MODELING PHYSICAL CHANGES IN HSV GENOMES THAT OCCUR DURING LYTIC AND LATENT INFECTIONS: THE ROLE OF ICP0.

by

Sara Ann Jackson

BS, Allegheny College, 2000

Submitted to the Graduate Faculty of

University of Pittsburgh in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2005

UNIVERSITY OF PITTSBURGH

FACULTY OF ARTS AND SCIENCES

This dissertation " Modeling physical changes in HSV genomes that occur during lytic and latent infections: the role of ICP0" was presented

by

Sara Ann Jackson

It was defended on

October 19, 2005

and approved by

Dr. Neal DeLuca, Ph.D.
Dissertation Director/Major Advisor
Department of Molecular Genetics
and Biochemistry

Dr. Stephen Phillips, Ph.D.
Department of Molecular Genetics
and Biochemistry

Dr. Donald DeFranco, Ph.D.
Department of Pharmacology

Dr. Richard Wood, Ph.D.
Department of Pharmacology

Dr. Saleem Khan, Ph.D.
Department of Molecular Genetics
and Biochemistry

MODELING PHYSICAL CHANGES IN HSV GENOMES THAT OCCUR DURING LYTIC AND LATENT INFECTIONS: THE ROLE OF ICP0

Sara Ann Jackson, Ph.D.

University of Pittsburgh, 2005

Herpes Simplex Virus (HSV) is a pervasive human pathogen that can establish both symptomatic productive infections and asymptomatic latent infections. During infection, the HSV genome undergoes physical changes that are regulated by cellular and viral proteins. These changes lead to either a template for genome replication during the productive cycle or a persistent stable genome configuration during latency. Changes in viral genomes and events leading to these changes during a particular life cycle are not clearly understood. Using both Gardella gel analysis for circular HSV genomes and restriction enzyme analysis for end-to-end linkages of HSV genomes, we show that HSV genomes circularize only in the absence of the HSV immediate early gene ICP0. In the presence of ICP0 genome circularization is inhibited. Although HSV replication has been previously thought to initiate by a theta mechanism from a circular genome template, these results suggest that HSV replication initiates from a linear genome template due to the presence of ICP0 during lytic infection. We also show that circular genomes are the stable form during long-term persistent infections that model latency. Because HSV genomes begin as linear, double-stranded DNA during infection, host cells may treat the ends of incoming genomes as DNA double strand breaks (DSB) and subsequently repair these ends by circularization of genomes. However, it is unclear if/how these DSB repair pathways contribute to the manipulation of HSV genome configurations during infection and how viral proteins, in particular ICP0, may alter/counteract this repair response to form a template for replication during the productive cycle. Here we show that the cellular DSB repair mechanism, non-homologous end-joining (NHEJ), is the major mechanism by which HSV genomes are circularized. ICP0 not only inhibits HSV genome circularization but also affects the abundance of proteins involved in NHEJ and the distribution of other repair proteins during infection. The study presented here begins to uncover how the interplay between host cell repair responses and the virus' reply to these responses contributes to forming either a genome template for replication during the productive cycle or a persistent stable configuration during latency.

TABLE OF CONTENTS

LIST OF FIGURES

1. INTRODUCTION

1.1. Overview

The primary goal of viruses is not to kill, but to reproduce resulting in progeny that will spread and again initiate the infectious cycle. Viruses lack the machinery to replicate independently, and subsequently require a host to provide many of the necessities required for replication. Viruses have evolved mechanisms to manipulate hosts into facilitating this replication during infection. However, many of the normal physiological processes of the host are compromised by the virus, and consequently the host attempts to inhibit many of the processes required for virus production. Viruses have adapted mechanisms to evade this host anti-viral response. One such escape mechanism is the ability of viral genomes to persist in cells in a non-toxic, quiescent state called latency until an appropriate environment for virus production is stimulated.

Latency is a hallmark of herpesvirus infections and is analogous to the well-studied lambda bacteriophage lysogenic state, during which most genes encoded by the integrated bacteriophage genome are silent and only few bacteriophage genes that maintain lysogeny are expressed (142). As with lysogenic bacteriophage infections, during latent herpesvirus infections only a limited group of viral genes are expressed, but the productive cycle can be readily stimulated. The cycling between productive and latent infection provides a continuous mechanism for herpesvirus production. During the productive, or lytic, cycle all herpesviruses share a general progressive cascade of gene expression that is executed through a combination of virus and host cell transcription and translation machinery. The earliest genes expressed in this cascade prepare the cell for further viral gene expression, which in turn promotes virus

replication and production of progeny virions. In the case of herpes simplex virus (HSV), this cascade of gene expression is initiated by a combination of host cellular factors and viral proteins provided by the infecting virion.

During HSV-1 productive infection, the tegument protein VP16 and host factors bind motifs present in HSV promoters and thereby initiate transcription of the first class of genes within the progressive cascade. Of the genes activated by this complex (ICP0, ICP4, ICP22, ICP27, and ICP47), four of them (ICP0, ICP4, ICP27, and ICP22) promote further viral gene expression. The ability of these genes to facilitate and in some cases, activate, viral gene expression largely depends on the cellular proteins/mechanism with which each protein interacts. Of these four genes, only ICP0 leads to indiscriminant transactivation of both cellular and viral genes. ICP0 is a major regulator of the balance between productive and latent infections, with its presence facilitating the productive cycle both during primary infections and reactivation from latency. ICP0 is the only HSV protein that induces gene expression from otherwise repressed HSV genomes during latency. One relatively well characterized activity of ICP0 results in degradation of cellular proteins. It follows that ICP0 is thought to create conditions permissive for viral gene expression and replication through inhibiting repressors of the productive cycle.

During infection, the HSV genome undergoes physical alterations that are regulated by both cellular and viral proteins. These changes ultimately lead to a template for genome replication during the productive cycle or a persistent stable genome configuration during latency. During the productive cycle HSV genomes are very dynamic: they actively express genes, replicate, and are packaged into progeny virions. During latency viral genomes are somewhat static, persisting in a stable transcriptionally repressed state. Changes in HSV genomes and events leading to these changes during a particular life cycle are not clearly

understood. This is largely due to the fact that little is known about how the host cell responds to incoming HSV genomes and how the virus manipulates this response. Because HSV genomes begin as linear, double-stranded DNA during infection, host cells may treat the ends of incoming genomes as DNA double-strand breaks (DSB). However, it is unclear if/how these DSB repair pathways contribute to manipulation of HSV genome configurations during infection and how viral proteins may alter/counteract this repair response. The study presented here begins to uncover how the interplay between host cell repair responses and the virus' reply to these responses contributes to forming either a genome template for replication during the productive cycle or a stable configuration during latency. It highlights the role of ICP0 as both a major facilitator to productive infection and a regulator of genome configuration.

1.2. Herpesviridae

Herpesviruses consist of a large family of viruses that infect all groups of vertebrates, including humans. There are eight known human herpesviruses: herpes simplex virus type-1 and type-2 (HSV1 and HSV2), human cytomegalovirus (HCMV), varicella-zoster virus (VZV), Epstein-Barr virus (EBV), and human herpes virus 6, 7, 8 (HHV6, HHV7, and HHV8). These viruses cause a variety of significant diseases in humans. For example, HSV1 and HSV2 cause severe facial and genital lesions, respectively; VZV causes chicken pox and then shingles during reactivation from latency; and EBV causes mononucleosis and B cell lymphomas. In immunocompromised patients, these infections can be more severe, with otherwise mild HCMV and HHV8 infections developing into retinitis and Kaposi's sarcoma, respectively. (162)

All members of the Herpesvirus family share structurally similar virions separated into layers containing a linear double-stranded DNA genome ranging from 120-230 KB at the core (162). The viral DNA is packaged into an icosohedral structure composed of 162 capsomer

proteins (162). Surrounding the capsid is an amorphous tegument region that can contain viral proteins responsible for initiation of the productive cycle. Surrounding the tegument is a lipid envelope from which glycoprotein spikes protrude to the outer surface (156). These glycoproteins facilitate interactions with host cells and many times designate the tropism of each virus for a particular tissue or cell (156).

Although all share the ability to form productive infections and persist in a latent state from which the productive cycle can be stimulated, herpesviruses can be divided into three groups: alpha, beta, and gamma (162). Classification into these three groups is based upon tissue tropism, pathogenicity, and properties during infection of cultured cells (162). Alphaherpesviruses can infect a broad range of cells and hosts, in which they replicate vary rapidly and can reside during latency in neurological tissues. Of the human herpesviruses, HSV1, HSV2, and VZV are classified as alphaherpesviruses. Betaherpesviruses and gammaherpesviruses infect a much more restricted host range, in which they replicate slowly in cells of glandular and/or lymphatic nature. HCMV, HHV6, and HHV7 are classified as betaherpesviruses, while EBV and HHV8 are classified as gammaherpesviruses. Understanding each herpesvirus within the context of its sub-group can be useful in understanding the functions of homologous viral genes during various herpesviral infections, albeit in different environments.

1.3. HSV pathogenesis

As with all herpesviruses, HSV can establish either a symptomatic productive (lytic) infection or an asymptomatic latent infection. HSV enters the host by infection of epithelial cells, causing painful vesicular lesions primarily on the face and genitals, which are the typical sites of human-to-human transmission (162). Primary infection with HSV can lead to life-long, latent infection of sensory neurons. Although latent infections are asymptomatic, periodic reactivation from

latency produces recurrent lytic infections, resulting in recurrent lesions. Common types of stresses, such as ultra-violet light, menses, malnutrition, excessive fatigue, and anxiety can induce reactivation (196). Recurrent lytic infections are highly prevalent in immunocompromised patients and patients receiving immunosuppressive drug therapy. These individuals are more vulnerable to the more serious consequences of HSV disease including blindness due to keratitis, organ failure due to visceral infections, and potentially even death due to encephalitis (196).

HSV disease begins with primary infections that result in productive replication in cells of the epidermis and dermis (162). At this point, progeny viruses may spread to nerves innervating the primary site of infection. HSV undergoes retrograde transport from nerve termini to the cell body where the viral genome can establish a latent state within the nuclei (32). During latency the HSV genome remains genetically intact and persists in a stable extrachromosomal state (126) that is associated with nucleosomes in a chromatin structure (40) from which gene expression is largely repressed (154, 165, 196) and the latency-associated transcript (LAT) is transcribed (196). Dynamic interactions between HSV, the host neuron, and the local immune system may contribute to maintaining latency, with $CD8^+$ T cells providing constant surveillance of infected neurons harboring HSV genomes that may express low-levels of productive cycle genes (97). These $CD8^+$ T cells may prevent the productive cycle and virion formation through IFN-γ production, which have inhibitory antiviral effects at various stages of virus replication (168), and through other yet undefined mechanisms (98). In response to environmental and in turn physiological stresses, repressed latent states can be reactivated by de-repression of the viral genome through unknown mechanisms that may be facilitated by ICP0 and involve the stress-induced loss of $CD8^+$ T cell surveillance and repression of HSV gene

expression (98). Progeny HSV then travels by anterograde transport down the axon of the nerve, spreading the infection to the primary site in which the innervating nerve was initially infected (32). Cycling between the productive and latent phases allows for a continual reservoir of virus that can cause recurrent lesions, resulting in life-long HSV disease.

1.4. HSV genome

Within virions, the HSV genome exists as a large (~152kb), linear double-stranded DNA molecule that contains single-strand nicks or gaps (161). The genome is divided into two regions of unique long (U_L) and unique short (U_S) sequences (Figure 1), which comprise approximately 82% and 18% of the total genome, respectively (99, 112, 122, 123). The U_L and U_S regions are each bracketed by inverted repeats (112). The terminal repeats of U_L and U_S occur in a direct orientation, while the joint repeats connecting the U_L and U_S regions occur in an inverted orientation with respect to their direct terminal repeats (112) (Figure 1). Although the repeat sequences are interrupted by some short unique sequences, for sake of simplicity, the repeats bracketing the U_L region have been designated *ab* and *b'a'*, while those bracketing the U_S region have been designated *ac* and *c'a'* (161) (Figure 1). The *a* repeat is approximately 250-500bp in length, depending on the HSV strain, and is located at the extreme termini of both U_L and U_S components and within the U_L/U_S junction (161). The *a* repeat occurs in only one copy at the U_S termini but in multiple copies at the U_L termini and within the joint repeats connecting the U_L and U_S regions (112).

Through recombination of the terminal direct repeats with the internal inverted repeats, the U_L and U_S regions may invert with respect to one another (36, 77). This recombination event may be initiated by multiple random double-strand breaks that arise during DNA replication across the inverted repeats of the genome or during cleavage events at the internal inverted "*a*"

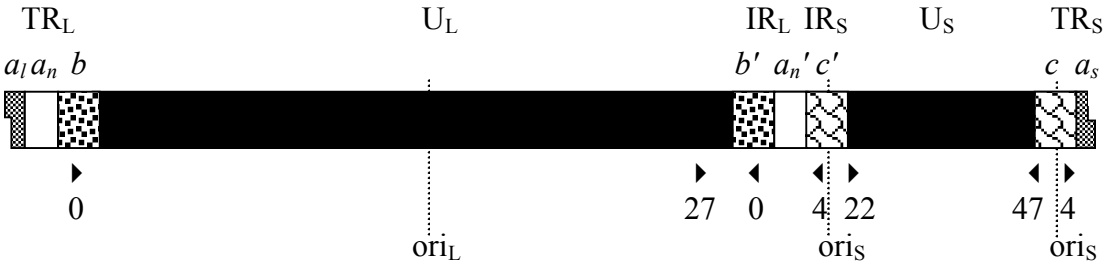


Figure 1. The HSV genome.

The unique-long (U_L) and unique-short (U_S) regions are bracketed by direct terminal repeats (TR_L and TR_S). Direct repeats are designated ($a_l a_n b$,) and ($c a_s$). These repeats are inverted within the internal repeats (IR_L and IR_S), which are designated (b', a_n', c') within the region connecting the U_L and U_S sequences. There are multiple '*a*' repeats where indicated (a_n). The one base overhang on the 3' strand of terminal *a* repeats is depicted by an incomplete rectangle (90). The position and direction of immediate early genes ICP 0, 4, 22, 27, and 47 are indicated, as are the positions of the origins of replication in each long and short region (ori_L and ori_S).

repeat sequences (114, 171, 179); viruses lacking inverted repeats do not invert (150). Subsequently, HSV genomes exist in four isomeric arrangements, in which the U_L region inverts, the U_S region inverts, both the U_L and the U_S regions invert, or neither the U_L nor the U_S region inverts. During infection, one isomer produces all four isomers in equimolar concentrations, suggesting a direct correlation between isomerization of viral genomes and replication (161).

The HSV genome contains approximately 80 genes, which are grouped into three classes based on their temporally regulated expression (161). As their names suggest, immediate early (IE), early (E), and late (L) genes are progressively expressed during HSV productive infection. The five IE genes, ICP 0, 4, 22, 27, and 47, cooperatively initiate the HSV productive cycle. These IE genes are largely located in the repeated sequences of the genome, while E and L genes are largely located in the U_L and U_S components (Figure 1). The genome contains two copies of the ICP0 and ICP4 genes within the repeats bracketing the U_L and U_S regions, respectively. In addition, the HSV genome contains three origins from which replication initiates. The U_L component contains one origin of replication and each *c* repeat region of the U_S component contains one origin of replication (Figure 1).

1.5. HSV productive infection

HSV recognizes potential host cells through interactions of glycoproteins that protrude from the lipid envelope of virions with glycoproteins on the cell surface, herparin sulfate proteoglycans, and cell surface receptors called herpesviral entry mediators (HVEM), which include HveA, HveB, and HveC (156). Following attachment, glycoproteins mediate the fusion of the virion lipid envelope with the cell plasma membrane, allowing the viral capsid to enter the cytoplasm of the cell. After entry, the capsid is transported to nuclear pores, where viral DNA is injected into the nucleus and may begin to express genes and replicate.

1.5.1. Cascade of viral gene expression

Viral gene expression progresses from immediate early genes (IE or alpha), to early genes (E or beta), and then to late genes (L or gamma), using host cell RNA polymerase II (RNA Pol II) and the host cell transcription machinery (79, 84, 120, 146). By definition, IE genes are expressed in the absence of *de novo* viral protein synthesis. Together, a tri-partite complex of VP16 supplied in the tegument of the infecting virion, the cellular octamer DNA-binding protein (oct-1), and the host cellular factor (HCF) protein initiate IE gene expression by binding to promoter elements (TAATGARAT) present in all HSV IE promoters (4, 17, 152). IE gene expression has been observed as early as 30 minutes postinfection in cell culture systems and peaks between 2 and 4 hours postinfection (83, 84). IE genes are the major regulators of viral gene expression and as such, have a variety of activities that all function in promoting the productive cycle. There are five IE genes expressed, which include infected cell polypeptides (ICP) 0, 4, 22, 27, and 47. Of the five IE genes, four are activators of viral gene expression. These gene products direct the expression of subsequent E and L viral genes

ICP4 is the main regulator of viral gene expression, activating both E and L viral genes (38, 52, 68, 69, 138, 155) through its interactions with the transcription machinery of the host (5, 20, 33, 71, 88, 180) and binding sites that recognize E and L promoters (5, 41). In the absence of ICP4, E and L genes are not transcribed and only the remaining IE genes are expressed (38). ICP4 also possesses the ability to repress viral gene expression (15, 38, 128, 138), which leads to over-expression of the remaining IE genes when ICP4 is absent (38). Like ICP4, ICP27 is required for the productive cycle and activates E and L gene expression (121, 166, 191). It also modulates mRNA processing on both the viral and cellular level, through regulating post-transcriptional modifications to cellular and viral RNA and the export of certain mRNA's from

the nucleus (74, 75, 122, 124, 125, 169). These modulations may contribute to ICP27's ability to shut-off off host protein synthesis. ICP22 facilitates L gene expression and is subsequently not necessary for virus replication. It affects the stability and processing of ICP0 transcripts (21) and the phosphorylated state of the RNA Pol II C-terminal domain (158). The phosphosphorylated state of RNA Pol II in turn affects the association and activity of RNA Pol II with viral promoters and thus transcription of these genes (158). ICP0 is an indiscriminant transactivator of gene expression and as such, is the only viral protein that activates all three classes of viral genes (24, 120). It affects proteins associated with a variety of cellular processes including those associated with ND10 structures (55, 116), cell cycle regulation (53, 94), transcription (95), translation (94), proteolysis (53, 55, 59), and DNA repair (107, 143). How these effects may underlie the ability of ICP0 to promote the productive cycle will be covered in detail in a section below, as it is a major focus of this report. Of the five IE genes, ICP47 is the only protein that does not induce cytotoxicity during infection and the only protein that does not direct transcriptional activation of other viral genes. Rather, ICP47 inhibits antigen presentation and subsequent recognition of infected cells by the immune system, in particular $CD8^+$ lymphocytes, through interactions with the transporter of antigen processing (TAP) molecule that disables transport of the major histocompatibility complex (MHC) class I and its associated antigens to the cell surface (2, 64, 80, 104, 190).

Aside from ICP47, IE gene products direct the expression of subsequent E and L viral genes. E gene expression peaks between 5 and 7 hours postinfection (83, 84), and these gene products mainly contribute to viral replication, encoding the viral DNA polymerase and other proteins necessary for replication, including the single-stranded DNA binding protein (ICP8), the DNA helicase-primase, the origin-binding protein (UL9), and enzymes involved in nucleotide

metabolism (10). After E protein synthesis begins, viral replication can occur as early as 3 hours postinfection and proceed up to 15 hours post infection (83, 84). Soon after replication initiates, L genes are expressed. L gene expression peaks at 8 hours postinfection, and the products of these genes mainly consist of structural proteins required for reassembly of mature progeny virions, which are released approximately 18 hours postinfection (83, 84).

1.5.2. Mechanism of replication

After IE and E gene expression, replication can proceed from three origins on the HSV genome, although none of these origins are uniquely required for replication (87, 113, 151, 181). A variety of DNA electrophoresis techniques have been used to examine the configuration of HSV genomes prior to and during the onset of replication, creating a model by which HSV replication initiates from a circular genome template. The first study to provide evidence for this template of genome replication was done by Poffenberger and Roizman (149). These investigators developed a non-inverting HSV genome by deleting internal inverted repeat sequences from the genome, subsequently creating a virus I358 that is held in one isomeric arrangement (149, 150). The authors have shown generation of end-to-end linkages of these viral genomes early during infection and in the presence of inhibitors to gene expression (149). They interpret the formation of these linkages to be indicative of intra-molecular joining of genome termini by circularization but admit that the formation of end-to-end linkages could also be indicative of inter-molecular joining of genome termini through concatemerization (149). Analysis for the progressive loss of genome termini has also suggested that HSV genomes circularize early during productive wild-type infection of CV-1 cells and both neuron-differentiated and undifferentiated rat-derived PC12 cells (186), but again, these results do not differentiate between concatemerization, circularization, and in these cases degradation of genome termini. Pulse field gel electrophoresis

(PFGE) has been used to differentiate between concatemerized and circularized HSV genomes. Early during productive infection, regardless of viral gene expression, HSV DNA is retained within PFGE wells, and this DNA is diminished for genome termini (65). Creating on average one double-strand nick in well-retained HSV DNA allows the HSV DNA to migrate a similar distance as one unit-length linear HSV genome, suggesting well-retained genomes are circular (65). However this study only demonstrated these unit-length circles in the absence of viral gene expression, not during the productive cycle when templates of replication are formed.

Together analyses for end-to-end linkages of genomes and PFGE studies have provided foundations for our current model of HSV replication, which initiates from a circular genome template via a theta mechanism and then proceeds into a rolling circle mechanism (162). This model is supported by the fact that only one viral genome is necessary for replication and the fact that the ends of viral genomes, which contain homologous direct *a* repeats, are suitable substrates for ligation or recombination of genome termini into circular genomes (162). Theta replication also eliminates loss of genetic material due to incomplete lagging-strand synthesis that would otherwise arise from successive cycles of semi-discontinuous replication of DNA genomes from linear templates. However, although the aforementioned studies have never clearly distinguished between circular and concatemeric forms during productive infection, they provide the only direct evidence for the initial theta mode of replication. This ambiguous evidence for the initial theta mode of replication has, in part, lead to a theory for the later rolling circle stage of replication, in which an initial circular template is necessary. Although there is evidence that genomes exist as highly branched concatemers during later stages of replication (7, 174, 175) and a rolling circle mechanism is predicted to produce concatemers of genomes (162), a more

thorough examination of the initial genome template for replication is required in order to understand the mechanism of replication at later stages.

In the present study, we challenge the previous model of HSV replication by providing for the first time evidence that replication is initiated from a linear genome template, while circular genomes are the stable configuration during latency (89). However, a very recent paper (185) has presented a rebuttal to our results. Using HSV viruses that lack '*a*' repeat sequences but contain parts of the HSV packaging signal at each termini, these authors suggest that end-to-end linkages of genomes indicative of circular genomes are present both early during infection in the presence of ICP0 and in the absence of HSV gene expression (185). However, there are many problems with the author's interpretation of their data, which arise from inherent complexities present within the technique these authors employed. These problems/complications will be addressed in Chapter 4, in which we provide further evidence that during the productive cycle ICP0 inhibits circularization of HSV genomes, providing a linear genome template from which HSV replication initiates.

1.5.3. Host cell death

After assembly and egress of mature progeny virions, HSV infected cells die. Cells accumulate damage to their cytoskeleton (42, 78) and chromatin (26, 73, 113, 139, 140, 182). They often fuse to form huge multi-nucleated cells and eventually round up. Cell death is in part due to the cytotoxic effects imposed by the activities and downstream effects of IE proteins. As described above, in order to carry out the productive cycle, IE proteins disrupt many of the normal processes of host cell metabolism, including those involved in transcription, translation, DNA repair, proteolysis, and cell cycle regulation. Although some of these disruptions are present to inhibit host anti-viral responses, such as antigen presentation, others are due to the virus'

requirement of cellular proteins/machinery. Viral factors coordinate the redistribution of necessary cellular proteins to sites of HSV replication and also shut-off host protein synthesis, freeing the translation machinery for viral protein synthesis. ICP27 has been shown to alter the export of host mRNA (74, 169, 170). In addition, the HSV virion host shut-off protein (*vhs*) that is provided in the tegument of the infecting virion affects mRNA stability by degrading ribosome associated mRNA (46, 101-103, 141). Together, these resulting changes in host cell metabolism are significant and likely to contribute to host cell death.

1.6. HSV latency

Latent HSV infections are established in sensory neurons that innervate the primary site of productive infection. During HSV latency, the productive cycle of viral gene expression is repressed (154, 165, 187) and only LAT's, whose function remains obscure, are transcribed (196). From a latent state, the productive cycle can be readily stimulated by various physiological stresses and also ICP0 (15, 16, 29, 196). In fact, ICP0 is the only HSV protein that can stimulate gene expression from repressed genomes. During latency HSV genomes persist in an extrachromosomal state (126) that is bound by nucleosomes in a chromatin arrangement (40), which may contribute to the stability and transcriptional repression of the genome. The ability of HSV genomes to adopt a repressed, stable state is necessary for its non-toxic persistence during these life-long infections. However, this process must be reversible for the productive cycle to occur during reactivation.

In order to understand how the physical characteristics of HSV genomes during latency may contribute to its stability and persistence, many studies have examined the configuration of HSV genomes during persistent infections *in vitro* and during latent infections *in vivo*. DNA isolated from the trigeminal ganglia of both latently infected mice and humans is depleted for

genome ends (44), suggesting that during latency the genome exists in an endless, potentially circular state. Neuronal-differentiated PC12 cells have provided an *in vitro* model system for latent HSV infections (9). During long-term infections of these cells with wild-type virus, HSV genomes become quiescent and do not produce progeny virus; the genomes persist in these cells in an endless state, suggesting that HSV genomes are circular during infections that model latency (186). However, terminal HSV genome fragments have been detected in some latently infected human trigeminal ganglia (44), and the termini of a replication-defective virus are relatively stable during long-term infections in differentiated PC12 cells (12). These studies have subsequently left some ambiguity with respect to HSV genome configurations during latency. Importantly, these studies have not differentiated between concatemerized and circularized genomes. In the present study, we provide unambiguous evidence that HSV genomes persist in a stable circular configuration during long-term quiescent infections that model latency.

1.7. The role of ICP0 during HSV infection

ICP0 is one of the five IE viral genes and is expressed within the first hour of infection. It is an indiscriminate transactivator of both viral and cellular gene expression, however it does not bind to promoters (92). Many of the properties of ICP0 have been deduced from phenotypic analyses of a series of ICP0-mutated viruses. ICP0 facilitates, but is not necessary for productive infection, as ICP0-deleted viruses can replicate, although inefficiently unless high doses of input viral particles are used (164, 183). ICP0 is necessary for derepression of gene expression during normally persistent, quiescent HSV infections that model latency (76, 165) and is important for efficient reactivation of latent infections in mice (15, 29, 109, 153). It follows that ICP0 is thought to indiscriminantly facilitate viral gene expression by inhibiting repression of viral gene

expression. This inhibition of repression could be how ICP0 facilitates gene expression both during initial infections and during reactivation from a latent state. Since these phenotypic analyses, ICP0 has been shown to interact with and/or affect a variety of cellular proteins involved in cell cycle regulation (53, 94), transcription (95), translation (94), proteolysis (53, 55, 59), and ND10 bodies (55, 116). The functions of these interactions have been largely interpreted in the context of ICP0's ability to inhibit productive cycle repressors. Although it is not completely understood how, these interactions underlie the ability of ICP0 to facilitate gene expression and the productive cycle.

1.7.1. Structure and function

Within the HSV genome, the ICP0 gene occurs in two copies, one located within each set of repeats flanking the U_L region (Figure 1). The ICP0 transcript is spliced into a 2.7KB mRNA that contains three exons (146, 147). Translation of this transcript results in a 775 amino acid protein with a molecular weight of 110 kDa. Both amino acid sequence and mutational/biochemical studies have revealed many of the molecular characteristics of ICP0 that may contribute to its function. ICP0 contains various potential sites for post-translational modifications, including several N-terminal casein kinase II sites, two central proline rich regions, a central serine-threonine tract, and a C-terminal serine-alanine rich sequence (147). ICP0 also contains several protein interaction domains, including a nuclear localization signal, an acidic N-terminal domain, a C-terminal dimerization domain, a cyclin D3 binding domain, a herpes-associated ubiquitin specific protease (HAUSP) binding domain, and a C_3HC_4 RING finger domain (47, 49, 59, 96, 135, 147). The RING finger is the only domain that is conserved in ICP0 homologues of other herpesviruses (27, 132, 133, 189, 201).

1.7.2. Protein stability and turnover

ICP0 has been shown to induce the degradation of: PML and Sp100 (23, 55, 116), which are constituents of ND10 bodies; the DNA-dependent Protein Kinase Catalytic Subunit (DNA PKcs) (107, 143), which is critical for VDJ recombination and DNA double-strand break repair via non-homologous end-joining (NHEJ) (30); and CENPA and CENPC (53), which are histone3-like centromere proteins. The ability of ICP0 to induce the degradation of cellular proteins may underlie the ability of ICP0 to promote the productive cycle. These proteins have been proposed to somehow be involved in cellular repression mechanisms that target the parental HSV genome.

ICP0-induced protein degradation has been linked to a cellular mechanism for protein turnover involving the ubiqutin-targeted degradation of proteins via the 26S proteasome, as inhibitors (MG132) of this pathway inhibit ICP0's effect (51). Ubiquitination is one of the various post-translational modifications that can result in protein instability (28). Ubiquitin is a 76kDa protein that associates with lysine residues of target proteins. Ubiquitin conjugating enzymes (E2 enzymes) catalyze the targeted transfer of ubiquitin onto target proteins, until polyubiquitin chains accumulate and bind to the 26S proteasome, leading to rapid degradation of the poly-ubiquitinated protein by proteasomal enzymes. Ubquitination is a reversible process, as ubiquitin may also be removed from protein substrates by ubiquitin specific proteases (USP).

Both ICP0's RING finger motif and the proteosome are required for ICP0-induced degradation of the aforementioned proteins (60). RING finger motifs are common to E3 ubiquitin ligases, which stimulate and probably regulate the activity of E2 ubiquitin conjugating enzymes, which transfer ubiquitin onto target proteins (63). Indeed, ICP0 has been shown to contain E3 ubiquitin ligase activity (13, 192). Hence, ICP0 is thought to direct the conjugation of ubiquitin onto specific proteins, targeting them for degradation via the proteasome. During HSV infection, ICP0 interacts with the herpes-associated ubiquitin specific protease termed

HAUSP, further associating ICP0 with ubiquitin-related protein stability (58, 59). Although HAUSP functions to remove ubiquitin from targeted proteins, ICP0 may use this interaction to free ubiquitin molecules for use in ubiquitination of other specific proteins (51). Although ICP0 induces degradation of proteins through the 26S proteasome and can induce conjugation of ubiquitin chains (13, 192), it has never been shown to specifically conjugate ubiquitin onto proteins, thereby inducing their degradation, either *in vitro* or *in vivo*. Nonetheless, ICP0 clearly induces the proteasome-dependent degradation of cellular proteins, which have since been thought to be somehow inhibitory to the productive cycle of the virus.

1.7.3. ND10

Early during infection ICP0 localizes to and then disrupts ND10 structures, which are conglomerates of nuclear proteins that form on average ten nuclear domains (ND10) or foci within dividing cells (115, 116). ND10 structures are composed of various proteins including the promyelocytic leukemia protein (PML) and SP100, which are both modified by a small ubiquitin-like molecule (SUMO-1) (118). ND10 bodies and their associated proteins have been shown to play roles in translation, transcription, DNA repair, DNA replication, RNA stability, and RNA transport (12). Although their precise functions are not known, ND10 bodies are modified during the cell cycle (56), disturbed by heat shock (119), disrupted by infections with various viruses (50).

During infection with HSV and many other DNA viruses, parental genomes localize to the periphery of ND10 structures (117). HSV may replicate at these sites after disruption of ND10 by ICP0, which is accomplished in part through the degradation of PML and SP100 (117). Both the RING finger domain and C-terminal domain of ICP0 are required for efficient disruption of and localization to ND10 (57, 115). These regions of ICP0 are critical for ICP0's

ability to facilitate viral gene expression and the productive cycle (47, 48, 57, 115). Accumulations of the ICP0-interacting protein HAUSP can also be found in or close to ND10 bodies (59). The heterochromatin protein (HP1) can be found at both ND10 structures and centromeres (54, 108, 173), which are also disrupted by ICP0 through degradation of the centromere proteins CENP-A and CENP-C, leading to disruption of centromere structure and mitotic failure (53). Although exactly how these proteins affect virus production and the lytic cycle is not understood, they are thought to be inhibitory to the productive cycle due to the ability of ICP0 to induce their degradation and subsequently disrupt the processes with which they are associated. Furthermore, the RING finger motif of ICP0 is both required for ICP0 to facilitate the productive cycle and necessary for the degradation of these proteins and subsequent disruption of these structures (60).

1.8. Host cell DNA repair pathways during HSV infection

Cells have developed a variety of mechanisms to repair breaks in DNA, because these breaks present potential loss and manipulation of vital genetic material. During infection, the HSV genome is presented as a foreign, double-stranded DNA molecule with unprotected DNA ends. These ends may be detected as DNA double strand breaks (DSB's) by host cells, which subsequently attempt to repair these breaks through DNA DSB repair pathways. The two primary pathways by which DSB's are repaired in mammalian cells are non-homologous end-joining (NHEJ) and homologous recombination (HR). Although these mechanisms are distinct, some repair proteins may play overlapping roles in both mechanisms. Repair of ends by HR requires exchange of homologous DNA sequences, while NHEJ involves direct ligation of DNA sequences with little sequence homology required.

Studies concerning HSV genome configurations during productive infection and during latency have suggested that at some point during the HSV life cycles viral genomes circularize (44, 65, 89, 149, 159, 185, 186). In the current study we present evidence that HSV genomes circularize in the absence of ICP0 and circular genomes serve as a stable configuration during persistent quiescent states that model latency (89). Circularization of HSV genomes could be a result of host cell DNA repair responses to unprotected genome ends present on linear double stranded HSV genomes. Through these repair responses, genome ends may be joined, creating circular genomes. Circularization is consistent with the structure of the genome, which contains direct *a* repeat sequences located at genome termini (Figure 1) (161). These repeats render the HSV genome a suitable substrate for circularization by either HR or NHEJ repair mechanisms.

1.8.1. Non-homologous end-joining (NHEJ)

Interestingly, NHEJ may be the dominant repair pathway of DSB's in non-dividing neurons (31, 66, 163), where latent HSV infections are established and where genomes may persist in an endless state, most likely as unit length circles (44, 159). DNA PKcs, Ku70, Ku80, XRCC4, and DNA Ligase IV are cellular proteins critical for NHEJ *in vivo* (30). Ku70 and Ku80 subunits dimerize, forming the regulatory subunits of the DNA PK holoenzyme (70). The Ku dimer binds DNA ends (8, 130), recruiting the catalytic subunit of DNA PK(cs) to sites of DNA DSB's (70). Once associated with DNA-bound Ku subunits, DNA PKcs catalyzes the end-joining reaction through a combination of phosphorylation events (127) that leads to recruitment of the XRCC4/DNA Ligase IV tetramer, which enzymatically ligates DNA ends together (198).

There is evidence that HSV manipulates NHEJ repair pathways during infections, potentially creating an environment that promotes the productive cycle. ICP0 induces the degradation of DNA PKcs during infection in a cell-type specific manner, inducing nearly

complete degradation in Hela S3 cells (107, 143), inducing degradation by less than 50% in Hep-2 cells (188), and not inducing degradation to observable levels in Vero cells (200). In Hep-2 cells, although DNA PKcs is somewhat degraded, complexes of DNA PKcs, Ku80, Ku70, and XRCC4 co-precipitate with the HSV single-stranded DNA binding protein, ICP8, at times in which ICP8 is located at HSV prereplicative sites and replication compartments, suggesting a direct and/or indirect interaction between these NHEJ repair proteins and viral genomes (188). In fact, Ku70 has been shown to bind some late HSV promoter sequences (148). During infection DNA PKcs and Ku80 are redistributed from their normally disperse nuclear localization to HSV replication compartments (188, 200). In mouse embryonic fibroblasts (MEFs) lacking Ku70 (188) and human glioblastoma cells lacking DNA PKcs (143), yields of replicated wild-type virus is increased up to approximately 50 and 100 fold respectively, suggesting that proteins involved in NHEJ are inhibitory to HSV replication. Although NHEJ proteins are clearly present at sites of HSV genomes, appear to affect HSV replication, and may be affected by ICP0, the exact role of NHEJ proteins and why/how the virus manipulates this repair mechanism during HSV infection remains unclear. NHEJ subsequently remains a likely mechanism by which genomes are circularized during infection.

1.8.2. Homologous recombination (HR)

Because the termini of HSV genomes contain direct *a* repeats, HR is another potential mechanism by which the cell may repair genome ends and subsequently circularize HSV genomes. Like NHEJ proteins, HR proteins localize to sites of HSV replication. WRN, BRCA1, BLM, and RPA, all of which may be involved in HR repair, associate with ICP8 (188) at prereplicative and replication compartments and redistribute from their normal nuclear localization to these sites during infection (188, 200). However, whereas virus growth is

increased in cells deficient in NHEJ, in cells deficient in WRN both virus growth and recombination is decreased, suggesting a non-essential but facultative role for WRN in virus replication (188). Rad51, which is the only protein absolutely required for HR, redistributes to HSV prereplicative sites and replication compartments during infection (200).

Like NHEJ proteins, HR proteins have the potential to participate in the repair of genome termini by circularization. However, unlike DNA PKcs degradation observed during infection, the steady state levels of examined HR repair proteins are not altered during HSV infection (200). In fact, HR activity increases during replication, a time in which genome isomerization occurs (161). HR proteins may be involved in isomerization and repair of damaged genomes during replication. There have been *in vitro* studies done aimed at determining if NHEJ or HR mechanisms circularize DNA during HSV infection (204, 205). However, these studies used linearized plasmids as substrates for repair in transfected cells that have been either infected with HSV or transfected with HSV expression vectors (204, 205). Although these studies have shown that both NHEJ and HR mechanisms can circularize linearized plasmid DNA during HSV infection, they suggest that HR is the major mechanism responsible for this circularization. Because the substrate for circularization was artificial in these investigations, additional studies examining the mechanism by which HSV genomes circularize using viral genomes as substrates for repair are required to differentiate between NHEJ and HR mechanisms.

1.8.3. Mre11/Rad50/NBS1 repair response

Additional proteins that may be involved in both NHEJ and HR repair are altered during HSV infection. A complex of proteins, Mre11/Rad50/NBS1 (M/R/N), has been implicated in cellular DNA double-strand break (DSB) repair processes by both NHEJ and HR mechanisms through its ability to recognize and in part process DSB's. During repair of DSB's, M/R/N complexes

localize to foci of the phosphorylated form (serine 139) of histone variant H2AX (γ-H2AX) (19, 144), which has been shown to co-localize with and act in the repair of DSB's (157, 160, 172). During HSV infection, both Mre11 and Rad50 either directly and/or indirectly associate with the HSV single-stranded DNA binding proteins, ICP8, which is localized to HSV replication compartments (188). In fact, M/R/N complexes relocate to replication compartments during infection (111, 188, 200). In addition, NBS1 is modified by phosphorylation during productive wild-type HSV infections (111, 178, 200). NBS1 phosphorylation occurs during DNA damage responses that are signaled through the ATM pathway (202).

M/R/N complexes act as sensors of DNA DSB's and may facilitate in ATM recruitment to DSB's and subsequent activation of ATM by inducing the dissociation of inactive ATM multimers into active ATM monomers, which in turn have downstream effects on DNA repair, cell cycle arrest, and apoptosis (1, 105). During ATM activation and recruitment to DSB's, many proteins including NBS1 are phosphorylated. Indeed the ATM pathway is activated during productive HSV infections (111, 178), with this repair response possibly recognizing replicating HSV genomes as abnormal structures. M/R/N complexes and ATM signaling may be beneficial for the productive cycle of the virus, because in some differentiated neurons, where a latent state can be established and this ATM repair response is compromised, there is a significant defect in HSV replication and replication compartment formation (111). However the exact role of M/R/N complexes and the ATM pathway during HSV infection remains largely uncharacterized, in part, due to the fact that it is not known whether this pathway is stimulated by events leading to and during replication or in response to incoming parental HSV genomes. The present study addresses these issues, suggesting that early during infection incoming HSV induces M/R/N complex formation independent of ATM activation.

2. RATIONALE

In host cells, the HSV genome undergoes physical alterations that are regulated by both cellular and viral proteins. During the lytic cycle HSV genomes are very dynamic, progressively changing throughout infection; viral genomes actively express genes, replicate, and are packaged, ultimately producing mature viral progeny. By contrast, during latency viral genomes persist in a transcriptionally repressed chromatin state in the nuclei of sensory nerves (40, 154, 187). Although many physical differences in HSV-1 genomes during the lytic and latent cycle have been characterized, little is known about how these characteristics develop during infection and furthermore, how these developing characteristics contribute to productive or latent life cycles.

Research that has examined the configuration of viral DNA during the various stages of lytic life cycle along with the known structural characteristics of the viral genome has led to a model of the progressive changes in HSV genomes during lytic infection, in which replication is initiated by a theta mechanism (12, 65, 149, 185). The template for theta replication is a circular HSV genome. Research that has examined the configuration of viral DNA during latency has led to a model in which HSV genomes persist in a stable circular configuration (12, 44). However, changes in viral genomes and events leading to these changes during a particular life cycle are not clear because it is not understood how the host cell responds to incoming HSV genomes and how the virus manipulates this response, ultimately leading either to a template for replication during productive lytic infections or to a stable configuration during persistent latent infections.

Because HSV genomes begin as linear, double-stranded DNA during infection, host cells may treat the ends of incoming genomes as DNA double strand breaks (161). The host cell may

subsequently attempt to repair these double-strand breaks via DNA double strand break repair pathways, resulting in intramolecular joining of genome termini through genome circularization. Some DNA repair proteins, including those involved in homologous recombination (HR), those involved in non-homologous end joining (NHEJ), and those that may be involved in both mechanisms are present at sites of HSV genomes during infection (111, 148, 178, 188, 200). However, it is not known how these repair proteins contribute to the manipulation of HSV genome configurations during infection and how viral proteins may alter or counteract this repair response. Furthermore, it is not known how the interplay between repair responses and the virus' reaction to these responses contributes to forming either a genome template for replication during the lytic cycle or a stable configuration during latency.

ICP0 promotes the lytic cycle both during primary infections and during reactivation from latency (15, 16, 29, 76, 92). ICP0 interacts with and/or affects cellular proteins associated with ND10 structures (55, 116), transcription (95), translation (94), cell cycle regulation (53, 94), DNA repair (107, 143), and proteolysis (53, 55, 59). These interactions are thought to underlie the process by which ICP0 promotes lytic infection. As a ubiquitin ligase, ICP0 may act by targeting proteins, most likely those that inhibit the productive cycle, for degradation via the proteosome (13, 192). However, the mechanism(s) by which ICP0 promotes the lytic cycle are far from being entirely understood.

In order to more clearly understand changes in the configuration of viral genomes during both productive and latent life cycles and events leading to these changes, we have used a variety of HSV mutant viruses to examine the configuration of viral genomes as persistent and productive infections are established; these events model changes leading to and during the latent and lytic life cycles, respectively. Because ICP0 is such a pivotal regulator in the balance

between the productive and latent cycles, with its presence facilitating lytic infection, it was of our interest to examine the effect of ICP0 on the configuration of viral genomes in order to understand if and how ICP0 alters genome configurations to promote the lytic cycle. Because cells may respond to incoming linear HSV genomes as double-strand breaks, we will examine the role of cellular DNA double-strand break repair pathways in altering the configuration of HSV genomes and determine how the virus may alter/counteract this repair response. More specifically the aims are: i) to determine the configuration of HSV genomes during productive infection, during persistent quiescent infection established by an HSV mutant, and as a function of ICP0 and ii) to determine cellular DNA repair pathways stimulated by input HSV, cellular DNA repair mechanisms responsible for altering the configuration of HSV genomes, and the effect of ICP0 on the cellular repair environment.

d109 will be used to model changes in HSV genome configurations during the establishment of latency. d109 lacks expression of all five immediate early (IE) viral genes (ICP 0, 4, 22, 27, 47) and subsequently forms an abortive infection in which the virus does not replicate without complementation (165). d109 is non-toxic to cells at even high multiplicities of infection and persists in cells in a quiescent state from which viral gene expression is largely repressed (165). d106 expresses ICP0 but lacks expression of the additional four IE genes and subsequently also forms an abortive infection (165). However, the expression of ICP0 inhibits a persistent, quiescent state from being established during d106 infection (165). d106 will be used to examine the effect of ICP0 on the configuration of genomes during abortive infections. The genome configuration will also be examined during productive infections, as a function of ICP0, using wild-type strain KOS and ICP0-deletion mutant d99 (165). During infection with these viruses, replication occurs but can be inhibited by phosphonoacetic acid (PAA), allowing all

events taking place prior to replication to evolve but inhibiting replication itself (85). In theory, PAA addition should allow the template for genome replication to be observed. The configuration of the aforementioned viruses will be examined using Gardella gel analysis. Gardella gels resolve large circular forms from their linear counterparts of the same molecular weight as discrete species within an agarose gel, as was first demonstrated in a study distinguishing persisting circular Epstein Barr Virus (EBV) episomes in Raji cells from their linear counterparts in virions (67).

Gardella gel analysis for circular genomes will be used in conjunction with an additional analysis for circularization, which has been previously demonstrated by Poffenberger and Roizman (149). Both analyses will be used to compare the configuration of a non-inverting HSV mutant I358 (149) to an ICP0 deleted version of I358 (d99/I2.3) that we will construct. Both I358 and d99/I2.3 are deleted for internal repeat sequences within their genomes and subsequently do not isomerize. Although a virus lacking the internal repeat sequences is not required for Gardella gel analysis, it is required for restriction enzyme analysis due to sequence homology within the internal repeats of the viral genome with the terminal repeats of the genome. During restriction enzyme analysis, DNA from infected cells is digested and then probed for a terminal genome fragment, which will also detect a novel fragment that includes both termini, if viral genomes are linked end-to-end, as they are when circularized. Novel restriction fragments containing end-to-end linkages of viral genomes can only be distinguished in the absence of homologous internal repeat sequences. Both Gardella gel analysis and restriction enzyme analysis will be used to assess HSV genome configurations during productive infections (I358), as a function of ICP0 (d99/I2.3).

Host cell DNA repair responses to incoming HSV genomes may alter the configuration of genomes, ultimately leading to either a template for genome replication during the productive cycle or a stable genome configuration during latency. We will examine both the effect of ICP0 on cellular DNA DSB repair pathways and the effect of these pathways on the configuration of HSV genomes. Although it is known that repair proteins localize to HSV replication compartments (111, 178, 188, 200), it is not known if they are recruited as a result of parental HSV genomes or due to events that occur during HSV replication. The localization and levels of repair proteins in response to incoming HSV will be examined during abortive and productive infections and as a function of ICP0, using the aforementioned viruses with immunofluorescence and Western blotting techniques. The effect of cellular DNA repair pathways on changes in HSV genome configurations will be assessed by Gardella gel analysis of HSV genome configurations in cells deleted for specific DNA repair proteins.

Using these approaches we will model changes that occur to viral genomes as both lytic and latent infections are established and will also begin to clarify how these changes evolve. For the first time, we will provide evidence for diverging physical changes in HSV genomes during the establishment of each life cycle of the virus and importantly show that the interplay between cellular responses to HSV genomes and ICP0 regulates these changes. In particular, we will provide evidence that host cell DNA repair pathways manipulate the configuration of HSV genomes and that ICP0 may alter these pathways, potentially promoting a template for replication during the lytic cycle. This ability of ICP0 to control the configuration of viral genomes is a newly identified mechanism by which ICP0 can promote the lytic cycle of the virus. Along with suggesting a revised model of HSV replication during productive infection,

we will also provide a mechanism by which HSV genomes can persist long-term in a stable configuration during latency.

3. RELATIONSHIP OF HERPES SIMPLEX VIRUS GENOME CONFIGURATION TO PRODUCTIVE AND PERSISTENT INFECTIONS.

3.1. Abstract

Infection of susceptible cells by herpes simplex virus can lead to productive infection, or latency, where the genomes persist in the nuclei of peripheral neurons in a quiescent state. Using the HSV strain d109, which does not express any viral genes and thus establishes a quiescent state in most cells, we observed that a fraction of genomes circularized upon infection. The expression of ICP0, which is known to be involved in reactivation from latency and the promotion of productive infection, inhibited the formation of circular genomes. Circular genomes were not observed upon infection of fully permissive cells by wild type virus, either in the presence or absence of viral DNA replication. However, productive infection in the absence of ICP0 resulted in the accumulation of a subpopulation of circular genomes. The proportion of circular genomes formed during infection with an ICP0 mutant was greater at low multiplicity of infection, a condition where ICP0 mutants replicate poorly. In the complete absence of viral gene expression, it was found that only circular genomes persisted in cells. These results suggest that circularization of the HSV genome may not occur early in the productive phase of wt HSV infection, but rather during establishment of a quiescent state or latency, providing a possible strategy for long-term persistence. Additionally, the circularization and possible fate of HSV genomes is regulated by an activity of ICP0.

3.2. Introduction

Herpes Simplex Virus Type 1 (HSV-1), can establish both productive and latent infections. Productive infection with HSV-1 results in epithelial lesions, such as cold sores, and can lead to latent infection in the trigeminal ganglia of neurons that innervate the primary site of infection (162). During latency the viral genome persists in a largely repressed, chromatin-associated state

(40) that can be derepressed, or reactivated, to produce recurrent productive infections. The HSV-1 immediate early (IE) protein ICP0 is crucial for reactivation (15, 29, 109).

The HSV genome is a linear double stranded DNA molecule existing as 4 isomers that differ with respect to the orientation of a long and a short region about a joint (36, 77). Both the long and short regions are bracketed by inverted repeats, and a 250-500 bp sequence (termed the "a" sequence) is directly repeated at the genomic termini (112, 176, 195, 197). Inverted copies of the "a" sequence are also present at the joint. During both productive and latent infection the genome assumes a configuration indicative of end-joining since genomic joint sequences increase in abundance relative to their homologous counterparts at the genomic termini (44, 65, 149, 159). End-joining can occur in the presence of inhibitors of viral protein synthesis, which has been interpreted as the circularization of the HSV genome as a function of cellular mechanisms (65, 149). Consequently, it is thought that circularization of viral genomes occurs prior to the onset of productive or latent infections, implying that this process occurs independent of the type of infection established. In addition, it provides the foundation for the current model that HSV-1 genomes replicate initially by a theta mechanism (161). However, an increase in joint regions can also result from the formation of concatemers, complicated branched structures that result during replication, or possibly from intermolecular ligation or recombination. Therefore, circularization of HSV-1 genomes has not been clearly demonstrated.

In the present study we differentiate between circular, linear and higher order genomic structures to test the hypothesis that the HSV genome circularizes in the absence of viral gene expression. The abundance and persistence of circular genomes during productive and quiescent infections was also determined. Quiescent infection was used as a model for latent infection and was achieved using an HSV-1 mutant, d109, which does not express the five HSV IE proteins,

and contains an EGFP transgene under control of the HCMV immediate early promoter (165). In cultured cells, d109 establishes a persistent and quiescent state, in which EGFP expression is largely repressed but can be derepressed by providing ICP0, in trans (82, 165). These characteristics are similar to events that occur during latency and reactivation from latency.

ICP0 is an E3 ubiquitin ligase (13, 192) and has been proposed to promote lytic infections by specifically destabilizing cellular proteins that inhibit the lytic viral life cycle. ICP0 is known to induce the degradation of proteins associated with ND10 bodies (116), which are discrete nuclear foci where HSV-1 genomes may localize early during infection (117). Interestingly, recent studies have suggested these foci to be sites of DNA double strand break repair (18). It is possible that the ends of linear HSV-1 genomes may be treated as double strand DNA breaks and subsequently repaired by circularizing genomes early during infection (161). Therefore, we also addressed whether ICP0 affects the circularization of the HSV genome, possibly providing an additional basis for its role as an effector of lytic and latent infections.

3.3. Materials and Methods

3.3.1. Cells and Viruses.

Vero, F06 (167), E11 (166) cells were cultured as previously described. Raji cells were similarly maintained in RPMI-1640 medium. The viruses, d109, d106, d99 (165), and n212 (15) (provided by Priscilla Schaffer, Harvard Medical School, Boston MA) are HSV-1 mutants derived from the wild type strain KOS. Each mutant was grown and assayed for infectivity (plaque forming units per milliliter (PFU/ml)) in the appropriate complementing cell lines (F06 and E11) as previously described (165). AdS.11E4(ICP0), an adenovirus vector that expresses ICP0, and AdS.11D, the empty adenoviral vector, were provided by Douglas Brough at GenVec, Inc., Gaithersburg, Md., and have previously been described (82).

Confluent monolayers of Vero cells were infected with viruses at the multiplicity of infection (moi) indicated. Where indicated, 400 ug/mL phosphonoacetic acid (PAA; Lancaster) was added to the cultures during viral infections. For each time course, viral infections were staggered such that all time points were harvested simultaneously. 0 hour time points were performed by incubating the virus with cells on ice for 1 hr prior to harvesting. In order to determine the configuration of d109 genomes during long-term persistent infections, d109 infected Vero cells were cultured in media with 2% FBS and incubated at 34º.

3.3.2. Gardella gels.

Gardella gels were performed essentially as described (67). Briefly, an approximately 25cm x 15cm horizontal agarose gel was poured such that the upper portion (~3cm), where the wells were molded, contained 2% SDS, 1mg/mL proteinase K, and 0.8% Type IIA: medium EEO agarose (Sigma) in TBE (0.09 M Tris-base, 0.09 M boric acid, 2 mM EDTA pH 8.0). The lower portion of the gel (~22cm) contained 0.75% agarose in TBE. Infected cells were washed with TBS (0.14 M NaCl, 2.7 mM KCl, 25 mM Tris-base, pH 7.4), scraped into TBS, and then pelleted by low-speed centrifugation. Nuclei were isolated as previously described (165). For each Gardella gel, either ($5x10^6$) infected cells or nuclei were resuspended in 50μL loading dye (15% Ficoll, 0.01% bromophenol blue) and then loaded into each well. Gels were run for 3 hr at 45V to efficiently lyse the cells, and then 38 hr at 120V to separate circular from linear viral genomes. Gels were analyzed by Southern blot as previously described (165). The hybridized filters were then exposed to x-ray film. For quantitative analysis, the filters were analyzed by a Storm 840 phosphorimager (Molecular Dynamics).

The probe used to detect d106 and d109 on Southern blots consisted of a 1.6-kb Pac I fragment of pucGFP (165), containing the EGFP gene under control of the HCMV IE promoter.

The probe used to detect KOS, d99 and n212 genomes was the 1.8-kb *Bam*HI-Y fragment of pKBY, which contains a portion of the ICP4 gene of HSV-1. The plasmid 1040 (provided by David Rowe, University of Pittsburgh), which contains the internal repeats of the EBV genome, was used to detect EBV genomes.

3.3.3. Electroelution.

Gel slices from 4 lanes of circular or 4 lanes of linear d109 DNA, separated from $5x10^6$ Vero cells per lane, were excised from Gardella gels. The locations of the bands in the gel were determined by conducting a Southern blot as described above on lanes from the same gel, and using the resulting autoradiogram as a guide to excise the bands from the remainder of the gel. DNA was eluted from each gel slice in 0.5x TBE in dialysis bags for 6 hr at 120V. The eluted DNA was digested with both *Bam*HI and *Sac*I (New England Biolabs) according to manufacturers instructions. The digested DNA was then analyzed by Southern blot using the BamHI Y fragment as probe, as described above.

3.4. Results

3.4.1. Accumulation of low-mobility, non-linear HSV genomes in the absence but not presence of ICP0.

Previous studies suggesting that the viral genome circularizes prior to viral DNA replication and during latency (65, 149, 159) did not rule out that the observed end-joining could be due to the formation of concatemers, or more complicated branched structures. Gardella gels resolve large circular forms from their linear counterparts as discrete species within the gel, as was first demonstrated in a study distinguishing persisting circular Epstein Barr Virus (EBV) episomes in Raji cells from their linear counterparts in virions (67). This analysis has also been used to

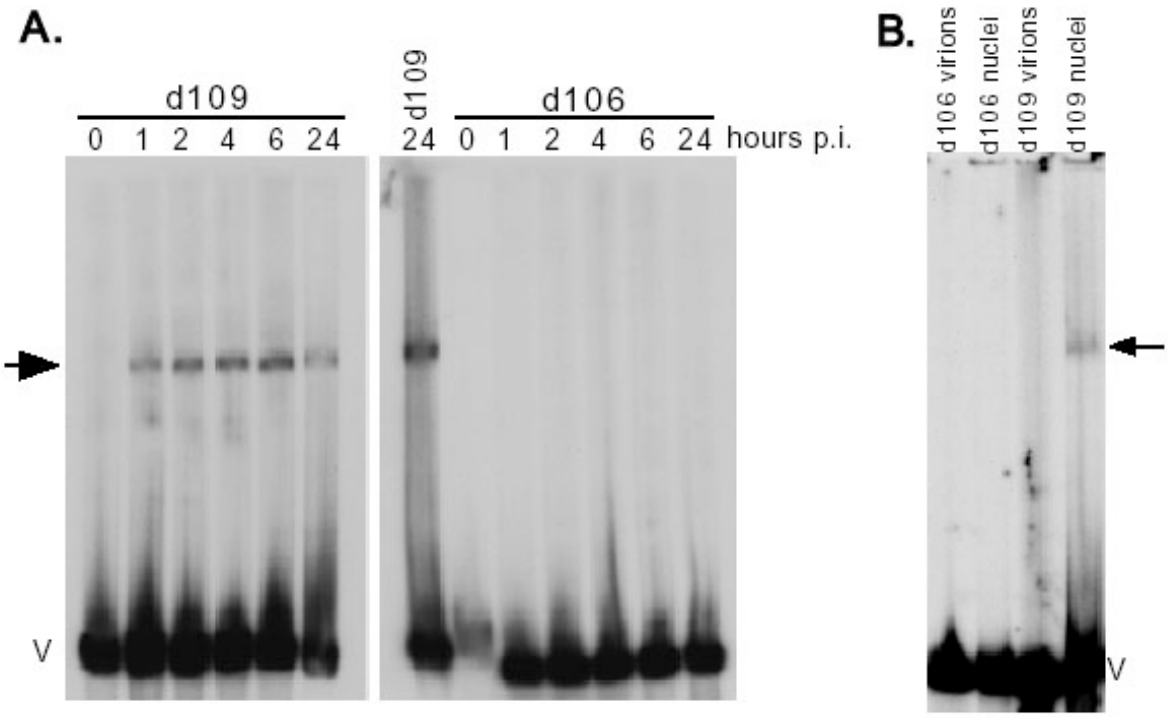


Figure 2. Gardella gel analysis of d109- and d106-infected cells.

(A) Monolayers of Vero cells were infected at a moi of 20 PFU per cell and incubated for the indicated times before analysis as described in *Materials and Methods*. **(B)** Vero cells were infected with d109 or d106 at a moi of 20 PFU per cell for 24 h. Nuclei were then isolated and run on the gels as described in *Materials and Methods*. In both **A** and **B**, the mobility of linear viral DNA is indicated by V and the low-mobility band is indicated by an arrow. The mobility of linear viral DNA was determined by examining the migration rate of virion DNA in samples where the virus was adsorbed to cells on ice (0 h; **A**), or where mock-infected cells were spiked with $5x10^6$ PFU of d109 or d106 virions (**B**).

establish that Varicella-Zoster Virus (VZV) circularizes shortly after infection (100). To examine changes in the HSV genome subsequent to infection and in the absence of viral gene expression, d109 infected cells were applied to a Gardella gel. Within 1h post infection a novel band of lower mobility than the linear virion DNA was observed, which increased in intensity up to 6 hours (Fig.2A). About 7-10% of input genomes were converted to this form. Similar results were obtained when nuclei were analyzed (Fig. 2B) implying that many genomes associated with nuclei did not circularize at this moi. In contrast, the lower mobility species was not observed with d106 infected cell or nuclei samples (Fig.2). d106 genetically differs from d109 in that the ICP0 gene is intact, and thus ICP0 is expressed in d106 infected cells (165). Early and late viral genes are poorly expressed if at all since d106, like d109, is deleted for both copies of ICP4 (38), and subsequently do not replicate. Material hybridizing to the probe was not observed in the wells of any of the samples.

3.4.2. Low-mobility HSV genomes represent circularized genomes.

A series of experiments were conducted to further characterize the novel low-mobility form of the d109 genome. Gardella gels are very sensitive for resolving large linear forms from their circular counterparts. They are not as sensitive for resolving linear or circular molecules of similar sizes (67). Therefore, a circular genome of similar size to HSV would have a similar mobility to the novel-low mobility form of the d109 genome, if this form was circular. The 178 kb EBV genome in Raji cells is circular and readily resolved from linear EBV genomes on Gardella gels (67). A comparison of the mobility of the low-mobility form of the d109 genome and the EBV genome in Raji cells revealed that the circular EBV genome had a slightly reduced mobility relative to the novel form of the d109 (~130 kb) genome (Fig. 3A). This suggests that

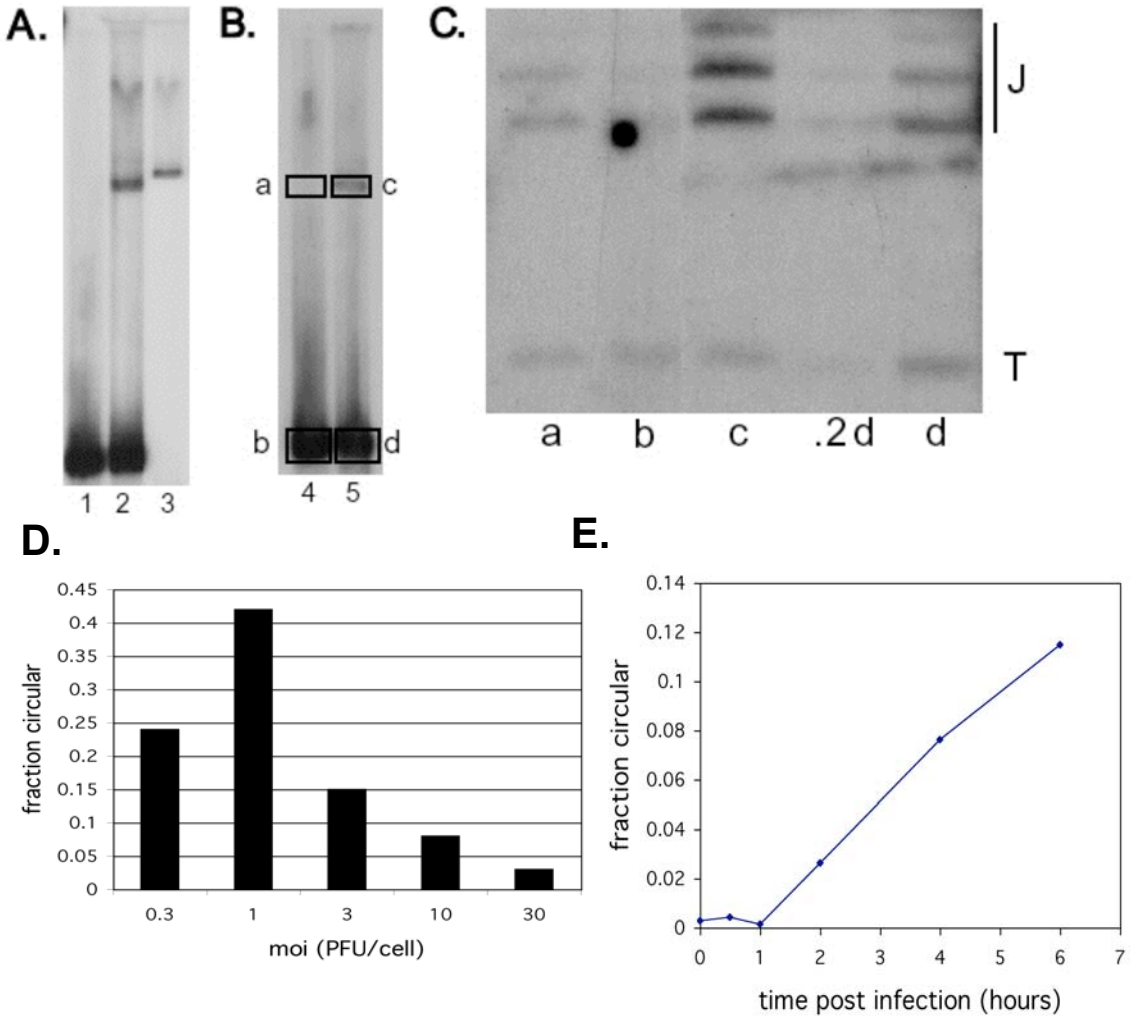


Figure 3. Characterization of the low-mobility form of the d109 genome.

(**A**) d109 virions (lane 1), d109-infected cells (lane 2), and Raji cells (lane 3) were analyzed on a Gardella gel and probed for HSV and EBV genomes. (**B**) Template for eluting regions a, b, c, and d from a Gardella gel of d109 virions (lane 4) and d109-infected cells (lane 5). (**C**) The DNA in samples a–d in **B** were analyzed as described in *Materials and Methods* to observe the abundance of joint (J) and terminal (T) fragments. Different amounts of d were loaded on the gel. (**D**) Vero cells were infected at the indicated moi of d109 for 24 h, run on a Gardella gel, subjected to Southern blot analysis, and quantified. (**E**) The kinetics of circularization of d109 genomes in Vero cells was determined as described in the text.

the novel form of the d109 genome is circular. To further test the hypothesis that the low-mobility form of the d109 genome was circular we eluted this material from a Gardella gel (Fig. 3B) and performed restriction enzyme digestions and Southern blots probing for genomic joints and termini (Fig. 3C). Circular genomes should have only genomic joints when blots are hybridized with a probe homologous to both termini and joints. The ratio of joint to terminal fragments was considerably greater for the low mobility form (c) relative to ratio observed for eluted linear DNA (b and d) (Fig. 3C). The small amount of terminal fragments detected in the material eluted from the low mobility band was most probably due to contaminating linear DNA in the region of the low mobility band since terminal fragments were detected when the same region was analyzed from the gel track containing virion DNA (a). Therefore based on the mobility relative to circular EBV, and the enrichment for joint regions relative to linear DNA, we conclude that the low mobility form of the d109 genome is circular.

3.4.3. Multiplicity dependence and kinetics of genome circularization during infection.

At high moi, only a relatively small proportion of input d109 genome circularized (Fig. 2A). Therefore, we next determined the fraction of d109 genomes that circularized as a function of moi. 45% of adsorbed d109 genomes circularized at a moi of 1 PFU/cell, and that this proportion decreased as a function of increasing moi (Fig. 3D). The degree of circularization at moi of 0.3 PFU/cell was also relatively high. The greater degree of circularization at a lower moi was also evident in the infections with the ICP0 mutant, d99 (Fig. 5). This result suggests that in Vero cells there is a limited capacity for the cell to support circularization. Similar results were observed in human embryonic lung cells (data not shown). However, given that circularization occurs in the absence of viral gene expression, it is likely that the capacity of a given cell type to support circularization of the HSV genome will vary.

Fig. 2A also suggests that circularization occurs within 6h post infection. In order to obtain an accurate view of the kinetics of circularization, 3 PFU/cell of d109 were adsorbed to a monolayer of Vero cells at 4º for 1 h, and then incubated at 37º for different times. Following adsorption, there was a 1-hour lag, after which time genomes began to circularize (Fig. 3E). At this moi about 15% of the genomes were circular after 24 h (Fig. 3D), so that the amount of circularization seen at 6 h post adsorption represents maximum circularization. These kinetics follow those for the previously published kinetics of HSV entry (37), however they are relatively slow considering that at 6 h post adsorption, progeny virions are beginning to be produced in a productive infection. Additionally, at 1 h post adsorption, physiologically significant levels of ICP0 are expressed in a productive infection (82).

3.4.4. Low-levels of ICP0 inhibit genome circularization.

The expression of ICP0 from the d106 genome precluded the formation of circular HSV genomes (Fig 2A). ICP0 is over expressed from the d106 genome relative to wt HSV, leading to aberrant alterations of host cell metabolism (81, 82). However, when driven from the E4 promoter in an adenovirus vector deleted for the E1, E3, and E4 regions, the expression of ICP0 is >1000 fold less than from d106, and is similar to that in the first hour of wt HSV infection (82). When cells were coinfected with d109 and the adenovirus expressing ICP0, the HCMV IE promoter-EGFP transgene on the d109 genome was quantitatively activated (Fig. 4A), as previously demonstrated (82). In addition, the d109 genomes did not circularize in cells infected with the adenovirus expressing ICP0 (Fig 4B), again demonstrating that the expression of ICP0 inhibits the circularization of the HSV genome. The empty adenovirus vector had no effect on EGFP expression from d109, or on the circularization of the d109 genome. The probe used in this experiment contains the HCMV IE promoter, allowing the simultaneous detection of both

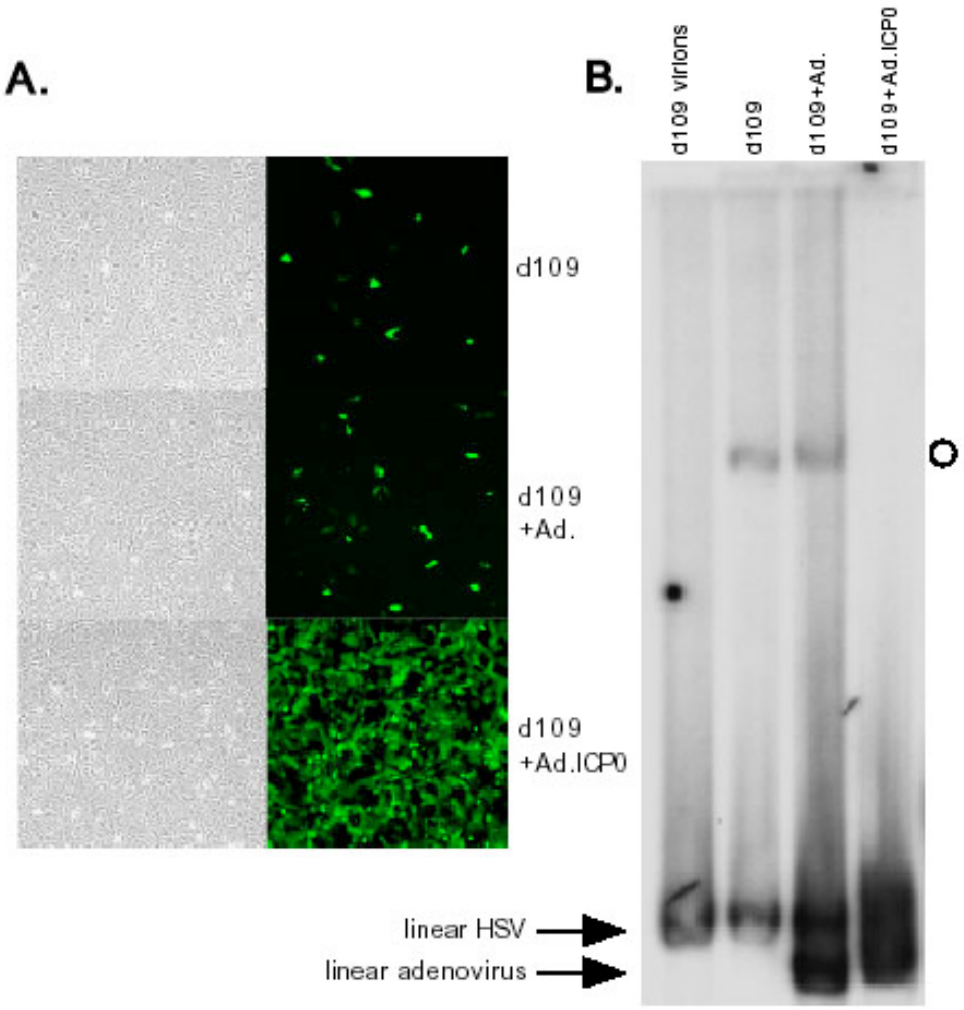


Figure 4. ICP0 expression from adenovirus inhibits circularization of d109 in trans.

(**A**) Phase-contrast and EGFP fluorescence images of Vero cells infected with d109. Cells infected with d109 were superinfected with AdS.11D (Ad.) or AdS.11E4(ICP0) (Ad.ICP0) at a moi of '50 PFU per cell directly postinfection with d109 where indicated. The infected cultures were analyzed 24h postinfection. (**B**) d109-infected cells were superinfected with the adenoviruses as described above and analyzed on Gardella gels.

d109 and the adenoviruses. Similar quantities of linear adenovirus genomes can be seen in the lanes containing samples from both adenovirus infections. The mobility of the linear adenovirus genome relative to the linear HSV genome is consistent with previous results (67). These results demonstrate that the very low levels of ICP0 are sufficient to inhibit circularization.

3.4.5. ICP0 inhibits genome circularization during productive infections.

Since ICP0 is abundantly synthesized prior to HSV DNA replication, the observation that the levels of ICP0 synthesized very early in infection can inhibit circularization of the genome presents a paradox in that previous studies examining end-joining have put forth the hypothesis that wt HSV genomes circularize, and that this circular form may serve as the template for genome replication (161). To address this paradox, samples of wt (KOS)- and ICP0 mutant (d99)- virus infected cells were analyzed on Gardella gels (Fig. 5). d99 is deleted for both copies of ICP0 (165). ICP0-defective mutants are replication competent, however they replicate slowly at low multiplicities of infection, and not every infectious center initiated by a single virion results in a plaque (164, 183). ICP0 is thought to function very early in infection, and increases the probability that an infection will proceed through the entire lytic cycle. The growth defect of ICP0 mutants is diminished at high multiplicities of infection (164, 183). Accordingly, cells infected at a moi of 20 (Fig. 5A) and 1 (Fig. 5B) PFU/cell were used for the analysis. In addition, samples of cells infected with d109 at the corresponding moi where included on each gel along with samples of cells infected with the test virus at the corresponding moi, but incubated in the presence of PAA to inhibit viral DNA replication.

At a moi of 20 PFU/cell hybridizing material began to accumulate in the wells beginning at 2h for KOS and 4h for d99 (Fig 5A). This is consistent with replicating DNA since this material was not observed in the presence of PAA. Moreover, there was an increase in the abundance of

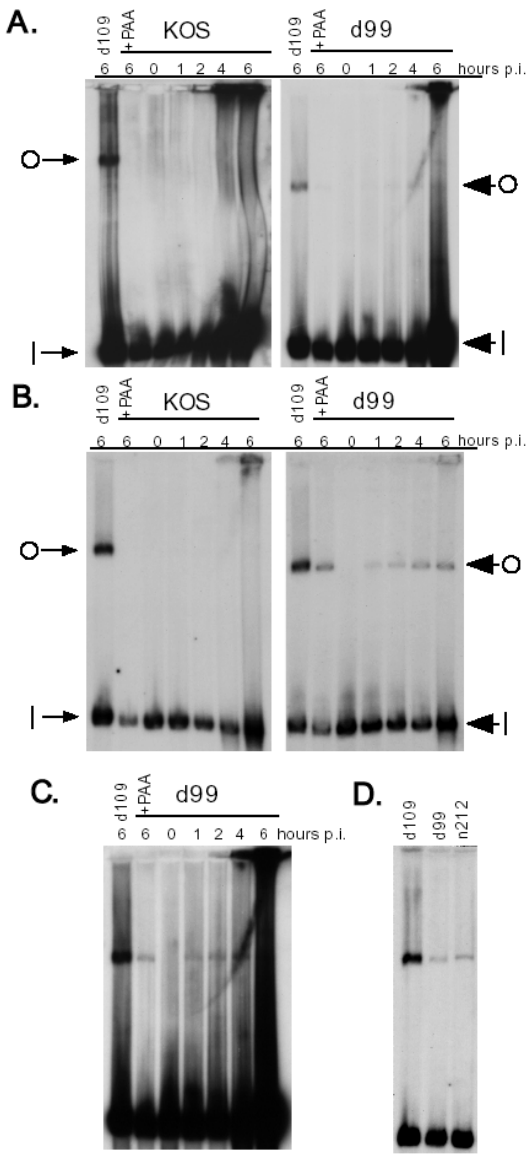


Figure 5. Circularization of the HSV genome during productive infection in the presence and absence of ICP0.

Vero cells were infected with wt virus (strain KOS) or d99 and incubated in the presence or absence of PAA for the indicated times before gel analysis. D109-infected cells were also included on all of the gels. (**A**) moi of 20 PFU per cell. (**B**) moi of 1 PFU per cell. (**C**) Overexposure of the d99 gel in A. (**D**) Vero cells were infected at moi of 3 PFU per cell of the indicated virus and incubated for 6 h in the presence of PAA before gel analysis.

linear forms beginning at 4h for KOS, and 6h for d99. These are inferred to be processed progeny genomes. However, in the case of KOS there was no evidence of the accumulation of material that comigrated with circular d109 genomes, in the presence or absence of PAA (Fig 5A). However, there was a low but detectable level of circular genomes in d99 infected cells, both in the presence and absence of PAA. An overexposure of the d99 (moi =20 PFU/cell) gel is shown in fig. 5C to clearly document the presence of circular genomes. Overexposure of the KOS gel failed to reveal the presence of circular genomes (data not shown). The difference between KOS and d99 was more pronounced at low moi (Fig. 5B). In KOS infected cells, replicative DNA (DNA in the wells) became evident by 4h post infection, however, there was no evidence of circular genomes, in the presence or absence of PAA. In contrast to KOS, a significant amount of circular genomes were seen in d99-infected cells (Fig. 5B). Circular genomes became evident very shortly after infection and increased in abundance up to 6h post infection. The proportion of circular relative to linear genomes accumulating in the presence of PAA in d99-infected cells was similar to that in d109-infected cells. Lastly, since d99 is entirely deleted for both copies of ICP0 (165), we tested another ICP0 allele, n212, to minimize the possibility that unknown genetic elements in the ICP0 region might be responsible for the observed effect. n212 exhibits no ICP0 activity by virtue of stop codons at residue 212 in both copies of ICP0 (15). Figure 5D demonstrates that the circularization of the n212 genome was similar to that of the d99 genome. Therefore, the accumulation of circles occurs in a productive infection in the absence of ICP0, and is more pronounced at low moi, a condition in which genome replication and virus production are limited.

3.4.6. Circular genomes are the persistent form during long-term quiescent infections that model latency.

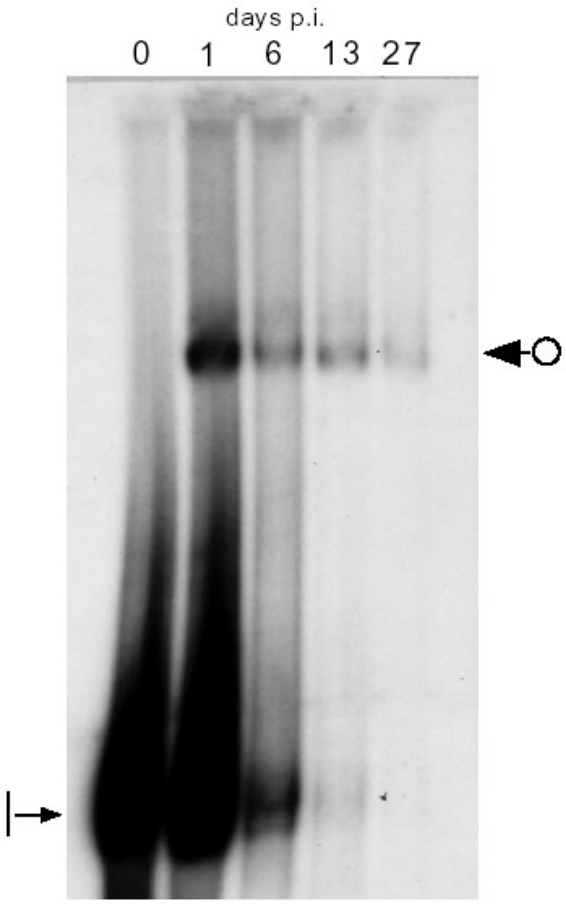


Figure 6. Stability of circular and linear d109 genomes during long-term persistent infection.

Vero cells were infected with d109 at 20 PFU per cell and incubated for the indicated time before analysis, as described in *Materials and Methods*.

At low multiplicities of infection, the abundance of linear KOS and d99 genomes remaining after 6h incubation in the presence of PAA was reduced relative to the amounts of input genomes and genomes present at early times post infection (Fig. 5B). This raises the possibility that linear non-replicating genomes may be somewhat unstable. d109, and other viruses limited in the expression of HSV IE proteins, are not toxic to cells, and their genomes have been demonstrated to persist in cultured cells in a quiescent state (154, 165). Therefore, to determine the relative stability of circular versus linear genomes, the fate of persisting d109 genomes in Vero cells was determined with respect to the abundance of linear and circular forms (Fig 6). Shortly after infection with d109 about 7-10% of the genomes circularized. The steady state level of circular d109 genomes decreased somewhat over the course of 27 days. However, the abundance of linear genomes decreased dramatically such that by 27 days post infection, only circular forms where detectable. Therefore, circular genomes are more stable, and are the predominant form of persisting HSV genomes in quiescent infections.

3.5. Discussion

Using Gardella gels we have demonstrated that a fraction of input HSV genomes circularized following infection in the absence of viral gene expression from the d109 genome (Fig. 2). This result is consistent with previous observations of end-joining (149) and "well DNA" in pulse field gels (65) of wt virus genomes in the presence of inhibitors of protein synthesis. However, the previously observed end-joining and "well DNA" in pulse field gels during productive infection with wt virus is not consistent with our observations, if it is interpreted to be due to circularization of the genome. We failed to observe circular wt (KOS) genomes during productive infection (Fig. 5). Therefore, the previously observed end-joining and "well DNA" in pulse field gels may represent replicative intermediates and/or recombination products. The

failure to observe circular genomes in wt HSV infected cells prior to the onset of DNA synthesis suggests that circular genomes are not the natural initial replication template.

Another observation that argues against the circularization of the genome prior to DNA replication is the lack of circularization during infections aborted prior to DNA replication. d106, which is similar to d109 except that its ICP0 genes are intact, did not circularize (Fig. 2). Circular genomes were also not observed during wt infection in the presence of PAA (Fig 5). PAA inhibits the activity of the HSV DNA polymerase (85). The failure to observe circular genomes in the presence of PAA is significant since all the viral proteins required for DNA replication are present, and all the events that occur up until DNA replication have occurred. Previous studies also failed to observe end-joining in the presence of PAA (149). These authors, like us, also concluded that the linear genomes present during wt virus infection in the presence of PAA were less stable. In contrast, it was recently reported that the ratio of genomic termini to joint regions decreases upon infection of CV-1 cells by wt virus in the presence of PAA (186). However, it is possible that this decrease may be due to exonucleolytic degradation of linear genomes, which would tend to decrease the termini to joint fragment ratio, and be consistent with the decreased stability of linear genomes observed by others (149) and us. Also consistent with our observation, was that the "well hybridization" to HSV sequences in pulse field gels of HSV infected cells was sensitive to PAA (65). These authors subsequently stated that they had not ruled out the possibility that DNA molecules other than circles might serve as initial templates for viral replication (65). In our studies, the temporal pattern of hybridization on Gardella gels proceeds from linear genomes, to DNA in the wells, and then to an increase in the abundance of linear genomes, without proceeding through a unit length circular intermediate (Fig. 5). Genome replication may proceed from the three origins on linear genomes producing

complicated branched structures that are modified and/or resolved by homologous recombination and by the viral cleavage/packaging machinery. Therefore, additional models to describe the initiation of HSV DNA replication should be tested.

ICP0 inhibited the formation of circular genomes (Figs. 2, 4, and 5). In particular, the level of ICP0 expressed from the adenovirus construct used in this study is sufficient for the activation of quiescent HSV genomes, the complementation of HSV ICP0 mutants, the inhibition of a virion-induced cellular antiviral response, and the disruption of PML (45, 82), all processes potentially relevant to the equilibrium between latency and productive infection. We propose that the inhibition of circularization by ICP0 is an additional mechanism for how ICP0 may regulate the balance between the productive and latent modes of the HSV life cycle. HSV genomes may circularize as a consequence of cellular mechanisms following infection. However, the expression of ICP0 very early in infection inhibits circularization. Interestingly, circles have been detected in Gardella gels of VZV infected cells (100), and by electron microscopy in pseudorabies virus (PRV) infection (90). However, the VZV Orf61 and PRV EP0, which both possess structural and function similarities to HSV ICP0 (132, 133), differ from HSV ICP0 in that they are expressed later in infection with these viruses. A fraction of VZV and PRV genomes may circularize prior to the synthesis of the ICP0 homologs. In the case of HSV, it may be that linear molecules serve as better templates for viral DNA replication. If this is case, the circular genomes accumulating during low moi productive infections with ICP0 mutants (Fig.5) may in part explain why ICP0 mutants are significantly impaired for replication at low moi. The relative efficiency with which circular and linear genomes serve as templates for replication and transcription will need to be examined to test this hypothesis.

The mechanism by which ICP0 inhibits circularization may involve the targeted degradation via the ubiquitin pathway of cellular proteins involved in end-joining, which may be due to either homologous recombination between the direct repeats at the genomic termini, or nonhomologous end-joining. The later process requires the DNA-dependant protein kinase (34), which has been shown by two groups to be targeted for degradation by ICP0 (107, 143). However since proteins involved in both processes (18), as well as ICP0 (57), can be found at ND10 structures, a number of possible mechanisms for the inhibition of circularization by ICP0 will need to be tested.

The circular form of the genome produced in the absence of ICP0 was more stable in cells relative to the linear structures, providing a mechanism for long-term persistence of genomes in cells. In d109-infected cells, the circular form was the only form persisting one month after infection (Fig. 6). Therefore, in an environment such as neurons, where the abundance and/or activity of ICP0 is regulated or reduced (25, 86), the genomes in some infected cells may circularize leading to persistence and latency, and in others remain linear, resulting in an abortive or lytic infection depending on the abundance of other viral regulatory and replication proteins.

4. ICP0 INHIBITS CIRCULARIZATION OF HSV GENOMES DURING PRODUCTIVE INFECTIONS: COMPARISON OF GARDELLA GEL ANLAYSIS FOR CIRCLES AND RESTRICTION ENZYME ANALYSIS FOR END-TO-END LINKAGES OF GENOMES.

4.1. Abstract

We have previously suggested that in the absence of the HSV immediate early protein ICP0 HSV-1 genomes circularize and that genome circularization is inhibited by ICP0 during productive infections. Here we provide further evidence that ICP0 inhibits genome circularization during productive infection with a non-inverting HSV-1 mutant, I358, by creating an ICP0 deleted version of I358. Both Gardella gel analysis for circular genomes and restriction enzyme analysis for end-to-end linkages of viral genomes consistently show that in the absence of ICP0 I358 genomes circularize, while in the presence of ICP0 I358 genomes are retained in a linear configuration, which may serve as a template for genome replication.

4.2. Note: Introduction/Results/Discussion

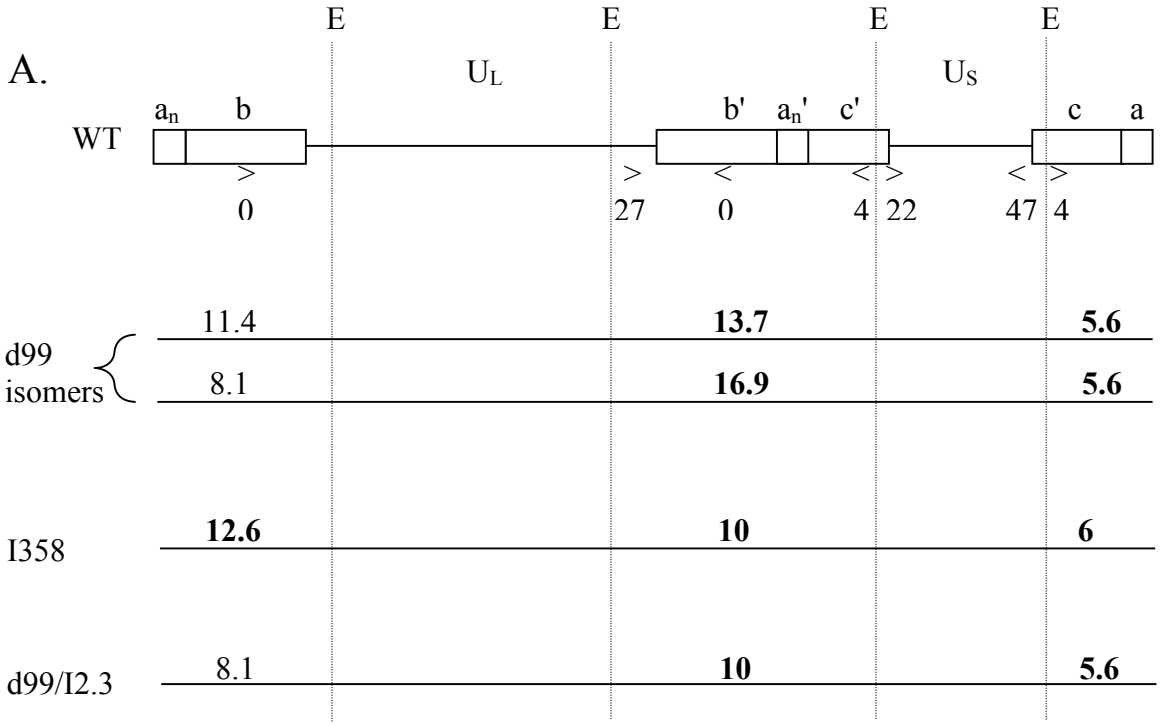
During infection, the HSV genome undergoes physical alterations in the nuclei of infected cells. These alterations are regulated by both cellular and viral proteins and ultimately lead to either a genome template for replication during the productive, lytic life cycle or to a stable genome configuration during the persistent, latent life cycle of the virus. Research that has examined the configuration of viral DNA during various stages of the virus life cycle along with the known structural characteristics of the viral genome has lead to a model of HSV DNA replication that is initiated by a theta mechanism (65, 149, 162, 185, 186). The template for theta mode replication is a circular viral genome. We have recently challenged this mechanism of replication by showing that the HSV immediate early protein, ICP0, inhibits genome circularization early during infection, thereby potentially preventing a theta mode of replication from occurring (89). In the absence of ICP0, HSV genomes circularize. In this note, we attempt

to clarify discrepancies between our results that suggest HSV genomes circularize only in the absence of ICP0 activity (89) and literature that suggests genomes circularize early during infection both in the presence and absence of viral gene expression, including ICP0 (65, 149, 185, 186). The ultimate goal of this work is to determine whether or not circular genomes are present early during productive infection and can subsequently serve as the initial template for genome replication.

We examined the configuration of a non-inverting HSV genome, I358 (courtesy of Bernard Roizman), early during infection, prior to the onset of replication. I358 is a non-inverting (non-isomerizing) HSV genome due to deletion of internal inverted a' and b' repeat sequences and truncation of internal inverted c' repeat sequences of the genome that subsequently retains the genome in one, prototypic, isomeric arrangement (149, 150). I358 forms novel end-to-end genome linkages during infection, as a result of either intramolecular linkages of single genomes (circularization) and/or intermolecular linkages of multiple genomes (concatemerization) (149). We examined circularization of I358 using both restriction enzyme analysis for end-to-end linkages and Gardella gel analysis in which large linear DNA molecules are separated from large circular DNA molecules of the same molecular weight through horizontal gel electrophoresis of whole cells with subsequent Southern blotting/hybridization to detect viral DNA (67, 89). In both analyses, unpurified virus stocks were prepared from the supernatants of cells (U20S or Vero) infected at low multiplicities and harvested when cytopathic effects were observed. Purified virus stocks were obtained by ultracentrifugation of unpurified stocks through a dextran T-10 gradient (37). Purified and unpurified stocks were used where indicated.

Using both analyses, we examined circularization of the non-inverting I358 virus in both the presence and absence of ICP0 by creating an ICP0 deleted version of the I358 virus. This was

A.



B.

Virus	Mutations	ICP0	ICP27	ICP4
d99	ICP0 deletion	-	16.9, 13.7	16.9, 13.7, 5.6
I358	a', b' deletion and truncated c'	12.6	10	6
d99/I2.3	ICP0, a', b' deletion and truncated c'	-	10	~5.6

C.

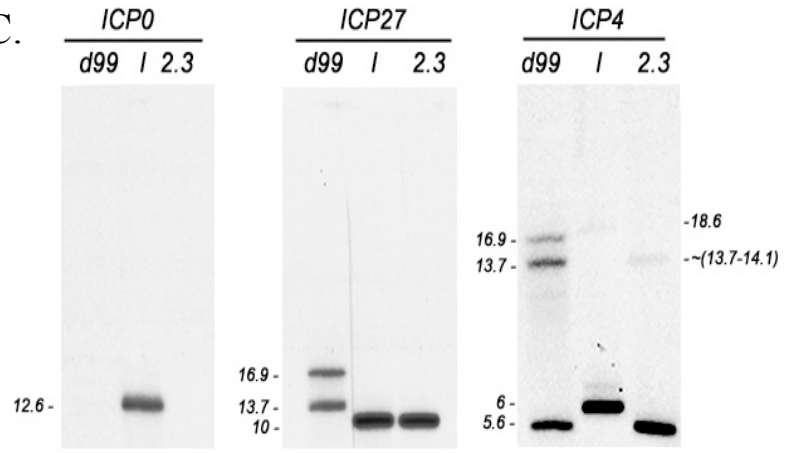


Figure 7. Analysis of d99/I2.3 genomes.

A. Wild type HSV (WT) genome showing U_L and U_S regions that are bracketed by direct repeats (ab, and ca, respectively). Direct repeats are inverted (b', a', c') within the region connecting the U_L and U_S sequences. There are multiple 'a' repeats where indicated (a_n). The position and direction of immediate early genes ICP 0, 4, 22, 27, and 47 are indicated, as are relevant EcoRI (E) sites. Below WT genome are representative relevant EcoRI maps of d99 isomer genomes and also I358 and d99/I2.3 genomes that are held in one isomeric arrangement. EcoRI fragments expected to hybridize to ICP 0, 4, or 27 sequences are in bold and indicated by fragment size in kilobases (KB). **B**. Table summarizing mutations of each virus, along with the expected sizes (KB) of EcoRI fragments after hybridization to each respective probe (ICP 0, 4, 27). **C**. DNA was isolated from d99, I358 (I), and d99/I2.3 (2.3) purified virions and then digested with EcoRI and resolved on a 0.8% agarose gel. After Southern blotting, DNA was hybridized to probes containing sequences for ICP0 (EcoRI/PstI fragment of pW3DHS8) (39), ICP4 (BamHI Y fragment of pKBY) (166), or ICP27 (pKHX-BH) (11).

done in U2OS cells through plaque purification of recombinants between I358 and another mutant derived in our laboratory, d99 (165), from which both copies of ICP0 have been deleted. Although ICP0 deficient viruses have severe growth defects (164, 183), they grow to normal levels in U20S cells, due to inherent activities within the cells that complement many activities of ICP0 (203). The subsequent virus d99/I2.3 contains the U_L terminus of d99, which is deleted for ICP0, and the U_L/U_S joint of I358, which is deleted for the other copy of ICP0 within the deleted U_L internal inverted repeats. Like I358, d99/I2.3 lacks internal inverted repeat sequences within the U_L/U_S junction of the genome and therefore does not isomerize. Figure 7A and 7B lists the expected sizes of EcoRI genome fragments generated from DNA isolated from purified virions of each respective virus. Figure 7C shows the subsequent Southern hybridization, performed as previously described (165), to ICP0 viral DNA sequences, confirming that I358 contains ICP0 sequences (12.6KB) while both d99 and d99/I2.3 lack ICP0. A similar EcoRI analysis with hybridization to ICP27 sequences confirms that d99/I2.3 contains the U_L/U_S joint sequence (10KB) of I358, not of d99 (13.7 or 16.9 KB) (Figure 7C). EcoRI analysis for ICP4 sequences indicates that like I358, d99/I2.3 lacks the internal ICP4 sequences that d99 contains within the U_S internal inverted repeats (13.7 or 16.9 KB); both I358 and d99/I2.3 only contain the U_S terminal copy of ICP4 (Figure 7C). However, the U_S terminus of d99/2.3 is similar in size to that of d99 (5.6KB) rather than I358 (6KB). The '*a*' repeats of d99 are approximately 250bp (165) while those of I358 are approximately 450bp (150). Recombination and/or cleavage and packaging events during generation of the d99/I358 recombinant, which occurs at the '*a*' repeats (161, 193), appears to have altered the size of the terminal U_S '*a*' repeat sequence, making the U_S terminal fragment of d99/I2.3 (~5.6KB) more similar to d99 (5.6KB) than I358 (6KB). Note, in DNA isolated from each respective purified virion prep, a small fraction of EcoRI

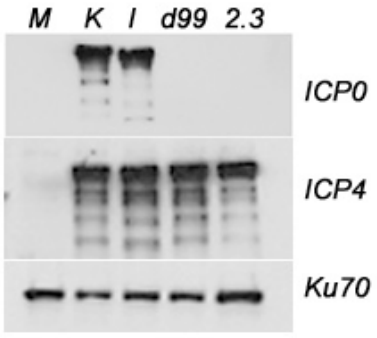


Figure 8. Western blot analysis of d99/I2.3 virus.

Western blot analysis for ICP0 (#1112; 1:5000; Goodwin Institute for Cancer Research, Inc., Plantation, Fla.), ICP4 (N15; 1:5000) (177), and Ku70 (1:5000; Santa Cruz Biotechnology) in mock (M), KOS (K), I358 (I), d99, and d99/I2.3 (2.3) infected Vero whole cell (moi 1) extracts at 24 hours post infection. Staining for Ku70 was used as a loading control.

fragments that are consistent with the expected size of U_L and U_S terminal junction fragments for both I358 (18.6KB) and d99/I2.3 (~13.7-14.1KB) hybridized to ICP4 probes (Figure 7C). This fraction of U_L and U_S junction fragments was not completely eliminated but greatly reduced by virion purification. Consistent with Southern analysis, Western blots (165) of whole cell extracts isolated from KOS (wild type), d99, I358, and d99/I2.3 infected Vero cells indicates that d99/I2.3 expresses ICP4 to similar levels of the other examined viruses but lacks expression of ICP0 even after 24 hours post infection, similar to d99 (Figure 8).

Using Gardella gel analysis, we examined circularization of I358 and d99/I2.3 during infection of Vero cells in the presence and absence of phosphonoacetic acid (PAA; Lancaster) to inhibit virus DNA replication (85). d109 has been used as a control for both the presence and migration rate of circular genomes in Vero cells during Gardella gel analysis (89). All infections were preformed on ice in TBS (+/- 400ug PAA) and then placed in fresh media (DMEM +/- 400ug PAA) at 37^0C, as previously described (89). Cells were harvested at the indicated times post absorption on ice; 0 hour time points correspond to cells harvested after 1 hour absorption on ice. During I358 infection of Vero cells, we observed a pattern of hybridization that proceeded from linear viral DNA to replicating well DNA after 6 hours post absorption on ice (Figure 9A). However, unlike during infection with d109 in which circular genomes were detected, during I358 infection circular genomes were not detected, importantly, in either the presence or absence of PAA. In fact, the pattern of hybridization observed during I358 infection was similar to that which we have previously observed for wild type strain KOS using this analysis, albeit with slower kinetics (89). These results suggest that I358 genomes do not circularize early during infection or during events leading up to replication.

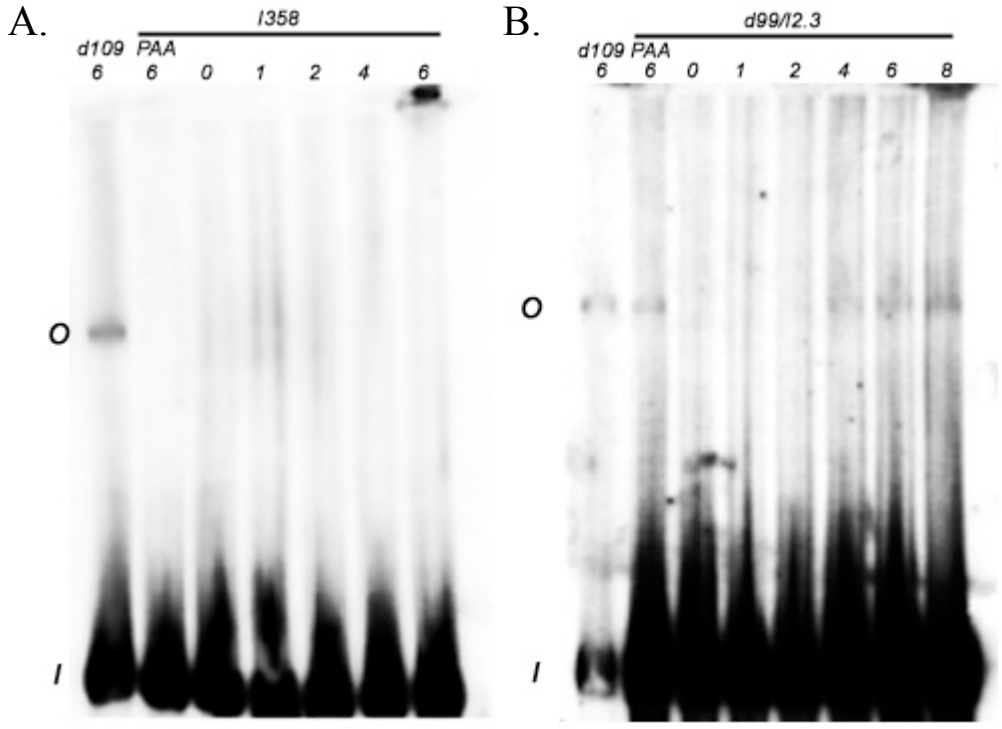


Figure 9. ICP0 inhibits HSV genome circularization during infection.

Gardella gel analysis of I358 (**A**) and d99/I2.3 (**B**) infected (moi 5) Vero cells, using unpurified virus. Cells were harvested at the indicated times post absorption and loaded into the wells of Gardella gels. After electrophoresis, gels were Southern blotted, hybridizing to the HSV BamHI Y fragment of pKBY. Infections were done in the presence of 400ug/mL PAA where indicated. d109 infected Vero cells were loaded as a control for detection of circular (O) and linear (l) genomes. Note that d109 infection in (**B**) was only done at moi 0.5.

During d99/I2.3 infection of Vero cells Gardella gel analysis shows a pattern of hybridization that proceeds from linear genomes to replicating well DNA at 8 hours post adsorption (Figure 9B). The comparative 2 hour delay in virus replication between d99/I2.3 and I358 can be attributed to the lack of ICP0, as ICP0 mutants show defects in virus replication and growth in non-complementing cells (164, 183). However, unlike during I358 infection, during d99/I2.3 infection circular genomes were detected in both the presence of virus replication, beginning at 4 hours post absorption, and the absence of virus replication inhibited by PAA (Figure 9B). A fraction of circular genomes was consistently observed during infection with d99/I2.3 at various multiplicities, including 1, 3, 5, and 10, using both purified and unpurified virus during infection in both the presence and absence of virus replication (data not shown). These results strongly suggest that ICP0 inhibits HSV genome circularization and that in the presence of ICP0 genomes remain in a linear state from which replication is initiated.

We also examined circularization of non-inverting HSV genomes in the presence (I358) and absence (d99/I2.3) of ICP0 using restriction enzyme digestion of DNA isolated from infected Vero cells with subsequent Southern analysis for the U_L terminal (J) fragment (Figure 10A). Because both I358 and d99/I2.3 are deleted for internal repeat sequences and do not isomerize, end-to-end linkages of genomes at the U_L (J) and U_S (L) terminal repeats form novel joint (JL) fragments, which should be indicative of circularized genomes (Figure 10A). Although the appearance of newly formed joint fragments does not rule out the possibility that new joints are formed through concatemerization of multiple genomes, the infections were done at a low multiplicity (moi 1) using purified I358 and purified d99/I2.3 virions to increase the probability that formation of novel joints is due to intramolecular joining of genome ends (circularization) rather than intermolecular joining of genome ends (concatemerization). All infections were

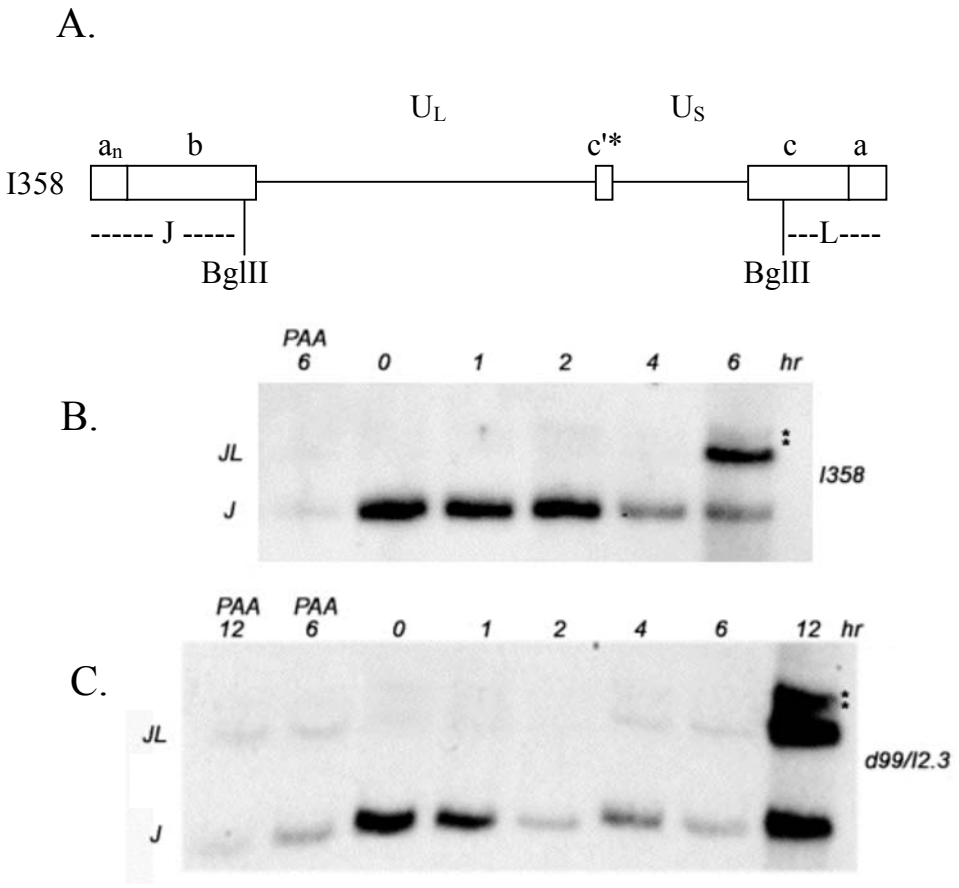


Figure 10. ICP0 inhibits formation end-to-end genome linkages during infection.

A. The I358 genome, showing deletion of internal inverted repeats a_n' and b' and truncation of c' (c'*). The relevant BglII sites are indicated and are similar in d99/I2.3. Vero cells were infected (moi 1) with purified I358 (**B**) or purified d99/I2.3 (**C**) virus stocks and harvested at the indicated times post absorption. 400ug/mL PAA was used to inhibit replication where indicated. DNA was isolated from cells, digested with BglII, and then resolved on a 0.8% agarose gel, which was Southern blotted and probed for HSV sequences that hybridize to the U_L (J) terminus and, subsequently the novel joint (JL) sequence. Defective genomes described by others are noted (**) (149). Note lane 2 in **C** was slightly underloaded, as observed on the ethidium bromide staining of gel prior to blotting (not shown).

preformed on ice in TBS (+/- 400ug PAA), placed in fresh media (+/- 400ug PAA) at 37^0C, and then harvested at the indicated times post absorption on ice; 0 hour time points correspond to cells harvested after 1 hour absorption on ice. However, only purified virus stocks were used to infect cells during restriction enzyme analysis. At the indicated times post absorption, DNA was isolated from infected cells, digested with BglII, and then resolved on an agarose gel. DNA was transferred to a membrane, which was then, hybridized to HSV-1 XbaI/BamHI sequences, spanning 10635-11819bp of the genome, that was isolated from a cloned EcoRI JK fragment. This 1.18kb XbaI/BamHI fragment was used to detect U_L termini (J) and subsequently joint (JL) viral DNA fragments during Southern analysis. These joint (JL) fragments should only be present in end-to-end linked viral genomes.

We first examined formation of novel joint fragments (JL) during I358 infection of Vero cells in both the presence and absence of replication inhibited by PAA (Figure 10B). Joint (JL) fragments were only observed at 6 hours post absorption of I358. In addition, at 6 hours post absorption for both I358 and d99/I2.3, we also observed a small fraction of bands that arise from 'defective genomes' above the joint (JL) fragment; these bands and 'defective genomes' have been previously observed and described by others (149). Importantly, joint fragments were not observed at 0 hours post absorption, from 1-4 hours post absorption, or moreover, at 6 hours post absorption when virus replication was inhibited by PAA (Figure 10B). Treatment of cells with PAA during infection allows all events leading up to replication to occur but inhibits replication itself, in theory, holding the viral genome in a configuration that will serve as a template for replication. Novel joint fragments that accumulate by 6 hours post absorption in the absence of PAA (JL; Figure 10B) correspond to replicative well DNA during Gardella gel analysis of I358 at 6 hours post absorption (Figure 9A). We have previously speculated that replicative well

DNA within Gardella gels is highly branched and/or concatemerized genomes that are byproducts of ongoing replication (89); the abundance of joint fragments during times in which well DNA is observed supports this theory. Consistent with the instability of linear genomes that we have previously observed during Gardella gel analysis of various HSV viruses (d109, KOS, and d99) (89) and others have noted (149, 185), the U_L terminus (J fragment) was unstable throughout infection with I358 both in the presence and absence of PAA (J; Figure 10B). This instability is more pronounced in restriction enzyme analysis than in Gardella gel analysis, most likely because a lower multiplicity of infection was used when end-to-end genome junctions were examined.

By contrast, upon BglII digestion and analysis for d99/I2.3 end-to-end genome junctions, we observed a small, but detectable, fraction of joint (JL) fragments beginning at 4 hours post absorption of Vero cells that became highly abundant by 12 hours post absorption (JL; Figure 10C). The highly abundant joint fragments at 12 hours post absorption correspond to replicative well DNA observed as early as 8 hours post absorption during Gardella gel analysis of d99/I2.3 (Figure 9B). Again, replication of d99/I2.3 is somewhat delayed with respect to I358 replication due to the lack of ICP0 and the U_L terminus (J fragment) was unstable throughout infection both in the presence and absence of PAA (Figure 10B, 10C). The appearance of joint fragments at 4 hours (Figure 10C) corresponds to the appearance of circular genomes that we observed during Gardella gel analysis at 4 hours post absorption (Figure 9B). Importantly, at later times (6 hr, 12 hr) post absorption, when replication was inhibited by PAA, a small fraction of joints was observed (PAA; Figure 10C), consistent with the small fraction of circular genomes that we have observed in the presence of PAA during Gardella gel analysis of d99/I2.3 (PAA, Figure 9B). However, the proportion of d99/I2.3 joint (JL) fragments, that appear early during infection (4hr)

and in the presence of PAA, to their relative U_L terminal (J) fragment appears high when compared to the proportion d99/I2.3 circular to linear genomes during Gardella gel analysis. A lower multiplicity of infection was used in analyses for end-to-end genome junctions than in the Gardella gel analyses. We have previously demonstrated that at lower multiplicities of infection a higher fraction of genomes circularize (89), which is consistent with the higher ratio of joint to terminal fragments in the end-to-end analysis as compared to the ratio of circular to linear genomes in Gardella gel analysis.

End-to-end linkages of HSV genomes, indicative of circular genomes, begin to form early during infection (4hr) in only the absence of ICP0 (d99/I2.3) (Figure 10), consistent with the detection of circular genomes in only the absence of ICP0 during Gardella gel analysis (Figure 9). These end-to-end linked and circular genomes were also observed in only the absence of ICP0 when DNA replication was inhibited. End-to-end genome junctions formed in both the presence and absence of ICP0 at later times during HSV replication corresponding to replicative well HSV DNA in Gardella gels and is most likely indicative of highly branched and/or concatemerized genomes that are intermediates of replication. Together, these results provide strong evidence that HSV genomes only circularize in the absence of ICP0 activity; in the presence of ICP0 genomes remain in a linear state from which replication is most likely initiated. Our BglII digest analysis for end-to-end linkages of viral genomes, in which we only observed linkages in the absence of ICP0, is inconsistent with similar studies by Poffenberger and Roizman in which end-to-end linkages of I358 were interpreted to be due to circularization of genomes early during infection, regardless of viral gene expression (149). Our results are also inconsistent with a very recent paper by Strang and Stow, in which the authors used HSV viruses that lack '*a*' repeat sequences but contain parts of the HSV packaging signal at each termini to

suggest that end-to-end linkages of genomes, indicative of circular genomes not concatemers, are present early during infection in the presence of ICP0 and in the absence of HSV gene expression (185). However, there are many problems with the interpretation of data in both of these papers, arising from inherent complexities present within the technique used by both authors to analyze end-to-end genome junctions. We also initially experienced many of these issues during our analysis of end-to-end linkages of genomes.

Because U_L and U_S terminal junction fragments are highly abundant during virus DNA replication, as we (Figure 10) and others (149, 185) have shown, these junctions are present in virus stocks grown in cell culture due to lysis of cells, where replicating viral DNA is present. Using unpurified virus in our restriction enzyme analysis for end-to-end linkages of genomes led to an abundance of U_L and U_S junction fragments within our unpurified virus stocks, even at 0 hours post absorption on ice (data not shown), during which the intensity of junctions increased with increasing multiplicity of infection. The end-to-end linkages of I358 noted by Poffenberger and Roizman may represent replicative intermediates and/or recombination products containing U_L and U_S terminal junctions derived from unpurified virus inoculum due to the high multiplicity of infection (40 PFU/cell) used (149). Unfortunately, 0 hour time points were not provided during their analysis to determine otherwise. During our analysis using unpurified I358, although this junction fragment was present at 0 hours, the intensity did not change throughout infection until 6 hours post absorption during replication (data not shown). In fact, even after virions were purified we observed a very small, but notable, fraction of U_L and U_S junction fragments within our purified virus stocks for both I358 and d99/I2.3, as we noted above (ICP4; Figure 7C). Importantly, when purified virus was used, this fraction was not present at 0 hours post absorption during analysis for end-to-end linkages of either I358 or d99/I2.3; U_L and U_S

junctions only formed early during d99/I2.3, not I358, infection and at later times in the presence of PAA during d99/I2.3, not I358, infection (Figure 10). In Strang and Stow's analysis, end-to-end linked genome junctions, in their case U_L(r) and U_L(l) junctions, are present in unpurified virion preps used to spike mock infected lysed cells and at 0 hours post absorption with unpurified virus (185). Furthermore, in Figure 5 of their paper, the fraction of U_L(r) and U_L(l) junctions detected in the presence of PAA throughout infection and in the absence of PAA early during infection was not much greater than that observed at 0 hours post absorption in each respective experiment. At 1 hour post absorption (+PAA), which shows the most dramatic increase in U_L(r) and U_L(l) junction fragments, the lane was over loaded, which is demonstrated by the relative increased stability of T1 and T2 fragments in the presence of PAA at this time as compared to the relative decreased stability of T1 and T2 fragments in the absence of PAA at this time. Also, the lane corresponding to 2 hours postabsorption (+PAA) was underloaded.

Differences in the observed presence of end-to-end genome linkages are most likely due to contaminating genome junctions, and we suggest these differences are resolved by using purified virus stocks. In our hands, both Gardella analysis and BglII analysis for end-to-end genome linkages, when purified virus is used, consistently suggest that HSV genomes circularize only in the absence of ICP0 and that ICP0 inhibits genome circularization. Moreover, these results strongly suggest that in the presence of ICP0 genomes remain in a linear state from which replication is initiated. Subsequently, an initial theta mechanism of HSV replication does not seem likely. Genome replication may proceed from the three origins of replication on linear genomes producing highly branched structures that are modified and/or resolved by recombination and by the viral cleavage/packaging machinery. Lagging strand synthesis of linear HSV genomes may be completed through mechanisms such as recombination-dependent

replication (RDR), a mechanism by which T4 phage lagging strand synthesis is accomplished (136, 137).

5. INHIBITION OF DNA REPAIR AND GENOME CIRCULARIZATION BY HSV ICP0

5.1. Abstract

We have recently shown that in the absence of immediate early protein ICP0 HSV-1 genomes circularize and that genome circularization is inhibited by ICP0 during productive infections. From these conclusions, we have hypothesized that the ends of input HSV genomes are treated as double strand breaks (DSB) that are repaired early during infection via circularization of viral genomes by cellular DNA DSB repair pathways and that ICP0 inhibits these pathways. We examined the effect of ICP0 on proteins involved in DNA repair events in order to study how ICP0 alters the cellular DNA repair environment, leading to inhibition of genome circularization. During DNA DSB repair, a complex of Mre11/Rad50/NBS1 (M/R/N) proteins form discrete nuclear foci that co-localize with phosphorylated H2AX (γH2AX) foci, which accumulate at DSB's. Early during abortive HSV infection (1hr) in the absence of IE gene expression, M/R/N and γH2AX foci were observed and colocalized but dissipated at later times (6 hr). In contrast, when ICP0 was expressed, M/R/N and γH2AX foci accumulated and persisted throughout abortive infection but did not colocalize. For the first time, these results suggest that incoming HSV stimulates cellular DNA repair processes that dissipate or are completed later during infection in the absence of ICP0. In the presence of ICP0 this repair response persists and is altered, as M/R/N and γH2AX foci do not co-localize. ICP0 does not affect the global level of M/R/N proteins during infection, and M/R/N complexes do not affect circularization of HSV genomes. However, ICP0 does induce the degradation of the nonhomologous end-joining (NHEJ) protein DNA PKcs in a cell type specific manner. We examined the effect of NHEJ proteins on circularization of HSV-1 genomes. In cells mutant for NHEJ proteins, genome circularization was greatly delayed and decreased. These results suggest a major role for NHEJ

in HSV genome circularization. Together, the results provide evidence for cellular DNA repair responses to incoming HSV genomes and alterations in these repair responses as a result of an activity of ICP0.

5.2. Introduction

Lytic infection with HSV-1 begins with the expression of five immediate early (IE) viral genes, designated infected cell polypeptide (ICP) 0, 4, 22, 27, and 47. IE proteins initiate a cascade of viral gene expression that leads to virus genome replication and production of progeny viruses. Latent HSV-1 infection occurs when the viral genome persists in a nucleosome-associated state (40) in which viral gene expression is largely repressed, thereby precluding virus replication. However, latent infections can reactivate to produce recurrent lytic infections.

ICP0 plays a pivotal role in balancing the lytic and latent life cycles by greatly enhancing the probability of the lytic cycle both during primary infection and reactivation events. Early during lytic infection, ICP0 localizes to and disrupts ND10 bodies (116), which are discrete nuclear foci where HSV-1 replication centers may be located (117). ND10 bodies play a role in a variety of cellular pathways including cellular stress responses and chromosomal DNA repair pathways (118). ICP0 has E3 ubiquitin ligase activity and subsequently induces the *in vivo* proteasome-dependent degradation of PML and Sp100 (23), which are constituents of ND10 bodies, of the DNA-dependent Protein Kinase Catalytic Subunit (DNA PKcs) (107) (143), which is critical for VDJ recombination and DNA double-strand break repair via non-homologous end-joining (NHEJ) (30), and of CENPA and CENPC (53), which are histone-3 like centromere proteins. Exactly how ICP0 activity promotes the lytic cycle remains unclear, although it ultimately leads to indiscriminate transactivation of viral gene expression.

We have recently shown that ICP0 also inhibits circularization of HSV-1 genomes (89). In the absence of ICP0 the HSV-1 genome circularizes. Viral genomes are thought to persist as circles during latency, where viral gene expression is largely repressed (44, 159). Therefore ICP0 activity may control the balance between the lytic and latent life cycles, in part, by regulating the configuration of viral genomes. We hypothesize that in the absence of ICP0 activity the ends of linear HSV-1 genomes are treated as double-strand DNA breaks that are repaired via circularization of viral genomes by cellular DNA double-strand break repair pathways. ICP0 could therefore inhibit circularization of HSV-1 genomes by inhibiting DNA repair pathways.

In mammalian cells, homologous recombination (HR) and non-homologous end-joining (NHEJ) are the two primary pathways by which double-strand breaks in DNA are repaired. Distinct proteins play a role in each NHEJ and HR repair, however some proteins, such as the Mre11/Rad50/NBS1 (M/R/N) complex, may have overlapping roles in both mechanisms (35). M/R/N complexes are sensors of DNA double-strand breaks and activate the ATM-mediated repair pathway (1, 105). Many proteins involved in HR have redundant functions and are subsequently somewhat disposable to the mechanism. However, one protein Rad51 appears to be necessary for HR (134). Ku70, Ku80, DNA PKcs, XRCC4, and DNA Ligase IV are critical for NHEJ *in vivo* (30). Ku70 and Ku80 subunits dimerize, forming the regulatory subunits of the DNA PK holoenzyme (70). The Ku dimer binds DNA ends (8, 130), recruiting the catalytic subunit of DNA PK (DNA PKcs) to sites of DNA double strand breaks (70). Once associated with DNA-bound Ku subunits, DNA PKcs catalyzes the end-joining reaction through a combination of phosphorylation events (127) that leads to recruitment of the XRCC4/DNA Ligase IV tetramer, which enzymatically ligates the DNA ends together (198). Little sequence

homology is required for NHEJ repair, while HR involves the exchange of homologous DNA sequences.

The HSV genome is a linear double stranded DNA molecule that is divided into unique long and unique short regions that are connected by a joint sequence (195) (197). Both long and short regions are bracketed by homologous inverted repeats that contain a 250-500 bp sequence, termed the 'a' sequence, in a directly repeating orientation at each termini but in an inverted orientation at the U_L/U_S junction (197) (161). Thus, the genome is a suitable substrate for either HR or NHEJ repair mechanisms.

HSV infection induces activation and signaling to proteins involved in DNA damage responses. In particular, during HSV infection ATM and Mre11 are necessary for activation and signaling that results in modification of repair proteins (ATM, Chk2, 53BP1, and NBS1) and many times redistribution of these repair proteins to HSV replication compartments (111, 178). Additional studies have also shown redistribution of other repair proteins (DNA PKcs, Ku80, Rad50, Rad51, BRCA1, WRN, BLM, MSH2, and RPA) involved in NHEJ and HR mechanisms to viral replication compartments (188, 200). However, whether these repair proteins are signaled as a result of input linear viral genomes, are recruited to repair the highly nicked HSV genome during replication, or play a role in inter- and intra- molecular recombination events that occur during viral replication has not been determined.

Our focus is to examine how the host cell responds to incoming HSV genomes and how the virus, in particular ICP0, may counteract or manipulate this response, ultimately leading to inhibition of genome circularization. Using replication defective mutant viruses, we examined the distribution of DNA repair proteins in response to input HSV and the effect of ICP0 on this distribution. We also examined the ability of DNA repair pathways to circularize HSV genomes

during infection. Together, the results provide evidence for cellular DNA repair responses to incoming HSV and an ICP0-induced effect on these responses. They also strongly suggest a major role for NHEJ in circularization of HSV-1 genomes.

5.3. Methods

5.3.1. Viruses.

The mutant viruses: d109, d105, d106, and d99 were derived from wild type strain KOS and used at the indicated multiplicities of infection (moi) (165). Where indicated, 400ug/mL phosphonoacetic acid (PAA; Lancaster Synthesis) was added to Tris-buffered saline (TBS; 0.14M NaCl, 2.7 mM KCl, 25mM Tris-base, pH 7.4) or the appropriate cell culture media during and after viral absorption to inhibit virus replication. Virus was allowed to absorb to HEL and Vero cells for one hour on ice in TBS in order to synchronize infections. Virus was allowed to absorb to other cells lines for one hour at room temperature in TBS. After all infections, cells were washed 1x with TBS and the appropriate media was placed on cells until indicated harvesting times post-infection.

5.3.2. Cells.

Human Embryonic lung (HEL) cells (ATCC) were used for all immunofluorescence experiments and for Western Blot analyses. HEL cells were maintained in DMEM supplemented with 10% FBS and grown in 5% CO_2 at 37^0C. Cells were used at low passage (<20). Similarly, Vero (ATCC) cells used in Western Blot analyses were maintained in DMEM supplemented with 5% FBS and grown in 5% CO_2 at 37^0C.

Cells mutated in genes involved in DNA doubles strand break repair used in Gardella gel analyses were cultured as follows. Primary fibroblasts from an apparently healthy human (NBS1+; Coriell Cell Repository) and primary fibroblasts from an individual homozygous for a

deletion of 5 nucleotides in exon 6 of the NBS1 gene (NBS-; Coriell Cell Repository), resulting in a truncated protein from a frameshift and subsequent premature termination at codon 218, were cultured in MEM Eagle-Earle BSS 2x concentration of essential and non-essential amino acids and vitamins with 2mM L-glutamine and 15% FBS under 5% CO_2 at 37^0C. MO59J, which lack DNA PKcs activity, and MO59K (both ATCC), which contain normal levels of DNA PKcs activity, cell lines isolated from the same human glioblastoma tumor were cultured in a 1:1 mixture of DMEM and Ham's F12 medium with 2.5 mM L-glutamine and supplemented with 0.5mM sodium pyruvate, 0.05mM non-essential amino acids and 10% FBS under 5% CO_2 at 37^0C. SV40 T-antigen transformed mouse embryonic fibroblasts (MEFs) lacking (-/-) or expressing (+/+) Ku80 were provided by Dr. Andre Nussenzweig (NIH/NCI). Both cell lines were cultured in DMEM with 10% FBS under 5% CO_2 at 37^0C. SV40 T-antigen transformed MEFs lacking both p53 and DNA ligase4 (p53-/-lig4-/-) and MEFs expressing DNA ligase 4 but lacking p53 (p53-/-) were provided by Dr. Frederick Alt (CBR Institute for Biomedical Research, Boston) and were cultured in DMEM supplemented with 10% FBS and grown under 5% CO_2 at 37^0C.

5.3.3. Immunofluorescence.

HEL cells were plated one day prior to infection on 18mm circular glass coverslips (VWR) at a densitly of $1.5x10^6$ cells per 60mm dish in media. Prior to infection, confluent HELs in 60mm dishes (4 coverslips per dish) were chilled on ice and rinsed with 4^0C TBS. Infections were done on ice for 1 hour with rocking, after which warm media (with 400ug/mL PAA for KOS and d99) was added. Cells were incubated immediately at 37^0C until the desired times post-infection. At desired times post-infection, coverslips were rinsed with warm TBS and rapidly submerged in -

20^0C methanol for exactly 5 minutes to fix the cells. Cells were allowed to dry thoroughly prior to beginning the indirect immunofluorescence protocol.

Cells were stained for various proteins using standard indirect immunofluorescence techniques. Antibody dilutions are as follows: rabbit anti-NBS1 (1:200; NOVUS Biologicals), mouse anti-Mre11 (1:100; GeneTex), mouse anti-PML (1:30; Santa Cruz Biotechnology), mouse anti-Rad50 (1:100; GeneTex), and rabbit anti-γH2AX (1:200; Upstate Biologicals). Primary antibodies were followed by secondary anti-mouse IgG-conjugated Alexa Fluor488 or anti-rabbit IgG-conjugated Alexa Fluor594 (both 1:500; Molecular Probes). Controls using only secondary antibodies were performed to ensure no non-specific staining or bleed-through was present (data not shown). Coverslips were mounted in gelvatol on glass slides and imaged at 100x power with a Nikon Diaphot 300 epifluorescent microscope. Images were collected using the Spot program and analyzed with Adobe Photoshop software.

5.3.4. Western Blots.

Standard Western Blot protocols were used to examine the levels of DNA repair proteins in whole cell extracts of HEL and Vero cells infected at the indicated multiplicities. Briefly, proteins were resolved on denaturing (SDS) Tris-HCl polyacrylamide gels and then transferred at 250mAmps (4^0C) onto Hybond-P membranes (Amersham Biosciences). Non-specific binding was blocked with 5% milk in PBS-0.5%Tween20. Membranes were washed with PBS-0.5%Tween20 3x10 minutes before and after primary antibody staining and 3x10 minutes after secondary antibody staining. To detect proteins, membranes were incubated for 1 hour with the following primary antibodies at the indicated dilutions in PBS-0.5%Tween20 with 3% milk: DNA PKcs (1:1000; Upstate Biotechnology), mouse monoclonal Ku80 (1:5000; Upstate Biotechnology), rabbit polyclonal Ku70-H308 (1:5000; Santa Cruz Biotechnology), mouse

monoclonal Rad51-3C10 (1:2000; Upstate Biotechnology), mouse monoclonal ICP0-1112 (1:5000; Goodwin Institute for Cancer Research, Inc., Plantation, Fla.), mouse polyclonal XRCC4 (1:1000; GeneTex), mouse monoclonal Rad50-13B3 (1:500; GeneTex), mouse monoclonal Mre11-12D7 (1:400; GeneTex), and rabbit polyclonal NBS1/p95/nibrin (1:10,000; Novus Biologicals). Primary antibodies were detected with secondary anti-mouse ($1:4x10^4$) or anti-rabbit ($1:1.5x10^4$) IgG HRP conjugated antibodies (Promega) diluted in PBS-0.5%Tween20 with 3% milk and were visualized by ECL plus Western Blotting Detection System (Amersham Biosciences) upon exposure of membrane to film.

5.3.5. Gardella gel analysis.

Gardella gels were assembled and preformed as previously described (89). After infection of $5x10^6$ cells at the indicated multiplicity of infection, cells were harvested at the indicated time points by washing with TBS, scraping into TBS, and then pelleting by low-speed centrifugation. Cell pellets were resuspended in 50uL loading dye (15% Ficoll, 0.01% bromophenol blue) and loaded into the wells of the Gardella gel. Gels were run at 45V for 3 hours to lyse cells and then 120V for 38 hours to separate circular from linear genomes of the same molecular weight. Southern blots were used to visualize viral DNA. The 1.8kb *Bam*HI-Y fragment of pKBY (166), which contains a portion of the ICP4 gene of HSV-1, was used as the probe to detect viral genomes during all Southern blot analyses.

5.4. Results

5.4.1. Formation of M/R/N complexes and γH2AX foci as a function of ICP0.

Foci of the phosphorylated (serine 139) form of histone variant H2AX (γH2AX) accumulate at sites of DNA double-strand breaks (DSB) (157, 160, 172). M/R/N foci colocalize with γH2AX foci during repair of DNA DSBs that occur as a result of ionizing radiation (IR) (19) (144).

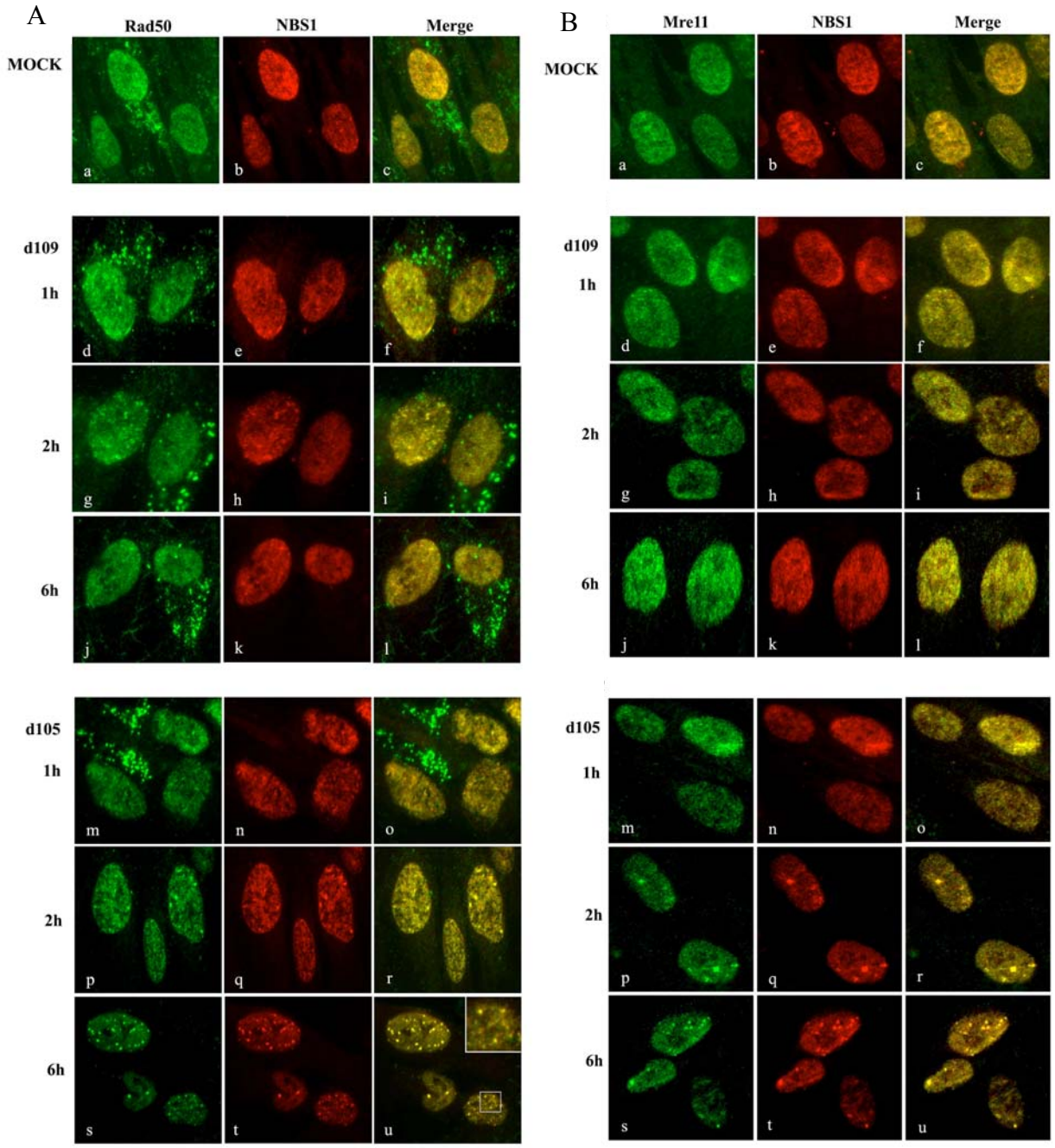


Figure 11. Formation of M/R/N foci as a function of ICP0 during abortive infection.

HEL cells were infected with d109 (**d-l**) or d105 (**m-u**) at an moi of 20 prior to fixation and indirect immunofluorescence (IF) staining at 1 hour (**d-f**; **m-o**), 2 hours (**g-i**; **p-r**), or 6 hours (**j-l**; **s-u**) post infection. **A**. Rad50 (green) and NBS1 (red) **B**. Mre11 (green) and NBS1 (red). For both (**A**) and (**B**), colocalization is shown in yellow and mock infected cells are shown (**a-c**). Highly resolute, colocalizing M/R/N foci can be seen in the presence (d105; inset **u**) but not absence (d109) of ICP0.

Using HSV-1 mutant strains d109 and d105, we examined M/R/N and γH2AX foci formation during viral infection of HEL cells as a function of ICP0. d109 is an HSV-1 mutant that lacks expression of all five IE viral genes and subsequently establishes a non-toxic persistent quiescent state from which gene expression can be de-repressed by ICP0 (165). d105 is an HSV-1 mutant that expresses ICP0 but lacks expression of the additional four IE viral genes (165). Both d109 and d105 form abortive infections in which neither mutant replicates due to lack of ICP4 expression.

We examined formation of M/R/N foci during abortive HSV infections as a function of ICP0. In the presence of ICP0, discrete and highly resolute foci of colocalizing Rad50 and NBS1 (d105, m-u; Figure 11A) proteins and colocalizing Mre11 and NBS1 (d105, m-u; Figure 11B) proteins were observed throughout infection, indicating that M/R/N complexes form in the presence of ICP0 during abortive infections. However the numerous, highly resolute M/R/N foci were not as evident in the absence of ICP0 (d109, d-l; Figure 11A and 11B) or during mock infections (mock, a-c; Figure 11A and 11B). These complexes accumulated and persisted up to 24 hours post infection with d105 (a-c and d-f; Figure 12). Co-localizing Mre11 and NBS1 foci were also observed during wild-type KOS (g-i; Figure 13) but not ICP0-mutant d99 (d-f; Figure 13) infections carried out in the presence of phosphonoacetic acid to inhibit virus replication but allow for IE gene expression (85). d99 lacks expression of ICP0, but expresses the additional four IE genes and thus replicates in the absence of PAA.

We next examined the formation of γH2AX foci and its colocalization with M/R/N foci during abortive infection as a function of ICP0. With closer inspection, at one hour post infection with d109, we observed some, yet less resolute, Mre11 foci (g; Figure 14). These Mre11 foci clearly co-localized with γH2AX foci (g-i; Figure 14). In the majority of cells, both

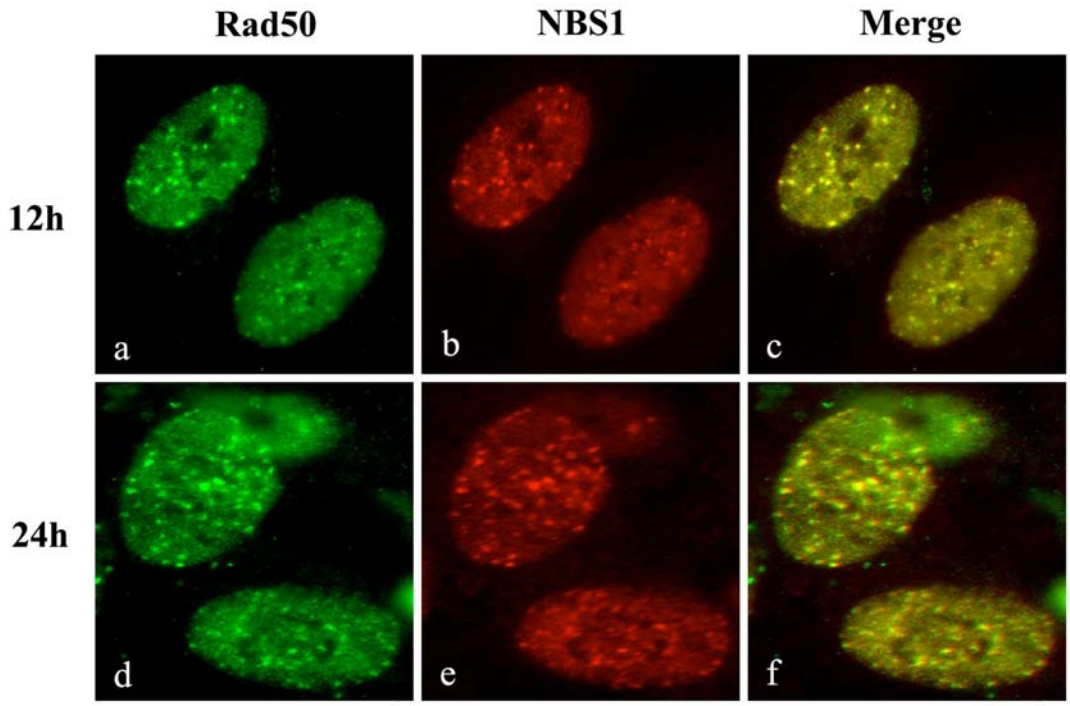


Figure 12. Rad50 and NBS1 foci accumulate and persist during abortive infection in the presence of ICP0.

HEL cells were infected with d105 at an moi of 20 and fixed at 12 (**a-c**) or 24 (**d-f**) hours post infection. Indirect Rad50 and NBS1 IF staining is shown in green and red, respectively. Colocalization is indicated in yellow.

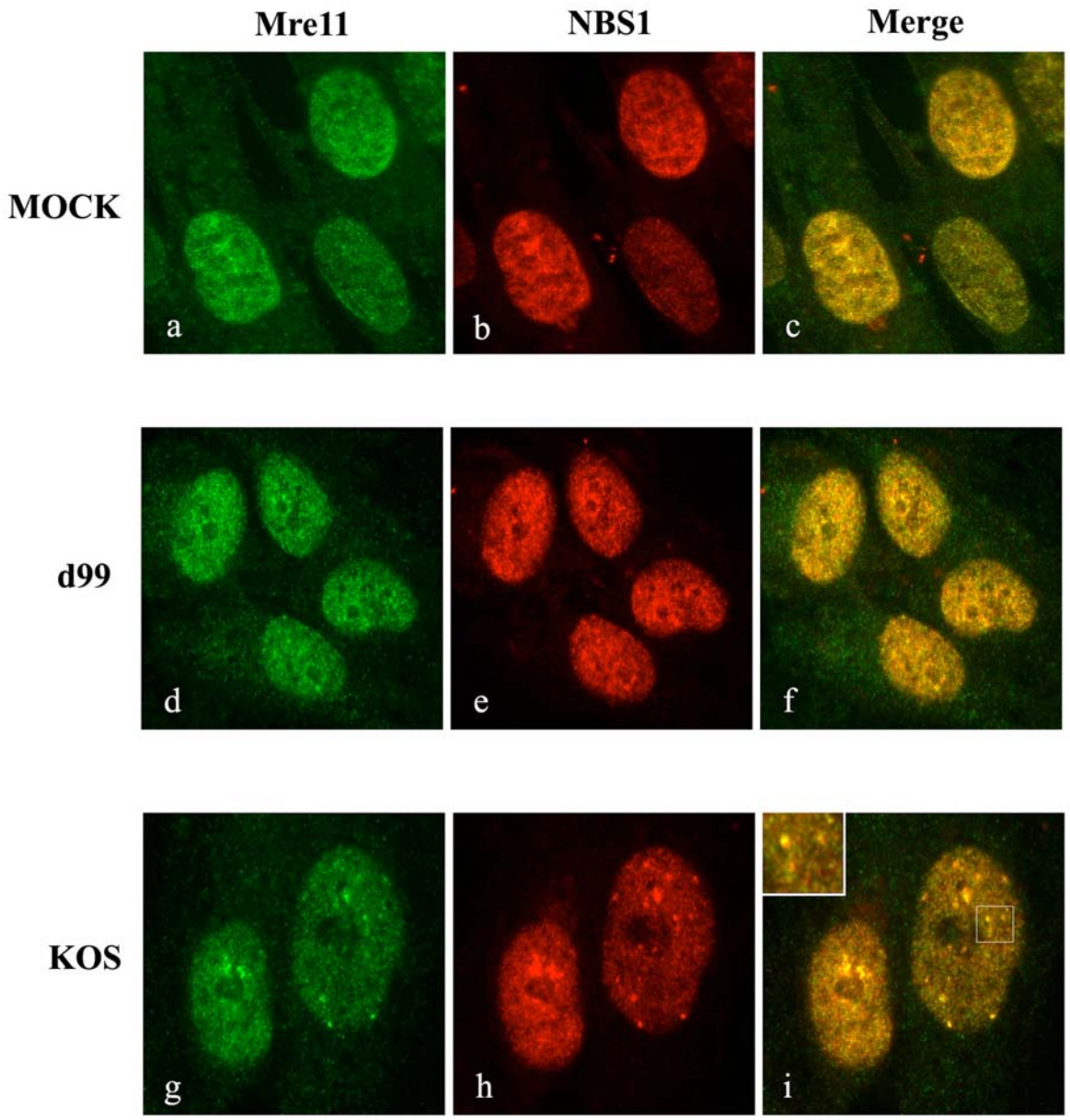


Figure 13. Formation of Mre11 and NBS1 foci as a function of ICP0 during productive infection in which replication is inhibited.

HEL cells were infected with KOS (**g-i**) or ICP0-mutant d99 (**d-f**) at moi of 10 and fixed 6 hours post infection at which time cells were stained for either Mre11 (green) or NBS1 (red) by indirect IF. Cells were infected in the presence of PAA to inhibit virus replication. Staining of mock infected cells (**a-c**) is shown, and colocalization of Mre11 and NBS1 is shown in yellow.

Mre11 and γH2AX foci dissipated by 6 hours post infection (j-l; Figure 14), with Mre11/γH2AX staining resembling the field of mock-infected cells (a-c; Figure 14). By contrast, when ICP0 was expressed, both Mre11 (m, p; Figure 14) and γH2AX (n, q; Figure 14) foci were observed throughout d105 infection, with many foci being strongly detected at 6 hours post infection (p-q; Figure 14), as in IR treated cells (d-e; Figure 14). Interestingly, in the presence of ICP0, M/R/N complexes do not co-localize with γH2AX foci (o, r; Figure 14) as they do in IR treated cells (f; Figure 14). These results suggest that infection with d109 induces a cellular DNA repair response early during infection that dissipates or is completed later in infection, as evidenced by the formation and then loss of co-localizing M/R/N and γH2AX foci. ICP0 appears to alter this repair response to incoming HSV in that M/R/N foci accumulate and persist throughout infection and do not colocalize with γH2AX foci.

5.4.2. Levels of DNA repair proteins as a function of ICP0.

We next examined the levels of DNA repair proteins as a function of ICP0 expression (Figure 15). For both infected HEL and Vero whole cell extracts, we compared the level of DNA repair proteins in the presence of ICP0 (d106, KOS) and in the absence of ICP0 (d109, d99) at 6 hours post abortive (d106, d109) and productive (KOS, d99) infection. d106, like d105, expresses ICP0 but lacks expression of the additional four IE genes (165). Levels of proteins involved in NHEJ (DNA PKcs, Ku70, Ku80, XRCC4) and HR (Rad51) were examined, as well as levels of proteins that constitute the M/R/N complex (Mre11, Rad50, NBS1). Of the proteins examined, only the level of DNA PKcs was dramatically reduced in the presence of ICP0 during both d106 and KOS infection of HEL cells, relative to that observed during mock, d109, or d99 infection of HEL cells. Consistently, it has been previously shown that DNA PKcs undergoes proteasome-

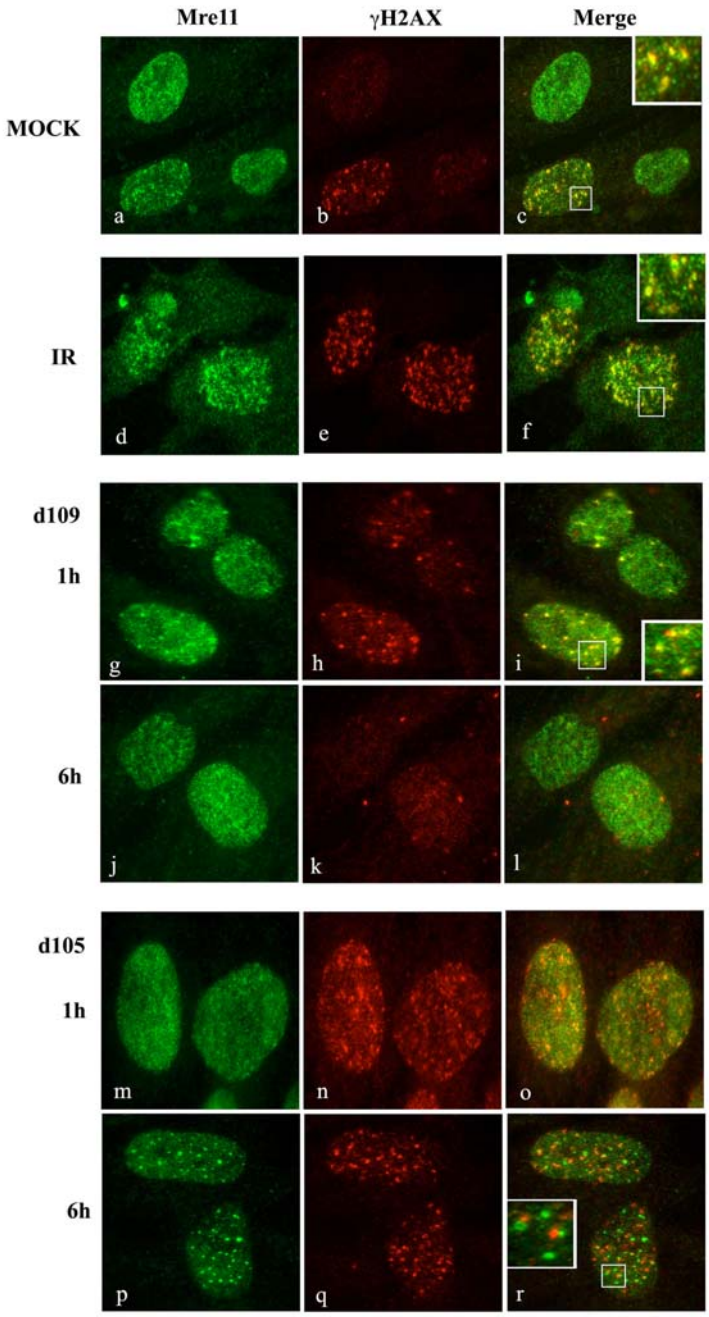


Figure 14. Colocalization of Mre11 and γH2AX foci in the absence but not presence of ICP0 during abortive infection.

HEL cells were infected with d109 (**g-l**) or d105 (**m-r**) at an moi of 30 and fixed 1 hr (**g-i**; **m-o**) or 6 hr (**j-l**; **p-r**) post infection. Indirect IF was used to detect Mre11 (green) and γH2AX (red); colocalization is shown in yellow. Cells in **a-c** were mock infected, with approximately only 5% of cells containing Mre11/γH2AX foci in nuclei (inset **c**). Cells in panels **d-f** were treated with 5 Gy ionizing radiation (IR) and allowed to recover for 30 minutes prior to fixation and staining. Colocalization of Mre11 and γH2AX can be seen in IR treated cells (inset **f**) and early during d109 infection (inset **i**) but not during d105 infection (inset **r**).

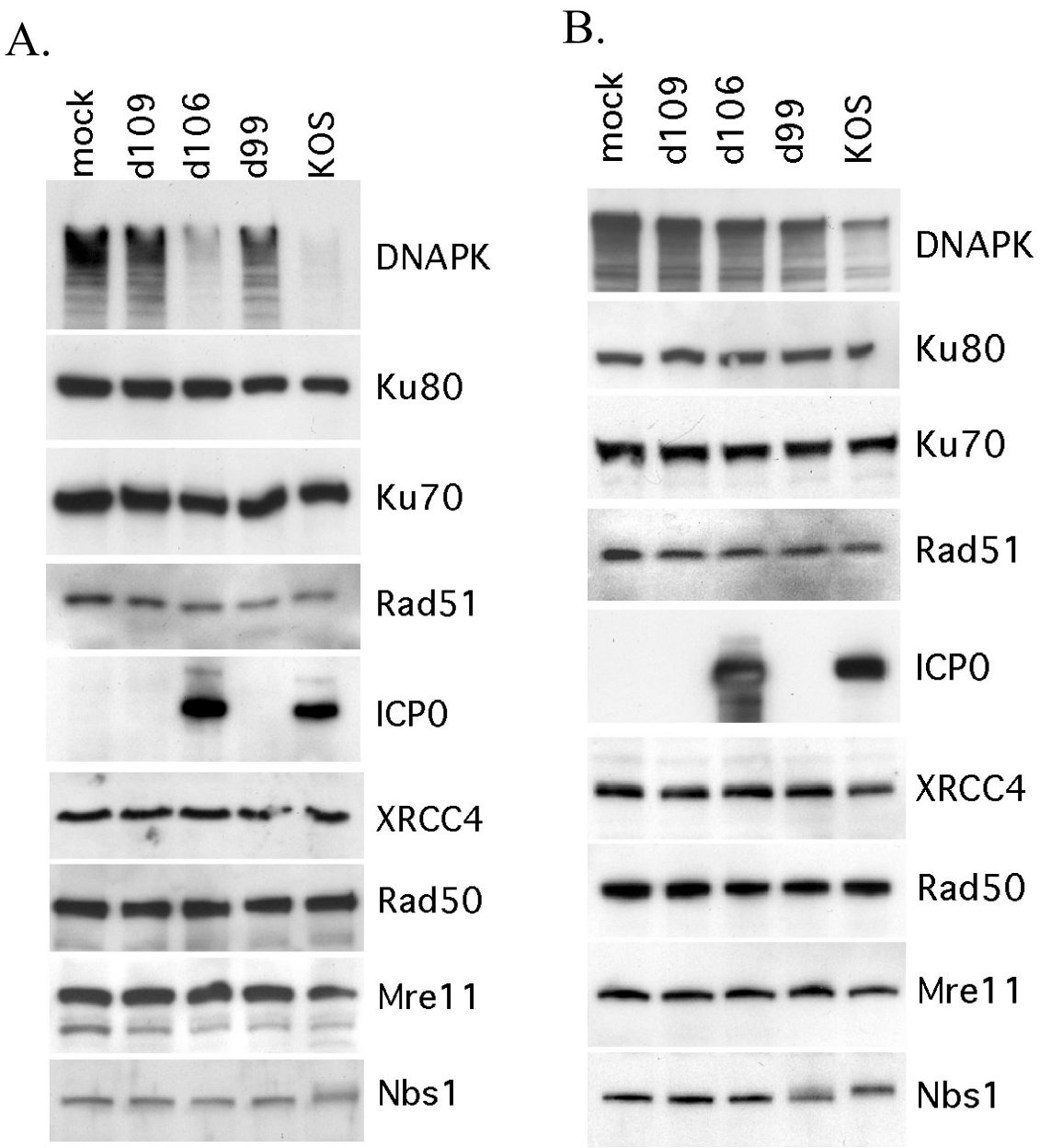


Figure 15. Global level of DNA repair proteins as a function of ICP0.

Western blot analysis of HEL (**A**) and Vero (**B**) whole cell extracts at 6hr post-infection with the indicated virus (moi = 10). The DNA repair proteins analyzed are indicated.

dependent degradation as a result of ICP0 expression in human-derived Hela (107) (143) but not monkey-derived Vero cell lines (200).

In addition, during KOS infection and to a lesser extent during ICP0-mutant d99 infection of both HEL and Vero cells, the mobility of NBS1 was slightly decreased (NBS1; Figure 15), which we project to be due to phosphorylation. Phosphorylation of NBS1 indicates induction of cellular ATM DNA repair processes (202) and has been observed by others during HSV infection (111, 200) (178). However, our study indicates that NBS1 modification does not occur as a function of ICP0 expression but rather occurs in response to additional factors that are present during both wild-type KOS and ICP0-mutant d99 infections.

5.4.3. NHEJ is involved in circularization of HSV-1 genomes

We hypothesized that DNA repair pathways play a role in circularizing HSV-1 genomes in the absence of ICP0 expression and that ICP0 inhibits circularization by inhibiting DNA repair pathways. Due to ICP0-induced degradation of DNA PKcs, which plays a major role in NHEJ, we examined circularization of d109 genomes in cells lacking NHEJ proteins using Gardella gel analysis (Figure 16). M059J cells are a human glioblastoma derived cell line that lack DNA PKcs activity (106). M059K cells are their complementing cell line that contain normal levels of DNA PKcs activity and were derived from the same human glioblastoma tumor (106). In M059K cells we detected circles in Gardella gels between 1 and 2 hours post infection, which is consistent with our previously determined kinetics of d109 circularization in Vero cells (Figure 16A). However, in M059J cells the kinetics of d109 circularization were severely delayed (Figure 16A). A similar delay in the kinetics of d109 circularization was also observed in mouse embryonic fibroblasts (MEF) lacking Ku80 expression relative to a complementing MEF cell line expressing Ku80 (Figure 16B) (194). In addition, the fraction of d109 genomes that

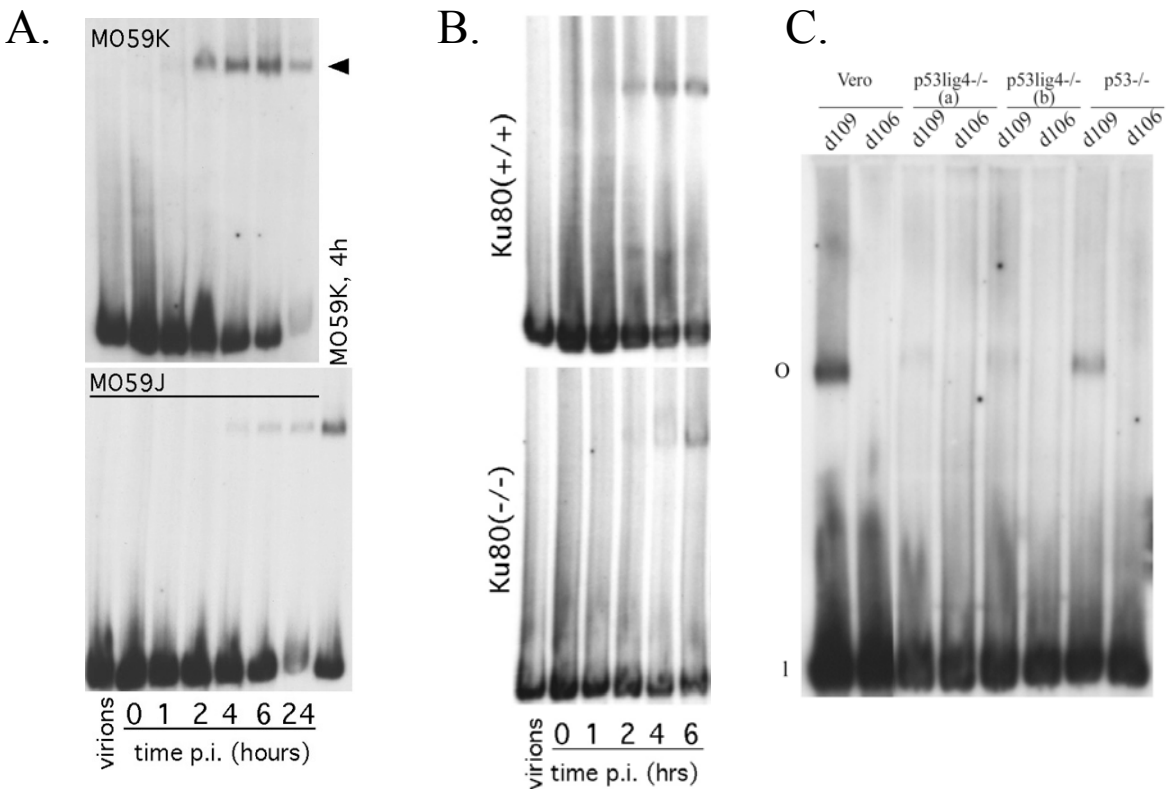


Figure 16. Circularization of HSV genomes in cells deficient in NHEJ.

A. MO59J (DNA PKcs -) or M059K (DNA PKcs +) human gliablastoma cells were infected with d109 (moi 20) for the indicated times and analyzed by Gardella gels. Arrow indicates position of circular genomes. **B**. Ku80+/+ or Ku80-/- MEFs were infected with d109 (moi 20) for the indicated times and analyzed by Gardella gels. **C**. Vero, ligase4-/-p53-/-(a) MEFs, ligase4-/-p53-/-(b) MEFs, and p53-/- MEFs were infected for 6 hours with d109 or d106 (moi 20) and then analyzed by a Gardella gel. Circular (O) and linear (l) genomes are indicated.

circularized was greatly decreased in cells lacking DNA PKcs (compare M059J and M059K 4hr; Figure 16A). Even more discernable is the reduction in circularized d109 genomes we also observed in two independent MEF cell lines lacking both DNA ligase4 and p53 (lig4-/-p53-/- (a), lig4-/-p53-/- (b)), relative to MEFs lacking only p53 (p53-/-) (Figure 16C). Deletion of both p53 and DNA ligase4 rescues the embryonic lethality conferred by DNA ligase4 deletion alone (3) (62) (93). Consistent with ICP0's ability to inhibit circularization of viral genomes (89), circular genomes were not detected during d106 infection of p53-/- or lig4-/-p53-/- (a) and (b) MEFs (Figure 16C). In lig4-/-p53-/- MEFs there was a small, but detectable, fraction of circular genomes observed in the absence of ICP0 (Figure 16C). These results provide strong evidence for a critical, but not absolute, role of the NHEJ pathway in circularizing HSV genomes in the absence of ICP0 expression.

Although ICP0 does not alter levels of proteins that constitute the M/R/N complex, it induces the accumulation and persistence of M/R/N foci (Figure 11, 12, 13) during HSV infection and inhibits co-localization of γH2AX foci with M/R/N complexes (Figure 14), suggesting that it alters DNA repair responses induced by incoming HSV. We thus examined circularization of d109 by Gardella gel analysis during infection of a human cell line derived from a Nijmegen Break Syndrome patient containing a severely truncated non-functional form of NBS1 and during infection of a cell line derived from a healthy, functional NBS1-expressing individual. There was no detectable difference in the kinetics of d109 circularization or the fraction of genomes that circularized in cells lacking functional NBS1, with respect to cells expressing functional NBS1 (Figure 17). Because M/R/N nuclear repair foci are not formed in cells lacking NBS1 (19) (43), the results suggest that processes localized to M/R/N nuclear foci do not play a significant role in circularizing d109 genomes.

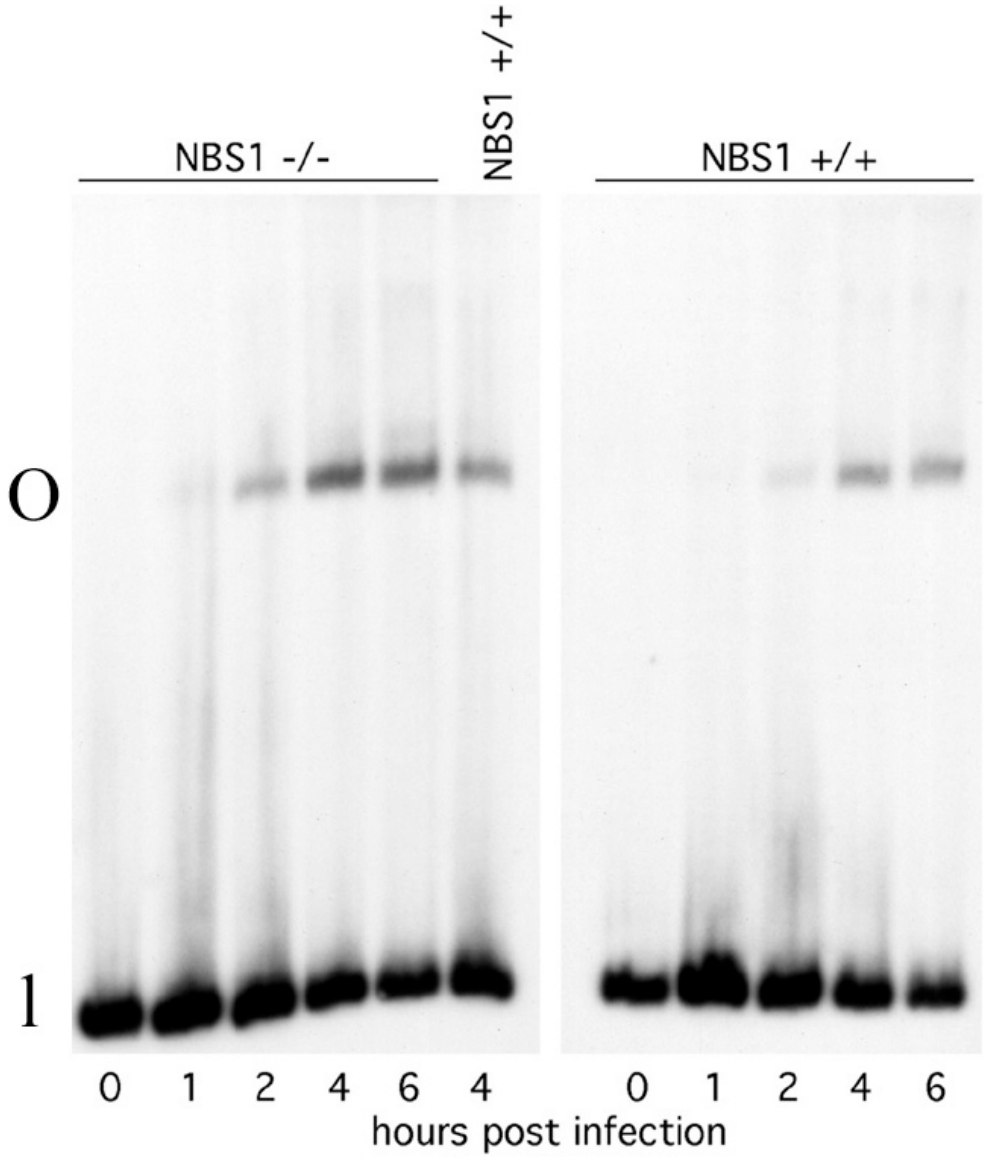


Figure 17. NBS1 does not affect HSV genome circularization.

Human cells lacking functional NBS1 (-/-) or expressing functional NBS1 (+/+) were infected with d109 at an moi of 10, harvested at the indicated times post infection, and analyzed by Gardella gels. Circular (O) and linear (l) genomes are indicated.

5.5. Discussion

We have previously shown that ICP0 inhibits HSV genome circularization (89) and have subsequently hypothesized that in the absence of ICP0 activity the ends of HSV genomes are treated as double-strand DNA breaks that are repaired via circularization of viral genomes by cellular DNA double strand break repair pathways. ICP0 could therefore inhibit circularization of HSV genomes by inhibiting DNA repair pathways. In this study we have examined both the effect of ICP0 on the cellular DNA DSB repair environment and the effect of repair pathways on circularization of HSV genomes. Incoming HSV appears to stimulate DNA repair events, and DNA repair pathways in particular NHEJ play a major role in HSV genome circularization. ICP0 both affects DNA repair events and inhibits HSV genome circularization.

M/R/N complexes are essential for DSB repair and genomic stability. These foci accumulate at sites of ongoing DNA repair (19, 144). Others have shown these foci accumulate at sites of HSV replication (111, 188, 200) and suggest that these repair proteins facilitate the HSV productive cycle (111). Here we demonstrate that in the presence of ICP0, M/R/N accumulate and persist both during abortive infection (d106) (Figure 11, 12) and, consistent with the results of others (200), during productive infection (KOS+PAA) when replication is inhibited by PAA (Figure 13). γH2AX foci, which are markers for ongoing DNA DSB repair (157, 160, 172), also accumulate during abortive infections in the presence of ICP0, as we have previously observed (72). However, unlike during DSB repair events, M/R/N and γH2AX foci do not colocalize and do persist when ICP0 is present (Figure 14). Although not as apparent in Figure 11, both M/R/N and γH2AX foci also form in the absence of ICP0 (d109) early (1hr) during abortive HSV infection (Figure 14). Importantly, γH2AX foci colocalize with M/R/N foci formed early during d109 infection, but at later times (6hr) both foci dissipate, which is similar to

the formation and then dissipation of these foci during DSB repair events. These results suggest that input HSV stimulates DNA repair events that dissipate or are completed at later times during infection in the absence of ICP0. Importantly, these repair events are affected by the presence of ICP0, as both γH2AX and M/R/N foci accumulate and persist and do not colocalize.

These results are the first to show that during abortive infections, both γH2AX foci and M/R/N complexes form in response to input HSV (Figure 11, 14) and that ICP0 promotes the accumulation and persistence of these complexes (Figure 12) but disables their association with each other (Figure 14). However, whether or not M/R/N complexes or γH2AX foci form at sites of input HSV genomes remains undetermined. We did not observe correlations between the number of γH2AX foci formed and the number of input HSV genomes during infections at various multiplicities (data not shown), which suggest that repair foci form at sites of cellular DNA. Early events during HSV-1 infection induce damage to host cell chromatin, including chromosome uncoiling, fragmentation, and margination (26, 139, 140, 145, 182). These events require HSV entry and IE gene expression (61, 140) and subsequently could be caused by ICP0 during abortive infections, thereby inducing the accumulation and persistence of M/R/N and γH2AX foci. Consistently, ICP0 induces the loss of kinetochore proteins CENPC and CENPA, which may lead to chromosome instability (53). However, M/R/N foci do not colocalize with γH2AX during infection, as would be expected during DSB repair events, suggesting that the repair process is somehow disrupted in the presence of ICP0.

The levels of M/R/N protein constituents are not affected in either the presence or absence of ICP0 during both productive and abortive infections (Figure 15). However, during productive infections, NBS1 is most likely modified by phosphorylation, as has been observed by others (200) (111) (178). M/R/N complexes are sensors to DNA DSB's and recruit/activate

ATM (1, 105). NBS1 is phosphorylated by ATM during activation of the ATM repair pathway (202). We have shown that this modification is not dependent on ICP0 but does require additional viral proteins other than ICP0; others have suggested it to be dependent on the presence of the HSV DNA polymerase HP66 (200). During abortive infections, incoming HSV appears to stimulate DNA repair events that induce M/R/N and γH2AX formation, but these events occur in the absence of detectable ATM activation, as NBS1 phosphorylation is not present during abortive infections (d109, d106; Figure 15). Others have also observed the absence of detectable ATM activation during abortive infections with replication incompetent virus (HSV amplicon vector) (178). Regardless of ICP0, the ATM repair pathway does not appear to be activated by input HSV. During HSV infection, additional factors must play a role in the activation of ATM. Lack of HP66 inhibits recruitment of repair proteins, including NBS1, to prereplicative sites and inhibits activation of ATM, as evidenced by the absence of NBS1 phosphorylation (200). Perhaps recruitment of NBS1 to prereplicative sites, which are not present during abortive infections, is required for ATM activation.

We have previously established that ICP0 inhibits HSV genome circularization (89) and have shown here that ICP0 alters DNA repair events involving M/R/N complexes that occur independent of ATM activation in response to incoming HSV. Although ICP0 appears to alter these DNA repair events, M/R/N complexes and the ATM pathway do not play a role in circularization of HSV genomes, as HSV circularization remains unaffected during abortive infections of cells lacking functional NBS1, in which M/R/N complexes do not form (Figure 17) (19, 43). Others have shown that M/R/N complexes and their associated processes may facilitate HSV replication (111). ICP0 may somehow direct this facilitation by promoting the

accumulation and persistence of M/R/N complexes and/or by potentially altering their nuclear distribution.

Of the proteins we examined, only the level of DNA PKcs was decreased in HEL cells but not Vero cells as a function of ICP0 (Figure 15). The levels of other examined proteins were not affected in either the presence or absence of ICP0 during both productive and abortive infections. Consistently, it has been previously shown that DNA PKcs undergoes proteasome-dependent degradation as a result of ICP0 expression in human-derived Hela (107) (143) but not monkey-derived Vero cell lines (200). Because of the cell type specific degradation of the global level of DNA PKcs, we suggest that ICP0 may have a localized effect, possibly only occurring where ICP0, viral genomes, and DNA repair proteins colocalize early during infection. Consistently, in Hep-2 cells DNA PKcs is somewhat degraded and complexes of DNA PKcs, Ku80, Ku70, and XRCC4 co-precipitate with the HSV single stranded DNA binding protein, ICP8, at times in which ICP8 is localized to HSV genomes, suggesting a direct and/or indirect interaction between these NHEJ repair proteins with viral genomes (188). Furthermore, only autophosphorylated forms of DNA PKcs, which are a very small fraction of the very abundant global level of DNA PKcs, localize to sites of DNA DSB's during repair events (22).

In cells deficient in NHEJ repair, HSV genome circularization is reduced (Figure 16). DNA PKcs and Ku80 play a critical but not absolute role in recognition and repair of DSB via NHEJ (30), which is consistent with the reduction and delay but not abrogation of circularization in cells lacking these proteins (Figure 16A, 16B). DNA Ligase4 is the most essential protein in the NHEJ mechanism due to its role in the enzymatic ligation of DNA ends (62, 198). Consistently, circularization is almost completely inhibited in cells lacking DNA Ligase4 (Figure

16B). These results provide strong evidence for a role of NHEJ in circularization of HSV genomes.

Infected cells appear to treat ends of linear double-stranded HSV genomes as breaks in DNA, and NHEJ serves as a mechanism to repair these breaks, subsequently circularizing HSV genomes. Linear un-integrated cDNA copies of retroviral genomes, including HIV, also undergo circularization (14, 91, 110) during infection and are substrates for the host cell NHEJ DNA repair pathway (91, 110). Some NHEJ proteins may be inhibitory to HSV replication (143, 188). HSV may evade host NHEJ pathways by expressing ICP0 to inhibit this pathway. In some cells types, ICP0 induces the global degradation of the NHEJ protein, DNA PKcs; in other cell types the effect of ICP0 may be more localized than global. This process would mirror that found during adenovirus (AV) infection, in which Mre11 promotes concatemerization of AV genomes, while AV E4 products inhibit concatemerization by promoting degradation of M/R/N proteins (184). Circularization of HSV genomes by NHEJ could result in a stable configuration during latency (44, 89, 159). In neurons, where HSV establishes latency, the abundance and/or activity of ICP0 may be reduced (25) (86) and NHEJ may be the predominant pathway by which DSB's in DNA are repaired (31, 66, 163). Competition between NHEJ mechanisms, which attempt to circularize HSV genomes, and the ability of ICP0 to inhibit circularization by altering/inhibiting the DNA repair environment, may in part affect if and how latency is established. When ICP0 prevails the balance may be swayed in favor of productive infections, when NHEJ processes prevail the balance may be swayed in favor of latency.

6. SUMMARY AND GENERAL DISCUSSION

6.1. Summary of results

The study presented here models changes in HSV genomes as they occur during the establishment of productive and latent infections. HSV mutant viruses deleted for specific subsets of viral genes were used to elucidate how host cells respond to incoming linear, double-stranded HSV genomes and how the virus manipulates this response to promote the productive cycle. The results of this study have important implications in understanding the mechanisms by which HSV genomes replicate during the productive cycle, the mechanisms by which stable genome configurations are established during latency, and the pivotal role that ICP0 plays in the balance between the productive and latent life cycles.

In Chapter 3, we have shown by Gardella gel analysis (67) that during infection HSV genomes circularize in the absence of ICP0 and that ICP0 inhibits genome circularization (89). d109 is an HSV mutant that lacks expression of all five IE viral genes and can subsequently persist in a non-toxic state in nuclei of infected cells (165). D109 forms an abortive infection in which the virus does not replicate and was used to model events leading to and during latency (165). During long-term infections that model latency, d109 genomes persist in a stable circular configuration. However, early during abortive infections, expression of ICP0 in *cis* or in *trans* can inhibit genome circularization. ICP0 also inhibits genome circularization early during productive infections in which HSV replicates and when productive replication is inhibited by phosphonoacetic acid (PAA). Circular genomes are only present during productive infections with ICP0 mutants. Because ICP0 is available to inhibit genome circularization within the first hour of wild-type infection and genome circularization occurs between 1 and 2 hours post infection in the absence of ICP0, circular genomes are not likely to be present during the lytic cycle. Furthermore, if circular genomes were required for replication during productive

infections, then all genomes that replicate would have to initially circularize. During infection, we detected as little as 2% of input genomes in a circular configuration by Gardella gel analysis, while at least 10% of these input genomes can replicate. If circularization were required for replication, then at least 10% of input genomes would have to circularize to serve as templates for replication and this fraction of circular genomes would have been detected by Gardella gel analysis, which is sensitive enough to detect as little as 2% of genomes in a circular state. Contrary to previous literature that suggests HSV replication is initiated from a circular genome template via a theta mechanism (65, 149, 185, 186), we suggest that HSV genomes replicate from a linear template, because ICP0 inhibits genome circularization and hence theta mode replication which requires circular genomes.

Because in Chapter 3 we have challenged the widely accepted concept that circular genomes serve as the initial template for HSV genome replication, we have employed/compared two different analyses to examine HSV genome configurations in Chapter 4. By comparing our method of Gardella gel analysis (67, 89) for circular genomes to other's methods of restriction enzyme analysis for end-to-end linkages of viral genomes (149, 185), we have clarified discrepancies between our results that suggest HSV genomes circularize only in the absence of ICP0 activity (89) and literature that suggests genomes circularize early during infection in both the presence and absence of viral gene expression, including ICP0 (65, 149, 184, 186). The ultimate goal of Chapter 4 has been to determine whether or not circular genomes are present early during productive infection and can subsequently serve as the initial template for genome replication.

In Chapter 4, we have examined circularization during productive infections with a non-inverting HSV mutant I358 (149, 150) and an ICP0-deleted version of I358 termed d99/I2.3.

Using these viruses, Gardella gel analysis has shown that circular genomes are present in the absence but not presence of ICP0 during productive infections. Consistently, using restriction enzyme analysis, we have also found that end-to-end linkages of viral genomes, indicative of circularized genomes, are only present early during infection, prior to replication, in the absence of ICP0 (d99/I2.3). End-to-end linkages of genomes formed at later times during replication in both the presence and absence of ICP0 correspond to replicative well DNA found in Gardella gels in both the presence and absence of ICP0 and are likely to be indicative of highly branched and/or concatemerized genomes generated during replication. Importantly, novel end-to-end linkages of viral genomes were only formed when purified virus stocks were used; when unpurified stocks were used end-to-end linkages were detected prior to viral entry, during infection, and in unpurified stocks. The use of unpurified virus stocks in this analysis for end-to-end linked genome junctions may result in the presence of contaminating end-to-end linked genome fragments, which could be misinterpreted as HSV genome circularization and account in part for inconsistencies between the results of other's (149, 185) with ours (89). Together both our Gardella gel analysis for circular genomes and our restriction enzyme analysis for end-to-end linkages of genomes using purified HSV stocks provide strong evidence that during productive infections HSV genomes only circularize in the absence of ICP0 and that ICP0 inhibits genome circularization. It follows that circular genomes are not likely the initial template for HSV replication, rather, HSV genomes may replicate from a linear template. In addition, the ability of ICP0 to inhibit genome circularization, thereby providing a linear template for HSV replication, is a newly defined mechanism by which ICP0 may promote the lytic cycle.

We have hypothesized that ends of linear, double-stranded HSV genomes are recognized as double-strand breaks (DSB) in DNA by host cells. In the absence of ICP0 activity, DNA DSB

repair mechanisms in host cells may repair the ends of HSV genomes by circularization. ICP0 may inhibit HSV genome circularization by inhibiting these repair pathways. In Chapter 5 we examined the effect of ICP0 on the cellular DSB repair environment and the effect of cellular DSB repair pathways on circularization of HSV genomes. Using mutant HSV viruses, we show that Mre11/Rad50/NBS1 (M/R/N) DSB repair complexes form in response to incoming HSV during abortive infections and that ICP0 promotes the accumulation and persistence of these complexes but disables their association with a marker for DSB's in DNA (γH2AX). This suggests that ICP0 somehow alters the DNA repair environment during infection. Although M/R/N complexes are sensors of DSB's and recruit/activate the ATM repair response (1, 105), the ATM pathway is not activated during abortive infections; it is activated independent of ICP0 during productive infections. We show that M/R/N complexes and the ATM pathway do not affect genome circularization; others have shown that they may facilitate HSV genome replication (111). Rather, non-homologous end-joining (NHEJ) mechanisms may circularize HSV genomes and may be inhibited in certain cell types by ICP0 (107, 143, 188). These results suggest that host cells respond to incoming parental HSV genomes as foreign DNA with DSB's. ICP0 may promote the productive cycle of the virus by inhibiting these DSB repair mechanisms, providing a linear genome template from which replication can initiate.

6.2. New models of HSV replication

After IE and E gene expression, replication can proceed from three origins of replication on the HSV genome. A model for HSV replication, in which only one HSV genome is required, has been deduced from both experimental evidence and the known structural characteristics of HSV genomes (10, 21). Many studies have provided evidence that circular genomes are present early during HSV infection, in both the presence and absence of viral gene expression (65, 149, 185,

186). All of these studies deduced circularization of genomes by showing either end-to-end linkages or loss of genome termini during infection. Because end-to-end linkages of viral genomes have been detected early during infection regardless of gene expression (65, 149, 185, 186), circles have been considered the default configuration of HSV genomes during infection. These circular genomes have been proposed to serve as templates for replication during the productive cycle (149, 185) and to serve as stable configurations during latency (44, 89, 159, 186).

HSV replication has been thought to proceed from this circular genome template by an initial theta and subsequent rolling circle mechanism (10, 162), producing genome concatemers during the replication process (6). This model is supported by the fact that only one viral genome is necessary for replication and the fact that the ends of viral genomes, which contain homologous direct *a* repeats, are suitable substrates for ligation or recombination of genome termini into circular genomes (162). The evidence for the initial theta mode of replication has, in part, led to theory for the later rolling circle stage of replication, which requires an initial circular template. There is evidence that genomes exist as highly branched concatemers during later stages of replication (6, 174, 175), and a rolling circle mechanism is predicted to produce concatemers of genomes (162), although not nearly as highly branched as those observed (174, 175).

However, in the present study we challenge the previous model of HSV replication by providing evidence that replication is initiated from a linear genome template, while circular genomes are the stable configuration during latency. Within Chapters 3 and 4 we clarify in detail how/why our results deviate from those interpreted by others. Many inconsistencies of our interpretations with others can be explained by technique (Chapter 4). Using multiple

techniques, including Gardella gel analysis for circular genomes and examining the formation of end-to-end linkages of genomes, we consistently show that HSV genomes circularize in the absence of ICP0 and that ICP0 inhibits genome circularization early during infection, providing a linear genome template from which replication is most likely initiated. For these reasons, a new model of replication in which replication is initiated from a linear not circular genome is required.

In light of our published results from Chapter 3 (89), studies have begun to consider alternative mechanisms by which HSV replicates from a linear genome template (136, 137). Replication of HSV may begin on a single, linear genome template from origins of replication that require HSV proteins to initiate and proceed into semi-discontinuous replication. However, replication of a linear HSV genome would encounter problems when the replication fork reaches genome termini, as removal of the RNA primer from the lagging-strand termini would create a gap that would progressively eliminate genetic material upon successive cycles of replication. One speculated mechanism by which HSV can circumvent this problem is through recombination-dependent replication (RDR), a mechanism by which T4 phage lagging strand synthesis is accomplished (129). In T4, the end of one linear genome invades into a homologous region of another linear genome, utilizing it as a template to complete lagging-strand synthesis (129). However, only one HSV genome is required for replication and thus itself must be a sufficient template from which replication proceeds. The homologous inverted repeats bracketing the U_L and U_S regions of the genome provide a way in which RDR can complete replication of a single linear HSV genome. HSV genome termini may use the homologous internal inverted repeats as templates for lagging-strand synthesis. During further rounds of replication, the end of one linear genome may use the homologous regions of another linear

genome or its own internal inverted repeats as templates for lagging-strand synthesis. RDR would generate highly branched replication intermediates that are commonly seen in HSV infected cells (174, 175) and we have observed in wells of Gardella gels during HSV replication (89). Furthermore, studies have reconstituted recombination-dependent DNA synthesis using HSV-1 enzymes (136, 137). These authors have also proposed that in addition to a means of replication, RDR may repair double-strand breaks that may occur during replication at collapsed replication forks and/or during isomerization events (136). RDR fits both empirical and theoretical observations thus far made for HSV genome configurations prior to and during replication and subsequently remains a likely mechanism by which HSV genomes replicate.

6.3. Pivotal role of ICP0 in promoting productive cycle.

ICP0 facilitates HSV production during both lytic infections and during reactivation from a repressed latent state. Although ICP0 is known to induce the degradation of cellular proteins (53, 107, 116, 143) and indiscriminantly transactivate gene expression, the mechanisms by which ICP0 promotes productive infections remains unclear. Because ICP0 is the only HSV protein capable of inducing gene expression from otherwise quiescent HSV genomes, ICP0 is likely to promote the lytic cycle by inhibiting repressors of virus gene expression and production. In the present study we provide evidence for a new mechanism by which ICP0 may promote the lytic cycle.

In Chapters 3 and 4, we show that ICP0 inhibits HSV genome circularization, providing a linear genome template from which replication may be initiated. HSV genomes may circularize as a consequence of cellular mechanisms following infection, but the expression of ICP0 very early in infection inhibits circularization. Although circular herpesvirus genomes have been detected in Gardella gels of VZV infected cells (100) and by electron microscopy in

pseudorabies virus (PRV) infection (90), this circularization may take place prior to the synthesis of their respective ICP0 homologs, as VZV Orf61 and PRV EP0 proteins, which both possess structural and function similarities to HSV ICP0 (132, 133), differ from HSV ICP0 in that they are expressed later during infection with these viruses. In the case of HSV, linear molecules may serve as more efficient templates for genome replication. This may in part explain why ICP0 mutants are significantly impaired for replication at low multiplicities of infection (164, 183), a condition in which a higher fraction of circular genomes accumulate (Chapter 3).

Exactly how ICP0 inhibits circularization remains unclear, however, it is likely that ICP0 inhibits DNA DSB repair pathways responsible for genome circularization. As a known inducer of protein degradation and with its activity as a ubiquitin ligase (13, 192), ICP0 may inhibit circularization by the targeted degradation of repair proteins via the proteasome pathway. In this study, we show that NHEJ repair pathways circularize HSV genomes; others have shown that proteins involved in NHEJ are inhibitory to the productive cycle of HSV (143, 188). NHEJ requires the DNA-dependant protein kinase (34), which has been shown by others and us to be targeted for proteasome-mediated degradation by ICP0 (107, 143, 188). The ability of NHEJ repair mechanisms to circularize HSV genomes and of ICP0 to inhibit this repair pathway would conceptually mirror that found during adenovirus (AV) infection, in which the M/R/N repair complex promotes concatemerization of AV genomes, while AV E4 gene products inhibit concatemerization by promoting degradation of M/R/N proteins (184).

Although ICP0 alters the DNA repair response of host cells to incoming HSV by altering the nuclear distribution of M/R/N complexes and their association with a marker for DNA DSB's, it does not induce the degradation of M/R/N constituents. M/R/N complexes can be found at ND10 bodies (18) and at sites of HSV replication (111, 178, 188, 200), which is likely

to initiate at the periphery of ND10 bodies (117). Studies have shown that M/R/N complexes may facilitate HSV replication (111). Early during infection, ICP0 localizes to and disrupts ND10 bodies by inducing the degradation of their major constituents PML and Sp100 (116, 117). Disruption of ND10 may release or make available proteins such as M/R/N complexes that may facilitate the lytic cycle. ICP0 also localizes to and disrupts centromeres by inducing the degradation of the centromere proteins CENPA and CENPC (53). The heterochromatin protein HP1 can be found at both centromeres and ND10 bodies during HSV infection (51, 53) and has been associated with transcriptional silencing (131). Proteins such as HP1 could lead to silencing of HSV genomes. In this case, disruption of ND10 and centromere structures may disable repressors of the HSV productive cycle. Through complex interactions with proteins at specific subnuclear sites and by targeting specific proteins for degradation, ICP0 may both inhibit repressors and facilitate activators. Much more research is required to delineate the exact role that each cellular protein affected by ICP0 plays in either promoting or inhibiting the productive cycle. Those that repress the productive cycle are likely to be inhibited by ICP0, while those that promote the productive cycle are likely to be facilitated by ICP0.

6.4. Circularization, latency, and reactivation

Latent HSV infections are established in sensory neurons that innervate the primary site of productive infection. During HSV latency, the productive cycle of viral gene expression is repressed and HSV genomes persist in an extrachromosomal state (126) that is bound by nucleosomes in a chromatin arrangement (40). The ability of HSV genomes to adopt a repressed, stable state is necessary for its non-toxic persistence during these life-long infections. Many studies have examined the configuration of HSV genomes during persistent infections *in vitro* and during latent infections *in vivo*. DNA isolated from the trigeminal ganglia of both

latently infected mice and humans lacks genome ends (44, 159). During long-term infection of neuronal-differentiated PC12 cells in which genomes become quiescent and do not produce progeny virus, HSV genomes persist in an endless state (186). In this study we have used Gardella gel analysis to show that HSV genomes persist as stable circles during persistent and quiescent infections that model latency (89). The presence of endless genomes during latency and the presence of circular genomes observed by us and endless genomes observed by others during models of latency provide strong evidence that HSV genomes persist as circles during life-long latent infections.

Linear un-integrated cDNA copies of retroviral genomes, including HIV, undergo circularization (14, 91, 110) during infection and are substrates for the host cell NHEJ DNA repair pathway (91, 110). During infection host cell DNA repair pathways also induce adenovirus concatemerizaion, which is inhibited by adenovirus proteins (111, 199). We have similarly hypothesized that host cells respond to incoming linear, double-stranded HSV genomes as DNA double-strand breaks (DSB) that are subsequently repaired by circularization via host cell DNA repair pathways. ICP0 may inhibit circularization by inhibiting these repair pathways. Consistently, in Chapter 5 we show that NHEJ circularizes HSV genomes and that ICP0 may be somewhat inhibitory to NHEJ. Interestingly, NHEJ may be the major mechanism of DSB repair in neurons (31, 66, 163), where HSV latency is established and the abundance and/or activity of ICP0 may be regulated or reduced (25, 86) during HSV infection. When productive infections spread to innervating neurons, these neurons may attempt to repair genome termini by NHEJ mechanisms. In this environment, genomes may circularize in some infected cells, where the level/activity of ICP0 is low, leading to persistence and latency, and in other infected cells,

where the level/activity of ICP0 is high, genomes may remain linear, resulting in an abortive or lytic infection depending on the abundance of other viral regulatory and replication proteins.

From a latent state, the productive cycle can be reactivated by various physiological stresses and also ICP0 (15, 29, 109). However, in the current study, we have provided evidence that HSV genome configurations diverge during diverging life cycles of HSV. During the productive cycle, we suggest that genomes replicate from a linear template, while during latency we suggest genomes persist as stable circles. During reactivation, stable circular genomes must either serve as genome templates for replication or must become linear to serve as templates for replication. How the configuration of genomes change during reactivation remains unclear. During reactivation, physiological stresses are thought to somewhat de-repress viral gene expression, allowing for expression of ICP0, which is thought to further de-repress viral gene expression by inhibiting cellular repressors (51). We have shown that ICP0 inhibits genome circularization, providing a linear template for genome replication during productive infections. It follows that ICP0 may also play a role in providing a genome template for replication during reactivation. Further experiments are required to determine whether or not circular or linear genomes serve as templates for replication during reactivation and if ICP0 plays a role in regulating this configuration during reactivation, potentially creating yet another mechanism by which ICP0 promotes the productive cycle.

BIBLIOGRAPHY

1. **Abraham, R. T., and R. S. Tibbetts.** 2005. Cell biology. Guiding ATM to broken DNA. Science **308:**510-1.
2. **Ahn, K., T. H. Meyer, S. Uebel, P. Sempe, H. Djaballah, Y. Yang, P. A. Peterson, K. Fruh, and R. Tampe.** 1996. Molecular mechanism and species specificity of TAP inhibition by herpes simplex virus ICP47. Embo J **15:**3247-55.
3. **Barnes, D. E., G. Stamp, I. Rosewell, A. Denzel, and T. Lindahl.** 1998. Targeted disruption of the gene encoding DNA ligase IV leads to lethality in embryonic mice. Curr Biol **8:**1395-8.
4. **Batterson, W., and B. Roizman.** 1983. Characterization of the herpes simplex virion-associated factor responsible for the induction of alpha genes. J Virol **46:**371-7.
5. **Beard, P., S. Faber, K. W. Wilcox, and L. I. Pizer.** 1986. Herpes simplex virus immediate early infected-cell polypeptide 4 binds to DNA and promotes transcription. Proc Natl Acad Sci U S A **83:**4016-20.
6. **Becker, Y., Y. Asher, E. Weinberg-Zahlering, S. Rabkin, A. Friedmann, and E. Kessler.** 1978. Defective herpes simplex virus DNA: circular and circular-linear molecules resembling rolling circles. J Gen Virol **40:**319-35.
7. **Ben-Porat, T., and S. A. Tokazewski.** 1977. Replication of herpesvirus DNA. II. Sedimentation characteristics of newly synthesized DNA. Virology **79:**292-301.
8. **Blier, P. R., A. J. Griffith, J. Craft, and J. A. Hardin.** 1993. Binding of Ku protein to DNA. Measurement of affinity for ends and demonstration of binding to nicks. J Biol Chem **268:**7594-601.
9. **Block, T., S. Barney, J. Masonis, J. Maggioncalda, T. Valyi-Nagy, and N. W. Fraser.** 1994. Long term herpes simplex virus type 1 infection of nerve growth factor-treated PC12 cells. J Gen Virol **75 (Pt 9):**2481-7.
10. **Boehmer, P. E., and I. R. Lehman.** 1997. Herpes simplex virus DNA replication. Annu Rev Biochem **66:**347-84.
11. **Bond, V. C., and S. Person.** 1984. Fine structure physical map locations of alterations that affect cell fusion in herpes simplex virus type 1. Virology **132:**368-76.
12. **Borden, K. L.** 2002. Pondering the promyelocytic leukemia protein (PML) puzzle: possible functions for PML nuclear bodies. Mol Cell Biol **22:**5259-69.
13. **Boutell, C., S. Sadis, and R. D. Everett.** 2002. Herpes simplex virus type 1 immediate-early protein ICP0 and is isolated RING finger domain act as ubiquitin E3 ligases in vitro. J Virol **76:**841-50.

14. **Butler, S. L., E. P. Johnson, and F. D. Bushman.** 2002. Human immunodeficiency virus cDNA metabolism: notable stability of two-long terminal repeat circles. J Virol **76:**3739-47.

15. **Cai, W., T. L. Astor, L. M. Liptak, C. Cho, D. M. Coen, and P. A. Schaffer.** 1993. The herpes simplex virus type 1 regulatory protein ICP0 enhances virus replication during acute infection and reactivation from latency. J Virol **67:**7501-12.

16. **Cai, W. Z., and P. A. Schaffer.** 1989. Herpes simplex virus type 1 ICP0 plays a critical role in the de novo synthesis of infectious virus following transfection of viral DNA. J Virol **63:**4579-89.

17. **Campbell, M. E., J. W. Palfreyman, and C. M. Preston.** 1984. Identification of herpes simplex virus DNA sequences which encode a trans-acting polypeptide responsible for stimulation of immediate early transcription. J Mol Biol **180:**1-19.

18. **Carbone, R., M. Pearson, S. Minucci, and P. G. Pelicci.** 2002. PML NBs associate with the hMre11 complex and p53 at sites of irradiation induced DNA damage. Oncogene **21:**1633-40.

19. **Carney, J. P., R. S. Maser, H. Olivares, E. M. Davis, M. Le Beau, J. R. Yates, 3rd, L. Hays, W. F. Morgan, and J. H. Petrini.** 1998. The hMre11/hRad50 protein complex and Nijmegen breakage syndrome: linkage of double-strand break repair to the cellular DNA damage response. Cell **93:**477-86.

20. **Carrozza, M. J., and N. A. DeLuca.** 1996. Interaction of the viral activator protein ICP4 with TFIID through TAF250. Mol Cell Biol **16:**3085-93.

21. **Carter, K. L., and B. Roizman.** 1996. Alternatively spliced mRNAs predicted to yield frame-shift proteins and stable intron 1 RNAs of the herpes simplex virus 1 regulatory gene alpha 0 accumulate in the cytoplasm of infected cells. Proc Natl Acad Sci U S A **93:**12535-40.

22. **Chan, D. W., B. P. Chen, S. Prithivirajsingh, A. Kurimasa, M. D. Story, J. Qin, and D. J. Chen.** 2002. Autophosphorylation of the DNA-dependent protein kinase catalytic subunit is required for rejoining of DNA double-strand breaks. Genes Dev **16:**2333-8.

23. **Chelbi-Alix, M. K., and H. de The.** 1999. Herpes virus induced proteasome-dependent degradation of the nuclear bodies-associated PML and Sp100 proteins. Oncogene **18:**935-41.

24. **Chen, J., and S. Silverstein.** 1992. Herpes simplex viruses with mutations in the gene encoding ICP0 are defective in gene expression. J Virol **66:**2916-27.

25. **Chen, X., J. Li, M. Mata, J. Goss, D. Wolfe, J. C. Glorioso, and D. J. Fink.** 2000. Herpes simplex virus type 1 ICP0 protein does not accumulate in the nucleus of primary neurons in culture. J Virol **74:**10132-41.

26. **Chenet-Monte, C., F. Mohammad, C. M. Celluzzi, P. A. Schaffer, and F. E. Farber.** 1986. Herpes simplex virus gene products involved in the induction of chromosomal aberrations. Virus Res **6:**245-60.

27. **Cheung, A. K.** 1989. DNA nucleotide sequence analysis of the immediate-early gene of pseudorabies virus. Nucleic Acids Res **17:**4637-46.

28. **Ciechanover, A.** 1994. The ubiquitin-proteasome proteolytic pathway. Cell **79:**13-21.

29. **Clements, G. B., and N. D. Stow.** 1989. A herpes simplex virus type 1 mutant containing a deletion within immediate early gene 1 is latency-competent in mice. J Gen Virol **70 (Pt 9):**2501-6.

30. **Collis, S. J., T. L. DeWeese, P. A. Jeggo, and A. R. Parker.** 2005. The life and death of DNA-PK. Oncogene **24:**949-61.

31. **Convery, E., E. K. Shin, Q. Ding, W. Wang, P. Douglas, L. S. Davis, J. A. Nickoloff, S. P. Lees-Miller, and K. Meek.** 2005. Inhibition of homologous recombination by variants of the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs). Proc Natl Acad Sci U S A **102:**1345-50.

32. **Cook, M. L., and J. G. Stevens.** 1973. Pathogenesis of herpetic neuritis and ganglionitis in mice: evidence for intra-axonal transport of infection. Infect Immun **7:**272-88.

33. **Cook, W. J., B. Gu, N. A. DeLuca, E. B. Moynihan, and D. M. Coen.** 1995. Induction of transcription by a viral regulatory protein depends on the relative strengths of functional TATA boxes. Mol Cell Biol **15:**4998-5006.

34. **Critchlow, S. E., and S. P. Jackson.** 1998. DNA end-joining: from yeast to man. Trends Biochem Sci **23:**394-8.

35. **D'Amours, D., and S. P. Jackson.** 2002. The Mre11 complex: at the crossroads of dna repair and checkpoint signalling. Nat Rev Mol Cell Biol **3:**317-27.

36. **Delius, H., and J. B. Clements.** 1976. A partial denaturation map of herpes simplex virus type 1 DNA: evidence for inversions of the unique DNA regions. J Gen Virol **33:**125-33.

37. **DeLuca, N., D. Bzik, S. Person, and W. Snipes.** 1981. Early events in herpes simplex virus type 1 infection: photosensitivity of fluorescein isothiocyanate-treated virions. Proc Natl Acad Sci U S A **78:**912-6.

38. **DeLuca, N. A., A. M. McCarthy, and P. A. Schaffer.** 1985. Isolation and characterization of deletion mutants of herpes simplex virus type 1 in the gene encoding immediate-early regulatory protein ICP4. J Virol **56:**558-70.

39. **DeLuca, N. A., and P. A. Schaffer.** 1987. Activities of herpes simplex virus type 1 (HSV-1) ICP4 genes specifying nonsense peptides. Nucleic Acids Res **15:**4491-511.

40. **Deshmane, S. L., and N. W. Fraser.** 1989. During latency, herpes simplex virus type 1 DNA is associated with nucleosomes in a chromatin structure. J Virol **63:**943-7.

41. **DiDonato, J. A., J. R. Spitzner, and M. T. Muller.** 1991. A predictive model for DNA recognition by the herpes simplex virus protein ICP4. J Mol Biol **219:**451-70.

42. **Dienes, H. P., A. Knoblich, and D. Falke.** 1985. Loss of surface fibronectin after infection of cultured cells by HSV-1 and 2. Arch Virol **86:**223-37.

43. **Dong, Z., Q. Zhong, and P. L. Chen.** 1999. The Nijmegen breakage syndrome protein is essential for Mre11 phosphorylation upon DNA damage. J Biol Chem **274:**19513-6.

44. **Efstathiou, S., A. C. Minson, H. J. Field, J. R. Anderson, and P. Wildy.** 1986. Detection of herpes simplex virus-specific DNA sequences in latently infected mice and in humans. J Virol **57:**446-55.

45. **Eidson, K. M., W. E. Hobbs, B. J. Manning, P. Carlson, and N. A. DeLuca.** 2002. Expression of herpes simplex virus ICP0 inhibits the induction of interferon-stimulated genes by viral infection. J Virol **76:**2180-91.

46. **Elgadi, M. M., C. E. Hayes, and J. R. Smiley.** 1999. The herpes simplex virus vhs protein induces endoribonucleolytic cleavage of target RNAs in cell extracts. J Virol **73:**7153-64.

47. **Everett, R. D.** 1988. Analysis of the functional domains of herpes simplex virus type 1 immediate-early polypeptide Vmw110. J Mol Biol **202:**87-96.

48. **Everett, R. D.** 1989. Construction and characterization of herpes simplex virus type 1 mutants with defined lesions in immediate early gene 1. J Gen Virol **70 (Pt 5):**1185-202.

49. **Everett, R. D.** 1987. A detailed mutational analysis of Vmw110, a trans-acting transcriptional activator encoded by herpes simplex virus type 1. Embo J **6:**2069-76.

50. **Everett, R. D.** 2001. DNA viruses and viral proteins that interact with PML nuclear bodies. Oncogene **20:**7266-73.

51. **Everett, R. D.** 2000. ICP0, a regulator of herpes simplex virus during lytic and latent infection. Bioessays **22:**761-70.

52. **Everett, R. D.** 1984. Trans activation of transcription by herpes virus products: requirement for two HSV-1 immediate-early polypeptides for maximum activity. Embo J **3:**3135-41.

53. **Everett, R. D., W. C. Earnshaw, J. Findlay, and P. Lomonte.** 1999. Specific destruction of kinetochore protein CENP-C and disruption of cell division by herpes simplex virus immediate-early protein Vmw110. Embo J **18:**1526-38.

54. **Everett, R. D., W. C. Earnshaw, A. F. Pluta, T. Sternsdorf, A. M. Ainsztein, M. Carmena, S. Ruchaud, W. L. Hsu, and A. Orr.** 1999. A dynamic connection between centromeres and ND10 proteins. J Cell Sci **112 (Pt 20):**3443-54.

55. **Everett, R. D., P. Freemont, H. Saitoh, M. Dasso, A. Orr, M. Kathoria, and J. Parkinson.** 1998. The disruption of ND10 during herpes simplex virus infection correlates with the Vmw110- and proteasome-dependent loss of several PML isoforms. J Virol **72:**6581-91.

56. **Everett, R. D., P. Lomonte, T. Sternsdorf, R. van Driel, and A. Orr.** 1999. Cell cycle regulation of PML modification and ND10 composition. J Cell Sci **112 (Pt 24):**4581-8.

57. **Everett, R. D., and G. G. Maul.** 1994. HSV-1 IE protein Vmw110 causes redistribution of PML. Embo J **13:**5062-9.

58. **Everett, R. D., M. Meredith, and A. Orr.** 1999. The ability of herpes simplex virus type 1 immediate-early protein Vmw110 to bind to a ubiquitin-specific protease contributes to its roles in the activation of gene expression and stimulation of virus replication. J Virol **73:**417-26.

59. **Everett, R. D., M. Meredith, A. Orr, A. Cross, M. Kathoria, and J. Parkinson.** 1997. A novel ubiquitin-specific protease is dynamically associated with the PML nuclear domain and binds to a herpesvirus regulatory protein. Embo J **16:**1519-30.

60. **Everett, R. D., A. Orr, and C. M. Preston.** 1998. A viral activator of gene expression functions via the ubiquitin-proteasome pathway. Embo J **17:**7161-9.

61. **Fortunato, E. A., and D. H. Spector.** 2003. Viral induction of site-specific chromosome damage. Rev Med Virol **13:**21-37.

62. **Frank, K. M., N. E. Sharpless, Y. Gao, J. M. Sekiguchi, D. O. Ferguson, C. Zhu, J. P. Manis, J. Horner, R. A. DePinho, and F. W. Alt.** 2000. DNA ligase IV deficiency in mice leads to defective neurogenesis and embryonic lethality via the p53 pathway. Mol Cell **5:**993-1002.

63. **Freemont, P. S.** 2000. RING for destruction? Curr Biol **10:**R84-7.

64. **Galocha, B., A. Hill, B. C. Barnett, A. Dolan, A. Raimondi, R. F. Cook, J. Brunner, D. J. McGeoch, and H. L. Ploegh.** 1997. The active site of ICP47, a herpes simplex virus-encoded inhibitor of the major histocompatibility complex (MHC)-encoded peptide transporter associated with antigen processing (TAP), maps to the NH2-terminal 35 residues. J Exp Med **185:**1565-72.

65. **Garber, D. A., S. M. Beverley, and D. M. Coen.** 1993. Demonstration of circularization of herpes simplex virus DNA following infection using pulsed field gel electrophoresis. Virology **197:**459-62.

66. **Garber, P. M., G. M. Vidanes, and D. P. Toczyski.** 2005. Damage in transition. Trends Biochem Sci **30:**63-6.

67. **Gardella, T., P. Medveczky, T. Sairenji, and C. Mulder.** 1984. Detection of circular and linear herpesvirus DNA molecules in mammalian cells by gel electrophoresis. J Virol **50:**248-54.

68. **Gelman, I. H., and S. Silverstein.** 1985. Identification of immediate early genes from herpes simplex virus that transactivate the virus thymidine kinase gene. Proc Natl Acad Sci U S A **82:**5265-9.

69. **Godowski, P. J., and D. M. Knipe.** 1986. Transcriptional control of herpesvirus gene expression: gene functions required for positive and negative regulation. Proc Natl Acad Sci U S A **83:**256-60.

70. **Gottlieb, T. M., and S. P. Jackson.** 1993. The DNA-dependent protein kinase: requirement for DNA ends and association with Ku antigen. Cell **72:**131-42.

71. **Gu, B., and N. DeLuca.** 1994. Requirements for activation of the herpes simplex virus glycoprotein C promoter in vitro by the viral regulatory protein ICP4. J Virol **68:**7953-65.

72. **Hadjipanayis, C. G., and N. A. DeLuca.** 2005. Inhibition of DNA repair by a herpes simplex virus vector enhances the radiosensitivity of human glioblastoma cells. Cancer Res **65:**5310-6.

73. **Hampar, B., and S. A. Ellison.** 1961. Chromosomal aberrations induced by an animal virus. Nature **192:**145-7.

74. **Hardwicke, M. A., and R. M. Sandri-Goldin.** 1994. The herpes simplex virus regulatory protein ICP27 contributes to the decrease in cellular mRNA levels during infection. J Virol **68:**4797-810.

75. **Hardy, W. R., and R. M. Sandri-Goldin.** 1994. Herpes simplex virus inhibits host cell splicing, and regulatory protein ICP27 is required for this effect. J Virol **68:**7790-9.

76. **Harris, R. A., R. D. Everett, X. X. Zhu, S. Silverstein, and C. M. Preston.** 1989. Herpes simplex virus type 1 immediate-early protein Vmw110 reactivates latent herpes simplex virus type 2 in an in vitro latency system. J Virol **63:**3513-5.

77. **Hayward, G. S., R. J. Jacob, S. C. Wadsworth, and B. Roizman.** 1975. Anatomy of herpes simplex virus DNA: evidence for four populations of molecules that differ in the relative orientations of their long and short components. Proc Natl Acad Sci U S A **72:**4243-7.

78. **Heeg, U., H. P. Dienes, S. Muller, and D. Falke.** 1986. Involvement of actin-containing microfilaments in HSV-induced cytopathology and the influence of inhibitors of glycosylation. Arch Virol **91:**257-70.

79. **Heine, J. W., R. W. Honess, E. Cassai, and B. Roizman.** 1974. Proteins specified by herpes simplex virus. XII. The virion polypeptides of type 1 strains. J Virol **14:**640-51.

80. **Hill, A., P. Jugovic, I. York, G. Russ, J. Bennink, J. Yewdell, H. Ploegh, and D. Johnson.** 1995. Herpes simplex virus turns off the TAP to evade host immunity. Nature **375:**411-5.

81. **Hobbs, W. E., 2nd, and N. A. DeLuca.** 1999. Perturbation of cell cycle progression and cellular gene expression as a function of herpes simplex virus ICP0. J Virol **73:**8245-55.

82. **Hobbs, W. E., D. E. Brough, I. Kovesdi, and N. A. DeLuca.** 2001. Efficient activation of viral genomes by levels of herpes simplex virus ICP0 insufficient to affect cellular gene expression or cell survival. J Virol **75:**3391-403.

83. **Honess, R. W., and B. Roizman.** 1974. Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. J Virol **14:**8-19.

84. **Honess, R. W., and B. Roizman.** 1975. Regulation of herpesvirus macromolecular synthesis: sequential transition of polypeptide synthesis requires functional viral polypeptides. Proc Natl Acad Sci U S A **72:**1276-80.

85. **Honess, R. W., and D. H. Watson.** 1977. Herpes simplex virus resistance and sensitivity to phosphonoacetic acid. J Virol **21:**584-600.

86. **Hsu, W. L., and R. D. Everett.** 2001. Human neuron-committed teratocarcinoma NT2 cell line has abnormal ND10 structures and is poorly infected by herpes simplex virus type 1. J Virol **75:**3819-31.

87. **Igarashi, K., R. Fawl, R. J. Roller, and B. Roizman.** 1993. Construction and properties of a recombinant herpes simplex virus 1 lacking both S-component origins of DNA synthesis. J Virol **67:**2123-32.

88. **Imbalzano, A. N., and N. A. DeLuca.** 1992. Substitution of a TATA box from a herpes simplex virus late gene in the viral thymidine kinase promoter alters ICP4 inducibility but not temporal expression. J Virol **66:**5453-63.

89. **Jackson, S. A., and N. A. DeLuca.** 2003. Relationship of herpes simplex virus genome configuration to productive and persistent infections. Proc Natl Acad Sci U S A **100:**7871-6.

90. **Jean, J. H., and T. Ben-Porat.** 1976. Appearance in vivo of single-stranded complementary ends on parental herpesvirus DNA. Proc Natl Acad Sci U S A **73:**2674-8.

91. **Jeanson, L., F. Subra, S. Vaganay, M. Hervy, E. Marangoni, J. Bourhis, and J. F. Mouscadet.** 2002. Effect of Ku80 depletion on the preintegrative steps of HIV-1 replication in human cells. Virology **300:**100-8.

92. **Jordan, R., and P. A. Schaffer.** 1997. Activation of gene expression by herpes simplex virus type 1 ICP0 occurs at the level of mRNA synthesis. J Virol **71:**6850-62.

93. **Karanjawala, Z. E., N. Adachi, R. A. Irvine, E. K. Oh, D. Shibata, K. Schwarz, C. L. Hsieh, and M. R. Lieber.** 2002. The embryonic lethality in DNA ligase IV-deficient mice is rescued by deletion of Ku: implications for unifying the heterogeneous phenotypes of NHEJ mutants. DNA Repair (Amst) **1:**1017-26.

94. **Kawaguchi, Y., R. Bruni, and B. Roizman.** 1997. Interaction of herpes simplex virus 1 alpha regulatory protein ICP0 with elongation factor 1delta: ICP0 affects translational machinery. J Virol **71:**1019-24.

95. **Kawaguchi, Y., M. Tanaka, A. Yokoymama, G. Matsuda, K. Kato, H. Kagawa, K. Hirai, and B. Roizman.** 2001. Herpes simplex virus 1 alpha regulatory protein ICP0 functionally interacts with cellular transcription factor BMAL1. Proc Natl Acad Sci U S A **98:**1877-82.

96. **Kawaguchi, Y., C. Van Sant, and B. Roizman.** 1997. Herpes simplex virus 1 alpha regulatory protein ICP0 interacts with and stabilizes the cell cycle regulator cyclin D3. J Virol **71:**7328-36.

97. **Khanna, K. M., R. H. Bonneau, P. R. Kinchington, and R. L. Hendricks.** 2003. Herpes simplex virus-specific memory CD8+ T cells are selectively activated and retained in latently infected sensory ganglia. Immunity **18:**593-603.

98. **Khanna, K. M., A. J. Lepisto, V. Decman, and R. L. Hendricks.** 2004. Immune control of herpes simplex virus during latency. Curr Opin Immunol **16:**463-9.

99. **Kieff, E. D., S. L. Bachenheimer, and B. Roizman.** 1971. Size, composition, and structure of the deoxyribonucleic acid of herpes simplex virus subtypes 1 and 2. J Virol **8:**125-32.

100. **Kinchington, P. R., W. C. Reinhold, T. A. Casey, S. E. Straus, J. Hay, and W. T. Ruyechan.** 1985. Inversion and circularization of the varicella-zoster virus genome. J Virol **56:**194-200.

101. **Kwong, A. D., and N. Frenkel.** 1989. The herpes simplex virus virion host shutoff function. J Virol **63:**4834-9.

102. **Kwong, A. D., and N. Frenkel.** 1987. Herpes simplex virus-infected cells contain a function(s) that destabilizes both host and viral mRNAs. Proc Natl Acad Sci U S A **84:**1926-30.

103. **Kwong, A. D., J. A. Kruper, and N. Frenkel.** 1988. Herpes simplex virus virion host shutoff function. J Virol **62:**912-21.

104. **Lacaille, V. G., and M. J. Androlewicz.** 1998. Herpes simplex virus inhibitor ICP47 destabilizes the transporter associated with antigen processing (TAP) heterodimer. J Biol Chem **273:**17386-90.

105. **Lee, J. H., and T. T. Paull.** 2005. ATM activation by DNA double-strand breaks through the Mre11-Rad50-Nbs1 complex. Science **308:**551-4.

106. **Lees-Miller, S. P., R. Godbout, D. W. Chan, M. Weinfeld, R. S. Day, 3rd, G. M. Barron, and J. Allalunis-Turner.** 1995. Absence of p350 subunit of DNA-activated protein kinase from a radiosensitive human cell line. Science **267:**1183-5.

107. **Lees-Miller, S. P., M. C. Long, M. A. Kilvert, V. Lam, S. A. Rice, and C. A. Spencer.** 1996. Attenuation of DNA-dependent protein kinase activity and its catalytic subunit by the herpes simplex virus type 1 transactivator ICP0. J Virol **70:**7471-7.

108. **Lehming, N., A. Le Saux, J. Schuller, and M. Ptashne.** 1998. Chromatin components as part of a putative transcriptional repressing complex. Proc Natl Acad Sci U S A **95:**7322-6.

109. **Leib, D. A., D. M. Coen, C. L. Bogard, K. A. Hicks, D. R. Yager, D. M. Knipe, K. L. Tyler, and P. A. Schaffer.** 1989. Immediate-early regulatory gene mutants define different stages in the establishment and reactivation of herpes simplex virus latency. J Virol **63:**759-68.

110. **Li, L., J. M. Olvera, K. E. Yoder, R. S. Mitchell, S. L. Butler, M. Lieber, S. L. Martin, and F. D. Bushman.** 2001. Role of the non-homologous DNA end joining pathway in the early steps of retroviral infection. Embo J **20:**3272-81.

111. **Lilley, C. E., C. T. Carson, A. R. Muotri, F. H. Gage, and M. D. Weitzman.** 2005. DNA repair proteins affect the lifecycle of herpes simplex virus 1. Proc Natl Acad Sci U S A **102:**5844-9.

112. **Locker, H., and N. Frenkel.** 1979. BamI, KpnI, and SalI restriction enzyme maps of the DNAs of herpes simplex virus strains Justin and F: occurrence of heterogeneities in defined regions of the viral DNA. J Virol **32:**429-41.

113. **Longnecker, R., and B. Roizman.** 1986. Generation of an inverting herpes simplex virus 1 mutant lacking the L-S junction a sequences, an origin of DNA synthesis, and several genes including those specifying glycoprotein E and the alpha 47 gene. J Virol **58:**583-91.

114. **Martin, D. W., and P. C. Weber.** 1996. The a sequence is dispensable for isomerization of the herpes simplex virus type 1 genome. J Virol **70:**8801-12.

115. **Maul, G. G., and R. D. Everett.** 1994. The nuclear location of PML, a cellular member of the C3HC4 zinc-binding domain protein family, is rearranged during herpes simplex virus infection by the C3HC4 viral protein ICP0. J Gen Virol **75 (Pt 6):**1223-33.

116. **Maul, G. G., H. H. Guldner, and J. G. Spivack.** 1993. Modification of discrete nuclear domains induced by herpes simplex virus type 1 immediate early gene 1 product (ICP0). J Gen Virol **74 (Pt 12):**2679-90.

117. **Maul, G. G., A. M. Ishov, and R. D. Everett.** 1996. Nuclear domain 10 as preexisting potential replication start sites of herpes simplex virus type-1. Virology **217:**67-75.

118. **Maul, G. G., D. Negorev, P. Bell, and A. M. Ishov.** 2000. Review: properties and assembly mechanisms of ND10, PML bodies, or PODs. J Struct Biol **129:**278-87.

119. **Maul, G. G., E. Yu, A. M. Ishov, and A. L. Epstein.** 1995. Nuclear domain 10 (ND10) associated proteins are also present in nuclear bodies and redistribute to hundreds of nuclear sites after stress. J Cell Biochem **59:**498-513.

120. **Mavromara-Nazos, P., S. Silver, J. Hubenthal-Voss, J. L. McKnight, and B. Roizman.** 1986. Regulation of herpes simplex virus 1 genes: alpha gene sequence requirements for transient induction of indicator genes regulated by beta or late (gamma 2) promoters. Virology **149:**152-64.

121. **McCarthy, A. M., L. McMahan, and P. A. Schaffer.** 1989. Herpes simplex virus type 1 ICP27 deletion mutants exhibit altered patterns of transcription and are DNA deficient. J Virol **63:**18-27.

122. **McGeoch, D. J., M. A. Dalrymple, A. J. Davison, A. Dolan, M. C. Frame, D. McNab, L. J. Perry, J. E. Scott, and P. Taylor.** 1988. The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. J Gen Virol **69 (Pt 7):**1531-74.

123. **McGeoch, D. J., A. Dolan, S. Donald, and D. H. Brauer.** 1986. Complete DNA sequence of the short repeat region in the genome of herpes simplex virus type 1. Nucleic Acids Res **14:**1727-45.

124. **McLauchlan, J., A. Phelan, C. Loney, R. M. Sandri-Goldin, and J. B. Clements.** 1992. Herpes simplex virus IE63 acts at the posttranscriptional level to stimulate viral mRNA 3' processing. J Virol **66:**6939-45.

125. **McMahan, L., and P. A. Schaffer.** 1990. The repressing and enhancing functions of the herpes simplex virus regulatory protein ICP27 map to C-terminal regions and are required to modulate viral gene expression very early in infection. J Virol **64:**3471-85.

126. **Mellerick, D. M., and N. W. Fraser.** 1987. Physical state of the latent herpes simplex virus genome in a mouse model system: evidence suggesting an episomal state. Virology **158:**265-75.

127. **Merkle, D., P. Douglas, G. B. Moorhead, Z. Leonenko, Y. Yu, D. Cramb, D. P. Bazett-Jones, and S. P. Lees-Miller.** 2002. The DNA-dependent protein kinase interacts with DNA to form a protein-DNA complex that is disrupted by phosphorylation. Biochemistry **41:**12706-14.

128. **Michael, N., and B. Roizman.** 1993. Repression of the herpes simplex virus 1 alpha 4 gene by its gene product occurs within the context of the viral genome and is associated with all three identified cognate sites. Proc Natl Acad Sci U S A **90:**2286-90.

129. **Miller, E. S., E. Kutter, G. Mosig, F. Arisaka, T. Kunisawa, and W. Ruger.** 2003. Bacteriophage T4 genome. Microbiol Mol Biol Rev **67:**86-156, table of contents.

130. **Mimori, T., and J. A. Hardin.** 1986. Mechanism of interaction between Ku protein and DNA. J Biol Chem **261:**10375-9.

131. **Moazed, D.** 2001. Common themes in mechanisms of gene silencing. Mol Cell **8:**489-98.

132. **Moriuchi, H., M. Moriuchi, H. Dean, A. K. Cheung, and J. I. Cohen.** 1995. Pseudorabies virus EPO is functionally homologous to varicella-zoster virus ORF61 protein and herpes simplex virus type 1 ICPO. Virology **209:**281-3.

133. **Moriuchi, H., M. Moriuchi, H. A. Smith, S. E. Straus, and J. I. Cohen.** 1992. Varicella-zoster virus open reading frame 61 protein is functionally homologous to herpes simplex virus type 1 ICP0. J Virol **66:**7303-8.

134. **Morrison, C., and S. Takeda.** 2000. Genetic analysis of homologous DNA recombination in vertebrate somatic cells. Int J Biochem Cell Biol **32:**817-31.

135. **Mullen, M. A., D. M. Ciufo, and G. S. Hayward.** 1994. Mapping of intracellular localization domains and evidence for colocalization interactions between the IE110 and IE175 nuclear transactivator proteins of herpes simplex virus. J Virol **68:**3250-66.

136. **Nimonkar, A. V., and P. E. Boehmer.** 2003. Reconstitution of recombination-dependent DNA synthesis in herpes simplex virus 1. Proc Natl Acad Sci U S A **100:**10201-6.

137. **Nimonkar, A. V., and P. E. Boehmer.** 2004. Role of protein-protein interactions during herpes simplex virus type 1 recombination-dependent replication. J Biol Chem **279:**21957-65.

138. **O'Hare, P., and G. S. Hayward.** 1985. Evidence for a direct role for both the 175,000- and 110,000-molecular-weight immediate-early proteins of herpes simplex virus in the transactivation of delayed-early promoters. J Virol **53:**751-60.

139. **O'Neill, F. J., and C. P. Miles.** 1969. Chromosome changes in human cells induced by herpes simplex, types 1 and 2. Nature **223:**851-2.

140. **O'Neill, F. J., and F. Rapp.** 1971. Early events required for induction of chromosome abnormalities in human cells by herpes simplex virus. Virology **44:**544-53.

141. **Oroskar, A. A., and G. S. Read.** 1989. Control of mRNA stability by the virion host shutoff function of herpes simplex virus. J Virol **63:**1897-906.

142. **Panet, A., E. Braun, A. Honigman, and I. Steiner.** 2005. Involvement of cellular death signals in the reactivation of herpes simplex virus type 1 and lambda bacteriophage from a latent state. J Theor Biol **236:**88-94.

143. **Parkinson, J., S. P. Lees-Miller, and R. D. Everett.** 1999. Herpes simplex virus type 1 immediate-early protein vmw110 induces the proteasome-dependent degradation of the catalytic subunit of DNA-dependent protein kinase. J Virol **73:**650-7.

144. **Paull, T. T., E. P. Rogakou, V. Yamazaki, C. U. Kirchgessner, M. Gellert, and W. M. Bonner.** 2000. A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. Curr Biol **10:**886-95.

145. **Peat, D. S., and M. A. Stanley.** 1986. Chromosome damage induced by herpes simplex virus type 1 in early infection. J Gen Virol **67 (Pt 10):**2273-7.

146. **Pereira, L., M. H. Wolff, M. Fenwick, and B. Roizman.** 1977. Regulation of herpesvirus macromolecular synthesis. V. Properties of alpha polypeptides made in HSV-1 and HSV-2 infected cells. Virology **77:**733-49.

147. **Perry, L. J., F. J. Rixon, R. D. Everett, M. C. Frame, and D. J. McGeoch.** 1986. Characterization of the IE110 gene of herpes simplex virus type 1. J Gen Virol **67 (Pt 11):**2365-80.

148. **Petroski, M. D., and E. K. Wagner.** 1998. Purification and characterization of a cellular protein that binds to the downstream activation sequence of the strict late UL38 promoter of herpes simplex virus type 1. J Virol **72:**8181-90.

149. **Poffenberger, K. L., and B. Roizman.** 1985. A noninverting genome of a viable herpes simplex virus 1: presence of head-to-tail linkages in packaged genomes and requirements for circularization after infection. J Virol **53:**587-95.

150. **Poffenberger, K. L., E. Tabares, and B. Roizman.** 1983. Characterization of a viable, noninverting herpes simplex virus 1 genome derived by insertion and deletion of sequences at the junction of components L and S. Proc Natl Acad Sci U S A **80:**2690-4.

151. **Polvino-Bodnar, M., P. K. Orberg, and P. A. Schaffer.** 1987. Herpes simplex virus type 1 oriL is not required for virus replication or for the establishment and reactivation of latent infection in mice. J Virol **61:**3528-35.

152. **Post, L. E., S. Mackem, and B. Roizman.** 1981. Regulation of alpha genes of herpes simplex virus: expression of chimeric genes produced by fusion of thymidine kinase with alpha gene promoters. Cell **24:**555-65.

153. **Preston, C. M.** 2000. Repression of viral transcription during herpes simplex virus latency. J Gen Virol **81:**1-19.

154. **Preston, C. M., and M. J. Nicholl.** 1997. Repression of gene expression upon infection of cells with herpes simplex virus type 1 mutants impaired for immediate-early protein synthesis. J Virol **71:**7807-13.

155. **Quinlan, M. P., and D. M. Knipe.** 1985. Stimulation of expression of a herpes simplex virus DNA-binding protein by two viral functions. Mol Cell Biol **5:**957-63.

156. **Rajcani, J., and A. Vojvodova.** 1998. The role of herpes simplex virus glycoproteins in the virus replication cycle. Acta Virol **42:**103-18.

157. **Redon, C., D. Pilch, E. Rogakou, O. Sedelnikova, K. Newrock, and W. Bonner.** 2002. Histone H2A variants H2AX and H2AZ. Curr Opin Genet Dev **12:**162-9.

158. **Rice, S. A., M. C. Long, V. Lam, P. A. Schaffer, and C. A. Spencer.** 1995. Herpes simplex virus immediate-early protein ICP22 is required for viral modification of host

RNA polymerase II and establishment of the normal viral transcription program. J Virol **69:**5550-9.

159. **Rock, D. L., and N. W. Fraser.** 1983. Detection of HSV-1 genome in central nervous system of latently infected mice. Nature **302:**523-5.

160. **Rogakou, E. P., D. R. Pilch, A. H. Orr, V. S. Ivanova, and W. M. Bonner.** 1998. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. J Biol Chem **273:**5858-68.

161. **Roizman, B.** 1979. The structure and isomerization of herpes simplex virus genomes. Cell **16:**481-94.

162. **Roizman, B., and Sears, A.E.** 1996. Herpes Simplex viruses and their replication, p. 2231-2295. *In* B. N. Fields, Knipe, D.M. & Howely, P.M. (ed.), Fields Virology, vol. 3. Lippincott-Raven, Philadelphia, PA.

163. **Rothkamm, K., I. Kruger, L. H. Thompson, and M. Lobrich.** 2003. Pathways of DNA double-strand break repair during the mammalian cell cycle. Mol Cell Biol **23:**5706-15.

164. **Sacks, W. R., and P. A. Schaffer.** 1987. Deletion mutants in the gene encoding the herpes simplex virus type 1 immediate-early protein ICP0 exhibit impaired growth in cell culture. J Virol **61:**829-39.

165. **Samaniego, L. A., L. Neiderhiser, and N. A. DeLuca.** 1998. Persistence and expression of the herpes simplex virus genome in the absence of immediate-early proteins. J Virol **72:**3307-20.

166. **Samaniego, L. A., A. L. Webb, and N. A. DeLuca.** 1995. Functional interactions between herpes simplex virus immediate-early proteins during infection: gene expression as a consequence of ICP27 and different domains of ICP4. J Virol **69:**5705-15.

167. **Samaniego, L. A., N. Wu, and N. A. DeLuca.** 1997. The herpes simplex virus immediate-early protein ICP0 affects transcription from the viral genome and infected-cell survival in the absence of ICP4 and ICP27. J Virol **71:**4614-25.

168. **Samuel, C. E.** 1991. Antiviral actions of interferon. Interferon-regulated cellular proteins and their surprisingly selective antiviral activities. Virology **183:**1-11.

169. **Sandri-Goldin, R. M., M. K. Hibbard, and M. A. Hardwicke.** 1995. The C-terminal repressor region of herpes simplex virus type 1 ICP27 is required for the redistribution of small nuclear ribonucleoprotein particles and splicing factor SC35; however, these alterations are not sufficient to inhibit host cell splicing. J Virol **69:**6063-76.

170. **Sandri-Goldin, R. M., and G. E. Mendoza.** 1992. A herpesvirus regulatory protein appears to act post-transcriptionally by affecting mRNA processing. Genes Dev **6:**848-63.

171. **Sarisky, R. T., and P. C. Weber.** 1994. Requirement for double-strand breaks but not for specific DNA sequences in herpes simplex virus type 1 genome isomerization events. J Virol **68:**34-47.

172. **Sedelnikova, O. A., E. P. Rogakou, I. G. Panyutin, and W. M. Bonner.** 2002. Quantitative detection of (125)IdU-induced DNA double-strand breaks with gamma-H2AX antibody. Radiat Res **158:**486-92.

173. **Seeler, J. S., A. Marchio, D. Sitterlin, C. Transy, and A. Dejean.** 1998. Interaction of SP100 with HP1 proteins: a link between the promyelocytic leukemia-associated nuclear bodies and the chromatin compartment. Proc Natl Acad Sci U S A **95:**7316-21.

174. **Severini, A., A. R. Morgan, D. R. Tovell, and D. L. Tyrrell.** 1994. Study of the structure of replicative intermediates of HSV-1 DNA by pulsed-field gel electrophoresis. Virology **200:**428-35.

175. **Severini, A., D. G. Scraba, and D. L. Tyrrell.** 1996. Branched structures in the intracellular DNA of herpes simplex virus type 1. J Virol **70:**3169-75.

176. **Sheldrick, P., and N. Berthelot.** 1975. Inverted repetitions in the chromosome of herpes simplex virus. Cold Spring Harb Symp Quant Biol **39 Pt 2:**667-78.

177. **Shepard, A. A., and N. A. DeLuca.** 1991. Activities of heterodimers composed of DNA-binding- and transactivation-deficient subunits of the herpes simplex virus regulatory protein ICP4. J Virol **65:**299-307.

178. **Shirata, N., A. Kudoh, T. Daikoku, Y. Tatsumi, M. Fujita, T. Kiyono, Y. Sugaya, H. Isomura, K. Ishizaki, and T. Tsurumi.** 2005. Activation of ataxia telangiectasia-mutated DNA damage checkpoint signal transduction elicited by herpes simplex virus infection. J Biol Chem **280:**30336-41.

179. **Smiley, J. R., C. Lavery, and M. Howes.** 1992. The herpes simplex virus type 1 (HSV-1) a sequence serves as a cleavage/packaging signal but does not drive recombinational genome isomerization when it is inserted into the HSV-2 genome. J Virol **66:**7505-10.

180. **Smith, C. A., P. Bates, R. Rivera-Gonzalez, B. Gu, and N. A. DeLuca.** 1993. ICP4, the major transcriptional regulatory protein of herpes simplex virus type 1, forms a tripartite complex with TATA-binding protein and TFIIB. J Virol **67:**4676-87.

181. **Smith, C. A., M. E. Marchetti, P. Edmonson, and P. A. Schaffer.** 1989. Herpes simplex virus type 2 mutants with deletions in the intergenic region between ICP4 and ICP22/47: identification of nonessential cis-acting elements in the context of the viral genome. J Virol **63:**2036-47.

182. **Stich, H. F., T. C. Hsu, and F. Rapp.** 1964. Viruses and Mammalian Chromosomes. I. Localization of Chromosome Aberrations after Infection with Herpes Simplex Virus. Virology **22:**439-45.

183. **Stow, N. D., and E. C. Stow.** 1986. Isolation and characterization of a herpes simplex virus type 1 mutant containing a deletion within the gene encoding the immediate early polypeptide Vmw110. J Gen Virol **67 (Pt 12):**2571-85.

184. **Stracker, T. H., C. T. Carson, and M. D. Weitzman.** 2002. Adenovirus oncoproteins inactivate the Mre11-Rad50-NBS1 DNA repair complex. Nature **418:**348-52.

185. **Strang, B. L., and N. D. Stow.** 2005. Circularization of the Herpes Simplex Virus Type 1 Genome upon Lytic Infection. J Virol **79:**12487-94.

186. **Su, Y. H., M. J. Moxley, A. K. Ng, J. Lin, R. Jordan, N. W. Fraser, and T. M. Block.** 2002. Stability and circularization of herpes simplex virus type 1 genomes in quiescently infected PC12 cultures. J Gen Virol **83:**2943-50.

187. **Taylor, T. J., M. A. Brockman, E. E. McNamee, and D. M. Knipe.** 2002. Herpes simplex virus. Front Biosci **7:**d752-64.

188. **Taylor, T. J., and D. M. Knipe.** 2004. Proteomics of herpes simplex virus replication compartments: association of cellular DNA replication, repair, recombination, and chromatin remodeling proteins with ICP8. J Virol **78:**5856-66.

189. **Telford, E. A., M. S. Watson, K. McBride, and A. J. Davison.** 1992. The DNA sequence of equine herpesvirus-1. Virology **189:**304-16.

190. **Tomazin, R., A. B. Hill, P. Jugovic, I. York, P. van Endert, H. L. Ploegh, D. W. Andrews, and D. C. Johnson.** 1996. Stable binding of the herpes simplex virus ICP47 protein to the peptide binding site of TAP. Embo J **15:**3256-66.

191. **Uprichard, S. L., and D. M. Knipe.** 1996. Herpes simplex ICP27 mutant viruses exhibit reduced expression of specific DNA replication genes. J Virol **70:**1969-80.

192. **Van Sant, C., R. Hagglund, P. Lopez, and B. Roizman.** 2001. The infected cell protein 0 of herpes simplex virus 1 dynamically interacts with proteasomes, binds and activates the cdc34 E2 ubiquitin-conjugating enzyme, and possesses in vitro E3 ubiquitin ligase activity. Proc Natl Acad Sci U S A **98:**8815-20.

193. **Varmuza, S. L., and J. R. Smiley.** 1985. Signals for site-specific cleavage of HSV DNA: maturation involves two separate cleavage events at sites distal to the recognition sequences. Cell **41:**793-802.

194. **Wachsberger, P. R., W. H. Li, M. Guo, D. Chen, N. Cheong, C. C. Ling, G. Li, and G. Iliakis.** 1999. Rejoining of DNA double-strand breaks in Ku80-deficient mouse fibroblasts. Radiat Res **151:**398-407.

195. **Wadsworth, S., R. J. Jacob, and B. Roizman.** 1975. Anatomy of herpes simplex virus DNA. II. Size, composition, and arrangement of inverted terminal repetitions. J Virol **15:**1487-97.

196. **Wagner, E. K., and D. C. Bloom.** 1997. Experimental investigation of herpes simplex virus latency. Clin Microbiol Rev **10:**419-43.

197. **Wagner, M. J., and W. C. Summers.** 1978. Structure of the joint region and the termini of the DNA of herpes simplex virus type 1. J Virol **27:**374-87.

198. **Wang, H., Z. C. Zeng, A. R. Perrault, X. Cheng, W. Qin, and G. Iliakis.** 2001. Genetic evidence for the involvement of DNA ligase IV in the DNA-PK-dependent pathway of non-homologous end joining in mammalian cells. Nucleic Acids Res **29:**1653-60.

199. **Weiden, M. D., and H. S. Ginsberg.** 1994. Deletion of the E4 region of the genome produces adenovirus DNA concatemers. Proc Natl Acad Sci U S A **91:**153-7.

200. **Wilkinson, D. E., and S. K. Weller.** 2004. Recruitment of cellular recombination and repair proteins to sites of herpes simplex virus type 1 DNA replication is dependent on

the composition of viral proteins within prereplicative sites and correlates with the induction of the DNA damage response. J Virol **78:**4783-96.

201. **Wirth, U. V., C. Fraefel, B. Vogt, C. Vlcek, V. Paces, and M. Schwyzer.** 1992. Immediate-early RNA 2.9 and early RNA 2.6 of bovine herpesvirus 1 are 3' coterminal and encode a putative zinc finger transactivator protein. J Virol **66:**2763-72.

202. **Wu, X., V. Ranganathan, D. S. Weisman, W. F. Heine, D. N. Ciccone, T. B. O'Neill, K. E. Crick, K. A. Pierce, W. S. Lane, G. Rathbun, D. M. Livingston, and D. T. Weaver.** 2000. ATM phosphorylation of Nijmegen breakage syndrome protein is required in a DNA damage response. Nature **405:**477-82.

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

204. **Yao, X. D., and P. Elias.** 2001. Recombination during early herpes simplex virus type 1 infection is mediated by cellular proteins. J Biol Chem **276:**2905-13.

205. **Yao, X. D., M. Matecic, and P. Elias.** 1997. Direct repeats of the herpes simplex virus a sequence promote nonconservative homologous recombination that is not dependent on XPF/ERCC4. J Virol **71:**6842-9.