Establishment of Latency in Mice by Herpes Simplex Virus 1 Recombinants That Carry Insertions Affecting Regulation of the Thymidine Kinase Gene

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Herpes simplex virus 1 recombinants carrying α -, β -, and late γ (γ_2)-regulated thymidine kinase (TK) genes were tested for the ability to establish latency in BALB/c mice inoculated by the eye route. The significant findings were as follows. Representatives of α - and γ_2 -regulated TK recombinants all established and maintained latent infections, but the efficiency was somewhat lower than that of wild-type virus. Of the three α TK recombinants tested, one (R316) spontaneously deleted portions of the inserted sequences which conferred α regulation to the TK gene. The viruses carrying these deletions expressed considerably lower TK activity than did wild-type virus, i.e., 2 to 40% of the levels expressed by the wild-type virus carrying the β TK gene. However, the ability of these viruses to establish latency was not related to the efficiency of expression of the TK gene. These results indicate the following: (i) conversion of the TK gene into an α or γ_2 gene did not preclude the establishment of latent infections; (ii) there was no correlation between the levels of TK activity expressed in cell culture and the ability to establish latency; and (iii) rearrangement of the genome by insertions or deletions which interrupt gene domains did not automatically result in an inability to establish latent infections.

In the current model of herpes simplex virus (HSV) latent infections, the virus invades nerve endings associated with dorsal root ganglia at the portal of entry into the body and ascends to the main cell body of the neuron, where it rests in a static (23) nonreplicating state. When triggered to multiply, the virus descends to a site at or near the portal of entry and infects the abutting cells. Although it is convenient to refer to the entire process as establishment of the latent state, it is clear that virus multiplication at the portal of entry may precede invasion of nerve endings and that the requirements for the initiation of the latent state may be different from those necessary to maintain it.

One of the major issues concerning this model is the requirement for viral gene expression during two phases of the process, i.e., initiation and maintenance of the latent state. The issue stems from the following considerations. It is difficult to conceptualize a total absence of viral gene expression from a competent viral genome resident in a cell capable of enabling it to multiply. Most DNA viral genomes, such as papovaviruses and Epstein-Barr virus, when resident in animal cells can and do express at least a subset of the genes when they encode (15, 16, 22). Inasmuch as HSV-1 produced in recurrent lesions is frequently the source of virus transmitted to susceptible individuals, the latent virus that gives rise to the recurrent lesion forms a significant reservoir for the maintenance and spread of the virus in the human population. The hypothesis that viral functions assist in the establishment of the latent state is therefore more likely to be true and certainly more amenable to testing than is the alternative that suggests that all functions required for this process are contributed by the host cell; the virus is likely to have developed functions to assist its dissemination in this manner. The major hazard in attempts to identify the viral genes putatively expressed during latency is the as-

sumption that they are a subset of genes expressed during a lytic infection; nevertheless, the latter is the sole guide to the experimental analysis of gene expression in the latent state.

In lytically infected cells, HSV gene expression consists of a series of highly ordered events leading to cell destruction and virus multiplication. The HSV-1 genes, for example, form at least three groups, α , β , and γ , whose expression is coordinately regulated and sequentially ordered in a cascade fashion (9, 10). At least the β and γ genes are nonhomogeneous, and subgroups (e.g., β_1 , β_2 , γ_1 , γ_2) which may be differentially regulated have been recognized (8, 17, 25). The genes responsible for lytic outcome of virus multiplication have not been identified, but the irreversible damage that is manifest in the cessation of host macromolecular metabolism, the margination of chromatin, and the disaggregation of the nucleolus has been associated with expression of β and γ genes (24). Although the events occurring in productive infections need not parallel those occurring in the latent state, it is more likely that the viral genes putatively required for the establishment of the latent state belong to the α group rather than to the groups expressed later in infection.

Because the number of neurons harboring virus in experimentally infected animals is small (12, 29), direct detection of viral gene products presents numerous technical problems compounded by the observations that α proteins are poorly soluble, made in relatively small amounts even in lytic infections, and generally difficult to quantitate (1, 2, 30). Nevertheless, the presence of one α protein, α_4 , in the absence of late proteins, has been reported in ganglia of rabbits harboring latent HSV-1 (6).

In an attempt to surmount these difficulties, we tested four HSV-1 recombinants carrying α - or γ -regulated genes whose products are readily assayable for the ability to establish latent infections.

Our comparison of the efficiency of latent infections by recombinants carrying thymidine kinase (TK) under the

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FIG. 1. DNA sequence arrangements of parental and recombinant viruses. The top two lines show the sequence arrangement of HSV-1 DNA, detailing the L and S components and terminal-reiterated sequences. HSV-1(F) is the parent virus of all of the recombinants; *Bam*HI restriction fragments are labeled. R314 carries the *Bam*HI Z fragment containing the $\alpha 4$ promoter inserted into the *Bgl*III site of the TK leader sequence so that the α promoter and TK are in the same transcriptional orientation; TK is regulated as an α gene (18). R316 is a similar construction, with the *Bam*HI N fragment containing the other copy of the $\alpha 4$ promoter inserted into the *Bgl*III site of TK (18). R3104 contains two copies of an $\alpha 27$ chimeric TK gene inserted into the reiterated sequences of the S component in *Bam*HI-P between the *a* sequence and the 3' end of the ICP4 gene, as well as a 700-base-pair deletion in the original TK gene (11). R3112 carries the *Bam*HI D' fragment containing a γ_2 promoter inserted into the *Bgl*III site of the TK gene; TK is regulated as a γ_2 gene (25). Ba, *Bam*HI; Bg, *Bgl*III; S, *Sac*I; P, *Pvu*II; Bs, *Bst*EII. P₂₇ refers to the promoter-regulatory region of the $\alpha 27$ gene which was linked to TK in R3104. *Bam*HI fragments contain the ICP4 promoters (N and Z), the TK gene (Q), and the γ_2 promoter (D'); P is the terminal fragment into which the $\alpha 27$ -TK gene was inserted in R3104.

control of α , β , or γ promoter-regulatory domains yielded three significant observations. (i) The perturbations introduced into the genome by the creation of α TK and γ TK chimeras reduced the frequency but did not preclude the establishment of latent infections by the recombinants. (ii) Only one of the four constructs tested was uniformly unstable in laboratory animals and gave rise to deletion mutants that colonized the ganglia. (iii) The efficiency of establishment of latent infections did not correlate with the level of TK expression.

MATERIALS AND METHODS

Cells. African green monkey kidney (Vero) cells were obtained from the American Type Culture Collection, Rockville, Md. Ltk⁻ cells were a gift from Saul Kit.

Viruses. HSV-1(F), an isolate passaged a limited number of times in culture (3), is the parent strain of all recombinants used in this study and the prototype HSV-1 strain used in this laboratory.

R314 and R316 recombinant viruses carry α TK genes constructed by the insertion of the *Bam*HI Z or N fragments, respectively, into the *Bgl*III site in the 5' transcribed noncoding sequences of the TK gene so that the $\alpha 4$ promoter-regula-

tory sequences and the TK coding sequences are in the same transcriptional orientation (18). R3112 is a chimeric γ_2 TK virus carrying the γ_2 promoter contained in the *Bam*HI D' fragment of HSV-1(F) (7) inserted into the *Bgl*III site of the TK gene (25).

R3104 carries two copies of a chimeric $\alpha 27$ -TK gene inserted into the reiterated sequences of the S component approximately 620 base pairs from the *a* sequence of the L-S junction (11).

TK assays. [³H]thymidine was used as a substrate for the TK assays done on cytoplasmic cell extracts as described by Post et al. (18). Ltk⁻ cells were infected at a multiplicity of 5 PFU per cell and harvested at various times after infection. For cycloheximide reversals, Ltk⁻ cells were incubated in medium containing 100 μ g of cycloheximide per ml for 1 h, then infected at a multiplicity of 10 PFU/ml in medium containing 100 μ g of cycloheximide per ml. At 5 h postinfection, 1 h before removal of the cycloheximide, the medium was replaced with medium containing 100 μ g of cycloheximide and 10 μ g of actinomycin D per ml. At 6 h postinfection, the cells were washed extensively in medium containing 10 μ g of actinomycin D per ml, incubated in the presence of actinomycin D for 1 h, and harvested. TK activity was expressed as counts per minute of [³H]TMP per microgram

of protein for each recombinant or ganglion-isolated strain. The percentage of wild-type levels of TK produced was calculated as the maximal level of TK produced by the recombinant virus divided by the maximal level of TK produced by HSV-1(F) in Ltk⁻ cells infected at a multiplicity of 5 PFU per cell.

Infection of animals. Five- to seven-week-old BALB/c AnN mice (Goodwin Institute for Cancer Research, Plantation, Fla.) were inoculated under sodium pentobarbital anesthesia injected intraperitoneally. After gentle scarification of the cornea with a hypodermic needle, the eyes were bathed with approximately 10⁷ PFU per eye contained in about 10 μ l of virus suspension. In different experiments, either the right eye or both eyes were infected.

Recovery of virus from infected animals. To recover latent virus, the mice were killed 4 to 5 weeks after inoculation and the trigeminal ganglia were removed. Ganglia were incubated in medium 199 with 5% calf serum at 37°C for 4 to 5 days and then homogenized and plated on Vero cell monolayers. Negative cultures were frozen and passaged again in Vero cells to recover very low quantities of virus.

RESULTS

The viruses used in these studies are listed in Fig. 1. The wild-type parent HSV-1(F) virus carries an unmodified β TK gene (14). R316 and R314 were constructed by insertion of sequences from the promoter-regulatory domains of each of the two copies of the α 4 genes, in the proper transcriptional orientation, into the *Bgl*II site of the transcribed domain of the TK gene (18). In the construction of these recombinants, the coding sequences and portions of the 5' transcribed noncoding domains of the TK gene were fused to portions of the 5' transcribed noncoding domain, capping site, and promoter-regulatory sequences of the α 4 genes. The chimeric genes are regulated as α genes. R3104 was constructed by fusion of the 5' transcribed noncoding and coding sequences of the TK gene to the 5' transcribed noncoding sequences, capping site, and promoter-regulatory domain of the α 27 gene. The chimeric gene was then inserted into the reiterated sequences of the S component between the *a* sequence and the 3' terminus of the α 4 gene. Because insertions or deletions in one copy of the S component inverted repeats are generally reflected in the other copy, the recombinant R3104 contains two copies of the α 27-TK chimeric gene (11). R3112 was constructed by insertion of a HSV-1(F) DNA fragment (*Bam*HI-D') which contains a γ 2 promoter (7) in the proper transcriptional orientation into the *Bgl*II site within the transcribed noncoding domain of the TK gene (25). The resulting chimeric gene is regulated as a γ 2 gene, i.e., its expression is stringently dependent on viral DNA synthesis.

Experimental design. BALB/c mice were inoculated with approximately 10⁷ PFU per eye into one or both eyes. The trigeminal ganglia were removed 4 to 5 weeks after inoculation, and the virus was recovered as described above. All of the recombinant viruses recovered from ganglia were examined for stability of the inserted sequences by restriction endonuclease analysis of their DNA. Because rearrangements in the DNA affecting TK expression may not be detectable by restriction endonuclease analysis, analyses of the regulation of TK genes expressed by the isolates from ganglia were done in cells in culture. Some of the ganglionic isolates were reexamined for the ability to establish latent infections by reinoculation in BALB/c mice. The results of these studies are listed in Table 1. Figures 2, 3, and 4 show

TABLE 1. Frequency of establishment of latency in trigeminal ganglia of BALB/c mice and properties of parent and progeny viruses

Virus	No. positive ganglia ^a /no. eyes infected	Latency (%)	Maximum TK (%) ^b	TK ^c regulation
HSV-1(F)	20/20 ^{d,e}	100	100	β
R314	10/19 ^e	53	ND	α
M314-1A	8/10	80	ND	α
M314-5A	4/10	40	ND	α
M314-10A	8/10	80	ND	α
R3104	7/20 ^e	35	ND	α
M3104-1A	5/10	50	ND	α
M3104-3A	7/10	70	ND	α
M3104-7A	5/10	50	ND	α
R316	6/25 ^{e,f}	24	57	α
M316-2	9/15 ^e	60	2	Not measurable
M316-6	15/15 ^e	100	23	Non- α
M316-10	14/15 ^e	93	2	Not measurable
M316-8D	0/19 ^e	0	42	α
R3112	9/20 ^e	45	35	γ

^a Ipsilateral to the inoculated eye.

^b Measured TK activity at 8 h postinfection in replicate cell cultures infected at the same multiplicity. All percentages are in relation to parental HSV-1(F) activity. ND, Not done.

^c Based on data from Table 2. The natural TK gene is regulated as a β gene (14).

^d One of many experiments; frequency of latent infections was always nearly 100%.

^e Virus inoculated in one eye only.

^f Combined figures from two separate experiments (3 of 10 and 3 of 15).

the results of the restriction analyses. Figure 5 and Table 2 show the results of analyses of the expression and regulation of the TK genes.

Establishment of latent infections by wild-type and recombinant viruses. The parent HSV-1(F) and the recombinant viruses R314, R316, R3104, and R3112 established latent infections in trigeminal ganglia when inoculated by the eye route (Table 1). The results for the parent virus involving 20 mice are those germane to this study. In both this and other studies (B. Meignier and B. Roizman, unpublished data), HSV-1(F) at 10⁷ PFU per eye established latency in nearly 100% of ganglia ipsilateral to the inoculated eye with little or no mortality. The incidence of latent infections in mice inoculated with the recombinant viruses ranged from 24 to 50% of the ganglia ipsilateral to the inoculated eye.

Stability of inserted sequences during passage of recombinant viruses in mice. Analyses of the restriction endonuclease patterns of the viruses recovered from ganglia revealed no changes in two of the four recombinants tested. Specifically, no changes were seen in recombinants R314 and R3112 carrying α 4TK and γ 2TK chimeric genes (Fig. 2 and 3). One of the seven isolates from ganglia of mice inoculated with R3104, which carries two copies of the α 27-TK chimeric gene, yielded a pattern identical to the virus into which the chimeric gene was inserted (M3104-5A; Fig. 2). Although this isolate could have arisen by deletion of the inserted sequences, it is most likely a contaminating parent TK⁻ virus.

An entirely different situation arose in the case of R316 carrying an α 4-TK chimeric gene constructed by the inser-

FIG. 2. DNA digests electrophoretically separated in agarose gels and stained with ethidium bromide. Latent virus was recovered from infected ganglia 35 days after eye inoculation. Cytoplasmic DNA was prepared from infected Vero cells and digested with *Bam*HI restriction endonuclease. M314-1A through M314-10A were isolated from mice infected with R314; M3104-1A through M3104-7A were isolated from mice infected with R3104. The arrows indicate the new bands formed by the insertion of *Bam*HI-Z into *Bam*HI (R314) or the chimeric α 27-TK fragment into *Bam*HI-P (R3104).

FIG. 3. DNA digests electrophoretically separated in agarose gels and stained with ethidium bromide. Latent viruses (M3112-1 through M3112-9) were recovered from infected ganglia 35 days after eye inoculation with R3112. Cytoplasmic DNA was prepared from infected Vero cells and digested with *Bam*HI restriction endonuclease. The arrow indicates the position of the band formed by the insertion of *Bam*HI-D' into *Bam*HI-Q in R3112.

tion of the HSV-1(F) *Bam*HI N fragment instead of the *Bam*HI Z fragment used in the construction of the R314 recombinant. In this instance all but one of the six isolates from two separate experiments showed deletions contained entirely within the domain of the fragment carrying the α promoter-regulatory sequences that had been inserted into the TK gene (Fig. 4). The deletions were not specifically related to latent virus; similar deletions were detected in virus isolated from the eye 2 and 4 days after inoculation (data not shown). Similar deletions were also reproduced in certain types of cells in culture, and both the left and right borders of the deleted domains seem to vary from one isolate to another (A. E. Sears and B. Roizman, *Virology*, in press).

M316-8D was the only isolate from ganglionic latent infection by R316 which had no demonstrable deletions. Unlike the other ganglionic isolates which grew as well as the parent virus in cell culture, M316-8D grew more slowly than the parent virus and may have undetected rearrangements or deletions in its genome.

Expression of TK by recombinants and ganglion-isolated strains. To determine the levels of TK expressed by the R316 parental and ganglion-isolated strains, Ltk^- cells were infected at a multiplicity of 5 PFU per cell, the cultures were harvested 2, 4, 6, or 8 h after infection, and the amount of TK was measured by methods previously described (18). To facilitate comparison, the maximum levels of TK gene expression of the recombinant and ganglionic isolates are given as percentages of the parental HSV-1(F) activity (Table 1). Figure 5 shows the TK activity obtained for HSV-1(F), R316, and the six isolates from the ganglia of mice infected with R316. Of these six isolates, M316-2 and

M316-10 expressed barely detectable levels of TK activity. M316-8D virus, which manifested no detected changes in the TK domain of its genome, accumulated only half as much TK as did HSV-1(F) 8 h after infection. This decrease may be due to the sluggish growth of this virus rather than to changes in the promoter-regulatory domain of the TK gene.

Ganglionic isolates of the α TK recombinants were tested for retention of α regulation of the TK gene (Table 2). Ltk⁻ cells were infected at multiplicities of 10 PFU per cell in the presence of cycloheximide. At 5 h postinfection, 1 h before removal of the cycloheximide, actinomycin D was added to the media. The cultures were maintained in actinomycin D and harvested 1 h after the cycloheximide was removed. The results were consistent with the observation that α TK chimeric genes other than that of R316 were stable. In the case of R316 and its ganglionic isolates, the TK genes of R316 and one of the isolates, M316-8D, were regulated as α genes, whereas those of M316-3D and M316-5D were not. Because of the low levels of expression of TK by M316-2 and

FIG. 4. DNA digests electrophoretically separated in agarose gels and stained with ethidium bromide. Ganglionic isolates M316-2, M316-6, and M316-10 were recovered from one experiment; the others, M316-3D, M316-5D, and M316-8D, were from another experiment. Latent virus was recovered from infected ganglia 28 days after eye inoculation with R316. Cytoplasmic DNA was prepared from infected Vero cells and digested with *Bam*HI restriction endonuclease. The arrow indicates the position of the band formed by the insertion of *Bam*HI-N into *Bam*HI-Q in R316. Note that the terminal fragments *Bam*HI-S and -P are heterogeneous; both fragments vary in size. The variation in the size of *Bam*HI-S is at least in part due to variation in the number of reiterations of the terminal *a* sequence.

FIG. 5. TK assays of Ltk⁻ cells infected with M316 ganglionic strains. Ltk⁻ cells were infected at a multiplicity of 5 PFU per cell with HSV-1(F), R316, or one of the six virus strains isolated from trigeminal ganglia of mice infected with R316. The cells were harvested at the indicated times after infection, and [³H]thymidine TK assays were done on cytoplasmic extracts of the infected or mock-infected cells. TK levels are given as counts per minute of [³H]TMP per microgram of protein.

M316-10, α regulation of TK by these viruses would have been undetectable.

The levels of expression and regulation of the TK genes of R3112 and the strains isolated from ganglia after inoculation with R3112 were also examined; TK assays of Ltk⁻ cells infected with these viruses in the presence and absence of phosphonoacetate (data not shown) indicated that the γ_2 regulation of R3112 TK was unaltered after passage in mice.

Reestablishment of latency by viruses isolated from murine ganglia. To verify the stability of the viruses upon passage in mice, several of the isolates recovered from ganglia were grown in Vero cells and reinoculated into mice. The results of these experiments (Table 1) show that progeny of R314 and R3104 were capable of establishing latent infections at the same or higher frequencies than those of the parental strains. In the case of the progeny of R316, three of the four tested, which carried deletions in the inserted sequences, were able to establish latency at frequencies superior to that of the parent virus stock with no further genomic rearrangement or changes in the level of expression or the regulation of the TK genes in the viruses recovered from ganglia (data not shown). However, the one isolate which exhibited no deletions by restriction endonuclease analysis (M316-8D) failed to establish and maintain latent infections in BALB/c mice. The striking feature of these results is the lack of

correlation between the levels of TK expressed by the recombinants and ganglionic strains and the ability to establish latent infections.

DISCUSSION

In this paper we present evidence that HSV-1 recombinants carrying either α - or γ_2 -regulated TK genes, as well as isolates producing extremely low levels of TK, were capable of establishing latent infections in mice. The salient features and significance of the results presented here are as follows.

(i) This study was the first attempt to establish latent infections with recombinant viruses carrying specifically engineered insertions or deletions. Although the site of the insertion of the DNA fragments into the domain of the TK gene did not affect the primary structure of the TK enzyme, it may have affected other genes reported to encompass that site (21). Although the chimeric TK gene of R3104 was inserted into a domain not known to have a function and which can be deleted (11), R3104 also carries a 700-base-pair deletion in the TK gene and its natural location. The observation that establishment of latency in this model was not seriously compromised by restructuring of the genome, at least to the extent tested, opens the way for systematic analyses of the function and expression of genes in latent infections. The observation that HSV-1 genomes carrying

α TK chimeric genes were able to establish latency suggests that either the α TK gene is not expressed during latency or mere capability of expansion in the deoxynucleoside triphosphate pools in neurons does not trigger the termination of the latent state. The α chimeric genes may help establish whether α genes are expressed during the course of latent infections, and deletion mutants may establish their function. It is noteworthy that recombinants carrying deletions in the $\alpha 22$ gene constructed by Post and Roizman (19) were able to establish latent infections (24a).

(ii) Numerous previous reports have established the role of the TK gene in productive infection of ganglia and brain tissue in laboratory animals (5, 20, 28). Many others have shown that TK⁻ viruses have greatly diminished abilities to establish latent infections (4, 5, 13, 20, 26, 27), and it has been postulated that this lack of latency is due to the inability of the viruses to replicate in the ganglia at the time of infection (26). It has also been shown that the levels of TK produced by a virus in cell culture correlate directly with the ability of the virus to replicate in ganglia and to the virulence of the virus (5, 28); it therefore might be supposed that levels of TK expression also correlate directly with the ability of a virus to establish latent infections.

Our results indicate that this is not the case. There was no relationship between the levels of TK produced by the viruses tested in these studies and the ability to establish latent infections. Furthermore, two of the strains tested, M316-2 and M316-10, produced so little TK that by the standards used in other studies (4, 28) they would have been classified as TK⁻, yet they were able to establish latent infections at high frequencies. Part of the discrepancy in the reported results may reflect the method for selection of some TK⁻ viruses, because secondary undetected mutations may contribute to the failure of some of these mutants to establish latent infections in experimental systems.

(iii) The observation that in murine tissues deletion mutants of R316 arise and grow preferentially was totally unexpected. The virus was originally isolated in Vero cells, in which deletions had not previously been observed (19). Subsequent studies revealed that similar deletions arise not only in murine cells but also in HEp-2 and human embryonic lung cells (Sears and Roizman, in press). It is clear from these results that the deletion mutants were not selected for by any requirement of latent infection. The apparent growth and stability of the R316 recombinant in Vero cells was in stark contrast to its instability in other cell lines and strains. Although the exact mechanism for the preferential growth of deletion mutants is not known, the results presented in this paper underscore the necessity of ensuring that the viruses recovered from experimental systems such as mice bear a reasonable likeness to those used in the original inoculations.

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TABLE 2. Verification of the α regulation of the TK gene of isolates recovered from trigeminal ganglia of mice inoculated with different recombinant strains

Expt no.	Virus	TK activity (cpm/ μ g)
1	HSV-1(F)	4
	R316	359
	M316-2	4
	M316-6	3
	M316-10	5
	M316-3D	3
	M316-5D	5
	M316-8D	66
2	Mock	5
	HSV-1(F)	4
	R314	64
	M314-1	121
	M314-2A	132
	M314-3A	91
	M314-4A	89
	M314-5A	109
	M314-6A	48
	M314-7A	47
	M314-8A	90
	M314-9A	63
	M314-10A	138
3	Mock	8
	HSV-1(F)	5
	R316	580
	R3104	11
	M3104-1A	13
	M3104-2A	11
	M3104-3A	13
	M3104-4A	14
	M3104-5A	6
	M3104-6A	15
	M3104-7A	13

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