A Cell-type Dependent Growth Defect in HSV-1 ICP27 Mutants

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DEDICATION

I would like to dedicate this thesis to my beloved wife Miesha and my outstanding children Amaya and Zariah. I couldn’t have done it without you.
Abstract

Herpes simplex virus type 1 (HSV-1) is a common human herpesvirus that has a sero-prevalence between 70-80% in the adult population and can lead to oral/genital lesions and viral encephalitis. Infected cell protein (ICP) 27 (ICP27) is an essential, 512-residue HSV-1 protein that performs many different functions during infection including but not limited to the regulation of viral gene expression, participating in viral mRNA export and modulating the cellular interferon response pathway. The majority of previous studies of this viral protein have been conducted in Vero cells, a line of African green monkey kidney cells. Here we have examined the phenotype of viral ICP27 mutants in other cell lines and primary cultures. We identified one mutant, d1-2, which shows a striking cell type-dependent growth defect in that it can partially replicate in Vero cells and some other cells but cannot replicate at all in many human cells including primary fibroblasts. Analysis of d1-2 infections showed that its restricted replication is associated with markedly decreased expression of the viral ICP8 gene and aberrant formation of viral replication compartments. These data suggest that viral DNA replication is tightly blocked in restrictive cells. Using a plasmid transfection/virus complementation strategy, we demonstrate that the cell-type dependent replication phenotype of HSV-1 mutants maps to the N-terminus of ICP27, specifically to residues 12-20. Together, our data indicate that ICP27 function is dependent on host cell type and that HSV-1 studies in Vero cells may not fully model natural human infections.
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INTRODUCTION

HSV-1 and the HSV-1 replication cycle.

Herpes Simplex Virus 1 or HSV-1, is an enveloped alphaherpesvirus with a dsDNA genome of approximately 150 kb (1, 2). It is an ubiquitous human pathogen with a seroprevalence that increases from childhood, reaching 70%-80% in adolescence. The major route of transmission is through saliva which leads to infection of epithelial cells, although there are alternative routes including transplacental and blood-borne transfer. In epithelial cells, the virus undergoes a lytic infection phase. The virus then spreads to the peripheral nervous system where it establishes a latent permanent infection in ganglia. While typically associated with non-life threatening oral and genital lesions, more severe pathology is possible. For example, HSV-1 is the most common cause of viral encephalitis in non-tropical areas and its incidence is estimated to be one case per million people per year (2).

HSV-1 Replication Cycle

The HSV-1 replication cycle begins through the action of several viral envelope proteins including at least glycoprotein B (gB), gC, gD, and a gH/gL heterodimer with the host cell membrane (3, 4). This initial interaction is mediated by the binding of gC to heparin sulfate moieties on the host cell surface. Virion attachment is then stabilized through interaction between gD and one of several possible cellular receptors collectively identified as Herpes virus entry (Hve) proteins. There are two protein families of which these cellular receptors are members: the tumor necrosis factor (TNF) family which includes HveA, and the immunoglobulin superfamily which includes HveB, HveC, and
Hl.gR. Fusion is proposed to be facilitated by gD binding to several of its cognate receptors including nectin-1, herpes virus entry mediator, and immunoglobulin-like type 2 receptor alpha (PILRα) (3). gD binding is next posited to trigger sequential conformational changes in gH/gL and gB, facilitating viral envelope fusion with the cell membrane (3). Following entry, the viral nucleocapsid and tegument proteins are released into the host cell cytoplasm. The nucleocapsid, along with tegument proteins VP16 and VP1-2, are carried to the nucleus by the microtubule network, while some tegument proteins such as vhs and US11 stay in the cytoplasm.

Once the HSV-1 DNA enters the nucleus, transcription of viral genes by host RNA polymerase II begins. HSV-1 gene expression and its regulation by various gene products have been studied extensively (5–9). HSV-1 viral gene expression consists of three phases that occur sequentially, leading to the production of immediate-early (IE or α), delayed-early (DE or β), and late (L or γ) gene products. In general, IE proteins, including ICP0, ICP4, ICP22, and ICP27, regulate the viral gene expression cascade; E proteins mediate viral DNA replication; and L proteins are structural proteins or proteins involved in assembly and maturation of virions.

HSV-1 protein synthesis is not required for IE transcription (10). Transcription of the IE genes occurs through recruitment of cellular transcriptional machinery to IE gene promoters. The tegument protein VP16 enhances IE gene expression through its interactions with HCF and Oct-1. Specifically, VP16 interacts with HCF in the cytoplasm and is translocated to the nucleus where it binds cellular protein Oct-1. This complex binds to the viral DNA at consensus TAATGArATT sites upstream of the RNA cap site.
This VP16 and Oct-1 complex enhances transcription of the IE genes by recruiting general transcription factors including TFIIB, TFIIH, TATA-binding protein, and TBP-associated factors (1). This interaction leads to the formation of the RNA pol II pre-initiation complex on IE promoters.

The IE gene proteins ICP4, ICP0, and ICP27 promote the expression of DE genes (6, 7, 9, 11, 12). ICP4 interacts with various cellular transcription factors to activate DE gene expression (12). ICP27, as will be discussed in more detail later, acts at both the transcriptional and posttranscriptional level to regulate the synthesis of some DE proteins (6, 7, 9, 11, 12). ICP0 interacts with cellular proteins to activate transcription and create a cellular environment suitable for viral replication (13).

Three IE proteins (ICP4, ICP0, and ICP27) are needed for expression of L genes, which are divided into two classes: leaky-late or $\gamma_1$, and true-late or $\gamma_2$ (6, 14–20). Expression of leaky late proteins can occur in the absence of viral DNA replication, but levels of these proteins are increased upon initiation of DNA replication. The true-late are only transcribed after viral DNA replication begins.

At the same time that it induces viral gene expression, HSV-1 shuts down host cell mRNA and protein synthesis through the action of a number of viral proteins including viral host shutoff protein (vhs) protein (15, 21, 22). Vhs facilitates the degradation of cellular mRNAs. ICP27 also has a role in host shutoff as will be discussed below. Another IE protein, ICP22, modifies host RNA polymerase II, possibly enhancing its ability to transcribe the viral genome at the expense of host genome transcription (23–28).
HSV-1 DNA replication takes place in the nucleus of infected cells in specialized structures called replication compartments (29). These structures form adjacent to cellular ND10 sites and then mature into replication compartments as viral DNA synthesis begins. Viral DNA replication can be initiated at any of three origins of replication on the HSV genome and is suggested to initially proceed via a theta replication mechanism (30). However, after DNA synthesis initiation, a rolling-circle mechanism likely comes into play to generate progeny genomes in the form of head-to-tail concatemers.

There are seven HSV-1 genes that are essential for viral DNA replication (31–42). The protein products of these seven genes are an origin binding protein (UL9), a single-stranded DNA binding protein ICP8 (UL29), a helicase-primase complex (UL5/UL8/UL52), and a DNA polymerase (UL30/UL42) (4). Cellular factors including DNA ligases and topoisomerases likely also assist in viral DNA replication. UL9 binds as a homodimer to the origin of replication at sites that contain the DNA sequence CGTTTCGCACCTT (33, 36). This leads to a bend in the DNA that distorts the DNA structure into a single-stranded stem loop structure. The ssDNA and UL9 are suggested to recruit ICP8, which then stimulates the helicase activity of UL9. ICP8 is essential for viral DNA synthesis and has not only been shown to stimulate the helicase activity of UL9, but also to promote the helicase-primase complex and to modify the polymerase activity of UL30 (33). Furthermore, ICP8 is required for viral replication compartment formation (33). The multifunctionality of ICP8 and its interaction with numerous viral replication proteins have led to the suggestion that it is a scaffolding protein able to recruit replication proteins to proper sites in the nucleus (33, 34, 37).
Viral capsid assembly requires synthesis of a number of late structural proteins and occurs within the nucleus (43–46). The mature HSV-1 capsid consists of an outer shell that has penton-shaped subunits of the major capsid protein, VP5, and hexons of VP5 and VP23. Capsid assembly can occur without any assistance from cellular factors, as this has been observed in vitro using purified viral protein components (46). Viral DNA is packaged in genome-length monomers forming the nucleocapsid. DNA cleavage and packaging are not completely understood, but a number of viral gene products are known to be involved in this process (47–50).

Virion envelopment proceeds through a process involving two envelopment steps (51–54). The initial nucleocapsid buds through the inner nuclear membrane where it receives some tegument proteins and a primary envelope. Next, the primary enveloped virion fuses with the outer nuclear membrane, and the free nucleocapid is released into the cytoplasm where it is re-enveloped by budding into the trans-Golgi network. These re-enveloped virions are then secreted in vesicles to the extracellular environment through the process of exocytosis.

The Multifunctional ICP27 Protein:

*Physical Properties of the Protein*

ICP27 is an HSV-1 protein with a predicted mass of 52 kDa. It is comprised of 512 amino acid residues (Fig. 1). On SDS-PAGE, ICP27 migrates as a 63 kDa protein (55, 56). The discrepancy between its predicted molecular weight and its observed size on SDS-PAGE may be due in part to the fact that it is extensively post-translationally modified as described below.
The best characterized post-translational modification that ICP27 undergoes is phosphorylation. Phosphorylation can regulate protein activities and interactions. A study by the Sandri-Goldin lab showed that mutation of ICP27 phosphorylation sites led to decreases in viral growth, DNA replication, and the transcription of an early and late gene (57). Three phosphorylation sites were shown to be important: serine residues 16 and 18, which are potential casein kinase 2 sites, and serine residue 114, which is a potential protein kinase A site (57). It is likely there are other functionally important phosphorylation sites that have not been uncovered yet.

A second form of ICP27 post-translational modification is methylation of three arginines in the RGG box sequence, at residues 138, 148, and 150 (5, 58–62). This region is responsible for ICP27’s ability to bind RNA and to act as an export adaptor for HSV-1 mRNA (5, 58–61, 63, 64). In an arginine-to-lysine substitution experiment, mutant forms of ICP27 were unable to bind the cellular proteins Aly/REF and SRPK1 and the viruses harboring these mutations were deficient in viral replication and growth (59).

RNA-binding activity

The RNA-binding activity of ICP27 was originally identified by Mears and Rice using a RNA homo-polymer binding assay (61). ICP27 was found to bind strongly to poly(G). The RNA-binding activity was mapped to an RGG box motif composed of 15 arginine and glycine residues. Mears and Rice also determined that the RGG box domain is essential for ICP27 biological function as a recombinant HSV-1 lacking an RGG box domain does not replicate efficiently in culture (65). Further work showed that the RGG box domain does not bind RNA G-quartet structures but rather interacts with flexible GC-rich
sequences (61, 62). Corbin-Lickfett et al. proposed that this preference for high GC content may allow ICP27 to distinguish between viral and cellular mRNA, given that the HSV-1 genome is 68% GC while the cellular genome is approximately 44% GC (62). They also suggested that ICP27's preference for flexible sequences may prevent it from interacting with some GC-rich cellular sequences, such as rRNAs which are highly structured. Consistent with these in vitro studies, Sandri-Goldin et al. found that ICP27 binds to viral mRNAs in vivo and that most of these targets are intronless (66).

Evidence for flexible structure

As will be discussed later, ICP27 interacts with a variety of cellular and viral proteins. Although many cellular and viral proteins have defined tertiary structures, recent studies indicate that other proteins, particularly those that interact with numerous partners, can be intrinsically disordered or have long, disordered regions (67). Consistent with this, NMR and bioinformatics studies have shown that N-terminal region of ICP27 has a high degree of flexibility and disorder (68). The disordered N-terminal region of ICP27 consists of a 160 amino acid region containing the leucine-rich nuclear export sequence, nuclear localization signal, and the RGG box (68). Several different partners of ICP27 bind in this region including RNA polymerase II, TAP/NXF1, Hsc70, Aly/REF, SRPK1, and RNA (68).

Functions: induction of E and L gene expression

Over the years, it has become clear that ICP27 is a multifunctional protein. This and the following sections summarize functions that have been attributed to ICP27.
ICP27 has a number of important functions relating to the regulation of viral gene expression. Importantly, ICP27 induces the expression of many of the E and L viral genes. The first experiments to characterize the effect of ICP27 on HSV-1 gene expression were transient expression assays and phenotypic studies of ICP27 temperature-sensitive mutants (6, 9). Subsequent work with viruses having deletions of the ICP27 gene showed that ICP27 is needed for robust expression of many DE and L genes (11, 69, 70). However, not all DE/L genes require ICP27 for their expression. One gene that appears to be expressed independently of ICP27 is ICP8 (11). Further work by the Knipe lab determined that other early genes including UL5 are expressed at near normal levels in the absence of ICP27 (11). This may explain why measurable levels of viral DNA replication (~10% of the wild-type level) occur in ICP27 deletion mutant infections (6, 11).

**Functions: mRNA export**

Substantial evidence indicates that ICP27 promotes viral gene expression by serving as an export factor for intronless viral mRNAs. Ordinarily, cellular mRNAs require splicing before they can be exported to the cytoplasm via the cellular mRNA system, which uses a central export factor known as TAP/NXF1. Specifically, a protein complex called the transcription export complex (TREX) binds the 5’ end of mRNAs during splicing. Aly/REF, a mRNA export adaptor that binds RNA and interacts with mRNA export receptor TAP/NXF1, is a constituent of the TREX complex. This complex is required for binding of mRNA to TAP/NXF1.
A role for ICP27 in mRNA export in HSV-1-infected cells was first suggested by \textit{in vivo} UV irradiation studies which showed that ICP27 could be cross-linked to poly (A)$^+$ RNA in both the nucleus and the cytoplasm (66). Further experiments showed that ICP27 interacts with both Aly/REF and TAP/NXF1, thus giving intronless mRNAs access to the mRNA export system (19, 20, 63, 66, 71–74). Some data suggest the interaction with TAP/NXF1 requires an intramolecular head-to-tail interaction between ICP27's N- and C-terminal regions (68, 75). The prevailing model for ICP27's role in mRNA export is that the RGG box domain of ICP27 allows it to bind to intronless viral mRNAs in the nucleus, and that its interactions with Aly/REF and TAP/NXF1 facilitate the escort of these mRNAs from the nucleus (60, 61, 63, 66, 72, 76).

\textit{Functions: inhibition of mRNA splicing}

Sandri-Goldin's lab initially reported that mRNA splicing is inhibited in HSV-1 infected cells, and that ICP27 is required for this effect (77). The majority of HSV-1 mRNAs are intronless, providing a rationale for why the virus may have evolved to suppress splicing (66). The mechanism by which ICP27 inhibits splicing is not fully understood. Splicing is known to be a highly regulated process that involves various RNA-RNA and RNA-protein interactions that occur at sequences on both the 5’ and 3’ splice sites, at the branch site, and within the exon itself (78). ICP27 may alter splicing through its ability to bind to the essential splicing factor SAP145 (73). This study also showed that that ICP27 colocalizes with SAP145 and provided evidence that splicing is inhibited prior to the initial catalytic step. ICP27 also interacts with the RNA splicing regulator p32, and this interaction could be important for splicing inhibition (74).
Additionally, ICP27 binds to a number of members of a family of serine-arginine-rich splicing factors referred to as SR proteins (73, 77, 79, 80). These proteins have many functions and play a particularly important role in spliceosome assembly (78). These SR proteins are regulated by phosphorylation and dephosphorylation. ICP27 has also been shown to recruit a cytoplasmic SR protein-specific kinase, SRPK1, to the nucleus to induce aberrant phosphorylation of SR proteins (79). It was proposed that this stalls spliceosome assembly.

*Functions: mRNA stabilization*

As mentioned earlier, cellular mRNAs are degraded during HSV-1 infection due to the action of the vhs viral RNase. However, there is some evidence that ICP27 also affects mRNA stability. Specifically, there is evidence that it binds to certain cellular mRNA transcripts that contain AU-rich elements (AREs) to stabilize them and prevent their degradation (20, 81). AREs have been discovered in several different mRNAs that code for inflammatory cytokines, transcription factors and growth factors. AREs promote rapid mRNA degradation and regulate translation (82). Ordinarily these transcripts are degraded rapidly but various factors such as proinflammatory cytokines and some types of viral infections lead to activation of the p38/MAPK pathway, which is known to stabilize ARE-containing transcripts by inhibiting their deadenylation (45, 82). Work in our lab using a doxycycline-inducible promoter system in HeLa cells determined that ICP27 expression alone is capable of inducing p38 signaling to levels comparable to those observed in wild-type HSV-1 infection (83). Thus, ICP27's effects on mRNA stability could be indirect, via its ability to stimulate p38 signaling.
Functions: interaction with RNA Pol II

The cellular RNA polymerase II holoenzyme is a large multisubunit complex comprised of the core RNA polymerase II and general transcription factors including TFIIB, TFIIE, TFIIF, and TFIIH (84). The core enzyme has sites for DNA-binding, RNA-binding, and catalysis and is expressed in all eukaryotic cells. It contains a region essential for cell viability known as the carboxyl-terminal domain (CTD), which consists of 52 tandem repeats of heptapeptide consensus sequence YSPTSPS. ICP27 binds the C-terminal domain of RNA polymerase II and both the N and C termini of ICP27 are required for this interaction (85, 86). The Knipe lab suggested that the interaction between ICP27 and RNA polymerase II involves RNA because RNase treatment led to reduction in coprecipitation with RNA polymerase II (86). However, under some conditions this association was resistant to RNase digestion, so they concluded that the interaction involves direct protein-protein interaction between ICP27 and RNA polymerase II. The Sandri-Goldin lab provided evidence that the interaction between ICP27 and RNA polymerase II is essential for recruitment of RNA polymerase II to viral replication centers (85).

Functions: stimulation of viral translation

There is evidence that ICP27 promotes the translation of some viral mRNAs (87–89). Translation involves a number of cellular factors working in concert. Translation initiation is facilitated by assembly of the eukaryotic initiation factor 4F (eIF4F) which consists of the m7G cap-binding protein eIF4E, scaffold and ribosome adaptor eIF4G, and the RNA helicase eIF4a located at the m7G cap (90). The cytoplasmic polyadenylate-
binding protein (PABP), by binding the poly(A) tail on mRNA, is also a key factor. PABP1, via bridging eIF4G and poly(A), may facilitate m$^7$G cap-poly(A) translation initiation (88). Interestingly, ICP27 co-precipitates with cellular translation initiation factors, PABP1, eIF3 and eIF4G (91). Larralde et al. also provided evidence of ICP27 interaction with polyribosomes (98). The ability for ICP27 to associate with polyribosomes suggests it has a functional role in mRNA stability or translation (92). Larralde et al. tested this interaction further in vivo in *Xenopus* oocytes using a tethered function assay to exam the effect of ICP27 on translation in vivo (92). They observed that ICP27 was sufficient to directly stimulate the translation of certain mRNAs and that it did not require other viral factors to achieve this effect. Larralde et al. also suggested that the ICP27 C-terminus was essential for translation stimulation. Finally, ICP27 was also shown to assist translation of the viral mRNA encoding the tegument protein VP16 (89, 93). The stimulation of VP16 translation was mapped to the C-terminus of ICP27, which is an area known to bind a variety of translation initiation factors.

*Functions: modulation of interferon responses*

ICP27 also plays a role in modulating the immune response. Type I interferon (IFNα/β) signaling is an immune antiviral mechanism and IFNα/β binding of IFNAR-1 and IFNAR-2 receptor subunits leads to heterodimerization and phosphorylation of these subunits (94). Activation leads to phosphorylation of Jak-1 and Tyk-2 which then phosphorylate signal transducers and activators of transcription (STATs) 1 and 2. These heterodimerize and translocate to the nucleus and associate with IRF-9, which enables them to bind to a cis-acting DNA element referred to as the interferon stimulated
response element, ISRE. This interaction leads to the transactivation of interferon-stimulated genes (94). ICP27 has been observed to downregulate STAT1 phosphorylation and prevent STAT1 accumulation in the nucleus (95, 96). ICP27 prevents STAT1 nuclear accumulation by inducing the expression of an unidentified small type interferon antagonizing protein (95, 96). The mechanism by which ICP27 regulates STAT1 phosphorylation and its induction of the small antagonizing protein remain to be elucidated.

Functions: activation of MAPKs

HSV-1 has been shown to stimulate the pathway of p38 and c-Jun mitogen-activated protein kinases (MAPKs) and ICP27 has been implicated in this stimulation (97). MAPKs are one of the cells pathways that respond to extracellular stimuli and both the c-Jun N-terminal kinase (JNK) and the p38 MAPK pathways are categorized as extracellular signal regulated kinases (98). The p38 and JNK pathways have both been found to be activated as early as 3 hpi by HSV-1, with peak activity at 6 to 8 hpi (83, 99). When these pathways are pharmacologically inhibited, HSV-1 viral yields are decreased, which suggests that these pathways are important for viral replication (83). The N-terminus of ICP27 is essential for the interactions with these pathways (83). Our lab has determined that ICP27 expression alone is sufficient for p38 activation, even in the absence of viral infection (83).
Inhibition of apoptosis by ICP27.

HSV-1 infection modules the apoptotic pathway in a myriad of ways to both induce and even prevent apoptosis during infection. Aubert et al. determined that DE and L gene expression was essential for apoptosis prevention (18). Aubert et al. found that HEp-2 cells infected with an ICP27 deletion mutant underwent apoptosis, suggesting that ICP27 blocks apoptosis in infected human cells (100). To further delineate which functions of ICP27 were essential to block infection-induced apoptosis, Aubert et al. tested several ICP27 mutant viruses (18). The N-terminus contains the protein’s RNA binding and nuclear/nucleolar localization sites, while the C-terminus contains regions that are required to enhance expression of later viral gene products. Sequences in both the N- and C-terminal segments of ICP27 were required to prevent apoptosis in HEp-2 cells (18). Aubert et al. proposed a model for an indirect antiapoptotic role of ICP27 in the viral life cycle through its ability to upregulate early and late antiapoptotic viral gene products (101).

Important functional sequences and domains in ICP27

Fig. 1 shows the identified functional regions of the ICP27 protein. ICP27 has several domains at its N-terminus that facilitate various functions including a nuclear localization signal (NLS), nuclear export signal (NES), a RGG box, and an acidic region (76). The C-terminus has three conserved herpesvirus domains. The C-terminus is required for its essential functions (102).
Statement of Thesis Question

To date most of the genetic studies focused on ICP27 have used the African green monkey kidney Vero cell line (21, 57, 68, 75, 76, 83, 95, 96, 103–107). These cells yield very high HSV-1 titers and the virus readily forms plaques on these cells. Vero cells are considered to be of epithelial origin, but they display fibroblastic properties (108–110). They are an immortal, non-transformed cell line that is IFN-β deficient (111). One paramount issue to consider is that because the virus can disseminate in the body, HSV-1 can potentially replicate in many cells other than epithelial cells. This raises the question of whether the phenotypes of ICP27 mutants are the same in other types of cultured cells. This is important because the function of ICP27 in many cases is inferred from the phenotype of viral mutants. Thus, in this work we set out to ask whether the replication ability of HSV-1 ICP27 mutants is the same in other types of cultured cells as it is in Vero cells.
MATERIALS AND METHODS

Cells, Viruses, Infections

The cell lines Vero, V27, CV-1, HEK293, U373, U2-OS, ARPE-19, HeLa, and HEp-2 were purchased from American Type Culture Collection or obtained from colleagues at the University of Minnesota. V27 cells (8) are Vero cells that have been stably transfected with the HSV-1 ICP27 gene. Human foreskin fibroblasts (HFF) and HFF-TERT (112) were kindly donated by Dr. Wade Bresnahan. TE286 are primary human tonsillar fibroblasts and were kindly donated by Dr. Peter Southern. Cells were grown in Dulbecco-modified Eagle medium containing 5% or 10%, depending on the cell line, heat-inactivated fetal calf serum (FCS), 50 U/ml penicillin, and 50 µg/ml streptomycin. The medium for V27 cells was the same, except that it also contained 300 µg/ml G418. All tissue culture reagents were purchased from Life Technologies/Invitrogen (Carlsbad, CA) except FCS, which was purchased from Atlas Biologicals (Fort Collins, CO).

The wild-type (WT) HSV-1 strain used was KOS1.1. The ICP27 mutants used were d1-2 and d27-1 and their construction has been described (8, 70, 76). The multiplicity of infection (MOI) used in experiments was 10 or 1 PFU per cell, as indicated. Infections were performed in phosphate-buffered saline (PBS) containing 0.1% glucose and 0.1% heat-inactivated newborn calf serum. Viral adsorption was for 1 hr at 37°C, after which time the viral inoculum was replaced with 199 medium containing 1% heat-inactivated newborn calf serum, 50 U/ml penicillin, and 50 µg/ml streptomycin. Infections were then incubated at 37°C. Infections destined for plaque assay analysis or
immunofluorescence were carried out in 12-well trays, while all other infections were carried out in 25 cm² flasks.

In the event that infections were to be analyzed via plaque assay, they were allowed to incubate for 72 hours before being processed. Processing consisted of removal of 199 media; permeabilization and fixation with methanol for 10 minutes; staining with 1% Giemsa stain (Sigma Aldrich) for at least 1 hr; rinsing of trays with DI water; and drying of trays overnight before analysis. Plaques were counted visually with the aid of a microscope and the counts were used to calculate the viral titer.

**Plasmid Construction**

Plasmids having specified mutations in a segment of the ICP27 gene were purchased from Genescript. The plasmids containing deletions were pLS12-20, pLS12-42, pLS43-63, pLS21-63, pLS21-42, and pLS12-63, with the numbers indicating the range of amino acid residues deleted from the N-terminus of ICP27. Plasmids that contained substitution mutations in the ICP27 gene were p15LSD-3A, p19DLD-3A, and p20L-A. The mutated ICP27 sequences were then introduced into the ICP27 gene on plasmid pBH27 via the following strategy. Both the mutant and the pBH27 vector were digested for 2 hours at 37°C with AgeI and DraIII. These digests were then run on a 1% agarose gel and after band separation, bands corresponding to the desired insert sizes were excised from the gel and purified using a Qiagen DNA purification kit as per the manufacturer’s instructors. The DNA concentration was determined via use of a Nanodrop spectrophotometer. Purified insert DNA containing the mutation was then ligated to the purified pBH27 vector backbone. The ligation products were then used to
transform competent DH5α E. coli and individual transformants were picked and grown overnight on a shaker at 37°C. DNA was obtained and purified from these transformants using a ProMega PureYield Plasmid Midiprep System kit. This purified transformant DNA was then digested for 2 hours at 37°C with AgeI and DraIII. These digests were ran on a 1% agarose gel and plasmids with the desired structure were isolated.

**Protein Analysis**

Isolation of protein samples and immunoblotting were performed as described previously (17). The primary antibodies used were mouse monoclonal antibodies to ICP0 (H1112), ICP27 (H1113), gD (H1103), ICP4 (H1114), and ICP8 (H1115) and were obtained from the Rumbaugh-Goodwin Institute for Cancer Research (Plantation, FL). ICP0, ICP27, gD, ICP4, and ICP8 antibodies were used at dilutions of 1:1,000, 1:1,000, 1:3000, 1:5000, and 1:300 respectively. VP5-specific mouse monoclonal primary antibody was obtained from Abcam (Cambridge, MA) and used at a dilution of 1:3200. A mouse monoclonal antibody specific to β-actin was purchased from Abcam (Cambridge, MA) and diluted 1:10,000. The secondary antibody used for immunoblot detection was a horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G, purchased from Jackson Immunoresearch (West Grove, PA) and was diluted 1:7500 in phosphate-buffered saline. Secondary antibodies were detected with enhanced chemiluminescence detection reagents as per the manufacturer’s specifications (Amersham).

**RNA Analysis**

Analysis of RNA via northern Blotting was conducted as described previously (76). Briefly, total RNA of infected cells was harvested at 7 hpi using Trizol (Life
Technologies) according to the manufacturer’s instructions. RNA was quantified and then run on a 1.5% agarose glyoxal gel as per the manufacturer’s directions. RNA was transferred to a nylon membrane and then probed for ICP8 mRNA using radioactively-labeled plasmid pE/3583 (113) (ICP8 probe). Finally, the radioactive signal was visualized by autoradiography.

**Immunofluorescence**

Cells plated on coverslips were fixed in 3.7% formaldehyde in phosphate-buffered saline for 10 min, followed by cold (-20°C) acetone permeabilization for 2 min. For fluorescent staining, coverslips were incubated at 37°C for 1 hr with one of two different primary antibodies. Primary antibodies used were a monoclonal mouse ICP4 (H1114) antibody (1:800) and a monoclonal mouse ICP27 (H1113) antibody (1:300). Both antibodies were diluted in PBS. The secondary antibody used was a Dylight 488-conjugated Affinipure Fab fragment Goat anti-mouse IgG(H+L) from Jackson Immunoresearch Laboratories diluted 1:300 in PBS. The secondary was also diluted in phosphate-buffered saline. Nuclei were stained with DAPI, which is a component of the mounting medium used (Fluoroshield, Sigma-Aldrich). Cells were examined using a confocal fluorescent microscope (Olympus Fluoview FV1000 BX2) linked to a video camera. Images were captured as TIFF files using the program Olympus Fluoview.

**Virus Complementation Experiments**

Subconfluent 25 cm² flasks of HEp-2 or Vero cells were transfected with 7.5 μg of ICP27-encoding plasmid using Lipofectamine 2000 (Invitrogen). At 24 hrs post transfection, cells were infected at an MOI of 1 with the ICP27-null mutant d27-1. At 2
hpi, flasks were subjected to an acid-glycine low pH wash step to inactivate any remaining extracellular virus. This step significantly decreased the virus background in this assay. Infections were then incubated at 37°C for 24 hrs. Infections were terminated by adding 5 mL of sterile non-fat dry milk. Viral yield was determined by plaque assay analysis on V27 cells. Virus was released by three cycles of freeze thawing.
RESULTS

ICP27 mutant d1-2 displays a cell-type dependent growth defect. Most previous analyses of ICP27 mutants have been carried out in Vero cells, an epithelial cell line originally isolated from African green monkey kidney tissue. Vero cells have been used extensively in HSV-1 research, in part because the virus replicates quite robustly in these cells and readily forms plaques on them. To see if ICP27 mutants grow similarly in a different cell type, we used HeLa cells, a transformed line of human cervical epithelial cells which have also been used in HSV-1 work. As mutations in the C-terminal half abrogate most or all of ICP27’s functions (102), we focused on mutants having short in-frame deletions in the N-terminal half of the ICP27 gene (Fig. 1) (65, 70, 76). Although some of these mutants are deficient for growth in Vero cells, they all replicate significantly greater than an ICP27 null mutant. To carry out the analysis, HeLa cells were infected at an MOI of 10 PFU/cell with WT HSV-1 or ICP27 deletion mutants and incubated for one day before being harvested. The amount of infectious progeny in each culture lysate was then determined by plaque assay on ICP27-complementing V27 cells (8) (Fig. 2A). The results showed that, with one exception, all mutants exhibited yields that are roughly comparable to that previously seen in Vero cells (see Fig. 1). The exception was d1-2, which has a deletion of codons 12-63. In Vero cells, this mutant shows a 10-100-fold replication defect, but in HeLa cells it is much more restricted, exhibiting a near 100,000-fold defect.

To confirm this finding, we next directly compared the growth of d1-2 in HeLa and Vero cells. We also included dAc, an ICP27 mutant that has a smaller deletion in the
same region of the ICP27 gene (codons 20-63) (Fig. 1). The results (Fig. 2B) confirmed that d1-2 replicates much more poorly in HeLa cells than it does in Vero cells. In contrast, dAc replicated similarly to the WT virus in both HeLa and Vero cells. Importantly, an independent isolate of d1-2, d1-2b (70), showed the same growth phenotype as d1-2 (data not shown), demonstrating that the inability of d1-2 to replicate in HeLa cells is not due to a secondary mutation, but rather results from the engineered ICP27 gene deletion.

To see if the growth restriction of d1-2 is limited to HeLa cells, we tested its growth in another human line, HEp-2 cells. The results (Fig. 2C) resembled those seen in HeLa cells, i.e., the growth of d1-2 was highly restricted. In this case, the results were even more dramatic, in that d1-2 replicated >10,000-fold less efficiently in these cells than in Vero cells. In contrast, dLeu and dAc showed only modest reductions in viral growth in HEp-2 cells compared to Vero cells. In summary, these results show that the ICP27 mutant d1-2 has a cell type-dependent growth phenotype, as it replicates considerably more poorly in HeLa and HEp-2 cells than it does in Vero cells. Amongst our collection of ICP27 N-terminal mutants, we did not identify any other ICP27 mutant with this same high degree of cell-type dependent replication.

Identification of other host cells that are highly restrictive for d1-2 replication. It is possible that the very poor replication of d1-2 in human HeLa and HEp-2 cells compared to monkey Vero cells is due to the species origin of the cells. However, there are several other differences between HeLa/HEp-2 and Vero cells. To understand the cellular factors that contribute to the cell-specific replication phenotype of d1-2, we
surveyed a variety of cell lines and primary cell cultures (Table 1) for their ability to serve as hosts for d1-2. Each cell line was infected in triplicate at an MOI of 10 with WT HSV-1 (KOS1.1), d27-1 (an ICP27 null mutant) or d1-2, and the infections were incubated for 24 hrs. The final viral yields were determined by plaque assay on V27 cells. The data are shown in Fig. 3. WT HSV-1 replicated quite well (>2 x 10^6 PFU/ml) in all the cell lines, while d27-1 completely failed to replicate in any of them (<1 x 10^1 PFU/ml). In contrast, the replication of d1-2 fell into two clear categories. Of the eleven cell lines/primary cell samples tested, four (Vero, CV-1, ARPE19, and 293) were semi-permissive, that is capable of supporting a modest level of d1-2 replication (>2 x 10^3 PFU/ml). The other seven were restrictive, failing to support any replication (<1 x 10^1 PFU/ml). Examination of the data with respect to cell type failed to reveal a simple correlation between the ability of d1-2 to replicate and the species, tissue origin, or transformation status of the host cells.

**Viral replication compartment formation is highly deficient in d1-2 and d27-1 infected HEp-2 cells.** The fact that d1-2 can replicate in some cells but not others suggest that an ICP27 function (or functions) defective in d1-2 is more important in certain cells than in others. However, it is not known what this function is. To provide some insight into this, we set out to determine why d1-2 completely fails to replicate in restrictive cells. In all experiments, we compared d1-2 infections in HEp-2 cells to those in Vero cells, where d1-2 can replicate, albeit semi-permissively.

One possibility for the restriction of d1-2 in HEp-2 cells is that ICP27 is expressed very poorly or is localized aberrantly. To test these possibilities, we used
immunofluorescence microscopy to examine ICP27's expression and localization. Thus, Vero and HEp-2 cells were infected with d1-2 at an MOI of 10. As controls, we also mock-infected cells, or infected them with KOS1.1 or d27-1. The cells were fixed and permeabilized at 4 and 8 hpi and subjected to immunofluorescence using a monoclonal antibody to ICP27 (Fig. 4). The results showed that d1-2 expresses ICP27 with WT kinetics in both HEp-2 and Vero cells. Additionally, at 4 hpi, d1-2 encoded ICP27 protein localizes similarly to WT protein, being strongly nuclear with a somewhat heterogeneous distribution. At 8 hpi, d1-2 ICP27 showed a much more compartmentalized distribution than WT ICP27. However, as this was observed in both the Vero and HEp-2 cells, this localization cannot explain the total failure of d1-2 to replicate in HEp-2 cells. This experiment indicates that the inability of d1-2 to replicate in HEp-2 cells is not due to a lack of expression of ICP27 or to a grossly altered localization in the cell.

We next examined the expression and localization of another IE protein, ICP4. ICP4 is an essential transcription factor that is required for the expression of nearly all DE and L genes. Early in infection (2-4 hpi), ICP4 localizes diffusely in the nucleus, but as infection proceeds, it begins to accumulate in globular structures known as viral replication compartments (RCs) (114). RCs are sites of viral DNA replication and possibly late gene expression, and their formation is dependent upon viral DNA replication (114). To examine ICP4 localization, HEp-2 or Vero cells were mock-infected or infected with WT, d27-1, or d1-2. The cells were fixed at 4 and 8 hpi and processed for ICP4 immunofluorescence (Fig. 5). At 4 hpi, ICP4 was expressed in all the infections and the localization patterns were similar, irrespective of virus or host cell. At this time
point, ICP4 exhibited a diffuse nuclear localization with some additional staining in small nuclear punctae. However, at 8 hpi, clear differences in ICP4 staining emerged. In the case of the WT virus, ICP4-containing RCs were clearly seen in numerous Vero and HEp-2 cells (white arrows in Fig. 5). In the d1-2 infection of Vero cells, RCs were also frequently observed, although only in a minority of the infected cells. However, in d1-2-infected HEp-2 cells, viral RCs were almost never observed. Interestingly, similar findings were seen in the d27-1 infections, in that nuclear RCs were frequently observed in Vero cells, but not in HEp-2 cells. A repeat experiment wherein the staining was performed at 12 hpi yielded a similar result (data not shown), i.e., RCs for d1-2 and d27-1 were seen in Vero but not HEp-2 cells.

The presence of replication compartments in Vero cells is not surprising as several studies have shown that in d1-2 and d27-1 infected cells, viral DNA is replicated in the absence of ICP27, although overall this results in low levels of replication such that DNA replication is reduced 60-90% (6, 8). The almost complete lack of RCs in d27-1-infected HEp-2 cells suggests that ICP27 is more critical for viral DNA replication in HEp-2 cells than it is in Vero cells. Moreover, these results suggest that d1-2 is unable to replicate significant amounts of viral DNA in HEp-2 cells.

**ICP8 expression is deficient in d1-2-infected HEp-2 cells.** Viral DNA replication is dependent on the induction of viral DE gene expression by the IE proteins. In particular, viral DNA replication is contingent upon expression of seven viral proteins that collectively make up the core viral DNA replication machinery. These consist of a single-stranded DNA-binding protein (ICP8), an origin-binding protein (UL.9), a two-
subunit viral DNA polymerase (UL30 and UL42), and a three-subunit helicase-primase complex (UL5, UL8, and UL52). To examine the expression of viral DE and L genes in d1-2-infected cells, we carried out an immunoblotting analysis. HEp-2 or Vero cells were mock-infected or infected at an MOI of 10 PFU/cell with KOS1.1, d1-2 or d27-1. Total protein extracts were prepared at 4, 8 and 12 hpi and subjected to immunoblotting using antibodies specific for ICP27, two DE proteins (glycoprotein D and ICP8), and an L protein (VP5) (Fig. 6). For ICP27, the results of this were consistent with the immunofluorescence experiments (Fig. 4) in that d1-2 was able to induce ICP27 expression similarly to the WT virus in both Vero and HEp-2 cells (note that the ICP27 molecule encoded by d1-2 migrates more rapidly than the WT protein as a result of its deletion). The induction of the gD protein was clearly deficient in d27-1-infected cells, indicating that its expression is ICP27-dependent. However, d1-2 was able to induce this gene efficiently in both Vero and HEp-2 cells, indicating that d1-2 is not greatly deficient in the ICP27 function needed for induction of this gene. The results for DE protein ICP8 were quite different, however. As has been previously reported, ICP8 induction was not ICP27-dependent in Vero cells (11). However, its expression was heavily dependent upon ICP27 in HEp-2 cells, as d27-1-infected cells showed a delay and reduction in ICP8 accumulation. Moreover, d1-2 closely resembled d27-1 in its ICP8 expression. Finally, we examined the expression of the L protein VP5. VP5 expression was dependent on ICP27 in both Vero and HEp-2 cells, based on the reduction in VP5 accumulation seen in the d27-1 infection. However, in both Vero and HEp-2 cells, d1-2 was able to express significantly more VP5 than d27-1, although the levels were still reduced compared to the
WT infection. These results indicate that the mutation in d1-2 weakens the function needed for VP5 expression.

Another experiment was conducted to determine whether ICP8 levels are reduced in d1-2 infections of other non-permissive cells. Thus, we infected restrictive cells, HFF, and a semi-permissive line, ARPE-19. Total proteins were harvested at 8 hpi, and analyzed for ICP8 expression by immunoblotting (Fig. 7). ICP8 protein expression levels were decreased in the d1-2 infected HFF cells, but not in d1-2 infected ARPE-19 cells. Thus, in the restrictive cells (HEp-2 and HFF) ICP8 expression is dependent upon ICP27, and does not occur efficiently in the d1-2 mutant. This indicates that the deletion in d1-2 weakens an ICP27 function that enhances ICP8 expression in HEp-2 and HFF cells.

**ICP8 mRNA expression is decreased in d1-2 infected HEp-2 cells.** In order to ascertain at which point in the gene expression pathway ICP8 protein levels were being reduced, we examined the expression of ICP8 mRNA. We infected either Vero or HEp-2 cells at an MOI of 10 with KOS, d1-2 and d27-1 and purified total RNA at 7 hpi. Equal amounts of the RNA were subjected to Northern blot analysis using a radioactive probe that binds to the ICP8 transcript (Fig. 8). In Vero cells, ICP8 mRNA levels were not dependent on ICP27, as has been noted before. However, in HEp-2 cells, both d1-2 and d27-1 displayed a significant decrease in ICP8 transcript levels. Thus, in HEp-2 cells, ICP8 mRNA expression is dependent on ICP27, and d1-2 is defective in the ICP27 function need to induce ICP8 mRNA expression.

**ICP27 residues 12-20 are essential for viral replication in HEp-2 cells.** The above results indicate that ICP27 residues 12-63 are absolutely essential for viral
replication in HEp-2 cells, although in Vero cells, they are not required for a significant level of viral growth. To narrow down the N-terminal residues involved in this cell-specific difference, we took advantage of the fact that plasmids expressing a cloned ICP27 gene can complement the growth of an HSV-1 ICP27 deletion mutant (8). Therefore, we constructed various ICP27 mutant plasmids that had a deletion or point mutations in the N-terminal region of ICP27 (Fig. 9) and asked whether transfection of the cloned genes into Vero and HEp-2 cells can complement growth of d27-1 (Fig. 10). A key mutant was pLS12-63, in which the deletion present in d1-2 was engineered. As expected, pLS12-63 significantly complemented d27-1 in Vero cells, but not in HEp-2 cells (Fig 10A). These results independently verify that the lethal phenotype of d1-2 in HEp-2 cells is not due to a secondary mutation. Plasmids pLS12-20 and pLS12-42 gave very similar results, i.e. they were completely unable to complement d27-1 in HEp-2 cells but were able to partially complement in Vero cells. Further experimentation with plasmids pLS21-42 and pLS21-63 indicated that deleted residues C-terminal to residue 20 does not lead to a lethal mutation in HEp-2 cells (Fig 10B). These results show that deletion of amino acids 12-20 leads to a lethal mutation in HEp-2 cells.

The above data implicate residues 12-20 as being critical for growth in HEp-2 cells. However, virus mutant dLeu, which lacks residues 6-19 (Fig. 9) grows to a significant extent in Vero cells. We therefore hypothesized that the sequence around residue 20 is a critical for growth in HEp-2 cells. To test this, we generated three ICP27 gene mutants that have point mutations at or near residue 20. 15LSD-3A has a triple alanine-substitution at residues 15-17, 19DLD-3A has a triple alanine-substitution at
residues 19-21, and 20L-A has a single alanine substitution at residue 20. When those mutants were tested in the complementation assay (Fig. 10D) 15LSD-3A, 19DLD-3A and 20L-A plasmids complemented the growth of d27-1 in both HEp-2 and Vero cells. However, the level of complementation differed significantly between cell lines. A student’s T-test revealed that there was a statistically significant difference between the ability of the 19DLD-3A plasmid to complement d27-1. This difference was observed in both cell lines but was most significant (p-value <.0001) in the HEp-2 cell line. Thus, although 19DLD-3A is not a lethal mutation, it does lead to a cell-type dependent phenotype.
DISCUSSION

The replication of ICP27 mutant d1-2 depends on the identity of the host cell.

In this work, we identified HSV-1 ICP27 mutant d1-2 as one that has a cell type-dependent growth phenotype, in that it replicates in Vero cells, albeit somewhat inefficiently, but is completely unable to replicate in HEp-2 cells. To explore the nature of this defect, we characterized the growth of d1-2 in a large panel of different cells (Table 1). The cells were chosen with regard to a number of different factors that could potentially be important for this phenotype. These include cell type, transformation status, and species origin. The results (Fig. 3) indicated that more than half of the cell lines or strains tested failed to support any d1-2 replication, while a few supported a significant amount of replication. Thus, the cells fell into two clear categories with respect to d1-2 replication. We failed to observe a correlation between the replication phenotype of d1-2 and the transformation status of the cells, as both primary cells (HFF, HFF-Tert, and TE286) and cancer cell lines (HeLa, HEp-2, U2-OS, U373) were restrictive for d1-2 growth. We were also able to rule out species origin as being critical to the phenotype, as two human cell lines (293 and ARPE19) and two monkey cell lines (Vero and CV-1) were semi-permissive for d1-2 growth. Additionally, the cell type did not appear to play a role, since we did not observe a correlation between cell type and restrictiveness. Human fibroblasts are a natural host cell of HSV-1 but were restrictive for d1-2 growth, while Vero cells, the model cell system for HSV-1 research, were semi-permissive.
With these results in mind, we suggest that Vero cells are not the best model of natural infection as they are not derived from the natural host for HSV-1 and they yield different results than cells which are. Our work thus emphasizes that cell type is a critical factor in studying HSV-1, particularly in regard to ICP27 function.

The data from these experiments suggests that one or more of ICP27 functions in HSV-1 infection is dependent on an as yet unknown factor (or factors) present in some cells but not others. There are two general possibilities for this. First, semi-permissive cells could express a factor that carries out a replication function that is lacking in the d1-2 ICP27 molecule. In this model, restrictive cells would lack that factor. Alternatively, restrictive cells could express a restriction factor that inhibits HSV-1 replication in the absence of the missing ICP27 function. In this model, semi-permissive cells would lack that factor. One experimental approach that may elucidate which of these models is correct is a host gene expression microarray analysis of d1-2 infected restrictive and semi-permissive cells. This might allow us to identify host genes that are up- or down-regulated during d1-2 infection in a manner that is correlated with the semi-permissive or restrictive nature of the infection. If so, such genes could be targeted by siRNA knockdown to test the role that the gene plays in replication under various conditions.

Identification of a short sequence in ICP27 that determines cell type-specific growth. The d1-2 mutant has a deletion of codons 12-63, indicating that the N-terminal region of ICP27 is critical for the host-range dependent phenotype. To home in on the most critical residues for this phenotype, we created various ICP27 deletion mutant gene constructs with shorter deletions in the region encompassing residues 12-63 (Fig. 9A).
These plasmids were then used in transfection-infection experiments to see which mutant genes supported the growth of an ICP27 deletion mutants in HEp-2 and Vero cells. We observed that deleting a 9 amino acid region, corresponding to residues 12-20, was sufficient to reproduce the replication defect of d1-2. (Fig. 10B). To study the relevant sequences further, we also engineered three plasmids encoding ICP27 molecules with point mutations in the region of interest. Of these, mutant 19DLD-3A was the only plasmid that resulted in a phenotype that resembled d1-2, i.e., it led to significantly less growth in Vero cells compared to HEp-2 cells (Fig. 9B). Thus, this mutant indicates that residues 19-21 are very important in the cell type-dependent replication phenotype. Future mutagenesis experiments should focus more intently on this region, both through deletions and substitution mutants.

**What is the block to replication in d1-2 infected HEp-2 cells?** We performed several experiments to understand why d1-2 is completely unable to replicate in HEp-2 cells. First, we examined the expression and localization of the d1-2 ICP27 protein. The ICP27 in d1-2 infections of both Vero and HEp-2 cells at 8hpi displayed a similar aberrant localization pattern which suggests that the abnormal localization cannot completely explain the failure of d1-2 to replicate in HEp-2 cells (Fig. 4). Second, we examined replication compartment formation as these structures are essential for HSV-1 DNA replication. Interestingly, we observed that replication compartments do not efficiently form in d1-2 infections of HEp-2 cells (Fig 5). This suggests that viral DNA is not replicated in d1-2-infected HEp-2 cells.
Since viral DNA replication depends on efficient DE protein synthesis, we next looked at protein accumulation at 4, 8 and 12 hpi, assaying proteins from all three viral gene classes. Of the proteins observed, only L protein VP5 and DE protein ICP8 were reduced in expression in d1-2 infected HEp-2 cells (Fig. 6). In the case of ICP8, expression was reduced quite significantly. This was unexpected because ICP8 expression is not dependent on ICP27 in Vero cells (11). We also found that ICP8 expression is reduced in another restrictive cell setting, HFF cells, but not in semi-permissive ARPE-19 cells (Fig. 7). Thus, there is a correlation between restrictiveness of the cell line for d1-2 growth and ICP8 expression. As ICP8 has been established as being essential for HSV-1 DNA replication, these results suggest an explanation for the failure of d1-2 to replicate DNA in HEp-2 and other restrictive cells (39).

Further investigation by northern blotting analysis revealed that ICP8 mRNA transcript expression is dramatically reduced in HEp-2 infections, but in Vero cells is only slightly diminished, consistent with work from the Knipe lab (11). Thus, deficient ICP8 protein expression in d1-2-infected HEp-2 cells appears to be due to a reduction at the level of its encoding mRNA. The level at which ICP27 regulates ICP8 mRNA expression in HEp-2 cells is unknown, but it is possible that it increases transcription of the ICP8 gene. It is also possible it stabilizes the mRNA, possibly through an effect on export of the mRNA.

Based on our results, we hypothesize that the reduction of ICP8 mRNA (and possibly in mRNA encoding other viral replication proteins) is responsible for the precipitous drop in genome replication in non-permissive cells. This would be in contrast
to the situation in Vero cells, where ICP27 mutants have diminished DNA replication but still replicate their genomes to a significant degree (11-38% of WT levels) (6, 70, 115). As seven viral gene products are involved in HSV-1 DNA replication, it is unknown if other viral replication proteins are similarly reduced in d1-2-infected HEp-2 cells. One way to address this is to ask whether exogenous expression of ICP8 in a restrictive cell line is sufficient to rescue the replication of d1-2. If so, then it would be possible to conclude that ICP8 insufficiency is the fundamental defect of d1-2 in HEp-2 cells. However, it is possible that other critical HSV-1 DE genes are expressed at reduced levels, and thus ICP8 alone would be unable to rescue the phenotype.

It should be pointed out that this work is consistent with work from the Blaho lab which indicated that certain ICP27 mutants including d1-2 induce apoptosis at late times in HEp-2 and other human cells, but not in Vero cells (100). Analysis of viral gene expression in infected HEp-2 cells suggested that this effect was due to the ability of ICP27 to stimulate expression of certain early and/or late genes that encode apoptosis suppressors (18). Our finding that d1-2 is deficient in early/late gene expression is consistent with this. However, as apoptosis occurs late in infection, it is unlikely to explain the complete lack of d1-2 growth in HEp-2 cells.

Is there a novel HEp-2 specific ICP27 function? ICP27, is a complex, multifunctional protein with a number of known functional domains. The region deleted in d1-2 includes a portion of the NES as well as the entire acidic region. However, we have determined that deletion of amino acid residues 12-20 is sufficient to replicate this phenotype. This suggest that there may be another functional domain within this region in
addition to the NES that has yet to be identified. Furthermore, it is possible that this function may only be required in certain, i.e. restrictive, cells. If so, it may be possible to create a mutant that is able to replicate to WT levels in Vero cells but is defective for growth in HEp-2 and other human cells. The mutant 19DLD-3A resembles such a mutant, although it is slightly defective for replication in Vero cells.

**Summary and conclusions.** The findings presented here show that one or more ICP27 functions is cell-type dependent. Specifically, we report that a specific ICP27 mutant, d1-2, shows a striking cell-type dependent growth defect wherein it replicates semi-permissively in monkey Vero cells but is completely restricted for replication in several human cells including HEp-2 cells and primary human fibroblasts. The failure of d1-2 to replicate in human HEp-2 cells appears to be linked to a reduction in expression of ICP8, a key DNA replication factor, at both the transcript and protein level. This suggests that expression of viral DE/L proteins are more dependent on ICP27 in certain cell lines, including primary human fibroblasts which are a better model of natural infections than are Vero cells. As most previous studies of ICP27 function have been carried out in Vero cells, our studies highlight the need to carry out future analyses in cells that can be considered more relevant to natural HSV-1 infections.
**Fig. 1. HSV-1 ICP27 mutants.** At the top is shown a schematic of the 512-residue ICP27 polypeptide, with notable functional sequences highlighted. Below are shown representations of the HSV-1 ICP27 N-terminal mutants used in this study. Numbers above the deletions indicate amino acids deleted in the various mutant polypeptides.
Fig. 2. Mutant d1-2 replication is more defective in HeLa and HEp-2 cells than in Vero cells. (A) Screening of mutants in HeLa cells. HeLa cells were infected in duplicate at an MOI of 10, incubated for one day, and harvested. The amount of viral progeny was then determined by plaque assay on V27 cells. The bars indicate the means of the duplicate infections and the bars denote the range of the two values. (B) Comparison of ICP27 mutant growth in HeLa and Vero cells. Details are as in (A), except that infections
were carried out in both HeLa (black bars) and Vero cells (white bars). (C) Comparison of ICP27 mutant growth in HEp-2 or Vero cells. Details as in (A), except that infections were carried out in both HEp-2 (cross-hatched bars) and Vero cells (white bars).
Figure 3. d1-2 replication is predominantly non-permissive in cell lines examined. The various cells were infected in triplicate with WT HSV-1, the ICP27 deletion mutant d27-1, or d1-2 at an MOI of 10 and incubated for one day. The progeny were then determined by plaque assay on V27 cells. Values shown represent mean titers and error bars denote standard error. Our limit of detection was $10^3$ PFU/mL as indicated by the yellow line. Indicated samples were considered semi-permissive for d1-2 infection if mean viral titers were >100-fold above the limit of detection and non-permissive if not.
Figure 4. ICP27 expression and localization appear similar in d1-2-infected Vero and HEp-2 cells. Detection of ICP27 in infected HEp-2 and Vero cells was carried out using confocal microscopy and an α-ICP27 mouse monoclonal antibody (green). Nuclei were stained with DAPI present in the mounting media (blue). Images were collected from a single coverslip at 4 and 8 hpi and are representative of expression in other cells observed on the coverslip.
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**Vero**

**Hep-2**
**Figure 5. Replication compartments do not form in d1-2-infected HEp-2 cells.**

Detection of ICP4 in infected HEp-2 and Vero cells was carried out by confocal microscopy using an α-ICP4 rabbit monoclonal antibody (green). Nuclei were stained with DAPI present in the mounting media (blue). Each image was collected from a single coverslip at 4, 8 and 12 hpi and is representative of expression in other cells on the coverslip.
Figure 6. Expression of viral proteins in d1-2 infections in HEp-2 and Vero cells. HEp-2 and Vero cells were infected with KOS, d1-2 and d27-1 at an MOI of 10. These samples were then harvested at 4, 8 and 12 hpi. These lysates were then fractionated by SDS-PAGE and finally transferred to nitrocellulose. Anti-ICP27, anti-ICP0, anti-ICP4, anti-ICP8, anti-VP5 and anti-gD antibodies were used for western blot analysis. β-actin levels were determined as the loading control.
Figure 7. ICP8 expression in infected HFF and ARPE-19. HFF and ARPE-19 cells were infected with KOS, d1-2 and d27-1 at an MOI of 10. These samples were then harvested at 8 hpi. The lysates were then fractionated by SDS-PAGE and transferred to nitrocellulose. Anti-ICP8 antibodies were used for western blot analysis. HnRNP L levels were analyzed as the loading control.
Figure 8. ICP8 mRNA levels are reduced in d1-2-infected HEp-2 cells. HEp-2 and Vero cells were infected with KOS, d1-2 and d27-1 at an MOI of 10. These samples were then harvested at 7 hpi. ICP8 mRNA transcript was detected using P32-labeled probes specific for the ICP8 genes. The Millennium RNA markers (Ambion) were used for transcript size determination.
Figure 9. Map of ICP27 plasmid mutants constructed in this study.

ICP27 plasmid mutants. Deletions or point mutations were engineered into ICP27 plasmids as shown.
Figure 10. Complementation of d27-1 replication by mutant ICP27 genes. (A-D) d27-1 complementation experiments. HEp-2 and Vero cells were transfected in triplicate with each of the respective plasmids using Lipofectamine 2000. After 24 hrs, the transfected cells were infected with d27-1. At 24 hpi, the infected samples were frozen in milk and freeze-thawed for viral titering on V27 cells. Values shown represent mean titers and error bars denote SEM. The limit of detection in the plaque assay was 10 PFU/mL.
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<td>-</td>
<td>Embryonic kidney</td>
<td>Epithelial</td>
</tr>
<tr>
<td>HeLa</td>
<td>X</td>
<td>Cervical adenocarcinoma</td>
<td>Epithelial</td>
</tr>
<tr>
<td>HEP2</td>
<td>X</td>
<td>Larynx carcinoma</td>
<td>Epithelial</td>
</tr>
<tr>
<td>U2-OS</td>
<td>X</td>
<td>Osteosarcoma</td>
<td>Epithelial</td>
</tr>
<tr>
<td>U373</td>
<td>X</td>
<td>Glioblastoma</td>
<td>Astrocytoid</td>
</tr>
<tr>
<td>TE286</td>
<td>X</td>
<td>Tonsil(primary)</td>
<td>Fibroblast</td>
</tr>
<tr>
<td>HFF</td>
<td>X</td>
<td>Foreskin (primary)</td>
<td>Fibroblast</td>
</tr>
<tr>
<td>HFF-Tert</td>
<td>X</td>
<td>Foreskin (primary, telomerase extended)</td>
<td>Fibroblast</td>
</tr>
</tbody>
</table>

**Table 1. Cells Used in This Study.** Asterisks indicate cells of African Green Monkey origin. An [x] in the lethal column indicates that d1-2 in this cell line/primary cell sample, while [-] indicates that it does. Morphology and tissue information was obtained from the American Type Culture Collection (ATCC) website for each of the cell lines obtained from ATCC, while morphology and tissue information for primary cell samples was provided by the respective donors of the cells.
REFERENCES


62. **Corbin-Lickfett KA, Souki SK, Cocco MJ, Sandri-Goldin RM.** 2010. Three arginine residues within the RGG box are crucial for ICP27 binding to herpes simplex virus 1 GC-rich sequences and for efficient viral RNA export. J. Virol. 84:6367–76.


68. **Hernandez F.** 2010. Head-to-tail intramolecular interaction of herpes simplex virus type 1 regulatory protein ICP27 is important for its interaction with cellular mRNA export receptor TAP/NXF1. MBio 1:1–9.


71. **Soliman T.** 1997. Shuttling of the herpes simplex virus type 1 regulatory protein ICP27 between the nucleus and cytoplasm mediates the expression of late proteins. J. Virol. **71:**9188–9197.


84. **Luse DS.** 2014. The RNA polymerase II preinitiation complex. Transcription 5:e27050.

85. **Dai-Ju J, Li L, Johnson LA, Sandri-Goldin RM.** 2006. ICP27 interacts with the C-terminal domain of RNA polymerase II and facilitates its recruitment to herpes simplex virus 1 transcription sites, where it undergoes proteasomal degradation during infection. J. Virol. 80:3567–3581.


