Immediate-Early Expression of the Herpes Simplex Virus Type 1 ICP27 Transcript Is Not Critical for Efficient Replication In Vitro or In Vivo


Immediate-Early Expression of the Herpes Simplex Virus Type 1 ICP27 Transcript Is Not Critical for Efficient Replication
In Vitro or In Vivo

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We constructed a promoter mutation altering the immediate-early expression of the herpes simplex virus type 1 (HSV-1) ICP27 transcript and its cognate wild-type rescue viruses in order to assess the role of the ICP27 protein in the earliest stages of viral infection by global transcriptional analysis with a DNA microarray. This mutant, ICP27/VP16, replaces the whole ICP27 promoter/enhancer with the VP16 promoter. It demonstrates loss of immediate-early expression of ICP27 according to the criteria expression in the absence of de novo protein synthesis and earliest expression in the kinetic cascade. Significant differences in relative transcript abundances between the mutant and wild-type rescue viruses were limited at the earliest times measured and not evident at all by 4 h after infection. Consistent with this observation, levels of some critical proteins were reduced in the mutant as compared to rescue virus infections at the earliest times tested, but were equivalent by 8 h postinfection. Further, both single and multistep levels of virus replication were equivalent with both mutant and rescue viruses. Thus, altering the immediate-early kinetics of ICP27 leads to a suboptimal quantitative lag phase in gene expression but without consequence for replication fitness in vitro. Infections in vivo also revealed equivalent ability of mutant and rescue viruses to invade the central nervous system of mice following footpad injections. Limitations to an immediate-early role of ICP27 in the biology of HSV are discussed in light of these observations.

The early phase of the well-characterized herpes simplex virus type 1 (HSV-1) cascade of transcript abundance has two components: immediate-early (α) and early (β). The former, originally defined by expression in the absence of de novo protein synthesis and characterized by promoter/enhancer elements (TATGARAT boxes) activated by the interaction between the virion-associated VP16 activator and cellular “adapter” DNA binding proteins (2, 3, 13, 21, 35, 41, 42), can be shown kinetically to be the earliest expressed in abundance by use of kinetic labeling and most completely by DNA microarray technology (39, 48). A requirement for very early expression of the HSV-1 α transcripts for efficient viral replication is buttressed by our recent use of DNA microarrays to demonstrate that a kinetically normal productive cascade can be induced in cells infected with a viral mutant lacking the VP16 activator of immediate-early transcription only when cells are stressed in such a manner as to lead to the expression of the immediate-early transcripts at the earliest stages of infection (43). The functions of most immediate-early transcripts are fully consistent with the timing of their expression; thus, expression of the extremely catholic transcriptional activator ICP4 is required for efficient expression of all other viral transcripts in the context of the viral genome (4, 9, 10, 24, 25). The requirement for ICP0 protein function is cell cycle and multiplicity of infection (MOI) dependent (8, 11) and has recently been shown to have a major role in HSV-1 genome circularization, potentially acting as a major switch in the productive/laten infection pathway in neurons (20). The function of the ICP22 protein also appears to be cell cycle dependent and have a role in the ability of virus to replicate efficiently in certain differentiated cell types (7, 26, 27, 30). Finally, the protein encoded by the ICP47 transcript interferes with major histocompatibility complex class I-mediated antigen presentation and thus can be envisioned as having a major role in the ability of HSV to establish long-term infections as well as augmenting reactivation from latency (12, 18, 47).

While transcriptional effects have been ascribed to the ICP27 protein, they have yet to be well characterized (28, 29, 31), and in light of the above discussion, the timing of expression of the immediate-early ICP27 protein stands as somewhat of a kinetic conundrum. Its well-characterized activities as a mediator of splicing inhibition and transport of unspliced transcripts from the nucleus to the cytoplasm are required throughout the replication cycle; however, while viral mutants lacking this gene express at least the majority of early transcripts at normal or above normal levels, the levels of many late transcripts are significantly reduced (15–17, 19, 23). In order to investigate functions of ICP27 requiring expression immedi-

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ately upon infection, we generated an HSV-1 mutant in which the timing of expression of the transcript was altered. This mutant, ICP27/VP16, substitutes the leaky-late (βγ) VP16 promoter for the entire ICP27 promoter. While it failed to express the ICP27 transcript with immediate-early kinetics, accumulation of viral transcripts as measured by DNA microarrays was equivalent to that of rescue virus by 3 to 4 h following low-MOI infection of several differentiated cultured cell lines. Protein levels were somewhat affected at the earliest times measured, but both mutant and rescue viruses replicated to equivalent titers with equivalent kinetics in single- and multistep growth experiments on several different primary cell lines. Further, the mutant displayed no significant alteration in the course of infection in mice injected in the footpad according to several parameters of viral neuropathogenesis, including the ability to establish a latent infection in dorsal root ganglia (DRG) and the ability to efficiently recover virus from such ganglia upon explant cocultivation. This rather surprising set of results suggests a number of testable hypotheses, which are discussed along with other implications of these findings.

**MATERIALS AND METHODS**

**Cells and viruses.** Human foreskin fibroblasts (HFFs) have been described in several previous publications (1, 36), and murine embryo fibroblasts (MEFs; NIH 3T3 cells) were obtained from the American Type Culture Collection. The cells were maintained at 37°C under 5% CO2 in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Cultures of 108 HFF or MEF cells in 150-cm² flasks or 100-mm-diameter dishes were used for infections—usually at an MOI of 1 PFU/cell or less. Virus was adsorbed for 30 min prior to the addition of fresh overlay medium consisting of Dulbecco's modified Eagle's medium containing 5% fetal bovine serum.

The ICP27 promoter mutant and its rescue were constructed on a background of the 1730+ strain of HSV-1. The modified promoter–containing fragment was cotransfected with infectious 1730+ DNA into rabbit skin cells, and recombinants were identified and isolated by hybridization screening and plaque purification as described previously (22, 40). The basic approach was to introduce the modified promoters, each containing a short sequence of bacterial DNA to use as a screening marker into a KOS-derived Sal EcoRI DNA fragment spanning bases 107379 to 110095 in which the BamHI site had been converted into an XbaI site. This converted site lies ca. 270 bases upstream of the ICP27 transcript cap site and essentially 100 bases upstream of a 115-bp fragment of HSV-1 DNA bound by SmaI sites containing the TatGARAT box (TATG TAGTGT). The VP16/ICP27 promoter mutation was made by substituting the wild-type (WT) sequences from the converted BamHI site to an AgeI site at +72 relative to the ICP27 cap site with the VP16 promoter (−286 relative to the VP16 cap site to +6). This promoter construct has been described previously and contains a 360-bp fragment of the bacterial β-galactosidase gene as a screening marker (14). Once recombinant viruses were purified, infectious DNA was isolated, and a rescue was generated by recombining the original KOS-derived 4,425-bp Sal to EcoRI fragment containing the converted XbaI site.

In one set of control experiments, transcript abundance of a GFP/ICP27-null mutant constructed on a background of HSV-1 strain KOS was compared to that of WT KOS virus. The ICP27-null mutant virus 27-GFP was isolated by marker transfer of a DNA fragment containing the GFP (green fluorescent protein) gene from plasmid NES-27-GFP (32). The GFP coding sequence was cloned into a 4-flanking-site derivative of pBluescript II (Stratagene, La Jolla, Calif.), and the construct was digested with BamHI and EcoRI and ligated into the unique HindIII and XhoI restriction sites of the HSV-1 shuttle plasmid pS1000 (32). The resulting plasmid was linearized with SacI and cotransfected with ICP27/CAT by Calcium phosphate-mediated transfection into 293 cells. Infected 293 cells were selected for CAT activity with 10 μg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) in medium containing 10% FBS for 72 h. Colonies of blue cells were isolated and purified, and the ICP27 transcript was measured by reverse transcription–PCR analysis. The mutant grows as efficiently as WT virus on 2-2 cells but has a titer that is 6 to 7 logs lower on Vero cells.

RNA preparation and generation of fluorescent- or biotin-labeled cDNA. Infected cells were harvested at various times after infection, and total RNA was extracted with Trizol reagent (Molecular Research Center, Inc., Cincinnati, Ohio) as described previously (1, 43). Random hexamer-primed fluorescent (Oligo Life Sciences)- or biotin (Enzo Diagnostics)-labeled cDNAs were synthesized from 50- to 250-ng aliquots of purified poly(A) RNA by reverse transcription using Superscript II reverse transcriptase (Gibco-BRL). Fluorescein–or biotin-labeled cDNA was purified by ultrafiltration through a Microcon centrifugal filter device column (YM-30; Millipore).

Generation of microarrays, hybridization, and scanning. The characteristics and construction of our HSV-1 oligonucleotide-based DNA microarray have been described previously (39, 43). In the present series of experiments, we used a two-color nucleic acid microarray resonance light-scattering-based (RLS) method (Genicon Sciences; http://www.invitrogen.com/content.cfm?pageid = 9912) and a MAUI hybridization system (BioMicro Systems, Inc.; http://www.biomicro.com/products/new_maui.html). This procedure, which is shown graphically in both the company's website and that of E.K.W. (http://darwin.bio.uci.edu/~faculty/wagner/hsv9new.html), utilizes nano-size gold and silver particles, which have the property of scattering polychromatic or white light, to tag the hybridization probe. The scattering is characterized by preferential radiation of a specific resonance wavelength for each metal tag. In comparative control experiments using RNA isolated from HSV-1-infected cells at various times after infection, we found that (a) 100-ng samples of poly(A)-containing RNA provided hybridization sensitivity comparable to that seen with 2 to 3 μg of such RNA by Cy3/Cy5 fluorescent labeling. In RLS, signal intensity is a function of exposure time to white light, and we found, again using comparative controls, that under conditions of exposure in which all samples provided signal intensities within range of the detector (ca. 500 to 40,000 arbitrary units), the relative levels of hybridization to individual oligonucleotide probes were entirely equivalent by the two methods.

For RLS detection, microarrays were prehybridized and hybridized with cDNA probes for 18 h and rinsed for scanning with a proprietary HiLight dual-color kit (QIAGEN, Genicon Sciences) at 52°C in a MAUI hybrid mixer assembly. All procedures were as described in the instructions with the labeling kit. After hybridization, the slides were washed, blocked, bound by the gold and silver RLS method, and carefully dried in a dust-free environment, and the arrays were sealed by dipping in archiving solution also supplied with the kit. Microarrays were scanned with a GSD-501 HiLight reader (designed for the RLS system; QIAGEN).

Net signals were calculated in a Microsoft Excel spreadsheet by subtracting the signal from a ring of area equal to each spotted probe immediately surrounding the probe data spot from the corresponding individual experimental spots. The median net value of each probe (spotted in triplicate) was taken as the experimental value. To compare data from the various experimental conditions, the net hybridization values were used in two ways. First, each experimental condition was repeated at least three times, the median values from those experiments were determined, and the 75th percentile rank for the total viral hybridization was calculated and expressed previously (39, 43). To estimate the relative transcript abundance for each experiment determined for the conditions being considered. These were then compared by Student's two-tailed t test, assuming unequal variance and with the null hypothesis being that the true values under those two conditions are identical. The original data as well as selected data not shown in the manuscript will be available (accession no. GGE-00030, GGE-00032, GGE-00033, and GGE-00034) in the MIAME compliant GTI expression database (GPDWeb; http://mendel.gti.ed.ca:8000/GPX/cgi_bin/gpx.cgi).

Analysis of the levels of representative viral proteins in infected cells. Samples of total protein extracted from 3T3 cells infected at 1 PFU per cell with the appropriate mutant and/or WT virus described were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes as described previously (34). The blots were then probed for the proteins indicated (ICP4, ICP0, ICP27, gD, and gC) with monoclonal antibodies and horseradish peroxidase–conjugated secondary antibody (Amersham). Signals were detected using an enhanced chemiluminescence reagent (Amersham).

Assay of in vivo replication of the ICP27 recombinants and rescue viruses following footpad inoculation of mice. Four- to 6-week-old ND4 mice (Harlan) were infected with 106 PFU (total per mouse) of each recombinant on both rear footpads as previously described (5, 6). The feet were injected subepithelially with 50 μl of a sterile 10% saline solution 4 h prior to infection. At 1, 3, and 5 days postinfection (p.i.), four mice per recombinant per time point were euthanized, and footpads, paws, and necks were dissected and snap-frozen in liquid nitrogen. Total intracellular virus present in each sample was determined as described previously (6). Briefly, the combined feet, spinal ganglia, and spinal cords for each time point were homogenized as 10% (wt/vol) suspensions

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TABLE 1. Loss of immediate-early expression of ICP27 with the ICP27/VP16 kinetic mutant

<table>
<thead>
<tr>
<th>Class*</th>
<th>Transcript</th>
<th>ICP27/VP16</th>
<th>ICP27/VP16R</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Median value</td>
<td>SD</td>
<td>Median value</td>
</tr>
<tr>
<td>Condition 1</td>
<td>ICP27</td>
<td>0</td>
<td>400</td>
<td>24,000</td>
</tr>
<tr>
<td>IE</td>
<td>ICP0</td>
<td>21,800</td>
<td>2,000</td>
<td>15,400</td>
</tr>
<tr>
<td>IE</td>
<td>ICP4</td>
<td>28,100</td>
<td>3,600</td>
<td>33,200</td>
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<tr>
<td>IE</td>
<td>ICP22</td>
<td>28,300</td>
<td>11,400</td>
<td>27,200</td>
</tr>
<tr>
<td>IE/E</td>
<td>ICP47/U310-12</td>
<td>22,200</td>
<td>5,800</td>
<td>16,700</td>
</tr>
<tr>
<td>E</td>
<td>U1,23</td>
<td>800</td>
<td>700</td>
<td>500</td>
</tr>
<tr>
<td>E</td>
<td>U1,29</td>
<td>300</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>E</td>
<td>U1,30</td>
<td>800</td>
<td>200</td>
<td>500</td>
</tr>
<tr>
<td>E</td>
<td>U1,39/40</td>
<td>7,200</td>
<td>6,400</td>
<td>4,900</td>
</tr>
<tr>
<td>Condition 2</td>
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<td>1,700</td>
<td>900</td>
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<td>200</td>
<td>1,000</td>
</tr>
<tr>
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<td>ICP4</td>
<td>3,700</td>
<td>1,300</td>
<td>8,200</td>
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<tr>
<td>IE</td>
<td>ICP22</td>
<td>6,100</td>
<td>1,900</td>
<td>4,200</td>
</tr>
<tr>
<td>IE/E</td>
<td>ICP47/U310-12</td>
<td>4,600</td>
<td>1,300</td>
<td>3,900</td>
</tr>
<tr>
<td>E</td>
<td>U1,23</td>
<td>900</td>
<td>500</td>
<td>600</td>
</tr>
<tr>
<td>E</td>
<td>U1,29</td>
<td>600</td>
<td>100</td>
<td>300</td>
</tr>
<tr>
<td>E</td>
<td>U1,30</td>
<td>1,000</td>
<td>300</td>
<td>500</td>
</tr>
<tr>
<td>E</td>
<td>U1,39/40</td>
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<td>600</td>
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<tr>
<td>E</td>
<td>U1,50</td>
<td>1,100</td>
<td>400</td>
<td>700</td>
</tr>
</tbody>
</table>

*Cells were preincubated for 60 min in the presence of cycloheximide prior to infection, and the drug was present during virus adsorption. IE, immediate-early; E, early. Condition 1, 3 h p.i. in the presence of 60-μg/ml cycloheximide; condition 2, 1 h p.i. at an MOI of 1 PFU/cell. Infection was initiated at a multiplicity of 1 PFU per cell. Only selected transcripts are shown. The original data (accession no. GXE-00030, GXE-00032, GXE-00033, and GXE-00034) is available in the MIAME-compliant GTI expression database, GXPxDb (http://mendel.gti.ed.ac.uk/8080/GPX_xgib/gpx.xml).

a SD, standard deviation.

b Absolute (nonnormalized) values of transcript levels for three separate infections at the various time points were compared by Student’s t test (MS-Excel based) as described in Materials and Methods. The null hypothesis is that the true values for the WT and mutant viruses are identical.

RESULTS

Promoter modifications result in loss of immediate-early kinetics of expression of the ICP27 transcript. As a preliminary experiment, we used Northern blots to confirm the loss of expression of ICP27 under conditions of cycloheximide blockage of de novo protein synthesis and the recovery of this expression with the cognate rescue viruses (data not shown). In order to carry out a global, quantitative analysis of the role of immediate-early ICP27 expression in all viral transcript levels, we used HSV-1 DNA microarrays to compare transcript abundance in cells infected with the ICP27/VP16 promoter mutants with that in their cognate rescue viruses. Table 1 includes data obtained following infections in PFU per cell for relative transcript abundance at 3 h p.i. in the presence of 60-μg/ml cycloheximide as well as at 1 h p.i. in untreated cells. Under conditions of inhibition of de novo protein synthesis, of the five immediate-early transcripts only ICP27 was significantly reduced in expression in mutant infections, ranging from undetectable to at least fivefold less in individual experiments. Also shown in Table 1, the expression of the early unique long (UL) transcripts U1,23, U1,29, U1,30, and U1,50 was essentially undetectable in both infections. The levels of U1,39/40 were somewhat more variable, consistent with the known leakiness of this transcript under marginally complete conditions of inhibition of protein synthesis, and this leakiness (especially in the VP16 promoter substitution) serves as an internal reference to the greatly reduced or absent expression of ICP27 under these conditions.

After 1 h without inhibitor present, the infection with the WT rescue viruses showed transcript abundance patterns typical for this time after infection (39): i.e., a preponderance of
the five immediate-early transcripts, levels of the early ribonucleotide reductase (U1,39/40) nearly as high as those of the immediate-early ones, and readily detectable levels of a number of other early transcripts, including that encoding thymidine kinase (U2,23). In the cells infected with the ICP27/VP16 mutant, only ICP27 of the immediate-early transcripts appeared reduced in relative abundance as compared to the rescue virus. Interestingly, levels of U1,29, U1,30, and U1,39/40 mRNAs were higher in the mutant than in the rescue virus, with marginal significance, while the overexpression of ICP0 in the mutant was significantly higher in the mutant than in the rescue virus infections (P = 0.007).

**Lack of immediate-early kinetics in ICP27 expression had minimal effects on the accumulation of HSV-1 transcripts by 2 h and none at later times p.i.** The complete absence of expression of the ICP27 protein is known to have profound effects on the accumulation of viral transcripts (cf. reference 33). As a control, we confirmed these effects in the context of the cell types currently used and the ability of the extremely high-sensitivity RLS detection of hybrids on DNA microarrays to provide an accurate quantitative measure of these changes by comparing the relative abundances of viral transcripts at 4 and 8 h p.i. with a full ICP27-null mutant (GFP/ICP27) to those in a WT infection (Table 2). Since the reciprocity between exposure time and signal strength is not linear as it is with fluorescent tags (39), we utilized relative transcript abundance at any given time as a measure of deviations from the WT patterns. We have highlighted those table entries where differences in these relative abundances are statistically highly significant (P ≤ 0.05 by t test). Values for those transcripts whose fractional abundance in the cells infected by mutant virus is increased by greater than a factor of 0.8 (mainly early) are shown in italic, while those whose relative abundance is decreased by this factor (mainly late) are shown in boldface. Since we are using a different chip, different cells, and polyadenylated versus total RNA in the present experiments, we did not attempt to fully correlate the transcript abundances seen with those reported in the earlier report, especially since the methods we are currently using are not applicable to measuring large differences in absolute transcript levels. Given these provisos, the transcript abundance patterns reported here are consistent with those in HeLa cells infected with a null mutant (ICP27lacZ) at a different MOI (36). This new null mutant has a significantly lower reversion frequency since the whole open reading frame is deleted from the viral genome.

As shown in Table 3, at 2 h after infection at an MOI of 0.1 PFU/cell, the relative abundance of only the U1,42 transcript was significantly different in a comparison between infections with the ICP27/VP16 mutant and its WT rescue virus, while differences in relative levels of ICP27, ICP4, ICP47/U30, and UL29, UL30, and UL39/40 mRNAs were higher in the mutant than in the WT virus, but as seen in Table 2, the relative abundance of the transcripts expressing these proteins is similar to that of the WT virus. At this MOI, the ICP27/VP16 mutant and its rescue virus behaved identically to the WT control at both 4 and 8 h, ICP4 and ICP0 were equal in the mutant and its rescue virus, and these levels were equivalent to WT levels.

**Lack of the ability of ICP27 RNA to accumulate in the absence of de novo protein synthesis has no effect upon levels of virus replication in cultured cells.** We examined the relative efficiency of replication of the ICP27 promoter mutant with its rescue virus after both single and multiple replication cycles in order to assess the effect of the transcriptional effects seen; these data are shown in Fig. 2. For single-cycle replication, aliquots of 1,000 PFU of the appropriate virus were used to infect replicate cultures of confluent MEFs, and virus titers were determined 12 and 24 h after virus adsorption. Based upon three replicate experiments, we found there was no significant difference in virus yield between the mutant and rescue viruses (Fig. 2A). In a second set of experiments, cultures were infected at an MOI of 0.1 PFU/cell and virus yields were determined 6, 24, 30, 48, and 54 h after virus adsorption. Here too, no significant difference in yield was seen between the mutant and rescue viruses (Fig. 2B). Finally, we confirmed the equivalent replication of the mutant as compared to its rescue and WT virus in a classic single-step growth experiment in which cells were infected at 10 PFU/cell and harvested 20 h.
TABLE 2. Effect of the absence of ICP27 expression on HSV-1 transcript abundance at 4 and 8 h.p.i.

<table>
<thead>
<tr>
<th>Class†</th>
<th>Transcript</th>
<th>WT 4 h.p.i.</th>
<th>ICP27(−)</th>
<th>p-value 4 h.p.i.</th>
<th>WT 8 h.p.i.</th>
<th>ICP27(−)</th>
<th>p-value 8 h.p.i.</th>
</tr>
</thead>
<tbody>
<tr>
<td>IE</td>
<td>ICP27</td>
<td>25,000</td>
<td>2,500</td>
<td>0.013 ± 0.017</td>
<td>800</td>
<td>1,600</td>
<td>0.002 ± 0.004</td>
</tr>
<tr>
<td>IE</td>
<td>ICP0</td>
<td>6,600</td>
<td>5,600</td>
<td>0.000 ± 0.002</td>
<td>2,500</td>
<td>2,100</td>
<td>0.006 ± 0.005</td>
</tr>
<tr>
<td>IE</td>
<td>ICP4</td>
<td>8,400</td>
<td>2,000</td>
<td>0.011 ± 0.006</td>
<td>7,900</td>
<td>8,300</td>
<td>0.017 ± 0.017</td>
</tr>
<tr>
<td>IE</td>
<td>ICP23</td>
<td>25,900</td>
<td>2,600</td>
<td>0.032 ± 0.017</td>
<td>5,100</td>
<td>7,200</td>
<td>0.011 ± 0.006</td>
</tr>
<tr>
<td>IE/E</td>
<td>ICP27/15</td>
<td>22,300</td>
<td>4,000</td>
<td>0.026 ± 0.015</td>
<td>13,600</td>
<td>5,100</td>
<td>0.029 ± 0.011</td>
</tr>
</tbody>
</table>

**Note:**

† IE, immediate early; E, early; L, late.

‡ Infection was initiated at a multiplicity of 1 PFU/cell. All data are based on three replicate experiments. SD, standard deviation. Boldface means that the fractional values in mutant infections are reduced by 0.8 or greater as compared to WT, where the differences are significant at P < 0.05. Italic means that fractional values in WT infections are reduced by 0.8 or greater as compared to the mutant, where the differences are significant at P < 0.05.

§ Fractional values are the medians of the individual fractional values calculated from the total viral signal for each experiment as described in Materials and Methods; the standard deviation is shown.

**Relative values of transcript levels were compared by Student’s t test as described in Materials and Methods. The null hypothesis is that the true values for the WT and mutant viruses are identical.**
later. No statistically significant differences in virus “burst” size was seen between any of the infections tested (data not shown).

The viral recombinants with altered temporal expression of ICP27 showed similar patterns of replication and spread in the mouse following footpad inoculation. Footpad inoculation of mice provides a sensitive means of assessing even subtle differences in virulence and viral replication (5). In order to assess whether the ICP27 promoter mutant under study displayed alterations in replication in vivo, mice were inoculated on both rear footpads with 10^5 PFU. Four mice per viral recombinant (or its rescue virus) per time point were infected, and mice were sacrificed at 1, 3, and 5 days p.i.. Feet, DRG, and spinal cords from the mice were dissected, and amounts of infection virus were determined. These analyses revealed that there were no significant differences in amounts of infectious virus detected when the ICP27 promoter mutants were compared with their cognate WT rescue viruses or the parental 17syn^- virus. All viruses showed similar yields of infectious virus in the feet throughout the course of the infection (data not shown). This was not surprising given the demonstrated ability of the virus to replicate normally in cultured fibroblasts. Measurement of virus yields in DRG and spinal cords provides an indication of whether the ICP27 kinetic mutants exhibited any alteration in their ability to replicate within neurons in vivo. Typically, viruses with alterations in the ability to replicate within neurons initially show a reduction in viral yields within the sensory neurons of the DRG (6, 37). As shown in Fig. 2C, the viruses tested yielded similar amounts of virus in the DRG assayed at 5 days p.i. and there was no statistically significant difference between the ICP27/VP16 recombinant and its rescue virus (P = 0.3112, respectively, two-tailed t test). Similarly, at day 5 p.i., all viruses were detected in similar amounts in the spinal cord (data not shown), demonstrating that the mutants were both capable of normal patterns of spread through the nervous system.

ICP27/VP16 both reactivate efficiently from mouse DRG following explant cocultivation. In order to assess whether the altered temporal expression of ICP27 might exert an influence on the relative ability of these recombinants to reactivate from latency, mice were infected with 500 PFU of the mutant and rescue viruses in order to establish a latent infection. Thirty days p.i., four mice per virus were sacrificed and individual DRG (six per mouse) were explanted (see Materials and Methods). Ganglia from both mice infected with ICP27/VP16 and its rescue virus began to show evidence of reactivation by day 5 and reactivated as efficiently (>90% of ganglia were explant positive by day 18) as their rescue viruses (data not shown).

DISCUSSION

The overall goal of the study described herein was to begin an analysis of possible functions of the immediate-early HSV-1 ICP27 transcript correlated with its kinetics of expression. It should be noted that these kinetics are somewhat different from those displayed by the other HSV-1 immediate-early transcripts. This has been measured both globally for relative levels of transcript abundance and directly by pulse-labeling (39, 48). Although ICP27 is expressed at the very outset of the infection cycle, rates of expression and relative transcript abundance remain high during the first 3 h or so following infections at moderate MOIs, while rates of synthesis and relative abundances of ICP4 and ICP0 are highest at the earliest times measured and rapidly decline thereafter, consistent with the known shutoff functions of the ICP4 protein. The continued expression of the ICP27 transcript fits well with the protein’s function in RNA transport.

While our results clearly demonstrate that abrogation of the immediate-early expression of ICP27 has an effect on the earliest patterns of transcript abundance in the viral replication...
### TABLE 3. Relative abundance of HSV-1 transcripts expressed by the ICP27/VP16 mutants at 2 and 4 h.p.i.

<table>
<thead>
<tr>
<th>Classa</th>
<th>Transcript</th>
<th>Median SD Fractionb</th>
<th>Median SD Fractionb</th>
<th>Median SD Fractionb</th>
</tr>
</thead>
<tbody>
<tr>
<td>IE</td>
<td>ICP27</td>
<td>7,500 800 0.031 ± 0.003</td>
<td>4,400 1,700 0.054 ± 0.014</td>
<td>24,500 4,800 0.036 ± 0.008</td>
</tr>
<tr>
<td>IE</td>
<td>ICP0</td>
<td>5,300 1,000 0.023 ± 0.005</td>
<td>1,700 300 0.018 ± 0.005</td>
<td>4,100 3,300 0.007 ± 0.004</td>
</tr>
<tr>
<td>IE</td>
<td>ICP9</td>
<td>8,900 1,100 0.038 ± 0.005</td>
<td>2,300 400 0.025 ± 0.005</td>
<td>4,000 1,900 0.006 ± 0.002</td>
</tr>
<tr>
<td>IE</td>
<td>ICP22</td>
<td>22,300 5,700 0.096 ± 0.024</td>
<td>6,700 2,400 0.072 ± 0.030</td>
<td>29,800 2,900 0.051 ± 0.007</td>
</tr>
<tr>
<td>IE/E</td>
<td>ICP24</td>
<td>18,400 1,300 0.076 ± 0.007</td>
<td>4,100 1,400 0.051 ± 0.040</td>
<td>26,700 3,000 0.036 ± 0.007</td>
</tr>
</tbody>
</table>

a) IE, immediate early; E, early; L, late.
b) Infection was initiated at a multiplicity of 0.1 PFU per cell. All data are based on three replicate experiments. SD, standard deviation. Italic means that fractional values in WT infections are reduced by 0.8 or greater as compared to the mutant, where the differences are significant at P ≤ 0.05.

c) Fractional values are the medians of the individual fractional values calculated from the total viral signal for each experiment as described in Materials and Methods; the standard deviation of these values is shown.

d) Relative values of transcript levels were compared by Student’s t-test as described in Materials and Methods. The null hypothesis is that the true values for the WT and mutant viruses are identical.
The observation of mutant of ICP27 demonstrate altered patterns of transcript cascade, it is not clear how vital the precise timing of this virus, given the potential for tissue-specific restriction subdomain. Genes Dev. 12:2566–2576.


