ICP0 Antagonizes ICP4-Dependent Silencing of the Herpes Simplex Virus ICP0 Gene

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Abstract

ICP0 is a regulatory protein that plays a critical role in the replication-latency balance of herpes simplex virus (HSV). Absence of ICP0 renders HSV prone to establish quiescent infections, and thus cellular repressor(s) are believed to silence HSV mRNA synthesis when ICP0 fails to accumulate. To date, an ICP0-antagonized repressor has not been identified that restricts HSV mRNA synthesis by more than 2-fold. We report the unexpected discovery that HSV’s major transcriptional regulator, ICP4, meets the criteria of a bona fide ICP0-antagonized repressor of viral mRNA synthesis. Our study began when we noted a repressive activity that restricted ICP0 mRNA synthesis by up to 30-fold in the absence of ICP0. When ICP0 accumulated, the repressor only restricted ICP0 mRNA synthesis by 3-fold. ICP4 proved to be necessary and sufficient to repress ICP0 mRNA synthesis, and did so in an ICP4-binding-site-dependent manner. ICP4 co-immunoprecipitated with FLAG-tagged ICP0; thus, a physical interaction likely explains how ICP0 antagonizes ICP4’s capacity to silence the ICP0 gene. These findings suggest that ICP0 mRNA synthesis is differentially regulated in HSV-infected cells by the virus-encoded repressor activity embedded in ICP4, and a virus-encoded antirepressor, ICP0. Bacteriophage λ relies on a similar repression-antirepression regulatory scheme to “decide” whether a given infection will be productive or silent. Therefore, our findings appear to add to the growing list of inexplicable similarities that point to a common evolutionary ancestry between the herpesviruses and tailed bacteriophages.

Introduction

During productive replication, ~75 proteins are synthesized from the herpes simplex virus (HSV) genome in a temporal cascade [1]. Virion protein 16 (VP16) in the tegument of HSV virions forms a complex with the cellular transcription factor Oct 1 to initiate a cascade of viral gene expression [2,3]. Only five immediate-early (IE) genes are initially induced based on the presence of VP16-responsive elements in their promoters [4]. Viral IE proteins such as infected cell proteins 0 (ICP0) and 4 (ICP4) are believed to play a key role in activating viral mRNA synthesis, and thus promoting the synthesis of ~70 early (E) and late (L) proteins that replicate and package HSV genomes into new virions.

ICP0 was first identified based on its capacity to transform HSV’s major transcriptional regulator, ICP4, from a weak transcriptional activator to a potent activator of mRNA synthesis; specifically, combinations of ICP0 and ICP4 are 20-fold more potent at driving mRNA synthesis than either ICP0 or ICP4 alone [5,6]. Functionally, synthesis of ICP0 causes HSV’s equilibrium to abruptly tip towards productive replication, whereas absence of ICP0 produces the opposite effect. Synthesis of ICP0 is sufficient to trigger HSV reactivation in trigeminal ganglion neurons and other models of latent HSV infection [7,8]. HSV ICP0− viruses replicate to nearly wild-type levels when cells are inoculated with more than 1 plaque-forming unit (pfu) of virus per cell. At multiplicities of infection (MOI) below 0.1 pfu per cell, the same ICP0− viruses establish quiescent infections in 99% of the cells they infect [9,10]. Such observations suggest that ICP0 antagonizes a repressor of HSV replication, whose repressive capacity can be saturated with high numbers of HSV genomes [10].

Preston and Everett first articulated the concept of an ICP0-antagonized repressor of HSV gene expression [11,12,13]. Because ICP0 is an E3 ubiquitin ligase [14], it was suggested that a repressor might silence HSV mRNA synthesis in the absence of ICP0, whereas synthesis of ICP0 would result in ubiquitination and/or proteasomal destruction of the repressor, thus allowing HSV mRNA synthesis to proceed unhindered [15]. Since the advancement of this hypothesis, many laboratories have joined in the search for the unidentified repressor(s) of HSV gene expression. Individual cellular proteins that co-localize with ICP0 have been probed for their capacity to function as repressors of HSV replication such as PML and Sp100 [16,17].Suspected pathways of silencing HSV genomes, such as epigenetic silencing, have been explored to determine if HSV ICP0− null viruses are more sensitive to these gene silencing mechanisms [18,19,20,21]. Based on such inquiries, the list of proteins that may function as ICP0-antagonized repressors of HSV gene expression continues to grow and currently includes PML [16,17,18,22], Sp100 [23], cyclin D3 [24], IRF-3 and IRF-7

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ICP0 protein (GFP) coding sequence and a stop codon in exon 2 of the ICP0 gene. In addition, we present the first characterization of HSV-1 viruses that contain a GFP reporter gene in HSV-infected cells such as presence or absence of biologically active ICP0.

Like many laboratories, we too have been interested in understanding why failure to synthesize ICP0 results in limited mRNA transcription from the HSV genome. Rather than interrogate specific proteins for their capacity to repress viral mRNA synthesis, we chose to establish an experimental system that would allow us to rapidly monitor repression of a single HSV IE gene, the ICP0 gene.

To this end, an ICP0+ virus was constructed that bore an ~750 bp insertion of green fluorescent protein (GFP) coding sequence and a stop codon in exon 2 of the ICP0 gene. The resulting virus, HSV-1 0−GFP, synthesized a 3.5 kb ICP0−GFP mRNA and a truncated ICP0−GFP peptide. Using the GFP fluorescent reporter as a screening tool, we probed for conditions that alleviated or exacerbated repression of the ICP0−GFP reporter gene in HSV-infected cells such as presence or absence of biologically active ICP0.

With the aid of these new reagents, we report the identification of a protein that satisfied four empirical criteria that should be expected of an ICP0-antagonized repressor of HSV mRNA transcription: specifically, the identified protein 1. was required to observe repression of ICP0 mRNA synthesis in HSV-infected cells; 2. was sufficient to repress ICP0 mRNA synthesis in the absence of ICP0; 3. was unable to silence ICP0 mRNA synthesis when ICP0 accumulated; and 4. physically interacted with ICP0.

We report the unexpected finding that the viral IE protein, ICP4, satisfied all of the criteria expected of a bona fide ICP0-antagonized repressor of HSV mRNA transcription. It is relevant to note that ICP4’s capacity to function as a repressor of HSV IE mRNA transcription is well established, particularly in the context of the ICP4 and I/ST genes [34,35]. Evidence has been presented both for and against the hypothesis that ICP4 represses the ICP0 gene in HSV-infected cells [36,37]. However, the perceived importance of the hypothesis may be measured in terms of the attention it has received; 14 years have elapsed since the last study was published that considered ICP4’s potential to repress the ICP0 gene [37].

We present new evidence that corroborates earlier findings that ICP4’s capacity to repress ICP0 mRNA synthesis is indeed modest when ICP0 accumulates. However, when ICP0 fails to accumulate, then ICP4 is capable of silencing the ICP0 gene. We present functional evidence that ICP0 antagonizes ICP4-dependent repression of the ICP0 gene. In addition, we present the first direct evidence that ICP0 and ICP4 physically interact in HSV-infected cells. Collectively, these data suggest an alternative view of HSV gene regulation, which is consistent with the biology of a virus that establishes latent infections: In the absence of ICP0, ICP4 functions as an efficient repressor of HSV IE mRNA transcription, whereas accumulation of ICP4’s binding partner, ICP0, converts ICP4 from a repressor to an activator of HSV mRNAs synthesis [5,6]. The findings that led us to these unexpected conclusions are presented below.

Results

Characterization of HSV-1 Viruses That Contain a GFP Reporter in ICP0− or ICP0+ Genes

HSV-1 recombinant viruses carrying a GFP coding sequence in each copy of the ICP0 gene were constructed for the purpose of studying how the ICP0 gene is regulated. An ICP0−GFP gene was constructed that encoded an ICP0 protein that bore a GFP insertion between amino acids 104 and 105 (Fig. 1A). An ICP0-null control gene, the ICP0−GFP gene, encoded the N-terminal 104 amino acids of ICP0 fused to GFP (Fig. 1A). The chimeric ICP0−GFP+ or ICP0−GFP− genes were introduced into the LAT-ICP0 locus of HSV-1 strain KOS by homologous recombination to yield the HSV-1 viruses 0−GFP and 0−GFP, respectively (Fig. 1B, Table 1).

The ICP0−GFP or ICP0−GFP reporter proteins encoded by HSV-1 0−GFP and 0−GFP were analyzed by two-color Western blot analysis. In cells infected with wild-type HSV-1 KOS or KOS-GFP, ICP0-specific monoclonal antibody 11060 labeled the 110 kDa protein species that is ICP0, as well as two protein species of ~40 and 55 kDa (red bands, Fig. 1C). In addition, the GFP-expressing recombinant virus, KOS-GFP [30], encoded an ~30 kDa GFP protein that was labeled by rabbit anti-GFP antibody (green band in Fig. 1C). As expected, HSV-1 0−GFP encoded a single ~53 kDa protein that was labeled by ICP0- and GFP-specific antibodies, whereas 0−GFP encoded an 140 kDa protein that was labeled with both ICP0- and GFP-specific antibodies (yellow bands in Fig. 1C).

The functionality of the ICP0−GFP protein was compared to wild-type ICP0. Wild-type HSV-1 strain KOS formed plaques with 100% efficiency in Vero cells relative to the number of plaques that formed in ICP0-complementing L7 cells (Fig. 1D). In contrast, only ~1% of HSV-1 0−GFP (ICP0−) viruses formed plaques in Vero cells (Fig. 1D). HSV-1 0−GFP formed plaques in Vero cells at 66% efficiency relative to the number of plaques that formed in ICP0-complementing L7 cells (Fig. 1D). Thus, the ICP0−GFP protein retained sufficient activity to promote HSV-1 0−GFP plaque formation with 45-fold greater efficiency than its ICP0−null control virus, HSV-1 0−GFP.

Inefficient ICP0−GFP mRNA Synthesis from HSV-1 0−GFP in the Absence of ICP0

ICP0 has been described as a promiscuous transcriptional activator [11,19,39]. We reasoned that ICP0 should act in a positive-feedback loop to promote mRNA synthesis from its own gene, the ICP0 gene. To test this inference, an ICP0-expressing adenovirus vector, Ad-ICP0, was compared to a control vector, Ad-n212 (Table 2), for its capacity to stimulate the expression of the ICP0−GFP reporter gene embedded in the native LAT-ICP0 locus of HSV-1 0−GFP.

In an initial test, cells were pre-treated with vehicle, Ad-n212, or Ad-ICP0, and were inoculated 12 hours later with 2.5 pfu per cell of HSV-1 0−GFP. When compared at 12 hours post-inoculation (p.i.), HSV-1 0−GFP expressed barely detectable levels of ICP0−GFP fluorescence in cells pre-treated with vehicle or Ad-n212 (Fig. 2A). In contrast, pre-treatment with Ad-ICP0 allowed HSV-1 0−GFP to express high levels of ICP0−GFP fluorescence (Fig. 2A).
Northern blot analysis was conducted to determine if adenovirus-encoded ICP0 stimulated ICP0-GFP mRNA accumulation in HSV-1 0-GFP-infected cells. Vero cells were inoculated with 0 to 10 pfu per cell of Ad-ICP0, and were inoculated 12 hours later with 2.5 pfu per cell of HSV-1 0-GFP. Northern blot analysis with a GFP-specific probe demonstrated that Ad-ICP0 stimulated a 5-fold increase in ICP0-GFP mRNA levels and did so in a dose-dependent manner (Fig. 2B, D). In contrast, Ad-n212 had no effect on ICP0-GFP mRNA levels (Fig. 2C, D). Therefore, exogenous ICP0 stimulated the accumulation of ICP0-GFP mRNA in cells inoculated with HSV-1 0-GFP.

Efficient ICP0-GFP mRNA Synthesis from HSV-1 0-GFP when Protein Synthesis Is Blocked

We were interested to determine how ICP0 influences the activity of the ICP0-GFP gene in cells inoculated with HSV-1 0-GFP. One possibility was that ICP0 induced mRNA synthesis from all of HSV-1 0-GFP's genes, including the ICP0-GFP gene (Fig. 3A). A second possibility was that absence of ICP0 allowed a repressor such as PML [17] or HDAC1/2-CoREST-REST [19] to silence all of HSV-1 0-GFP's genes including the ICP0-GFP gene (Fig. 3B, red line). Under this latter hypothesis, ICP0 might stimulate ICP0-GFP mRNA synthesis by antagonizing the repressor of the ICP0-GFP gene (Fig. 3B, green line). An experiment was conducted to evaluate the relative likelihood of these two possibilities.

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

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

As expected, cells pre-treated with Ad-ICP0 expressed high levels of ICP0-GFP mRNA despite the cycloheximide block to the synthesis of other HSV proteins (Fig. 3F, H). However, to our surprise, a similar result was observed in Ad-n212-treated cells treated with cycloheximide (Fig. 3G, H). Thus, when protein synthesis was blocked in HSV-infected cells, ICP0 was no longer required for the efficient synthesis of ICP0-GFP mRNA (Fig. 3G). These findings argued against the hypothesis that ICP0 was required to actively induce ICP0-GFP mRNA synthesis (Fig. 3A).

Rather, the results suggested that ICP0 antagonized a repressor of the ICP0-GFP gene that was synthesized de novo in HSV-infected cells (Fig. 3B, green line). Further tests were conducted to explore the validity of this hypothesis.
Testing the Predictions of a Repression-Antirepression Model of ICP0 Gene Regulation

Three predictions followed from an hypothesis that HSV-1 ICP0 gene activity was regulated by a process of repression and antirepression: i. HSV-1 ICP0 - and ICP0+ viruses should express high and equivalent levels of ICP0 mRNA when the repressor accumulated in HSV-infected cells (Fig. 3B, blue line); ii. an HSV-1 ICP0- virus should efficiently express ICP0 mRNA when the repressor accumulated in HSV-infected cells (Fig. 3B, green line); and iii. an HSV-1 ICP0- virus should exhibit little to no ICP0 mRNA synthesis when the repressor accumulated in the absence of ICP0 (Fig. 3B, red line).

As an initial test of these predictions, the relative expression of ICP0-GFP or ICP0+GFP fluorescent reporter proteins was compared in cells inoculated with 2.5 pfu per cell of HSV-1 0+GFP (ICP0+) versus HSV-1 0-GFP (ICP0-). In vehicle-treated cells, HSV-1 0-GFP expressed barely detectable levels of ICP0-GFP fluorescence, whereas HSV-1 0+GFP expressed ICP0+GFP fluorescence to readily detectable levels (Fig. 4A). In contrast, when protein synthesis was inhibited for the first 6 hours of infection with cycloheximide, then both ICP0-GFP and ICP0+GFP fluorescent proteins accumulated to high levels by 6 hours post-release from the cycloheximide block (Fig. 4B). Quantification by flow cytometry [40] demonstrated that ICP0-GFP and ICP0+GFP fluorescent proteins accumulated to high and equivalent levels following release from a cycloheximide block (Fig. S1).

The relative expression of ICP0-GFP or ICP0+GFP mRNA was compared in cells inoculated with HSV-1 0-GFP versus 0+GFP. When protein translation was allowed to occur, HSV-1 0-GFP produced levels of ICP0-GFP mRNA that were barely detectable between 3 and 12 hours p.i. (Fig. 4C). When cycloheximide was used to block protein synthesis, ICP0-GFP mRNA levels accumulated to levels that were ~90 times background in HSV-1 0-GFP-infected cells (Fig. 4C, E). In contrast, the onset of protein translation did not prevent an ICP0+ virus, HSV-1 0+GFP, from expressing ICP0+GFP mRNA to levels that were ~20 times background (Fig. 4D, E). When protein synthesis was inhibited with cycloheximide, HSV-1 0+GFP expressed ~5-fold higher levels of ICP0+GFP mRNA (Fig. 4D, E). As predicted, cycloheximide treatment allowed HSV-1 0-GFP and 0+GFP to express high and equivalent levels of ICP0+GFP mRNA (Fig. 4E). Control experiments verified that cycloheximide blocked HSV-1 L mRNA synthesis (i.e., glycoprotein D mRNA), but had only a negligible effect on cellular GAPDH mRNA levels (Fig. S2).

These findings were consistent with the predictions of a repression-antirepression model of ICP0 gene regulation (Fig. 3B). However, questions remained about the identity of the de novo repressor that inhibited ICP0 mRNA synthesis in HSV-1 0-GFP-infected cells. Alternatively, it was possible that cycloheximide stimulated synthesis of all HSV mRNAs including ICP0+GFP mRNA in a non-specific manner [41]. Further experiments were conducted to differentiate between these possibilities.

### Table 1. Description of HSV-1 viruses.

| Virus/Genotype | Phenotype/Properties | Reference |
|----------------|----------------------|-----------|
| KOS (wild-type)| Wild-type laboratory strain of HSV-1 (12 passages removed from isolation). | [84] |
| n122 (ICP0 null)| Replicates with 1% efficiency at MOIs below 0.1 pfu per cell; virus synthesizes the first 211 of 775 amino acids of ICP0. | [43] |
| 0+GFP (ICP0+ null)| Replicates with 1% efficiency at MOIs below 0.1 pfu per cell; virus synthesizes the first 104 amino acids of ICP0 coupled to GFP. This study |
| 0-4BS- (ICP0- null)| Replicates with 66% efficiency at MOIs below 0.1 pfu per cell; virus synthesizes full-length ICP0 with GFP inserted between amino acids 104 and 105. | This study |
| 0-4BS+ (ICP0+) Replicates with 66% efficiency at MOIs below 0.1 pfu per cell; virus synthesizes full-length ICP0 with GFP inserted between amino acids 104 and 105. | This study |
| 0-FLAG (ICP0) Replicates with 100% efficiency at MOIs below 0.1 pfu per cell; virus synthesizes full-length ICP0 with FLAG epitope inserted between amino acids 23 and 24. | This study |
| RP4 (VP16+ null)| Replicates with 1% efficiency at MOIs <0.1 pfu per cell; virus synthesizes VP16 protein lacking half of the acidic transactivation domain (Δaa. 413–454 (of 490)). | [44] |
| n12 (ICP4 null)| Incapable of replication unless ICP4 is provided in trans; virus synthesizes the first 252 of 1298 amino acids of ICP4. | [50] |
| tsB21 (ICP4+) Replication-competent at 34°C; replication-defective at 39.5°C; virus synthesizes a temperature-sensitive ICP4 protein (lesion maps to the C-terminal third). | [47] |
| d27-1 (ICP27 null)| Incapable of replication unless ICP27 is provided in trans; virus bears a 1.6 kb deletion that removes 80% of the ICP27 ORF. | [48] |
| 22/m199 (ICP22- null)| Virus replicates like wild-type in cell culture but fails to replicate in animals; virus synthesizes the first 198 of 420 amino acids of ICP22. | [49] |

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### Table 2. Description of ΔE1–E3 adenovirus vectors.

| Virus | Promoter/Gene product | Reference |
|-------|-----------------------|-----------|
| Ad-null | TRE promoter/no gene product | [7] |
| Ad-ICP0 | TRE promoter/wild-type ICP0 (775 amino acids) | [7] |
| Ad-n212 | TRE promoter/N-terminus of ICP0 (211 of 775 amino acids) | [7] |
| Ad-ICP4 | TRE promoter/wild-type ICP4 (1298 amino acids) | [7] |
| Ad.CMV-TetOn | CMV promoter/TetOn (rTA) protein | [7] |

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Cycloheximide Stimulates HSV ICP0 Gene Expression in a VP16-Dependent Manner

VP16 carried in HSV virions binds six VP16-responsive elements in the ICP0 promoter at the outset of infection (Fig. 5A), and thus VP16 induces ICP0 mRNA synthesis [2,42]. An experiment was conducted to determine if cycloheximide’s effect on steady-state ICP0 mRNA levels was the result of a VP16-dependent process, or rather was due to a myriad of non-specific effects that should operate with equal efficiency in the presence or absence of VP16.

ICP0 mRNA accumulation was compared in cells inoculated with 2.5 pfu per cell of wild-type HSV-1 KOS, the ICP0− virus n212 [43], or the VP16+ virus RP4 [44] (Table 1). When protein synthesis was allowed to occur, wild-type HSV-1 expressed ICP0 mRNA to levels that were 28-fold above background, whereas an ICP0− virus and VP16+ virus expressed ICP0 mRNA to levels that were only 4-fold above background (Fig. 5B, C). When protein synthesis was blocked with cycloheximide, the VP16− viruses exhibited a robust increase in ICP0 mRNA levels; consequently, wild-type HSV and the ICP0− virus expressed high and equivalent levels of ICP0 mRNA in cycloheximide-treated cells (Fig. 5B, C). In contrast, the VP16− virus failed to efficiently express ICP0 mRNA in either vehicle- or cycloheximide-treated cells (Fig. 5C). Thus, cycloheximide was incapable of stimulating ICP0 mRNA accumulation in the absence of VP16. These data argued against the possibilities that cycloheximide might increase ICP0 mRNA abundance by (1) decreasing the rate of ICP0 mRNA turnover, or by (2) stimulating a non-specific increase in the rate of transcription of all genes, including the ICP0 gene. Rather, the data were consistent with an hypothesis that cycloheximide blocked the formation of a de novo repressor of the ICP0 gene that was dominant over the HSV-specific inducer of the ICP0 gene, VP16.

De Novo Repression of HSV ICP0 Gene Expression Is ICP4-Dependent

There is evidence that inhibition of protein synthesis causes all of HSV’s IE mRNAs to be overexpressed [45]. One interpretation that has been offered is that cycloheximide’s effect on HSV mRNA synthesis is global and non-specific [41]. However, there is a second, biologically important hypothesis that has not been adequately considered. As well as HSV ICP4’s function as an essential activator of E and L mRNA transcription [46], ICP4 may function as a repressor of IE mRNA transcription [47]. To determine if ICP4 contributed to de novo repression of the ICP0 gene, ICP0 mRNA accumulation was compared in cells inoculated with HSV-1 mutants deficient in the IE regulatory proteins ICP0, ICP27, ICP22, or ICP4 (Table 1).

Regardless of their different genotypes, 2.5 pfu per cell of each HSV-1 VP16− virus yielded high and equivalent levels of ICP0 mRNA in cycloheximide-treated cultures (Fig. 6A). The ICP0− virus, n212, expressed very low levels of ICP0 mRNA when protein translation was allowed to occur, whereas each of the ICP0+ viruses expressed ICP0 mRNA to levels that were readily detectable in vehicle-treated cells (Fig. 6A). Wild-type HSV-1 KOS expressed 3.5±0.4-fold lower levels of ICP0 mRNA in vehicle-treated cells relative to cycloheximide-treated controls (Fig. 6A, B). Likewise, the ICP27+ and ICP22+ viruses, d27-1 [48] and 22/n199 [49], expressed 4- to 5-fold less ICP0 mRNA in vehicle-treated cells relative to cycloheximide-treated controls (Fig. 6A, B). These results suggested that the de novo repressor of ICP0 mRNA synthesis formed efficiently in the absence of ICP22, ICP27, or the ~70 HSV E and L proteins whose synthesis was ICP27-dependent [48]. In contrast, an ICP4− virus, n12 [50],...
expressed high and equivalent levels of ICP0 mRNA in vehicle- and cycloheximide-treated cells (Fig. 6A, B). Therefore, de novo repression of ICP0 mRNA accumulation appeared to be dependent upon the synthesis of ICP4.

An independent test was conducted to corroborate this interpretation. Vero cells were inoculated with 2.5 pfu per cell of HSV-1 KOS (ICP4+), HSV-1 tsB21 (ICP4ts), which bears a temperature-sensitive lesion in the C-terminus of ICP4; this mutation renders ICP4 non-functional at a temperature of 39.5°C [47]. At 34.0°C, 37.0°C, and 39.5°C, wild-type HSV-1 KOS expressed ~3-fold less ICP0 mRNA in vehicle-treated cells relative to cycloheximide-treated controls (Fig. 6C, D).

Figure 3. ICP0 de-represses HSV ICP0-GFP gene expression. (A and B) Alternative models by which ICP0 may stimulate ICP0-GFP gene activity. (A) An ICP0 induction model predicts that ICP0 will be required to induce the ICP0-GFP gene to its full activity (green line), whereas efficient ICP0-GFP gene expression will not occur in the absence of ICP0 (blue line). (B) An ICP0 antirepression model predicts that i. a repressor will silence the ICP0-GFP gene when ICP0 fails to accumulate (red line), ii. ICP0 prevents the repressor from silencing the ICP0-GFP gene (green line), and iii. ICP0 is not required for efficient ICP0-GFP gene expression when the repressor is absent (blue line). (C, F) Northern blots of ICP0-GFP mRNA isolated from Vero cells inoculated with MOIs of 0.1 to 2 pfu per cell of HSV-1 0-GFP (RNA harvested 12 hours p.i.; 10 μg per lane). Vero cells were pre-treated with 10 pfu per cell of (C, F) Ad-ICP0 or (D, G) Ad-n212 whose TRE promoters were induced with 20 pfu per cell of Ad.CMV-TetOn and 10 μM doxycycline. Cells were treated with (C, D) vehicle or (F, G) 200 μM cycloheximide at the time of HSV-1 0-GFP inoculation. (E, H) ICP0-GFP mRNA levels are plotted as a function of the MOI of HSV-1 0-GFP used to inoculate Vero cells treated with (E) vehicle or (H) cycloheximide.

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Likewise, HSV-1 tsB21 expressed 2.9- and 2.3-fold less ICP0 mRNA in vehicle-treated cells at 34.0°C and 37.0°C, respectively, relative to cycloheximide-treated controls (Fig. 6C, D). In contrast, at the non-permissive temperature of 39.5°C, HSV-1 tsB21 expressed equivalent levels of ICP0 mRNA in both vehicle- and cycloheximide-treated cells (Fig. 6C, D). Collectively these results indicated that de novo repression of ICP0 mRNA accumulation was dependent upon the synthesis of a biologically active form of HSV’s major transcriptional regulator, ICP4.

Figure 4. De novo repression of HSV ICP0GFP mRNA accumulation is antagonized by a GFP-tagged ICP0 protein. (A and B) Photomicrographs of ICP0GFP and ICP0+GFP reporter protein fluorescence in Vero cells 12 hours after inoculation with 2.5 pfu per cell of HSV-1 0′GFP or 0′GFP (20× magnification). Cells were treated with either (A) medium containing no drug (vehicle) from 0 to 12 hours p.i., or (B) 200 µM cycloheximide from −0.5 to 6 hours p.i. followed by vehicle from 6 to 12 hours p.i. (C and D) Representative Northern blots of (C) ICP0GFP mRNA or (D) ICP0+GFP mRNA harvested between 3 and 12 hours after inoculation with 2.5 pfu per cell of HSV-1 0′GFP or 0′GFP, respectively. Cells were treated with vehicle or 200 µM cycloheximide until the indicated time of RNA harvest (10 µg per lane). (E) Mean±sd of ICP0GFP and ICP0+GFP mRNA levels plotted as a function of time of RNA harvest (n=2 per time point per group).

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Figure 5. Cycloheximide increases ICP0 gene expression in a VP16-dependent manner. (A) Schematic of ICP0 promoter, which indicates the positions of six distal VP16-responsive elements (VREs), a proximal ICP4-binding site (4BS), and the TATA-box of the ICP0 gene. (B) Representative Northern blot of ICP0 mRNA in Vero cells that were uninfected (UI) or were inoculated with 2.5 pfu per cell of HSV-1 KOS, n212 (ICP0′), or RP4 (VP16′), and which were treated with vehicle or 200 µM cycloheximide from −0.5 to 12 hours p.i., at which time total RNA was harvested (10 µg per lane). (C) Mean ± sem of ICP0 mRNA levels in KOS, n212, or RP4-infected cells (n=3 per group). Asterisks denote significant differences between vehicle- and cycloheximide-treated cells inoculated with the same virus (p<0.01, two-tailed Student’s t-test).

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De Novo Repression of HSV ICP0\textsuperscript{GFP} Reporter Genes Is ICP4-Binding-Site-Dependent

A consensus ICP4-binding site (ATCGTC) occurs 40-bp upstream of the TATA box in the ICP0 promoter [37,51]. To determine if ICP4 binding to the ICP0 promoter contributed to de novo repression of ICP0\textsuperscript{GFP} mRNA synthesis, the recombinant viruses HSV-1 \textsuperscript{0\textsuperscript{4BS}} and \textsuperscript{0\textsuperscript{4BS}} were constructed which were equivalent to HSV-1 \textsuperscript{0\textsuperscript{GFP}} and \textsuperscript{0\textsuperscript{GFP}}, respectively, but which bore a 4-bp deletion in the ICP0-binding site in the ICP0 promoter (Fig. 7A).

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

To corroborate this interpretation, accumulation of the 3.5 kb ICP0\textsuperscript{GFP} RNA species was compared in cells inoculated with 5 pfu per cell of HSV-1 \textsuperscript{0\textsuperscript{GFP}}, \textsuperscript{0\textsuperscript{4BS}}, \textsuperscript{0\textsuperscript{4BS}}, or \textsuperscript{0\textsuperscript{4BS}} (Fig. 7D). Each virus yielded high levels of ICP0\textsuperscript{GFP} mRNA in cycloheximide-treated cultures (Fig. 7D). HSV-1 \textsuperscript{0\textsuperscript{4BS}} exhibited a 132-fold repression of ICP0\textsuperscript{GFP} mRNA synthesis in vehicle-treated cells relative to cycloheximide-treated controls (Fig. 7D, 7E). Disruption of the ICP4-binding site in the ICP0 promoter alleviated de novo repression by 4-fold, and thus HSV-1 \textsuperscript{0\textsuperscript{4BS}} exhibited only a 3.3-0.5-fold reduction in ICP0\textsuperscript{GFP} mRNA in vehicle-treated cells (Fig. 7D, 7E). Disruption of the ICP4-binding site in the ICP0\textsuperscript{GFP} gene increased the efficiency of ICP0\textsuperscript{GFP} mRNA synthesis to the same extent as synthesis of a biologically active ICP0 protein; thus, HSV-1 \textsuperscript{0\textsuperscript{4BS}} and \textsuperscript{0\textsuperscript{4BS}} both exhibited a 3.3-fold reduction in ICP0\textsuperscript{GFP} mRNA levels in vehicle-treated cells relative to their respective cycloheximide-treated controls (Fig. 7D, 7E). Disruption of the ICP4-binding site in the ICP0 promoter of HSV-1 \textsuperscript{0\textsuperscript{4BS}} resulted in a significant de-repression; thus ICP0\textsuperscript{GFP} mRNA synthesis was 2.1-0.2-fold lower than cycloheximide-treated controls (Fig. 7D, 7E). Collectively, these observations indicated that the magnitude of de novo repression of ICP0\textsuperscript{GFP} mRNA synthesis was significantly dependent upon the ICP4-binding site proximal to the transcriptional start site of the ICP0 gene (Fig. 7A).

Adenovirus-Encoded ICP4 Substitutes for the De Novo Repressor of the HSV ICP0\textsuperscript{GFP} Gene

Our results raised the possibility that the de novo repressor of ICP0 mRNA synthesis was not a cellular protein, but rather was the HSV ICP4 protein because 1. loss of ICP4 function de-repressed ICP0 mRNA synthesis to the same extent as cycloheximide treatment (Fig. 6), and 2. a 4-bp deletion that removed a single ICP4-binding site significantly alleviated de novo repression of ICP0 mRNA synthesis (Fig. 7). Based on this hypothesis, it was predicted that pre-treatment of Vero cells with an ICP4-expressing adenovirus should negate cycloheximide's
ICP0 Physically Interacts with the Repressor of Its Gene, ICP4, in HSV-Infected Cells

A physical interaction between ICP0 and ICP4 might begin to explain how ICP0 influences ICP4’s activity as a transcriptional regulator. The available evidence from far-Western blotting [32] and co-localization studies [53] suggested that ICP0 may physically interact with ICP4. However, direct evidence was lacking to address whether or not ICP0 and ICP4 physically interact in the context of HSV-infected cells.

To address this gap in knowledge, a recombinant virus was constructed that bore a FLAG epitope between amino acids 23 and 24 of ICP0, and which is referred to as HSV-1 0′FLAG24 (Fig. 9A). Southern blot analysis validated that the FLAG coding sequence was inserted in both copies of the ICP0 gene, and that HSV-1 0′FLAG24 grew with wild-type efficiency (not shown). Immunofluorescent staining verified that HSV-1 0′FLAG24 expressed a FLAG-tagged protein that exhibited a nuclear-staining pattern typical of ICP0 during the IE phase of HSV infection (Fig. 9B; 10 hours in cycloheximide).

Immunoprecipitation experiments were performed to determine if ICP4 physically interacted with FLAG-tagged ICP0. Vero cells were inoculated with vehicle or 5 pfu per cell of HSV-1 KOS or 0′FLAG24 in the presence of cycloheximide (0–10 hours p.i.) followed by actinomycin D (10–16 hours p.i.), such that only viral IE proteins were efficiently synthesized. Input levels of ICP4 and ICP0 in each immunoprecipitation reaction were equivalent, as verified by Western blot analysis with monoclonal antibodies 58S and 110B3, respectively (Fig. 9C, top panel). The remainder of each protein sample was immunoprecipitated with an anti-FLAG antibody-agarose conjugate under conditions of medium or high stringency, and immunoprecipitates were analyzed for the presence of ICP4.

Figure 7. De novo repression of ICP0GFP gene expression is ICP4-binding-site-dependent. (A) Schematic of the ICP4 DNA-binding site (4BS) in HSV-1 0′ GFP and 0′ GFP versus the mutated 4BS-element in HSV-1 0′ 4BS- and 0′ 4BS- . All four viruses transcribe 3.5 kb ICP0GFP mRNAs, but HSV-1 0′ 4BS- and 0′ 4BS- are deleted of bases −43 to −40 relative to the TATA box of the ICP0GFP gene, which disrupts the consensual ICP4-binding site, 5′-ATCGTC-3′. (B and C) Photomicrographs of ICP0GFP and ICP0GFP fluorescent protein accumulation in Vero cells 12 hours after inoculation with 5 pfu per cell of (B) HSV-1 0′ GFP versus 0′ GFP or (C) HSV-1 0′ GFP versus 0′ 4BS- (20× magnification). (D) Representative Northern blots of ICP0GFP mRNA accumulation in Vero cells that were uninfected (UI) or were inoculated with 5 pfu per cell of HSV-1 0′ GFP (ICP0 ), 0′ 4BS- (ICP0 ), 0′ GFP (ICP0 ), or 0′ 4BS- (ICP0 ), and which were treated with vehicle or 200 μM cycloheximide from −0.5 to 12 hours p.i., at which time total RNA was harvested (10 μg per lane). (E) Mean ± sem fold-reduction in ICP0GFP mRNA levels in vehicle-treated cells relative to cycloheximide-treated controls (fold-reduction = mRNA GFP mRNA levels between the ICP0 viruses, HSV-1 0′ GFP versus 0′ 4BS− (p<0.001), and between the ICP0 viruses, HSV-1 0′ GFP versus 0′ 4BS− (p<0.01; two-tailed Student’s t-test). doi:10.1371/journal.pone.0008837.g007
Discussion

Identification of an ICP0-Antagonized Repressor of HSV mRNA Synthesis

HSV routinely establishes latent infections in its human host. During the latent phase of infection, most of the HSV genome is transcriptionally silent. It is unclear how the HSV genome is silenced, but it is generally agreed that synthesis of ICP0 destabilizes repression and thus triggers reactivation of mRNA synthesis from latent HSV genomes [7,8,54].

The search for an ICP0-antagonized transcriptional repressor has led investigators to consider the possibility that PML and Sp100 [17], IRF-3 and IRF-7 [25], and centromere proteins CENP-B and CENP-C [26,27] may represent the repressors of the HSV genome that are antagonized by ICP0. Although ICP0 may alter the subcellular distribution, stability, or function of these proteins, there is no direct evidence that these proteins are capable of restricting mRNA synthesis from the HSV genome. Likewise, it has been postulated that epigenetic silencing may be the ICP0-antagonized repressive mechanism that is capable of silencing the HSV genome [20,55]. However, no specific histone deacetylase or histone mark has been associated with more than a 2-fold reduction in HSV mRNA synthesis [18,19,56,57].

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

The results clarify that ICP4 is capable of silencing mRNA synthesis from the ICP0 gene when ICP0 fails to accumulate. At low MOIs, HSV ICP0+ viruses establish quiescent infections in 99% of infected cells [9,10]. Intriguingly, ICP4 accumulates to detectable levels in >85% of cells that become quiescently infected with HSV ICP0- viruses [58]. Thus, further studies will be required to determine if ICP4 routinely accumulates in the absence of ICP0 in HSV quiescently infected cells, and if so, to determine if ICP4 plays a causal role in restricting mRNA synthesis from quiescent HSV genomes [59,60,61].

Differential Regulation of ICP0 mRNA Synthesis in HSV-Infected Cells

There is anecdotal evidence that the ICP0 gene is differentially regulated in HSV-infected cells, but it remains unclear how such regulation is achieved. The HSV latency-associated transcripts (LATs) have been discussed for their potential to repress ICP0 gene expression [62,63]. Likewise, VP16’s capacity to function as a transactivator of IE genes has been considered as a potential target of differential regulation [64,65,66]. However, it remains unclear that LATs or VP16 are sufficient to explain how the ICP0 gene is regulated.

The ICP4-binding site in the ICP0 promoter was identified 20 years ago as a potential means by which ICP4 might regulate ICP0 mRNA synthesis [51]. When studied in the context of HSV ICP0+ viruses, some investigators concluded that ICP4’s effect on ICP0 gene expression was negligible [36]. Other investigators noted that ICP4 produced a modest decrease in ICP0 mRNA levels in the context of ICP0+ viruses [37], but ICP4’s full potential to silence ICP0 gene expression went unrecognized.

The current study demonstrates that the ICP0 gene is differentially regulated by three HSV proteins, VP16, ICP4, and...
ICP0, whose precise relationship to one another does not appear to be fully resolved. Functionally, the results suggest that VP16 and ICP4 ‘push’ ICP0 mRNA synthesis in opposite directions, and accumulation of ICP0 dictates which way this equilibrium will tilt. When ICP0 fails to accumulate, ICP4 is capable of silencing ICP0 mRNA synthesis despite VP16 in the tegument of virions (Fig. 3–6; ICP0− treatments). When formation of the de novo repressor is blocked with cycloheximide, VP16’s full capacity to transactivate the HSV-1 ICP0 gene is revealed (Fig. 5). If ICP0 accumulates, ICP4 fails to efficiently repress ICP0 mRNA synthesis (Fig. 3–6; ICP0+ treatments). These findings suggest that a hierarchy exists in the HSV regulatory scheme in which i. ICP0-dependent antirepression of the ICP0 gene is dominant over ii. ICP4-dependent repression of the ICP0 gene, which is dominant over iii. VP16-dependent induction of the ICP0 gene. Therefore, while VP16 induces transcription of both the ICP4 and ICP0 genes, the opposing processes of ICP4-dependent repression and ICP0-dependent antirepression appear to dictate whether the ICP0 gene remains ON or OFF in HSV-infected cells (Fig. 3B).

ICP4-Dependent Silencing of the ICP0 Gene: A Cell Type-Specific Phenomenon?

Less than 1 HSV ICP0− virus per 10,000 pfu is able to form plaques in monolayers of HEL cells [67]. In contrast, 1 HSV ICP0− virus per 100 pfu forms plaques in Vero cell monolayers [9], and nearly 100% of HSV ICP0− viruses form plaques in U2OS cell monolayers [52]. The highly variable permisiveness of host cells for HSV ICP0− viruses raises the question, “Is ICP4’s capacity to silence ICP0 gene expression in Vero cells representative of what occurs in other cell types?” Therefore, it is noteworthy that HSV ICP0− viruses form plaques with an efficiency that is indistinguishable from Vero cells in 11 of 15 other cell lines tested to date. Specifically, 0.5–2% of HSV ICP0− viruses form plaques in monolayers of human 293 cells, mouse 3T3 cells, hamster BHK-21 cells, bovine BIEC cells, bovine BUVEC cells, human Caco-2 cells, human HeLa cells, monkey MA104 cells, canine MDCK cells, pig PK-15 cells, and human WiDr cells (unpublished data of W. Halford). Further testing will be required to determine if the phenomenon of ICP4-dependent silencing of the ICP0 gene varies in proportion to the degree with which HSV ICP0− viral replication is restricted in different cell lines.

ICP0 as a Regulatory Subunit of ICP4: A Missing Lynchpin in HSV Gene Regulation?

The 1298-amino-acid ICP4 protein functions as an essential activator that recruits cellular TATA-binding protein complexes to HSV genomes, and thus stimulates mRNA synthesis from HSV E and L genes [46]. ICP4 also possesses a second activity as a repressor of HSV IE genes, which requires the binding of 350 kDa ICP4 homodimers to consensus ATCGTC sequences [68,69]. To date, it remains unclear how the balance between ICP4’s opposing transcriptional repressor versus activator functions is controlled.

Since the discovery that ICP0 re-organizes PML nuclear bodies [29], ICP4 and ICP0 have been predominantly studied in isolation from one another. However, ICP0 was initially discovered as a factor that stimulated a 20-fold increase in ICP4-dependent mRNA synthesis from HSV E and L promoters [6]. The results of the current study establish that ICP0 and ICP4 participate in a robust physical interaction in HSV-infected cells (Fig. 9). We postulate that ICP0’s physical interaction with ICP4 is likely regulatable in nature, and hence may be relevant in explaining how ICP0 potentiates ICP4-dependent E and L mRNA synthesis [5,6],
as well as how ICP0 antagonizes ICP4-dependent repression of ICP0 mRNA synthesis (Fig. 3–5). Therefore, we propose that the accumulation of ICP0 in HSV-infected cells (or lack thereof) may dictate whether ICP4 functions predominantly as an activator, or a repressor, of HSV mRNA synthesis. Further studies will be required to test the validity of this important hypothesis.

**How Does ICP0 Antagonize ICP4-Dependent Repression?**

What specific protein-DNA complexes explain how ICP0 antagonizes ICP4-dependent silencing of the ICP0 gene? The mechanisms that underlie the phenomenon documented herein are unknown, and further study will be required to differentiate between many possibilities. To illustrate this point, we present two molecular models that are each consistent with our findings, but which suggest two completely different interpretations for the significance of an interaction between ICP0 and ICP4 in the biology of HSV infections.

The first model, the ICP0-ICP4 Interference Model, is the most intuitively obvious interpretation and may reflect the mental model that many readers have invoked up to this point in the manuscript to make sense of the results. Under an ICP0-ICP4 Interference Model, it could be predicted that I. ICP4 binding to HSV dsDNA serves the sole purpose of repressing mRNA synthesis from adjacent HSV genes, and that 2. ICP0 blocks ICP4's capacity to bind dsDNA, and thus ICP0 blocks ICP4-dependent repression of the HSV ICP0 gene. However, synthesis of ICP4 is required for synthesis of viral E and L mRNAs, and mutations that disrupt ICP4's DNA-binding domain also render HSV incapable of synthesizing its ~70 E and L mRNAs that are required for HSV replication [50,70,71]. Therefore, it is difficult to envision how an ICP0-based activity that dislodges ICP4 from dsDNA would help explain how ICP0 potentiates ICP4-dependent synthesis of HSV E and L mRNAs [5,6].

An ICP0-ICP4 Synergy Model is less intuitively obvious, but is more consistent with ICP0’s capacity to enhance ICP4-dependent synthesis of HSV E and L mRNAs. Under this latter model, it may be envisioned that I. the functional purpose of ICP4 and the >50 ICP4-binding sites that span the HSV genome is to allow ICP4 to form a histone-like scaffold that coats HSV genomes; 2. the 1298 amino-acid ICP4 protein possesses numerous domains that allow ICP4 to interact with cellular proteins [72,73] and viral proteins (Fig. 9) which influence whether ICP4 stimulates or represses mRNA synthesis from the underlying HSV DNA; and 3. the nature of ICP0’s interaction with ICP4 relates to potentiating ICP4-dependent activation of mRNA synthesis from HSV DNA. Clearly, further investigation will be required to determine if the specific protein-DNA interactions that occur in HSV-infected cells are consistent with an ICP0-ICP4 Synergy Model of HSV gene regulation.

**Conclusion**

The herpesviruses and temperate bacteriophage are large dsDNA viruses that share the capacity to alternate between productive and silent infections. Many similarities have emerged in the mechanisms that herpesviruses and the tailed bacteriophage employ to replicate and encapsidate their large dsDNA genomes [74,75,76,77]. Such observations point to a common evolutionary ancestry between these two ancient families of viruses [78,79]. We conclude that the results of the current study appear to add to this growing list of inexplicable similarities.

The parallel capacity of HSV and bacteriophage λ to alternate between productive and silent infections may not be a coincidence. Rather, both viruses may control the onset of viral replication through a system of repression and antirepression of a few IE proteins whose accumulation, or lack thereof, dictates the probability of replication. In λ, the phase, the ‘decision’ between productive versus silent infections hinges upon whether the λ cro antirepressor gene is expressed or repressed [80,81,82]. Thus, important questions follow from the observation that HSV encodes its own transcriptional repressor function, embedded in ICP4, which is capable of silencing the HSV antirepressor gene that encodes ICP0. Further study will be required to determine if, in fact, repression-antirepression of the ICP0 gene is relevant to the natural process by which HSV ‘decides’ whether a given infection will be productive or silent.

**Materials and Methods**

**Cells and Viruses**

Vero cells and 293 cells were obtained from the American Type Culture Collection (Manassas, VA). ICP4-complementing E5 and ICP0-complementing L7 cell lines were kindly provided by Neal Deluca (University of Pittsburgh; Ref. [83]). ICP27-complementing V27 cells and HSV-1 d27-1 (ICP27+) virus were a kind gift of Steve Rice (University of Minnesota Medical School, Minneapolis; Ref. [48]). Cell lines were propagated in Dulbecco’s Modified Eagle’s medium supplemented with 5% fetal bovine serum and antibiotics.

Table 1 summarizes the HSV-1 recombinant viruses used in this study, which are derivative of low passage strains of wild-type HSV-1 strain KOS [84], HSV-1 KOS, HSV-1 22/n199 (ICP22-; Ref. [49]), HSV-1 0′GFP (ICP0+GFP), and HSV-1 0′BS− (ICP0−GFP) were propagated in Vero cells. The HSV-1 ICP4− virus n12 [50] and the ICP4 temperature-sensitive virus HSV-1 tsB21 [47] were propagated in ICP4-complementing E5 cells. The HSV-1 ICP0− viruses n212 [43], 0′GFP (ICP0−GFP), and 0′BS− (ICP0−GFP) were propagated and titered in ICP0-complementing L7 cells, as was the HSV-1 VP16+ virus RP4 [44] that was kindly provided by Steve Triezenberg (Michigan State University, East Lansing).

The salient features of each mutation in the ICP0 gene are summarized, as follows: i. the HSV-1 ICP0− null virus n212 contains a 14 bp translational stop insertion (ctacagctactg) in codon 212 of the ICP0 gene; hence, the n212 virus encodes the N-terminal 211 of 775 amino acids of ICP0 [43]; ii. the HSV-1 ICP0− null viruses 0′GFP and 0′BS− each contain a GFP open-reading frame and translational stop inserted in codon 105 of the ICP0 gene; hence, HSV-1 0′GFP and 0′BS− encode the N-terminal 104 amino acids of ICP0 fused to GFP; and iii. the HSV-1 ICP0− viruses 0′GFP and 0′BS− each encode a chimeric protein in which GFP is inserted between amino acids 104 and 105 of ICP0.

Table 2 summarizes the ΔE1–ΔE3 adenovirus vectors used in this study, which were propagated in human embryonic kidney 293 cells in complete DMEM, as described previously [7]. Adenovirus titers were determined by a limiting dilution analysis in 96-well cultures of 293 cells to establish the titer of infectious virus in terms of 50% tissue-culture infectious dose (TCID50). One TCID50 unit is equivalent to 1 pfu, but the TCID50 assay is less prone to underestimate adenovirus vector titers (unpublished data of W. Halford).

**Plasmid Precursors of HSV-1 Recombinant Viruses**

The construction of plasmid precursors of the HSV-1 recombinant viruses used in this study, which were propagated in human embryonic kidney 293 cells in complete DMEM, as described previously [7]. Adenovirus titers were determined by a limiting dilution analysis in 96-well cultures of 293 cells to establish the titer of infectious virus in terms of 50% tissue-culture infectious dose (TCID50). One TCID50 unit is equivalent to 1 pfu, but the TCID50 assay is less prone to underestimate adenovirus vector titers (unpublished data of W. Halford).
Corporation, Carlsbad, CA). All mutations in the ICP0 gene were subcloned into this plasmid, pCRII-HSV118-125, and plasmids bearing the desired mutation were co-transfected with HSV-1 strain KOS to obtain each of the recombinant viruses described below. The mutagenesis steps taken to create each mutant allele of the ICP0 gene are summarized, as follows.

i. **p0°-GFP**: The plasmid p0°-GFP was created by a multi-step sub-cloning procedure, which inserted a polylinker, GFP coding sequence, and stop codon derived from the plasmid pGFP-N1 (Clontech Laboratories) into a Xho I site in codon 104 of the ICP0 gene. The resulting plasmid, p0°-GFP, encoded the N-terminal 104 amino acids of ICP0 fused to GFP by a 14-amino-acid linker. Homologous recombination between HSV-1 KOS and the plasmid p0°-GFP yielded the recombinant virus HSV-1 0°-GFP (Table 1).

ii. **p0°-FLAG24**: The plasmid p0°-FLAG24 was derived by subcloning a dsDNA linker containing BrsGI and Xho I sites (gtagat agatag ctcgag) into the 3’ end of the GFP coding sequence in p0°-GFP. This sub-cloning step deleted 7 bp, thus removing a TAA terminator codon and placing the GFP and ICP0 coding sequences in the same open-reading frame. The resulting plasmid, p0°-FLAG24, encoded the ICP0-GFP reporter protein, which bears a GFP insertion between amino acids 104 and 105 of ICP0. Homologous recombination between HSV-1 KOS and p0°-FLAG24 yielded the recombinant virus HSV-1 0°-GFP (Table 1).

iii. **p0°-4BS** and **p0°-4BS**: The plasmids p0°-4BS- and p0°-4BS+ were created as follows. A plasmid, pmin0°-GFP, contains the ICP0 promoter region and a unique Tfi I restriction site that overlaps the ICP4 DNA-binding site (4BS) element in the ICP0 promoter. Thus, mutation of the 4BS element was achieved by linearizing pmin0°-GFP with Tfi I, using mung bean exonuclease to remove ssDNA overhangs, and religating with T4 DNA ligase. The 4-bp deletion in the 4BS element was confirmed by the loss of the Tfi I restriction site and DNA sequencing (Fig. 7A). The ICp4-binding site mutation was transferred into the larger plasmids p0°-4BS- and p0°-4BS+. Homologous recombination between HSV-1 KOS and these plasmids yielded the HSV-1 recombinant viruses 0°-4BS- and 0°-4BS+ (Table 1).

iv. **p0°-FLAG24**: The plasmid p0°-FLAG24 was created as follows. A plasmid, p0°-FLAG24, contained a Bgl II restriction site-GFP coding sequence-Spe I restriction site insertion between amino acids 23 and 24 of the ICP0 open-reading frame. A dsDNA oligonucleotide linker of the following sequence was synthesized, CCCAGATCTGGACTACAAGGACGTAGCAGACAAACTAGTCTG, digested with Bgl II and Spe I, and subcloned in place of the GFP sequence to create p0°-FLAG24. The resulting gene encoded a FLAG-tagged protein that bore the amino acid sequence LDYKDDDKDL between amino acids 23 and 24 of ICP0. Homologous recombination between HSV-1 KOS and p0°-FLAG24 yielded the HSV-1 recombinant virus 0°-FLAG24 (Table 1).

**Construction of Recombinant HSV-1 Viruses**

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

Recombinant HSV-1 viruses were generated by co-transfecting the following DNA species into a 60 mm dish containing 8×10^5 ICP0-complementing L7 cells: 1. 1.8 μg infectious HSV-1 KOS DNA, 2. 0.2 μg of an ICP0-expressing plasmid (which promotes HSV-1 replication), and 3. 1.0 μg of each ICP0-GFP reporter gene plasmid described above. Lipofectamine 2000 (Invitrogen Corporation) was used as the transfection reagent. After 12 hours, co-transfection medium was replaced with complete DMEM containing 1% methylcellulose and GFP+ plaques were selected on the stage of a TE2000 fluorescent microscope (Nikon Instruments, Lewisville, TX). GFP+ recombinant viruses were repeatedly passed in ICP0-complementing L7 cells until a uniform population of virus was obtained that produced 100% GFP+ plaques, at which time Southern blot analysis was used to confirm that the anticipated ICP0-GFP reporter gene was transferred into the HSV-1 recombinant virus.

**Western Blot Analysis**

Vero cell cultures were established at a density of 3×10^5 cells per well in 12-well plates, and were infected at an MOI of 5 pfu per cell. After 18 hours, proteins were harvested using mammalian protein extraction reagent (Pierce Chemical Co., Rockford, IL) supplemented with 1 M dithiothreitol and protease inhibitor cocktail set I (Calbiochem, La Jolla, CA). After heat denaturation, 20 μg of each protein sample and MagicMark™ XP protein MW markers (Invitrogen, Carlsbad, CA) were resolved in a 10% polyacrylamide gel with 4% stacking gel, and were transferred to nitrocellulose membranes. Protein blots were blocked in phosphate-buffered saline (PBS) containing 5% nonfat dry milk, and were incubated overnight at 4°C in PBS +0.1% Tween-20 +5% nonfat dry milk containing a 1:100 dilution of primary antibodies. Specifically, the primary antibodies were either the mouse monoclonal 11060 antibody specific for a peptide between amino acids 20 to 105 of ICP0 (Santa Cruz Biotechnology, Santa Cruz, CA; Ref. [85]), mouse monoclonal H1083 antibody specific for a peptide between amino acids 395 to 775 of ICP0 (EastCoast Bio, CA; Ref. [85]), mouse monoclonal H1083 antibody specific for a peptide between amino acids 950 to 775 of ICP0 (EastCoast Bio, North Berwick, MA; Ref. [86]), mouse monoclonal 58S antibody specific for a peptide between amino acids 700 to 1298 of ICP4 (Santa Cruz Biotechnology Clone 10F1; Ref. [87]), or a rabbit polyclonal anti-GFP antibody (Clontech Laboratories Inc.). Following incubation with primary antibodies, membranes were washed four times with PBS +0.1% Tween-20 (PBS-T), and were then incubated for 1 hour with secondary antibodies diluted 1:20,000 in PBS +0.1% Tween-20 + nonfat 5% dry milk. The secondary antibodies were goat anti-rabbit IgG and goat anti-mouse IgG that were respectively conjugated to the infrared fluorescent dyes IRDye® 680 and IRDye® 800CW (LI-COR Bioscience, Lincoln, NE). Protein blots were washed three times in PBS-T, rinsed in PBS (to remove Tween-20), and were scanned for two-color fluorescence using the Odyssey Infrared imaging system (LI-COR Bioscience). Data were analyzed using Odyssey application software version 3.0.16 (LI-COR Bioscience).
Northern Blot Analysis

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

Drug Treatments

In all experiments in which cycloheximide was used, 200 µM cycloheximide (Sigma Chemical Co., St. Louis, MO) was added to Vero cells 30 minutes prior to HSV-1 inoculation, and was maintained in cultures until the time of RNA harvest, or until the time at which protein translation was restored by removing cycloheximide, rinsing cultures twice, and thereafter incubating cells in complete DMEM containing 10 µg/ml actinomycin D (Sigma Chemical Co.).

Immunofluorescent Staining of HSV-Infected Cells

The subcellular localization of FLAG-tagged ICPO in HSV-1 0 FLAG24-infected cells was determined using an adaptation of a staining protocol that was generously provided by Roger Everett (MRC Virology Unit, Glasgow, Scotland). Cells were fixed with PBS containing 2% formaldehyde and 2% sucrose for 10 minutes and were permeabilized with 90% methanol for 10 minutes. HSV-1 Fc-γ receptors (glycoprotein E-I heterodimers; Ref. [88]) were blocked along with all other non-specific protein-binding sites by incubating fixed cells in a solution of PBS containing 0.5% fetal bovine serum and 10 µg/ml each of the γ-globulin fractions of human, donkey, and goat serum (PBS-F-Ig). Fixed and blocked cells were incubated for 16 hours with a 1:1000 dilution of mouse FLAG-specific mouse monoclonal FLAG-M2 antibody (Sigma Chemical Co.). Excess antibody was removed by washing with PBS-F-Ig solution, and cells were incubated for 1 hour in 1:1000 Alexa Flour 594-conjugated goat α-mouse IgG secondary antibody (Molecular Probes, Eugene, OR). Excess secondary antibody was removed by washing with PBS-F-Ig solution, and cells were photographed at 20 × magnification using a TE2000 inverted fluorescent microscope (Nikon Instruments, Lewisville, TX) and an Olympus DP72 digital camera (Olympus America Inc., Center Valley, PA).

Co-Immunoprecipitation of FLAG-Tagged ICPO and ICPO

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

Supporting Information

Figure S1 Quantitative comparison of the accumulation of ICPO GFP and ICPO GFP protein accumulation following cycloheximide release. (A and B) Photomicrographs of Vero cells infected with 2.5 pfu per cell of (A) HSV-1 0 GFP or (B) HSV-1 0 GFP at 6, 12, and 18 hours p.i., as visualized by fluorescence microscopy (20 × magnification). Vero cells were treated with vehicle or 200 µM cycloheximide (CHX) from −0.5 to 6 hours p.i. (red box denotes CHX treatment), and cultures were released into medium containing no inhibitor from 6 to 18 hours p.i. (C) ICPOGFP protein abundance between 3 and 18 hours p.i., as determined by flow cytometry. Each point represents the mean ± SEM of ICPOGFP abundance in n = 3 cultures x n = 25,000 cells per culture, where protein abundance was defined as the mean GFP fluorescence intensity scaled relative to background fluorescence, as defined by n = 12 uninfected cultures. The details of this methodology for quantifying GFP fluorescence are described in detail elsewhere [40].

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Figure S2 Replicate blots of HSV-1 0 GFP and 0 GFP RNA samples shown in Figure 4 probed for (A) HSV-1 glycoprotein D mRNA or (B) cellular GAPDH mRNA, which encodes the glycolytic enzyme glyceraldehyde 3-phosphodehydrogenase. RNA was harvested from uninfected Vero cells (0 hours) or from cells between 3 and 12 hours after inoculation with 2.5 pfu per cell of HSV-1 0 GFP or HSV-1 0 GFP. Cells were treated with vehicle or 200 µM cycloheximide until the indicated time of total RNA harvest (10 µg per lane).

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Acknowledgments

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Author Contributions
Conceived and designed the experiments: ML WPH. Performed the experiments: ML EG. Analyzed the data: ML EG EES WPH. Contributed reagents/materials/analysis tools: ML BR EG CMW OL EES. Wrote the paper: ML WPH.

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