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Herpes Simplex Virus 1 Immediate-Early and Early Gene Expression during Reactivation from Latency under Conditions That Prevent Infectious Virus Production

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The program of gene expression exhibited by herpes simplex virus during productive infection of cultured cells is well established; however, less is known about the regulatory controls governing reactivation from latency in neurons. One difficulty in examining gene regulation during reactivation lies in distinguishing between events occurring in initial reactivating cells versus events occurring in secondarily infected cells. Thus, two inhibitors were employed to block production of infectious virus: acyclovir, which inhibits viral DNA synthesis, and WAY-150138, which permits viral DNA synthesis but inhibits viral DNA encapsidation. Latently infected murine ganglia were explanted in the presence of either inhibitor, and then amounts of RNA, DNA, or infectious virus were quantified. In ganglia explanted for 48 h, the levels of five immediate-early and early RNAs did not exhibit meaningful differences between acyclovir and WAY-150138 treatments when analyzed by in situ hybridization or quantitative reverse transcription-PCR. However, comparative increases in viral DNA and RNA content in untreated ganglia suggested that virus was produced before 48 h postexplant. This was confirmed by the detection of infectious virus as early as 14 h postexplant. Together, these results suggest that high levels of viral gene expression at 48 h postexplant are due largely to the production of infectious virus and subsequent spread through the tissue. These results lead to a reinterpretation of previous results indicating a role for DNA replication in immediate-early and early viral gene expression; however, it remains possible that viral gene expression is regulated differently in neurons than in cultured cells.

A remarkable feature of herpes simplex virus (HSV) is its ability to establish a lifelong latent infection in hosts following acute disease. This latent stage and subsequent reactivations of infections, causing recurrent disease at peripheral sites, account for the high transmission rate and prevalence of HSV in the general population and are an attractive target for drug therapy. However, the molecular events that promote and regulate the shift from latency to reactivation are not well understood.

In the mouse eye model of infection, virus applied to the cornea gains access to axon termini and establishes latency in neurons of the trigeminal ganglia (TG) (44). In most mouse models, infectious virus is not detected during latency (38). The viral genome is detectable at the level of one to several thousand copies per neuron (15, 29, 31), but evidence of viral antigens during latency is rare (10, 16, 32). Only the LAT (latency-associated transcript) region of the viral genome produces abundant transcripts (39), although transcripts from other genes can be detected by sensitive reverse transcription-PCR (RT-PCR) assays (2, 3, 19, 43).

Compared with latency, the productive replication cycle of HSV in fully permissive cells is robust and dynamic, ending in destruction of host cells (reviewed in reference 30). The virus directs a regulated cascade of gene expression that can be grouped into three main kinetic classes (14). Upon entry into a cell, immediate-early (IE) genes are induced by a combination of virion components and cellular transcription factors. These genes, including *ICP27*, *ICP4*, and *ICP0*, act in part to up-regulate the early (E) genes. ICP8 and thymidine kinase (TK), along with the products of other early genes, carry out functions involved in viral DNA synthesis. The third gene class, the late (L) genes, is dependent upon DNA replication for maximal expression. Although this regulated program of gene expression has been studied extensively during in vitro productive infection, its relevance to events during reactivation from latency remains unclear.

Several investigations focusing on this issue have suggested that the progression of gene expression in neurons during initial infection and/or reactivation differs from that in permissive cells. Previous studies of ours, which measured gene expression during a 48-h explant reactivation from murine TG by in situ hybridization (ISH), observed that inhibition of viral DNA synthesis by pharmacologic inhibitors or genetic mutations resulted in an unexpected decrease in IE and E gene expression, events which precede viral DNA synthesis in productive infection (17). Thus, these results suggested that DNA synthesis might play a key role during reactivation. In uninhibited explants, the progressive detection of IE, E, and L genes over a 48-h period conformed to other reports suggesting that the reactivation process took approximately 48 h to complete (36, 37, 47). This, coupled with the detection of LATs in the same cells as IE, E, and L gene RNA hybridization, made it seem

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likely that the chronicled events were occurring during a single, initial replicative cycle. However, the possibility that secondary infections could occur within this time period and account for the high gene expression levels was not ruled out. Therefore, whether the decreased gene expression was a direct result of blocking DNA synthesis or was due to the attendant blocking of production of infectious virus and spread to neighboring cells was not resolved.

Nevertheless, more data in support of a role for DNA replication in gene expression in neurons were reported. In vitro studies comparing levels of HSV gene expression in Vero cells and rat peripheral neurons found that inhibition of viral DNA synthesis greatly reduced IE and E gene expression in neurons but not in Vero cells (26). Inhibition of DNA synthesis was also reported to reduce IE and E promoter activity in primary cells but not in Vero cells (41). In addition, it has been reported that during explant reactivation from murine TG, E and leaky late transcripts are detected before IE transcripts (43). On the other hand, a recent investigation of this question with rabbit TG found no deviation from the canonical regulatory order (28).

To enable examination of gene expression during reactivation in more detail, we have employed acyclovir (ACV), a well-established inhibitor of viral DNA replication, and WAY-150138, a newer inhibitor of viral DNA cleavage and packaging (45). The latter compound appears to preclude the incorporation of the UL6 portal protein and UL15 into capsids (25). Because WAY-150138 inhibits progeny virus production at a stage subsequent to viral DNA replication, it allows examination of a possible role of viral DNA synthesis in reactivation without the confounding effects of infectious virus production and spread. Here, we describe the use of this inhibitor in individual, explanted TG to distinguish between gene expression events confined to initial reactivating neurons and those events associated with spread through explanted tissue.

MATERIALS AND METHODS

Cells and viruses. Strain Patton wild-type and 138^R/5 drug-resistant viruses were gifts from M. van Zeijl and T. R. Jones, Wyeth Research. Procedures for propagation and assay of HSV on Vero cell monolayers have been described previously (7).

Compounds. Acyclovir (acycloguanosine; Sigma) and WAY-150138 (a gift from M. van Zeijl and T. R. Jones, Wyeth Research) were dissolved in dimethyl sulfoxide (DMSO). Because WAY-150138 requires DMSO for solubility, all media were supplemented with 0.5% DMSO.

Plaque reduction assay. Antiviral activity was assessed essentially as described previously (7), with the following modifications. After adsorption of virus, Vero cell monolayers were overlaid with media containing 2% newborn calf serum, 0.8% methylcellulose, 0.5% DMSO, and WAY-150138. After 3 days of incubation, the cells were fixed and then stained with crystal violet.

Dot blot assay for viral DNA replication. Following a published method (20) with slight modifications, Vero cells in 24-well plates were infected at a multiplicity of infection of 5 or mock infected. After 1 h, cells were washed three times with cold phosphate-buffered saline (PBS) and then incubated a further 2 or 14 h in media containing 5 to 40 μg/ml WAY-150138, 0.5% DMSO; 200 μM ACV, 0.5% DMSO; only 0.5% DMSO; or no additives. After lysis in 0.4 N NaOH-10 mM EDTA, lysates were applied to a nylon membrane (GeneScreen Plus; PerkinElmer Life Sciences) with a dot blot apparatus (Schleicher & Schuell) and hybridized with a ^{32}P-labeled *ICP0* oligonucleotide probe, ICP0-3 (oligonucleotide sequence can be found in the supplemental material at http://coen.med.harvard.edu). The amount of hybridization was quantified with a Personal Molecular Imager FX and Quantity One software (Bio-Rad) and compared with that obtained with a dilution series of lysates from untreated, infected cells.

Mouse infection. Male ICR mice (Harlan) were infected as described previously (21), with minor changes. Eight-week-old mice were anesthetized with 85 mg sodium pentobarbital/kg of body weight (Nembutal; Abbott Laboratories) or a mixture delivering 120 mg/kg ketamine and 18 mg/kg xylazine (Sigma). Either 2×10^5 or 2×10^6 PFU of virus was dropped onto both eyes in a volume of 4 μl. Animal housing and all procedures involving mice were approved by the Harvard Medical School Institutional Animal Care and Use Committee in accordance with federal guidelines.

Explant reactivation. At a minimum of 30 days postinfection, mice were sacrificed, and TG were removed, bisected, and placed in 1.5 ml reactivation media (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 200 U/ml penicillin, 200 μg/ml streptomycin, 0.25 μg/ml amphotericin B [Fungizone], and 0.5% DMSO) with or without inhibitors (17, 37). Within 70 min, TG were transferred to a 37°C incubator. Hours postexplant were counted from the time of sacrifice.

In situ hybridization. Explanted TG were serially cryosectioned lengthwise to a thickness of 8 μm and hybridized with DNA probes (17) labeled with α-^{35}S-dATP and α-^{35}S-dCTP, as described previously (12).

RNA/DNA isolation. Explanted TG were quickly rinsed in cold PBS, frozen in liquid nitrogen, and stored at −80°C. Frozen TG were homogenized in 650 μl of guanidine thiocyanate solution (4). For DNA analysis, 1/10 volume of the homogenate was removed and mixed with 150 μl water, 20 μl 3 M sodium acetate, and 600 μl ethanol. Precipitates were treated with proteinase K as described previously (19), except that samples were vigorously vortexed every 30 min and the addition of Tween and the 80°C incubation were omitted. Aliquots were taken directly for real-time PCR. RNA was purified from the remaining 9/10 of the TG homogenate essentially as described previously (4).

DNA and RNA standards. (i) Mouse DNA standards. Mouse brain DNA was isolated and treated with RNase as described previously (40) and quantified by spectrophotometry at 260 nm. Diluted aliquots were used directly in real-time PCRs. (ii) HSV DNA standards. Transfection-quality HSV DNA was purified from strain Patton-infected Vero cells, as described previously (22). Concentrations were measured by comparison to mass standards electrophoresed on an agarose gel. Tenfold serial dilutions were mixed with 1/10 of a mock-infected TG lysate and then processed along with the experimental sample lysates, as described above. (iii) Mouse RNA standards. Mouse brain RNA was purified in a scaled-up version of the protocol for ganglionic RNA, and concentrations were determined by spectrophotometry at 260 nm. Serial dilutions of this RNA were subjected to DNase, cDNA synthesis, and PCR protocols, as described below. (iv) HSV RNA standards. Plasmid pKS + 5′ICP8 (kindly provided by M. F. Kramer) was constructed by cloning a 2.3-kb BamHI fragment from plasmid p8B-S (11), containing sequences in the 5′ region of the *ICP8* gene, into the BamHI site of pBluescript II KS (Stratagene), downstream from the T3 RNA polymerase promoter. Similar transcription plasmids containing *ICP0*, *ICP4*, or *tk* sequences have been described previously (19). Synthetic viral transcripts of approximately 1.5 to 2.6 kb were generated from these plasmids via in vitro transcription using T7 or T3 RNA polymerases (Promega) as described previously (19), with the following modifications. Linearized plasmids were transcribed for 4 h at 37°C in the presence of 600 Ci/mmol α-^{35}S-CTP (MP Biomedicals) and an RNase inhibitor, SUPERase•In (Ambion). Afterwards, reactions were treated with DNase and RNA was purified with an RNeasy kit (QIAGEN). The percentage of α-^{35}S-CTP incorporated into trichloroacetic acid-precipitable material was used to calculate RNA concentration (19). Synthetic HSV RNAs representing intended target genes were combined and serially diluted. Several aliquots of each dilution were mixed with 5 μg of mouse brain RNA to generate replicate standard sets. These standards were then subjected to DNase, cDNA synthesis, and PCR protocols along with TG samples, as described below. In some cases, preparation of standards was scaled up while ensuring that efficiency and recovery were not altered.

DNase digestion. To completely digest the large amount of viral DNA resulting from reactivated virus, the following DNase protocol was devised. Purified TG RNA or RNA standards (20 μl) were mixed with 10 μl 10× buffer, 5 μl SUPERase•In, and 5 μl TURBO DNase (all Ambion reagents) in a total volume of 95 μl. The reactions were incubated at 37°C for 90 min, with the addition of 5 μl more DNase after 45 min. The reactions were stopped by adding 10.7 μl of 58 mM EDTA–850 μg/ml baker's yeast tRNA (as carrier; Roche)–11 μl 2 M sodium acetate, pH 4; and 120 μl acid phenol:chloroform (5:1). These mixtures were extracted once and then back-extracted following the addition of 80 μl 0.2 M sodium acetate, pH 4. The pooled aqueous layers were extracted with 200 μl chloroform:isoamyl alcohol (49:1) and precipitated with ethanol at −80°C.

RT-PCR. DNase-treated, precipitated RNA was collected by centrifugation, washed with 70% ethanol, air dried for 8 min, and resuspended in water: SUPERase•In (100:1). Annealing of reverse primers and reverse transcription

were carried out essentially as described previously (19). Each annealed sample was split equally between reaction mixtures with and without avian myeloblastosis virus reverse transcriptase (Promega). Mouse cDNA was assayed by real-time PCR, as described below. PCR assays for HSV cDNA were performed according to the general parameters described previously (19), except that the reaction volumes were 50 μl and included 2 or 3 μl of cDNA, depending on the gene assayed. Conditions for the *ICP8* assay were as follows: 3 mM $MgCl_2$, 10% glycerol, and a 5 min, 94°C incubation followed by 30 cycles comprising 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. Primer sequences are reported in the supplemental material (http://coen.med.harvard.edu). Conditions and primer sequences for other assays have been described previously (3, 19). Replicate sets of cDNA standards were subjected to PCR alongside batches of experimental TG samples.

Real-time PCR. Real-time PCR assays, based on those developed by M. F. Kramer (unpublished data), were performed as follows to quantify viral DNA, mouse DNA, and mouse cDNA; primer and probe sequences can be found in the supplemental material (http://coen.med.harvard.edu). Viral DNA was assayed in 20 μl using 2.5 μl of TG DNA or standards, 0.1 μM primers for the *tk* gene, and SYBR green PCR master mix (PerkinElmer Life Sciences). Mouse DNA was assayed in 20 μl using 2 μl of purified TG DNA or standards, 0.1 μM primers for the mouse glyceraldehyde-3-phosphate dehydrogenase (*mGAPDH*) gene, and SYBR green PCR master mix. Mouse RNA was assayed in 20 μl using 1 μl of cDNA made from TG or mouse RNA standards, 0.1 μM *mGAPDH* primers, 0.1 μM 6-carboxyfluorescein/6-carboxytetramethylrhodamine dual-labeled probe (Integrated DNA Technologies), and a Brilliant QPCR core reagent kit (Stratagene). All reactions were performed by an ABI Prism 7700 sequence detection system. Semilog plots of threshold cycle (fractional cycle at which an arbitrary amount of product is detected) versus input DNA were analyzed by linear regression, and the nucleic acid contents of unknown samples were interpolated (see the supplemental material at http://coen.med.harvard.edu).

Gel transfers and dot blots. Southern blotting of polyacrylamide gels was performed as described previously (19). Dot blotting was performed essentially as recommended by the GeneScreen manufacturer. In brief, membranes were soaked in 0.4 M Tris-HCl, pH 7.5, for 5 min and briefly subjected to a vacuum before 20 μl of each PCR premixed with 80 μl cold 0.5 N NaOH-12.5 mM EDTA was loaded onto the dot blot apparatus (see above). After 25 min, vacuum was applied to empty the wells, followed by a 5-min incubation with 200 μl 0.4 N NaOH and final vacuum. Blots were neutralized in 2× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]) and cross-linked in a UV Stratalinker (Stratagene). Gel blots and dot blots were incubated with ^{32}P-end-labeled oligonucleotide probes as previously described (19). *ICP8* probe sequence can be found in the supplemental material (http://coen.med.harvard.edu); *ICP4*, *tk*, and *ICP0* probes have been published (3, 19). Membranes were exposed to phosphor screens for quantification of hybridized probe. Log-log plots of hybridization signal (in phosphorimager units) versus input RNA copies were analyzed by linear regression, and the RNA contents of unknown samples were interpolated. Statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, Calif.).

Detection of infectious virus after explant. After explant incubations, TG were washed twice on ice with PBS, 0.5% DMSO for 10 min to remove replication inhibitors. (Washes at 25°C for 15 to 60 min were detrimental because they allowed a fraction of TG to support reactivation after the explant period). TG were then frozen in culture media at −80°C, thawed at 37°C, homogenized, and sonicated as previously described (21). The homogenates were centrifuged for 15 min at 1,300 × *g*, and the supernatants were plated on Vero cell monolayers. After 1 h of incubation, methyl cellulose-containing medium was overlaid, and plaques were allowed to form for 3 days.

RESULTS

To investigate if viral DNA synthesis could account for the elevated gene expression observed with previous ISH experiments (17), a method to allow viral DNA synthesis to proceed while blocking production of infectious virus was sought. Attempts to address this question by using a temperature-sensitive HSV mutant blocked at a late stage of replication were hindered by deterioration of the ganglion tissue (unsuitable for ISH) and inhibition of reactivation of wild-type virus at elevated temperatures (D. A. Garber and D. M. Knipe, unpublished data). As the explant model is well suited for pharmacological studies, the development of an inhibitor of a late event in viral replication provided a solution. This compound, WAY-150138, along with the wild-type strain Patton virus and a WAY-150138-resistant derivative, 138^R/5 (with a resistance-conferring mutation mapped to the UL6 gene), were kindly provided by T. R. Jones and M. van Zeijl, Wyeth Research (45).

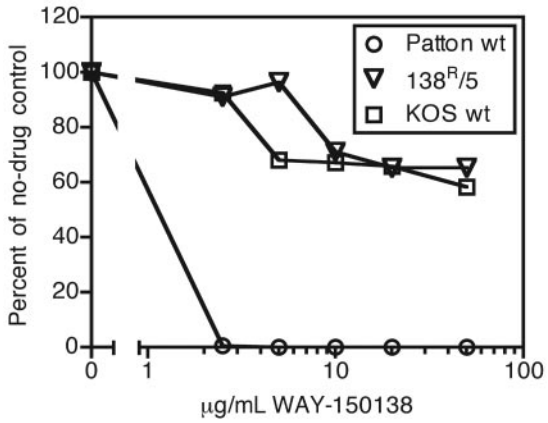

FIG. 1. Plaque reduction assay with WAY-150138. Patton wild-type (wt); 138^R/5 mutant, which is resistant to WAY-150138; and KOS wt viruses were assayed for plaque formation in the presence of WAY-150138. Average plaque numbers from triplicate wells were calculated as percentages of plaques formed relative to DMSO vehicle treatment.

Specificity and efficacy of WAY-150138 in vitro. WAY-150138 inhibits the replication of some strains of HSV-1, including Patton, with a 50% inhibitory concentration of 0.2 to 0.7 μg/ml (45). Plaque reduction assays demonstrated that wild-type strain Patton was prevented from forming plaques in 10 μg/ml WAY-150138, while the drug-resistant derivative and wild-type strain KOS were subject to no more than a 40% reduction in plaque number in up to 50 μg/ml (Fig. 1).

The value of WAY-150138 in our proposed experiments lay in its reported ability to block production of progeny virus while allowing viral DNA replication to proceed (45). To confirm that this compound had little or no effect on viral DNA replication, the viral DNA content of cells infected in the presence of WAY-150138 was measured by dot blotting and Southern hybridization (Fig. 2). Both wild-type strain Patton and the 138^R/5 resistant mutant virus exhibited no more than a 35% reduction in viral DNA levels when treated with WAY-150138 (Fig. 2). This effect, which did not correlate with the difference in susceptibility of these two viruses, was minor compared to the inhibition achieved by acyclovir (greater than 95%) (Fig. 2) and to the effect of WAY-150138 on plaque formation by wild-type strain Patton. In addition, two groups have reported finding no effect of WAY-150138 on synthesis of certain viral proteins (25, 45).

Specificity and efficacy of WAY-150138 and behavior of Patton viruses in vivo. In moving from in vitro tests to in vivo studies, we needed to assess (i) the ability of the wild-type Patton strain to establish latency and subsequently reactivate in our murine eye model and (ii) the efficacy of WAY-150138 when used to treat whole ganglion tissue. Conditions for animal studies were designed to achieve reactivatable latent infections that were comparable to those achieved via our stan-

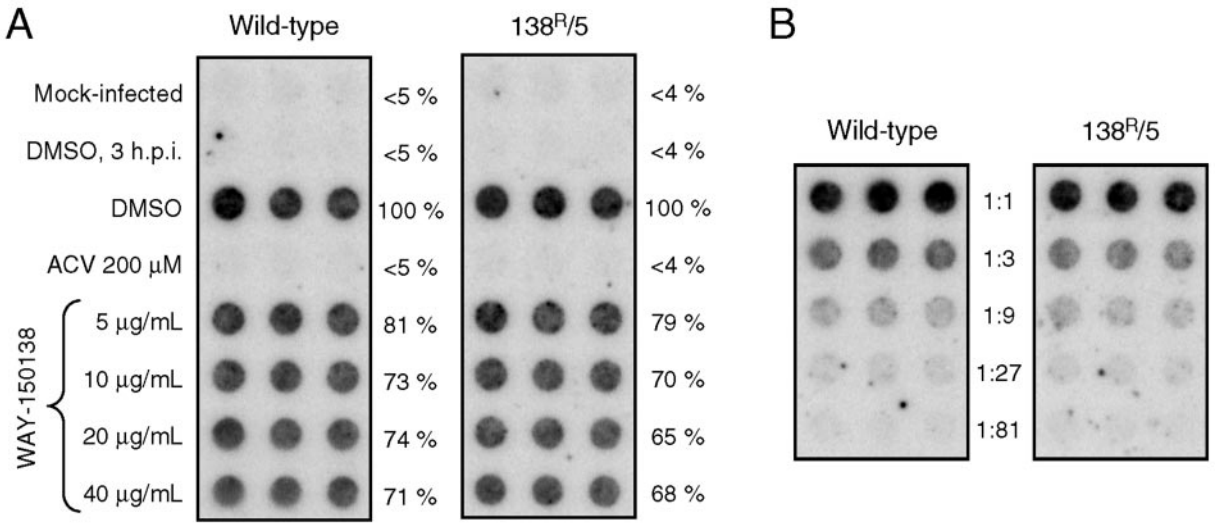

FIG. 2. Effects of WAY-150138 and ACV on viral DNA replication. (A) Cells were mock infected (first row) or infected (all other rows) with wild-type virus (left) or 138^R/5 virus (right) in triplicate and incubated for 3 h (second row) or 15 h (all other rows) in media containing DMSO alone, ACV (plus DMSO), or WAY-150138 (plus DMSO), as indicated at the far left. Lysates were applied to a dot blot and hybridized with a probe specific for viral DNA sequences. The mean percentage of hybridization, representing the amount of viral DNA synthesized under each treatment relative to the DMSO-treated control, is labeled to the right of each blot. (B) Amounts of hybridization were quantified using 1:3 serial dilutions of triplicate, untreated, infected cell lysates. For wild-type and 138^R/5 infections, the lowest detectable serial dilutions represented 5% and 4% of the DMSO-treated controls, respectively. h.p.i., hours postinfection.

dard protocol using KOS strain virus while limiting the mortality of the more virulent Patton viruses (unpublished data). To assess the ability of these viruses to reactivate and the ability of the drugs to prevent reactivation, latently infected TG were harvested and incubated for 48 h with or without drugs and then scored for reactivation by detection of infectious virus in TG homogenates (Table 1). TG latently infected with wild-type virus or 138^R/5 virus and not subjected to drug treatment reactivated 100% of the time, indicating that all TG were latently infected and reactivation could be readily and reliably induced over 48 h. Six latently infected TG (frozen in liquid nitrogen directly after harvest) yielded no virus, demonstrating that detected virus was a product of reactivation (data not shown). In initial experiments (Table 1, third column), reactivation by wild-type virus showed sensitivity to both ACV and WAY-150138. However, reactivation by 138^R/5 showed sensitivity only to ACV, with 100% of TG yielding virus when treated with WAY-150138. Although reactivated virus was observed for 4 of 15 wild-type-infected TG treated with ACV and 3 of 15 treated with WAY-150138, only a few PFU were detected for each TG (while we estimated that >1,500 PFU were produced by each untreated TG). Further testing indicated these few PFU were likely produced after the explant incubation, during procedures to wash out the drugs before freezing (data not shown). In subsequent experiments in which mice were infected with 10-fold-lower inocula (to reduce mortality), inhibitor concentrations were doubled and shorter washes were performed on ice. Under these conditions, complete inhibition of wild-type virus production was reproducibly achieved by both compounds (Table 1, last column), confirming that the drugs penetrated the tissue and were effective for the duration of explant.

In situ hybridization of explanted TG in the presence of WAY-150138. To evaluate the possible contribution of secondary infections to viral gene expression in reactivation experiments, we used wild-type Patton or 138^R/5 virus to infect mice and WAY-150138 or ACV to inhibit viral multiplication. Latently infected TG were explanted for 48 h in the presence or absence of these inhibitors and then frozen and cryosectioned. ISH was performed using probes for RNA transcripts from a marker of latent infection (LATs), an immediate-early gene (*ICP27*) or an early gene (*ICP8*). Hybridization with the LAT probe yielded an average of 4.0 to 7.0 signals (positive cells) per section under all three treatment conditions (data not shown), consistent with past experiments using strain KOS (17). Untreated wild-type-infected and 138^R/5-infected TG displayed high levels of hybridization with both productive-

TABLE 1. Performance of drugs and viruses in 48-h explants

Virus	Drug	Fraction of TG reactivated	
		Earlier conditions[a]	Later conditions[b]
Wild type	None	15/15	5/5
Wild type	ACV	4/15	0/21
Wild type	WAY-150138	3/15	0/28
138^R/5	None	5/5	ND[c]
138^R/5	ACV	0/3	ND
138^R/5	WAY-150138	7/7	ND
Mock	None	0/8	0/4

[a] PFU inoculated, 2 × 10^6; 100 μM ACV or 10 μg/ml WAY-150138; 15- to 60-min washes at 25°C; data collated from three experiments.
[b] PFU inoculated, 2 × 10^5; 200 μM ACV or 20 μg/ml WAY-150138; 5- to 15-min washes at 4°C; data collated from four experiments.
[c] ND, not done.

TABLE 2. In situ hybridization of TG undergoing reactivation in the presence of inhibitors

Virus	Drug	No. of TG	*ICP27* probe			*ICP8* probe		
			Sections	Signals[a]	Signals/section	Sections	Signals[a]	Signals/section
Wild type	None	3	48	439	9.1	43	708	16
Wild type	ACV	4	48	4	0.08	60	5	0.08
Wild type	WAY-150138	5	48	10	0.21	56	16	0.29
138^R/5	None	1	18	176	9.8	32	353	11
138^R/5	WAY-150138	2	26	559	22	24	619	26

[a] Each signal corresponds to hybridization observed in a single cell.

cycle gene probes: 9.1 and 9.8 signals/section, respectively, for *ICP27*; and 16 and 11 signals/section, respectively, for *ICP8* (Table 2). High signal levels were also observed for WAY-150138-treated, 138^R/5 samples (Table 2), confirming that the mutant virus was exhibiting resistance to WAY-150138 and that the drug did not cause any nonspecific suppression of gene expression in the tissue.

Consistent with the results of Kosz-Vnenchak et al. (17), ACV treatment resulted in a large reduction in hybridization for *ICP27* and *ICP8* probes: only 0.08 signals per section were counted for both, more than a 2-log reduction compared with untreated sections (Table 2). However, a large reduction was also observed for samples treated with WAY-150138, the encapsidation inhibitor (Table 2). These data were bolstered by observation of the intensity of hybridization: in the drug-treated, wild-type virus sections, only weak hybridization in individual neurons was seen, suggestive of restricted viral activity, whereas in the untreated sections, neurons contained strong hybridization signals, similar to the results of Kosz-Vnenchak et al. (17).

Viral DNA quantification in explanted TG. This ISH study suggested that similarly low levels of IE and E RNA were expressed over 48 h of reactivation when infectious virus production and viral spread were blocked. Therefore, for more sensitive and quantitative assessments of viral gene activity during reactivation, explanted TG were analyzed for DNA and RNA content by using real-time PCR and RT-PCR. Following infection and explant protocols similar to those used for the ISH study, individual TG homogenates were divided for use in DNA and RNA quantification procedures.

For DNA analyses, viral and mouse DNA standards were amplified by real-time PCR alongside DNA from experimental ganglia, using primers for sequences within the viral *tk* or mouse *GAPDH* genes. Standard curves were used to interpolate the amounts of DNA in unknown samples and furnish a measure of amplification efficiency (detailed in the supplemental material at http://coen.med.harvard.edu). To account for differential recovery, for each TG, viral DNA was normalized to mouse DNA detected and measured in a similar manner.

The levels of viral DNA achieved with ACV-treated and untreated TG were similar for wild-type and mutant viruses, providing evidence that they established comparable latent infections. For wild-type virus, the mean viral DNA levels in the presence of ACV over 48 h did not rise above latent levels (0 explant hours), which confirmed the activity of ACV in blocking viral DNA synthesis during explant (Fig. 3). A statistically significant sevenfold increase in DNA content seen for TG treated with WAY-150138 for 48 h likely represented the amount of viral DNA replicated in cells stimulated to reactivate. By comparison, there was a nearly-50-fold difference in DNA content between WAY-150138-treated and untreated ganglia, which could be evidence of secondary infections within 48 h. In 138^R/5-infected TG, the amount of DNA detected in the presence of WAY-150138 was similar to that for the no-drug control. This was expected since 138^R/5 displays considerable resistance to the concentrations of WAY-150138 used in this assay (Fig. 1). Similar results were obtained when mice were infected with a 10-fold-lower inoculum and TG were treated with doubled concentrations of inhibitors (unpublished data).

Viral RNA quantification in explanted TG. To confirm and extend the RNA expression data gathered in the ISH study, we measured the levels of two IE RNAs (*ICP4* and *ICP0*) and two

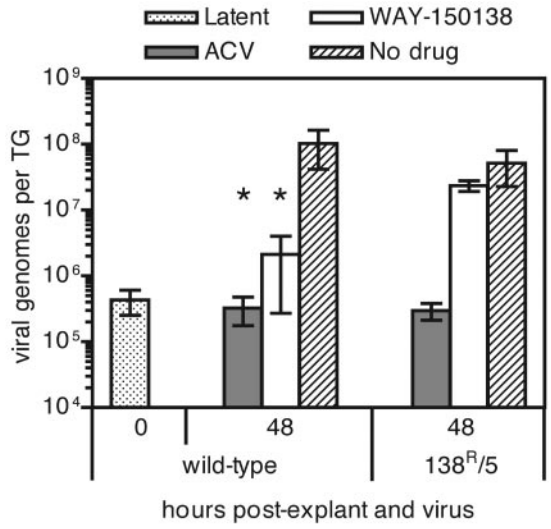

FIG. 3. Viral genome copies per TG during explant reactivation. TG from mice infected with 2×10^6 PFU wild-type Patton or 138^R/5 virus were harvested and either frozen directly (latent; 0 h) or incubated for 48 h with 100 μM ACV, 10 μg/ml WAY-150138, or no drug. Viral DNA content was measured by quantitative, real-time PCR and normalized to mouse DNA content. Means ± standard deviations are plotted. For wild type, $n = 6$ for 0 h, and $n = 9$ for 48 h; for 138^R/5, $n = 3$. Mock-infected TG were negative for viral DNA. *, $P = 0.0104$ by two-sided Student's t test. The nine WAY-150138-treated, wild-type-infected ganglia comprised seven ganglia with viral DNA levels above those present in any of the ACV-treated, wild-type-infected ganglia, and two ganglia whose lower viral DNA contents resulted in relatively large error bars for this group.

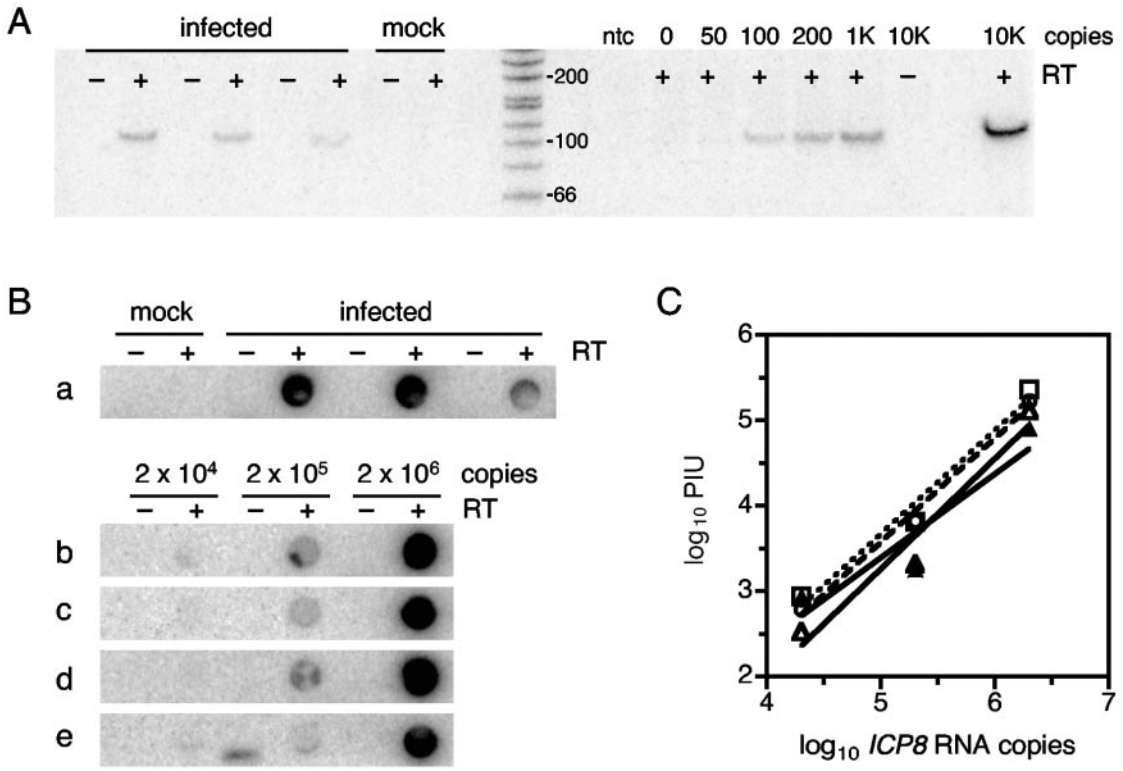

FIG. 4. Quantitative *ICP8* RT-PCR. (A) Southern blot of polyacrylamide gel containing *ICP8* RT-PCR products. (Left) TG samples (either infected or mock infected, as indicated). (Right) RNA standards representing the indicated number of copies per 90% of a TG. RT indicates whether reverse transcriptase was added (+) to one-half of the cDNA synthesis reaction or omitted (−). The DNA size marker is shown in the center, and the sizes of certain DNA markers are labeled in bp. ntc, no template control for PCR. (B) Portions of dot blots with RT-PCR products from TG samples (either infected or mock infected, as indicated in panel a) and standards. Panels b to e contain replicate sets of RNA standards representing the indicated number of RNA copies per 90% of a TG. RT indicates whether reverse transcriptase was added (+) to one-half of the cDNA synthesis reaction or omitted (−). (C) Standard curve of *ICP8* RT-PCR products. Data are derived from panel B in the following manner: filled and open triangles/solid lines represent data from the same cDNA series subjected to separate PCRs and applied to separate blots (panels c and e, respectively); circles/dashed line (panel b) and squares/dotted line (panel d) represent two further, independent cDNA series, each loaded on one of the two blots described above. This standard range encompassed all experimental samples. PIU, phosphorimager units.

E RNAs (*ICP8* and *tk*) for the same TG analyzed in Fig. 3. Total RNA was isolated from individual TG and reverse transcribed with a battery of gene-specific primers. Every RNA sample was divided into two aliquots, one to which reverse transcriptase was added and one where it was omitted. Each cDNA sample was then divided among several PCRs for amplification of viral transcripts or mouse *GAPDH* transcripts. The assays for *ICP0*, *ICP4*, and *tk* have been described previously (3, 19), but the *ICP8* assay was newly developed. All assays were optimized to yield a single major amplified species, confirmed by gel electrophoresis and Southern blot hybridization (Fig. 4A). This permitted quantification of large numbers of samples by using dot blots of the PCR products (Fig. 4B). For all four PCR assays, the primer regions and intervening cDNA sequences contained no mismatches when comparing Patton viral DNA to the plasmids used to make the RNA standards. Standard curves generated from *ICP8* (Fig. 4C) and *mGAPDH* (see the supplemental material at http://coen.med.harvard.edu) RT-PCR displayed a linearity and reproducibility typical of all of the RT-PCR assays.

The mean numbers of copies of viral RNAs were measured for latently infected TG and for TG that had been explanted for 48 h in the presence of ACV, WAY-150138, or no drug (Fig. 5). Wild-type-infected TG left untreated and 138^R/5-infected TG left untreated or treated with WAY-150138 exhibited the highest RNA contents. For some of these TG, RNA levels were beyond the linear range of the RT-PCR assays, which were designed to optimize detection of possible differences between the lower RNA levels expected with drug-treated samples. These TG were assigned an RNA content equivalent to the upper quantification limit of the assay, yielding values that represent minimum mean RNA copies per TG. Thus, this experiment likely underestimates the increase in RNA levels in untreated versus treated or latently infected ganglia.

For wild-type-infected TG (Fig. 5A), all IE and E RNA species showed a clear increase over latent levels during explant. However, compared to TG in which viral DNA replication was blocked (ACV treated), TG in which viral DNA replication was free to proceed (WAY-150138 treated) did not exhibit measurable increases in IE or E gene expression. This result is similar to those observed with cell culture, in which immediate-early and early gene expression were not reduced by inhibition of viral DNA synthesis (14). Compared to results for these drug-treated ganglia, increases were readily apparent with ganglia that were untreated and allowed to freely reacti-

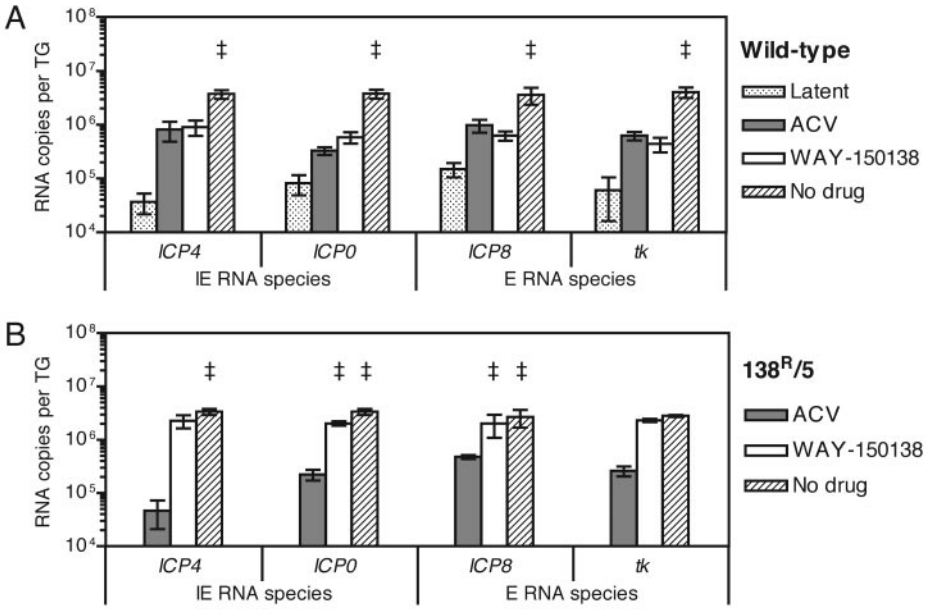

FIG. 5. Viral RNA copies per TG in latent and reactivating TG. TG from mice infected with 2×10^6 PFU wild-type Patton (A) or $138^R/5$ (B) were harvested and either frozen directly (latent) or incubated for 48 h with 100 μM ACV, 10 μg/ml WAY-150138, or no drug. Indicated viral RNA species were measured by quantitative RT-PCR. Undetectable samples were calculated as zero. Means ± standard errors of the means are plotted. (A) $n = 6$ for latent, and $n = 11$ for others; (B) $n = 3$. Mock-infected TG were negative for viral RNA. ‡, representation of minimum values, because one or more TG contained RNA levels greater than the linear range of the assay.

vate over a 48-h period, substantiating the ISH results and showing again that both ACV and WAY-150138 were restricting some source of viral transcripts.

The same assays were also performed on TG latently infected with the drug-resistant $138^R/5$ virus. Figure 5B shows that levels of RNA expressed in the presence of ACV were similar to those achieved by the wild-type virus with the same drug and that all four RNAs displayed a marked increase in expression in the presence of WAY-150138. These results indicated that WAY-150138 did not exhibit nonspecific, suppressive effects on the expression of IE and E RNAs.

Timing of replication cycle during reactivation. The ISH, PCR, and RT-PCR studies all suggested that secondary infection could be under way by 48 h postexplant (h p.e.). To investigate how early infectious virus could be detected during reactivation in our system, we explanted TG latently infected with either wild-type Patton or KOS virus for between 2 and 26 h and then measured virus yield. Both strains of virus achieved 100% reactivation by 24 h p.e. (Table 3), much earlier than the 4 days previously reported for 100% reactivation in a similar explant model (37), on which interpretations of earlier ISH data had been based (17). Additionally, virus could be reproducibly detected in a fraction of TG explanted for only 14 h: 27% of Patton-infected TG and 22% of KOS-infected TG. Although none of the KOS-infected TG were positive for virus at 16 h p.e., two independent experiments yielded TG positive for virus at 14 h p.e. The difference in the fractions of positive TG at 14 and 16 h p.e. is not statistically significant. Overall, the data support the authenticity of these early reactivation events. In addition, this early detection of reactivated virus is concordant with similar experiments using heat shock for induction of reactivation in vivo, in which virus was also detected at 14 h postinduction (34).

The ranges of PFU found in individual explanted TG are depicted in Fig. 6. To test the recovery of infectious virus in this protocol, we spiked uninfected TG with 10 to 50 PFU of virus just before homogenization and continued to process as usual. The number of PFU recovered from spiked sam-

TABLE 3. Infectious virus production during explant reactivation

Virus production in TG	Result for virus strain or mock infection at indicated h p.e.													
	KOS					Mk[a]	Patton							Mk
	2	14	16	18	24	24	2	14	16	18	20	24	26	26
No. positive	1	4	0	4	6	0	0	6	10	4	7	4	6	0
No. negative	19	14	18	4	0	3	8	16	12	4	1	0	0	2
Total no. tested	20	18	18	8	6	3	8	22	22	8	8	4	6	2
% Positive	5	22	0	50	100	0	0	27	46	50	88	100	100	0

[a] Mk, mock infected.

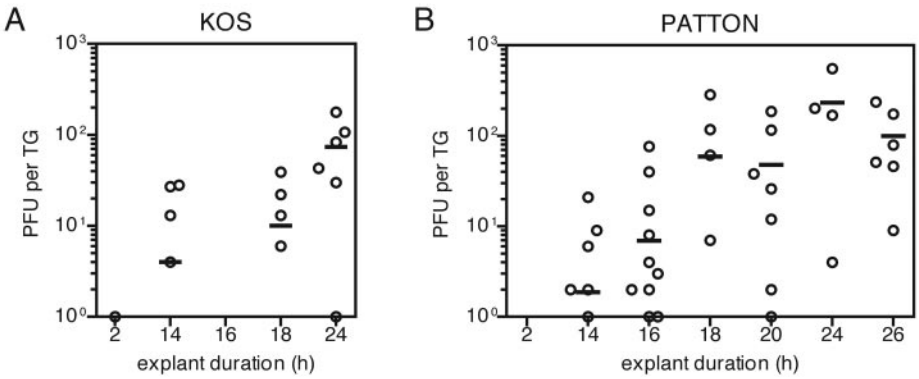

FIG. 6. Infectious virus production during explant reactivation. TG latently infected with wild-type KOS (A) or Patton (B) virus (inoculated with 2×10^6 and 2×10^5 PFU per eye, respectively) were harvested and incubated for the indicated times before being washed, frozen, and processed for infectious virus assay. Circles represent only TG which were positive for infectious virus, but bars represent mean values which include zeros for negative TG. Data are from two experiments for each viral strain.

ples was no more than 50% lower than the titer of the spiked virus (data not shown). Virus could have been lost during the freeze/thaw, homogenization, or sonication procedures or by association with membranes in the spun-out cellular debris. This protocol aimed to ensure that all TG cells were destroyed, preventing any from assembling virus after drugs were removed. Numbers of PFU from TG that produced virus ranged from 1 to 552, with no obvious differences in distribution between KOS and Patton. Although very low amounts of PFU were detected in some TG even at 24 h p.e., there is clearly a time-dependent increase in mean PFU produced. The yield of infectious virus at 24 h p.e. was lower than that produced by TG explanted for 48 h, which typically achieved complete obliteration of the monolayer in the same protocol (>1,500 PFU). This suggested that by 48 h p.e., virus had time to significantly multiply and spread within TG. We intended the 2-h explant treatment to be an additional negative control but were surprised to find 1 PFU of virus detected in a single TG for strain KOS (Table 3 and Fig. 6). Possible sources of this virus are considered in Discussion.

The demonstration that virus was reproducibly present in some explanted TG as early as 14 h p.e. and present in all TG by 24 h p.e. suggested that reactivating virus could complete two or even three rounds of replication within 48 h. This was in agreement with the data from ISH and RT-PCR studies, which indicated that removing blocks to DNA replication and packaging led to much higher levels of expression over 48 h, whereas comparing ACV treatment to WAY-150138 treatment showed little or no difference. Viral DNA content of explanted TG also displayed large increases in untreated TG samples compared to drug-treated samples. Taken together, these data strongly suggest that production of infectious virus, followed by viral spread, accounts for the bulk of increase in RNA expression detected after 24 h p.e. in untreated, explanted TG.

DISCUSSION

In early, classic studies, investigators used metabolic inhibitors of DNA replication, transcription, or protein synthesis to probe the gene expression patterns of HSV in cell culture (14, 27, 42, 46). Such pharmacological interference can identify events that are critical to measured variables and avoid the complexity of overlapping activities. In this report, we employed ACV and WAY-150138 to test the hypothesis that DNA replication augments IE and E gene expression during reactivation from latency over 48 h and to distinguish events occurring in initial reactivating cells from those presumably resulting from secondary infections.

While viral DNA levels displayed a significant increase between ACV and WAY-150138 treatments at 48 h p.e., suggestive of viral DNA that is replicated in primary reactivating cells, IE and E gene RNA levels measured by ISH or RT-PCR exhibited little or no differences. However, there were large increases in viral DNA and RNA in untreated ganglia compared to latent or drug-treated ganglia, which strongly suggest that progeny virus initiated secondary infections well before 48 h p.e. This was confirmed by the reproducible detection of infectious virus as early as 14 h p.e.

We have repeated and extended the results of Kosz-Vnenchak et al. (17). In those ISH studies, when untreated TG were compared to TG in which viral DNA replication was inhibited, a large decrease in IE and E gene expression was observed and interpreted as evidence that DNA replication potentiated gene expression during reactivation. Our use of WAY-150138 in ISH and RT-PCR studies provides strong evidence that the great majority of gene expression detected by ISH in untreated tissues at 48 h p.e. is due not to viral DNA replication in primary reactivating cells but to productive infection of secondary cells by reactivated virus. The ISH signals were much less intense in cells from drug-treated TG than in cells from untreated TG (data not shown), which suggests restricted expression in primary reactivating cells and more typically robust, productive infections in secondary cells. This is consistent with the notion that expression from the latent, nucleosomal genome is limited, perhaps by the absence of VP16 or other facilitating factors. Kosz-Vnenchak et al. (17) had disfavored the possibility of spread for several reasons. It had been reported that viral DNA replication (36), production of infectious virus (37), and staining of multiple, adjacent cells with anti-HSV-1 sera (47) were not observed before 48 h p.e., sug-

gesting that 48 h marked the end of the first cycle of viral replication. The inability to detect DNA replication or HSV antigens at times before 48 h p.e. was likely due to a lack of sensitivity, but the inability to detect virus at 24 h p.e. in a nearly identical system is harder to explain, because we easily detected virus between 14 and 24 h p.e. The hybridization of both LATs and productive-cycle genes in the same ganglion cells in studies by Kosz-Vnenchak et al. (17) was also offered as evidence that events were occurring in initial reactivating cells. It was assumed that LATs would decrease after initial reactivation and adopt a pattern wherein they would be regulated as a late gene in secondarily infected cells, as occurs during productive infection of cell culture (35; S. A. Rice and D. M. Knipe, unpublished data). Instead, the finding of relatively strong hybridization of LATs in the same cells as IE and E genes interestingly suggests that the division between latent and reactivating gene programs is not a hard line and leaves the door open for LATs to play a role in the reactivation process.

In addition to supporting our ISH results, the RT-PCR experiments provided quantitative information on RNA expression levels in individual TG. Whereas some studies have relied on pooling ganglia (24, 28, 43) and have rarely detected productive-cycle gene expression during latency (9, 24, 28, 43), our sensitive assays have detected genes from all kinetic classes in individual TG in this study and others (2, 3, 18, 19). Although the variability observed between individual TG can impede achievement of statistical significance for certain comparisons, it provides a clear picture of the natural variation among latent and reactivating ganglia.

We had previously detected *ICP4*, *ICP0*, and *tk* RNA in ganglia latently infected with strain KOS (2, 3, 19) and herein have extended these analyses to strain Patton. To our knowledge, this is the first reported detection and quantification of *ICP8* RNA during HSV latency and reactivation, although transcripts from the varicella-zoster virus *ICP8* homolog have been detected in human ganglia latently infected with varicella-zoster virus (23). It is interesting to note that *ICP8* was expressed at the highest levels of any productive-infection gene during latent infection. ICP8 is essential for productive infection, playing vital roles in DNA replication as well as transcription (1, 8, 13).

Increases in viral DNA by 24 h p.e. (9) or 48 h p.e. (36) have been reported previously for similar systems; however, these data were not strictly quantitative and could not distinguish between primary reactivation and spread. The use of WAY-150138 permitted such a distinction. The increases measured in viral DNA and RNA over 48 h in the presence of WAY-150138 could be due to accumulation of molecules in a few initial reactivating cells or to accruing numbers of reactivating cells. Similarly, the upward trend in PFU over time in untreated TG could result from either a few cells with large burst sizes or many cells with small burst sizes. Immunohistochemical studies of explanted, ACV-treated ganglia have indicated that the number of single, HSV-antigen-staining neurons increases over the course of 4 days of explant (33). Thus, at least a portion of the increased expression is likely due to new neurons being induced to reactivate over time.

Production of infectious virus was shown to occur reproducibly as early as 14 h p.e. Although this contradicts one report of a similar explant system (37), it concurs with another (33) and with HSV reactivation induced by hyperthermia (34). We were surprised to detect a single PFU produced by a KOS-infected TG explanted for only 2 h. Although contamination is a possible source of the virus in this sample, homogenates in other wells on the same 6-well plate were negative for virus and additional negative controls revealed no PFU. Survival of a latently infected cell which produced virus after the explant period is unlikely because such a cell would have had to withstand freezing, homogenization, and sonication. Three other potential explanations are spontaneous reactivation, a new infection (perhaps transmitted by a cage-mate with reactivated virus), or evolution of a mutated virus unable to maintain latency. These all would be controversial, in that 20 years of studies have failed to find evidence for infectious virus in murine TG latently infected with most viral strains (enumerated in reference 19). However, two TG latently infected with strain 17*syn*$^+$ were recently reported to each yield a single PFU (32) (although these TG were neither frozen nor sonicated, making the survival of a latently infected cell more plausible, as described above). Various avenues of study have suggested that low levels of HSV antigens exist in latently infected TG (2, 10, 16, 19). Based on these results, we are intrigued by the possibility that, in rare instances, 2 h is sufficient time for infectious virus to be assembled in reactivating neurons where viral genes have been expressed. Studies with a temperature-sensitive packaging mutant in cell culture have shown that approximately 1 h permits recovery of virus after downshift from a nonpermissive temperature to a permissive one (6). Follow-up studies indicate that viral DNA can be packaged even in the presence of ACV, suggesting that active DNA replication is not a requirement for genome packaging (5). Thus, we suggest that a latently infected cell could be primed for virus production with low but sufficient levels of progeny genomes and virion components synthesized and thus could theoretically complete assembly within 2 h of the stimulus. As interesting as the possibilities are, in all cases this event would be considered extremely rare, and we have not observed it more than once.

Although our results have precipitated a reinterpretation of the results of Kosz-Vnenchak et al. (17), the model they put forth for novel regulatory mechanisms and a possible role for viral DNA replication in gene expression in neurons still may be viable at early times following reactivation stimuli. There is already supporting evidence for the ideas they presented, with several other reports suggesting a novel program of gene expression during reactivation (9, 43) and supporting a role for viral DNA replication in potentiating gene expression in certain cells (26, 41). Tal-Singer et al. (43) detected E and leaky L genes (*tk*, *ICP6*, and *VP5*) at 4 h p.e., before IE genes (*ICP4*, *ICP0*, *ICP27*, *ICP22*, and *ICP47*), during HSV reactivation from latently infected TG. Devi-Rao et al. (9) also detected *VP5* early, by 1 to 4 h p.e., but detected *ICP4* and *ICP27* at 2 and 4 h p.e., respectively. Working in vitro, Nichol et al. (26) found that ACV inhibited IE and E gene expression in primary neuronal cultures but not in Vero cells, while Summers and Leib (41) observed decreased IE and E promoter activity in primary cells (mouse embryo fibroblasts and dissociated ganglia) but not Vero cells when both were treated with DNA

synthesis inhibitors. The explanations for these observations and how to reconcile such results remain to be elucidated.

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AUTHOR'S CORRECTION

Herpes Simplex Virus 1 Immediate-Early and Early Gene Expression during Reactivation from Latency under Conditions That Prevent Infectious Virus Production

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Volume 79, no. 23, p. 14516–14525, 2005. Page 14523, column 1, first full paragraph, lines 11 to 15: The sentence beginning "Taken together" should read "Taken together, these data strongly suggest that production of infectious virus, followed by viral spread, for which evidence has recently been provided at 36 h p.e. (33), accounts for the bulk of increase in RNA expression detected after 24 h p.e. in untreated, explanted TG."

Page 14525: The following should be added to the Discussion. "After these studies were completed, Sawtell and Thompson (33) presented immunohistochemical evidence of spread at 36 h p.e. These data and their detection of infectious virus by 22 h p.e. led those authors to suggest that earlier studies addressing transcriptional events in explanted ganglia from 24 to 48 h p.e. should be interpreted with caution."