Restricted Expression of Herpes Simplex Virus Lytic Genes during Establishment of Latent Infection by Thymidine Kinase-Negative Mutant Viruses

MAGDALENA KOSZ-VNENCHAK,^{1†} DONALD M. COEN,² AND DAVID M. KNIPE^{1*}

*Departments of Microbiology and Molecular Genetics¹ and Biological Chemistry and Molecular Pharmacology,²
Harvard Medical School, 200 Longwood Avenue, Boston, Massachusetts 02115*

Received 5 June 1990/Accepted 14 August 1990

Infection of cells by herpes simplex virus (HSV) can lead to either lytic, productive infection or nonlytic, latent infection. The factors influencing this infection pathway decision are largely unknown. Thymidine kinase-negative mutant viruses can establish latent infection in neurons of mouse trigeminal ganglia but do not replicate productively in these cells. We show that during the early stages of establishment of latency by these mutants, expression of viral lytic genes is drastically reduced or undetectable as assayed by *in situ* hybridization. Thus, establishment of latent infection by HSV can occur despite severely restricted levels of lytic gene expression. This suggests that the block to productive replication during establishment of latent infection by HSV occurs before or early during the expression of α genes.

Herpes simplex virus (HSV) can establish lytic, productive infection at its initial site of inoculation in a host organism or in cultured cells. In contrast, when the virus spreads to sensory neurons in a host, it can establish a latent infection. Later stimuli can lead to reactivation of infectious virus, release of virus from the neuron and a recrudescence lesion (38).

Expression of HSV gene products during lytic infection is tightly regulated (33). Initially after infection, the α (immediate-early) genes are expressed. The products of these genes induce and regulate the expression of later viral genes. The next class of gene products, the β (delayed-early) gene products, is largely involved in replication of viral DNA. Following viral DNA replication, expression of γ (late) gene products is maximal. In contrast, during latent infection, the only viral gene product expressed abundantly is the latency-associated transcript (LAT; 3, 4, 13, 27, 31, 39, 40). LAT is not essential for establishment of latent infection (16) but appears to play a role in reactivation (15, 17, 37). Although its complete function is not known, LAT expression has provided a marker for latent infection by HSV (2, 23, 42).

The events that occur during the initial infection of neurons, allowing establishment of latent infection, have not been defined. Several hypotheses have been raised to explain the restricted gene expression by HSV during latent infection: (i) lack of necessary cellular transcription factors for viral α gene expression in certain neurons (32); (ii) lack of transport of the virion *trans*-inducing factor to the neuron nucleus, leading to reduced expression of viral α gene products (32); (iii) presence of an inhibitor of α gene expression in neuronal cells (18); (iv) dominance of viral negative regulatory gene products in neurons leading to reduced expression of α or β gene products (20, 26, 41); or (v) inability of the viral replication cycle to progress beyond the expression of α gene products (14), possibly because of lack of the necessary cellular factors. There has been little information with which to discriminate among these models.

One difficulty in the study of the early events that lead to establishment of HSV latency in neurons is that, in most animal models of latency, some productive viral replication is always apparent in the sensory ganglia. Thus, it is difficult to distinguish the events occurring in lytic versus latent infections in cells within these tissues. We and others have reported recently that although thymidine kinase-negative (TK⁻) HSV mutant viruses do not replicate in murine sensory ganglia, they do establish latency in these ganglia (2, 8, 23, 42). Therefore, infection of murine sensory ganglia with TK⁻ mutant viruses provides a system in which the initial events leading to latent infection can be studied without simultaneous lytic infection in the tissue.

MATERIALS AND METHODS

Viruses and infection of mice. The wild-type KOS strain and the TK⁻ mutant viruses *dl*sptk and *dl*sactk have been described previously (2). Methods for corneal inoculation of virus have been described (21).

***In situ* hybridization.** Methods for *in situ* hybridization using ³H- and ³⁵S-labeled DNA probes have been described previously (22). Hybridization data are reported as numbers of positive ganglia, because it was difficult to define individual cells in ganglia infected with wild-type virus owing to the silver grains obscuring cell boundaries (e.g., see Fig. 1 and 2).

Plasmids. Plasmid pK1-2 (5) was used as a probe for ICP4 gene transcripts. Plasmid pBH27 (29) was used as a probe for ICP27 gene transcripts. Plasmid pSP64-ICP8 (E. Villarreal and D. Knipe, submitted for publication) was used as a probe for ICP8 gene transcripts. Plasmid pKOSBamQ (10) was used as a probe for polymerase gene transcripts. The *Eco*RI-*Bam*HI I-I HSV DNA fragment (9, 11) was used as a probe for glycoprotein C gene transcripts. Plasmid pIPH (22) was used as a probe for LAT transcripts. Plasmid pSHZKB was constructed by subcloning the 0.75-kilobase *Kpn*I-*Bam*HI viral DNA fragment from plasmid pSHZ (25, 28) and used as a probe for ICP0 gene transcripts.

* Corresponding author.

† Present address: Institute of Molecular Biology, Jagiellonian University, Cracow, Poland.

FIG. 1. In situ hybridization detection of viral transcripts in infected ganglion tissue. Mice were infected with wt or TK⁻ virus or mock infected. At 3 days postinoculation of the virus into the animals, the trigeminal ganglia were removed and in situ hybridization was performed on sections of the tissue. Panels: A, mock infection and α ICP27 gene probe; B, mock infection and β ICP8 gene probe; C, wt virus infection and ICP27 gene probe; D, wt virus infection and ICP8 gene probe; E, *dlsptk* TK⁻ virus infection and ICP27 gene probe; F, *dlsptk* TK⁻ virus infection and ICP8 gene probe.

RESULTS

TK⁻ mutants of HSV can establish latent infection in mouse trigeminal ganglia with no apparent productive replication (2, 8, 23, 42). To define the events occurring during establishment of latent infection by these mutant viruses, we examined viral gene expression in the trigeminal neurons by using in situ hybridization. Mice were infected by corneal inoculation with HSV type 1 wild-type (wt) strain KOS or TK⁻ deletion mutant virus KOS *dlsptk* or KOS *dlsactk* at 2×10^6 PFU per eye. At 24 to 72 h postinfection, trigeminal ganglia were removed and frozen sections were prepared and hybridized with individual labeled DNA probes. DNA probes for α genes (ICP4, ICP0, and ICP27), β genes (ICP8, polymerase), γ genes (glycoprotein C[gC]), and LAT were used. None of the ganglia removed at 24 h postinoculation

showed hybridization with any of the probes (data not shown). Hybridization with the probe for α gene product ICP4 (Fig. 1C), β gene product ICP8 (Fig. 1D), or γ gene product gC (Fig. 2C) was detected in neurons of ganglia from mice infected with wt virus by 2 to 3 days postinoculation. These probes showed cytoplasmic and nuclear hybridization in neurons. No hybridization with these probes was observed on sections from mock-infected animal tissues (Fig. 1A and B and 2A and B). In addition, the LAT probe showed nuclear and cytoplasmic hybridization in neurons from mice infected with wt virus (Fig. 2D). This subcellular distribution of LAT is consistent with previous observations that LAT is expressed as a cytoplasmic transcript in lytically infected cells (43). Numerous ganglia were examined for hybridization with the various probes, and nearly all ganglia from mice

FIG. 2. In situ hybridization detection of viral transcripts in infected ganglion tissue. The methods used are described in the legend to Fig. 1. Panels: A, mock infection and γ gC gene probe; B, mock infection and LAT probe; C, wt virus infection and gC gene probe; D, wt virus infection and LAT probe; E, *dlsptk* TK^{-} virus infection and gC gene probe; F, *dlsptk* virus infection and LAT probe.

infected with wt virus showed nuclear and cytoplasmic hybridization with each of the probes (Table 1).

Most ganglia from animals infected with the TK^{-} mutant viruses showed hybridization of the LAT probe (Table 1), but grains in neurons were evident only in the nuclei (Fig. 2F). The nuclear distribution of the LAT probe hybridization to tissues from these early times postinfection was similar to that seen with the LAT probe in neurons latently infected with these TK^{-} viruses (2). Furthermore, in striking contrast to the results obtained with wt virus, very few of the neurons from animals infected with the TK^{-} mutant viruses showed hybridization with the α ICP27 gene probe (Fig. 1E), the β ICP8 gene probe (Fig. 1F), or the γ gC gene probe (Fig. 2E). Table 1 summarizes the virtual lack of hybridization with any of the lytic gene probes to all of the ganglia examined from animals infected with the two TK^{-} mutants. Thus, the pattern of hybridization to these tissues from TK^{-} virus-

TABLE 1. Compiled data on HSV gene expression in ganglionic tissues of mice infected with wt or TK^{-} virus

Virus	No. of positive ganglia/total no. of ganglia examined with the following in situ hybridization probe: ^a						
	ICP4	ICP8	ICP27	pol ^b	ICP8	gC	LAT
KOS wt	3/4	7/8	7/8	6/8	13/14	6/8	11/12
<i>dlsptk</i>	0/8	1/6	1/10	0/10	0/14	0/9	8/14
<i>dlsactk</i>	0/9	1/6	0/8	0/3	0/17	0/8	15/19

^a At 2 or 3 days postinoculation of the virus into the mice, the trigeminal ganglia were removed and analyzed for viral transcript expression by in situ hybridization with DNA probes for the genes listed. With each DNA probe, 3 to 10 sections from each ganglion were analyzed and at least one section was from the first, middle, and last in the series from each ganglion.

^b pol, Polymerase.

FIG. 3. In situ hybridization detection of transcripts from the ICP0 coding region in tissues infected with TK⁻ virus. Mice were mock infected or infected with *dlsptk* TK⁻ virus. Viral transcripts were detected as described in the legend to Fig. 1. A limited number of ganglia from the animals infected with the TK⁻ viruses showed hybridization. Panels: A, mock infection and ICP0 gene probe; B, *dlsptk* virus infection and ICP0 gene probe.

infected animals at early times was similar to that of a latent infection.

Hybridization of the ICP0 gene probe in a few ganglia (2 of 12; Table 1) may not reflect ICP0 mRNA but rather a large transcript hypothesized to serve as the precursor of the LAT (7). Consistent with the hypothesis that this was a precursor RNA molecule, hybridization of the ICP0 gene probe to tissues from animals infected with TK⁻ viruses was almost exclusively nuclear (Fig. 3), in contrast to the nuclear and cytoplasmic distribution of hybridization seen with all of the lytic gene probes in ganglia infected with wt virus (Fig. 1 and 2; data not shown).

The ICP27 gene probe showed no hybridization to 17 of the 18 ganglia from TK⁻ virus-infected animals (Table 1), but we observed a density of silver grains over one neuron in the remaining ganglion that may represent a weakly positive signal (Fig. 4). If this represents a significant signal, then the ICP27 gene probe is the only lytic gene probe, other than the ICP0 gene probe, that hybridized to TK⁻ virus-infected ganglia (Table 1). Thus, although LAT was readily detected

in TK⁻ virus-infected tissue, transcripts from lytic genes were present at very low levels or undetectable. We conclude that nuclear accumulation of the LAT transcript, characteristic of latent infection, could occur in these neurons even though expression of lytic genes was drastically reduced.

The two independently isolated TK⁻ viruses showed identical phenotypes, with reduced expression of lytic gene products (Table 1) and nuclear accumulation of LAT RNA. This strongly suggests that the phenotype is due to the mutations in the gene for thymidine kinase and not to secondary mutations. We conclude that either the TK⁻ mutant viruses establish latent infection earlier than the wt virus or it is more readily apparent without the possible masking effect of lytic gene expression.

Expression of viral gene products in mice infected with reduced amounts of wt virus. The observation that a defect in a β gene product, thymidine kinase, led to decreased levels of expression of α gene products was surprising and paradoxical. It seemed possible that reduced amounts of TK⁻

FIG. 4. Detection of possible expression of transcripts from the ICP27-coding region in ganglia from animals infected with TK⁻ virus. Panels: A, mock infection and ICP27 gene probe; B, *dlsptk* TK⁻ virus infection and ICP27 gene probe.

FIG. 5. Detection of viral transcripts after inoculation of various doses of wt virus. Mice were inoculated with 2×10^6 , 2×10^4 , or 2×10^2 PFU of wt virus. At 3 days postinoculation of virus, the trigeminal ganglia were removed and transcripts were analyzed by in situ hybridization. Panels: A, 2×10^4 PFU of wt virus and ICP4 gene probe; B, 2×10^2 PFU of wt virus and ICP4 gene probe; C, 2×10^4 PFU of wt virus and LAT probe; D, 2×10^2 PFU of wt virus and LAT probe. The results obtained with 2×10^6 PFU of virus were identical to those obtained with the same dose shown in Fig. 1 and 2.

virus reached the ganglia although normal amounts of virus were shed from the eyes (2). The reduced levels of TK^- virus reaching the trigeminal ganglia might lead to reduced expression of α gene products. To test the hypothesis that a reduced amount of virus reaching the ganglia was responsible for the differential expression of LAT and lytic gene products by the TK^- virus, we introduced lower amounts of wt virus onto mouse eyes, a situation previously shown to result in lower levels of infectious virus in the ganglia (21). We removed the ganglia at 3 days postinoculation and assayed for LAT and ICP4 gene transcripts by in situ hybridization. With 100-fold less wt virus introduced (a situation that gives 1,000-fold less virus in the ganglia; 21), we observed expression of both LAT and ICP4 gene transcripts (Fig. 5). With 10,000-fold less virus introduced, expression of neither LAT nor ICP4 gene transcripts was observed. Thus, reduced levels of wt virus inoculation and resulting reduced ganglionic virus levels did not mimic the situation observed with TK^- virus. We conclude that the phenotype observed with the TK^- mutant viruses was due to some novel behavior of the mutant viruses and not simply the result of less virus reaching the ganglia.

DISCUSSION

In this report, we have demonstrated that during the first few days following their inoculation on mouse corneas, TK^-

mutant strains of HSV express nuclear LAT RNA in ganglionic neurons without detectable expression of many of the viral lytic genes. We and others have previously shown that such mutants establish latent infection (2, 8, 23, 42). These results indicate that these HSV mutants can establish latent infection despite virtually undetectable lytic gene expression. Thus, the latent infection pathway can diverge from the lytic infection pathway before or early in the process of expression of α gene products. A recent study (36), published after this study was completed, reported that an HSV VP16 mutant at least partially defective for lytic α gene expression could establish and reactivate from latent infection. Our results are consistent with that work and extend it by showing that with different mutant viruses, expression of α and other lytic genes is actually reduced in neurons during establishment of latent infection. Thus, the phenotype of establishment of latent infection despite reduced lytic gene expression is not unique to a VP16 mutant.

Divergence from the lytic pathway at very early stages by TK^- viruses raises the possibility that the wt virus also establishes latency under conditions in which α gene expression is minimal or absent. Productive replication by wt HSV in other neurons or cell types might obscure the latent infection events during wt virus infection. The lack of lytic gene expression in cells during establishment of the latent

infection would be reminiscent of latent infection by another herpesvirus, Epstein-Barr virus, which directly establishes latent infection with expression of latency gene products and no expression of lytic gene products (1, 19). A unified hypothesis for establishment of latent infection by herpesviruses would involve circularization of the viral genome, little or no expression of lytic genes, and expression of latency gene products.

Although we have not detected expression of several lytic genes by TK⁻ viruses in neurons, some expression of these genes may occur, and other, more sensitive approaches may detect their expression. For example, limited expression of certain α gene products capable of negative regulation of viral gene expression, such as ICP4 (6, 44) or ICP27 (24, 30, 34, 35, 41), might lead to restricted viral gene expression. Others (16) have detected β -galactosidase expression from a *lacZ* gene located adjacent to a viral lytic promoter in a TK⁻ virus during acute infection of the trigeminal ganglion. Thus, it may be possible to detect expression of viral lytic genes during acute infection by TK⁻ viruses. However, it is difficult to compare our results with those of Ho and Mocarski (16) because their report provided no quantitative comparisons between the amounts of expression in ganglia infected with TK⁺ and TK⁻ viruses and no evidence that the *lacZ* transcripts in neurons originated from a viral lytic promoter. Regardless, our data show that the level of viral lytic gene transcripts expressed by TK⁻ viruses during early stages of infection of trigeminal ganglion neurons is far below the normal level of transcripts expressed by wt virus.

As described in the introduction to this report, several models have been advanced to explain the nonpermissive infection of neurons by HSV which leads to latent infection. Our results lead us to favor models in which expression of α gene products is severely reduced or absent. These include (i) lack of necessary cellular transcription factors, (ii) presence of an inhibitor of α gene expression, (iii) lack of transport of the virion *trans*-inducing factor to the neuron nucleus, and (iv) dominance of viral negative regulatory functions in neurons. Tests need to be devised to determine the requirement for viral gene products in latent infection to test the active role of these gene products in this process. Recent tests using very sensitive polymerase chain reaction assays to detect viral DNA have shown that viral genomes can associate with the ganglia despite lack of replication at the site of inoculation (17a). This is consistent with the apparent limited requirement for lytic viral gene expression during initiation of latent infection.

The observation that a defect in a β gene product, thymidine kinase, leads to a reduction in α gene expression, an earlier event in the lytic cascade, was a surprising result and remains to be explained. Possible models that could explain this observation include the following: (i) The mechanisms controlling viral gene expression in neurons are different from those in cultured cells. This could also mean that the virus possesses a mechanism to sense the suitability of the intracellular environment for replication and, if it is not suitable, down regulates the expression of α genes. (ii) The TK⁻ phenotype leads to reduced multiplicity of infection of neurons, a situation which could favor the latent infection pathway. (iii) The TK⁻ phenotype restricts the spread of the virus within the eye or from the eye to the ganglion such that the TK⁻ virus has access largely or solely to a population of sensory neurons in the trigeminal ganglion, where expression of α gene products is restricted. (iv) The lytic gene transcripts detected in wt virus-infected ganglia are actually transcripts from the large portions of the genome observed at

late times of infection (12, 45). These transcripts do not accumulate in infected cultured cells (P. J. Godowski, Ph.D. thesis, Harvard University, Cambridge, Mass., 1985), but RNA metabolism could be different in neurons in infected animals. We do not favor model ii, because by decreasing the dose of wt virus, we were unable to recreate the phenotype of the TK⁻ viruses. Moreover, the ranges of the amounts of HSV DNA in ganglia latently infected with wt or TK⁻ mutants overlap (17a). By model iii, TK⁻ viruses might provide a probe for defining a particular type(s) of sensory neuron in which HSV latent infection can occur. Further work is needed to test these hypotheses, but an explanation of this observation is likely to shed further light on the mechanism(s) by which latent infection is established.

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