Gene regulation and function of ICP0 in herpes simplex virus infected cells

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GENE REGULATION AND FUNCTION OF ICP0
IN HERPES SIMPLEX VIRUS INFECTED CELLS

by

Mingyu Liu
B.S., China Agricultural University, 2006

A Dissertation
Submitted in Partial Fulfillment of the Requirements for the
Doctor of Philosophy.

Molecular Biology, Microbiology and Biochemistry Graduate Program
Department of Medical Microbiology, Immunology and Cell Biology
Southern Illinois University School of Medicine at Springfield
May 2010
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GENE REGULATION AND FUNCTION OF ICP0
IN HERPES SIMPLEX VIRUS INFECTED CELLS

By
Mingyu Liu

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in the field of Molecular Biology, Microbiology and Biochemistry

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March 25th, 2010
AN ABSTRACT OF THE DISSERTATION OF

MINGYU LIU, for the Doctor of Philosophy degree in MOLECULAR BIOLOGY, MICROBIOLOGY and BIOCHEMISTRY, presented on March 25th 2010, at Southern Illinois University School of Medicine at Springfield.

TITLE: GENE REGULATION AND FUNCTION OF ICP0 IN HERPES SIMPLEX VIRUS INFECTED CELLS

MAJOR PROFESSOR: Dr. William P. Halford

Herpes simplex virus (HSV) is a clinically important virus, whose life cycle alternates between productive replication and latency. Infected cell protein 0 (ICP0) is generally believed to play a key role in determining the outcome of HSV infections. Synthesis of ICP0 promotes the productive replication of HSV, whereas absence of ICP0 renders HSV prone to establish latent infections.

In the first part of the dissertation, I attempt to address the question how is ICP0 gene regulated. To tackle this question, we constructed recombinant HSV that encodes GFP-tagged ICP0 so that the regulation of ICP0 gene can be visualized in real time. Using this reagent, we found that ICP0 gene was subject to potent repression immediately following infection. Surprisingly, HSV’s major transcriptional regulator, ICP4, was necessary and sufficient to repress ICP0 gene, and did so in an ICP4-binding-site dependent manner. Synthesis of ICP0
alleviated the ICP4-dependent repression of ICP0 gene. ICP4 co-immunoprecipitated with FLAG-tagged ICP0, thus, a physical interaction between ICP0 and ICP4 likely explains how ICP0 antagonizes ICP4’s capacity to silence the ICP0 gene. Therefore, our findings suggest that ICP0 gene is differentially regulated by virus-encoded repressor ICP4 and virus-encoded antirepressor ICP0.

In the second part of the dissertation, I attempt to address the question what function does ICP0 assume. Since the discovery of ICP0, the nuclear function of ICP0 has been the focal point of studies, whereas the cytoplasmic function of ICP0 is unknown. While pursuing our first study, we unexpectedly found that GFP-tagged ICP0 was predominantly localized to the cytoplasm during infections. Taking advantage of live-cell imaging, we found that ICP0 translocated from nucleus to cytoplasm during early phase of HSV infections, where it bundled and dispersed microtubules. Synthesis of ICP0 was proved to be necessary and sufficient to dismantle microtubules in HSV-infected and transfected cells. Therefore, our findings suggest ICP0 might play a previously unrecognized role in the cytoplasm through dismantling microtubule networks of the host cells. Furthermore, our study represents the first report showing a virus-encoded E3 ligase disrupts host cell microtubule networks, thus suggests a general function of many other viral E3 ligases.
DEDICATION

I would like to dedicate this work to my parents, Zhu Yang and Yinjiang Liu, whose constant love and unwavering support have accompanied me throughout my academic career. I would like to thank my mentor, Dr. William P. Halford, who sparked my interest in virology, took me from Big Sky Country to Land of Lincoln, fostered my confidence in writing and presenting, and kept me on track through graduate school. I would like to thank my colleague, Brandon Rakowski, for his excellent technical support. Finally, I would like to extend my gratitude to my Ph.D. committee members: Dr. Kounosuke Watabe, Dr. Donald S. Torry, Dr. Edward Gershburg and Dr. Andrew Wilber for their generous advice and support.
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Herpes simplex virus (HSV) infection is a clinically important virus, which is carried by 80% of the population in the world (1). Although there is only 2% chance that a given HSV infection will progress into recurrent herpetic diseases, the actual number of people suffering from herpetic disease is estimated to be 100 million due to the high prevalence of HSV (1).

HSV life cycle alternates between productive replication and latency. Although the mechanisms by which HSV establishes, maintains and reactivates from latency are not well understood, it is generally believed that ICP0 plays an important role in the regulation of the balance between productive and latent infections (2, 3). Synthesis of ICP0 tips the balance toward productive infection, whereas absence of ICP0 tilts the balance toward latent infection (2, 3). Therefore, it would be of extreme interest to understand the questions 1) how is ICP0 gene regulated, and 2) what function does ICP0 assume during HSV infection. The specific introduction to each question is detailed below:

1.1. GENE REGULATION OF ICP0

During productive replication, ~75 proteins are synthesized from the herpes simplex virus (HSV) genome in a temporal cascade (4). Virion protein 16 (VP16) in the tegument of HSV virions forms a complex with the cellular transcription factor Oct 1 to initiate a cascade of viral gene expression (5, 6). Only five
immediate-early (IE) genes are initially induced based on the presence of VP16-responsive elements in their promoters (7). Viral IE proteins such as infected cell proteins 0 (ICP0) and 4 (ICP4) are believed to play a key role in activating viral mRNA synthesis, and thus promoting the synthesis of ~70 early (E) and late (L) proteins that replicate and package HSV genomes into new virions.

ICP0 was first identified based on its capacity to transform HSV’s major transcriptional regulator, ICP4, from a weak transcriptional activator to a potent activator of mRNA synthesis; specifically, combinations of ICP0 and ICP4 are 20-fold more potent at driving mRNA synthesis than either ICP0 or ICP4 alone (8, 9). Functionally, synthesis of ICP0 causes HSV’s equilibrium to abruptly tip towards productive replication, whereas absence of ICP0 produces the opposite effect. Synthesis of ICP0 is sufficient to trigger HSV reactivation in trigeminal ganglion neurons and other models of latent HSV infection (10, 11). HSV ICP0− viruses replicate to nearly wild-type levels when cells are inoculated with more than 1 plaque-forming unit (pfu) of virus per cell. At multiplicities of infection (MOI) below 0.1 pfu per cell, the same ICP0− viruses establish quiescent infections in 99% of the cells they infect (12, 13). Such observations suggest that ICP0 antagonizes a repressor of HSV replication, whose repressive capacity can be saturated with high numbers of HSV genomes (13).

Preston and Everett first articulated the concept of an ICP0-antagonized repressor of HSV gene expression (3, 14). Because ICP0 is an E3 ubiquitin ligase (15), it was suggested that a repressor might silence HSV mRNA synthesis in the absence of ICP0, whereas synthesis of ICP0 would result in
ubiquitination and/or proteasomal destruction of the repressor, thus allowing HSV mRNA synthesis to proceed unhindered (16). Since the advancement of this hypothesis, many laboratories have joined in the search for the unidentified repressor(s) of HSV gene expression. Individual cellular proteins that co-localize with ICP0 have been probed for their capacity to function as repressors of HSV replication such as PML and Sp100 (17, 18). Suspected pathways of silencing HSV genomes, such as epigenetic silencing, have been explored to determine if HSV $ICP0^-$ null viruses are more sensitive to these gene silencing mechanisms (19-22). Based on such inquiries, the list of proteins that may function as ICP0-antagonized repressors of HSV gene expression continues to grow and currently includes PML (17-19, 23), Sp100 (24), cyclin D3 (25), IRF-3 and IRF-7 (26), centromeric proteins CENP-B and CENP-C (27, 28), HDAC1/2-CoREST-REST (19), DNA-dependent protein kinase (29), and class II histone deacetylases (22).

The PML protein is among the most carefully scrutinized of the potential ICP0-antagonized repressors of HSV replication. In support of the PML repressor hypothesis, ICP0 and HSV genomes co-localize with PML nuclear bodies in cells (30, 31), synthesis of ICP0 triggers the dispersal of PML nuclear bodies (30), and PML is strongly induced by interferon treatments that are known to impede HSV replication (32). Weaknesses in the PML repressor hypothesis include the fact that overexpression of PML does not inhibit HSV gene expression and does not interfere with the replication of wild-type HSV or $ICP0^-$ null viruses (33); unpublished data of W. Halford]. Although siRNA-knockdown of both PML and Sp100 results in an ~40-fold increase in the replication of HSV
ICP0− null viruses in human fibroblasts, this represents less than a 1% complementation of the >10,000-fold repression that occurs in these cells (18). Finally, there is no direct evidence of a physical interaction between ICP0 and PML (34).

Like many laboratories, we too have been interested in understanding why failure to synthesize ICP0 results in limited mRNA transcription from the HSV genome. Rather than interrogate specific proteins for their capacity to repress viral mRNA synthesis, we chose to establish an experimental system that would allow us to rapidly monitor repression of a single HSV IE gene, the ICP0 gene. To this end, an ICP0− virus was constructed that bore an ~750 bp insertion of green fluorescent protein (GFP) coding sequence and a stop codon in exon 2 of the ICP0 gene. The resulting virus, HSV-1 0′GFP, synthesized a 3.5 kb ICP0GFP mRNA and a truncated ICP0GFP peptide. Using the GFP fluorescent reporter as a screening tool, we probed for conditions that alleviated or exacerbated repression of the ICP0GFP reporter gene in HSV-infected cells such as presence or absence of biologically active ICP0.

With the aid of these new reagents, we report the identification of a protein that satisfied four empirical criteria that should be expected of an ICP0-antagonized repressor of HSV mRNA transcription; specifically, the identified protein 1. was required to observe repression of ICP0 mRNA synthesis in HSV-infected cells; 2. was sufficient to repress ICP0 mRNA synthesis in the absence of ICP0; 3. was unable to silence ICP0 mRNA synthesis when ICP0 accumulated; and 4. physically interacted with ICP0.
We report the unexpected finding that the viral IE protein, ICP4, satisfied all of the criteria expected of a *bona fide* ICP0-antagonized repressor of HSV mRNA transcription. It is relevant to note that ICP4’s capacity to function as a repressor of HSV IE mRNA transcription is well established, particularly in the context of the *ICP4* and *L/ST* genes (35, 36). Evidence has been presented both for and against the hypothesis that ICP4 represses the *ICP0* gene in HSV-infected cells (37, 38). However, the perceived importance of the hypothesis may be measured in terms of the attention it has received; 14 years have elapsed since the last published study that considered ICP4’s potential to repress the *ICP0* gene (38).

We present new evidence that corroborates earlier findings that ICP4’s capacity to repress ICP0 mRNA synthesis is indeed modest when ICP0 accumulates. However, when ICP0 fails to accumulate, then ICP4 is capable of silencing the *ICP0* gene. We present functional evidence that ICP0 antagonizes ICP4-dependent repression of the *ICP0* gene. In addition, we present the first direct evidence that ICP0 and ICP4 physically interact in HSV-infected cells. Collectively, these data suggest an alternative view of HSV gene regulation, which is consistent with the biology of a virus that establishes latent infections: *In the absence of ICP0, ICP4 functions as an efficient repressor of HSV IE mRNA transcription, whereas accumulation of ICP4’s binding partner, ICP0, converts ICP4 from a repressor to an activator of HSV mRNA synthesis* (8, 9). The findings that led us to these unexpected conclusions are presented below.
1.2. FUNCTION OF ICP0

ICP0’s mechanism of action is unknown, but the possibilities are constrained by four facts: 1. ICP0 potentiates ICP4’s function as a transcriptional activator of HSV mRNA synthesis (8, 9, 39); 2. ICP0 is a RING-finger E3 ubiquitin ligase (15, 40); 3. ICP0 is essential for HSV’s resistance to the innate interferon response of animals (2, 41); and 4. ICP0’s E3 ubiquitin ligase activity triggers the dispersal of pro-myelocytic leukemia (PML) nuclear bodies (30, 42), which may contribute to the formation of adjacent, sub-nuclear replication compartments (43, 44).

Point mutations in ICP0’s RING-finger domain (amino acids 116 to 156) destroy ICP0’s E3 ligase activity and destroy ICP0’s capacity to promote HSV replication (40). It remains unclear which substrate(s) explain how ICP0’s E3 ligase activity promotes HSV replication and spread. Although ICP0 triggers the efficient dispersal of PML nuclear bodies, ICP0 does not ubiquitinate the PML protein in an *in vitro* E3 ligase assay (45).

In the first part of the dissertation, we clarifies that ICP0 physically interacts with HSV’s major transcriptional regulator, ICP4, and suggests that ICP0 influences whether ICP4 functions predominantly as an activator or a repressor of HSV mRNA synthesis (39). However, ICP0’s interaction with ICP4 does not explain 1. how ICP0 triggers the dispersal of PML nuclear bodies and centromere proteins (27, 42), nor does it explain 2. why synthesis of ICP0 causes cells to arrest in the G2/M phase of the cell cycle (46, 47). These latter observations suggest that ICP0 must interact with at least one cellular protein. Rather than
interrogate specific proteins for their capacity to interact with ICP0, we chose to use live-cell imaging to determine if new clues might be obtained by tracking the distribution of a green fluorescent protein (GFP)-tagged form of ICP0 in HSV-infected cells over time.

Three ICP0+ viruses were constructed that bore an ~750 bp insertion of GFP coding sequence inserted in the ICP0 gene. The resulting recombinant viruses, HSV-1 0+GFP12, HSV-1 0+GFP24, and HSV-1 0+GFP105 each synthesized a 3.5 kb ICP0+GFP mRNA and a 140 kDa protein. Each GFP-tagged ICP0 protein retained much of ICP0’s activity, and was visible in HSV-1 infected cells by fluorescent microscopy. Contrary to our initial expectations, the majority of GFP-tagged ICP0 was observed in the cytoplasm of HSV-1 infected cells. Subsequent tests verified that wild-type ICP0 also accumulated to much higher levels in the cytoplasm than in the nucleus of virus-infected cells.

Since the discovery that ICP0 potently stimulates HSV mRNA synthesis (8, 9), sporadic reports have documented the presence of ICP0 in the cytoplasm of HSV-infected cells, or have described ICP0 translocating from the nucleus to the cytoplasm of HSV-infected cells (48-52). The biological significance of cytoplasmic ICP0 remains obscure, and it remains possible that cytoplasmic ICP0 represents an artifact of ICP0 overexpression (48, 49). Therefore, ICP0 continues to be discussed predominantly in terms of its potential function(s) in the nucleus of HSV-infected cells (17, 27, 53, 54).

The results of the current study clarify that ICP0 is an exclusively nuclear protein during the IE phase of HSV infection, but translocates to the cytoplasm.
during the early (E) phase. Once in the cytoplasm of HSV-infected cells, ICP0 efficiently bundles and disperses host cell microtubules. Microtubule networks are known to be disrupted in HSV-infected cells (55), but the effectors that mediate this process are unknown. The HSV-1 tegument protein VP22 bundles microtubules when overexpressed in transfected cells (56), but this is not observed in HSV-infected cells (57). Our results demonstrate that ICP0 is necessary and sufficient to trigger the complete disassembly of the host cell microtubule network. This finding adds to a growing list of proteins encoded by plant and animal viruses that reorganize microtubules (58-61).

This is the first report of a viral E3 ligase that regulates microtubule stability. Intriguingly, cellular E3 ligases such as the anaphase-promoting complex and cullin 3 orchestrate massive reorganizations of microtubules during mitosis (62-64). ICP0-like E3 ligases are not unique to herpes simplex virus, but are encoded by at least 20 other α-herpesviruses that infect primates, dogs, pigs, frogs, fish, and other species (65, 66). Thus, our results raise the possibility that an ancestral herpesvirus may have stolen an E3 ligase from its host cell, and modified this regulator of microtubule stability to suit the needs of the virus. Specifically, our results imply that ICP0 possesses a mitosis-related regulatory activity that disassembles microtubules upon ICP0’s translocation to the cytoplasm, and hence prepares the host cell for the synthesis and/or egress of newly formed HSV virions.
CHAPTER 2: MATERIALS AND METHODS

2.1. CELLS AND VIRUSES

Vero cells and 293 cells were obtained from the American Type Culture Collection (Manassas, VA). ICP4-complementing E5 and ICP0-complementing L7 cell lines were kindly provided by Neal Deluca (University of Pittsburgh; (67)). ICP27-complementing V27 cells and HSV-1 d27-1 (ICP27) virus were a kind gift of Steve Rice (University of Minnesota Medical School, Minneapolis; Ref. (68)). Cell lines were propagated in Dulbecco’s Modified Eagle’s medium (DMEM) supplemented with 5% fetal bovine serum and antibiotics.

Table 1 summarizes the HSV-1 recombinant viruses used in this study, which are derivative of low passage strains of wild-type HSV-1 strain KOS (69). HSV-1 KOS, HSV-1 22/n199 (ICP22; Ref. (70)), HSV-1 0+GFP12 (ICP0+GFP-12), HSV-1 0+GFP24 (ICP0+GFP-24), HSV-1 0+GFP105 (ICP0+GFP-105) and HSV-1 0+4BS- (ICP0+GFP-105) were propagated in Vero cells. The HSV-1 ICP4 virus n12 (71) and the ICP4 temperature-sensitive virus HSV-1 tsB21 (72) were propagated in ICP4-complementing E5 cells. The HSV-1 ICP0 viruses n212 (73), 0+GFP (ICP0+GFP), 0-4BS- (ICP0-GFP) and 0(ΔRING) (ICP0ΔRING) were propagated and titered in ICP0-complementing L7 cells, as was the HSV-1 VP16 virus RP4 (74) that was kindly provided by Steve Triezenberg (Michigan State University, East Lansing).
HSV-1 strain KOS was used as the parental wild-type virus in this study. KOS and all of the HSV-1 recombinant viruses used in this study were propagated in Vero cells, L7 cells, or E5 cells cultured in complete DMEM. KOS-GFP is a recombinant virus derived from HSV-1 strain KOS that expresses GFP from a CMV promoter cassette inserted in the intergenic region at the 3' ends of the UL26 and UL27 genes (75, 76). To construct HSV-1 recombinant viruses, a 7.3 kb DNA fragment which encompasses the entire LAT-ICP0 locus was first subcloned from HSV-1 strain KOS into a pCRII plasmid vector (Invitrogen Corporation, Carlsbad, CA). Mutations were introduced into the ICP0 gene of this plasmid, as described below.

i. p0+GFP12: A GFP coding sequence was amplified from plasmid peGFP-N1 (Clontech Laboratories, Mountain View, CA) using PCR primers that added Ncol and Bsu36I restriction sites to the 5’ and 3’ ends of the GFP coding sequence. Following PCR amplification with a high fidelity mixture of thermostable DNA polymerases, the GFP sequence was subcloned into Ncol and Bsu36I sites that occur in codons 1 and 11 of the ICP0 coding sequence. The resulting plasmid, p0+GFP12, encoded ICP0+GFP-12 protein. The genetic identity of this and all GFP insertions in the ICP0 gene was confirmed by DNA sequencing. Homologous recombination between HSV-1 KOS and the p0+GFP12 plasmid yielded the recombinant virus HSV-1 0+GFP12.

ii. p0+GFP24: The plasmid p0+GFP24 was created by PCR amplification of three DNA fragments that were ligated in series to create a GFP insertion at the 5’ end of exon 2 of the ICP0 gene (Fig. 1A). Specifically, 1. the 3’ end of intron 1
of the *ICP0* gene; 2. a *GFP* coding sequence; and 3. the 5’ half of exon 2 of the *ICP0* gene were PCR-amplified, ligated together, sequenced, and the resulting BamHI - Xhol fragment was subcloned into the BamHI - Xhol sites in the *ICP0* gene. The resulting plasmid, p0+GFP24, encoded ICP0+GFP-24 protein in which GFP is inserted between amino acids 23 and 24 of ICP0. Homologous recombination between HSV-1 KOS and the p0+GFP24 plasmid yielded the recombinant virus HSV-1 0+GFP24.

**iii. p0-GFP:** The plasmid p0-GFP gene was created by inserting a polylinker, *GFP* coding sequence, and stop codon derived from the plasmid peGFP-N1 (Clontech Laboratories) into a XhoI site in codon 104 of the *ICP0* gene. The resulting plasmid, p0-GFP, encoded the N-terminal 104 amino acids of ICP0, a 14 amino acid linker, and GFP. Homologous recombination between HSV-1 KOS and the p0-GFP plasmid yielded the recombinant virus HSV-1 0-GFP.

**iv. p0+GFP105:** The plasmid p0+GFP105 was derived by subcloning a dsDNA linker containing BsrGI and XhoI sites (*tgtaca agatat ctcgag*) in the 3’ end of the *GFP* coding sequence in p0-GFP. Thus, a TAA terminator codon was removed and the *GFP* and *ICP0* coding sequences were placed in the same open-reading frame. The resulting plasmid, p0+GFP105, encoded ICP0+GFP-105 protein in which GFP is inserted between amino acids 104 and 105 of ICP0. Homologous recombination between HSV-1 KOS and p0+GFP105 yielded the virus HSV-1 0+GFP105.
v. **p0ΔRING**: The plasmid p0ΔRING was derived by deletion of codons 105-229 of the *ICP0* gene from p0^+^GFP^105_. Homologous recombination between HSV-1 KOS and the p0ΔRING plasmid yielded the recombinant virus HSV-1 0ΔRING.

vi. **p0^−^4BS^−^ and p0^+^4BS^−^**: The plasmids p0^−^4BS^−^ and p0^+^4BS^−^ were created as follows. A plasmid, pmin0^−^GFP, contains the *ICP0* promoter region and a unique Tfil restriction site that overlaps the ICP4 DNA-binding site (4BS) element in the *ICP0* promoter. Thus, mutation of the 4BS element was achieved by linearizing pmin0^−^GFP with Tfil, using mung bean exonuclease to remove ssDNA overhangs, and religating with T4 DNA ligase. The 4-bp deletion in the 4BS element was confirmed by the loss of the Tfil restriction site and DNA sequencing (Figure 1A). The ICP4-binding site mutation was transferred into the larger plasmids p0^−^GFP and p0^+^GFP^105_ by subcloning a XhoI - EcoRI restriction fragment to yield the plasmids p0^−^4BS^−^ and p0^+^4BS^−^_. Homologous recombination between HSV-1 KOS and these plasmids yielded the HSV-1 recombinant viruses 0^−^4BS^−^ and 0^+^4BS^−^ (Table 1).

vii. **p0^+^FLAG^24_**: The plasmid p0^+^FLAG^24_ was created as follows. A plasmid, p0^+^GFP^24_, contained a BgIII restriction site-GFP coding sequence-Spel restriction site insertion between amino acids 23 and 24 of the ICP0 open-reading frame. A dsDNA oligonucleotide linker of the following sequence was synthesized, CCCAGATCTGGACTACAAGGACGATGACGACAAACTAGTCTG, digested with BgIII and Spel, and subcloned in place of the *GFP* sequence to create p0^+^FLAG^24_. The resulting gene encoded a FLAG-tagged protein that bore
the amino acid sequence LDYKDDDDK between amino acids 23 and 24 of ICP0. Homologous recombination between HSV-1 KOS and p0\(^+\)FLAG\(_{24}\) yielded the HSV-1 recombinant virus 0\(^+\)FLAG\(_{24}\) (Table 1).

Table 2 summarizes the ΔE1-ΔE3 adenovirus vectors used in this study, which were propagated in human embryonic kidney 293 cells in complete DMEM, as described previously (10). Adenovirus titers were determined by a limiting dilution analysis in 96-well cultures of 293 cells to establish the titer of infectious virus in terms of 50% tissue-culture infectious dose (TCID\(_{50}\)). One TCID\(_{50}\) unit is equivalent to 1 pfu, but the TCID\(_{50}\) assay is less prone to underestimate adenovirus vector titers (unpublished data).

### 2.2. CONSTRUCTION OF RECOMBINANT HSV-1 VIRUSES

Infectious HSV-1 DNA was prepared by a protocol that relies upon dialysis to minimize shearing of genome-length HSV-1 DNA; this is a modification of a protocol that was generously provided by Karen Mossman (McMaster University, Hamilton, Ontario). Five 100 mm dishes of Vero cells (3x10\(^7\) cells) were inoculated with 5 pfu per cell of HSV-1 strain KOS. After 24 hours, cells were scraped, centrifuged, rinsed with PBS, resuspended in 7.0 ml of 200 mM EDTA pH 8.0, and transferred into a 15 ml conical. Proteinase K (75 µl of 10 mg/ml) and 375 µl of 10% SDS were added to virus-infected cells, and the tube was incubated in a rotisserie oven with slow rotation at 50ºC for 16 hours. Proteins were removed by phenol : chloroform extraction, and DNA was transferred into a 0.5 – 3.0 mL Slide-a-lyzer cassette (10,000 MW cutoff; Pierce Chemical Co.,
Rockford, IL) and dialyzed against 0.1x standard saline citrate for 24 hours. Following dialysis, infectious HSV-1 DNA was aliquoted and frozen at -80°C.

Recombinant HSV-1 viruses were generated by co-transfection of 2 µg infectious HSV-1 KOS DNA and 1 µg each plasmid into a 60 mm dish containing 8 x 10^5 ICP0-complementing L7 cells. After 12 hours, medium was replaced with complete DMEM containing 1% methylcellulose and GFP^+ plaques were selected on a Nikon TE2000 fluorescent microscope (Nikon Instruments, Lewisville, TX). GFP^+ recombinant viruses were repeatedly passed in ICP0-complementing L7 cells until a uniform population of viruses was obtained that produced 100% GFP^+ plaques, at which time Southern blot analysis was used to confirm that the anticipated mutation had been introduced into the ICP0 gene of HSV-1.

2.3. SOUTHERN BLOT ANALYSIS

Vero cell cultures were established at a density of 1.5 x 10^6 cells per 100 mm dish and were inoculated with viruses at an MOI of 5 pfu per cell. DNA was harvested from virus-infected cells or uninfected controls at 24 hours p.i. using a standard DNA extraction procedure and Southern blot analysis was performed as previously described (77, 78). Oligonucleotide probes specific for intron 1 of the ICP0 gene (5’-cccctagatgctgtagtaaggggggagctgtag-3’) were used to probe restriction fragments of HSV-1 viruses.
2.4. NORTHERN BLOT ANALYSIS

Cultures of L7 or Vero cells were established at a density of 1.5 x 10^6 cells per plate in 60 mm dishes, and inoculated with MOIs of 0.1 to 5 pfu per cell. RNA was isolated using Ultraspec RNA isolation reagent (Biotecx Inc., Houston, TX). Equal amounts of total RNA (10 μg) were electrophoretically separated on 1% formaldehyde agarose gels, blotted onto Zeta Probe GT nylon membranes (Biorad Laboratories, Hercules, CA), and hybridized with radiolabeled oligonucleotides specific for exon 3 of HSV-1 ICP0 mRNA (5'-ggagtcgtgctgactatgggggtctctgttgtttgcaagg-3'), GFP mRNA (5'-atagacgttgtggctgttgtagttactccagcttgtgc-3'), HSV-1 glycoprotein D mRNA (5'-aggccccagagacttgtgtgtgaggacattcggtgtactc-3') or cellular GAPDH mRNA (5'-tgacctggccagggtgctaa-gcagttgtggtgagca-3'). Oligonucleotides were end-labeled with [α-32P] dATP using terminal deoxynucleotidyl transferase (Promega Corporation, Madison, WI) and were hybridized to their target sequence via 16 hours of hybridization at 37°C in a solution containing 5 ng/ml labeled probe, 7% SDS, 120mM NaH2PO4, and 250mM NaCl. Excess probe was removed from membranes by sequential rinses in 0.1x standard saline citrate containing 0.1% SDS. Blots were exposed to phosphor screens, which were scanned and analyzed with a Cyclone PhosphorImager and OptiQuant software (Perkin Elmer, Boston, MA).
2.5. WESTERN BLOT ANALYSIS

Vero cell cultures were established at a density of $3 \times 10^5$ cells per well in 12-well plates, and were infected at an MOI of 10 pfu per cell. After 18 hours, proteins were harvested using mammalian protein extraction reagent (Pierce Chemical Co., Rockford, IL) supplemented with 1 mM dithiothreitol and protease inhibitor cocktail set I (Calbiochem, La Jolla, CA). After heat denaturation, 20 μg of each protein sample and MagicMark™ XP protein MW markers (Invitrogen Corporation, Carlsbad, CA) were resolved in a 10% polyacrylamide gel with a 4% stacking gel, and were transferred to nitrocellulose membranes. Protein blots were blocked in phosphate-buffered saline (PBS) containing 5% nonfat dry milk, and were incubated overnight at 4°C in PBS + 0.1% Tween-20 + 5% nonfat dry milk containing a 1:1000 dilution of mouse monoclonal H1083 antibody specific for amino acids 395 to 775 of ICP0 (79-81) (EastCoast Bio, North Berwick, MA) and a 1:500 dilution of rabbit polyclonal anti-GFP antibody (Clontech Laboratories Inc.). Following incubation with primary antibodies, membranes were washed four times with PBS + 0.1% Tween-20 (PBS-T), and were incubated for 1 hour with 1:20,000 dilution of goat anti-rabbit IgG and goat anti-mouse IgG conjugated, respectively, to the infrared fluorescent dyes IRDye® 680 and IRDye® 800CW (LI-COR Bioscience, Lincoln, NE). Protein blots were washed three times in PBS-T, and were scanned for two-color fluorescence using the Odyssey Infrared imaging system (LI-COR Bioscience). Data were analyzed using Odyssey application software version 3.0.16 (LI-COR Bioscience).
2.6. IMMUNOFLUORESCENT STAINING IN VERO CELLS

Immunofluorescent staining in Vero cells was performed using an adaptation of a staining protocol that was generously provided by Roger Everett (MRC Virology Unit, Glasgow, Scotland). Glass coverslips were placed on the bottom of 6-well dishes and Vero cells were seeded at a density of $1 \times 10^6$ cells per well. After allowing 8 hours for cell attachment, cultures were inoculated with HSV-1 viruses using the conditions defined in each Figure Legend and Results sub-section. At the indicated time of harvest, glass coverslips were removed from culture wells, and were fixed with PBS containing 3% formaldehyde and 2% sucrose for 10 minutes, followed by permeabilization with 90% methanol for 10 minutes. HSV-1 Fc-γ receptors (glycoprotein E-I heterodimers; Ref. (82)) were blocked along with all other non-specific protein-binding sites by incubating fixed cells in a solution of PBS containing 0.5% fetal bovine serum (FBS), as well as 10 µg / ml each of the γ-globulin fractions of human, donkey, and goat serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Fixed cells were incubated in PBS + FBS + γ-globulin block solution for 10 minutes, and the same solution was used as the diluent for primary and secondary antibodies. Each protein of interest was labeled by incubating fixed cells in a 1:1000 dilution of primary antibody for 16 hours. Excess primary antibody was removed by washing with PBS + FBS + γ-globulin block solution, and cells were then incubated for 1 hour in PBS + FBS + γ-globulin block containing a 1:1000 dilution of each secondary antibody as well as 10 ng / ml of Hoechst 33342 (Calbiochem, La Jolla, CA). Excess secondary antibody was removed by washing with the
PBS + FBS + γ-globulin block solution, and coverslips were mounted on glass slides using FluorSave Reagent (Calbiochem, Gibbstown, NJ). Cells on coverslips were photographed using an Olympus BX41 microscope equipped with Olympus DP70 digital camera (Olympus America, Center Valley, PA). Images of green, red, and blue fluorescence were captured at exposure times of 500, 200, and 20 ms, respectively.

The primary antibodies were mouse α-ICP0 monoclonal antibody H1083 (EastCoast Bio), mouse anti-FLAG monoclonal antibody (Sigma Chemical Co.), rabbit anti-α-tubulin polyclonal antibody ab18251 (Abcam, Cambridge, MA), rabbit anti-β-COP polyclonal antibody ab2899 (Abcam, Cambridge, MA), rabbit anti-calreticulin polyclonal antibody ab4 (Abcam, Cambridge, MA). The secondary antibodies were Alexa Fluor 594-conjugated goat α-mouse IgG (Molecular Probes, Eugene, OR), fluorescein-conjugated goat α-mouse IgG (Jackson ImmunoResearch), or DyLight 594-conjugated donkey α-rabbit IgG (Jackson ImmunoResearch).

2.7. CO-IMMUNOPRECIPITATION

Replicate 100 mm dishes containing $10^7$ Vero cells were inoculated with vehicle or 5 pfu per cell of HSV-1 KOS or HSV-1 0+FLAG24 in the presence of 200 µM cycloheximide. At 10 hours p.i., cycloheximide-containing medium was replaced with 10 µg/ml actinomycin D, and 6 hours was allowed for translation of accumulated IE mRNAs. At 16 hours p.i., 100 mm dishes of Vero cells were washed with ice-cold phosphate-buffered saline, and cells were lysed in either
0.5 ml of an NP40-based buffer or RIPA buffer. The NP40-based buffer contained 50 mM Tris (pH 7.4), 150 mM sodium chloride, 2 mM EDTA, 1% NP40, and 1x Halt protease inhibitor cocktail (Pierce Chemical Co.). RIPA buffer consisted of 50 mM Tris (pH 8.0), 150 mM sodium chloride, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, and 1x Halt protease inhibitor cocktail. Cell lysed for 2 hours at 4°C on a rotisserie, cell debris was removed by centrifugation, and 10% of each lysate was reserved for Western blot comparison of relative levels of ICP0 and ICP4. Supernatants were pre-cleared by incubation with 1 μg normal mouse IgG and Protein A/G agarose beads (SantaCruz Biotechnology) for 30 min at 4°C. Pre-cleared supernatants were incubated with 15 μl of FLAG-M2 agarose beads (Sigma Chemical Company) overnight at 4°C. Immunocomplexes were washed four times with NP-40 buffer containing 500 mM NaCl or RIPA buffer, and were prepared for Western blot analysis by boiling in 2x Laemmli loading buffer prior to electrophoresis on an 8% polyacrylamide gel. Transfer and detection of blotted proteins on PVDF membranes was performed as described above.

2.8. TRANSFECTION OF VERO CELLS

Vero cells were transfected with plasmid DNA (0.75μg / well in 12-well plates) using Lipofectamine 2000 (Invitrogen Corporation, Carlsbad, CA) in complete DMEM at 37°C. Medium was replaced at 4 hours post-transfection to avoid toxicity associated with the transfection reagent, and cells were incubated at 37°C in complete DMEM until cells were fixed for immunofluorescent staining.
Table 1

Description of HSV-1 viruses

<table>
<thead>
<tr>
<th>Virus / Genotype</th>
<th>Phenotype / Properties</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>KOS (wild-type)</td>
<td>Wild-type laboratory strain of HSV-1 (12 passages removed from isolation).</td>
<td>(69)</td>
</tr>
<tr>
<td>n212 (ICP0 null)</td>
<td>Replicates with 1% efficiency at MOIs below 0.1 pfu per cell; virus synthesizes the first 211 of 775 amino acids of ICP0.</td>
<td>(73)</td>
</tr>
<tr>
<td>0'GFP (ICP0 null)</td>
<td>Replicates with 1% efficiency at MOIs below 0.1 pfu per cell; virus synthesizes the first 104 amino acids of ICP0 coupled to GFP.</td>
<td>this study</td>
</tr>
<tr>
<td>0'4BS (ICP0 null)</td>
<td>Replicates with 1% efficiency at MOIs below 0.1 pfu per cell; virus synthesizes the first 104 amino acids of ICP0 coupled to GFP. A 4-bp deletion disrupts the ICP4 DNA-binding site in the ICP0 promoter.</td>
<td>this study</td>
</tr>
<tr>
<td>0'GFP12 (ICP0+)</td>
<td>Replicates with 66% efficiency at MOIs below 0.1 pfu per cell; virus synthesizes full-length ICP0 with GFP in lieu of the first 11 amino acids.</td>
<td>this study</td>
</tr>
<tr>
<td>0'GFP24 (ICP0+)</td>
<td>Replicates with 66% efficiency at MOIs below 0.1 pfu per cell; virus synthesizes full-length ICP0 with GFP inserted between amino acids 23 and 24.</td>
<td>this study</td>
</tr>
<tr>
<td>0'GFP105 (ICP0+)</td>
<td>Replicates with 66% efficiency at MOIs below 0.1 pfu per cell; virus synthesizes full-length ICP0 with GFP inserted between amino acids 104 and 105.</td>
<td>this study</td>
</tr>
<tr>
<td>0ΔRING (ICP0 null)</td>
<td>Replicates with 1% efficiency at MOIs below 0.1 pfu per cell; virus synthesizes ICP0 with GFP in lieu of amino acids 105 and 229.</td>
<td>this study</td>
</tr>
<tr>
<td>0'4BS (ICP0+)</td>
<td>Replicates with 66% efficiency at MOIs below 0.1 pfu per cell; virus synthesizes full-length ICP0 with GFP inserted between amino acids 104 and 105. A 4-bp deletion disrupts the ICP4 DNA-binding site in the ICP0 promoter.</td>
<td>this study</td>
</tr>
<tr>
<td>0'FLAG24 (ICP0+)</td>
<td>Replicates with 100% efficiency at MOIs below 0.1 pfu per cell; virus synthesizes full-length ICP0 with FLAG epitope inserted between amino acids 23 and 24.</td>
<td>this study</td>
</tr>
<tr>
<td>RP4 (VP16 null)</td>
<td>Replicates with 1% efficiency at MOIs &lt;0.1 pfu per cell; virus synthesizes VP16 protein lacking half of the acidic transactivation domain (Δa.a. 413-454 [of 490]).</td>
<td>(74)</td>
</tr>
<tr>
<td>n12 (ICP4 null)</td>
<td>Incapable of replication unless ICP4 is provided in trans; virus synthesizes the first 252 of 1298 amino acids of ICP4.</td>
<td>(83)</td>
</tr>
<tr>
<td>tsB21 (ICP4 null)</td>
<td>Replication-competent at 34°C; replication-defective at 39.5°C; virus synthesizes a temperature-sensitive ICP4 protein (lesion maps to the C-terminal third).</td>
<td>(72)</td>
</tr>
<tr>
<td>d27-1 (ICP27 null)</td>
<td>Incapable of replication unless ICP27 is provided in trans; virus bears a 1.6 kb deletion that removes 80% of the ICP27 ORF.</td>
<td>(68)</td>
</tr>
<tr>
<td>22/n199 (ICP22 null)</td>
<td>Virus replicates like wild-type in cell culture but fails to replicate in animals; virus synthesizes the first 198 of 420 amino acids of ICP22.</td>
<td>(70)</td>
</tr>
</tbody>
</table>
Table 2

Description of ΔE1-E3 adenovirus vectors

<table>
<thead>
<tr>
<th>Virus</th>
<th>Promoter / Gene product</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad-null</td>
<td>TRE promoter / no gene product</td>
<td>(10)</td>
</tr>
<tr>
<td>Ad-ICP0</td>
<td>TRE promoter / wild-type ICP0 (775 amino acids)</td>
<td>(10)</td>
</tr>
<tr>
<td>Ad-n212</td>
<td>TRE promoter / N-terminus of ICP0 (211 of 775 amino acids)</td>
<td>(10)</td>
</tr>
<tr>
<td>Ad-ICP4</td>
<td>TRE promoter / wild-type ICP4 (1298 amino acids)</td>
<td>(10)</td>
</tr>
<tr>
<td>Ad.CMV-TetOn</td>
<td>CMV promoter / TetOn (rtTA) protein</td>
<td>(10)</td>
</tr>
</tbody>
</table>
CHAPTER 3: RESULTS

3.1. GENE REGULATION OF ICP0

3.1.1. Characterization of HSV viruses that express GFP-tagged ICP0

Three $ICP0^{+GFP}$ genes were constructed that encoded GFP-tagged ICP0 in which 

- $i$. GFP replaced amino acids 1 to 11 of the 775-amino acid ICP0 protein ($ICP0^{+GFP-12}$); 
- $ii$. GFP was inserted between amino acids 23 and 24 of ICP0 ($ICP0^{+GFP-24}$); or 
- $iii$. GFP was inserted between amino acids 104 and 105 of ICP0 ($ICP0^{+GFP-105}$) (Fig. 1A). An ICP0-null control gene, the $ICP0^{-GFP}$ gene, encoded only the N-terminal 104 amino acids of ICP0 fused to GFP (Fig. 1A). Chimeric $ICP0^{GFP}$ genes were introduced into the $LAT-ICP0$ locus of HSV-1 strain KOS by homologous recombination to yield HSV-1 $0^{-GFP}$, $0^{+GFP-12}$, $0^{+GFP-24}$, or $0^{+GFP-105}$ (Fig. 1B). Northern blot analysis verified that each of these viruses synthesized the predicted 3.5 kb $ICP0^{GFP}$ mRNA (Fig. 1C). GFP-tagged ICP0 proteins were analyzed by two-color Western blot analysis. In cells infected with wild-type HSV-1 KOS or KOS-GFP, ICP0-specific monoclonal antibody H1083 labeled the 110 kDa ICP0 protein (red band in Fig. 1D). In addition, the GFP-expressing recombinant virus, KOS-GFP (75), also encoded an ~30 kDa GFP protein that was labeled by rabbit anti-GFP antibody (green band in Fig. 1D). HSV-1 $0^{-GFP}$ encoded a truncated ~55 kDa protein that was only labeled by the GFP-specific antibody, whereas the $0^{+GFP}$ viruses encoded ~140 kDa $ICP0^{+GFP}$. 
proteins that were labeled with both ICP0- and GFP-specific antibodies (yellow bands in Fig. 1D).

The functionality of ICP0+GFP-12, -24, and -105 proteins was compared to ICP0. Wild-type HSV-1 formed plaques with 100% efficiency in Vero cells relative to the number of plaques that formed in ICP0-complementing L7 cells (Fig. 1E). In contrast, only ~1% of HSV-1 0’GFP (ICP0') viruses formed plaques in Vero cells (Fig. 1E). Each HSV-1 0’GFP virus formed plaques in Vero cells at ~66% efficiency relative to the number of plaques that formed in ICP0-complementing L7 cells (Fig. 1E). Thus, ICP0+GFP-12, -24, or -105 protein each retained sufficient activity to promote HSV-1 plaque formation with 45-fold greater efficiency than the ICP0' null control virus, HSV-1 0’GFP.
Figure 1

Characterization of HSV-1 viruses that express GFP-tagged ICP0.

(A) Schematic of the wild-type or chimeric ICP0 genes in wild-type HSV-1 strain KOS or the recombinant viruses, HSV-1 0^+GFP_{12}, 0^+GFP_{24}, and 0^+GFP_{105}, and 0^-GFP.  (B) Southern blot analysis of the ICP0 locus of HSV-1 KOS versus HSV-1 recombinant viruses based on hybridization of an ICP0 intron1-specific probe to
DNA digested with StuI and HpaI (ICP0 gene), StuI and BamHI (exon 1 fragment), or PshAI and BssHII (exon 2 fragment). (C) Northern blot analysis with an ICP0- versus GFP-specific probes hybridized to 10 µg total RNA isolated from Vero cells that were uninfected (no virus) or were infected with the specified HSV-1 viruses (MOI=10; RNA harvested 12 hours p.i.). (D) Two-color Western blot analysis of cells that were uninfected (no virus) or were infected with the specified HSV-1 viruses (MOI=10; protein harvested 18 hours p.i.). (E) Efficiency of plaque formation by HSV-1 KOS, 0^GFP, 0^GFP_{12}, 0^GFP_{24}, or 0^GFP_{105} in Vero cells relative to the number of plaques that formed in ICP0-complementing L7 cells (mean ± sem; n=3 cultures per group).

3.1.2. Inefficient ICP0^{GFP} mRNA synthesis from HSV-1 0^GFP in the absence of ICP0

ICP0 has been described as a promiscuous transcriptional activator (3, 20, 84). We reasoned that ICP0 should act in a positive-feedback loop to promote mRNA synthesis from its own gene, the ICP0 gene. To test this inference, an ICP0-expressing adenovirus vector, Ad-ICP0, was compared to a control vector, Ad-n212 (Table 2), for its capacity to stimulate the expression of the ICP0^{GFP} reporter gene embedded in the native LAT-ICP0 locus of HSV-1 0^GFP.

In an initial test, cells were pre-treated with vehicle, Ad-n212, or Ad-ICP0, and were inoculated 12 hours later with 2.5 pfu per cell of HSV-1 0^GFP. When compared at 12 hours post-inoculation (p.i.), HSV-1 0^GFP expressed barely
detectable levels of ICP0-GFP fluorescence in cells pre-treated with vehicle or Ad-n212 (Figure 2A). In contrast, pre-treatment with Ad-ICP0 allowed HSV-1 0’GFP to express high levels of ICP0-GFP fluorescence (Figure 2A).

Northern blot analysis was conducted to determine if adenovirus-encoded ICP0 stimulated ICP0-GFP mRNA accumulation in HSV-1 0’GFP-infected cells. Vero cells were inoculated with 0 to 10 pfu of Ad-ICP0, and were inoculated 12 hours later with 2.5 pfu per cell of HSV-1 0’GFP. Northern blot analysis with a GFP-specific probe demonstrated that Ad-ICP0 stimulated a 5-fold increase in ICP0-GFP mRNA levels and did so in a dose-dependent manner (Figure 2B, D). In contrast, Ad-n212 had no effect on ICP0-GFP mRNA levels (Figure 2C, D). Therefore, exogenous ICP0 stimulated the accumulation of ICP0-GFP mRNA in cells inoculated with HSV-1 0’GFP.
Figure 2

ICP0 stimulates HSV ICP0-GFP gene expression.

(A) Photomicrographs of ICP0-GFP protein fluorescence in Vero cells inoculated with 2.5 pfu per cell of HSV-1 0°GFP at 12 hours p.i., which were pre-treated with either vehicle or 10 pfu per cell of Ad-n212 or Ad-ICP0 for 12 hours (20x magnification). The TRE promoters in Ad-n212 and Ad-ICP0 were induced with 20 pfu per cell of Ad.CMV-TetOn and 10 µM doxycycline. (B and C) Northern blot comparison of ICP0-GFP mRNA levels in cells that were uninfected (UI) or were inoculated with 2.5 pfu per cell of HSV-1 0°GFP and 0 to 10 pfu per cell of (B) Ad-ICP0 or (C) Ad-n212 (10 µg RNA per lane; RNA harvested at 12 hours p.i.). (D) ICP0-GFP mRNA levels plotted as a function of the MOI of adenovirus in the pre-treatment.
3.1.3. Efficient ICP0-GFP mRNA synthesis from HSV-1 0’GFP when protein synthesis is blocked

We were interested to determine how ICP0 influences the activity of the ICP0-GFP gene in cells inoculated with HSV-1 0’GFP. One possibility was that ICP0 induced mRNA synthesis from all of HSV-1 0’GFP’s genes, including the ICP0-GFP gene (Figure 3A). A second possibility was that absence of ICP0 allowed a repressor such as PML (18) or HDAC1/2-CoREST-REST (20) to silence all of HSV-1 0’GFP’s genes including the ICP0-GFP gene (Figure 3B, red line). Under this latter hypothesis, ICP0 might stimulate ICP0-GFP mRNA synthesis by antagonizing the repressor of the ICP0-GFP gene (Figure 3B, green line). An experiment was conducted to evaluate the relative likelihood of these two possibilities.

The effect of Ad-ICP0 versus Ad-n212 was compared in cells inoculated with MOIs of 0.1 to 2.0 pfu per cell of HSV-1 0’GFP. As expected, HSV-1 0’GFP expressed ICP0-GFP mRNA at all MOIs so long as ICP0 was provided in trans from Ad-ICP0 (Figure 3C). In cells treated with the Ad-n212 control vector, HSV-1 0’GFP failed to synthesize detectable levels of ICP0-GFP mRNA at MOIs below 0.9 pfu per cell (Figure 3D). At an MOI of 0.9 pfu per cell of HSV-1 0’GFP, 34-fold higher levels of ICP0-GFP mRNA were noted in cells treated with Ad-ICP0 relative to cells treated with Ad-n212 (Figure 3E). Thus, efficient ICP0-GFP mRNA accumulation was highly dependent on ICP0 at low multiplicities of HSV infection.

The mechanism by which ICP0 promoted ICP0-GFP mRNA accumulation was unclear. Two possibilities included ICP0-dependent induction of ICP0-GFP
mRNA synthesis (Figure 3A) or ICP0-dependent antirepression of ICP0\textsuperscript{GFP} mRNA synthesis (Figure 3B). To differentiate between these possibilities, cells were inoculated with Ad-ICP0 or Ad-n212 for twelve hours, and then cells were treated with cycloheximide at the time of HSV-1 0\textsuperscript{GFP} inoculation. Thus, Ad-ICP0’s capacity to stimulate ICP0\textsuperscript{GFP} mRNA accumulation could be evaluated in the relative absence of other activators and/or repressors that formed in HSV-1 infected cells.

As expected, cells pre-treated with Ad-ICP0 expressed high levels of ICP0\textsuperscript{GFP} mRNA despite the cycloheximide block to the synthesis of other HSV proteins (Figure 3F, H). However, to our surprise, a similar result was observed in Ad-n212-treated cells treated with cycloheximide (Figure 3G, H). Thus, when protein synthesis was blocked in HSV-infected cells, ICP0 was no longer required for the efficient synthesis of ICP0\textsuperscript{GFP} mRNA (Figure 3G). These findings argued against the hypothesis that ICP0 was required to actively induce ICP0\textsuperscript{GFP} mRNA synthesis (Figure 3A). Rather, the results suggested that ICP0 antagonized a repressor of the ICP0\textsuperscript{GFP} gene that was synthesized \textit{de novo} in HSV-infected cells (Figure 3B, green line). Further tests were conducted to explore the validity of this hypothesis.
Figure 3

ICP0 de-represses HSV ICP0-GFP gene expression.
(A and B) Alternative models by which ICP0 may stimulate \( ICP0^{\text{GFP}} \) gene activity. (A) An ICP0 induction model predicts that ICP0 will be required to induce the \( ICP0^{\text{GFP}} \) gene to its full activity (green line), whereas efficient \( ICP0^{\text{GFP}} \) gene expression will not occur in the absence of ICP0 (blue line). (B) An ICP0 antirepression model predicts that \( i. \) a repressor will silence the \( ICP0^{\text{GFP}} \) gene when ICP0 fails to accumulate (red line), \( ii. \) ICP0 prevents the repressor from silencing the \( ICP0^{\text{GFP}} \) gene (green line), and \( iii. \) ICP0 is not required for efficient \( ICP0^{\text{GFP}} \) gene expression when the repressor is absent (blue line). (C, D, F, G) Northern blots of \( ICP0^{\text{GFP}} \) mRNA isolated from Vero cells inoculated with MOIs of 0.1 to 2 pfu per cell of HSV-1 \( 0^{\text{GFP}} \) (RNA harvested 12 hours p.i.; 10 \( \mu \)g per lane). Vero cells were pre-treated with 10 pfu per cell of (C, F) Ad-ICP0 or (D, G) Ad-n212 whose TRE promoters were induced with 20 pfu per cell of Ad.CMV-TetOn and 10 \( \mu \)M doxycycline. Cells were treated with (C, D) vehicle or (F, G) 200 \( \mu \)M cycloheximide at the time of HSV-1 \( 0^{\text{GFP}} \) inoculation. (E, H) \( ICP0^{\text{GFP}} \) mRNA levels are plotted as a function of the MOI of HSV-1 \( 0^{\text{GFP}} \) used to inoculate Vero cells treated with (E) vehicle or (H) cycloheximide.
3.1.4. Testing the predictions of a repression-antirepression model of ICP0 gene regulation

Three predictions followed from an hypothesis that HSV-1 ICP0 gene activity was regulated by a process of repression and antirepression:  

- **i.** HSV-1 ICP0⁻ and ICP0⁺ viruses should express high and equivalent levels of ICP0 mRNA when synthesis of the repressor was blocked with cycloheximide (Figure 3B, blue line);  
- **ii.** an HSV-1 ICP0⁺ virus should efficiently express ICP0 mRNA when the repressor accumulated in HSV-infected cells (Figure 3B, green line);  
- **iii.** an HSV-1 ICP0⁻ virus should exhibit little to no ICP0 mRNA synthesis when the repressor accumulated in the absence of ICP0 (Figure 3B, red line).

As an initial test of these predictions, the relative expression of ICP0-GFP or ICP0⁺GFP-105 fluorescent reporter proteins was compared in cells inoculated with 2.5 pfu per cell of HSV-1 0⁻GFP (ICP0⁻) versus HSV-1 0⁺GFP₁₀⁵ (ICP0⁺). In vehicle-treated cells, HSV-1 0⁻GFP expressed barely detectable levels of ICP0⁻GFP fluorescence, whereas HSV-1 0⁺GFP₁₀⁵ expressed ICP0⁺GFP-105 fluorescence to readily detectable levels (Figure 4A). In contrast, when protein synthesis was inhibited for the first 6 hours of infection with cycloheximide, then both ICP0⁻GFP and ICP0⁺GFP-105 fluorescent proteins accumulated to high levels by 6 hours post-release from the cycloheximide block (Figure 4B). Quantification by flow cytometry (85) demonstrated that ICP0⁻GFP and ICP0⁺GFP-105 fluorescent proteins accumulated to high and equivalent levels following release from a cycloheximide block (not shown).
The relative expression of ICP0\(^{\text{GFP}}\) or ICP0\(^{\text{GFP-105}}\) mRNA was compared in cells inoculated with HSV-1 0\(^{-}\)GFP versus 0\(^{+}\)GFP\(_{105}\). When protein translation was allowed to occur, HSV-1 0\(^{-}\)GFP produced levels of ICP0\(^{\text{GFP}}\) mRNA that were barely detectable between 3 and 12 hours p.i. (Figure 4C). When cycloheximide was used to block protein synthesis, ICP0\(^{\text{GFP}}\) mRNA levels accumulated to levels that were ~90 times background in HSV-1 0\(^{-}\)GFP-infected cells (Figure 4C, E). In contrast, the onset of protein translation did not prevent an ICP0\(^{+}\) virus, HSV-1 0\(^{+}\)GFP\(_{105}\), from expressing ICP0\(^{+\text{GFP-105}}\) mRNA to levels that were ~20 times background (Figure 4D, E). When protein synthesis was inhibited with cycloheximide, HSV-1 0\(^{-}\)GFP\(_{105}\) expressed ~5-fold higher levels of ICP0\(^{+\text{GFP-105}}\) mRNA (Figure 4D, E). As predicted, cycloheximide treatment allowed HSV-1 0\(^{-}\)GFP and 0\(^{+}\)GFP\(_{105}\) to express high and equivalent levels of ICP0\(^{\text{GFP}}\) mRNA (Figure 4E). Control experiments verified that cycloheximide blocked HSV-1 L mRNA synthesis (i.e., glycoprotein D mRNA), but had only a negligible effect on cellular GAPDH mRNA levels (not shown).

These findings were consistent with the predictions of a repression-antirepression model of ICP0 gene regulation (Figure 3B). However, questions remained about the identity of the de novo repressor that inhibited ICP0 mRNA synthesis in HSV-1 0\(^{-}\)GFP-infected cells. Alternatively, it was possible that cycloheximide stimulated synthesis of all HSV mRNAs including ICP0\(^{\text{GFP}}\) mRNA in a non-specific manner (16). Further experiments were conducted to differentiate between these possibilities.
**Figure 4**

*De novo repression of HSV ICP0\(^{\text{GFP}}\) mRNA accumulation is antagonized by a GFP-tagged ICP0 protein.*

(A and B) Photomicrographs of ICP0\(^{-\text{GFP}}\) and ICP0\(^{+\text{GFP-105}}\) reporter protein fluorescence in Vero cells 12 hours after inoculation with 2.5 pfu per cell of HSV-1 0\(^{-\text{GFP}}\) or 0\(^{+\text{GFP-105}}\) (20x magnification). Cells were treated with either (A) medium containing no drug (vehicle) from 0 to 12 hours p.i., or (B) 200 µM cycloheximide from -0.5 to 6 hours p.i. followed by vehicle from 6 to 12 hours p.i.
(C and D) Representative Northern blots of (C) ICP0-GFP mRNA or (D) ICP0+GFP-105 mRNA harvested between 3 and 12 hours after inoculation with 2.5 pfu per cell of HSV-1 0´GFP or 0+GFP105 respectively. Cells were treated with vehicle or 200 µM cycloheximide until the indicated time of RNA harvest (10 µg per lane). (E) Mean ± sd of ICP0-GFP and ICP0+GFP-105 mRNA levels plotted as a function of time of RNA harvest (n=2 per group).

3.1.5. Cycloheximide stimulates HSV ICP0 gene expression in a VP16-dependent manner

VP16 carried in HSV virions binds six VP16-responsive elements in the ICP0 promoter at the outset of infection (Figure 5A), and thus VP16 induces ICP0 mRNA synthesis (5, 86). An experiment was conducted to determine if cycloheximide’s effect on steady-state ICP0 mRNA levels was the result of a VP16-dependent process, or rather was due to a myriad of non-specific effects that should operate with equal efficiency in the presence or absence of VP16.

ICP0 mRNA accumulation was compared in cells inoculated with 2.5 pfu per cell of wild-type HSV-1 KOS, the ICP0- virus n212 (73), or the VP16- virus RP4 (74) (Table 1). When protein synthesis was allowed to occur, wild-type HSV-1 expressed ICP0 mRNA to levels that were 28-fold above background, whereas an ICP0- virus and VP16- virus expressed ICP0 mRNA to levels that were only 4-fold above background (Figure 5B, C). When protein synthesis was blocked with cycloheximide, the VP16+ viruses exhibited a robust increase in ICP0 mRNA levels; consequently, wild-type HSV and the ICP0- virus expressed
high and equivalent levels of ICP0 mRNA in cycloheximide-treated cells (Figure 5B, C). In contrast, the VP16 virus failed to efficiently express ICP0 mRNA in either vehicle- or cycloheximide-treated cells (Figure 5C). Thus, cycloheximide was incapable of stimulating ICP0 mRNA accumulation in the absence of VP16. These data argued against the possibilities that cycloheximide might increase ICP0 mRNA abundance by 1. decreasing the rate of ICP0 mRNA turnover, or by 2. stimulating a non-specific increase in the rate of transcription of all genes, including the ICP0 gene. Rather, the data were consistent with a hypothesis that cycloheximide blocked the formation of a de novo repressor of the ICP0 gene that was dominant over the HSV-specific inducer of the ICP0 gene, VP16.
Cycloheximide increases *ICP0* gene expression in a VP16-dependent manner.

(A) Schematic of *ICP0* promoter, which indicates the positions of six distal VP16-responsive elements (VREs), a proximal ICP4-binding site (4BS), and the TATA-box of the *ICP0* gene. (B) Representative Northern blot of ICP0 mRNA in Vero cells that were uninfected (UI) or were inoculated with 2.5 pfu per cell of HSV-1 KOS, n212 (*ICP0*), or RP4 (*VP16*), and which were treated with vehicle or 200 µM cycloheximide from -0.5 to 12 hours p.i., at which time total RNA was harvested (10 µg per lane). (C) Mean ± sem of ICP0 mRNA levels in KOS, n212, or RP4-infected cells (n=3 per group). Asterisks denote significant differences between vehicle- and cycloheximide-treated cells inoculated with the same virus (p<0.01; two-tailed Student’s t-test).
3.1.6. De novo repression of HSV ICP0 gene expression is ICP4-dependent

There is evidence that inhibition of protein synthesis causes all of HSV’s IE mRNAs to be overexpressed (87). One interpretation that has been offered is that cycloheximide’s effect on HSV mRNA synthesis is global and non-specific (16). However, there is a second, biologically important hypothesis that has not been adequately considered. As well as HSV ICP4’s function as an essential activator of E and L mRNA transcription (88), ICP4 may function as a repressor of IE mRNA transcription (72). To determine if ICP4 contributed to de novo repression of the ICP0 gene, ICP0 mRNA accumulation was compared in cells inoculated with HSV-1 mutants deficient in the IE regulatory proteins ICP0, ICP27, ICP22, or ICP4 (Table 1).

Regardless of their different genotypes, 2.5 pfu per cell of each HSV-1 VP16+ virus yielded high and equivalent levels of ICP0 mRNA in cycloheximide-treated cultures (Figure 6A). The ICP0- virus, n212, expressed very low levels of ICP0 mRNA when protein translation was allowed to occur, whereas each of the ICP0+ viruses expressed ICP0 mRNA to levels that were readily detectable in vehicle-treated cells (Figure 6A). Wild-type HSV-1 KOS expressed 3.5 ± 0.4-fold lower levels of ICP0 mRNA in vehicle-treated cells relative to cycloheximide-treated controls (Figure 6A, B). Likewise, the ICP27- and ICP22- viruses, d27-1 (68) and 22/n199 (70), expressed 4- to 5-fold less ICP0 mRNA in vehicle-treated cells relative to cycloheximide-treated controls (Figure 6A, B). These results suggested that the de novo repressor of ICP0 mRNA synthesis formed efficiently in the absence of ICP22, ICP27, or the ~70 HSV E and L proteins whose
synthesis was ICP27-dependent (68). In contrast, an ICP4+ virus, n12 (71), expressed high and equivalent levels of ICP0 mRNA in vehicle- and cycloheximide-treated cells (Figure 6A, B). Therefore, de novo repression of ICP0 mRNA accumulation appeared to be dependent upon the synthesis of ICP4.

An independent test was conducted to corroborate this interpretation. Vero cells were inoculated with 2.5 pfu per cell of HSV-1 KOS (ICP4+) or HSV-1 tsB21 (ICP4ts), which bears a temperature-sensitive lesion in the C-terminus of ICP4; this mutation renders ICP4 non-functional at a temperature of 39.5 °C (72). At 34.0°, 37.0°, and 39.5°C, wild-type HSV-1 KOS expressed ~3-fold less ICP0 mRNA in vehicle-treated cells relative to cycloheximide-treated controls (Figure 6C, D). Likewise, HSV-1 tsB21 expressed 2.9- and 2.3-fold less ICP0 mRNA in vehicle-treated cells at 34.0° and 37.0°C, respectively, relative to cycloheximide-treated controls (Figure 6C, D). In contrast, at the non-permissive temperature of 39.5°C, HSV-1 tsB21 expressed equivalent levels of ICP0 mRNA in both vehicle- and cycloheximide-treated cells (Figure 6C, D). Collectively these results indicated that de novo repression of ICP0 mRNA accumulation was dependent upon the synthesis of a biologically active form of HSV’s major transcriptional regulator, ICP4.
**Figure 6**

**De novo repression of HSV ICP0 gene expression is ICP4-dependent.**

(A) Representative Northern blot of ICP0 mRNA in Vero cells that were uninfected (UI) or were inoculated with 2.5 pfu per cell of HSV-1 KOS (wild-type), n212 (ICP0−), dl27-1 (ICP27−), 22/n199 (ICP22−), or n12 (ICP4−), which were treated with vehicle or 200 µM cycloheximide from -0.5 to 6 hours p.i., at which time total RNA was harvested (10 µg per lane). (B) Mean ± sem fold-reduction in ICP0 mRNA levels in vehicle-treated cells relative to cycloheximide-treated controls (fold-reduction = mRNA<sub>CHX</sub> + mRNA<sub>VEH</sub>; n=4 per group). (C) Representative Northern blot of ICP0 mRNA in Vero cells inoculated with 2.5 pfu per cell of KOS (ICP4+) or HSV-1 tsB21 (ICP4<sup>ts</sup>). Vehicle- and cycloheximide-treated cultures were incubated in CO2 chambers at 34.0, 37.0, or 39.5º C, and total RNA was harvested at 6 hours p.i. (10 µg per lane). (D) Mean ± sem fold-reduction in ICP0 mRNA levels plotted as a function of incubation temperature.
(fold-reduction = mRNA\textsubscript{CHX} ÷ mRNA\textsubscript{VEH}; n=4 per group). In panels B and D, asterisks denote fold-reductions in ICP0 mRNA that significantly differ from the value of 1, which is predicted by a null hypothesis that cycloheximide will have no effect on ICP0 mRNA accumulation in HSV-infected cells (p<0.01; two-tailed Student’s t-test).

3.1.7. De novo repression of HSV ICP0\textsuperscript{GFP} reporter genes is ICP4-binding-site-dependent

A consensus ICP4-binding site (ATCGTC) occurs 40-bp upstream of the TATA box in the ICP0 promoter (38, 89). To determine if ICP4 binding to the ICP0 promoter contributed to de novo repression of ICP0\textsuperscript{GFP} mRNA synthesis, the recombinant viruses HSV-1 0\textsuperscript{-}4BS\textsuperscript{-} and 0\textsuperscript{+}4BS\textsuperscript{-} were constructed which were equivalent to HSV-1 0\textsuperscript{-}GFP and 0\textsuperscript{+}GFP\textsubscript{105}, respectively, but carried a 4-bp deletion in the ICP4-binding site in the ICP0 promoter (Figure 7A).

A test was conducted to determine if disruption of the ICP4-binding site might alleviate de novo repression of the ICP0\textsuperscript{GFP} gene. Vero cells were inoculated with 5 pfu per cell of HSV-1 0\textsuperscript{-}GFP or 0\textsuperscript{-}4BS\textsuperscript{-}, and ICP0\textsuperscript{-}GFP fluorescence was compared at 12 hours p.i. (Figure 7B). In cells inoculated with HSV-1 0\textsuperscript{-}GFP, ICP0\textsuperscript{-}GFP fluorescence was barely visible (Figure 7B). In contrast, ICP0\textsuperscript{-}GFP fluorescence was readily detected in cells inoculated with HSV-1 0\textsuperscript{-}4BS\textsuperscript{-} (Figure 7B). Likewise, ICP0\textsuperscript{+}GFP\textsubscript{-105} fluorescence accumulated to appreciably higher levels in cells inoculated with HSV-1 0\textsuperscript{+}BS\textsuperscript{-} relative to HSV-1 0\textsuperscript{+}GFP\textsubscript{105} (Figure 7C). These observations suggested that disruption of the proximal ICP4-
binding site in the *ICP0* promoter significantly alleviated *de novo* repression of the *ICP0*\(^{\text{GFP}}\) and *ICP0*\(^{\text{GFP-105}}\) genes.

To corroborate this interpretation, accumulation of the 3.5 kb ICP0\(^{\text{GFP}}\) RNA species was compared in cells inoculated with 5 pfu per cell of HSV-1 0\(^{-}\)GFP, 0\(^{-}\)4BS\(^{-}\), 0\(^{+}\)GFP\(_{105}\), or 0\(^{+}\)4BS\(^{-}\) (Figure 7D). Each virus yielded high levels of ICP0\(^{-}\)GFP or ICP0\(^{+}\)GFP\(_{105}\) mRNA in cycloheximide-treated cultures (Figure 7D). HSV-1 0\(^{-}\)GFP exhibited a 13 ± 1-fold repression of ICP0\(^{-}\)GFP mRNA synthesis in vehicle-treated cells relative to cycloheximide-treated controls (Figure 7D, 7E). Disruption of the ICP4-binding site in the *ICP0* promoter alleviated *de novo* repression by 4-fold, and thus HSV-1 0\(^{-}\)4BS\(^{-}\) exhibited only a 3.3 ± 0.5-fold reduction in ICP0\(^{-}\)GFP mRNA in vehicle-treated cells (Figure 7D, 7E). Disruption of the ICP4-binding site in the *ICP0*\(^{\text{GFP}}\) gene increased the efficiency of ICP0\(^{\text{GFP}}\) mRNA synthesis to the same extent as synthesis of a biologically active ICP0 protein; thus, HSV-1 0\(^{-}\)4BS\(^{-}\) and 0\(^{+}\)GFP\(_{105}\) both exhibited a 3.3-fold reduction in ICP0\(^{\text{GFP}}\) mRNA levels in vehicle-treated cells relative to their respective cycloheximide-treated controls (Figure 7D, 7E). Disruption of the ICP4-binding site in the *ICP0* promoter of HSV-1 0\(^{+}\)4BS\(^{-}\) resulted in a significant de-repression; thus ICP0\(^{+}\)GFP\(_{105}\) mRNA levels in vehicle-treated cells infected with HSV-1 0\(^{+}\)4BS\(^{-}\) were only 2.1 ± 0.2-fold lower than cycloheximide-treated controls (Figure 7D, 7E). Collectively, these observations indicated that the magnitude of *de novo* repression of ICP0\(^{\text{GFP}}\) mRNA synthesis was significantly dependent upon the ICP4-binding site proximal to the transcriptional start site of the *ICP0* gene (Figure 7A).
Figure 7

De novo repression of ICP\textsubscript{0}GFP gene expression is ICP4-binding-site-dependent.

(A) Schematic of the ICP4 DNA-binding site (4BS) deletion in HSV-1 0’GFP and 0\textsuperscript{+}GFP\textsubscript{105}. All four viruses transcribe 3.5 kb ICP\textsubscript{0}GFP mRNAs, but HSV-1 0’4BS\textsuperscript{-} and 0\textsuperscript{+}4BS\textsuperscript{-} are deleted of bases -43 to -40 relative to the TATA box of the ICP\textsubscript{0}GFP gene, which disrupts the consensus ICP4-binding site, 5’-ATCGTC-3’.

(B and C) Photomicrographs of ICP\textsubscript{0}GFP\textsuperscript{-} and ICP\textsubscript{0}GFP\textsubscript{105} fluorescent protein accumulation in Vero cells 12 hours after inoculation with 5 pfu per cell of (B) HSV-1 0’GFP versus 0-4BS\textsuperscript{-} or (C) HSV-1 0\textsuperscript{+}GFP\textsubscript{105} versus 0\textsuperscript{+}4BS\textsuperscript{-} (20x).
magnification). (D) Representative Northern blots of ICP0\textsuperscript{GFP} mRNA accumulation in Vero cells that were uninfected (UI) or were inoculated with 5 pfu per cell of HSV-1 0\textsuperscript{−}GFP (ICP0\textsuperscript{−}), 0\textsuperscript{−}4BS\textsuperscript{−} (ICP0\textsuperscript{−}), 0\textsuperscript{+}GFP\textsubscript{105} (ICP0\textsuperscript{+}), or 0\textsuperscript{+}4BS\textsuperscript{−} (ICP0\textsuperscript{+}), and which were treated with vehicle or 200 µM cycloheximide from -0.5 to 12 hours p.i., at which time total RNA was harvested (10 µg per lane). (E) Mean ± sem fold-reduction in ICP0\textsuperscript{GFP} mRNA levels in vehicle-treated cells relative to cycloheximide-treated controls (fold-reduction = mRNA\textsubscript{CHX} ÷ mRNA\textsubscript{VEH}; n=3 per group). Asterisks denote significant differences in fold-reduction in ICP0\textsuperscript{GFP} mRNA levels between the ICP0\textsuperscript{−} viruses, HSV-1 0\textsuperscript{−}GFP versus 0\textsuperscript{−}4BS\textsuperscript{−} (p<0.001), and between the ICP0\textsuperscript{+} viruses, HSV-1 0\textsuperscript{+}GFP\textsubscript{105} versus 0\textsuperscript{+}4BS\textsuperscript{−} (p<0.01; two-tailed Student’s t-test).

3.1.8. Adenovirus-encoded ICP4 substitutes for the de novo repressor of the HSV ICP0\textsuperscript{GFP} gene

Our results raised the possibility that the de novo repressor of ICP0 mRNA synthesis was not a cellular protein, but rather was the HSV ICP4 protein because 1. loss of ICP4 function de-repressed ICP0 mRNA synthesis to the same extent as cycloheximide treatment (Figure 6), and 2. a 4-bp deletion that removed a single ICP4-binding site significantly alleviated de novo repression of ICP0 mRNA synthesis (Figure 7). Based on this hypothesis, it was predicted that pre-treatment of Vero cells with an ICP4-expressing adenovirus should negate cycloheximide’s capacity to stimulate ICP0\textsuperscript{GFP} mRNA synthesis in HSV-1 0\textsuperscript{−}GFP-infected cells.
To test this prediction, Vero cells were infected with 0 to 250 pfu per cell of Ad-ICP4 or a null control vector, and cells were inoculated 12 hours later with HSV-1 0'-GFP in the presence of cycloheximide. As expected, cells that received no adenovirus vector and which were inoculated with HSV-1 0'-GFP in the presence of cycloheximide expressed high levels of ICP0'-GFP mRNA (Figure 8A). However, when Vero cells were pre-treated with Ad-ICP4, cycloheximide’s capacity to stimulate ICP0'-GFP mRNA synthesis dissipated and this inhibition was adenovirus dose-dependent (Figure 8A). Despite cycloheximide treatment, the highest MOI of Ad-ICP4 caused a 42-fold reduction in ICP0'-GFP mRNA levels in HSV-1 0'-GFP-infected cells (Figure 8C). This was not a non-specific effect of the adenovirus vector, as MOIs of 10 to 250 pfu per cell of Ad-null did not reduce the efficiency of ICP0'-GFP mRNA synthesis in HSV-1 0'-GFP-infected cells treated with cycloheximide (Figure 8A).

To verify that Ad-ICP4’s capacity to repress ICP0'-GFP mRNA synthesis was ICP4-binding-site-dependent, the experiment was performed in parallel with HSV-1 0'-4BS'. Ad-ICP4 had a modest effect on ICP0'-GFP mRNA synthesis in HSV-1 0'-4BS'-infected cells (Figure 8B). However, the potency of inhibition was 10-fold less than that observed in HSV-1 0'-GFP-infected cells (Figure 8C). Specifically, an MOI of 250 pfu per cell of Ad-ICP4 produced only a 4.4-fold reduction in ICP0'-GFP mRNA levels in HSV-1 0'-4BS'-infected cells treated with cycloheximide (Figure 8C). Therefore, adenovirus-encoded ICP4 was sufficient to substitute for the de novo repressor of the ICP0'-GFP gene (Figure 8C).
Exogenous ICP4 substitutes for the *de novo* repressor of the ICP0-GFP gene.

Representative Northern blots of ICP0-GFP mRNA levels in Vero cells treated with 200 µM cycloheximide and inoculated with 5 pfu per cell of (A) HSV-1 0’GFP or (B) HSV-1 0’4BS- (10 µg total RNA per lane; time of harvest = 12 hours p.i.).

Twelve hours prior to HSV-1 inoculation, cells were pre-treated with 0, 10, 50, or 250 pfu per cell of Ad-ICP4 or Ad-null whose TRE promoters were induced with 20 pfu per cell of Ad.CMV-TetOn and 10 µM doxycycline. (C) Mean ± sem fold-
reduction in ICP0\textsuperscript{GFP} mRNA levels in HSV-1 0\textsuperscript{GFP} or 0\textsuperscript{4BS\textsuperscript{-}} infected cells as a function of the MOI of Ad-ICP4 in the pre-treatment (n=3 per group). Asterisks denote significant differences in Ad-ICP4-induced reductions in ICP0\textsuperscript{GFP} mRNA levels between HSV-1 0-GFP and 0\textsuperscript{4BS\textsuperscript{-}} (p < 0.001; two-way analysis of variance).

3.1.9. ICP0 physically interacts with the repressor of its gene, ICP4, in HSV-infected cells

A physical interaction between ICP0 and ICP4 might begin to explain how ICP0 influences ICP4’s activity as a transcriptional regulator. The available evidence from far-Western blotting (81) and co-localization studies (80) suggested that ICP0 may physically interact with ICP4. However, direct evidence was lacking to address whether or not ICP0 and ICP4 physically interact in the context of HSV-infected cells.

To address this gap in knowledge, a recombinant virus was constructed that bore a FLAG epitope between amino acids 23 and 24 of ICP0, and which is referred to as HSV-1 0\textsuperscript{FLAG\textsubscript{24}} (Figure 9A). Southern blot analysis validated that the FLAG coding sequence was inserted in both copies of the ICP0 gene, and that HSV-1 0\textsuperscript{FLAG\textsubscript{24}} grew with wild-type efficiency (not shown). Immunofluorescent staining verified that HSV-1 0\textsuperscript{FLAG\textsubscript{24}} expressed a FLAG-tagged protein that exhibited a nuclear-staining pattern typical of ICP0 during the IE phase of HSV infection (Figure 9B; 10 hours in cycloheximide → 6 hours in actinomycin D).
Immunoprecipitation experiments were performed to determine if ICP4 physically interacted with FLAG-tagged ICP0. Vero cells were inoculated with vehicle or 5 pfu per cell of HSV-1 KOS or 0^+/FLAG_{24} in the presence of cycloheximide (0 - 10 hours p.i.) followed by actinomycin D (10 - 16 hours p.i.), such that only viral IE proteins were efficiently synthesized. Input levels of ICP4 and ICP0 in each immunoprecipitation reaction were equivalent, as verified by Western blot analysis with monoclonal antibodies 58S and H1083, respectively (Figure 9C, top panel). The remainder of each protein sample was immunoprecipitated with an anti-FLAG antibody-agarose conjugate under conditions of medium or high stringency, and immunoprecipitates were analyzed for the presence of ICP4.

Significant amounts of ICP4 co-immunoprecipitated with FLAG-tagged ICP0 from lysates of HSV-1 0^+/FLAG_{24}-infected cells (Figure 9C, middle panel). Non-specific pull down of ICP4 was negligible from lysates of uninfected Vero cells or KOS-infected cells (Figure 9C). Therefore, the α-FLAG agarose only appeared to pull down ICP4 when the FLAG epitope was present in ICP0. Consistent with this interpretation, re-probing of the same blot with an ICP0-specific monoclonal antibody, H1083, verified that anti-FLAG agarose efficiently pulled down the 110 kDa FLAG-tagged ICP0, but did not pull down wild-type ICP0 (Figure 9C, lower panel). We conclude that ICP0 and ICP4 do indeed physically interact in HSV-infected cells, and propose that this interaction is likely relevant in explaining how ICP0 antagonizes ICP4’s capacity to silence ICP0 mRNA synthesis from the HSV genome.
Figure 9

ICP4 co-immunoprecipitates with FLAG-tagged ICP0.

(A) Schematic of 30-bp FLAG coding sequence insertion in exon 2 of the ICP0 gene in the HSV-1 recombinant virus, 0*FLAG24, in which a FLAG tag is inserted between amino acids 23 and 24 of ICP0. (B and C) Vero cells were inoculated with 5 pfu per cell of HSV-1 KOS (wild-type) or HSV-1 0*FLAG24, which were treated with 200 µM cycloheximide from -0.5 to 10 hours p.i., released into actinomycin D (10 µg / ml) from 10 to 16 hours p.i., and harvested for immunofluorescent staining or immunoprecipitation. (B) Immunofluorescent staining with a FLAG-specific antibody and AlexaFluor 594-conjugated secondary antibody. (C) Cells were harvested in an NP40-based buffer containing 150 mM NaCl (left panel) or RIPA buffer (right panel). The relative abundance of ICP4 and ICP0 was compared in 5% of total cell lysates (top panel), and the remainder was immunoprecipitated with anti-FLAG agarose. Immunoprecipitates were...
washed in 500 mM NaCl (left panel) or RIPA buffer (right panel) prior to electrophoresis and blotting, and were probed with ICP4-specific monoclonal antibody 58S (middle panel), followed by re-probing with ICP0-specific monoclonal antibody H1083 (bottom panel). The relative locations of ICP4 and ICP0 are denoted by asterisks and open circles.

3.2. FUNCTION OF ICP0

3.2.1. GFP-tagged ICP0 is predominantly observed in the cytoplasm of HSV-infected cells

In cells transfected with the plasmids p0⁺GFP₁₂, p0⁺GFP₂₄, or p0⁺GFP₁₀⁵, the ICP0⁺GFP⁻₁₂, -₂₄, or -₁₀⁵ proteins each accumulated in the nuclei of transfected cells in a nuclear domain 10 (ND10)-like pattern (Fig. 10A-C; Ref. (30, 42)). In contrast, the truncated ICP0⁻GFP protein was distributed in a nuclear and perinuclear pattern in cells transfected with the plasmid p0⁻GFP (Fig. 10D). When expressed in the context of HSV-1 infection, ICP0⁺GFP⁻₁₂, -₂₄, or -₁₀⁵ protein was predominantly observed in the cytoplasm of virus-infected cells. For example, ICP0⁺GFP⁻₁₂ was predominantly observed in the cytoplasm of cells at the outer edge of plaques formed by HSV-1 0⁺GFP₁₂ (white arrow in Fig. 10E). Likewise, the ICP0⁺GFP⁻₂₄ and ICP0⁺GFP⁻₁₀⁵ proteins were primarily detected in the cytoplasm of cells at the outer edge of plaques formed by HSV-1 0⁺GFP₂₄ and HSV-1 0⁺GFP₁₀⁵, respectively (white arrows in Fig. 10F, 10G). In contrast, the
ICP0\textsuperscript{GFP} peptide expressed by HSV-1 0\textsuperscript{+}GFP accumulated throughout virus-infected cells (Fig. 10H).

The subcellular distribution of ICP0\textsuperscript{+GFP-24} protein was monitored in Vero cells inoculated with 10 pfu per cell of HSV-1 0\textsuperscript{+}GFP\textsubscript{24}. At 3 hours p.i., GFP-tagged ICP0 was evident in the nuclei of many cells (Fig. 11A). However, ICP0\textsuperscript{+GFP-24} only accumulated to high levels after 5 hours p.i., at which time the protein had translocated to the cytoplasm of most HSV-1 0\textsuperscript{+}GFP\textsubscript{24}-infected cells (Fig. 11A). To determine if the translocation of ICP0\textsuperscript{+GFP-24} was replication-dependent, an HSV-1 \textit{ICP4}\textsuperscript{−} null virus was constructed, HSV-1 0\textsuperscript{+}GFP\textsubscript{24}Δ4. In cells inoculated with HSV-1 0\textsuperscript{+}GFP\textsubscript{24}Δ4, the nuclear-to-cytoplasmic translocation of ICP0\textsuperscript{+GFP-24} occurred with kinetics similar to the \textit{ICP4}\textsuperscript{+} virus (Fig. 11B). ICP0\textsuperscript{+GFP-24} was observed in bright perinuclear lines and globular bodies when overexpressed from the \textit{ICP4}\textsuperscript{−} null virus (Fig. 11B). Intriguingly, this pattern of cytoplasmic ICP0 was equivalent to the findings of Knipe and Smith (1986) (48), who first described ICP0’s cytoplasmic distribution in cells inoculated with an HSV-1 ICP4 mutant. Live-cell imaging of HSV-1 0\textsuperscript{+}GFP\textsubscript{24}Δ4-infected cells demonstrated that the cytoplasmic bodies in which ICP0\textsuperscript{+GFP-24} protein accumulated were highly mobile. In addition, cytoplasmic ICP0\textsuperscript{+GFP-24} was often observed in linear arrays that encircled the nucleus (denoted by asterisks in Fig. 11B).

These observations raised questions both about the requirements for ICP0’s nuclear-to-cytoplasmic translocation, and what function ICP0 might fulfill in the cytoplasm of HSV-1 infected cells.
GFP-tagged ICP0 is predominantly cytoplasmic in HSV-1 infected cells.
(A) ICP0^{+GFP-12}, (B) ICP0^{+GFP-24}, (C) ICP0^{+GFP-105}, or (D) ICP0^{-GFP} as seen in Vero
cells 12 hours post transfection with the plasmids p0^{+GFP}_{12}, p0^{+GFP}_{24},
p0^{+GFP}_{105}, or p0^{-GFP}, respectively. Each plasmid was co-transfected with the
plasmid pBHAd.CMV-VP16 to induce the ICP0 promoter and expression of GFP-
tagged ICP0. (E) ICP0^{+GFP-12}, (F) ICP0^{+GFP-24}, (G) ICP0^{+GFP-105}, or (H) ICP0^{-GFP}
as seen in Vero cells 40 hours post inoculation with the HSV-1 recombinant
viruses 0^{+GFP}_{12}, 0^{+GFP}_{24}, 0^{+GFP}_{105}, or 0^{-GFP}, respectively. In each panel, an
entire plaque is shown on the right, and one edge of the plaque is magnified on
the left. White arrows denote HSV-1 infected cells in which GFP-tagged ICP0
was abundant in the cytoplasm, but was not evident in the nuclei of HSV-1
infected cells. Scale bar = 10 µm.
Figure 11

ICP0*GFP-24 translocates to the cytoplasm of HSV-1 infected cells in the presence or absence of ICP4.
ICP0\textsuperscript{GFP-24} protein in Vero cells inoculated with 10 pfu per cell of HSV-1 0\textsuperscript{GFP-24} (left column) or HSV-1 0\textsuperscript{GFP-24-Δ4} (right column) between 3 and 8 hours p.i. White asterisks denote cells in which ICP0\textsuperscript{GFP-24} was observed in a linear pattern encircling the nucleus of HSV-1 infected cells. Scale bar = 10 µm.

3.2.2. Cytoplasmic translocation of ICP0 is an early event in the HSV-1 replication cycle

To determine if cytoplasmic translocation of GFP-tagged ICP0 was dependent upon the synthesis of other HSV-1 IE, E, or late (L) proteins, chemical inhibitors were used to subdivide HSV-1 protein synthesis into its different phases. Vero cells were treated with cycloheximide to block protein translation, and cells were inoculated with 5 pfu per cell of HSV-1 0\textsuperscript{GFP-105}. After allowing 6 hours for the accumulation of viral IE mRNAs, cycloheximide was removed and cultures were released into medium containing either actinomycin D (mRNA synthesis inhibitor, Ref. (90)), acyclovir (DNA synthesis inhibitor, Ref. (91)) or vehicle (no inhibitor).

In HSV-1 0\textsuperscript{GFP-105}-infected cells released into medium containing actinomycin D, ICP0\textsuperscript{GFP-105} remained in the nuclei of >90% of cells at 1 and 4 hours post-release from a cycloheximide block (Fig. 12A). Thus, translocation of ICP0\textsuperscript{GFP-105} failed to occur when only viral IE proteins were efficiently expressed. When HSV-1 0\textsuperscript{GFP-105}-infected cells were released into medium containing acyclovir (IE and E proteins expressed), ICP0\textsuperscript{GFP-105} was initially observed in nuclei, but translocated to the cytoplasm of most cells by 4 hours post-release (Fig. 12A). Likewise, when cycloheximide treatment was followed with vehicle,
ICP0\textsuperscript{+GFP-105} translocated to the cytoplasm with similar kinetics (Fig. 12A).

Parallel tests with HSV-1 0\textsuperscript{+GFP\textsubscript{12}}, 0\textsuperscript{+GFP\textsubscript{24}}, and 0\textsuperscript{+GFP\textsubscript{24-Δ4}} yielded equivalent results (not shown). These results suggested that the nuclear-to-cytoplasmic translocation of ICP0\textsuperscript{+GFP-105} occurred during the E phase of HSV-1 replication.

To determine if these results were relevant to wild-type ICP0, similar tests were performed with wild-type HSV-1 strain KOS (69). When cycloheximide treatment was followed with actinomycin D, wild-type ICP0 was retained in the nuclei of KOS-infected cells at 4 hours post-release (Fig. 12B). In contrast, when cycloheximide treatment was followed with acyclovir or vehicle, wild-type ICP0 translocated to the cytoplasm by 4 hours post-release and was observed in globular and linear structures that encircled the nuclei of >90% of KOS-infected cells (Fig. 12B). Therefore, both GFP-tagged ICP0 and wild-type ICP0 were predominantly nuclear during the IE phase of viral infection, but rapidly translocated to the cytoplasm when viral E protein synthesis was allowed to occur.
ICP0 translocates to the cytoplasm during early phase of HSV-1 replication.

(A) ICP0^{GFP-105} protein in Vero cells inoculated with HSV-1 0^{GFP}{_{105}} following release from cycloheximide block. Cultures were inoculated with 5 pfu per cell of 0^{GFP}{_{105}}, treated with 200 μM cycloheximide from -0.5 to 6 hours p.i., and released into medium containing actinomycin D (10 μg / ml), 300 μM acyclovir, or vehicle. Cultures were photographed at 7 and 10 hours p.i. (B) ICP0 in HSV-1 KOS-infected Vero cells following release from cycloheximide block. Cultures were inoculated with 5 pfu per cell of KOS, treated with cycloheximide from -0.5 to 6 hours p.i., released into medium containing actinomycin D, acyclovir, or vehicle, and fixed for immunofluorescent staining at 7 and 10 hours p.i. White asterisks denote cells in which ICP0 was observed in a linear pattern encircling the nucleus of HSV-1 infected cells. Scale bar = 10 μm.
3.2.3. ICP0\(^{\text{GFP-105}}\) disperses linear structures in the cytoplasm of HSV-1 infected cells

The cytoplasmic structures in which ICP0 accumulated did not appear to be mitochondria, lysosomes, Golgi apparatus, or endoplasmic reticulum (ER) based upon the failure of ICP0 to co-localize with MitoTracker dye (92), LysoTracker dye (93), Golgi marker β-COP (94), or the ER marker calreticulin (95). Moreover, in early attempts to isolate the fluorescent-ICP0 labeled cytoplasmic bodies by density gradient sedimentation, it was noted that the bodies rapidly dispersed upon homogenization of cells in ice-cold buffer (not shown). To gather further clues about the nature of these structures, live-cell imaging was used to study the dynamics of accumulation of ICP0\(^{\text{GFP-105}}\) in the cytoplasm of HSV-1 0\(^{+}\)GFP\(_{105}\)-infected cells.

Cycloheximide was used to alleviate ICP4-dependent repression of the ICP0\(^{\text{GFP}}\) gene (39), such that ICP0\(^{\text{GFP-105}}\) mRNA could accumulate to high levels and be synchronously translated upon cycloheximide release (39). At 1 hour post-release, ICP0\(^{\text{GFP-105}}\) was observed exclusively in the nuclei of HSV-1 0\(^{+}\)GFP\(_{105}\)-infected cells (Table 3). Between 2 and 4 hours post-release, ICP0\(^{\text{GFP-105}}\) had partially or completely translocated to the cytoplasm of >90% of HSV-1 0\(^{+}\)GFP\(_{105}\)-infected cells (Table 3). At 4 hours post-release, ICP0\(^{\text{GFP-105}}\) was observed in linear cytoplasmic structures in 16 ± 1% of 0\(^{+}\)GFP\(_{105}\)-infected cells (Table 3). However, by 6 hours post-release, ICP0\(^{\text{GFP-105}}\) was rarely observed in linear cytoplasmic structures (Table 3).
We considered the possibility that ICP0\textsuperscript{+GFP-105} might trigger the dispersal of cytoplasmic structures in much the same manner that ICP0 triggers the dispersal of PML nuclear bodies (30, 42). To test this hypothesis, a recombinant virus was constructed, HSV-1 0ΔRING, which encoded a GFP-tagged ICP0\textsuperscript{ΔRING} protein that was deleted of amino acids 105 – 221, and thus lacked ICP0’s RING-finger domain (15). When cycloheximide-release experiments were performed in cells inoculated with HSV-1 0ΔRING, the ICP0\textsuperscript{ΔRING} protein accumulated in linear cytoplasmic structures between 2 and 6 hours post-release (Table 3). Intriguingly, ICP0\textsuperscript{ΔRING} was rarely observed in globular bodies, but rather the protein was observed almost exclusively in linear cytoplasmic structures (Table 3). These findings suggested that ICP0\textsuperscript{ΔRING} stably accumulated in linear cytoplasmic structures, whereas ICP0\textsuperscript{+GFP-105} triggered the dispersal of these structures upon accumulating at these sites.

To test this hypothesis, live-cell imaging was used to track the fate of linear structures in which ICP0\textsuperscript{+GFP-105} accumulated. Cells were inoculated with HSV-1 0\textsuperscript{+GFP-105} and time-lapse photography was conducted between 4.0 and 4.5 hours post-release from a cycloheximide block. Linear, cytoplasmic arrays of ICP0\textsuperscript{+GFP-105} that initially encircled the nucleus were found to be unstable, and dispersed into smaller, globular bodies over a 10- to 20-minute time frame (Fig. 13A). In contrast, experiments with HSV-1 0ΔRING demonstrated that the ICP0\textsuperscript{ΔRING} protein stably accumulated in linear cytoplasmic structures which grew visibly in length and thickness between 4.0 and 4.5 hours post-release (Fig. 13B). This trend continued until 6 hours post-release, at which time the
experiment was terminated (not shown). Therefore, ICP0\textsuperscript{GFP-105} translocated to the cytoplasm, accumulated in linear cytoplasmic structures, and then triggered the dispersal of these structures in a RING-finger-dependent manner.

### Table 3

**Subcellular distribution of ICP0\textsuperscript{GFP} in HSV-1 infected cells at times after release from a cycloheximide block\textsuperscript{a}**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Hours</th>
<th>Subcellular distribution\textsuperscript{b}</th>
<th>Cytoplasmic pattern\textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ICP0\textsuperscript{GFP} in nucleus</td>
<td>ICP0\textsuperscript{GFP} in cytoplasm</td>
</tr>
<tr>
<td></td>
<td></td>
<td>only</td>
<td>in cytoplasm</td>
</tr>
<tr>
<td>0\textsuperscript{GFP-105}</td>
<td>1</td>
<td>100 ± 0\textsuperscript{d}</td>
<td>0 ± 0\textsuperscript{d}</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>9 ± 1</td>
<td>91 ± 1</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2 ± 0</td>
<td>98 ± 0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2 ± 0</td>
<td>98 ± 0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>2 ± 0</td>
<td>98 ± 0</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>1 ± 0</td>
<td>99 ± 0</td>
</tr>
<tr>
<td>0\DeltaRING</td>
<td>1</td>
<td>100 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>40 ± 2</td>
<td>60 ± 2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>39 ± 1</td>
<td>61 ± 1</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>34 ± 1</td>
<td>66 ± 1</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>26 ± 1</td>
<td>74 ± 1</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>22 ± 1</td>
<td>78 ± 1</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Vero cells were inoculated with 5 pfu per cell of HSV-1 0\textsuperscript{GFP-105} or HSV-1 0\DeltaRING in the presence of 200 μM cycloheximide from -0.5 to 10 hours p.i., and were released into medium containing no drugs. Cultures were photographed.
between 1 and 6 hours post-release to record the position of all nuclei (visualized with Hoechst 33342) and the subcellular localization of ICP0^{GFP-105} or ICP0^{ΔRING}.

b Frequency of cells in which ICP0^{GFP-105} or ICP0^{ΔRING} was observed exclusively in the nucleus (left column) versus cells in which proteins were observed in either the cytoplasm and nucleus or just in the cytoplasm (right column).

c Frequency of cells (mean ± sem) in which ICP0^{GFP-105} or ICP0^{ΔRING} was observed exclusively in the nucleus. The frequency reported at each time point was calculated as 100 x number of cells in which ICP0^{GFP} was observed solely in the nucleus ÷ number of ICP0^{GFP+} cells. Each value is based on n = 4 independent cultures, and each estimate of frequency was based on n=400 Hoechst^{+} cells. Between 4 and 6 hours post-release, 95 ± 1% of cells in HSV-1 inoculated cultures were positive for the ICP0^{GFP-105} or ICP0^{ΔRING} proteins.

d Frequency of cells (mean ± sem) in which ICP0^{GFP-105} or ICP0^{ΔRING} was observed in the cytoplasm and nucleus, or just in the cytoplasm. Frequencies were calculated by the method described in footnote c.

e Frequency of ICP0^{GFP-105} or ICP0^{ΔRING} accumulating in linear arrays (left column) versus globular patterns (right column) in the cytoplasm of HSV-1 infected cells.

f Frequency of cells (mean ± sem) in which ICP0^{GFP-105} or ICP0^{ΔRING} were observed in linear cytoplasmic arrays that encircled Hoechst^{+} nuclei. The frequency reported at each time point was calculated as 100 x number of cells containing linear arrays of ICP0^{GFP} protein ÷ number of cells in which ICP0^{GFP} was evident in the cytoplasm. Each value is based on n = 4 independent cultures, and each estimate of frequency was based on n=400 Hoechst^{+} cells.

f Frequency of cells (mean ± sem) in which ICP0^{GFP-105} or ICP0^{ΔRING} were observed in globular, cytoplasmic bodies, which was calculated as described in footnote f.
Figure 13

ICP0\textsuperscript{+GFP-105} disperses linear cytoplasmic structures in a RING-finger-dependent manner.
Vero cells were inoculated with 5 pfu per cell of (A) HSV-1 0^+GFP_{105} or (B) HSV-1 0ΔRING in the presence of 200 μM cycloheximide from -0.5 to 10 hours p.i., and were released into medium containing no drugs. Between 4.0 and 4.5 hours post-release, time-lapse photographs were collected of the ICP0^+GFP^{-105} or ICP0^ΔRING proteins that encircled the nucleus of HSV-1 infected cells. Scale bar = 10 µm.

3.2.4. ICP0 co-localizes with disrupted microtubule networks in HSV-1 infected cells

The linear cytoplasmic structures in which ICP0^+GFP^{-105} transiently accumulated were reminiscent of microtubule bundles formed by HSV-1’s VP22 protein (56). Therefore, we considered the possibility that ICP0^+GFP^{-105} and ICP0^ΔRING proteins might associate with host cell microtubules. To test this hypothesis, cycloheximide-release experiments were performed in cells inoculated with HSV-1 0ΔRING, HSV-1 0^+GFP_{105}, or wild-type HSV-1 KOS. At 4 hours post-release, cells were fixed and immunofluorescently stained for α-tubulin and ICP0.

In uninfected Vero cells, α-tubulin staining revealed a normal network of microtubules that radiated from the microtubule-organizing center at the periphery of the nucleus (Fig. 14). In cells inoculated with HSV-1 0ΔRING, α-tubulin staining revealed extensive thickening of microtubules into elongated bundles that circumscribed the nucleus, and co-localized with the ICP0^ΔRING protein (Fig. 14). In cells inoculated with HSV-1 0^+GFP_{105}, α-tubulin staining
revealed that the microtubule network had dispersed into small $\alpha$-tubulin$^+$, globular bodies that co-localized with ICP0$^{\text{GFP-105}}$ (Fig. 14). In cells inoculated with wild-type HSV-1 KOS, the host cell microtubule network was also disrupted, and $\alpha$-tubulin was dispersed into $\alpha$-tubulin$^+$ globular bodies that co-localized with ICP0 (Fig. 14). Although the ICP0$^{\text{RING}}$ protein bundled microtubules, it failed to trigger their dispersal into globular bodies (Fig. 14). These results raised the possibility that HSV’s E3 ligase, ICP0, might contribute to microtubule disassembly in HSV-infected cells (55). Further experiments were conducted to test the validity of this hypothesis.
ICP0 co-localizes with reorganized microtubules in HSV-1 infected cells.

Immunofluorescent staining of Vero cells that were uninfected (no virus) or that were inoculated with 5 pfu per cell of HSV-1 0ΔRING, HSV-1 0^+GFP_{105}, or wild-
type HSV-1 strain KOS. Cells were inoculated in the presence of 200 μM cycloheximide from -0.5 to 10 hours p.i., and were released into medium containing no drugs. At 4 hours post-release, uninfected cells and KOS-infected cells were fixed and stained with antibodies against α-tubulin (rabbit IgG, Alexa Fluor 594) and ICP0 (mouse IgG, fluorescein). Nuclei were counterstained with the DNA-binding dye Hoechst 33342. HSV-1 ΔRING and 0^ΔGFP105-infected cells were fixed and stained for α-tubulin, and the ICP0^ΔRING and ICP0^ΔGFP105 proteins were visualized using their GFP fluorophores. Scale bar = 10 μm.

3.2.5. ICP0^ΔRING protein accumulates in linear structures that are nocodazole-sensitive

If microtubules did indeed provide the underlying scaffold on which ICP0^ΔRING protein accumulated in linear arrays (Fig. 13, 14), then inhibition of microtubule polymerization with nocodazole (96) should recapitulate the effect of ICP0’s RING finger domain. Specifically, nocodazole treatment, like wild-type ICP0, should trigger microtubule disassembly and cause linear accumulations of ICP0^ΔRING protein to disperse into smaller bodies. To test this prediction, cells were infected with HSV-1 ΔRING in the presence of cycloheximide for 10 hours, and were released into medium containing no inhibitor. Four hours later, the stability of linear arrays of ICP0^ΔRING protein was compared in the presence or absence of nocodazole.

In the absence of nocodazole, ICP0^ΔRING protein stably accumulated in linear structures that encircled the nucleus of HSV-1 ΔRING-infected cells.
between 4.0 and 4.5 hours post-release (Fig. 15A). In contrast, nocodazole treatment caused linear accumulations of ICP0\(^{\Delta RING}\) protein to disperse into small globular bodies within 10 to 30 minutes, and the time required for dispersal varied in proportion to the thickness of each bundle (Fig. 15B). Nocodazole-induced, ICP0\(^{\Delta RING+}\) globular bodies were identical in appearance to \(\alpha\)-tubulin\(^+\) globular bodies that co-localized with ICP0 and ICP0\(^{\ast GFP-105}\) (Fig. 14). Independent tests verified that nocodazole-induced, ICP0\(^{\Delta RING+}\) globular bodies were also \(\alpha\)-tubulin\(^+\) (not shown).

These observations indicated that the linear cytoplasmic structures in which ICP0\(^{\Delta RING}\) protein accumulated were indeed microtubule bundles. This finding was also consistent with our prior observation that ICP0\(^{\ast GFP-105}\)-containing bodies rapidly dispersed upon homogenization in ice-cold buffer; a condition known to cause depolymerization of microtubules (97).
ICP0ΔRING accumulates in linear structures that are nocodazole-sensitive.

Vero cells were inoculated with 5 pfu per cell of HSV-1 0ΔRING in the presence of 200 μM cycloheximide from -0.5 to 10 hours p.i., and were released into
medium containing no drugs. At 4 hours post-release, a representative field of view was chosen, and cells were treated with (A) vehicle or (B) 10 µg/ml nocodazole. Between 4.0 and 4.5 hours after cycloheximide release, time-lapse photographs were captured at 10-minute intervals. White asterisks denote cells in which ICP0\(^{\text{RING}}\) was observed in a continuous, linear pattern encircling the nucleus of HSV-1 infected cells. Scale bar = 10 µm.

3.2.6. ICP0 is necessary for efficient dispersal of microtubules in HSV-1 infected cells

It is well established that the host cell microtubule network is disrupted during the course of HSV-1 infection (55, 98, 99). However, it is unclear what process triggers microtubule reorganization in HSV-1 infected cells. We questioned whether this event might be ICP0-dependent. To test this hypothesis, \(\alpha\)-tubulin and ICP0 staining were compared in Vero cells that were uninfected (UI) or were inoculated with HSV-1 KOS, HSV-1 \(0^+\text{GFP}_{105}\), or an \(ICP0^-\) null virus, HSV-1 \(0^-\text{GFP}\). Cells were inoculated with 5 pfu per cell of each virus in the presence of cycloheximide for 10 hours to allow high and equivalent levels of ICP0\(^+\) and ICP0\(^-\) mRNAs to accumulate (39). At 1 and 4 hours post-release, a normal distribution of \(\alpha\)-tubulin staining was noted in uninfected Vero cells; specifically, microtubules radiated from the microtubule-organizing center on one side of the nucleus (Fig. 16A). At 1 hour post-release, ICP0 and ICP0\(^{+\text{GFP}-105}\) were observed in the nuclei of cells infected with HSV-1 KOS and \(0^+\text{GFP}_{105}\) (Fig. 16A). Importantly, \(\alpha\)-tubulin staining was always normal when
ICP0 or ICP0$^{\text{GFP-105}}$ were confined to the nucleus (Fig. 16A). However, at 4 hours post-release, ICP0 and ICP0$^{\text{GFP-105}}$ had translocated to the cytoplasm and this event coincided with dispersal of microtubules in KOS- and 0$^{\text{GFP-105}}$-infected cells, respectively (Fig. 16B). Specifically, microtubule-organizing centers were no longer discernible, and α-tubulin was dispersed into globular bodies that co-localized with ICP0 or ICP0$^{\text{GFP-105}}$ (Fig. 16B). In contrast, at 1 and 4 hours post-release, cells inoculated with HSV-1 0-GFP retained an intact microtubule network that radiated from a perinuclear microtubule-organizing center (Fig. 16A, 16B). Likewise, at 6 and 8 hours post-release, microtubule networks remained intact in HSV-1 0-GFP-infected cells (not shown). Therefore, synthesis of ICP0 appeared to be necessary for the efficient dismantling of microtubule networks that normally occurs in HSV-1 infected cells (55).
ICP0 is necessary for the dissolution of microtubule networks in HSV-1 infected cells.

Vero cells were uninfected or were inoculated with 5 pfu per cell of HSV-1 strain KOS, HSV-1 0\(^{+}\)GFP\(_{105}\), or HSV-1 0\(^{-}\)GFP in the presence of 200 \(\mu\)M cycloheximide from -0.5 to 10 hours p.i., and were released into medium containing no drugs until the time of fixation. At (A) 1 hour post-release or (B) 4 hours post-release, uninfected and KOS-infected cells were fixed and stained with antibodies against
α-tubulin (rabbit IgG) and ICP0 (mAb H1083). HSV-1 0’GFP\textsubscript{105} or 0’GFP-infected cells were fixed and stained for α-tubulin, and ICP0\textsuperscript{+GFP-105} and ICP0\textsuperscript{-GFP} proteins were visualized using their GFP fluorophores. Nuclei were counterstained with the DNA-binding dye Hoechst 33342. Scale bar = 10 µm.

3.2.7. ICP0 is sufficient to dismantle microtubule networks in transfected cells

The HSV-1 proteins US11 (100), UL34 (101), VP26 (102) and VP22 (56) interact with host cell microtubules. Thus, ICP0 might be one of many HSV proteins that is required to disperse the intracellular ‘freeway system’ that is the cell’s microtubule network (103). Alternatively, synthesis of ICP0 alone might be sufficient to disperse cellular microtubules. To differentiate between these possibilities, Vero cells were transfected with pICP0, p0\textsuperscript{+GFP-105}, or p0\textsuperscript{ΔRING} to determine if synthesis of ICP0 proteins affected the subcellular distribution of α-tubulin, one of the principal structural subunits of the hollow, polymeric tubes known as microtubules (104).

A normal distribution of α-tubulin staining was noted in mock-transfected Vero cells at 12 and 24 hours post-transfection. Specifically, microtubules radiated from the organizing center on one side of the nucleus, and α-tubulin staining was not observed in the nuclei of mock-transfected Vero cells (Fig. 17A, 17B). Consistent with prior results (Fig. 10A-10C), wild-type ICP0 and ICP0\textsuperscript{+GFP-105} were observed in the nuclei of cells transfected with pICP0 and p0\textsuperscript{+GFP-105} at 12 hours post-transfection (Fig. 17A). At this early time, a normal distribution of
α-tubulin staining was noted in the cytoplasm, but to our surprise significant amounts of α-tubulin were present in the nuclei and co-localized with wild-type ICP0 and ICP0^{+GFP-105} (Fig. 17A). Control staining with individual antibodies verified that the appearance of co-localization was not an unintended consequence of bleedover between the red and green channels (not shown). In cells transfected with p0ΔRING, the ICP0^{ΔRING} protein was also observed in the nuclei of cells at 12 hours post-transfection, and a normal distribution of α-tubulin staining was noted in the cytoplasm. However, large amounts of α-tubulin were present in the nuclei of transfected cells, and α-tubulin co-localized with the ICP0^{ΔRING} protein (Fig. 17A).

At 24 hours post-transfection, wild-type ICP0 and ICP0^{+GFP-105} were observed in the nuclei and cytoplasm of cells transfected with pICP0 and p0^{+GFP105}, respectively (Fig. 17B). Translocation of ICP0 or ICP0^{+GFP-105} to the cytoplasm consistently correlated with 1. dissolution of the host cell microtubule-organizing center and microtubule network, and 2. co-localization of α-tubulin with ICP0 or ICP0^{+GFP-105} in small, globular bodies in the cytoplasm (Fig. 17B). Likewise, 24 hours after transfection with p0ΔRING, ICP0^{ΔRING} protein co-localized with α-tubulin in elongated microtubule bundles (Fig. 17). Therefore, synthesis of the ICP0^{ΔRING} protein was sufficient to trigger bundling, but not dispersal of microtubules. In contrast, wild-type ICP0 was sufficient to trigger a complete dispersal of host cell microtubules.
ICP0 is sufficient to trigger the dissolution of host cell microtubule networks.

Vero cells were mock-transfected or were transfected with a VP16-expressing plasmid and pICP0, p$0^+$GFP$_{105}$, or p$0\Delta$RING. At (A) 12 hours post-transfection or (B) 24 hours post-transfection, mock- and pICP0-transfected cells were fixed and stained with antibodies against $\alpha$-tubulin (rabbit IgG) and ICP0 (mAb H1083). Cells transfected with p$0^+$GFP$_{105}$ or p$0\Delta$RING were fixed and stained to visualize
α-tubulin, and the ICP0\textsuperscript{RING} and ICP0\textsuperscript{GFP-105} proteins were visualized using their GFP fluorophores. Scale bar = 10 µm.

3.2.8. ICP0 translocation and microtubule dispersal in a single HSV-1 plaque

High MOIs and cycloheximide-release experiments allowed robust visualization of ICP0 and co-localization of ICP0 with microtubule breakdown products. These tests left unaddressed the question of whether or not ICP0 actually translocates and/or dismantles microtubule networks during the normal progression of HSV-1 infection. To address this issue, Vero cells were inoculated with wild-type virus at an MOI of 0.0001 pfu per cell, and the distribution of ICP0 and α-tubulin was analyzed in isolated HSV-1 plaques at 40 hours p.i. A single, representative plaque is considered to illustrate our findings (Fig. 18).

With the enhanced sensitivity of immunofluorescent staining (versus direct imaging of GFP fluorescence, Fig. 10), low levels of ICP0 were detectable in the nuclei of cells at the outermost, advancing edge of HSV-1 plaques (● symbols, Fig. 18A). In those HSV-1 infected cells in which ICP0 was observed solely in the nucleus, the perinuclear pattern of microtubule staining remained normal (● symbols, Fig. 18B). Behind this advancing front, a second row of HSV-1 infected cells was observed in which ICP0 was far more abundant and predominantly localized to the cytoplasm of HSV-1 infected cells (X symbols, Fig. 18A). When ICP0 accumulated in the cytoplasm of HSV-1 infected cells, gross reorganization
of the cellular microtubule network was observed (X symbols, Fig. 18B).

Moreover, α-tubulin was frequently observed reorganized into linear structures or globular bodies that co-localized with wild-type ICP0 (white arrows, Fig. 18).

Therefore, we conclude that regardless of MOI, a nuclear-to-cytoplasmic translocation of ICP0 routinely occurs in HSV-1 infected cells, and the timing of ICP0’s translocation to the cytoplasm coincides with a massive reorganization of the host cell’s microtubule network.
Figure 18

Translocation of ICP0 and disruption of host cell microtubules in a single HSV plaque.

Vero cells were inoculated with wild-type HSV-1 KOS virus at an MOI of 0.0001 pfu per cell, and cells were fixed and stained at 40 hours p.i. using antibodies against (A) ICP0 and (B) α-tubulin. Nuclei were counterstained with the DNA-binding dye Hoechst 33342. Cells in which ICP0 was observed solely in the
nucleus are denoted by a circle (●) to the right of the nucleus. Cells in which ICP0 was prominent in the cytoplasm are denoted with an ‘X’ to the right of the nucleus. White arrows denote cells in which α-tubulin was not only dispersed, but also co-localized with ICP0. Scale bar = 10 µm.
CHAPTER 4: DISCUSSION

At the beginning of the dissertation, I set out to address the questions 1) how is ICP0 gene regulated, and 2) what function does ICP0 assume during HSV infection. Regarding to the first question, the results of our study suggest that ICP0 gene is differentially regulated by virus-encoded repressor ICP4 and virus-encoded antirepressor ICP0, which hints herpesviruses might deploy bacteriophage λ-like strategy to regulate their genes. Regarding to the second question, the results of our study show that ICP0 dismantles the microtubule networks of HSV-infected cells, which indicates ICP0 might play a previously unrecognized role in the cytoplasm, and suggests a general function of many other viral E3 ligases. The specific discussion regarding each question is detailed below:

4.1. GENE REGULATION OF ICP0

4.1.1. Identification of an ICP0-antagonized repressor of HSV mRNA synthesis

HSV routinely establishes latent infections in its human host. During the latent phase of infection, most of the HSV genome is transcriptionally silent. It is unclear how the HSV genome is silenced, but it is generally agreed that
synthesis of ICP0 destabilizes repression and thus triggers reactivation of mRNA synthesis from latent HSV genomes (10, 11, 105).

The search for an ICP0-antagonized transcriptional repressor has led investigators to consider the possibility that PML and Sp100 (18), IRF-3 and IRF-7 (26), and centromere proteins CENP-B and CENP-C (27, 28) may represent the repressors of the HSV genome that are antagonized by ICP0. Although ICP0 may alter the subcellular distribution, stability, or function of these proteins, there is no direct evidence that these proteins are capable of restricting mRNA synthesis from the HSV genome. Likewise, it has been postulated that epigenetic silencing may be the ICP0-antagonized repressive mechanism that is capable of silencing the HSV genome (21, 53). However, no specific histone deacetylase or histone mark has been associated with more than a 2-fold reduction in HSV mRNA synthesis (19, 20, 53, 106).

Although an ICP0-antagonized repressor has not been identified, it would be reasonable to expect that the repressive entity: 1. should be necessary and sufficient to restrict the synthesis of one or more HSV mRNAs; 2. should be unable to silence HSV mRNA synthesis when ICP0 accumulates; and 3. may physically interact with ICP0. The results of the current study establish that HSV’s major transcriptional regulator, ICP4, meets these criteria, and thus appears to be the first bona fide ICP0-antagonized repressor of HSV mRNA synthesis.

The results clarify that ICP4 is capable of silencing mRNA synthesis from the ICP0 gene when ICP0 fails to accumulate. At low MOIs, HSV ICP0− viruses
establish quiescent infections in 99% of infected cells (12, 13). Intriguingly, ICP4 accumulates to detectable levels in >85% of cells that become quiescently infected with HSV ICP0− viruses (107). Thus, further studies will be required to determine if 1. ICP4 often accumulates in the absence of ICP0 in HSV quiescently infected cells (108-110), and if 2. ICP4 may contribute to quiescent HSV infections by restricting mRNA synthesis from HSV IE genes.

4.1.2. Differential regulation of ICP0 mRNA synthesis in HSV-infected cells

There is anecdotal evidence that the ICP0 gene is differentially regulated in HSV-infected cells, but it remains unclear how such regulation is achieved. The HSV latency-associated transcripts (LATs) have been discussed for their potential to repress ICP0 gene expression (111, 112). Likewise, VP16’s capacity to function as a transactivator of IE genes has been considered as a potential target of differential regulation (113-115). However, it remains unclear that LATs or VP16 are sufficient to explain how the ICP0 gene is regulated.

The ICP4-binding site in the ICP0 promoter was identified 20 years ago as a potential means by which ICP4 might regulate ICP0 mRNA synthesis (89). When studied in the context of HSV ICP0+ viruses, some investigators concluded that ICP4’s effect on ICP0 gene expression was negligible (37). Other investigators noted that ICP4 produced a modest decrease in ICP0 mRNA levels in the context of ICP0+ viruses (38), but ICP4’s full potential to silence ICP0 gene expression went unrecognized.
The current study demonstrates that the ICP0 gene is differentially regulated by three HSV proteins, VP16, ICP4, and ICP0, whose relationship to one another is not resolved. Functionally, the results suggest that VP16 and ICP4 ‘push’ ICP0 mRNA synthesis in opposite directions, and accumulation of ICP0 dictates which way this equilibrium will tilt. When ICP0 fails to accumulate, ICP4 is capable of silencing ICP0 mRNA synthesis despite VP16 in the tegument of virions (Figure 3-6; ICP0− treatments). When formation of the de novo repressor is blocked with cycloheximide, VP16’s full capacity to transactivate the HSV-1 ICP0 gene is revealed (Figure 5). If ICP0 accumulates, ICP4 no longer represses ICP0 mRNA synthesis (Figure 3-6; ICP0+ treatments). These findings suggest that a hierarchy exists in the HSV regulatory scheme in which i. ICP0-dependent antirepression of the ICP0 gene is dominant over ii. ICP4-dependent repression of the ICP0 gene, which is dominant over iii. VP16-dependent induction of the ICP0 gene. Therefore, while VP16 induces transcription of both the ICP4 and ICP0 genes, the opposing processes of ICP4-dependent repression and ICP0-dependent antirepression appear to dictate whether the ICP0 gene will remain ON or OFF in HSV-infected cells (Figure 3B).

4.1.3. ICP0 as a regulatory subunit of ICP4: a missing lynchpin in HSV gene regulation?

The 1298-amino-acid ICP4 protein functions as an essential activator that recruits cellular TATA-binding protein complexes to HSV genomes, and thus stimulates mRNA synthesis from HSV E and L genes (88). ICP4 also possesses
a second activity as a repressor of HSV IE genes, which requires the binding of 350 kDa ICP4 homodimers to consensus ATCGTC sequences (116, 117). To date, it remains unclear how the balance between ICP4’s opposing transcriptional repressor versus activator functions is controlled.

Since the discovery that ICP0 re-organizes PML nuclear bodies (30), ICP4 and ICP0 have been predominantly studied in isolation from one another. However, ICP0 was initially discovered as a factor that stimulated a 20-fold increase in ICP4-dependent mRNA synthesis from HSV E and L promoters (9). The results of the current study establish that ICP0 and ICP4 participate in a robust physical interaction in HSV-infected cells (Figure 9). We postulate that ICP0’s physical interaction with ICP4 is likely regulatory in nature, and hence may be relevant in explaining how ICP0 potentiates ICP4-dependent E and L mRNA synthesis (8, 9), as well as how ICP0 antagonizes ICP4-dependent repression of ICP0 mRNA synthesis (Figure 3 - 5). Therefore, we propose that the accumulation of ICP0 in HSV-infected cells (or lack thereof) may dictate whether ICP4 functions predominantly as an activator, or a repressor, of HSV mRNA synthesis. Further studies will be required to test the validity of this important hypothesis.

4.1.4. How does ICP0 antagonize ICP4-dependent repression?

What specific protein-DNA complexes explain how ICP0 antagonizes ICP4-dependent silencing of the \textit{ICP0} gene? The mechanisms that underlie the phenomenon documented in this study are unknown, and further study will be
required to differentiate between scores of possibilities. In an effort to illustrate this point, we present two distinct molecular models that are compatible with the results of the current study.

The first model, the ICP0-ICP4 Interference Model, is the most intuitively obvious interpretation and may reflect the mental model that many readers have invoked up to this point in the manuscript to make sense of the results. Under an ICP0-ICP4 Interference Model, it could be predicted that 1. ICP4 binding to HSV dsDNA serves the sole purpose of repressing mRNA synthesis from adjacent HSV genes, and that 2. ICP0 blocks ICP4’s capacity to bind dsDNA, and thus ICP0 blocks ICP4-dependent repression of the HSV ICP0 gene. However, synthesis of ICP4 is required for synthesis of viral E and L mRNAs, and mutations that disrupt ICP4’s DNA-binding domain also render HSV incapable of synthesizing its ~70 E and L mRNAs that are required for HSV replication (71, 83, 118). Therefore, it is difficult to envision how an ICP0-based activity that dislodges ICP4 from dsDNA would help explain how ICP0 potentiates ICP4-dependent synthesis of HSV E and L mRNAs (8, 9).

An ICP0-ICP4 Synergy Model is less intuitively obvious, but is more consistent with ICP0’s capacity to enhance ICP4-dependent synthesis of HSV E and L mRNAs. Under this latter model, it may be envisioned that 1. the functional purpose of ICP4 and the >50 ICP4-binding sites that span the HSV genome is to allow ICP4 to form a histone-like scaffold that coats HSV genomes; 2. the 1298 amino-acid ICP4 protein possesses numerous domains that allow ICP4 to interact with cellular proteins (119, 120) and viral proteins (Figure 9).
which influence whether ICP4 stimulates or represses mRNA synthesis from the underlying HSV DNA; and 3. the nature of ICP0’s interaction with ICP4 relates to potentiating ICP4-dependent activation of mRNA synthesis from HSV DNA. Clearly, further investigation will be required to determine if the specific protein-DNA interactions that occur in HSV-infected cells are consistent with an ICP0-ICP4 Synergy Model of HSV gene regulation.

4.1.5. Repression-antirepression model of \textbf{ICP0} gene regulation: evolutionarily conserved gene regulation scheme?

The herpesviruses and temperate bacteriophage are large dsDNA viruses that share the capacity to alternate between productive and silent infections. Many similarities have emerged in the mechanisms that herpesviruses and the tailed bacteriophage employ to replicate and encapsulate their large dsDNA genomes (121-124). Such observations point to a common evolutionary ancestry between these two ancient families of viruses (125, 126). We conclude that the results of the current study appear to add to this growing list of inexplicable similarities.

The parallel capacity of HSV and bacteriophage \( \lambda \) to alternate between productive and silent infections may not be a coincidence. Rather, both viruses may control the onset of viral replication through a system of repression and antirepression of a few IE proteins whose accumulation, or lack thereof, dictates the probability of replication. In \( \lambda \) phage, the ‘decision’ between productive versus silent infections hinges upon whether the \( \lambda \) cro antirepressor gene is
expressed or repressed (127-129). Thus, important questions follow from the observation that HSV encodes its own transcriptional repressor function, embedded in ICP4, which is capable of silencing the HSV antirepressor gene that encodes ICP0. Further study will be required to determine if, in fact, repression-antirepression of the ICP0 gene is relevant to the natural process by which HSV ‘decides’ whether a given infection will be productive or silent.

4.2. FUNCTION OF ICP0

4.2.1. ICP0 serves distinct roles in the nucleus and cytoplasm of HSV-infected cells

Studies of ICP0 have focused on the protein’s role in the nucleus (17, 27, 53, 54). Cytoplasmic ICP0 has been repeatedly observed (48-52), but its significance remains obscure for two reasons. First, the kinetics of ICP0’s nuclear-to-cytoplasmic translocation has been poorly defined. Thus, it has not been apparent that ICP0’s function in the cytoplasm is kinetically delayed and is only manifest once ICP0 fulfills its IE function in the nucleus (8, 9, 39). Second, the role that ICP0 fulfills in the cytoplasm of HSV-infected cells has not been clearly articulated. The results of the current study address these gaps in knowledge.

The results of the current study demonstrate that once E proteins accumulate, ICP0 translocates to the cytoplasm (Fig. 12) and there dismantles the host cell’s microtubule network. Five observations support this conclusion: 1.
Microtubule dispersal is ICP0-dependent in HSV-infected cells (Fig. 16); 2. Synthesis of ICP0 is sufficient to trigger microtubule dispersal (Fig. 17); 3. Dispersal of microtubule bundles is dependent upon ICP0’s RING finger domain (Fig. 14); 4. The timing of microtubule dispersal coincides with ICP0’s translocation to the cytoplasm (Fig. 16); and 5. α-tubulin+ breakdown products co-localize with ICP0 (Fig. 16).

4.2.2. GFP-tagged ICP0 yields new insights into the biology of ICP0

HSV-1 viruses that encoded chimeric ICP0*GFP proteins formed plaques with ~66% efficiency relative to wild-type HSV-1 (Fig. 1E). Using this new tool, we were able to observe GFP-tagged ICP0 in the act of bundling and/or dispersing microtubules in the cytoplasm of HSV-infected cells. These findings would be difficult to ascertain from analysis of fixed cells because ICP0’s cytoplasmic pattern is pleomorphic in HSV-infected cells. However, direct observation of a single HSV-infected cell in real time reveals the fluid process by which GFP-tagged ICP0 triggers the disassembly of subcellular structures, which proved to be part of the microtubule network (Figs. 13 and 14; Table 3).

4.2.3. Virus-induced reorganization of host cell microtubules

Many animal and plant viruses use host cell microtubule networks during their replication cycle (59-61). For example, movement protein of tobacco mosaic virus (130) and transmission factor of cauliflower mosaic virus (131) modify microtubules in plant cells. The human immunodeficiency virus Rev
protein forms dimers that bind microtubule ends, and inhibit their polymerization (132). The 3C protease of foot-and-mouth disease virus cleaves microtubule-associated protein 4, and thus excludes microtubules from cytoplasmic replication compartments (58, 133). Expression of Epstein-Barr virus’s LMP-1 protein or SV40’s large T antigen causes formation of aberrant microtubule structures via the respective modulation of microtubule-stabilizing proteins RASSF1 A (134) and TACC2 (59). While many viruses modify the microtubule network during their replication cycles, the functional significance of these interactions is often unclear. Thus, there is no clear virological precedent that explains why HSV should encode a protein, ICP0, that dismantles the microtubule network.

4.2.4. ICP0: a viral E3 ligase that orchestrates microtubule disassembly

The current study provides the first example of a viral E3 ligase that triggers microtubule disassembly. Most of the ~20 α-herpesviruses whose genomes have been sequenced encode RING-finger E3 ligases (65). These include varicella-zoster virus (135), pseudorabies virus (136), and channel catfish virus (137). Based on our results, we predict that each of these RING-finger E3 ligases will also 1. fulfill dual roles in the nucleus and cytoplasm of virus-infected cells, and 2. will dismantle microtubule networks upon their translocation to the cytoplasm. Clearly, further testing is required to test the validity of this prediction.

The α-herpesviruses are not alone in their use of E3 ligases as regulatory molecules. The Rta protein of Kaposi’s sarcoma herpesvirus possesses intrinsic
E3 ligase activity despite the absence of a canonical RING finger domain (138). Intriguingly, both ICP0 and Rta E3 ligases are sufficient to trigger reactivation of latent herpesviral infections (10, 139). Most viruses are too small to encode their own E3 ligases. Thus, smaller RNA and DNA viruses exploit ubiquitination as a regulatory mechanism by encoding adapter proteins such as adenovirus E1b, papillomavirus E6 and E7, or paramyxovirus V proteins that redirect cellular E3 ligases to substrates that benefit the virus (138, 140-143). It will be of interest to determine in coming years if any of these other viral E3 ligases reorganize host cell microtubule networks.

Many cellular E3 ligases play a prominent role in coordinating the complex events that transpire during mitosis, and which include disassembly of the microtubule network (63). For example, cullin 3 is an E3 ligase that regulates the activity of a microtubule-severing protein known as katanin (i.e., ‘sword’ in Japanese; Ref. (63, 64, 144)). Given the propensity of viruses to steal useful cellular functions (138, 145), our results raise the possibility that ICP0 may be derived from one of the many cellular E3 ligases that influences cell-cycle progression via regulating the disassembly and reassembly of microtubule networks (63). Specifically, our results imply that ICP0 possesses a mitosis-related regulatory activity that is manifest upon its translocation to the cytoplasm. Further investigation will be required to test the validity of this important hypothesis.
4.2.5. Is α-tubulin a substrate of ICP0’s E3 ligase activity?

The discovery of ICP0’s E3 ligase activity by Everett and colleagues (15, 16) arose from a need to explain why PML nuclear bodies were dispersed shortly after ICP0 arrived in these sub-nuclear structures (30, 42, 43). Although it seemed likely that PML would prove to be a direct substrate of ICP0’s E3 ligase activity, the development of a robust in vitro assay for ICP0’s E3 ligase activity demonstrated that this was not the case (15, 45).

It is possible that α-tubulin may be a direct substrate of ICP0’s E3 ligase activity, but the history of ICP0 research suggests that such speculation is premature. What should be considered, however, is the extraordinarily robust co-localization of α-tubulin and ICP0. For example, in transfected cells, large amounts of α-tubulin co-localized with ICP0 in the nucleus, which is not normally observed in uninfected cells (Fig. 17). These results suggest that newly synthesized ICP0 may be tethered to α-tubulin so rapidly in the cytoplasm that the ICP0-containing complexes that are imported into the nucleus may also contain sizable amounts of α-tubulin. Moreover, ICP0 co-localized with dispersed α-tubulin+ structures for hours after disassembly of the microtubule network (Fig. 14, 16, 17). In contrast, ICP0’s nuclear interactions with PML (30, 43) and ICP4 (39, 80) appear to be far more transient in nature. Clearly, further work is needed to define how ICP0’s E3 ligase activity triggers microtubule disassembly in host cells, and to define why ICP0’s interaction with α-tubulin appears to be so much more stable than any other cellular protein identified to date.
4.2.6. ICP0-induced disassembly of microtubules versus G2/M cell cycle arrest

Synthesis of ICP0 causes dividing cells to arrest in the G2/M phase of the cell cycle (27, 46, 47). Two explanations for this phenomenon have been offered including ICP0-induced degradation of centromere protein-C (27), and ICP0-induced activation of a DNA damage response pathway (146). The current study suggests a third possibility; ICP0-induced disassembly of microtubules produces the same type of G2/M arrest that is caused by nocodazole (147).

The rationale for this proposal is explained. The microtubule bundles in which ICP0<sup>ΔRING</sup> stably accumulated (Figs. 13 and 14) were rapidly destabilized upon treatment with nocodazole (Fig. 15). The resulting ICP0<sup>ΔRING+</sup> globular bodies that were dispersed upon nocodazole treatment were α-tubulin<sup>+</sup>, and were indistinguishable in appearance from the ICP0<sup>+</sup> α-tubulin<sup>+</sup> globular bodies that formed in HSV-infected cells (Fig. 18). Thus, our results suggest that, like nocodazole, ICP0 triggers the rapid disassembly of microtubules.

Nocodazole causes cells to arrest in the G2/M phase of the cell cycle by blocking mitotic spindle formation, which is needed to align chromosomes on the metaphase plate prior to their segregation into daughter cells (147). Thus, nocodazole treatment causes cell division to arrest in prometaphase (148). Likewise, synthesis of ICP0 has nocodazole-like effects on cell division (46, 47). Specifically, chromosome condensation occurs normally in cells treated with ICP0-expressing viruses (27) or nocodazole (148), but in both cases the
condensed chromosomes fail to align on metaphase plates (27, 148). Further work will be required to test the validity of this interpretation, and determine if ICP0-induced disassembly of microtubules explains why synthesis of ICP0 causes cell division to stall during mitosis.

4.2.7. Functional consequences of host cell microtubule networks disruption by herpesviruses

In recent years, the list of viruses that interact with cellular microtubules has grown in length, but the general significance of the phenomenon remains unclear. Herpesviruses rely on microtubules as a ‘conveyor belt’ to carry their incoming virions from the cell membrane to the nuclear pore (103, 149). Therefore, it is conceivable that disruption of this conveyor belt may be critical for virion egress, such that newly formed herpesvirus virions may flow efficiently in the reverse direction, from the nucleus back to the cell membrane. Of course, many other possibilities exist and warrant equal consideration. For example, ICP0-induced disruption of microtubules may elicit massive changes in the cell’s metabolism that may be needed late in infection to produce the vast quantities of energy and protein required during the logarithmic phase of virion synthesis (150). Likewise, if microtubule disassembly is linked to ICP0’s capacity to disrupt mitosis, it is conceivable that microtubule disassembly could provide signals that would cause the host cell to respond by producing a whole suite of DNA repair enzymes that might be critical for the synthesis, recombination, and/or packaging of HSV DNA (54). Whatever the correct explanation proves to be, it should
account for the fact that modulation of microtubules is not HSV-specific, but rather is likely critical to the herpesviruses as a group. In light of these considerations, it will be of particular interest to determine in coming years if viral E3 ligase-dependent dismantling of microtubule networks is unique to the herpesviruses, or rather is paralleled in the replication strategies of other plant and animal viruses (151-153).
CHAPTER 5: CONCLUSION

Two questions were posed at the beginning of the dissertation: 1) how is \textit{ICP0} gene regulated, and 2) what function does ICP0 assumes. Regarding to the first question, we found that \textit{ICP0} gene is subject to potent ICP4-dependent repression immediately following HSV infection. ICP0 physically interacts with ICP4, and antagonizes ICP4’s repressive activity. Therefore, we conclude that \textit{ICP0} gene is differentially regulated by virus-encoded repressor ICP4 and virus-encoded antirepressor ICP0. The balance between ICP0 and ICP4 likely determines the “ON” and “OFF” of \textit{ICP0} gene, which contributes to the lytic versus latent decision of HSV.

Our pursuit of the second question began as we unexpectedly found that ICP0 is predominantly localized to the cytoplasm of HSV-infected cells. Further investigations showed that ICP0 translocates from nucleus to cytoplasm during the early phase of infection, where it bundles and disperses host cell microtubule networks. The RING-finger domain, which confers E3 ligase activity to ICP0, is required to the microtubule-dispersing activity, not the microtubule-bundling activity. Our study represents the first report of a virus-encoded E3 ligase that disrupts microtubule networks of the host cell. Since nearly all the \(\alpha\)-herpesviruses encode an E3 ligase, our findings hint a general function of virus-encoded E3 ligase, which is to dismantle microtubule networks.
Finally, we conclude that ICP0 assume dual functions during the life cycle of HSV: 1) In the very early stage of HSV replication, ICP0 is an exclusively nuclear protein, which promotes HSV gene expression through antagonizing ICP4’s repressive activity; 2) In the very late stage of HSV replication, ICP0 translocates to the cytoplasm, where it dismantles host cell microtubule networks, which may facilitate viral spread.


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