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A Herpes Simplex Virus Type 1 Mutant Containing a Nontransinducing Vmw65 Protein Establishes Latent Infection In Vivo in the Absence of Viral Replication and Reactivates Efficiently from Explanted Trigeminal Ganglia

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Vmw65, a herpes simplex virus type 1 (HSV-1) tegument protein, in association with cellular proteins, transactivates viral immediate early genes. In order to examine the role of Vmw65 during acute and latent infection in vivo, a mutant virus (*in*1814), containing a 12-base-pair insertion in the Vmw65 gene, which lacks the transactivating function of Vmw65 (C. I. Ace, T. A. McKee, J. M. Ryan, J. M. Cameron, and C. M. Preston, J. Virol. 63:2260–2269, 1989) was examined in mice. Following corneal inoculation, the parental virus (17^+) and the revertant (1814R) replicated effectively in eyes and trigeminal ganglia with 30 to 60% mortality. At either equal PFU or equal particle numbers, *in*1814 did not replicate in trigeminal ganglia and none of the infected mice died. Although *in*1814 did not replicate following corneal inoculation, it established latent infection in trigeminal ganglia. HSV-1 *in*1814 reactivated at explant as efficiently and rapidly as did 17^+ and 1814R. Even low amounts of inoculated *in*1814 (10^2 PFU) were sufficient to establish latent infection in some animals. Since infectious *in*1814 was not detected at any time in mouse trigeminal ganglia, *in*1814 provided a unique opportunity to determine how soon after primary infection latency begins. Latent *in*1814 infection was detected shortly after virus reached the sensory ganglia, between 24 to 48 h postinfection. Thus, though Vmw65 may be required for lytic infection in vivo, it is dispensable for the establishment of and reactivation from latent infection. These data support the hypotheses that the latent and lytic pathways of HSV-1 are distinct and that latency is established soon after infection without a requirement for viral replication. However, the levels of Vmw65 reaching neuronal nuclei may be a critical determinant of whether HSV-1 forms a lytic or latent infection.

A distinguishing feature of herpesvirus infections is the ability to persist for long periods in the host in a nonreplicative or latent state. Herpes simplex virus type 1 (HSV-1) establishes latent infection in human peripheral sensory ganglia and can reactivate to produce recurrent mucocutaneous lesions in the innervated dermatome (for reviews, see references 3, 21, 46). Operationally, the pathogenesis of herpesvirus infections can be divided into several distinct stages which can be studied individually in experimental animal models: acute viral replication, establishment of latency, maintenance, and reactivation (21, 46). HSV-1 replicates at the site of inoculation and is transported to sensory ganglia. Replication at the periphery or in sensory ganglia, measured by viral titers of tissue homogenates, may increase the amount of virus that has the potential to establish latent infection. During latent infection, HSV-1 DNA can be detected in infected tissues (15, 16, 45), but infectious virus cannot (9, 24). This latent state is often maintained for the life of the host. A variety of stimuli (such as febrile illness and X-irradiation) can interrupt the latent state and cause the reappearance of infectious virus or reactivation.

Most of the information about acute viral replication and gene expression has been obtained in tissue culture systems. The HSV-1 lytic replication cycle has been described as a coordinated process that involves the temporal regulation of at least three viral gene classes: α, β, and γ (for a review, see reference 46). Five immediate-early (IE) (α) genes have been identified that are first expressed in infected cells prior to viral protein synthesis. Two of these genes, ICP4 and ICP27, are essential for viral replication in cell culture (12, 48). The α genes activate β genes, many of which are enzymes involved in nucleotide metabolism and DNA replication, leading in turn to activation of γ genes, many of which are structural components of the mature virus particle. Following corneal inoculation, a broad spectrum of HSV-1 genes from the α, β, and γ gene classes can be detected by in situ hybridization and Northern (RNA) blot analysis during the acute phase of viral replication in mouse trigeminal ganglia (9, 51).

There has been recent progress in characterizing HSV-1 gene expression during latent infection. After the first 5 to 6 days postinfection in mice, HSV-1 gene expression in trigeminal ganglia is limited (51). This correlates with the decline of infectious virus in trigeminal ganglia (24, 53). As early as 4 days postinfection, the HSV-1 latency-associated transcripts (LATs; 50, 55) begin to accumulate in trigeminal

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ganglia (51). The LATs are present predominantly in neuronal cell nuclei (9, 10, 54–56) and are not extensively polyadenylated (50, 58). These 2.0-, 1.5-, and 1.45-kilobase (kb) transcripts are encoded by diploid genes within the repeat regions of the viral genome. The smaller LATs are spliced (59, 61) either from the 2.0-kb LAT or from a larger primary transcript (13; W. J. Mitchell, R. P. Lirette, and N. W. Fraser, J. Gen. Virol., in press). Characterization of LAT minus viruses in vivo (13, 53) and promoter constructs in vitro (62) indicates that LAT promoter regulatory elements are located at a considerable distance upstream of the 2.0-kb LAT 5′ terminus (58, 61). The 2.0-kb LAT is detectable at low levels in infected tissue culture cells (25, 50, 52, 54, 59) and appears to be regulated differently than any of the previously defined classes of HSV-1 genes (52).

The function of the LATs, or λ genes (52), has only recently been examined; genetic analysis indicates that the λ genes are not required for the establishment of latent infection (22, 29, 53) but that they might play a role in the reactivation process (29, 53). Although HSV-1 deletion mutants which do not express the LATs establish latent infection in mouse trigeminal ganglia, they reactivate in explanted ganglia much more slowly (53) or less efficiently (29) than does wild-type virus. During the reactivation process in explanted ganglia, there is a lag period before the synthesis of viral RNA, DNA, or infectious virus can be detected (51). The levels of the LATs decline about twofold during this period, even when reactivation is blocked by inhibitors. Presently, there is little information about the switch to lytic or latent HSV-1 gene expression during the initial stages of infection or from latent to lytic transcriptional programs during the reactivation process. It is likely that both viral and cellular transcription factors are important determinants.

Transcription of the HSV-1 IE genes is not detectable during latency (9, 50, 54, 55). However, in tissue culture, IE gene expression is a prerequisite for viral replication (12, 42, 46, 48). Transcription of the IE genes is transinduced by an HSV-1 protein (4, 6, 37, 41, 43), Vmw65 (transinducing factor-α, VP16), that is a component of the virion (4, 6, 20, 43). Vmw65 does not bind directly to HSV-1 DNA but mediates transinduction by association with cellular proteins to form a complex which interacts with the IE regulatory element TAATGARAT (18, 33, 36, 44). Since the expression of IE genes is a critical factor in the outcome of HSV-1 infection in tissue culture cells, the presence or absence of functional Vmw65 might be an important determinant of productive and latent infection in vivo (1, 46).

An HSV-1 mutant, *in*1814 (1), which contains a 12-base-pair insertion in the coding region of Vmw65, is unable to transinduce IE gene expression, but the altered Vmw65 protein is incorporated into mature virions. Replication of *in*1814 during infection is dependent on the multiplicity of infection (MOI). The Vmw65 defect is partially overcome by infection at high multiplicities. At a high MOI (10^2 to 10^3 particles per cell), the expression of ICP0 and ICP27 is significantly reduced, ICP22 is slightly reduced, and ICP4 expression is unaffected (1). At a lower MOI (1 to 10 particles per cell) of *in*1814, the expression of HSV-1 thymidine kinase, an indicator β gene, is profoundly reduced, suggesting that IE gene expression is insufficient to activate the viral replication cycle (1).

In this study, *in*1814 was used to examine the role of Vmw65 in vivo during primary infection, establishment of latent infection, and explant reactivation from latently infected mouse trigeminal ganglia. While the parental strain, 17^+, and the revertant, 1814R, replicated in trigeminal ganglia of mice infected via the cornea, *in*1814 replication was not detected. Despite this lack of viral replication, *in*1814 established latent infection within 24 h of infection and reactivated as well as 17^+ and 1814R did. Thus, although Vmw65 is not required for the establishment of latent infection, it may play a pivotal role in vivo in determining whether or not viral replication will be initiated. Moreover, these data indicate that latent infection can be established as soon as HSV-1 reaches the trigeminal ganglia and that the pathway to latent infection is independent of the lytic replication cycle.

MATERIALS AND METHODS

Cell culture, virus titration, and preparation of virus stocks. Subconfluent monolayers of baby hamster kidney (BHK)-21 clone 13 cells were infected with HSV-1 strain 17^+ (5), insertion mutant *in*1814 (1), or revertant 1814R (1) to produce virus stocks for the infection of mice. The titers of the viruses were determined on BHK cells, and virus particle concentrations were determined by electron microscopy with latex bead standards. The viral stocks used in PFU/ml (particles per ml) were as follows: 17^+, 5×10^8 (3.1×10^9); 1814R, 5×10^8 (5.1×10^9); and *in*1814, 1.3×10^7 (1.2×10^{11}).

Comparison of viral titers on cells expressing Vmw65. The titers of HSV-1 strain 17^+, *in*1814, and 1814R were determined by using MTX5 cells, which are derived from L TK^- cells and which express Vmw65 (23). The titers determined by using CV-1, L TK^-, and MTX5 cells were compared to determine which cell line would be most sensitive for plaque assay of *in*1814 during acute infection and for explant reactivation from trigeminal ganglia. Although the MTX5 cells provided Vmw65 in *trans* and did complement *in*1814 when compared with L TK^- cells, the titers of *in*1814 were not greater when using MTX5 cells than they were when using CV-1 (data not shown). Moreover, *in*1814 did not form distinct plaques with MTX5, which made the determination of viral titers less exact and more difficult than with CV-1. Thus, CV-1 cells were used as indicator cells in most experiments. In order to increase the sensitivity of this assay, UV-irradiated *ts*K virus was absorbed to CV-1 cells at 0.1 PFU per cell (based on titer before UV irradiation) and titers of *in*1814 from the primary stock as well as trigeminal ganglia homogenates were determined with these cells. This procedure increases the plaque formation ability of *in*1814 by 3 logs (1) so that the particle-to-PFU ratio was similar to that of the wild-type virus. The UV-inactivated *ts*K did not form plaques at 37°C.

Infection of mice and viral titers during acute infection. Following corneal scarification, 4- to 6-week-old female BALB/cBYJ mice (Jackson Laboratory) or BALB/c mice (Harlan Sprague Dawley) were infected with approximately 10^5 PFU per eye of 17^+, 1814R, or *in*1814 (Table 1). Starting at 1 day postinfection, mice were sacrificed by cervical dislocation, corneas were swabbed under sterile conditions with cotton-tipped applicators, and the applicators were incubated with CV-1 cells. Trigeminal ganglia and eyes were removed aseptically and homogenized in 1 ml of media without serum, and the titers of infectious HSV-1 with CV-1 or with MTX5 cells were determined, as described above.

Explant reactivation. (i) At a minimum of 4 weeks after being infected, latently infected mice were sacrificed and the trigeminal ganglia were removed and incubated with monolayers of CV-1 cells. Mice were from a group infected at 10^5

TABLE 1. Reactivation of latent *in*1814, 1814R, and 17^+ from mice infected with equal PFU or equal number of particles

Virus	PFU	Virus particle concn[a]	Reactivation[b]
17^+	1.3×10^5	7.8×10^5	14/14
1814R	1.3×10^5	1.5×10^6	24/24
*in*1814	1.3×10^5	1.2×10^9	19/20
*in*1814	1.6×10^2	1.5×10^6	4/14
*in*1814	1.6×10^0	1.5×10^4	0/14

[a] As determined by electron microscopy with latex bead standards.
[b] Reactivation-positive trigeminal ganglia divided by the total number of trigeminal ganglia explanted for cocultivation.

PFU per eye with 17^+, 1814R, or *in*1814 or at equal particle numbers of approximately 10^6 per eye (Table 1). Another infection was done with 10^4 particles per eye of *in*1814. The monolayers were inspected daily for signs of cytopathic effect. Every 4 to 6 days, ganglia were transferred to new monolayers of cells and observed until reactivation occurred or for a maximum of 35 days. After reactivation, the virus-containing media were removed and saved for DNA extraction. As a latency control, titers of virus from ganglia, which were explanted at 5 and 7 weeks postinfection and immediately homogenized, were shown to be zero.

(ii) In order to determine when *in*1814 latency was established, trigeminal ganglia were removed at the indicated times postinfection from mice infected with *in*1814 for explant reactivation and for determination of the titers of infectious virus in ganglion homogenates. As a control, trigeminal ganglia from mice infected with 17^+ were explanted, and the monolayer of cells was observed daily for cytopathic effects.

DNA extraction from reactivated virus. Individual plaques of reactivated virus were used to infect CV-1 cells and to grow viral stocks. Nucleoprotein-associated HSV-1 DNA was prepared from cytoplasmic fractions of infected cells as described by Pignatti et al. (38). Briefly, infected cells were lysed by 0.25% Triton X-100, 10 mM EDTA, 10 mM Tris hydrochloride (pH 7.9) (final concentration, 1.5×10^7 cells per ml) and were incubated at room temperature for 10 min with gentle mixing. NaCl was then added to a final concentration of 0.2 M, and the mixture was centrifuged at $100 \times g$ at 4°C for 10 min. The supernatant was incubated with 100 μg of proteinase K per ml and 0.2% sodium dodecyl sulfate at 37°C for 2 h, and DNA was extracted with phenol, phenol-chloroform, and chloroform, followed by ethanol precipitation. DNA amounts were measured by A_{260}.

DNA analysis. DNA was cut with restriction enzyme *Bam*HI, resolved by 0.8% agarose gel electrophoresis, Southern blot transferred to nitrocellulose, hybridized with ^{32}P-labeled HSV-1 (strain F) restriction fragment *Bam*HI F, and washed by standard procedures (45). The filters were autoradiographed with XAR-5 film at −70°C with intensifying screens (Du Pont Co.).

Preparation of ^{32}P-labeled probes. Total HSV-1 DNA was isolated from virions and purified by CsCl gradient centrifugation. The *Bam*HI F restriction fragment of HSV-1 (strain F) cloned into pBR322 was a generous gift of B. Roizman (40). Restriction enzymes were purchased from Boehringer Mannheim Biochemicals and were used as recommended by the manufacturer. DNA probes were nick translated by standard procedures (32). The probes were separated from unincorporated nucleotides by passage through Sephadex G-50 columns (Quick Spin; Boehringer). The specific activities of the probes were at least 0.8×10^8 cpm/μg of DNA.

TABLE 2. Virus-positive corneal swabs following corneal infection

Days postinfection	No. of virus-positive corneas/total corneas		
	17^+	1814R	*in*1814
1	8/8	8/8	8/8
2	8/8	8/8	3/8
3	8/8	7/8	0/8
4	7/8	6/8	0/8
5	4/8	4/8	0/8
7	0/8	0/8	0/8

RESULTS

***in*1814 did not replicate in trigeminal ganglia of mice. (i) Corneas and eyes.** Following corneal inoculation with the parental virus 17^+ or the revertant 1814R, infectious virus was detected in eight of eight corneal swabs for 5 days postinfection (Table 2) while *in*1814 was detected in eight of eight corneal swabs only on day 1 postinfection and in three of eight on day 2 postinfection. Both 17^+ and 1814R reached titers of about 10^4 PFU per eye (Fig. 1), with the peak titers occurring on days 3 and 2 postinfection, respectively. The titers of *in*1814 in eye homogenates were approximately 10^2 PFU on day 1 postinfection and dropped below detection by day 3. To examine the possibility that the infectious *in*1814 virus present in eye homogenates was due to the viral inoculum rather than viral replication, the viral stock used to infect mice was incubated at 37°C and titers were determined daily. On each of the 3 days, the titer of *in*1814 incubated at 37°C was greater than the titer in eye homogenates of *in*1814-infected mice (data not shown).

There was a 3 log difference in particle-to-PFU ratio between 17^+ (or 1814R) and *in*1814 (Table 1). To increase the sensitivity of detection of *in*1814, Vmw65 was provided in *trans* by an UV-inactivated HSV-1 strain, *ts*K, which re-

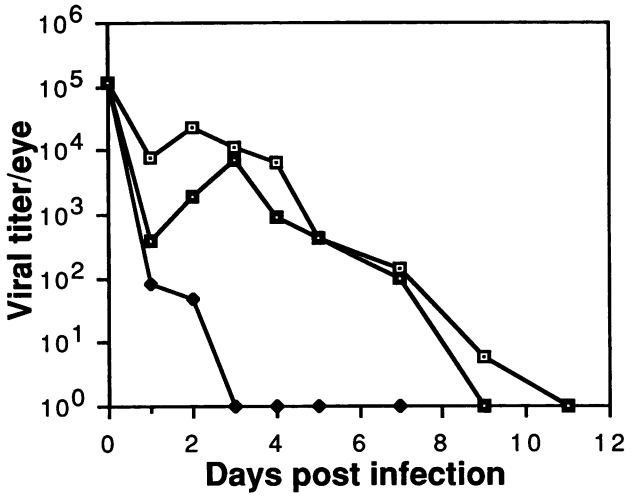

FIG. 1. HSV-1 titers in mouse eyes during acute infection. Each point represents the geometric mean titer determined from eight individual eyes at the indicated time (days) postinfection from two experiments. The titers are plotted on a logarithmic scale as PFU per eye. ◆, *in*1814; ⊡, 17^+; ■, 1814R.

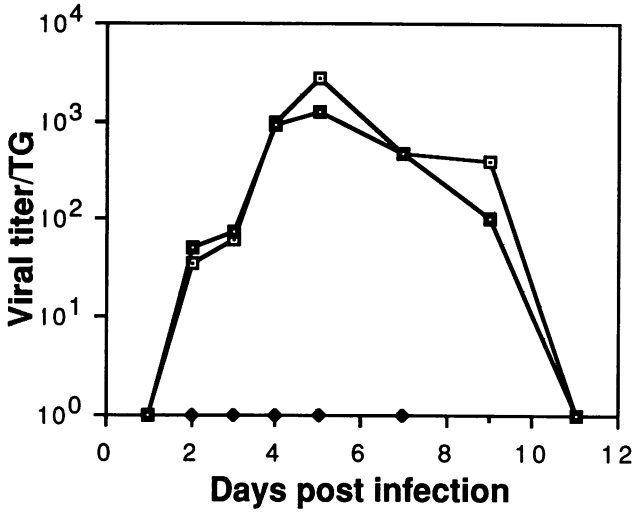

FIG. 2. HSV-1 titers in mouse trigeminal ganglia during acute infection. Each point represents the geometric mean titer determined from eight individual ganglia at the indicated time (days) postinfection from two experiments. The titers are plotted on a logarithmic scale as PFU per trigeminal ganglia (PFU/TG). ⊡, Strain 17^+; ◘, revertant 1814R. No infectious *in*1814 was detected during acute infection (◆).

stored the particle-to-PFU ratio of *in*1814 to that of the wild-type virus (1). While the titer of the *in*1814 stock virus used to infect mice was increased 1,000-fold by *ts*K, no infectious *in*1814 was detected in eye homogenates obtained during the peak of wild-type virus replication, 3 to 5 days postinfection.

(ii) Trigeminal ganglia. The peaks of viral replication in the trigeminal ganglia for 17^+ and 1814R were on day 5 postinfection, and viral titers declined until day 11 (Fig. 2). No infectious *in*1814 was detectable in trigeminal ganglia throughout this period when the titers were determined with CV-1 cells (Fig. 2). To increase the sensitivity of the assay, entire trigeminal ganglia homogenates from *in*1814-infected mice were incubated with CV-1 cells in six-well plates. Infectious *in*1814 virus was not detected by this procedure. In another set of experiments, Vmw65 was provided in *trans* to the CV-1 cells with MTX5 cells, which constitutively express Vmw65 (23), or by prior infection of CV-1 cells with UV-inactivated *ts*K virus. No infectious *in*1814 was detected in trigeminal ganglia homogenates during days 3 to 5 postinfection by these methods.

(iii) Mortality. While mortality rates of mice infected with 17^+ and 1814R were similar and ranged between 35 and 60% in independent experiments, none of 127 *in*1814-infected mice died. Thus, the lack of *in*1814 replication in mice following corneal inoculation correlates with its lack of virulence.

***in*1814 establishes latent infection in trigeminal ganglia and reactivates as efficiently as does 17^+ and 1814R.** There was a 3-log difference in particle-to-PFU ratio between 17^+ (or 1814R) and *in*1814 (Table 1). We therefore examined the ability of *in*1814, 17^+, and 1814R to form a latent infection with equal PFU or equal numbers of inoculated particles.

(i) Mice infected at equal PFU. Reactivation of latent HSV-1 was assayed at 28 to 37 days postinfection by incubating explanted ganglia with monolayers of susceptible cells (CV-1) and inspecting them daily for cytopathic effects. In all mice infected with strain 17^+ (seven mice, 14 of 14 ganglia), reactivation was detectable between 5 and 6 days postexplant (Table 1). Similarly, in the trigeminal ganglia of mice infected with 1814R, reactivation occurred in all animals examined (24 of 24 ganglia) between days 5 and 9 postexplant. Reactivation of latent *in*1814 from trigeminal ganglia was detected in all latently infected mice (19 of 20 trigeminal ganglia) between 5 and 10 days postexplant. Thus, Vmw65 does not play a role in the establishment of HSV-1 latent infection or in the reactivation process. No infectious virus was detectable in latently infected ganglia at explant, as measured by virus titers of ganglionic homogenates.

(ii) Mice infected at equal particle numbers. In mice infected with 1.45×10^6 particles (1.6×10^2 PFU), *in*1814 reactivation from latent infection was apparent at 6 to 8 days postexplant in 4 of 14 trigeminal ganglia. However, reactivation was not observed from the trigeminal ganglia of seven mice infected with 1.45×10^4 particles (1.6 PFU) of *in*1814. Thus, *in*1814 can form latent infection in mouse trigeminal ganglia at PFU equivalent to those of 17^+ and 1814R but establishes latent infection less efficiently at equal particle numbers.

Analysis of reactivated viral DNA. To confirm that the insertion in the Vmw65 gene of *in*1814 remained unchanged during latent infection, DNAs isolated from reactivated 17^+, 1814R, and *in*1814 were hybridized with ^{32}P-labeled *Bam*HI restriction fragment F of HSV-1, which encodes Vmw65. An additional *Bam*HI restriction site introduced by the 12-base-pair insertion into the Vmw65 gene is diagnostic of *in*1814 DNA (1). The Southern blot data demonstrate that the 12-base-pair insertion in *in*1814 was preserved during latency and reactivation (Fig. 3). Two *Bam*HI restriction fragments of 5 and 3 kb were observed in reactivated isolates from *in*1814-infected trigeminal ganglia, whereas a single 8-kb band was present with 17^+ and 1814R DNA. The profiles of reactivated 17^+, 1814R, and *in*1814 were the same as those previously described and were identical to the patterns of the viruses used for infection (1).

When is latency established? Since infectious *in*1814 was not detected in mouse trigeminal ganglia but latent infection was established, *in*1814 provided a unique opportunity to determine when latency begins. Operationally, a latently infected tissue is defined by the absence of infectious virus and by the capacity to reactivate infectious virus (for a review, see reference 21). Trigeminal ganglia from mice infected with *in*1814 were explanted starting at 12 h postinfection. Reactivated virus could be detected in 7 of 14 ganglia at 24 h postinfection, in 9 of 10 at 36 h postinfection, in 13 of 14 at 48 h postinfection, and in all explanted ganglia from day 3 postinfection (Fig. 4). The elapsed time after explant before detection of reactivated virus was similar for all explant time points, ranging from 5 to 10 days postexplant. This was similar to the time required to detect reactivated virus from trigeminal ganglia explanted at 1 month postinfection. No infectious virus was detectable in any *in*1814 trigeminal ganglia homogenates during this time period (Fig. 2). In contrast, trigeminal ganglia explanted from 17^+ acutely infected mice at days 2 and 3 postinfection contained infectious virus (Fig. 2) and caused cytopathic effects within 2 to 3 days postexplant, which was faster than reactivation of latent virus from ganglia explanted from latent 17^+-infected mice at 1 month postinfection (5 to 6 days; see above). It seems, therefore, that *in*1814 establishes latent infection as soon as the virus reaches the trigeminal ganglia, within 24 to 48 h following infection in mice.

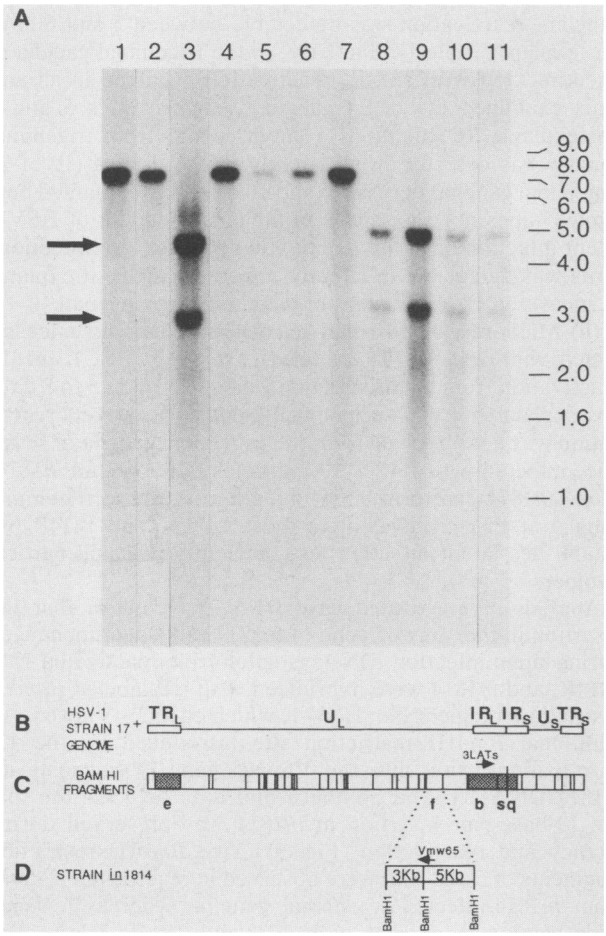

FIG. 3. (A) Southern blot analysis of DNA from reactivated virus. Reactivated virus DNA was restricted with *Bam*HI, and the nitrocellulose filters were hybridized with ^{32}P-labeled nick-translated HSV-1 restriction fragment *Bam*HI F. Lanes: 1, strain 17$^+$; 2, revertant 1814R; 3, *in*1814; 4 through 11, plaque-purified reactivated virus from the trigeminal ganglia of mice latently infected with 17$^+$ (4 and 5), 1814R (6 and 7), and *in*1814 (8 through 11). A single 8-kb HSV-1 band was present in DNA of reactivated virus from trigeminal ganglia infected with 17$^+$ and 1814R. Two bands (arrows), 5 and 3 kb in size, were present in DNA of reactivated virus from trigeminal ganglia infected with *in*1814. The positions of DNA markers are labeled in kilobases on the right. (B) The HSV-1 genome illustrating the unique long and short (U_L and U_S) regions of the genome bounded by the internal (IR) and terminal (TR) repeat regions. (C) The positions of *Bam*HI restriction fragments e, f, b, s, and q. The HSV-1 *Bam*HI DNA fragments positive by in situ hybridization to viral RNA during latency (b, e, and s and q) are shaded. Also presented is the location of the three LATs. (D) Map of *Bam*HI f showing the location and orientation of Vmw65 mRNA and the position of the 12-base-pair insertion in Vmw65, which creates a novel *Bam*HI restriction site.

DISCUSSION

Vmw65 is an HSV-1 virion protein present in the tegument layer between the capsid and the envelope (20). Vmw65 stimulates the transcription of HSV-1 IE genes during lytic virus replication (4, 6, 43). This study examines the consequences of the presence and absence of functional Vmw65 protein in vivo with respect to viral replication and latency. A hypothesis for the mechanism of latent HSV-1 infection that includes a role for this protein is proposed.

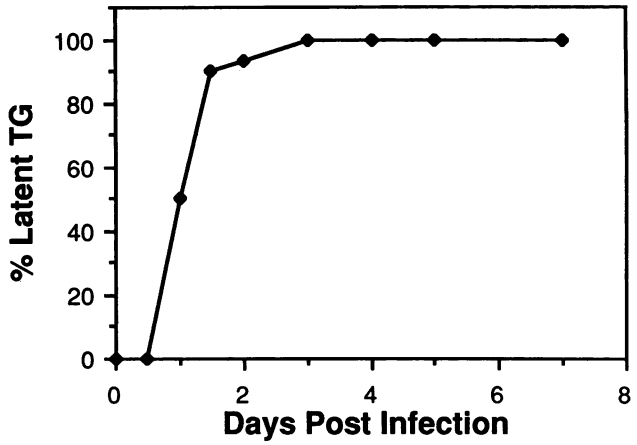

FIG. 4. Time of establishment of HSV-1 latent *in*1814 infection in the trigeminal ganglia of mice. A trigeminal ganglion (TG) was scored positive for latency when cytopathic effects of reactivated virus were detected in the CV-1 monolayer. The numbers are cumulative data from three different experiments. Data are given in percentage of latently infected ganglia at each time point, 10 to 14 ganglia per point.

Vmw65 is required for HSV-1 productive infection in vivo. Productive replication of *in*1814, an HSV-1 mutant which lacks functional Vmw65, was not detected in the eyes or the trigeminal ganglia of mice (Fig. 1 and 2). This correlates with its avirulent behavior and 100% survival rate in mice infected via the cornea. *in*1814 was also avirulent in mice following intraperitoneal or intracerebral inoculation (1). However, we can not completely exclude the possibility that *in*1814 replicated at a very low level, below detection, in mouse eyes or trigeminal ganglia.

Several factors may have contributed to the inability of *in*1814 to replicate well in eyes and trigeminal ganglia. The production of ICP27, an IE gene essential for HSV-1 replication in cell culture (48), is significantly reduced in the absence of Vmw65 (1) and might not be sufficient for virus replication in vivo. In addition, a combined decrease of ICP27, ICP0, and ICP22 (all expressed at reduced levels in cell culture in the absence of Vmw65; 1) might result in a failure to activate the normal viral replication cycle in vivo. Moreover, the situation for *in*1814 in vivo infection in some respects might resemble infection at low MOI in tissue culture cells; while the level of ICP27 expression is sufficient for replication of *in*1814 at high MOI in cell culture, at low MOI in cell culture, the expression of HSV-1 thymidine kinase, an indicator of β gene expression, is dramatically reduced compared with wild-type virus (1).

Viral replication and Vmw65 are not required for establishment of latent HSV-1 infection. Even though replication of *in*1814 could not be detected, the viral genome did reach trigeminal ganglia, probably via axoplasmic transport (26), and established latent infection (Table 2). Even lower amounts of *in*1814 (approximately 10^2 PFU) were capable of establishing a latent infection in some trigeminal ganglia. Despite the fact that *in*1814 did not replicate in trigeminal ganglia, latent infections were established and virus was reactivated from explanted ganglia with normal kinetics. In contrast, experiments with IE (30) and thymidine kinase (7, 14) HSV-1 mutants indicate that these viruses are reactivation defective (see below). These results demonstrate that Vmw65 is not essential for the establishment of latent infection or reactivation at explant.

Previous information on the role of HSV-1 replication at the site of inoculation and in sensory ganglia on the outcome of latent infection is not definitive. With deletion mutants of ICP4 and ICP27, Leib et al. were unable to detect viral replication in corneal swabs or trigeminal ganglia of mice, viral DNA in ganglia, or reactivated virus (30). Since both ICP4 and ICP27 are essential for viral replication, the possibility that the input virus led to latent viral DNA below the level of detection, even though it could not be reactivated, can not be excluded. HSV-1 thymidine kinase deletion mutants were capable of replication at the peripheral site of inoculation but were unable to replicate in sensory ganglia, yet did establish latent infection (7, 14). A number of HSV-1 temperature-sensitive (*ts*) mutants were latency competent at the nonpermissive body temperature of the mouse (2, 31, 34, 60). Since (i) *ts* mutants may replicate slightly at nonpermissive temperatures, (ii) the nonpermissive temperature of some *ts* mutants is higher than the normal temperature of the mouse, and (iii) high doses of inoculated virus may contain wild-type revertants (46), the data derived with such mutants must be interpreted with caution. Our findings, on the basis of the ability of *in*1814 to establish latent infection despite the fact that replication was not detected in vivo, support the hypothesis that HSV-1 replication during the acute stage of primary infection is not a prerequisite for establishment of latent infection. Similarly, it has been shown that failure to synthesize ICP4 and inhibition of the early stages of HSV-1 replication do not prevent establishment of latent infection in vitro (47).

Latency is established as soon as HSV-1 reaches the sensory ganglia. When mice are infected with HSV-1, an acute infection in peripheral sensory ganglia peaks around days 4 to 5 postinfection and then rapidly declines (24, 53). By 14 to 16 days postinfection, no virus can be detected in ganglia homogenates. However, by explant cocultivation, latent virus can be detected. Since infectious *in*1814 was not detected during the first days postinfection in mouse trigeminal ganglia, they were explant reactivated, and it was determined that latency was established between 12 and 48 h postinfection (Fig. 5). This is compatible with estimates of the rate at which herpesviruses travel along peripheral nerves (2 to 10 mm/h) and the distance from the eye to the trigeminal ganglion of the mouse (about 1 cm; 8, 21). Our findings are in accordance with those of Sekizawa et al. (49), in which mice were passively immunized in order to inhibit the acute phase of infection. In their experiments, latency was established within 48 to 96 h postinfection, and perhaps even sooner, since earlier time points were not examined. These data support the hypothesis that latency may be established as soon as the HSV-1 reaches the ganglia. Although operationally latency is established within 12 to 48 h postinfection in mouse trigeminal ganglia, a comparison of events at the molecular level, i.e., the structure of HSV-1 DNA (15, 45) and gene expression at these early times would be informative. It has been observed that the 2.0-kb HSV-1 LAT has been detected as early as 4 days postinfection (51) and may be present even earlier (Spivack and Fraser, unpublished observations).

The mechanisms for establishment of HSV-1 latency and reactivation. Figure 5 illustrates a model for HSV-1 latency that incorporates the present findings with host and viral factors which might influence the outcome of infection.

Host factors. Several cellular factors probably participate in the process of establishment of HSV-1 latency. Within the promoter region of the viral IE genes, there are one to three copies of the TAATGARAT element that mediate the induction of IE genes by Vmw65. Vmw65 associates with these elements but does not bind DNA directly (33, 44, 57). These sites bind cellular, not viral, proteins (27, 28), which suggests that the transinducing function of Vmw65 is dependent on one or more host proteins. These host proteins include an octamer binding protein (36) that interacts with sites contained in IE gene promoter-regulatory domains and also interacts with Vmw65. The functional importance of cellular factors for HSV-1 replication has been demonstrated in a cell line that constitutively expresses a mutant form of Vmw65 lacking *trans*-activating activity (17). These cells have a decreased ability to support HSV-1 replication that correlates with decreased IE gene transcription. Friedman et al. (17) suggested that the mutant Vmw65 competes with the native form contained in the input virions for cellular factors that mediate IE transactivation. Possibly, these cellular factors are present in vivo in cells that become acutely infected and are absent in those neurons that become latently infected. Another possibility is that there might also be cellular factors that repress HSV-1 replication by binding IE promoter-regulatory elements or through other mechanisms. In the latter case, Vmw65 may enable the virus to

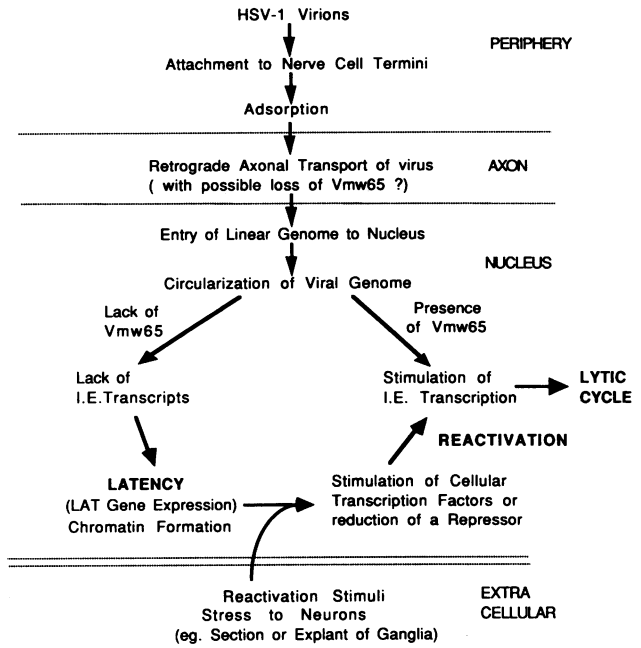

FIG. 5. A model for the mechanism of HSV-1 latency. Virion particles attach to the neuronal cell membrane at the peripheral site of inoculation. It has been postulated that virus is transported to the neuronal cell nucleus with the possible loss of tegument proteins before or at entry to the nucleus (46). Viral DNA enters the nucleus, and lytic or latent infection results, depending on the amount of the IE gene transactivating factor Vmw65 that is transported as part of the tegument. In the presence of Vmw65, the IE genes are induced and an acute infection occurs. During the very early stages of infection, the genome circularizes (39), but the time sequence in relation to HSV-1 transcription is not yet known. In the absence of Vmw65, the IE genes are not expressed and the genome becomes associated with nucleosomes (11) and is maintained in a nonintegrated form (35) with production of the LATs (50, 55). The LATs are not essential for latency but may participate in reactivation (29, 53). Reactivation may occur when the neuron is induced to produce factors which complement the function of Vmw65 and induce IE expression, or when inhibitor levels decline.

overcome a host state that is nonpermissive for viral replication.

Triggering stimuli (X-irradiation, febrile illness, or trauma) may cause reactivation by induction of cellular factors which complement the transinducing activity of Vmw65 and lead to expression of IE genes and viral replication. Since Vmw65 is not expressed during latency (9, 10, 50, 55) and since the IE genes would be expressed during reactivation prior to the synthesis of Vmw65 (a γ gene), it seems unlikely that Vmw65 plays a role in the initial events of the reactivation process. An example of viral gene expression that can be stimulated by host transcription factors is activation of human immunodeficiency virus gene expression in monocytes following induction of the transcription factor NF-kB (19).

Viral factors. In the initial stages of primary infection at the periphery, virus, regardless of its capacity to replicate, enters the nerve terminals. Capsids are transported to the neuronal nucleus by rapid retrograde axonal transport (26). Prior to or at the portal of entry to the nucleus, most of the tegument material, including Vmw65, may be lost (46). Those particles entering the nucleus without sufficient Vmw65 to initiate the transcription of IE genes may enter a latent state directly, as early as 1 day postinfection, without HSV-1 replication. Virions retaining sufficient Vmw65 to activate the IE genes may initiate a productive infection, which eventually will be cleared. Thus, after transport of virions to neuronal nuclei, there are two possibilities: either viral replication or establishment of latency will follow.

Gene expression during latency is restricted and consists of the LATs (9, 50, 55), which can, in part, be detected as early as 4 days postinfection (51). They belong to a new class of viral genes, the λ genes (52), indicating that the transcription patterns during productive infection and latency are different. The LATs are not essential for the establishment or maintenance of latency but appear to have a role during reactivation (29, 53). Thus, two viral factors appear to play an important role in HSV-1 infections in vivo; Vmw65 levels influence initiation of the lytic replication cycle during primary infection, and the λ genes (LATs) modulate the reactivation of latent infection.

In conclusion, although functional Vmw65 is required for normal levels of HSV-1 replication in mice following infection via the eye, Vmw65 is not necessary for establishment of or reactivation from latent infection. Perhaps more importantly, detectable HSV-1 replication during primary infection is not a requirement for the establishment of latent infection in peripheral sensory ganglia. Latent HSV-1 infection can be established as soon as virus reaches the ganglia. These data provide further evidence that productive and latent infections may represent two separate modes of interaction between HSV-1 and the host.

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