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# RNA from an immediate early region of the type 1 herpes simplex virus genome is present in the trigeminal ganglia of latently infected mice

(neurovirology/*in situ* hybridization/restricted herpesvirus gene expression/viral pathogenesis)

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**ABSTRACT** **Transcription of the type 1 herpes simplex virus (HSV-1) genome in trigeminal ganglia of latently infected mice was studied using *in situ* hybridization. Probes representative of each temporal gene class were used to determine the regions of the genome that encode the transcripts present in latently infected cells. Probes encoding HSV-1 sequences of the five immediate early genes and representative early (thymidine kinase), early-late (major capsid protein), and late (glycoprotein C) genes were used in these experiments. Of the probes tested, only those encoding the immediate early gene product infected-cell polypeptide (ICP) 0 hybridized to RNA in latently infected tissues. Probes containing the other immediate early genes (ICP4, ICP22, ICP27, and ICP47) and the representative early, early-late, and late genes did not hybridize. Two probes covering ≈30% of the HSV-1 genome and encoding over 20 early and late transcripts also did not hybridize to RNA in latently infected tissues. These results, with probes spanning >60% of the HSV-1 genome, suggest that transcription of the HSV-1 genome is restricted to one region in latently infected mouse trigeminal ganglia.**

A characteristic of the herpesviruses is the ability to remain latent indefinitely in the host. A latent infection has the following three stages: establishment, maintenance, and reactivation. Maintenance of latent herpesvirus infections has been studied using the mouse eye model system because the virus does not reactivate spontaneously as it does in humans and other animal model systems (1–3). Mice are inoculated after corneal scarification with the neurotropic type 1 herpes simplex virus (HSV-1) (strain F). An acute infection spreads through the peripheral and central nervous systems. By 15 days after inoculation, infectious virus can no longer be detected in cell-free homogenates. During the latent state, the complete viral genome can be detected in the trigeminal ganglia and brain by Southern blot hybridization of DNA isolated from these tissues (4). Infectious virus can be reactivated from the trigeminal ganglia by explant cocultivation (3).

The expression of HSV-1 genes during latency remains a central issue in understanding the establishment and maintenance of the latent state. The technique of *in situ* hybridization has been used to detect HSV-1 RNA in the central nervous system of latently infected mice (3, 5), and type 2 herpes simplex virus (HSV-2) transcripts have been detected in guinea pigs (6) and human sensory ganglia (7, 8). Thus, some herpes simplex virus (HSV) gene expression may occur during latency. During an acute infection, the three major groups of HSV genes are expressed sequentially in a temporally regulated fashion (9). The immediate early (IE) genes [infected-cell polypeptides (ICP) ICP0, ICP4, ICP22, ICP27, and ICP47] are the first to be expressed and are transcribed in the absence of protein synthesis (9). We detected (3) hybridization to RNA in latently infected central nervous system tissues using a probe corresponding to 12% of the genome encoding IE genes. To determine the regions encoding latent HSV-1 transcripts, we have carried out *in situ* hybridization experiments using cloned HSV-1 DNA probes that encode sequences representative of all temporal gene classes. The data presented using probes covering >60% of the HSV-1 genome suggest that transcription is restricted to one region during latency.

## MATERIALS AND METHODS

Preparation of HSV-1 (F) stocks for animal inoculation, infection of mice, and preparation of uninfected or mock-infected, acutely infected (5 days after inoculation), and latently infected (≥1 month after inoculation) tissues were performed as described (4, 5). Two trigeminal ganglia from each mouse were embedded together. Each block was cut into 150 sections, 5 μm thick. On average, 10 sections were examined per probe per mouse. *In situ* hybridization was performed as described (5, 10).

**Probes.** Plasmid pBR322 containing *Bam*HI fragment a′ (KOS) (11) that corresponds to *Bam*HI fragment b′ (F) was obtained from E. K. Wagner and R. H. Costa (University of California, Irvine, CA); plasmid pBR322 containing the *Eco*RI/*Bam*HI fragment I-I (KOS) was obtained from R. J. Frink (University of California, Irvine, CA) (12); plasmids pRB112 (*Bam*HI fragment B), pRB114 (*Bam*HI fragment N), pRB124 (*Bam*HI fragment X), pRB134 (*Bam*HI fragment E), pRB113 (*Bam*HI fragment Y), and pRB115 (*Bam*HI fragment PS) were obtained from B. Roizman (University of Chicago, Chicago, IL) (13). Plasmid LE578, obtained from L. Enquist (DuPont, Wilmington, DE), contains a 3.4-kilobase (kb) *Bam*HI fragment of HSV-1 (Patton) (14), which encodes the thymidine kinase (*tk*) gene and corresponds to the *Bam*HI fragment Q in HSV-1 (F). pMC123 (*Xba* I fragment E) and pMC122 (*Xba* I fragment F) (KOS) DNA were supplied by M. Challberg (National Institutes of Health) (15). HSV-1 DNA inserts were separated from the plasmid DNA, purified, and nick-translated (16). The specific activities of the $^{35}$S-labeled probes were 1–2 × $10^8$ cpm/μg.

## RESULTS

**HSV-Specific RNA Was Detected by *In Situ* Hybridization.** To establish that viral RNA was being detected, *in situ* hybridization experiments were performed with tissues from

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Abbreviations: HSV, herpes simplex virus; HSV-1 and HSV-2, type 1 and type 2 HSV, respectively; IE, immediate early; ICP, infected-cell polypeptide; tk, thymidine kinase.

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uninfected (or mock-infected) mice in parallel with acutely and latently infected mice because HSV-1 sequences can cross-hybridize with murine (17–19) and human cellular DNA sequences (17, 19–23). Mouse brain DNA and RNA (1 mg/ml of each) were added to the hybridization mixture, which was prehybridized for at least 1 hr, and hybridized under high-stringency conditions [45% (vol/vol) formamide at 50°C] to minimize potential cross-hybridization. None of the HSV-1-specific probes used in this study hybridized at detectable levels with the tissues from uninfected or mock-infected mice.

An HSV-1 DNA probe hybridized strongly to RNA in acutely and latently infected trigeminal ganglia but not in tissues treated with RNase before hybridization (data not shown). An adenovirus DNA probe and a probe containing globin sequences did not hybridize to sequences in the trigeminal ganglia from uninfected, acutely infected, or latently infected mice (data not shown), further demonstrating that the hybridization detected was not to DNA.

*In situ* hybridization was performed using cloned probes from regions of the HSV-1 genome that contain sequences from IE, early, early-late, or late gene temporal classes to study viral transcription in latently infected trigeminal ganglia. No infectious virions were detectable in the latently infected tissues at the time of explant, and latent HSV-1 could be reactivated from trigeminal ganglia by explant cocultivation with susceptible cells, as demonstrated (for review see ref. 1). *In situ* hybridization experiments using probes that were positive and probes that were negative for hybridization with latently infected tissues were performed routinely with tissues from the same latently infected animal. Probes considered negative for hybridization with latently infected tissues did not hybridize to any cells (except where indicated); whereas those probes that did hybridize to RNA in latently infected tissues hybridized to 4–35 infected cells per section.

**_Bam_HI Probes Hybridizing to RNA in Latently Infected Trigeminal Ganglia.** We detected (3) hybridization to RNA in latently infected central nervous system tissues using a probe corresponding to 12% of the genome, which overlaps the junction-repeat region and encodes IE genes. The *Bam*HI restriction fragments PS, B, and E (which encode IE genes ICP0, ICP4, and ICP27, as well as the γ34.5 late gene) (Fig. 1) were used as probes to determine whether the transcripts present in trigeminal ganglia tissues from latently infected animals were homologous to these regions of the HSV-1 genome. The *Bam*HI B and E (regions encoding duplicated ICP0 sequences) and *Bam*HI PS probes hybridized strongly to RNA in trigeminal ganglia from both acutely and latently infected mice (Fig. 2 for *Bam*HI B only).

There was no detectable hybridization in most of the latent tissue sections probed with *Bam*HI fragment N, which encodes ICP22 and <40 nucleotides of ICP4, and with *Bam*HI fragment X, which encodes part of ICP47. One or two strongly positive cells were detected in a few tissue sections

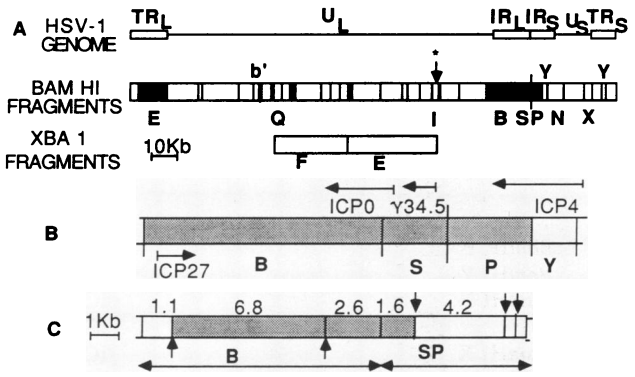


FIG. 1. (*A*) Structure of the HSV-1 DNA. The unique, long ($U_L$) region is bounded by the terminal repeat ($TR_L$) and the internal repeat ($IR_L$). The unique short ($U_S$) region is bounded by the terminal repeat ($TR_S$) and the internal repeat ($IR_S$). The *Bam*HI restriction map of HSV-1 (F) DNA (13) is shown, but only the restriction fragments used as probes are labeled. The starred arrow indicates the map position of the *Eco*RI/*Bam*HI fragment I-I (KOS). The map positions of *Xba* I (KOS) fragments E and F are also shown (15). The fragments shaded indicate the regions of the genome showing positive hybridization to latent trigeminal ganglia. (*B*) The junction repeat region is enlarged to show the location and direction of the transcripts mapped to the region of *Bam*HI fragments B, PS, and Y. *Bam*HI fragments B and E encode the same repeat sequences so only *Bam*HI B is pictured. (*C*) The junction repeat region of *Bam*HI fragments B and PS as pictured in *B* is shown with restriction sites of *Sal* I (arrows below the line) and *Sac* I (arrows above the line). The numbers above the line indicate the size in kb of the *Sal* I and *Sac* I subfragments of *Bam*HI fragments B and PS, respectively. The areas shaded indicate regions of positive hybridization.

of latently infected mice; however, positive cells were not detected in every tissue section examined from the same mouse (Table 1). Thus, these transcripts may be present at very low levels and/or expressed in only a very small fraction of the latently infected cells.

As illustrated in Fig. 1, four transcripts map to the regions of *Bam*HI fragments B, E, and PS, regions which show positive hybridization with latently infected tissues. To determine more specifically which of the regions encoding these four transcripts are transcribed during latency, *Bam*HI fragment Y, *Sal* I fragments of *Bam*HI B, and *Sac* I fragments of *Bam*HI PS were used as probes to separate the three IE gene regions. The 2.6-kb and 6.8-kb *Sal* I fragments of *Bam*HI B and the 1.6-kb *Sac* I fragment of *Bam*HI PS encode ICP0 sequences. *Bam*HI Y, the 1.1-kb *Sal* I fragment of *Bam*HI B, and the 4.2-kb *Sac* I fragment of *Bam*HI PS encode ICP4, ICP27, and γ34.5 sequences, respectively. Of these probes, only those encoding ICP0—the 1.6-kb *Sac* I fragment of *Bam*HI PS and the 2.6-kb (Fig. 3 *A* and *C*) and the 6.8-kb *Sal* I fragments of *Bam*HI B—hybridized significantly to both acutely and latently infected trigeminal ganglia (Table 1). *Bam*HI Y (Fig. 3 *D–F*), the 4.2-kb *Sac* I fragment of *Bam*HI

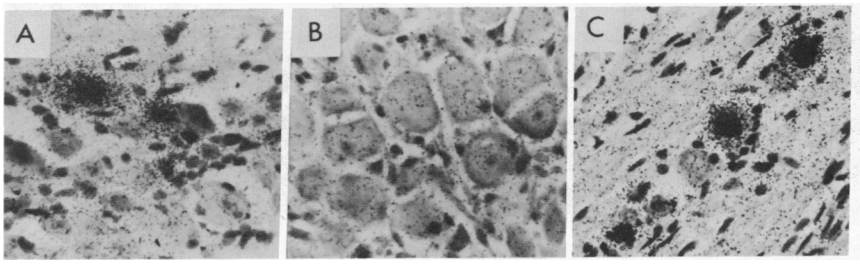


FIG. 2. *In situ* hybridization experiments of mouse trigeminal ganglia tissues with *Bam*HI fragment B probe. (*A*) Tissue from an acutely infected mouse 5 days after infection. (*B*) Tissue from an uninfected mouse. (*C*) Tissue from a latently infected animal (1 month after infection). Exposure time was 3 days. (×105.)

Table 1. Summary of *in situ* hybridization results

| Probe* | Mapped transcripts | Refs. | Positive latent mice/ total mice examined†, no./no. |
|---|---|---|---|
| *Bam*HI B | ICP0 (IE mRNA-1) | 24, 25 | 4/4 |
| | ICP27 (IE mRNA-2) | 24, 25 | |
| *Bam*HI PS | ICP0 (IE mRNA-1) | 24, 25 | 4/4 |
| | ICP4 (IE mRNA-3) | 24, 25 | |
| | γ34.5 | 26 | |
| *Bam*HI E | ICP0 (IE mRNA-1) | 24, 25 | 4/4 |
| *Bam*HI Y | ICP4 (IE mRNA-3) | 24, 25 | 0/9 |
| *Bam*HI N | ICP4 (IE mRNA-3) | 24, 25 | 0/10‡ |
| | ICP22 (IE mRNA-4) | 24, 25 | |
| *Bam*HI X | ICP47 (IE mRNA-5) | 24 | 0/10§ |
| 1.6-kb *Sac* I fragment of *Bam*HI PS | ICP0 (IE mRNA-1) | 24, 25 | 5/5 |
| 6.8-kb *Sal* I fragment of *Bam*HI B | ICP0 (IE mRNA-1) | 24, 25 | 7/7 |
| | ICP27 (IE mRNA-2) | | |
| 2.6-kb *Sal* I fragment of *Bam*HI B | ICP0 (IE mRNA-1) | 24, 25 | 4/4 |
| 4.2-kb *Sac* I fragment of *Bam*HI PS | γ34.5 | 26 | 0/5 |
| 1.1-kb *Sal* I fragment of *Bam*HI B | ICP27 (IE mRNA-2) | 24, 25 | 0/4 |
| *Bam*HI a' (KOS) | Vp5 βγ 155 (MCP)¶ | 11 | 0/5 |
| *Eco*RI/*Bam*HI I-I (KOS) | Glycoprotein C, γ69, γ50 | 12 | 0/4 |
| LE578 (Patton) = *Bam*HI Q (F) | TK β 43 | 14 | 0/4 |
| | 3KBL (γ), 4 early RNAs | 27 | |
| | gH 90K | 28 | |
| *Xba* I E (KOS) | Many | 29 | 0/5 |
| *Xba* I F (KOS) | Many | 29 | 0/5 |

*HSV-1 (F) DNA restriction fragments unless otherwise specified.
†Positive tissue sections had more than four positive cells. Negative sections did not have any except where indicated.
‡Three different animals showed a total of one or two strongly positive cells out of all tissue sections examined.
§One animal showed one strongly positive cell in four tissue sections examined.
¶Major capsid protein.

PS, and the 1.1-kb *Sal* I fragment of *Bam*HI B did not hybridize to uninfected, mock-infected, or latently infected trigeminal ganglia tissues, although positive hybridization was detected with acutely infected tissues (Table 1). Thus, it appears that the region encoding ICP0 was actively transcribed during latency, but not the regions encoding ICP4, ICP27, ICP22, and ICP47.

**HSV-1 Probes That Did Not Hybridize to RNA in Latently Infected Ganglia.** *In situ* hybridization was also performed using probes encoding sequences of select early, early-late, and late gene classes. The LE578 probe, corresponding to the *Bam*HI fragment Q of HSV-1 (F) and containing the *tk* gene (14), hybridized to HSV-1-specific transcripts in acutely infected trigeminal ganglia (Fig. 4*A*) but not to transcripts in uninfected (Fig. 4*B*) or latently infected trigeminal ganglia (Fig. 4*C*). Other transcripts that also map to this region include a late transcript (3KBL), four minor early transcripts (27), and the gH transcript (28).

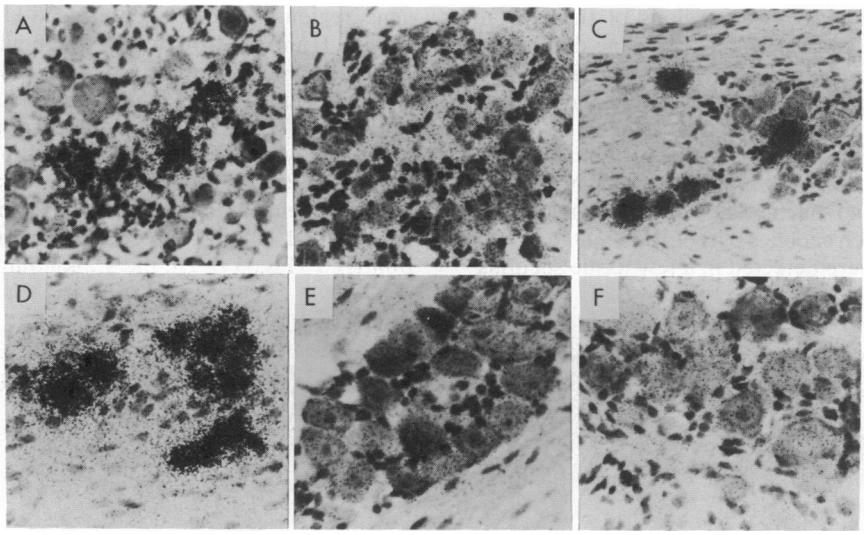


FIG. 3. *In situ* hybridizations with mouse trigeminal ganglia using the 2.6-kb *Sal* I subfragment of *Bam*HI fragment B (*A–C*) and *Bam*HI fragment Y (*D–F*). Trigeminal ganglia from acutely infected mice (*A* and *D*). Trigeminal ganglia from uninfected mice (*B* and *E*). Trigeminal ganglia from latent mice 12 months after infection (*C* and *F*). Exposure time was 4 days for *A*, *B*, *C*, *D*, and *F* and 8 days for *E*. (*B* and *C*, ×70; *A*, *D*, *E*, and *F*, ×112.)

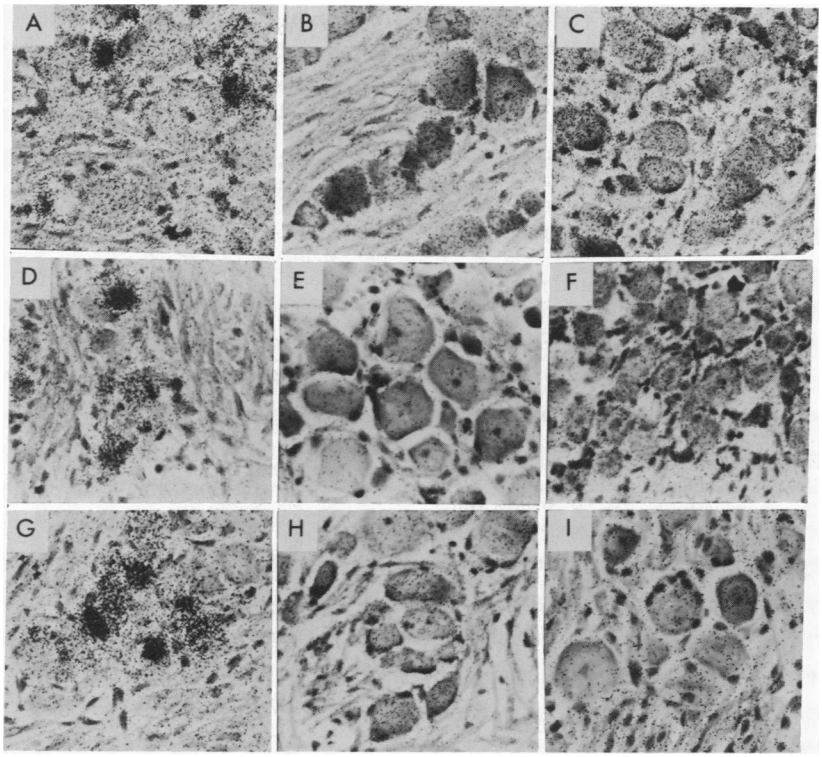


FIG. 4. *In situ* hybridizations with mouse trigeminal ganglia hybridized with LE578 probe (*A–C*), *Bam*HI fragment a′ (*D–F*), and *Eco*RI/*Bam*HI fragment I-I (*G–I*). Trigeminal ganglia from acutely infected mice are shown in *A*, *D*, and *G*. Trigeminal ganglia from uninfected mice are shown in *B*, *E*, and *H*. Shown also are trigeminal ganglia from latently infected mice 20 months after infection (*C*) and 1 and 3 months after infection (*F* and *I*, respectively). Exposure time was 2 days for *D* and *F* and 3 days for all other panels. (×100.)

*In situ* hybridization results were also negative with the probes designed specifically to contain sequences of Vp5, the major capsid protein (an early-late gene), and glycoprotein C (a late gene). These probes hybridized strongly to transcripts in the trigeminal ganglia from acutely infected mice but not to transcripts in trigeminal ganglia from uninfected or latently infected mice (Fig. 4 *D–I*).

In addition, probes of *Xba* I fragments (15) were used in *in situ* hybridization experiments to further substantiate the restricted transcription during HSV-1 latency. *Xba* I fragments E and F (see Fig. 1) that cover ≈30% of the HSV-1 genome (>50% of the long, unique region) and encode over 20 early and late transcripts did not hybridize to RNA in trigeminal ganglia from latently or mock-infected mice, although positive cells were detected in acutely infected tissues (Table 1). In total approximately two-thirds of the genome has been analyzed.

Table 1 summarizes the data obtained from the *in situ* hybridization experiments.

## DISCUSSION

The data presented in this study show that the HSV-1 RNA detectable during latency may be limited to a specific region of the genome known to encode ICP0. No hybridization was detected using probes of select early, early-late, and late genes with latently infected trigeminal ganglia tissues. In addition, no hybridization was detected using probes covering >50% of the long, unique region of the HSV-1 genome and encoding >20 early and late transcripts.

These findings are consistent with two possible transcriptional programs during latency. (*i*) The RNA may be latency specific and, thus, not yet identified or mapped. (*ii*) The RNA detected during latency may be from a gene(s) expressed during both latency and the lytic cycle. Transcripts mapping to the regions of positive hybridization include the IE RNAs of ICP0, ICP4, ICP27, and the late RNA γ34.5. Of the probes tested, only the subfragment probes encoding ICP0 sequences (1.6-kb *Sac* I fragment of *Bam*HI fragment PS and the 2.6-kb and 6.8-kb *Sal* I fragments of *Bam*HI fragment B) hybridized to RNA in latently infected trigeminal ganglia. Although these results suggest that the ICP0 transcript is present in latently infected tissues, we have not eliminated the other possibility that there are other transcripts that also map in this region. In fact, Gelman and Silverstein (30) reported a transcript in the region overlapping the 5′ end of ICP0.

In studies of transcription and gene expression during HSV-2 latency, Tenser *et al.* (6) detected HSV-2 RNA in latently infected trigeminal ganglia of guinea pigs using *in situ* hybridization. Galloway *et al.* (8) reported that the majority of transcription from the HSV-2 genome in human paravertebral ganglia was limited during latency to the left-hand 30% of the HSV-2 genome that encodes genes such as *tk* (8). RNA homologous to the IE regions was not detected in these tissues. It is possible that the transcripts detected are different in the two systems or that the HSV-2 virus was in the process of reactivation. In the latently infected ganglia analyzed in the present study, we did not detect one of the most abundant late transcripts in a lytic HSV-1 infection (Vp5) arguing against the possibility of reactivation in mouse tissues.

Green *et al.* (31) reported the detection of the ICP4 gene product in latently infected rabbit ganglia by an indirect immunofluorescence assay using a monospecific antibody. If ICP4 mRNA is present in latently infected mouse ganglia, it is below the level of detection. However, it is possible that the ICP4 protein is produced during the establishment of latency and remains thereafter. Sears *et al.* (32) demonstrated that the ability of HSV-1 to express *tk* is not related to its

ability to establish latent infections. By *in situ* hybridization, we did not detect tk RNA in latently infected mouse ganglia. However, Yamamoto *et al.* (33) demonstrated tk activity in the sensory ganglia of latently infected mice, and Tenser *et al.* (34) and Tenser and Dunstan (35) demonstrated the importance of the expression of tk in latent infections of mice and guinea pigs, respectively, using explant cocultivation. It is possible that tk expression is important for reactivation but may not be required during the maintenance of the latent viral state.

Our data, indicating the transcription of a limited region of the HSV-1 genome during latency, suggest the significance of this region in the establishment and/or maintenance of a latent HSV-1 infection.

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