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Quantitative Polymerase Chain Reaction Analysis of Herpes Simplex Virus DNA in Ganglia of Mice Infected with Replication-Incompetent Mutants

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To study the roles of viral genes in the establishment and maintenance of herpes simplex virus (HSV) latency, we have developed a polymerase chain reaction assay that is both quantitative and sensitive. Using this assay, we analyzed the levels of viral DNA in trigeminal ganglia of mice inoculated corneally with HSV mutants that are defective for virus replication at one or more sites in mice and for reactivation upon ganglionic explant. Ganglia from mice infected with thymidine kinase-negative mutants, which replicate at the site of inoculation and establish latency but do not replicate acutely in ganglia or reactivate upon explant, contained a range of levels of HSV DNA that overlapped with the range found in ganglia latently infected with wild-type virus. On average, these mutant-infected ganglia contained one copy of HSV DNA per 100 cell equivalents (ca. 10^4 molecules), which was 50-fold less than the average for wild-type virus. Ganglia from mice infected with a ribonucleotide reductase deletion mutant, which is defective for acute replication and reactivation upon ganglionic explant, also contained on average one copy of HSV DNA per 100 cell equivalents. We also detected substantial numbers of HSV DNA molecules (up to ca. 10^3) in ganglia of mice infected with an ICP4 deletion mutant and other replication-negative mutants that are severely impaired for viral DNA replication and gene expression. These results raise the possibility that such mutants can establish latency, which could have important implications for mechanisms of latency and for vaccine and antiviral drug development.

Herpesviruses, like retroviruses, papillomaviruses, and hepadnaviruses, establish latent infections in their hosts, forming lifelong reservoirs of recurrent disease that resist cure (1). Herpes simplex virus (HSV) latency (12, 27) is preceded by productive infection at the periphery of a mammalian host. The virus enters nerve endings and migrates to ganglionic nuclei, where productive infection can again ensue. With time, latency develops, in which viral DNA (9, 23) but no infectious virus is present. However, infectious virus can be reactivated by certain stimuli or by explant of ganglia.

The roles of productive infection processes in the establishment and maintenance of HSV latency have not been determined. Considerable understanding of the productive infection cycle of HSV and the roles of specific genes has been gained through the use of engineered mutants with defects in functions important for virus replication and gene expression (24). When such replication-defective mutants have been tested in a mouse model of latency, they have failed to reactivate from explanted ganglia (14, 16). It has not been possible to determine whether the failure of these mutants to reactivate is due solely to the replication defect during reactivation or also to a requirement for specific viral productive infection processes in the establishment and maintenance of latency. Such processes could include DNA replication or steps in the regulatory cascade of virus gene expression.

Certain mutants that are replication competent in cell culture fail to reactivate from explanted ganglia in a mouse model. These include thymidine kinase-negative (tk^-) mutants and certain thymidine kinase-deficient mutants. These mutants do establish and maintain latency, as evidenced by expression of latency-associated transcripts (5, 17, 29) and their ability to rescue replication-negative virus following superinfection of dissociated ganglia (5) or to be rescued by superinfection with wild-type virus (8), demonstrating the presence of biologically active virus genomes. However, in at least two cases (8; K. Hicks, D. Yager, and D. Coen, unpublished results), *tk* mutant genomes were not detected reliably by blot hybridization methods.

To quantify the number of viral genomes in ganglia from mice infected with various HSV mutants, we have developed a quantitative polymerase chain reaction (PCR) assay for HSV DNA. This has allowed us to begin to address the question of whether certain productive infection processes are required for establishment and maintenance of latency by determining how much, if any, viral DNA is resident in ganglia of mice infected with replication-incompetent mutants.

MATERIALS AND METHODS

Cells and viruses. The viruses used in this study are listed in Table 1. The wild-type strain was HSV type 1 KOS, which was propagated and assayed on Vero cells, maintained as described previously (31). Mutants *dl*sptk and *dl*sactk (5) engineered to contain 360- and 4-base-pair deletions in the HSV *tk* gene, respectively, were propagated and titers were determined on Vero cells. Mutant ICP6Δ (11) contains a 2.9-kilobase-pair deletion in the gene encoding ICP6, the large subunit of HSV ribonucleotide reductase. This mutant and the D14 cells (10) on which it was propagated and its titer determined were kindly provided by D. Goldstein and S. Weller. High-titer stocks of mutants *d*120 and *n*12, which contain a 4.1-kilobase-pair deletion and a nonsense mutation in the gene encoding ICP4, respectively (6, 7), were generously provided by N. DeLuca and P. Schaffer. A high-titer

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TABLE 1. Amount of HSV DNA in ganglia of mice infected with HSV mutants

Virus	Gene altered	Avg no. of[a]:		Range (copy per cell)[b]
		Copy per cell	Molecules per ganglion	
KOS	None	0.5	5×10^5	0.01–1
*dl*sactk	Thymidine kinase	0.01	10^4	0.0001–0.1
*dl*sptk	Thymidine kinase	0.01	10^4	0.0001–0.03
ICP6Δ	Ribonucleotide reductase	0.01	10^4	0.0001–0.03
*d*120	ICP4	0.0001	10^2	0–0.001
*n*12	ICP4	0.0001	10^2	0–0.0005
*d*27-1	ICP27	0.0001	10^2	0–0.0005
ΔX14	DNA polymerase	0.0001	10^2	0–0.001

[a] Values shown are the averages of measurements analyzed as in Fig. 1 to 4 on at least six ganglia from mice infected with each virus. Measurements were included only when they came from experiments in which all of at least three negative controls yielded no HSV-specific signal. The average number of molecules per ganglion value was calculated on the basis of about 10^6 cells per ganglion.

[b] Values represent the ranges of measurements obtained from individual ganglia analyzed as described in footnote *a*.

stock of mutant *d*27-1 (22), which contains a 1.6-kilobase-pair deletion in the gene encoding ICP27, was graciously provided by S. Rice and D. Knipe. Mutant ΔX14 (18), which contains a 1.2-kilobase-pair deletion in the gene encoding the HSV DNA polymerase, and the DP6 cells on which it is propagated and its titer determined (18) were kindly provided by A. Marcy.

Infections of mice. Seven-week-old CD-1 mice (Charles River Breeding Laboratories, Kingston, N.Y.) were mock inoculated or inoculated at the cornea with wild-type virus or the mutants as described before (16, 28) at a dose of 2×10^6 PFU/eye with the exception of mutant *d*120, which was inoculated at 10^7 PFU/eye.

Isolation of ganglionic DNA. To prepare ganglionic DNA, ganglia were removed from mice >30 days after infection, using instruments that had been treated with DNase and autoclaved to avoid introduction of contaminants. Only ganglia from animals infected with a given mutant were taken on any given day. Methods for DNA extraction, including additional precautions to avoid contamination, have been described in detail (3). Briefly, each ganglion was placed in a screw-cap microcentrifuge tube and digested overnight at 50°C with 100 μg of proteinase K per ml in proteinase digestion buffer (20 mM Tris chloride [pH 7.4], 20 mM EDTA [pH 8], 0.5% sodium dodecyl sulfate). The sample was gently mixed and phenol-chloroform extracted, the organic phase was back-extracted once with proteinase digestion buffer, and the pooled aqueous phases were chloroform extracted twice. Ammonium acetate was added to the final aqueous phase to 2.5 M, and the DNA was precipitated with ethanol, washed once with 70% ethanol, and suspended in 10 mM Tris chloride–1 mM EDTA, pH 7.5. The intactness and concentration of the DNA were estimated by agarose gel electrophoresis alongside known amounts of standard DNA. By performing extraction procedures gently, the vast majority of the DNA migrated as high-molecular-weight species.

PCR. The standard PCR, using *Taq* DNA polymerase (25), was modified to permit quantification of HSV DNA. The details of the procedure, including measures to preclude contamination with exogenous DNA sequences, are presented elsewhere (3). Briefly, 100-ng samples of ganglionic DNA or, as standards, 100-ng samples of mouse tail DNA spiked with known amounts of HSV DNA were mixed with 50 pmol each of two pairs of primers. One primer-pair, CT TAACAGCGTCAACAGCGT and CAAAGAGGTGCGGG AGT, was specific for the HSV *tk* gene (13, 19, 30) and the other, AGTGTGCGGGGATGCAGT and ACGCGAGAGC CCCACGTA, was specific for the single-copy mouse adipsin gene (20). These DNAs were then assembled into 100-μl reactions containing 50 mM KCl, 10 mM Tris chloride (pH 8.4), 4.5 mM $MgCl_2$ (which was found to be optimal for this primer-template combination), 100 μg of gelatin per ml, 200 μM concentrations of each deoxynucleoside triphosphage, and 4 U of *Taq* DNA polymerase. PCR amplification was then performed for 30 cycles, with denaturation for 1 min at 94°C, annealing for 2 min at 55°C, and extension for 3 min at 72°C, with a final additional extension of 7 min.

Analysis of PCR products. A 10-μl portion from each PCR amplification was electrophoresed on a 12% nondenaturing polyacrylamide gel, visualized by ethidium bromide staining, transferred to a nylon filter (Gene-Screen Plus; New England Nuclear-Dupont), and UV cross-linked as described before (2). The filter was then prehybridized at 50°C for 2 h in 6× SSPE (0.54 M NaCl, 60 mM NaH_2PO_4 [pH 7.4], 60 mM EDTA [pH 7.4])–1% sodium dodecyl sulfate–5× Denhardt solution–200 μg of native salmon sperm DNA per ml–200 μg of denatured salmon sperm DNA per ml–10% dextran sulfate. The filter was then hybridized in the same solution with an oligonucleotide probe specific for the HSV PCR product, CAGATCTTGGTGGCGTG, radiolabeled with T4 polynucleotide kinase as described, for 2 h at 50°C. The filter was washed four times at room temperature (5 min each time) with 6× SSPE–1% sodium dodecyl sulfate, four times at 50°C (10 min each time) with 6× SSPE–1% sodium dodecyl sulfate, and once at room temperature with 6× SSPE. The filter was then exposed to Kodak XAR-5 film. When intensifying screens were used, the film was preflashed. For certain experiments, nylon filters were stripped of the HSV-specific probe by boiling in 0.015 M NaCl–0.0015 M sodium citrate–1% sodium dodecyl sulfate for 20 min and then reprobed as above with radiolabeled oligonucleotide specific for the mouse adipsin PCR product, AGTCGAAGGTGTGG TTAC. Autoradiographic signals were analyzed by densitometry with an LKB laser scanner.

RESULTS

Development of a quantitative PCR assay. Initial slot blot hybridization experiments to quantify HSV DNA in ganglia from mice infected with certain HSV mutants proved to be too insensitive to determine whether these ganglia contained HSV DNA (16; Hicks et al., unpublished results). We therefore converted the PCR, an assay capable of detecting single molecules of DNA (25), to an assay that can quantify rare DNAs. This was accomplished by varying several of the parameters of the standard PCR assay. Figure 1 shows the results obtained in a reconstruction experiment designed to test the quantitative aspects of the PCR assay. Various amounts of HSV DNA were mixed with 100 ng of mouse DNA and two pairs of primers. One primer pair was specific for the HSV *tk* gene (13, 19, 30) and the other was specific for the single-copy mouse gene adipsin (20). The mixtures were subjected to 30 cycles of PCR, and the resulting products were separated on a polyacrylamide gel and transferred to a nylon filter. When the filter was probed with a radiolabeled oligonucleotide specific for the HSV *tk* PCR product, the autoradiographic signal of this product decreased monotonically with increasing dilution of the amount of virus DNA

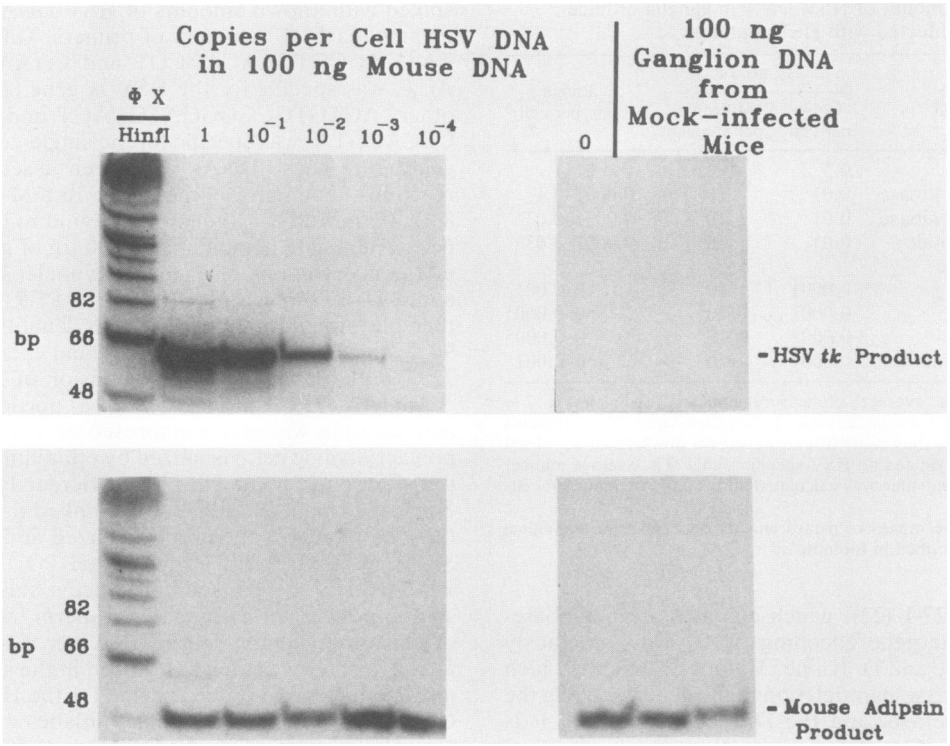


FIG. 1. Quantitative PCR analysis of HSV DNA. (Top) Quantitative detection of HSV DNA. Tenfold dilutions of HSV-1 strain KOS DNA were mixed with 100 ng of DNA from the tails of uninfected mice so that the viral DNA was present at 1 copy per mouse cell equivalent (0.33 pg of HSV DNA), 0.1 copy, etc., as indicated at the top of each lane. As negative controls, 100 ng of mouse tail DNA alone and 100 ng of DNA from each of two trigeminal ganglia of mock-infected mice were also included. These samples were amplified by PCR, using oligonucleotide primers specific for a portion of the HSV *tk* gene and a second set of primers specific for the mouse adipsin gene. The PCR products were electrophoresed on a polyacrylamide gel alongside radiolabeled *Hin*fI-digested ϕX174 DNA, which served as size markers. The DNA was transferred to a nylon filter and probed with a radiolabeled oligonucleotide specific for the HSV PCR product. (Bottom) Detection of the internal control mouse PCR product. The PCR products shown were analyzed by stripping the filter and probing with an oligonucleotide specific for the mouse adipsin gene PCR product.

(Fig. 1, top). In contrast, the PCR signal corresponding to the mouse adipsin product, which could be visualized routinely by ethidium bromide staining (not shown), remained relatively constant. This is shown as detected by probing with an appropriate radiolabeled primer (Fig. 1, bottom). When densitometric analysis of the HSV signal from similar reconstruction experiments was performed, a nearly linear log-log relationship between signal intensity and the amount of viral DNA was obtained (see Fig. 3B for an example). This empirically derived relationship was convenient in permitting analysis of a wide range of amounts of HSV DNA.

The assay could readily detect amounts of HSV DNA mixed with mouse DNA at 10^{-4} copies per cell equivalent (most obvious in Fig. 2B), which corresponds to two molecules of HSV DNA (based on Poisson distribution principles, it is fairer to say that the amount of HSV DNA present in this mixture is one or a few molecules). A zero-copy reconstruction control and ganglionic DNA prepared from two mock-infected mice yielded similar mouse adipsin signals, but no detectable HSV-specific signal (Fig. 1). These three negative controls validated the rigorous measures used to preclude contamination (3) and were similarly negative in all experiments reported below. The mouse adipsin internal control both verified true negatives and allowed standardization of PCRs that varied in efficiency or amount of input DNA. Indeed, increasing or decreasing the amount of a mixture of HSV and mouse DNA as much as 10-fold did not change the ratio of the HSV and mouse PCR signals (not shown).

Quantification of DNA from wild-type and *tk* mutant latently infected ganglia. We next used the quantitative PCR assay to measure the amounts of HSV DNA in trigeminal ganglia from CD-1 mice following corneal inoculation with wild type HSV-1 strain KOS and various mutants of this strain. Thirty days or more after inoculation, at which time the wild-type virus has established a reactivatable latent infection (16), ganglia were harvested. DNA was prepared and measured for its content of HSV DNA by using the assay. Ganglia from mice latently infected with wild-type virus contained on average 0.5 copy per cell equivalent of HSV DNA (ca. 5×10^5 molecules per ganglion), although the amount of HSV DNA per ganglion ranged from below 0.1 copy per cell to about 1 copy per cell in individual ganglia (Fig. 2A, Table 1). This variation evidently is a property of the ganglia themselves; it could not be ascribed to variability in the PCR assay as repeat assays of different samples from individual ganglia yielded similar results (not shown). Both the average value and the range were very similar to those obtained for ganglia of CD-1 mice following corneal inoculation with wild-type strain KOS, using slot-blot hybridization methods (16).

Figure 2B shows the results obtained with ganglia from mice infected with mutant *dl*sactk. This mutant and mutant

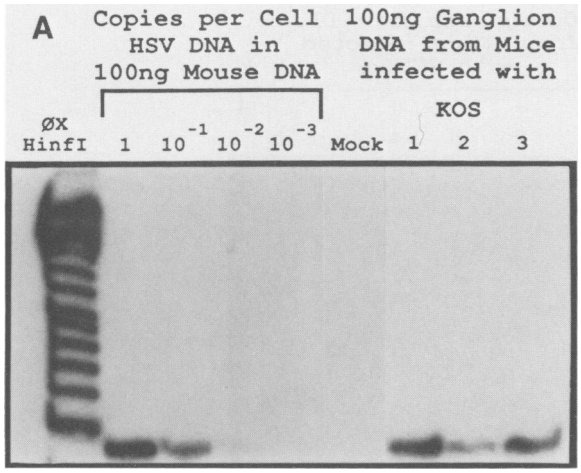
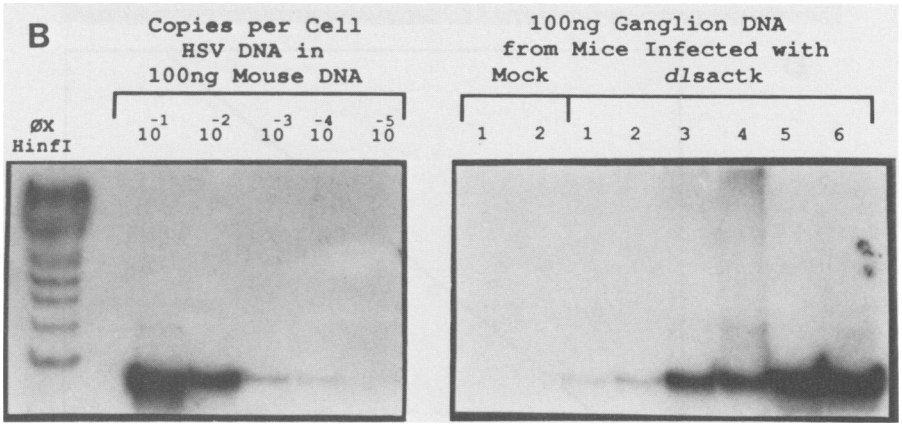


FIG. 2. Quantitative PCR analysis of HSV DNA in trigeminal ganglia from mice infected with wild type-strain KOS (A) and a tk^- mutant (B). The indicated mixtures of HSV DNA and uninfected mouse DNA and the samples of trigeminal ganglion DNA from mock-infected mice or mice infected with KOS and mutant *dl*sactk were analyzed as described in the legend to Fig. 1.



*dl*sptk, which contain deletions in the HSV *tk* gene and do not express thymidine kinase enzyme, grow to wild-type titers in the mouse eye following corneal inoculation, but do not replicate detectably in trigeminal ganglia (5). They do not reactivate upon ganglionic explant, but do establish latent infections, as evidenced by expression of latency-associated transcripts and their ability to rescue replication-negative virus following superinfection of dissociated ganglia (5). As expected from these results, ganglia from mice latently infected with the tk^- mutants contained substantial numbers of HSV DNA molecules, ranging up to 0.1 copy per cell (ca. 10^5 molecules per ganglion) (Fig. 2B). Although, as was the case with wild-type virus, there was substantial variation in the amounts of HSV DNA in individual ganglia, the average amount of HSV DNA present in ganglia of mice infected with either tk^- mutant was 0.01 copy per cell (Table 1). Thus, the average amount of DNA in ganglia from tk^- mutant-infected mice was less than that from KOS-infected mice, but the ranges of values overlapped.

Quantification of DNA from mice infected with a ribonucleotide reductase mutant. Figure 3 shows the results obtained with ganglia from mice infected with mutant ICP6Δ. This deletion mutant fails to specify HSV ribonucleotide reductase activity (11). Although it can replicate in many cell types in culture (11), it is highly defective for replication in mouse cells at 38°C (14). Following corneal inoculation, ICP6Δ replicates very poorly in the eye, achieving barely detectable titers. It fails to achieve detectable titers in trigeminal ganglia during the 4 days following inoculation or to reactivate from these ganglia upon explant 30 days after inoculation (14). Despite the replication defects of ICP6Δ, levels of HSV DNA averaging 0.01 copy per cell equivalent (ca. 10^4 molecules per ganglion) could be detected in ganglia of mice infected with this mutant (Fig. 3, Table 1). Thus, minimal replication at the site of inoculation suffices for tens of thousands of viral DNA molecules to reach the trigeminal ganglion and be maintained there stably.

ICP4, ICP27, and *pol* mutants. Based on these results, we next asked whether any viral replication is necessary for viral DNA to reach the trigeminal ganglion and be maintained there. We therefore tested several mutants that are replication negative because of defects in viral gene expression and DNA replication. These mutants include *d*120 (6), which contain a large deletion in the gene encoding the major regulatory protein, ICP4; *n*12 (7), which contains a nonsense mutation in the ICP4 gene; *d*27-1 (22), which contains a deletion in the gene encoding the regulatory protein, ICP27; and ΔX14 (18), which contains a deletion in the HSV DNA polymerase gene. The ICP4 mutants are severely restricted in the expression of early and late genes, while the ICP27 and DNA polymerase mutants are severely restricted in the expression of late genes. All of the mutants fail to synthesize detectable levels of viral DNA except the ICP27 mutant, which nonetheless is very restricted in its DNA synthesis (22). These replication-incompetent mutants are routinely propagated on cell lines containing viral genes that can complement the mutations; importantly, mutants *d*120, and *d*27-1 carry deletions that cannot be rescued by the genes resident in the cell lines. As a result, no detectable wild-type

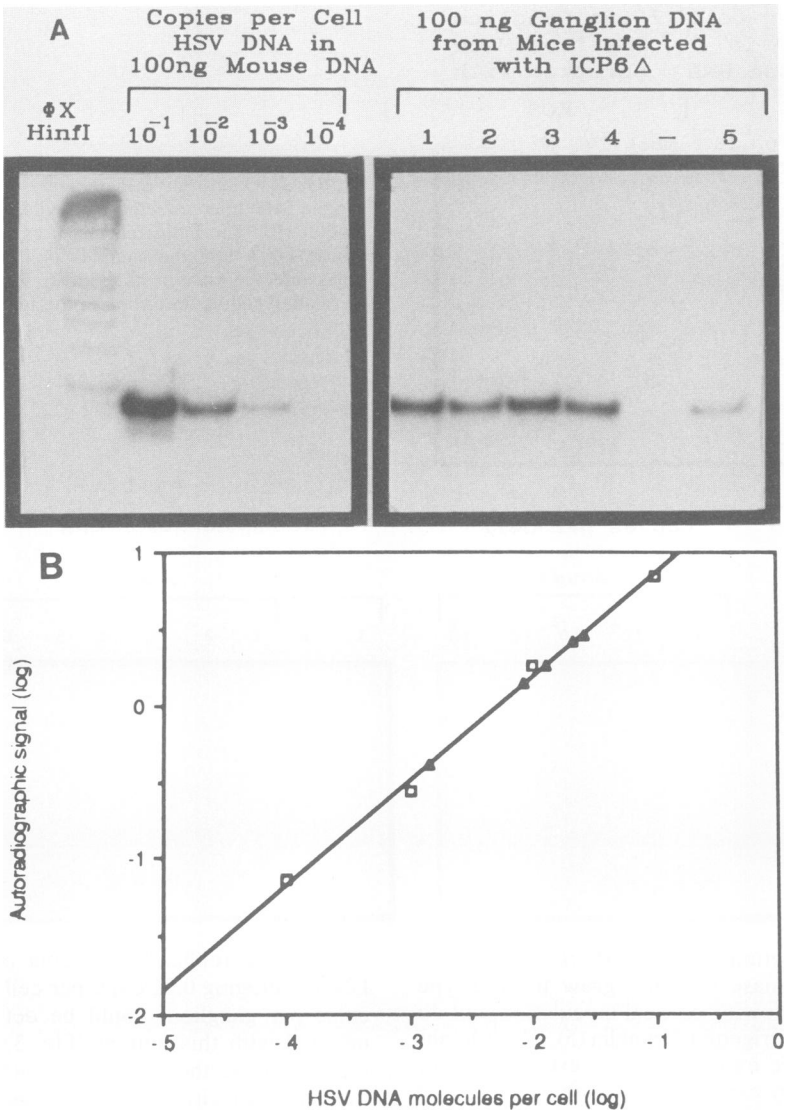


FIG. 3. Quantitative PCR analysis of HSV DNA in trigeminal ganglia from mice infected with a ribonucleotide reductase mutant. (A) The indicated mixtures of HSV DNA and uninfected mouse DNA and the samples of trigeminal ganglion DNA from mice infected with HSV mutant ICP6Δ were analyzed as described in the legend to Fig. 1. (B) Densitometric analysis of quantitative PCR. The autoradiographs shown in panel A were scanned on an LKB laser scanner, and integrals of the signals were computed. The logs of the computed signals were plotted relative to the logs of the amounts of HSV DNA present in the mixtures with mouse DNA, as indicated by the open squares. The signals from the ganglia are shown as closed triangles. The mouse adipsin signals were indistinguishable in all samples.

virus is present in stocks of these mutants (6, 22; N. DeLuca, S. Rice, and D. Knipe, personal communications).

Despite the severe defects of the mutant viruses, HSV DNA could be detected readily in ganglia from mice infected 30 days or more previously with the mutants (Fig. 4, Table 1). As stated previously, data were used only from experiments such as that shown in Fig. 4 in which all negative controls (at least three per experiment) gave no HSV signal, but gave similar mouse adipsin signals (not shown). Different aliquots of ganglionic DNA from mice infected with these mutants could contain no detectable DNA or varying amounts of DNA. In particular, up to 0.001 copy per cell (ca. 10^3 molecules per ganglion) was found in the ganglia of mice that had been infected with *d*120 (Fig. 4). Repeated analyses of DNA from specific ganglia revealed that certain samples that scored negative in one PCR assay often scored positive in others, which would be expected from a Poisson distribution of HSV DNA molecules among aliquots of ganglionic DNA. Taking into account all of the assays, levels of HSV DNA averaging 0.0001 copy per cell (ca. 10^2 molecules per ganglion) were detected in ganglia of mice that had been infected with any of the replication-negative mutants.

DISCUSSION

We describe here the development of a quantitative PCR assay for HSV DNA and its use in quantifying DNA in ganglia of mice infected with HSV mutants. As summarized in Table 1, we found substantial amounts of HSV DNA not only in ganglia of mice infected with tk^- mutants known to establish latent infections (5) but, more surprisingly, in mice infected with the replication-defective mutant, ICP6Δ, and

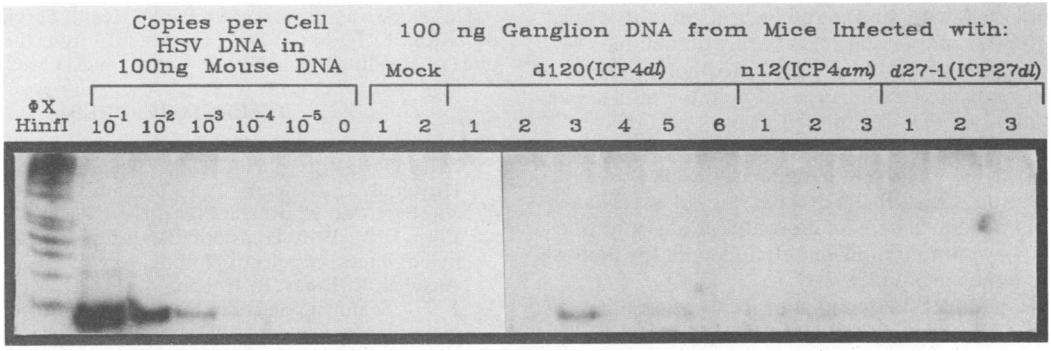


FIG. 4. Quantitative PCR analysis of HSV DNA in trigeminal ganglia from mice infected with immediate-early regulatory gene mutants. The indicated mixtures of HSV DNA and uninfected mouse DNA and samples of trigeminal ganglion DNA from mock-infected mice or mice infected with HSV mutant *d*120, *n*12, or *d*27-1 were analyzed as described in the legend to Fig. 1.

even in mice infected with replication-negative mutants. Below we discuss aspects of the quantitative PCR assay, how the amounts of HSV DNA found in ganglia relate to biological properties of the tk^- and ICPΔ mutants, and the possible implications for mechanisms of latency and for vaccine and antiviral drug development of our finding of HSV DNA in ganglia of mice infected with replication-negative mutants.

Quantitative PCR assay for HSV DNA. To develop the quantitative PCR assay, the most important modifications to the standard assay (25) were optimizing the $MgCl_2$ concentration, increasing the amount of DNA polymerase, and limiting the amount of template DNA. These and other parameters are discussed in more detail elsewhere (3). Under the conditions described here, the nearly linear log-log relationship between autoradiographic signal and amount of DNA in the range of roughly 10^{-4} and 1 copy per cell sometimes breaks down above 10^{-1} copy per cell; however, decreasing the number of PCR cycles allows linearity in the range of 10^{-1} through 10 copies per cell (E. Pelosi and D. Coen, unpublished results).

For ganglia from mice infected with wild-type virus (Fig. 2A, Table 1) and for ICP0 deletion mutants (not shown), we obtained values for HSV DNA content with the PCR assay that were very similar to those obtained with slot blot hybridization (16). These results also help establish the validity of the PCR assay. The PCR assay was nevertheless far more sensitive than slot blot hybridization, with which it was difficult to distinguish signal from background below 0.01 to 0.1 copy per cell (16) and which failed to determine whether ganglia from mice infected with mutant *n*12 contained HSV DNA (16). The PCR assay answered that question (Fig. 4).

The use of PCR to detect viral nucleic acids in clinical specimens is growing rapidly. Two features of the assay described here may be useful in clinical settings. The internal control (in clinical specimens this could be a single-copy human gene) is invaluable both in eliminating false-negatives and in aiding quantification. The quantitative assay is helpful in assessing the presence of contaminating sequences and the likelihood that a positive result is due to contamination. In our hands, when contamination occurred, it was usually at the level of one or a few molecules per sample, which would be easy to distinguish from true positives in many clinical situations.

After the present study was completed, a PCR assay to quantify human immunodeficiency virus DNA was reported (21). This assay, which uses end-labeled PCR primers and thus does not require blotting and hybridization steps, appeared to be as quantitative as the assay described here, but somewhat less sensitive and more likely to detect nonspecific products.

Levels of HSV DNA in ganglia latently infected with tk^- mutants. Our finding that the ranges of the amounts of DNA found in ganglia of mice infected with wild-type strain KOS and the tk^- mutants overlapped supports our previous conclusion (5) that these mutants establish latency. Nevertheless, there was on average 50-fold less DNA per ganglion from the mutant-infected mice than from wild-type-infected mice. In contrast, in situ hybridization experiments detected three- to fivefold fewer latency-associated transcript-positive cells in tk^- mutant latently infected ganglia than in those of wild type (5). The autoradiographic signal per cell was similar between KOS and tk^- mutant-infected ganglia (5); still, there could be fewer HSV genomes per cell in mutant-infected ganglia. It is also possible that more HSV DNA is found in non-neuronal cells in KOS-infected ganglia than in mutant-infected ganglia. These possibilities are under investigation.

Two other reports have estimated the amount of DNA in ganglia latently infected with *tk* mutants. Efstathiou et al. (8), in agreement with our findings, reported that with Southern blot hybridization such ganglia contained much less HSV DNA (undetectable) than did ganglia from wild-type-infected mice. In contrast, Leist et al. (17), using slot blot hybridization, reported that such ganglia when pooled contained amounts of HSV DNA similar to those found in wild-type-infected ganglia, although a comparison of the hybridization signals with known amounts of HSV DNA was not reported.

The differences between these results could be due to differences in mouse strains, routes of inoculation, and/or the *tk* mutants used. Efstathiou et al. (8) and we engineered deletions in *tk* protein-coding sequences away from the UL24 gene, which can be important for virus growth (15). The mutants did not exhibit detectable TK activities (<0.4% of wild type). The mutant analyzed by Leist et al. (17) deleted the *tk* promoter and much of the UL24 gene without deleting *tk* protein-coding sequences. The TK activity of this mutant could not be distinguished from that of mock-infected cells; however, in the assay used, this was 14% that of wild-type-infected cells. We note that levels of TK that are 5 to 10% those of wild-type virus are sufficient to allow acute replication in ganglia and other biological activities (4).

HSV DNA in ganglia of ICP6Δ-infected mice. We were surprised to find levels of HSV DNA averaging 0.01 copy per

cell in mice infected with mutant ICP6Δ, a mutant whose replication at the site of inoculation is barely detectable (14). This average amount of DNA is similar to that of the *tk*$^-$ mutants, which replicate to wild-type titers in the mouse eye and establish latency. This comparison raises two possibilities: (i) barely detectable levels of replication at the site of inoculation deliver HSV DNA to ganglia as well as wild-type levels of replication, and (ii) the ICP6Δ mutant establishes latent infections despite its replication and reactivation defects. Studies to examine ICP6Δ-infected ganglia for biologically active genomes are under way.

HSV DNA is resident in ganglia of replication-negative mutants: possible implications for virus latency and for vaccine and antiviral drug development. The results of Fig. 4 show that neither productive infection, viral DNA replication, nor the normal regulatory cascade of gene expression is required for hundreds or thousands of viral DNA molecules to reach the trigeminal ganglion and be maintained there stably, a critical component of the establishment and maintenance of latency. Although we cannot comment on the physical state or biological activity of the HSV DNA in ganglia from mice infected with replication-negative mutants, our results raise the possibility that mutants that are severely restricted for productive virus replication and gene expression can establish latency. This is consistent with recent studies (M. Kosz-Vnenchak, D. M. Coen, and D. M. Knipe, submitted for publication) that show that HSV *tk* deletion mutants can establish latency in the face of severely restricted lytic gene expression. They also are consistent with results from Steiner et al. (26), who studied an HSV mutant defective in the virion transactivating factor Vmw65 (α-transinducing factor, VP16). This mutant replicated poorly in the mouse eye (but evidently not as poorly as ICP6Δ [14]) and not detectably in mouse ganglion, yet could reactivate from latency with fair efficiency. Keeping in mind the reservations stated above, these studies taken together suggest that establishment and maintenance of latency do not require lytic pathways of gene expression and that viral replication itself merely permits an increase in the number of virus genomes that can gain access to neuronal nuclei. This contrasts with models in which latency is established by a change in the balance of regulatory activities that operate during productive infection. We think that consideration should be given to two hypotheses. (i) As yet undescribed viral regulatory genes promote the latency pathway. (ii) Establishment of HSV latency is a passive process that is governed by neuronal factors rather than by any de novo synthesized viral gene product.

There is abundant evidence that prevention of HSV replication by vaccines or antiviral drugs can greatly reduce reactivatable latent infections (12). Based on the discussion above, we suggest that this is due solely to decreasing the number of virus genomes that are capable of establishing latent infections. Our results therefore lead to the speculation that chemotherapeutic or immunoprophylactic strategies targeted against lytic functions of the virus may be unable to prevent latency completely.

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