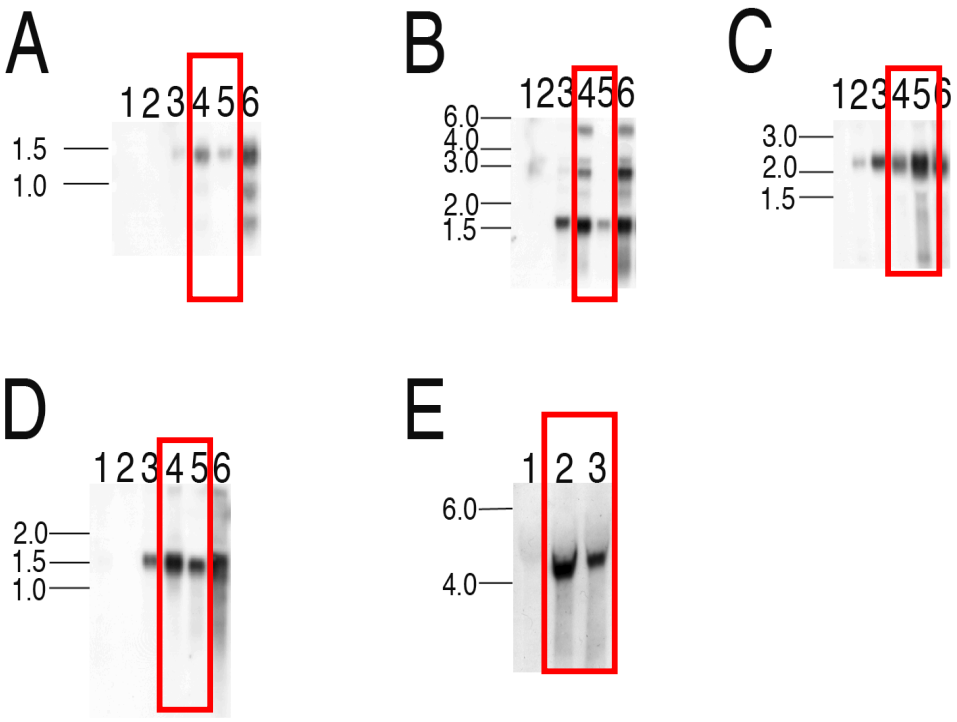


Figure 4: Northern Blot depicting a time course of gene expression in SA8-infected VERO cells in the presence and absence of cycloheximide. The sizes of the molecular weight markers (kb) are indicated. A, US10/US11/US12 (851/996/1266 bp mRNAs, respectively); B, UL52/UL53/UL54 (5963/2850/1570 bp mRNAs, respectively); C, RL02 (2323 bp mRNA); D, US01 (1352 bp mRNA). E, RS01 (3560 bp mRNA). Expected mRNA sizes were determined from the reported start codon to the poly(A) signal, excluding known introns. For A-D, the lanes are: lane 1, mock infected cells; lane 2, 1 hpi; lane 3, 2 hpi, lane 4, 4 hpi; lane 5, 4 hpi with cycloheximide; lane 6, 8 hpi. For E, the lanes are: lane 1, mock infected cells; lane 2, 5 hpi; lane 3, 5 hpi with cycloheximide (Sevenhusen et al. Submitted for publication)

Rationale

In HSV-1, multiple genes act in concert to activate and regulate gene expression. Of the IE genes, ICP4 and ICP27 are essential for viral replication. ICP0 on the other hand, contributes to activation of gene expression; however, it is not essential as demonstrated by the multitude of ICP0 mutant studies done in HSV-1. HSV-1 mutants lacking ICP0 display an attenuated phenotype that is still capable of replication and infection. These mutants have an increased particle to plaque forming unit (pfu), substantially lower yield and decreased levels of α gene expression yet this phenotype is overcome in a multiplicity of infection (moi) and a cell type dependent manner. From previous work in our laboratory it appears that in Simian viruses ICP0 is the only true IE early gene and it may be the single global transactivator of gene expression. As a result, ICP0 may be needed for the full expression of the homologues of the other IE genes in SA8, HVP2 or B virus. To test the hypothesis that the other IE genes in simian require the presence of ICP0 for expression, we can provide ICP0 in trans in cells infected in the presence of cycloheximide and observe if the other IE gene transcription is induced to the levels observed in HSV-1. SA8 virus was chosen over HVP-2 and B virus as the representative for the simian simplexviruses in large part because it can be used for infection in a CL-2 lab as well as the fact it was selected as the candidate virus for the earlier work done by our lab.

Objectives

1. To clone SA8 ICP0 form SA8 and develop a system for transient expression in Vero cells.
2. To determine if SA8 ICP0 is the single, global transactivator of gene expression by examining if ICP0 provided in trans is sufficient to activate expression of the other putative IE genes and make their transcription insensitive to the protein synthesis cycloheximide, as is the case in HSV-1.

Materials and Methods

Tissue Culture, Virus Propagation and Virus Stock

African Green Monkey Kidney Cells (Vero) cells were obtained from ATCC (strain CCL-81) and cultured as monolayers in T150 culture flasks Cells were incubated at 37ºC with 3.5% CO_2. Cells were supplied with an MEM growth medium containing, 2mM L-glutamine, 1% sodium bicarbonate and 5400mg glucose. During maintenance of cells, 5% foetal calf serum was added, but for cells that were intended for transfection and infection experiments, 2% calf-serum was added. The subculturing protocol started by removing and discarding culture medium into autoclaved waste bottles, followed by briefly rinsing the cell layer with 5ml of a trypsin-HBSS solution to remove traces of serum, which contains trypsin inhibitor. Next, the remaining 5ml of trypsin-HBSS solution was added and the flask was placed at 37ºC for 4 minutes. Once removed from the incubator, the sides of the flask were tapped to - encourage the detachment of the cells. Next, 7ml of fresh growth medium was added to the flask to neutralize the trypsin. The cells were thoroughly re-suspended to ensure a homogenous solution before seeding either new T150 flasks or 6-well TC treated plates generally using a passage ratio of 1:4. Cells were maintained and split every 4 -5 days.

For the purposes of this work, a new batch of SA8 virus was propagated from a stock routinely used in our lab. Five T150 flasks with 100% cell confluency were infected at an MOI of 0.1PFU/cell. To begin the infection process, growth media was

removed and discarded in an autoclaved waste bottle. The appropriate dilution of virus was added to each flash while flasks were laid flat. Virus adsorption was carried out over an hour at 37ºC and flasks were tilted every 20min to evenly distribute the virus before supplementing each flask with fresh media. Flasks were incubated in 5% CO2 until 100% CPE was observed, which in this case took 24hrs. At this time, media was removed and the infected cells were rinsed with 5ml of cold PBS. This PBS was discarded and a fresh 5ml of PBS was added to the flasks. Using a cell scraper (when necessary) cells were collected into 50ml Falcon tubes. Tubes were centrifuged at 4°C for 10min at 500 rcf. At this time the supernatant was discarded and the cells were resuspended in cold PBS. Virus was released from cells by freeze-thawing the pellet in an acetone and dry ice bath and 37°C water bath. Tubes were then centrifuged at 4°C for 10min at 2000 xg rcf. The supernatant carrying the virus was aliquoted into freezer tubes and stored at -80°C until required for an experiment. The virus titer was determined by performing a plaque assay. VERO cells were seeded into 100mm dishes three days prior to performing the assay to achieve 100% confluence. The cells were infected with serial dilutions of the virus stock, ranging from 10^5 and 10^9. After 1h virus adsorption at 37C, 15ml (2X MEM/ 1.5% agar mixture) overlay was added to cover the cells. Plates were incubated for 48h and the serial dilution which produced a countable number of plaques was used to determine the viral titer. Viral titer was calculated by multiplying the average number of plaques obtained between the replicates by the serial dilution, then dividing by the dilution volume.

Subcloning SA8-RL02

RL02 was excised from the Blue Heron vector using EcoRI and HindIII restriction enzymes. A quantity of 1ug of plasmid DNA was restriction digested in reaction volume of 50uL. The reaction vessel was incubated at 37ºC for 2hrs, and the reaction was terminated by heat-inactivation at 65°C for 20mins. A volume of 18uL of the restriction digest was used in gel electrophoresis and run on a 0.8% agarose gel in 0.5% TBE buffer solution. The band of appropriate size was excised from the gel under a UV lamp. DNA was purified from the gel using a QIAquick gel extraction kit (Qiagen). A portion of the extracted DNA was submitted for sequencing to confirm the gene sequence. The RL2 fragment was then inserted into an untagged (pcDNA3.1) or a tagged (CMV-Myc) vector. The ligation reactions were prepared in a ratio of 3:1 using T4 DNA ligase and a 1X DNA ligase buffer, the reaction was incubated at 16°C overnight. Chemically competent E. coli cells (OneShot Top 10- Invitrogen) were transformed with the ligation reaction according to the manufacturer guidelines. Transformed cells were plated on warmed Luria-Bertani (LB) agar plates with 100mg/ml ampicillin (AMP). Colonies were screened using a Qiagen Mini QIAprep System (Qiagen) and purified clones were sequenced for conformation by the DNA Core Facility (National Microbiology Laboratory, Winnipeg). The successful clones were selected stored in 80% glycerol at -80C for future use.

The pCMV-Myc vector backbone was modified with an adapter containing HingdIII prior to the subcloning of RL2. The two oligos (24bp in length) designed for the adapter were purified but had no modifications. The oligos were phosphorylated using

T4 kinase. The reaction was set-up as per the manufacturers protocol, which included the addition of ATP. The final reaction volume was 25ul, and it was incubated at 37°C for 10 minutes. The kinase was inactivated by the addition of 5mM EDTA (because kinase is not susceptible to heat inactivation). Following phosphorylation, the single-stranded DNA was annealed together to form a double-stranded adapter fragment. A volume of 10ul of each of the phosphorylated oligos, were heated to 85°C for 5 minutes. The vector backbone was cut at the BglII site, which also flanked both ends of the adapter. The modified vector was double digested with EcoRI and HindIII and gel purified using the gel extraction kit (Qiagen) and this was followed by general subcloning procedures described above for RL2; the only difference was that the newly synthesized adapter was used in much higher ratio, 50:1 with the vector. One clone containing the HindIII site was used for the subsequent subcloning of RL2, and a 3:1 insert to vector ratio was used in the ligation reaction. As before, clones were sequenced to confirm the insert was in-frame.

Transfection

The transfection experiments were conducted on VERO cells. Cells were maintained in a MEM medium, with 5% CS, L-glutamine, sodium carbonate. Cells were split the same day as transfection using a solution of HBSS (Hanks Balanced Salt Solution) and 0.01% Trypsin. Cell monolayers were grown in 10cm tissue culture treated TC flat bottom plates. Each well was seeded with approximately 5.5 x 10^5 cells and the plates were incubated at 37°C for three hours. At the 2.5 hour mark, the transfection

solution was prepared in order to provide sufficient time for the DNA-lipid complexes to be formed. For each plate, a volume of 18ul of transfection reagent, FuGENE (Roche) was diluted in 1.5ml of serum-free medium, OptiMEM. This solution was incubated at RT for 5 minutes. Next, a quantity of 3ug of plasmid DNA was added to the transfection reagent so no trypsinization was required prior to transfection. Time course of RNA samples were taken, RNA cell protect reagent was immediately added to each aliquot and frozen at -80°C until the time course was complete (0 to 48 hrs). The following day, total RNA was harvested from the transfected cells using RNeasy Kit (Qiagen).

Transfection /Infection

VERO cells were seeded into 10cm tissue culture plates three hours prior to transfection. Transfection was carried out as described above using 3ug plasmid DNA and 18ul FuGENE 6 per plate. Transfected cells were incubated at 37°C, 5% CO2 for 48 hours. Infection at 24h post transfection and 48h post transfection did not appear to show any differences in viral gene expression but since cells were more confluent at 48h this was the timeframe used in all experiments onwards. At 48hrs post-transfection, cells were then infected with SA8 virus at an MOI of 5 PFU/cell. The infection was performed in the presence and absence of cycloheximide at a concentration of 100ug/ml. A working solution of cycloheximide (100mg/mL) was made by dissolving 0.200g of cycloheximide powder in 2mL of dimethyl sulfoxide (DMSO). Drug was added to 2% CS as needed for a final concentration of 100ug/mL. Before infection, the cells were synchronized. Growth medium was decanted from cell monolayer, and the monolayer of each plate was rinsed

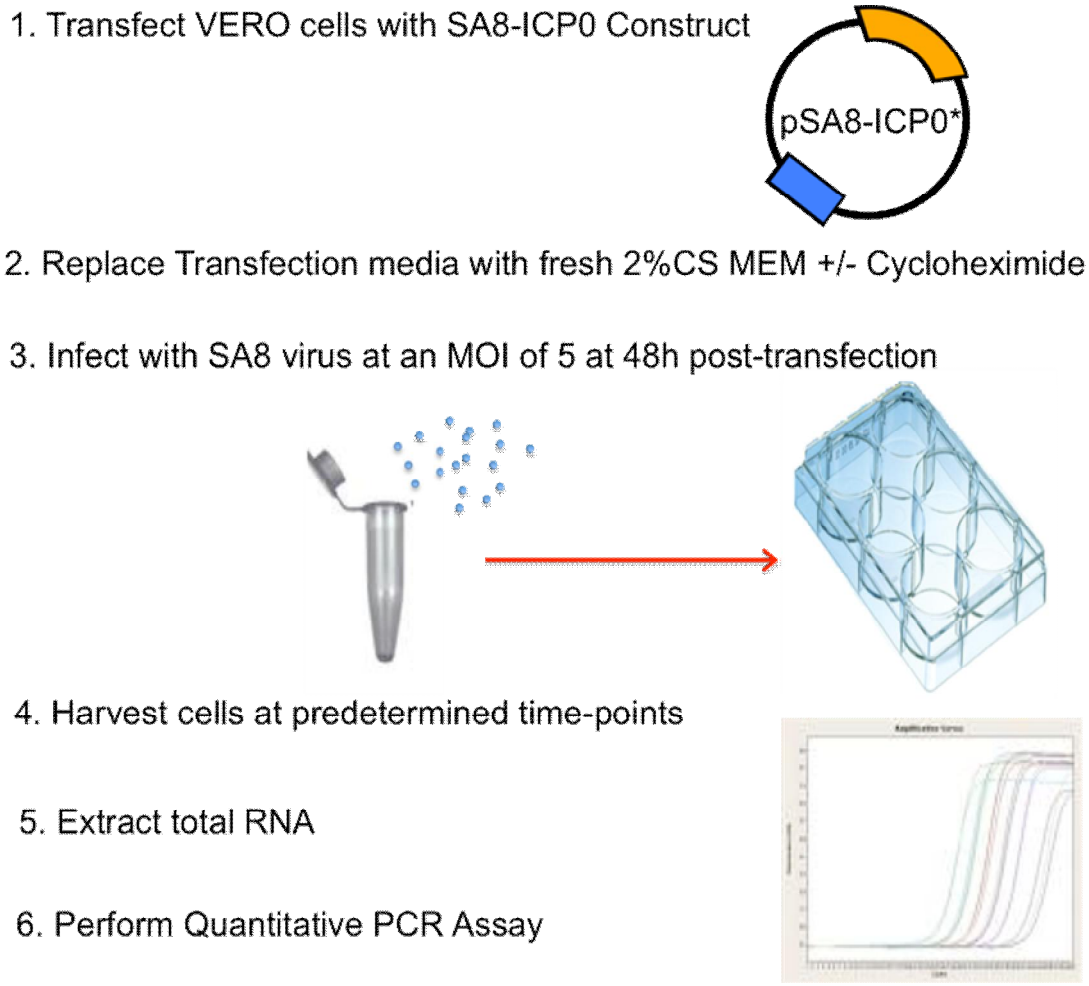
with 2mL of MEM or MEM with cycloheximide as necessary. Next, an additional 5mL of MEM or MEM with cycloheximide was added to each plate. To ensure even distribution of the medium, the plates were gently rolled back and forth for 30s. The plates were laid flat to chill in the refrigerator for 30 minutes. During this time virus stock was diluted in MEM or MEM with cycloheximide. Cells were almost fully confluent at the time of infection, so there were approximately 7.0X10^6 cells in a 100% confluent 100mm dish. This number was used to calculate the required number of particle forming units for an MOI of 5 PFU/cell. In general, the total virus needed for the entire experiment would be diluted in a final volume of 12.8 mL so that a volume of 800uL could be aliquoted per dish. Virus adsorption took place on ice over 1 hr. The diluted virus was added to each well and the plates were remained at 4°C, with gentle rocking every 15 min to promote even dispersal of virus.

RNA Extraction

Cells were harvested at fixed times during time-course experiments. First, the growth medium was decanted and placed in a labelled 15mL Falcon tube. Then cells were rinsed with 1mL of a trypsin-EDTA-HBSS solution, which was then added to the same Falcon tube. A fresh 2mL volume of trypsin-EDTA-HBSS was added to the cells and the plate was placed at 37°C for 4 minutes. The detached cells were collected and again, added to the Falcon tube. The well was rinsed with 1.5 mL of PBS which was also added, and the entire volume was centrifuged at 125 x g for 5 minutes, at 4°C. The supernatant was removed and discarded, and the cell pellet was rinsed with 2mL of PBS

before a second centrifugation at the same speed. Again the supernatant was discarded and the remaining cell pellet was re-suspended in 750uL (max volume that can be loaded on the extraction columns) of buffer RLT containing (10uL/mL of B-mercaptoethanol). Samples were then processed using RNeasy Plus Mini Kits (QIAGEN), which includes an on-column gDNA removal treatment. Obtained RNA was tested for quality and quantity using a Nanodrop1000.

Figure 5: Flowchart of the infection/transfection procedure



DNA Contamination and RNA Clean-Up

On-column gDNA elimination was not entirely sufficient so additional methods were required. RNA was subsequently treated with Turbo DNase-Free (Ambion). An amount of 5000ng of RNA was arbitrarily selected for this second DNase treatment. The reaction was carried out in a volume of 50ul which contained 5 uL of 10X DNase buffer, 2U of the recombinant DNase enzyme and 43ul of RNA and DEPC-treated water. The reaction was done in 0.5mL (autoclaved) Eppendorf tubes. The reaction was held at 37°C for 40 minutes, at which point an additional 1U of DNase was added to the reaction. After the second 40 minute incubation at 37°C 13uL of the patented DNase-Inactivation enzyme (Ambion) was added. As per the manufacturer protocol the tubes were gently mixed for at least 5 minutes at RT. After the inactivation step, the tubes were placed in 1.5mL Eppendorf tubes and centrifuged for 1.5 minutes at 6000 rpm. Following the centrifugation, tubes were removed and placed in a rack. If centrifugation was done properly, two distinct liquid layers were observed. The top layer containing the "clean" RNA was aspirated and placed in a clean, new tube. Aspiration was to be done in a single attempt and care was taken to ensure that bottom liquid layer was not touched or disrupted. The bottom layer contains various cations and other potential RT-PCR inhibitors.

Generation of cDNA Using Random Hexamers

The DNase-treated RNA was again run on the Nanodrop spectrophotometer to get an approximate concentration and purity of the RNA. This RNA was used in an RT-PCR reaction to generate cDNA. An amount of 1200ng of RNA was used in the RT-PCR reaction. The reaction volume of 20ul contained 4ul of a 5X VILO reaction mix (an optimized mixture containing $MgCl_2$, dNTPs and random primers), 2ul of a 10X Superscript III enzyme mix (an optimized mixture containing an engineered RT enzyme and RNaseOUT recombinant ribonuclease inhibitor) in addition to 14ul of a combination of DEPC-treated water and RNA as needed. The reaction was gently mixed before running on a conventional PCR-amplification machine. The protocol was as follows; 25°C for 10 minutes, 42°C for 120 minutes and 85°C for 5 minutes. The cDNA was stored at -20°C until needed for qPCR.

Quantitative PCR Using Specific Oligos

The quantitative PCR was performed using the LightCycler 480 SYBR Green I Master (Roche). The reaction was set-up in a final volume of 18ul plus 2ul of template cDNA. The cycling conditions were; 95°C for 5 mins, followed by 45 cycles of 95°C for 10s, 67°C for 10s and 72°C for 15s. The cDNA was diluted 1 in 20 before being used as a template in qPCR. Previously designed primers were used to amplify RL02, US01, US12, UL54 and US06 from SA8 and are listed in Table 2.

Table 2: Oligonucleotides Used for Nucleic Acid Amplification

Virus	Gene	Forward Sequence 5'-3'	Reverse Sequence 5'-3'
HSV-1	RL02	TGCGCTGCGACACCTTC	GGGATGGTGCTGAACGACC
HSV-1	US01	GGAACCCGTGTGCAAGCTT	GAGCGTGTGGTCCGAACC
HSV-1	UL54	GCTGTGCTGGATAACCTCGC	TGGCCAGAATGACAAACACG
HSV-1	US12	ATGTCGTGGGCCCTGGA	CGCCCCCTTTTATTGATCTCA
HSV-1	RS01	GCGGCGACGACGACGATAAC	CGGCGAGTACAGCACCACCA
HSV-1	US06	ACGGTGGACAGCATCGGGATG	CCAGTTTGGTGGGATTTGCGG
SA8	RL02	TCGACGAGACCCAGCTCT	ATGGTGCTGTACGAGCCG
SA8	US01	CGTCTGGTCCGCGACTGCTA	AAACTCCCGCCGCAGCCT
SA8	UL54	CCCGAAAACATCGACCAG	GCACATCTTGCACCACGA
SA8	US12	CGAGGTGCGAGTGGTTGG	GCGTTCCTACGGAATCCG
SA8	US06	GTGCCCGTCGCCGTGTACT	GCATGAGGAACCCCAGGTCG

Western Blots

VERO and GP2-293 cells were seeded into 6-well plates and transfected with the SA8- RL2 construct containing the c-myc (9E10) tag. At each of the prescribed time points, growth media was removed and discarded and cells were harvested using 500uL of PBS (pH 7.4) 1% SDS lysis buffer. The cell solution was collected in a 1.5mL Eppendorf tube, and the tube was heated to 95°C for 5 minutes. The tubes were then placed on ice for several minutes to cool the solution, before loading it onto a QIAshredder column for homogenization. The loaded column was centrifuged at 11270 x g for 2 minutes. The flow-though was aliquoted into volumes of 50uL and stored at -80°C until needed for Western Blots.

Protein samples were separated on both 10% and 12% pre-cast Ready gels (Bio-Rad). 7uL of the Benchmark (Invitrogen) pre-stained ladder was mixed with 15uL of the MagicMarker (Invitrogen). Samples were prepared by mixing 25uL of protein with 25uL

of 1X Lameli loading buffer (Bio-Rad). The entire sample was heated to 95°C for 3 minutes, and between 15uL and 20uL of the sample was loaded per well depending on the gel. The gel was run at 110V for 1.5 h. A 1X Running buffer was prepared by diluting a 10X commercial stock (Bio-Rad) in dH20. A 1X Transfer buffer was prepared by diluting a 10X commercial stock (Bio-Rad) in dH20 and pure methanol. Once the gels were run to completion, they were removed from the cassettes and prepared for transfer to a PVDF membrane (Millipore). The PVDF membrane was activated by a 10s wash in methanol, then rinsed in dH20 before being included in the transfer stack. The transfer system was placed in the cold room overnight. For two gels, an ice block was added to the transfer chamber, and the transfer was run with a constant current of 160mA.

The next morning, the membrane was placed in a small dish for immunological detection. First the membrane was blocked with a PBS (pH 7.4) 5% skim milk and 0.1% Tween solution for 2 h. As there is no commercial antibody against SA8-ICP0, a monoclonal anti c-Myc tag antibody was used to confirm the presence of the protein. The primary antibody was diluted 1:200. The primary wash was done for 2h followed by 3 X 5 minutes washes with PBS 0.1% Tween. The secondary goat anti-mouse antibody was diluted 1:10000 and the membrane was incubated with the secondary for 45 minutes. This was followed by an additional 3 X 5 minute washes with PBS 0.1% Tween. The GE ECL™ western blotting detection kit was used for chemiluminescent visualization of protein bands. The kit required the membrane to be covered with equal amounts of solution A and B for one minute then stored in a clean piece of plastic wrap. The membrane was then exposed to hyperfilm ECL™ film (GE Healthcare) for 1- 10 minutes and the film was developed using the Feline™ 14 x-ray film processor (Fisher Scientific).

Indirect Fluorescent Antibody (IFA) Assay

Immunoflourescence was performed on cells transfected with the SA8-RL2 construct containing the myc-tag. High-performance no.1 $^{1/2}$ glass coverslips (Zeiss) were added to each well of TC plate. At established time points, growth media was removed and discarded. Cells were rinsed once with PBS and fixed using 4% paraformaldehyde in PBS for 10 minutes at RT. Fixation mixture was removed and cells were washed three times with PBS. Fixed coverslips were stored in PBS at 4°C until the immunological steps were performed. At this time, coverslips were removed from PBS and placed on a clean, hard surface covered with parafilm. Cells were permeabilized with 0.25% Triton X-100 in PBS for 5 minutes at RT. Cells were washed three times with PBS for 30s, before a generous amount of a 5% BSA PBS blocking solution was added. Cells were incubated with the blocking solution for close to 2h at RT. In the meantime, the primary mouse anti-myc antibody (Santa Cruz # sc-40) was diluted 1:20 in the blocking solution. When blocking was completed, cells were washed three times for 5 minutes. 100uL of the diluted primary Ab was added to the surface of cells and left to incubate at RT for > 1 hour. The area of the washes was kept moist by keeping the coverslips under a lid in between manipulation. The primary Ab solution was removed and cells were rinsed with three 10 minute washes with PBS. Next, the secondary goat-anti-mouse IgG antibody (Alexafluor 488) was diluted 1:500. A volume of 100uL of the diluted Ab was added to the surface of the cells and incubated (protected from light) at RT for 45 minutes. The secondary Ab solution was removed and the coverslips were washed with an additional three 10 minute rinses. Next, Hoescht was diluted 1:1000 in PBS and placed on the

surface of the cells. Cells were incubated with Hoescht for 10 minutes at RT before being removed. A final series of three 10 minute rinses with PBS, excess liquid was aspirated off and coverslips were placed cell-side down onto a microscope slide. A minute amount of mounting solution was used to keep slips bound to the slide. Slides were laid flat to dry at 4°C, protected from light. The following day, slides were checked under a florescent microscope to observe antibody activity.

Short-Interfering RNA (siRNA)

The sequence for the SA8-RL02 gene was provided to Fisher Scientific as a target in order to generate custom siRNA duplexes. Information on the nature of virus, and the exact type of cells that were to be used in the experiments were also provided. Fischer created 4 pairs of siRNA duplexes targeted towards RL02 and the sequences are listed in Table 3.

Table 3: Sequences for Custom siRNA Duplexes

Name	Sense Sequence	Length (bp)
siRNA1	GCUCAGACGCAGACCCAGAUU	21
siRNA2	CGAGGAAGUGUGCGCGGAAUU	21
siRNA3	CCGAGAGAGCACACGGAUCUU	21
siRNA4	UCUCGAGCGUCGUCGCCAUUU	21

A quantity of 20nmol of each siRNA duplex was resuspended in a 1X siRNA buffer (Dharmacon) and were slowly pipeted a few times to mix. Each solution was placed on a shaker for 30 minutes at room temperature to properly reconstitute the oligos. After incubation, the four different siRNA stocks were aliquoted in small working volumes to avoid freeze-thawing the samples. VERO cells were plated into 12-well plates in the morning and siRNA transfection was carried out in the afternoon. The siRNA was diluted with Hyperfect siRNA Transfection reagent (Qiagen) and the solution was incubated at room temperature for 5-10 minutes prior to the addition to cells. The siRNA transfection was carried out overnight and cells were infected with the SA8 virus the following afternoon. Cells were collected at 5hpi and samples were run on the same qPCR assay as in the previous experiments.

Statistical Analysis

Data obtained from all experiments was entered into Excel (Microsoft) and the arithmetic mean (average) and standard deviations were calculated using the software functions. The values obtained for averages and standard deviations were used to perform statistical analysis on GraphPad Prism® software. A one-way ANOVA test was performed to establish whether the values for transcript accumulations were statistically different by generating a P value summary. Tukey's test was selected for the Post-Hoc test to compare which pairs of data were significantly different, in order to determine if RL02 provided in trans had any true effect.

3.0 Results

3.1 Codon optimization of the RL2 gene from SA8

Attempts to amplify the RL2 genes from cDNA or from genomic SA8 DNA were fruitless, probably because of the high G/C content of this DNA region (79%). An artificial RL2 coding region was derived from the published SA8 sequence[83](GenBank accession number AY714813) and the synthesis and cloning of the gene was commissioned to Blue Heron Biotechnology.

The cDNA sequences were taken from GenBank, and the introns were removed manually. The cDNA gene sequence is long (1893bp) and comprises long stretches of repeats so the sequence required further manipulation before submission. The predicted cDNA sequence was run on a program designed to compute codon optimization (GeneMaker, Blue Heron Biotechnology), in order to reduce the G/C content and to optimize the codons for expression in mammalian cells SA8-RL2. The program generated two optimized coding sequences in which the G/C was reduced from 79 to 64.3%, and, after careful investigation one was selected to be created de novo. The original cDNA sequence for RL2 was aligned with the optimized sequence of RL2 using the Align-X program (VectorNTI) and the image is included as figure 6.

Figure 6: Sequence Alignment of SA8-RL2 Gene Before and After Optimization

```
                1                                                  50
 cDNA RL2      (1) ATGGAGCCCTCGCGCGGCCTCGGAGCCCCGGGTCGCTCCTCCGCCCAGGC
Optimized      (1) ATGGAACCTTCCCGCGGCCTCGGCGCTCCCGGAAGATCTAGCGCACAAGC
                   51                                                 100
 cDNA RL2     (51) GGCGGGGGACGCCCTCCTGGACGAGGAGCTCCTGATGCTCTCCCGCTACT
Optimized     (51) AGCGGGGGACGCCCTGCTGGACGAGGAGTTGCTGATGCTGAGCAGGTACT
                   101                                                150
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 cDNA RL2     (101) TTTTCCCCGACTCCTCCGACGACGACTCGGACGTCGACGACGACGACGAC
Optimized     (101) TCTTCCCAGACTCTAGTGATGACGATAGCGATGTGGATGATGATGATGAC
                    151                                             200
 cDNA RL2     (151) GGGGGCCGCGACCGCCCCGCCTCCGACGCCGACACGGACGCCGAGCTCTG
Optimized     (151) GGAGGCCGCGATCGGCCCGCCTCTGACGCTGACACAGACGCTGAGTTGTG
                    201                                             250
 cDNA RL2     (201) CGAGCCCGGGCCCGCCTCCCGGGGGCCGCGGGCCCCCTCTCCCCCGCCCC
Optimized     (201) TGAACCGGGGCCCGCCAGTCGAGGTCCAAGGGCACCCTCTCCTCCGCCGC
                    251                                             300
 cDNA RL2     (251) CGCCCCGCGAGGTCTGCGCCGTCTGCACGGAGCGCATCGACGAGACCCAG
Optimized     (251) CCCCTCGGGAGGTTTGTGCTGTGTGCACAGAAAGGATCGATGAGACTCAG
                    301                                             350
 cDNA RL2     (301) CTCTGCGCCGCCTTCCCCTGCCTGCACCGCTTCTGCATCCCCTGCCTCAA
Optimized     (301) CTGTGCGCGGCCTTCCCTTGCTTGCACAGATTCTGCATCCCCTGCCTTAA
                    351                                             400
 cDNA RL2     (351) GACCTGGCTCCCCATGCGCAACAGCTGCCCCCTCTGCAACGCCGTGGTGGG
Optimized     (351) GACTTGGCTGCCTATGCGGAACTCATGTCCTTTGTGTAACGCGGTGGTCG
                    401                                             450
 cDNA RL2     (401) CCTATCTCTCATCGTGGGCGTGAAGCCCGACGGCTCGTACAGCACCATCCCG
Optimized     (401) CCTACTTGATTGTCGGGGTGAAACCCGACGGCTCTTATTCTACTATTCCT
                    451                                             500
 cDNA RL2     (451) GTGATCAACGACCCGCGCACGCGCGCCGAGGCCGAGGAGGCGGTGCGCGC
Optimized     (451) GTTATCAACGACCCCAGAACAAGAGCAGAAGCAGAGGAGGCTGTGCGAGC
                    501                                             550
 cDNA RL2     (501) CGGCACCGCCGTGGACTTCATCTGGACGCACCGCCTCCCCGGGGAGGCGG
Optimized     (501) AGGCACTGCAGTGGATTTCATCTGGACACATAGGCTGCCTGGCGAAGCCG
                    551                                             600
 cDNA RL2     (551) CCCCGGCCTCCGTCACCCTCGGGGGCCGCACCGTGCGCGCCCTCTCCCCG
Optimized     (551) CGCCAGCAAGTGTTACACTGGGGGGAAGAACCGTGCGCGCCCTGAGCCCC
                    601                                             650
 cDNA RL2     (601) CCCGCCCGCATGGGCCAGCCCGCGCCCCGAGGCGGCGCCGCGGCGCGCGC
Optimized     (601) CCCGCTAGAATGGGACAACCTGCACCCCGCGGCGGCGCGGCTGCCAGAGC
                    651                                             700
 cDNA RL2     (651) GCCTCGCGCCGCGGCCCGCGTCGCGCGCCCGCCCCCGCCGACTCGCCCA
Optimized     (651) CCCTAGGGCCGCGGCTAGGGTCGCGCGCCCACCTCCGGCCGACTCTCCGA
                    701                                             750
 cDNA RL2     (701) TCCTGATCGCCGACTCGCCGCCCGCCTCCCCCGCCGCCCCCCGGCCGTC
Optimized     (701) TACTGATCGCTGACAGCCCTCCCGCCAGTCCGCGCAGACCACCAGCCGTG
                    751                                             800
 cDNA RL2     (751) TCGGGGCCCCCCGTGGCCCCGGTGGCCCCGCGGCCGCGAGCCGCCATGCC
Optimized     (751) TCAGGCCCACCCGTGGCTCCGGTCGCCCCTAGACCCAGAGCAGCCATGCC
                    801                                             850
 cDNA RL2     (801) CCGCCCACCCGCCCAGGCCCGGCCCCCGGCCCTGACGCAGGCCCAGGCCCC
Optimized     (801) AAGGCCACCTGCGCAGGCCAGACCACCAGCACTCACCCAGGCTCAGGCAC
                    851                                             900
 cDNA RL2     (851) AGACGCAGGCCCGGAGTCAAGCCCGGGCCCAGGCGGCCCTGGCCCAGGCC
Optimized     (851) AGACACAGGCCAGAAGCCAGGCGCGGGCCCAGGCAGCCCTGGCACAAGCC
                    901                                             950
 cDNA RL2     (901) CTGGCCCAGGCGCTGGGCCGGGGCGCCGCCCGCCCCGCGCCGCCGCAGCA
Optimized     (901) CTCGCACAGGCACTGGGCCGGGGCCCCCCTCGCCCTGCCCCACCTCAGCA
                    951                                            1000
 cDNA RL2     (951) CGCCCACACCCAGACCCCGCCCCGGGCCCAGGCGCAGACCCAGACCCAGA
Optimized     (951) TGCACATACCCAGACGCCACCGCGGGGCGCAAGCGCAAACACAGACTCAGA
                   1001                                            1050
 cDNA RL2    (1001) CCCCGACTCAGGCGCGGGCCCAGACCCGGGCCCAGACTCAGGCTCAGACC
Optimized    (1001) CGCCTACCCAGGCACGGGCCCAAACTCGCGCACAGACGCAAGCCCAGACT
                   1051                                            1100
```

```
 cDNA RL2    (1051) CAGACCCAGGCCCAGGCCCAGGCCCAGGCCCAGGCCCAGACTCAGACCCA
Optimized    (1051) CAAACGCAGGCTCAAGCACAAGCACAGGCTCAGGCCCAGACACAGACACA
                    1101                                          1150
 cDNA RL2    (1101) GACCCAGGCCCGGCCTGAAACCCAGACCCAGGCCCAGACCCAGGCCCGGG
Optimized    (1101) GACCCAGGCCAGGCCTGAGACACAGACCCAAGCCCAGACGCAGGCTAGAG
                    1151                                          1200
 cDNA RL2    (1151) CTCAGACGCAGACCCAGACCCAGGCCCGCAAGCGCCCGGCCTCGGGCGCC
Optimized    (1151) CCCAGACCCAGACTCAGACGCAGGCTCGCAAAAGACCTGCCTCCGGAGCA
                    1201                                          1250
 cDNA RL2    (1201) GGGGGCGCCTCCGGCTCGCGGGGACCCAAGCGGGCCTCGCTGCCCGCGCCC
Optimized    (1201) GGCGGAGCATCTGGCTCCAGGGGACCCAAACGAGCAAGCCTGCCTGCTCCC
                    1251                                          1300
 cDNA RL2    (1251) CCCGACGCGCCGGCCCGCCGGCGCAGCTCCCCCGGCACCTCCGCTCG
Optimized    (1251) ACCAGATGCACCCGCCCGACCCGCCCAGCTTCCTCCTGCCCCTCCACTGG
                    1301                                          1350
 cDNA RL2    (1301) CGGCCGCCGCTCCGCCCCCGCTCCTCCGCCGCCTCCCGCGTCGTCGGCT
Optimized    (1301) CCGCAGCGGCACCACCTCCAGCCCCTCCTCCCCCCCTGCTTCATCAGCT
                    1351                                          1400
 cDNA RL2    (1351) CCTCGGGGCTCCGCCGCTCCGCCTCCGCCCGCCGCTCCGCCCCCTGCCGA
Optimized    (1351) CCCAGGGGATCTGCCGCTCCCCCACCTCCCGCTGCTCCACCCCCCGCTGA
                    1401                                          1450
 cDNA RL2    (1401) GAGAGCACACGGATCCTCCCTCGGCCCCCGCCCCCGCCGAGCGGGGGCCGA
Optimized    (1401) GCGCGCACACGGCTCCTCTCTGGGCCCACGCCCTGCAGAACGGGGACCTC
                    1451                                          1500
 cDNA RL2    (1451) GGAAGTGTGCGCGGAAGACCCACCACGTGGACGCCGACCGCGCCCCCGCG
Optimized    (1451) GCAAGTGTGCTCGAAAGACTCATCACGTGGACGCTGATAGAGCCCCCGCT
                    1501                                          1550
 cDNA RL2    (1501) GCGTCCGGCCCCACGCGCTACCTCCCCATCTCGGGGGTCTCGAGCGTCGT
Optimized    (1501) GCGTCAGGACCTACAAGGTATTTGCCAATTAGTGGGGGTTTCATCCGTGGT
                    1551                                          1600
 cDNA RL2    (1551) CGCCATGGCGCCCTACCTCAACAAGACCGTCACGGGCGACTGCCTGCCGGG
Optimized    (1551) TGCCATGGCGCCCTACTTGAACAAAACTGTTACTGGCGATTGCCTGCCAAG
                    1601                                          1650
 cDNA RL2    (1601) TCCTCGACATGGAGACGGGCGCCATCGGGGCCTACGTGGTCCTCGTGGGGG
Optimized    (1601) TTCTGGACATGGAAACAGGCGCCATCGGTGCATACGTGGTGCTCGTCGGGG
                    1651                                          1700
 cDNA RL2    (1651) CGCGACTGCAACCTGGCGCGCTGCCTGGCCGACGCGGAGCCGCAGTGGGC
Optimized    (1651) AGGGATTGTAATTTGGCTAGATGTCTTGCCGACGCAGAACCTCAGTGGGC
                    1701                                          1750
 cDNA RL2    (1701) CCGCCGCTCCCGCCTCCCCGAGGCCGCCCCCGGGTGCGTGTCCCCGCCCCG
Optimized    (1701) TAGGCGCTCACGACTGCCGGAAGCGGCCCCCGGGTGTGTGTCTCCACCTG
                    1751                                          1800
 cDNA RL2    (1751) AGTACCCGGGAGACCCCGCCCACGGCCTCTGGATGACCCCGGTGGGCGGC
Optimized    (1751) AATACCCCGGCGATCCGGCACATGGTCTGTGGATGACGCCCGTGGGCGGA
                    1801                                          1850
 cDNA RL2    (1801) ATGCTCTTCGAGCAGGGCGCGCTGCTGGGCGGCCGCAGCTTCCACAGCCT
Optimized    (1801) ATGCTTTTTGAGCAGGGCGCGCTGCTGGGGGGTAGATCCTTTCACTCTCT
                    1851                                    1893
 cDNA RL2    (1851) GGACTCGCGCCACCCCCTGGACCCCCGCCGAGGGCGACCCGTAG
Optimized    (1851) CGACAGCAGACACCCCTGGACCCCCGCCGAGGGCGATCCTTAG
```

The cDNA sequence for RL02 gene from the SA8 virus was obtained from Genbank. Original G+C content was 79%. After codon-optimization, the G+C content was reduced

to 64.3%. The alignment of the original sequence to the codon-optimized sequence was performed on Vector NTI using Align-X. Identical regions are highlighted in yellow.

In order to facilitate cloning the optimized gene into various expression vectors, two restriction sites, EcoRI and Hind III, were added to the cloned RL2 gene at the 5' and 3' ends respectively. This clone obtained from Blue Heron is named pMR-OPTRL2.

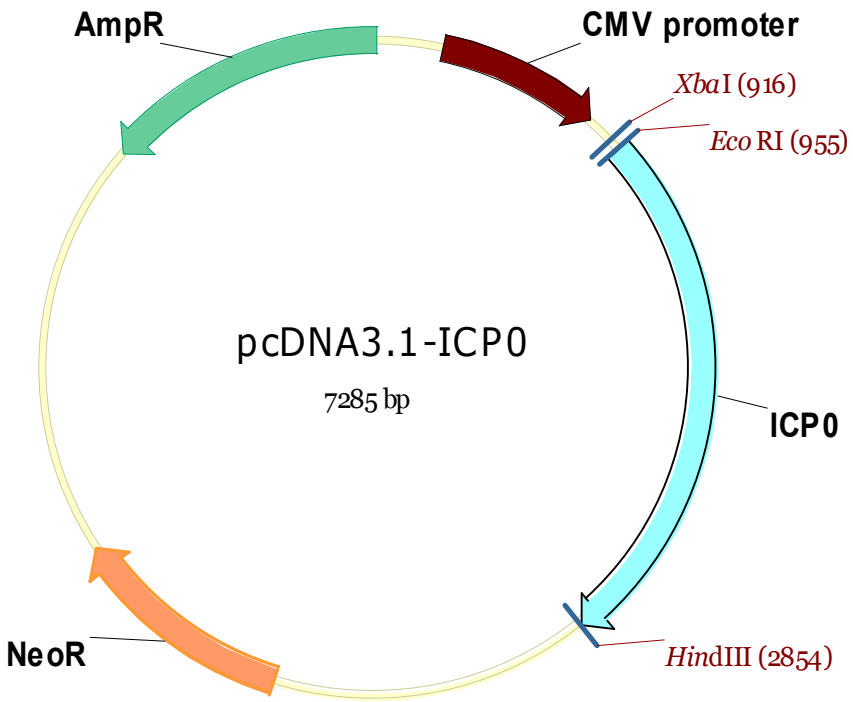
The optimization of the RL2 gene enabled easy differentiation between the viral RL2 transcript (non-optimized) from the transfected RL2 transcript (optimized). Different pairs of PCR primers were designed to amplify each transcript. In earlier experiments designed to estimate levels of expression of the transfected RL2, a TaqMan PCR assay was used. In later experiments involving transfection and an infection, the SYBR green based PCR assay was used to quantify levels of the viral RL2 transcript which was used as the positive control in those experiments.

3.2 Subcloning of the RL2 Gene into Various Vectors

There are no commercial antibodies available against SA8 ICP0 protein and therefore the RL2 gene was cloned into two different expression vectors, pcDNA3.1 (which is untagged) and pCMV-Myc (which contains a myc-tag). While the pCMV-Myc clone was used in order to study the expression and cell distribution of the expressed ICP0, the pDNA3.1-ICP0 clone expressing an untagged ICP0 was used to study the effects of ICP0 on transcription and nuclear structure. It was important to have both of these expression vectors in order to avoid possible interference effects of the myc tag on the transfection and infection experiments.

The codon-optimized RL2 gene was excised from the pMR-OPTRL2 by digestion with the terminal restriction enzymes EcoRI and HindIII and the fragment was cloned in the corresponding restriction site of the eukaryotic expression vector pcDNA3.1 under the control of a CMV promoter. The untagged vector, pcDNA3.1(-) had both of the compatible restriction sites available in the MCS so this cloning was straightforward. Many colonies were obtained in the first attempt and eight of the twenty clones which were selected for screening contained appropriately sized bands on the agarose gel, indicating the insertion of the SA8-RL2 gene. Six of the eight prospective clones that were submitted for sequencing had the RL2 inserted in the correct orientation, in-frame. Figure 7 displays a vector map for pcDNA3.1-ICP0, the untagged clone.

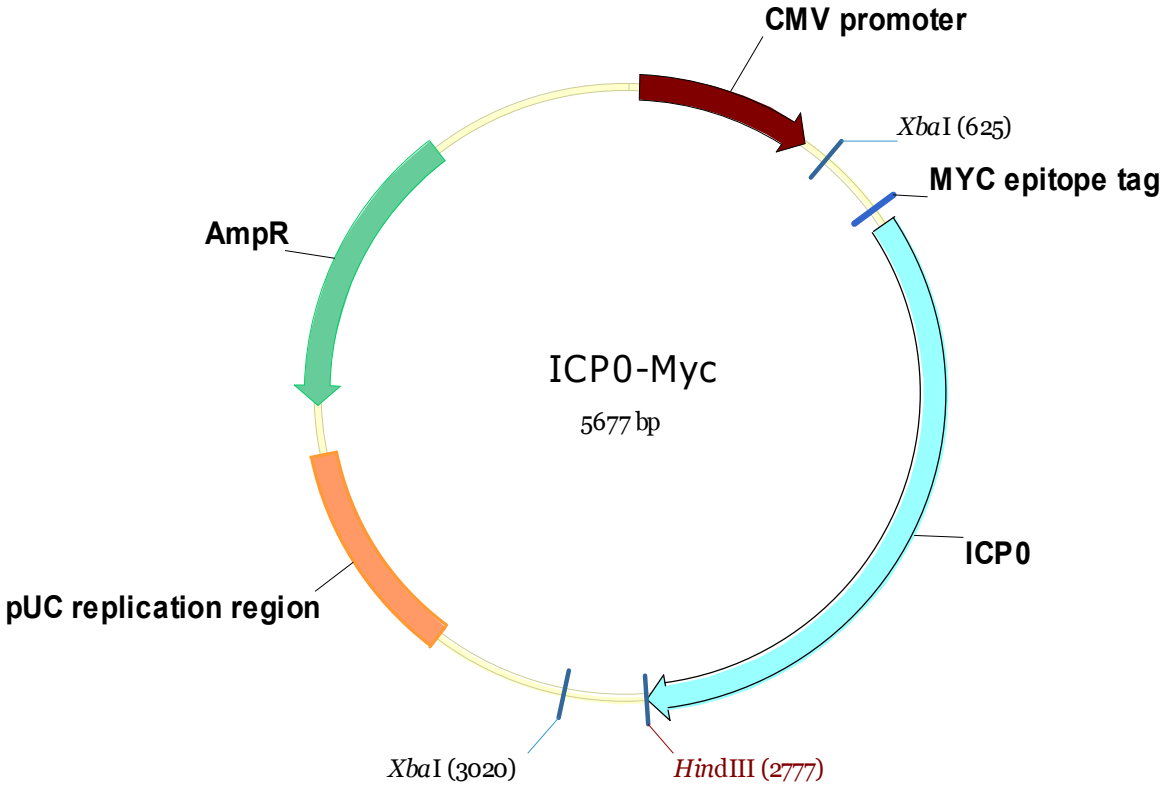
Figure 7: Schematic representation of the pcDNA3.1(-) expression vector (Invitrogen) containing the RL02 gene insert.



The second expression clone was generated using the expression vector pCMV-Myc. This vector contains the c-myc-tag for which specific monoclonal antibodies exist (c-Myc 9E10: sc-40, Santa Cruz Biotechnology) with minimal cross-reactivity with cellular cMyc. The pCMV-Myc vector lacked a HindIII site, so an adapter was inserted into the MCS to make this site available for cloning. The adapter was created by making two, complementary, oligonucleotides that contained the Hind III site and was flanked by Blg II sites. The sequence of adapter and the modified MCS site is depicted in figure 8B. The oligos were phosphorylated before the individual strands were annealed together. The newly synthesized adapter required slow and steady cooling to RT to maximize renaturation of the strands. Even and gradual cooling was achieved by removing the

entire heat-block and placing it on the lab bench. The tagged expression vector, pCMV-ICP0, is depicted in Figure 8.

8A)



8B) Multiple Cloning Site Modifications

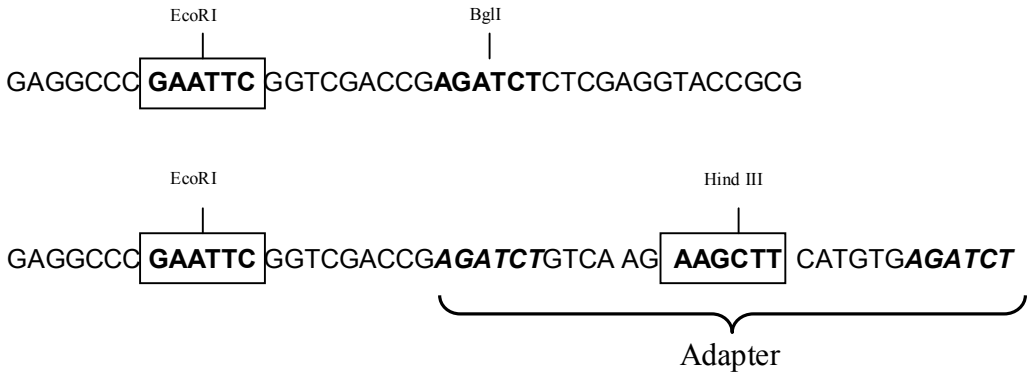


Figure 8: (A) Schematic representation of the pCMV-Myc expression vector (Clontech) comprising the RL02 gene insert. (B) The modified sequence of the multiple cloning site region to show features relevant to cloning. In bold are the necessary restriction sites and the bracket highlights the HindIII adapter that was used to modify the vector backbone.

3.3 Optimization of Transfection

The goal from optimizing the transfection procedure was to determine the duration of transfection to achieve maximal expression of ICP0 with minimal detriment to the cells. VERO cells were first transfected with a commercial construct expressing green fluorescent protein (GFP). The GFP clone serves not only as a positive control in the transfection assays, but its transfection efficiency was determined by estimating the percentage of fluorescent cells after 48h. During this time transfected cells would attach and grow to 50% confluency.

Optimal transfection efficiency of GFP was obtained by transfecting Vero cells within 2-3 hours of splitting the cells, before the attachment process took place. The cells were transfected using the FuGENE transfection reagent in OptiMEM medium as

described in Materials and Methods. Transfection efficiency after 24 hours was low, about 10-15%, and transfection carried out to 48 hours revealed significantly improved transfection efficiency to about 50%.

Attempts at transfecting attached VERO cells were unsuccessful, using the transfection reagents Lipofectamine 2000 (Invitrogen), Effectene (Qiagen), and FuGENE (Roche), at ratios between 2:1 and 9:1.

Figure 9 displays the expression of transfected GFP at 48 hours. This image is one of several taken and it reflects a consistent transfection efficiency obtained during each experiment. The transfection protocol that was optimized using the GFP construct was used in all experiments involving the transfection and expression of RL2 expressing clones.

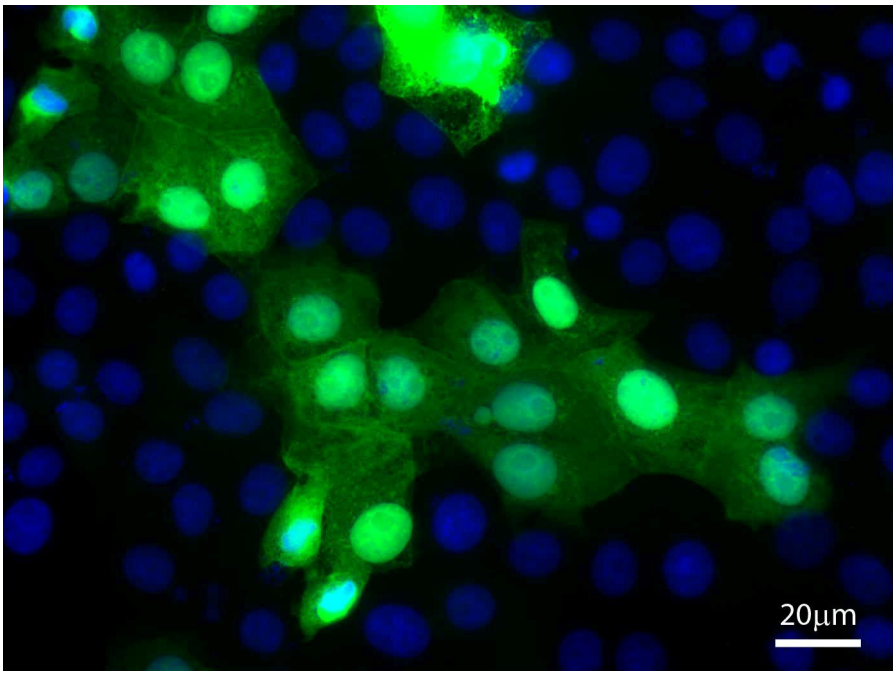


Figure 9: Expression of GFP in VERO cells 48 hours post-transfection. Cells were transfected using FuGENE (Roche). Nuclei (in blue) are stained with Hoechst and transfected GFP protein is represented in green.

3.4 Quantitative Polymerase Chain Reaction (qPCR)

Vero cells were transfected with the untagged SA8-RL2 construct. Cells were harvested at various time points, in order to determine if RL2 was successfully being expressed. A quantitative PCR (qPCR) TaqMan (Applied Biosystems) assay was used to analyze the mRNA transcript for SA8 RL2. The qPCR data revealed that RL2 transcript is detected as early as 18hours post-transfection. The first few experiments involving the transfection of RL2 revealed some gDNA contamination, as the mock-transfected and 0 hours-post transfection samples generated a signal above what was determined to be background signal, so more extensive DNase treatments were necessary. Total RNA was extracted using RNeasy Plus (Qiagen), which includes an on-column genomic DNA

elimination step during the extraction process. Since this was not sufficient to remove all the gDNA contamination in the samples, a second DNase treatment using Turbo-DNA-Free (Ambion) was performed after the extraction process. Amplification of a dilution series of the untagged SA8-RL02 construct in order to produce a standard curve allowed conversion of the Ct values into gene copies per microgram of total RNA.

Table 4: Ct Values Obtained Following qPCR for Transfected RL02 Transcript

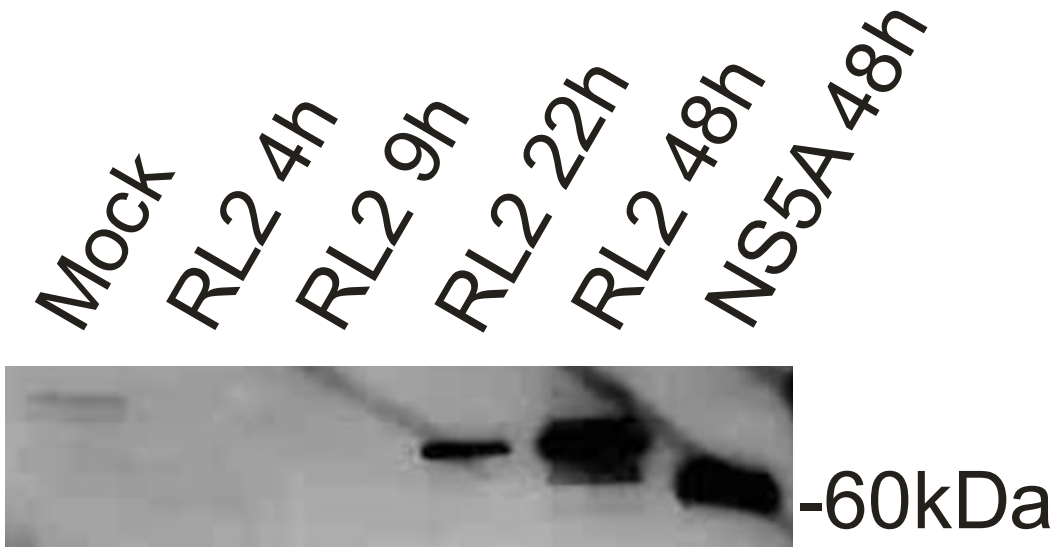
Time of Cell Harvest (Hours Post-Transfection)	Average Ct Values
Mock	40.00
0	38.90
4	35.75
8	33.55
18	23.80
24	18.00
48	21.90

3.5 Expression of the exogenous ICP0

Qualitative Western blots were performed on samples of transfected GP2-293 as well as VERO cells. Using the optimized protocol, VERO cells were transfected with the myc-tagged RL02 construct. Cells were harvested at various time points, and the crude extracts were separated in SDS-PAGE. The western blots were performed as a qualitative

investigation into how the transfected RL02 was expressed by a primary anti-myc antibody. Western blot analysis was performed to see if RL02 was successfully being expressed and to determine during what timeframe ICP0 was being expressed. Western blots were also useful to demonstrate if the ICP0 protein was accumulating over time, or if the protein was experiencing degradation. As shown in Fig. 10 the appearance of a single band at the appropriate size of 67kDa suggests that the RL02 protein is detectable in the VERO cells. RL02 expression is detected as early as 22 hours post-transfection. The blot also reveals an accumulation of protein between 22 and 48 hours post-transfection. The absence of any smaller bands below RL02 suggests that the transfected RL02 protein is not being degraded which is an important observation when designing the transfection-infections experiments that we will see later on.

Figure 10: Western Blot Analysis of ICP0 Expression in VERO cells. Cells were transfected with vector expressing myc-tagged RL02. Cells were harvested at various time points, and a crude lysate was obtained. Right-most lane is a myc-tagged control. Samples were run on a 12% SDS-PAGE gel (Biorad). Images were prepared using ECL reagent (GE Healthcare).

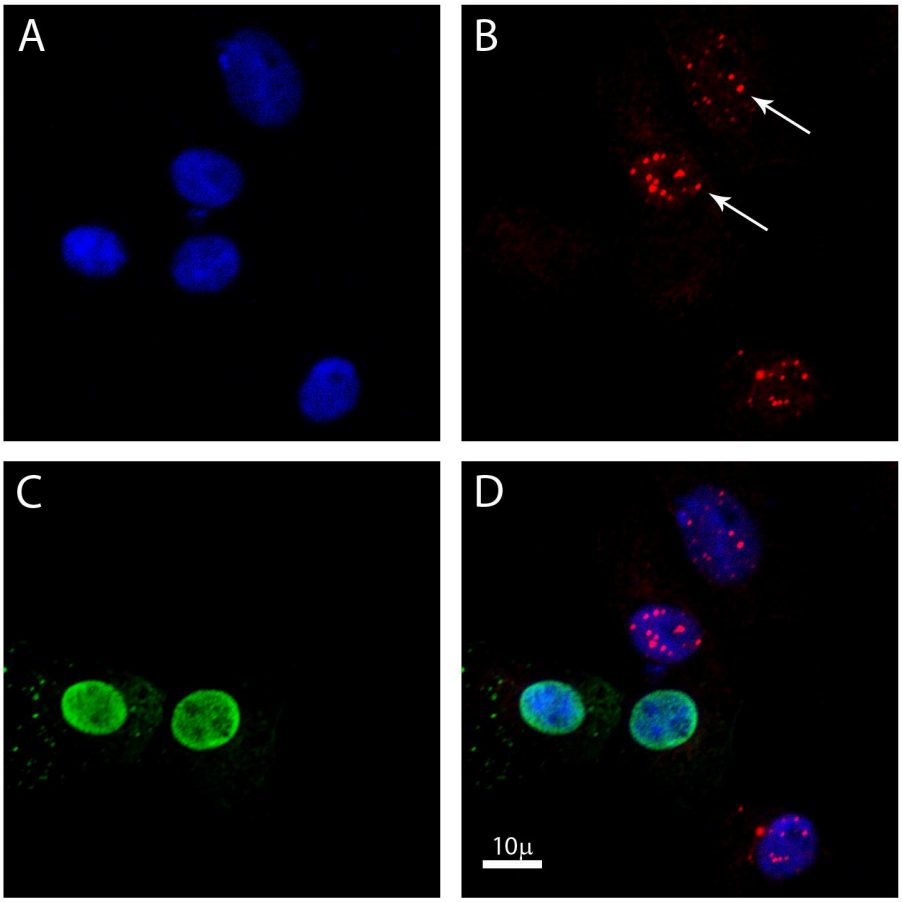


3. 6 Intracellular localization of the expressed ICP0 protein

Indirect Fluorescent Antibody (IFA) was performed to examine the intracellular localization of the transfected RL02. Using the optimized protocol, VERO cells were transfected with the myc-tagged SA8-RL02 construct. This experiment was repeated in triplicate, and several slides were produced during each run in order to compile a pool of slides to examine using a confocal microscope. This is important because, RL02 displays

different functions early during its nuclear sojourn and later, during its cytoplasmic duration. In this case, coverslips were placed in the bottom of the tissue-culture flasks before cells were seeded to facilitate the fixation process. Cells were fixed at 48 hours post-transfection before immunological work was carried out. Staining the nuclei with Hoechst revealed that the transfected SA8-RL02 was primarily localized in the nucleus. Compatible with the literature on ICP0 from HSV-1, once ICP0 is expressed following transfection, it fills the entire nuclear space. Cells fixed at later time points show that the ICP0, while still more pronounced in the nucleus, begins to translocate to cytoplasm, also consistent with how this protein behaves during a typical infection. The red dye represents the Promyelotic Leukemia Protein (PML). The primary antibody, a rabbit polyclonal antibody, targets all isoforms of PML. PML is an endogenous protein that should be present in all cells under normal conditions. From the literature, this protein typically aggregates to the discreet nuclear substructures, called ND10 [37, 66]. Expression of PML is typically visualized as punctate clusters within the nucleus. Interestingly, in cells that are transfected or infected and expressing ICP0, the PML is absent. This data suggest that RL02 might be resulting in the degradation of PML within the nucleus. ICP0-induced dispersal and degradation of PML during HSV infection is a well-reported function of this protein [23,25,32]. Each of the slides examined displayed the same pattern, whereby cells expressing ICP0 no longer show the presence of PML. These results were obtained from four independent experiments.

Figure 11: Intracellular Fluorescent Antibody Displaying Distribution of ICP0. A) Vero cell nuclei stained in blue with Hoechst B) Promyelotic Leukemia Protein (PML) structures displayed in red by goat anti-rabbit . C) Transfected ICP0 protein displayed in green by anti-mouse D) Represents the merged imaged showing regions where PML was displaced by ICP0 from the nuclei of Vero cells.



3.7 Effect of transfected ICP0 on the transcription of SA8 immediate early genes

In SA8 infected cells, cycloheximide, an inhibitor of protein synthesis, inhibits the expression of the immediate early genes, but not of ICP0. In contrast, none of the 5 IE genes of HSV-1 are inhibited by cycloheximide. In order to test the hypothesis that ICP0 is required for the expression of the other IE in SA8, VERO cells were transfected with either the untagged SA8-RL02 construct or the empty pcDNA3.1 vector. Transfection was carried out for 48 hours and then infection with SA8 was carried out for 4 hours in the presence or absence of cycloheximide, an inhibitor of protein synthesis. Cells were harvested and the level of RNA for the viral ICP0 as well as the immediate early genes US54, US12 and US01 was measured by RT-PCR. As a control the RNA level of the early gene US6 was also measured. Early genes transcription is completely inhibited by cycloheximide, since it requires the synthesis of immediate early transcription factors.

3.7.1 Effect of transfected ICP0 on the transcription of ICP0 from infection with SA8

Figure 12A shows the levels of ICP0 (RL2) transcripts in cells infected with SA8 for 4 hours. Cells were transfected 48 hours before with the ICP0 expression vector, an empty expression vector, or not transfected. ICP0 transcription was induced as expected after 4h of infections and the levels were not significantly different in cells transfected with ICP0, transfected with an empty vector, or not transfected. In the presence of cycloheximide, the level of ICP0 transcripts was enhanced on average by 1334%, regardless of the transfection treatment. This was also expected and it is due to the lack of

synthesis of ICP4, which acts as a repressor of immediate early genes, including RL2. The levels of ICP0 transcripts in the presence of cycloheximide were also not significantly affected by the transfection treatment.

As a control, the levels of US6 transcripts were also measured. US6 is a late gene that requires de novo synthesis of many viral proteins for expression. As expected, cycloheximide completely inhibited US6 expression, and transfection with ICP0 had no effect (Figure 12 B).

The effects of adding cycloheximide to the culture medium reflected our expectations in that it enhanced the accumulation of the RL02 transcript and it reduced the accumulation of the UL54, US12, US01 and US06 transcripts. In cells that were only subjected to an infection with SA8 virus, the degree of enhancement (RL02) and reduction (all remaining genes under investigation) paralleled results obtained in earlier experiments. On average, the levels of the RL02 transcript in the presence of cycloheximide were enhanced by 1334% when compared to the treatment with DMSO. It should be noted that the relative expression levels obtained for RL02 were on the order of 100-fold higher than the other immediate-early genes, an observation which may partially be attributed to the integrity and efficiency of the qPCR assay developed for this gene. The representative late gene, US06, which is used as a positive control in these experiments similarly reflect the results obtained in the earlier experiments. As a late gene, US06 should be entirely wiped out in the presence of cycloheximide and this is exactly what was observed. In cells only treated with infection in the presence of DMSO, transcript levels of US06 accumulated on the order of 10,000-fold. The US06 transcript, particularly susceptible to the effects of cycloheximide, reflected consistent reduction

values between 98-100% in the experimental condition. In the infection only treatment, the remaining immediate-early genes under investigation were also reduced in the presence of cycloheximide, with reduction values ranging between 77.2%, 63.4% and 79.2% on average, for UL54, US12 and US01 respectively. The values calculated for percent reduction were similarly consistent for UL54 and US01 transcripts, however US12 reflected a slightly-higher standard deviation. Results for the effects of cycloheximide on the expression of the various IE transcripts are shown in figures 12A-E.

3.7.2 Effects of transfected ICP0 on the putative IE genes UL54, US01 and US12

To test the hypothesis that ICP0 is required for the expression of the other SA8 immediate early genes, the level of RNA from the US54, US01 and US12 genes was measured in cells transfected with ICP0 and infected as described for the experiment in Fig. 12A. If expression of these genes requires ICP0, providing ICP0 in trans by transfection would results in transcription even in the presence of cycloheximide.

The results, presented in Fig 12 B, C, and D for UL54, US12 and US01, respectively, show that in untransfected cells the transcripts of these genes are induced after 4h of infection, but induction is significantly inhibited in the presence of cycloheximide. These data confirm previous results obtained in our laboratory (described in the introduction) and strongly suggest that full expression of SA8 immediate early genes other than RL2, require de novo protein synthesis. This is unlike the situation in

HSV-1, where all 5 immediate early genes transcription is not inhibited, but actually enhanced, by cycloheximide.

Fig 12 C to E also show that transfection with the ICP0 expression vector does not significantly increase the transcription of the 3 IE genes in the presence of cycloheximide. In cells transfected with the ICP0 vector or the empty vector the RNA levels in the presence or absence of cycloheximide is no longer significantly different, unlike the situation in non transfected cells. This may be attributed to a reduction of transcription in the absence of cycloheximide, rather that stimulation of transcription in the presence of cycloheximide, although the differences are not statistically significant compared to non transfected cells. This observation may be caused by effect of the transfection procedure itself. Whatever the reason, it is clear that transfection with ICP0 does not have a measurable effect on the transcription of IE genes of SA8.

The effect of transfecting cells with ICP0 prior to the addition of infection does not appear to affect the levels of gene transcription of the other immediate-early genes. The level of RL2 accumulation displays a similar pattern of gene expression in the cells that were transfected with ICP0 or the empty vector. There was an average increase in viral RL2 transcript accumulation in transfected-infected cells but this increase is attributed to effects of cycloheximide. Results revealed a 631% increase in gene expression when transfected with ICP0 and a 671% increase when transfected with the empty vector. In both cases, the degree of transcript accumulation was lower than in the cells that were infected only. One-way ANOVA test indicated that there was a significant difference between samples in data set, with $p < 0.001$. A Tukey's post-hoc test was performed to determine which pairs of data displayed statistically different values. The

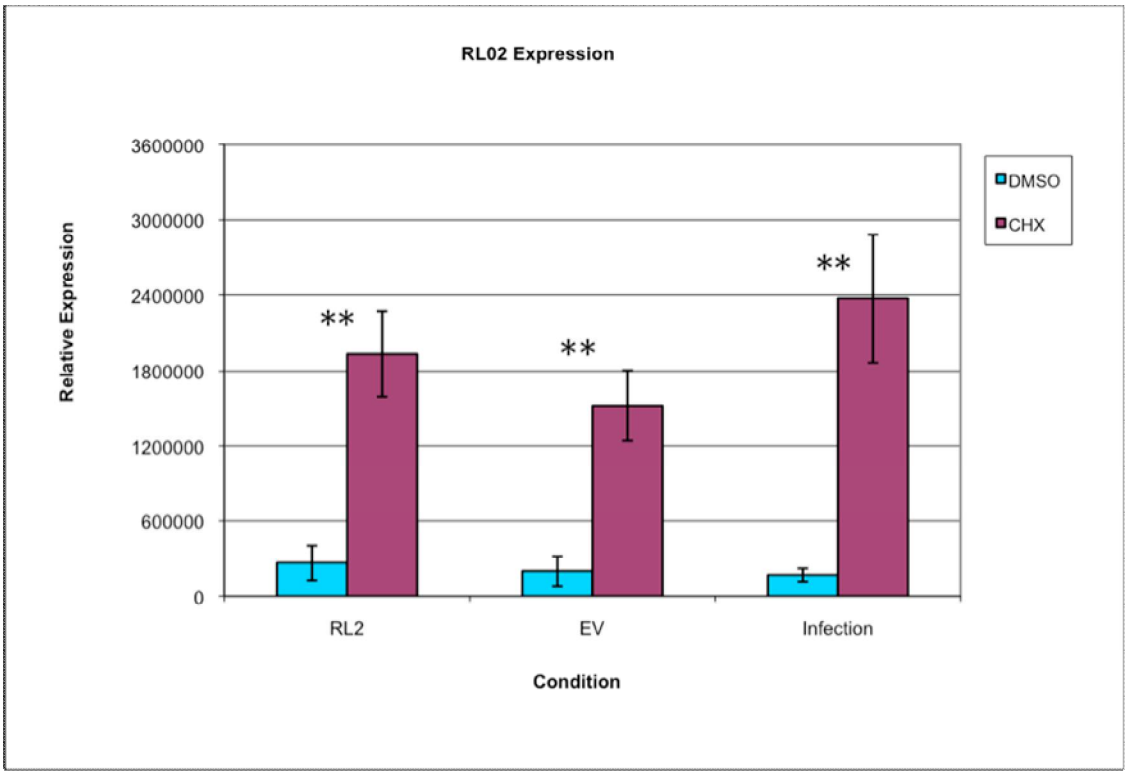
test revealed that all of the treatments with DMSO were significantly different than all treatments with CHX. The degree of variability between samples and between treatment conditions was lower than the variability calculated for all of the other genes under investigation. The level of US06 accumulation in cells that were transfected with ICP0 or the empty vector similarly did not differ. Both transfection scenarios with cycloheximide experienced a complete reduction of transcript accumulation, as was observed in cells that were only subjected to infection. Degree of reduction in cells was very similar in the cells transfected with ICP0 or the empty vector, with 98% and 99% respectively. The expression of RL2 and US06 behaved similarly across all treatment options.

The remaining immediate-early genes behaved somewhat differently. UL54 levels were reduced in the presence of cycloheximide in both transfection conditions, but this reduction was to a lesser extent than was observed in cells that were infected only. Cells receiving ICP0 prior to transfection showed a reduction in the levels of UL54 expression by 30% in the presence of cycloheximide whereas cells that received the empty vector were reduced by 20%. A similar observation was made for the US12 transcript and the US01 transcript. The US12 transcript was reduced by 20% in the presence of cycloheximide, and it displayed a slight overall increase in transcript accumulation in cells transfected with the empty vector (10% increase). US12 displayed the largest standard deviation out of all the genes under investigation. The 1-way ANOVA test demonstrated that there was no statistical difference between any of the treatment conditions for the US12 transcript. The US01 transcript levels obtained in cells that were transfected with ICP0 experienced a 32% reduction in cells and it experienced a 56%

reduction in cells transfected with the empty vector. In all three of these immediate-early genes; UL54, US12 and US01, the variability between sample conditions and between experiments was high. In general, transfecting cells with ICP0 prior to infection does not appear to have any specific effect on the levels of IE transcript accumulation.

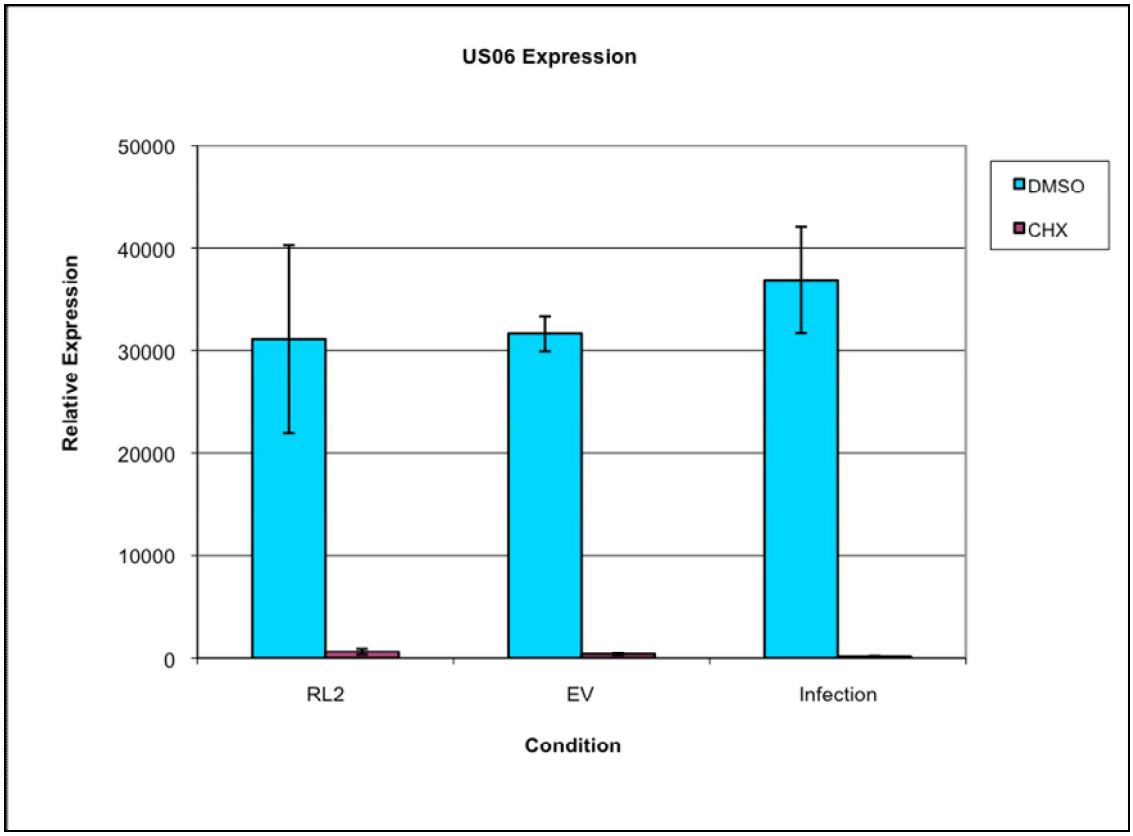
Conversely, the transfection procedure itself appears to exert some off-target effects. There does not appear to be any differences when cells are transfected with ICP0 or the empty vector, however, the act of transfecting appears to be affecting the transcriptional state of the cells in some way. It is possible that the transfection is harming the cells such that they are unable to sustain a complete infection for extended periods. During transfection it should be noted that no overt cytopathic effect was observed. The effects of transfection prior to infection are summarized in the figures 12A-E.

Figure 12A: Effect of Transfected ICP0 on Levels of Accumulated RL02 Transcript



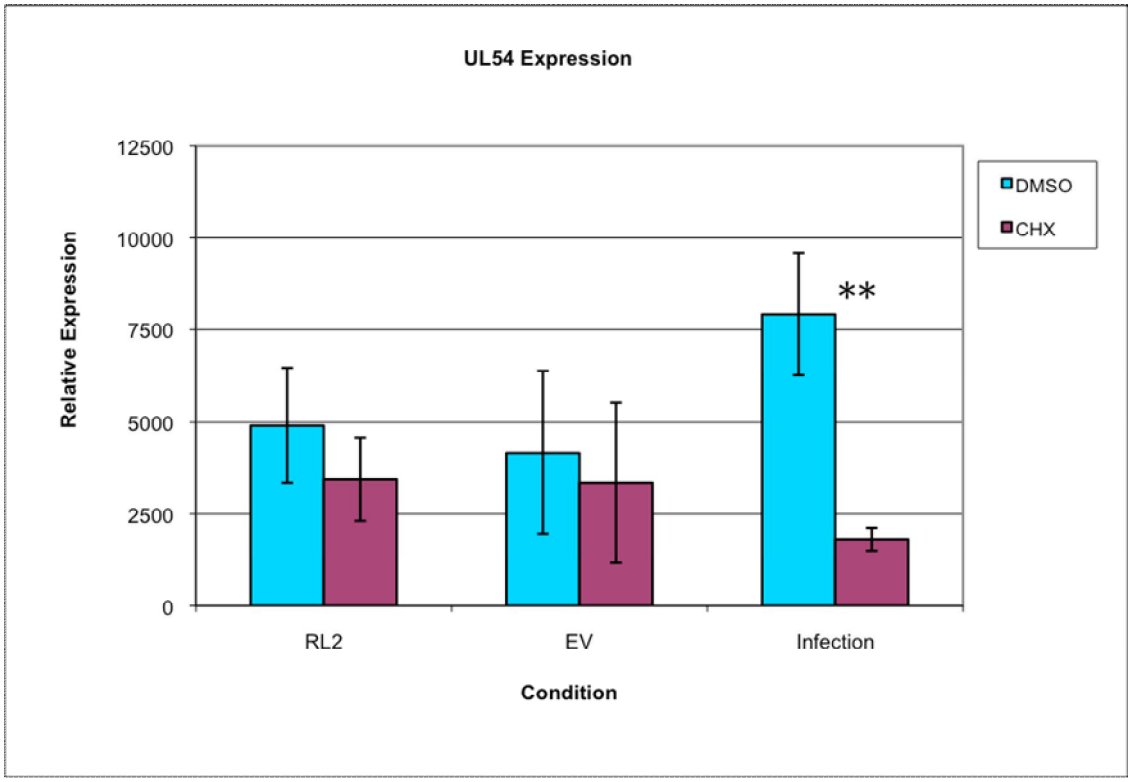
Graphical interpretation of RL02 transcript levels following transfection. Blue bars indicate that DMSO was added during the experiment. Red bars indicate that cycloheximide was added during the experiment. The first pair of bars represents VERO cells that were transfected with ICP0 for 48h then subsequently infected with the SA8 virus at an MOI of 5. The second pair of bars represents cells that were transfected with the empty vector for 48h then infected. The final pair of bars represents cells that were not transfected; subjected only to infection under the same conditions. Y-error bars were included to demonstrate the calculated standard deviation. A 1-way ANOVA test was performed on all six values ($p < 0.0001$) and Tukey's multiple comparison Post-Hoc test was performed to determine statistical significance between pairs. ** Equals a p value <0.001.

Figure 12B: Effect of Transfected ICP0 on Levels of Accumulated US06 Transcript



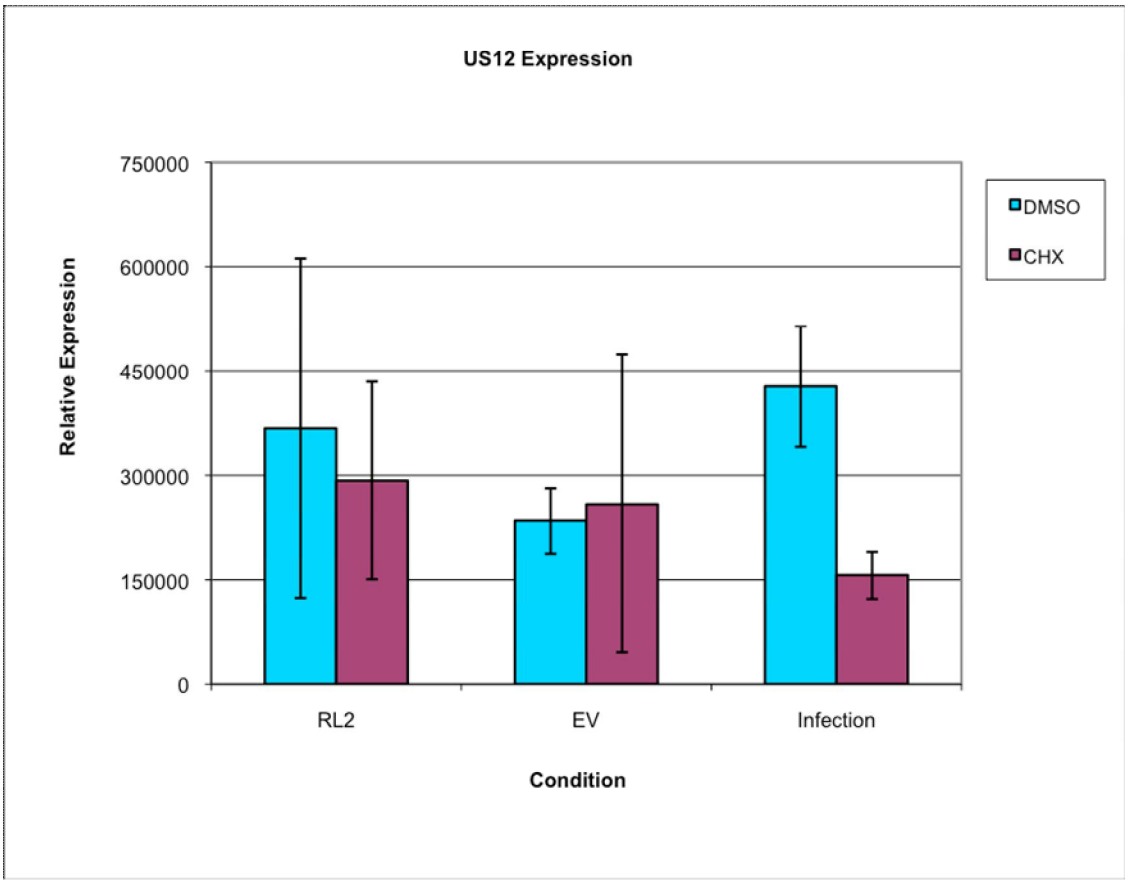
Graphical interpretation of a typical late-gene, US06, transcript levels following transfection. Blue bars indicate that DMSO was added during the experiment. Red bars indicate that cycloheximide was added during the experiment. The first pair of bars represents VERO cells that were transfected with ICP0 for 48h then subsequently infected with the SA8 virus at an MOI of 5. The second pair of bars represents cells that were transfected with the empty vector for 48h then infected. The final pair of bars represents cells that were only subjected to infection under the same conditions. Y-error bars were included to demonstrate the calculated standard deviation.

Figure 12C: Effect of Transfected ICP0 on Levels of Accumulated UL54 Transcript



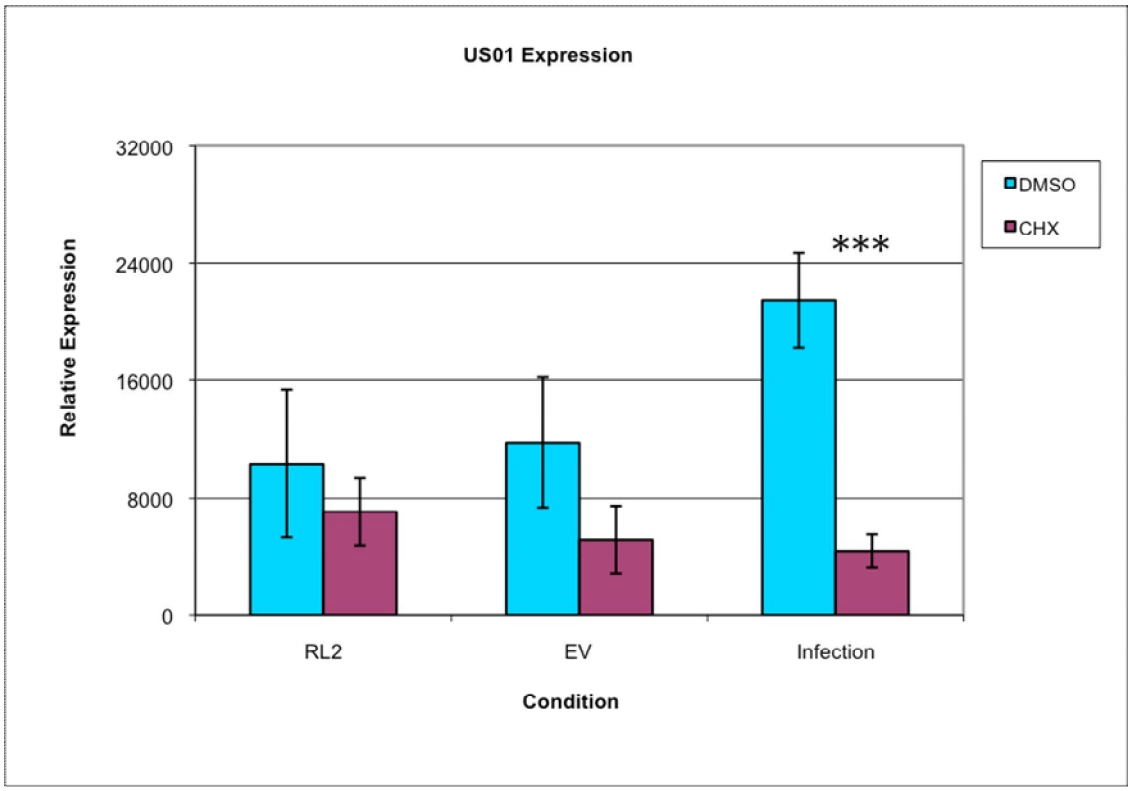
Graphical interpretation of a typical late-gene, UL54, transcript levels following transfection. Blue bars indicate that DMSO was added during the experiment. Red bars indicate that cycloheximide was added during the experiment. The first pair of bars represents VERO cells that were transfected with ICP0 for 48h then subsequently infected with the SA8 virus at an MOI of 5. The second pair of bars represents cells that were transfected with the empty vector for 48h then infected. The final pair of bars represents cells that were only subjected to infection under the same conditions. Y-error bars were included to demonstrate the calculated standard deviation. A 1-way ANOVA test was performed on all six values (p of 0.0128) and Tukey's multiple comparison Post-Hoc test was performed to determine statistical significance between pairs. ** equals a p value <0.001.

Figure 12D: Effect of Transfected ICP0 on Levels of Accumulated US12 Transcript



Graphical interpretation of a typical late-gene, US12, transcript levels following transfection. Blue bars indicate that DMSO was added during the experiment. Red bars indicate that cycloheximide was added during the experiment. The first pair of bars represents VERO cells that were transfected with ICP0 for 48h then subsequently infected with the SA8 virus at an MOI of 5. The second pair of bars represents cells that were transfected with the empty vector for 48h then infected. The final pair of bars represents cells that were only subjected to infection under the same conditions. Y-error bars were included to demonstrate the calculated standard deviation. A 1-way ANOVA test was performed on all six values ($p = 0.3511$) and was values were shown not to be statistically different.

Figure 12E: Effects of Transfected ICP0 on Levels of Accumulated US01 Transcript



Graphical interpretation of a typical late-gene, US01, transcript levels following transfection. Blue bars indicate that DMSO was added during the experiment. Red bars indicate that cycloheximide was added during the experiment. The first pair of bars represents VERO cells that were transfected with ICP0 for 48h then subsequently infected with the SA8 virus at an MOI of 5. The second pair of bars represents cells that were transfected with the empty vector for 48h then infected. The final pair of bars represents cells that were only subjected to infection under the same conditions. Y-error bars were included to demonstrate the calculated standard deviation. A 1-way ANOVA test was performed on all six values (p of 0.004) and Tukey's multiple comparison Post-Hoc test was performed to determine statistical significance between pairs. ** equals a p value <0.0001.

3.8 Transfection with siRNA Targeting ICP0

This series of experiments was performed to explore if the reduction of ICP0 would cause an effect on the other immediate-early homologues. A group of four siRNA oligonucleotides were generated towards the cDNA gene sequence in an attempt to reduce either the levels of mRNA or the level of ICP0 protein. The siRNAs were commercially designed. The results for three independent siRNA experiments are summarized in figures 13A-C.

The oligonucleotides were mixed with hyperfect (Qiagen) siRNA transfection reagent, and after a brief 8 minute incubation period, freshly seeded Vero cells were transfected. The transfection was carried out for 24 hrs before infecting the cells with SA8 virus. Cells were harvested at 5 hrs post infection. None of the 4 siRNA oligos appeared to have an effect on the levels of RL2 mRNA (Figure 13A). A one-way ANOVA generated a p value of 0.05. Typically in siRNA experiments the levels of mRNA as well as the levels of the protein itself would be checked to see if either experienced a reduction following treatment. As no antibody is currently available for SA8-ICP0, quantitative PCR measuring mRNA was the only method we had to evaluate how the siRNA performed. It is possible that the levels of ICP0 protein were reduced even though mRNA was not, so two of the other immediate-early genes were tested to see if the effect was felt downstream. Figure 13B displays qPCR data for the US12 transcript and Figure 13C displays data for the US01 transcript. Neither of these genes

seemed to experience a positive, nor a negative effect as values under all conditions did not reveal any statistically significant differences.

Figure 13A: Levels of RL2 Transcript following siRNA Treatment

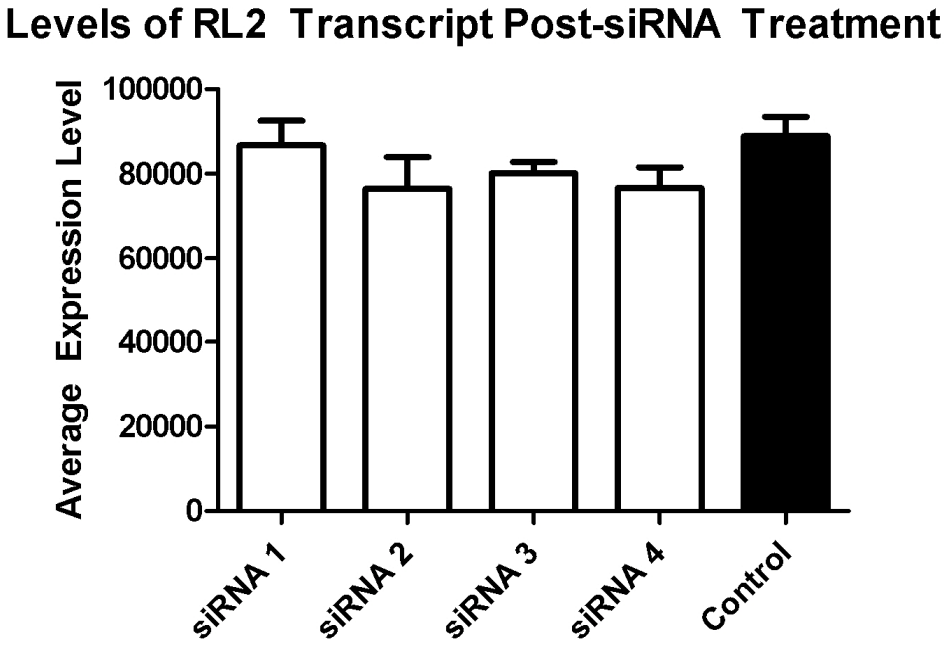


Figure 13B: Levels of US12 Transcript Following siRNA Treatment

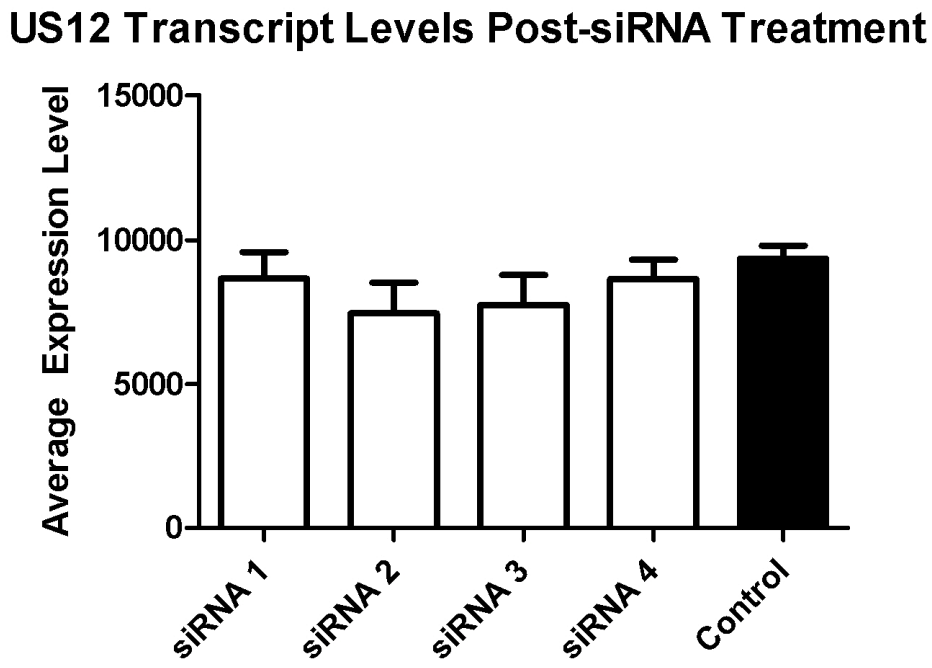
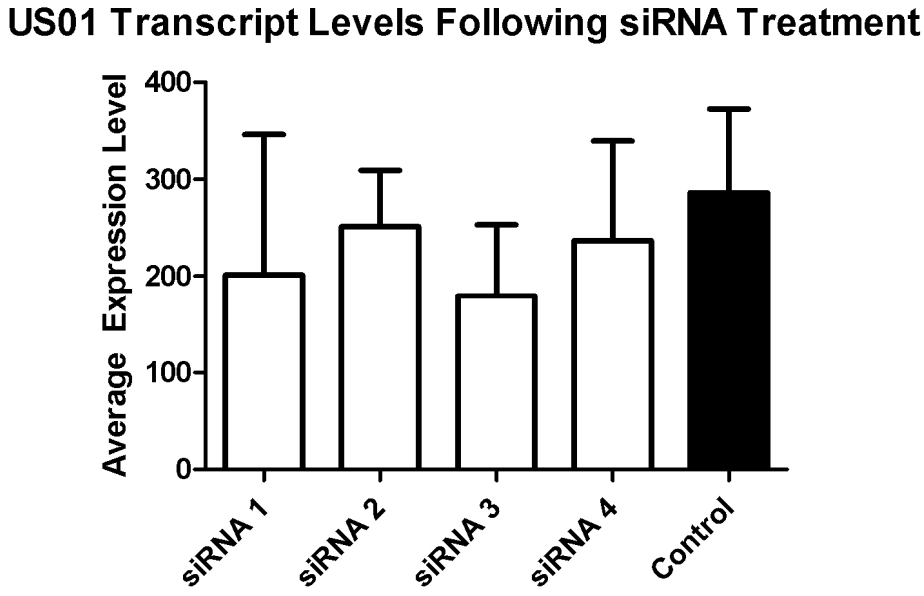


Figure 13C: Levels of US01 Transcript Following siRNA Treatment



3.9 Characterization and Comparison of IE Promoters in HSV-1 and SA8

The promoter region of immediate-early gene transcripts in HSV-1 are known to comprise a consensus activator sequence, a feature which is absent in the promoter regions of early and late genes. As reported in the literature, the immediate-early promoter sequence motif is described as (5'-NCGyATGnTAATGArATTCyTTGnGGG-3') and also called TAATGARAT consensus sequence. Due to the observed differences in gene expression pattern of immediate-early gene transcripts in HSV-1 and SA8, it was important to investigate if the promoter regions could be a contributing factor. The regions upstream (up to +700bp) from the transcriptional start site were screened for the presence of the complete or partial consensus sequence. As this sequence provides some nucleotide flexibility, regions that contained a minimum of ten compatible bases were considered to be a match. In HSV-1, the promoters of all the immediate-early genes comprised the consensus sequence and in some cases, contained multiple copies of the sequence. All of the consensus sequences were located within a 500bp range upstream from the ATG codon.

In SA8, the promoter regions of the IE genes were quite different. The promoters for both copies of the RL02 genes revealed several copies of the consensus sequence, as did the promoters for both copies of RS01. The promoters for UL54, US12 and US01 lacked the ten base compatibility criteria. The promoter of UL54 loosely displayed the consensus, with an eight base consensus motif but this was only observed 1kb upstream from the ATG, so it is not likely that this region is actually involved in the activation of UL54 transcription and it is more likely to be an unrelated sequence in the genome. The

promoters of US12 and US01 lacked any appreciable identity with the consensus sequence. In general, the promoters in HSV-1 showed a great sequence identity to the consensus and also, more copies of the consensus sequence. In SA8, only RL02 and RS01 demonstrated the presence of the consensus while the promoters from the other immediate-early genes showed no evidence of this motif. The characterization of the immediate-early promoters from HSV-1 and SA8 are summarized in Table 5.

Virus	Gene	Presence of Consensus	Sequence Similarity
HSV-1	RL02	Yes 3 copies	G**G**T**ATG**G**TAATGA**GT**TTC**TTCGGG G**G**C**ATG**C**TAATGG**GG**TTC**TTTGGG T**G**C**ATG**C**TAATGA**TA**TTC**TTTGGG
	UL54	Yes 2 copies	CAT**ATG**C**AAATGA**AA**ATC**GGTCCC GAT**ATG**C**TAATTA**AA**TAC**ATGCCA
	US12	Yes 2 copies	**C**G**C**GGGGCTCGT**ATCTCATTA**C**C**G **CC**T**G**CCCGTTCCTC**GT**T**A**GC**A**TG**C**G
	US01	Yes 2 copies	T**C**GGC**G**G**TAATGA**GA**TAC**GAGCCC
	RS01	Yes 1 copy	C**G**GGC**G**G**TAATGAGAT**G**C**CAT**G**C**G**
SA8	RL02	Yes 3 copies	CCC**ATG**C**TAAT**T**AAA**AG**C**GTG**GGG** CCT**ATG**C**T**G**AT**T**AAA**AAATCGAT**G** AT**ATG**C**T**G**ATGG**GA**CGCCGCTC**GG**
	UL54	No	
	US12	No	
	US01	No	
	RS01	Yes 2 copies	C**G**C**ATG**C**TAATG**C**GAT**CCTCGAC**G** C**G**C**ATG**C**TAATG**C**GAT**CCTCGAC**G**

Table 5: Characterization of the Immediate-Early Promoters from HSV-1 and SA8. HSV-1 and SA8 genomes were screened for the consensus motif (5'NCGyATGnTAATGArATTCyTTGnGGG-3') in regions upstream from promoter regions of each of the immediate-early genes using VectorNTI software.

4.0 Discussion

In this work I have expressed a complete ICP0 protein from the RL2 gene SA8 virus in mammalian cells and I have tested its effects on the activation of transcription of the SA8 genes which are homologous to the immediate early genes of HSV1. Previous experiments in our laboratory had shown that in SA8 the transcription of RS01, US01, UL54 and US12, but not of RL1 (ICP0), is inhibited rather that enhanced by cycloheximide, an inhibitor of protein synthesis. This suggested that full expression of these SA8 genes requires de novo synthesis of an additional factor(s) for and we hypothesised that ICP0 could be that factor. Using the approach of providing SA8-ICP0 in trans by transient transfection, we investigated if the protein was sufficient to enhance the expression of these genes in SA8 infecting the cells in the presence of cycloheximide. Since antibodies against SA8 of ICP0 are not available, in order to demonstrate production of ICP0 in transiently transfected Vero cell, an N terminus myc tag was fused to the protein so that ICP0 could be detected by anti myc monoclonal antibodies. Western blot experiments demonstrated production of the full size proteins.

During infection, ICP0-myc displays a nuclear localization at early times and then it migrates into the cytoplasm at later times during infection [1,48,81]. To the extent of the degree of expression and protein accumulation, and most significantly, with respect to intracellular localization, the transfected ICP0 displayed the features we expected it would. Additionally, the indirect fluorescent antibody assay demonstrated that the transfected ICP0 was residing in the nucleus at early times following transfection, but that 24hrs post-transfection, the protein began migrating out to the cytoplasm.

The role of ICP0 in HSV-1 has been extensively researched and it is clear that ICP0 is involved in several aspects of productive infection. ICP0 was coined a "promiscuous" transactivator, yet, it carries out this function indirectly as no physical association to DNA has been demonstrated [13,22,30,93]. Using the E3 ubiquitin ligase function encoded by exon 2, ICP0 disperses and degrades ND10 substructures [25,30,32,33,81]. Proteins like PML, hDaxx, sp100, which help control and regulate important cellular events, become targeted for proteosomal degradation. The IFA images obtained from VERO cells transfected with ICP0 are consistent with this reported function of ICP0. At both 24h and 48h post-transfection, cells expressing ICP0 no longer show the punctate presence of PML that is observed in all neighbouring cells lacking ICP0, as it has been previously shown in HSV-1 [25]. These results strongly suggest that ICP0 is active in disrupting ND10, an effect that has been linked to the gene activation activity of ICP0 [23].

We have therefore developed a reliable system in which the effects of ICP0 may be tested in VERO cells. The system we developed also provides a useful method which may be applied more generally to investigate the effects other viral proteins of interest in VERO cells. This research supports the notion that ICP0 from SA8 behaves like ICP0 from HSV-1. Not only do the two ICP0 proteins resemble one another at level of nucleotide sequence, but the translated proteins appear to have parallels as well. The IFA experiments demonstrated ICP0 from SA8 to localize in a similar way to what has been demonstrated in HSV-1. Furthermore, the dispersal of ND10 structures in the presence of ICP0 is another commonality between ICP0 of both viruses.

Results for the transfection-infection experiments investigating SA8 gene expression in the presence and absence of cycloheximide reproduced the data previously obtained in our laboratory. RL02 was the only gene to be enhanced in the presence of cycloheximide while US01, US12, UL54 were inhibited in the presence of cycloheximide, albeit to varying degrees. The late gene US06 served as a control, was transcribed during SA8 infection and completely inhibited in the presence of cycloheximide, regardless of the presence or absence of the transfected ICP0. Presence of transfected ICP0 failed to significantly enhance the levels of transcription of US01, US12 and UL54 in the presence of cycloheximide. As per our hypothesis that RL02 is the major regulator of transcription activation in simian simplexviruses, we expected RL02 to be sufficient to enhance the levels of gene transcription of the "early" genes which are homologous to immediate-early genes in HSV-1.

Based on this work, it appears that SA8 ICP0 is not sufficient to activate and enhance gene expression in the presence of cycloheximide. Several potential explanations come to mind after analyzing the results. The first explanation is that ICP0 is unable to successfully direct the transcriptional activation of the immediate-early homologues genes without the cooperation of another factor(s). In HSV-1, immediate-early promoters are characterized by the TAATGARAT consensus sequence [16,27,39,65,79]. Each of the immediate-early promoters has one to several copies of this sequence present which mediates the specific interaction of viral protein VP16, and cellular proteins Oct-1 and HCF with the IE promoter to make transcription possible. Should this be the case, it would present another difference in transcriptional regulation between the human and simian simplexviruses. This explanation does not exclude ICP0 from taking part in this

process, but simply suggests that it requires the help of other proteins to do the job. We would predict these alternative factors to be cellular in nature, and these proteins are not likely to be ones that recognize the TAATGARAT sequence as the promoters for the UL54,US12 and US01 genes did not have any copies of this consensus sequence present.

The transfection procedure itself appears to have effects on the VERO cells. As observed in the case of UL54, US12 and US01, transfection itself lowers the transcription in the absence of cycloheximide and removes the inhibitory effect of CHX. Additionally, the transfection might disturb the harmony within the cells, rendering them weak and unable to sustain the onslaught of a complete infection, although transfection does not seem to have any effect on the transcription of the late gene UL06. The TAATGARAT consensus sequence itself might play a role in the differential effect transfection seemed to have on the immediate-early genes compared with US06. Since US06 is the ONLY gene under investigation that has a promoter which lacks this consensus sequence, perhaps it is this consensus in particular which activates cellular defense mechanisms or, alternatively, makes the genes more sensitive to the cycloheximide-induced inhibition.

The transfection process could have altered the intracellular environment. For example, heat shock proteins (HSPs), which typically become expressed when cells experience a stress (in this case, transfection), might be a possible candidate for the other proteins which may be necessary or involved in the transactivation process. The heat shock response is a highly conserved mechanism, from yeast to humans that becomes induced following exposure of cells to extreme conditions that cause acute or chronic

stress. Heat-shock factors (HSFs) function as inducible transcriptional regulators that bind to cis-acting elements to activate heat-shock proteins (HSPs) which function as molecular chaperones to preserve cellular homeostasis and promote survival of the cell. This is achieved by preventing the accumulation of non-native proteins through a rapid triggering a rapid, transient, and global reprogramming of gene expression[2,4]. The mediation of the heat shock response through HSFs was initially characterized in response to thermal stress but has since been observed to respond to oxidative stress, heavy metals, toxins and infections, indicating a much broader role in the stress response pathway through different types of mechanisms including chromatin remodeling and trapping of transcription and splicing factors[2].

Briefly, heat shock factor 1 (HSF-1) localizes and accumulates rapidly forming HSF-1 granules that parallels its activation and the transient induction of heat shock gene transcription. These granules concentrate near transient subnuclear organelles that are named nuclear stress bodies (NSBs) because they are only rarely detectable in unstressed cells[4]. During recovery from heat shock, HSF1 granules are no longer detected, but HSF1 rapidly relocalizes to the same structures upon subsequent reexposure to stress. Recognizing HSFs as an efficient way to control gene expression in a cell and stimulus specific manner to orchestrate the differential upstream signalling and target-gene networks creates a condition that might be involved in regulation of a viral infection such as HSV.

Heat shock response correlates with a global shut-down of transcription and with an alteration of splicing functions it is not entirely clear whether it affects the majority of pre-mRNAs, whether all transcripts are affected to a similar degree, or whether heat

shock targets only specific subsets of pre-mRNAs[2]. It seems possible that the heat shock system was activated in some capacity during this experimentation. The transfection procedure itself is a prime candidate for disrupting the harmony within the Vero cells. Additionally, based on what we know about the function of the SA8-ICP0 protein during infection, it seems plausible that it could have activated the HSFs. If this was indeed the case, the unexpected results from the transfection-infection gene expression assays might be contributed (at least in part) to the heat-shock type of response.

It also possible that if the population of cells that were transfected and expressing RL02 and subsequently infected was too small, the effects of RL02 would not be detectable in our system. This does not seem likely because transfection efficiency was over 50% at the time of infection, and the infection was carried out at a multiplicity of 5 which, due to the high ratio of virus particles to number of cells, typically results in all cells becoming infected.

In addition to providing ICP0 in trans in the presence of cycloheximide, we attempted to inhibit the function of the viral ICP0 during the infection using the short interfering RNA technique (siRNA). The siRNA were specifically targeted towards the viral ICP0 transcript. Our system was not ideal for experiments of this type, given the lack of antibody available towards ICP0 as well as the lack of positive control for our cell line. Due to these circumstances, I tried experiments using four commercially generated siRNA sequences targeted toward ICP0 were used. Results were analyzed using qPCR and but unfortunately none of the sequences demonstrated any reduction in levels of ICP0 mRNA. Lack of specific antibodies against SA8 ICP0 prevented an assessment of

the effect of the siRNAs on the actual levels of viral ICP0. Regardless, siRNAs did not have an effects of the transcription of US01.

When attempting to investigate the specific contribution of ICP0 to the kinetics of gene expression in SA8, we realized there were two general approaches we could take. What we have come to understand regarding the immediate-early genes in HSV-1 highlights the IE promoters and the special sequence motifs and consensus sequences as important in the cascade of gene expression. As such, we contemplated approaching the research from this angle, by exploring, what (if any) distinct roles these promoters have and whether these promoters could be sufficient on their own to produce the observed pattern of gene expression. By using a prototype IE promoter from HSV to direct gene expression of other (non-IE) genes, we might be able to understand more about the important role these promoters play in the gene expression process. The other approach, and the approach we ultimately went with, was to provide the protein of interest *in trans* to determine if it was sufficient to activate gene expression of other SA8 genes in the presence and absence of cycloheximide. The latter approach, using a whole virus (as opposed to simply an IE promoter) more closely resembles the setting of a natural infection so it was the preferred method. Once we more properly establish the role ICP0 from SA8 plays in gene expression, it would be a natural progression to narrow the focus in on the promoter region.

4. 1 Future Directions

I have created useful clones and have established a methodology to investigate the gene expression patterns in simplexviruses. Despite the fact that RL2 does not appear to

be sufficient to stimulate the expression of the immediate-early homologues, it does not rule out RL2 as being necessary in this process. During these experiments the lack of commercial antibody was overcome by including a myc-tag to the ICP0 protein. While this was useful and acceptable during the localization assays, the siRNA experiments would be vastly improved with a means to check the affect of the siRNA on the levels of protein. Additionally, the integrity of the siRNA experiments would also be improved with positive and negative siRNA targets controls. While the experimental procedure itself is fairly straightforward, it would be much more worthwhile if siRNAs were available to specifically target a simian cellular gene. The next step would be to investigate the effects of targeting either cellular or viral factors with siRNA. It might be easier to target cellular factors for example, targeting a component of the ND10 nuclear structures. PML could be a candidate, however, many isoforms exist and each isoform is thought to be present during specific events so it might be difficult to select which isoform would be most appropriate. Sp100 is another significant protein that is affected by ICP0-dependent ND10 dispersal but it has a few isoforms as well. UHAUSP7? Any other protein that is known to associate with ICP0?

It would be interesting to investigate what proteins or factors ICP0 associates with a various stages of infection. Using a tandem-affinity purification (TAP) approach, we would be able to determine both viral and cellular factors that contribute to ICP0's observed effect in cells. The approach involves cloning ICP0 into an appropriate vector so that once expressed, ICP0 would possess a His-tag. Formaldehyde cross-linking fixes the expressed protein (and any proteins that become directly associated to it) to facilitate purification. Samples are analyzed on the mass spectrometer to determine which proteins

are present. The creation of SA8 mutant viruses is an interesting possibility that our lab has discussed but has yet to explore. Creating a strain with a truncated version of ICP0 seems appealing, but without overemphasizing the observations in our lab, we would expect this strain to struggle more than an HSV ICP0 mutant. It would be interesting to explore if this type of an SA8 mutant could replicate, would its phenotype be similarly cell-type dependent and multiplicity of infection dependent like the HSV ICP0 mutant? If it was not able to progress through a complete infection, would providing ICP0 in trans recover a wild-type phenotype? Our lab has also discussed conducting experiments using the promoters from the immediately-early genes in HSV-1 and SA8. One direction could be to put a reporter gene under the control of the promoters from both viruses to see if expression could be detected. A different angle could be to swap the IE promoters from HSV-1 to control the IE homologues in SA8. This approach could potentially reconcile the differences in gene expression if the experiment revealed the change in promoters to be sufficient to get the IE homologues in SA8 to be expressed.

4. 2 Concluding Remarks

We have demonstrated an effective and reliable method to investigate the role of ICP0 in Vero cells during an SA8 infection. We believe this method may also be applied more generally to investigate the role other viral proteins have during SA8 infection in this type of cell. We acknowledge the limitation of the lack of antibody directed specifically against ICP0 and we attempted to address this by cloning ICP0 into a vector for bacterial expression. Earlier work in our lab experienced similar difficulties in the expression of some herpes viruses in bacterial cells. Developing antibodies directed

against ICP0 would definitely enhance the integrity of many of the assays performed during this research and it should be investigated.

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