In Vivo and In Vitro Reactivation Impairment of a Herpes Simplex Virus Type 1 Latency-Associated Transcript Variant in a Rabbit Eye Model

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Many recent studies of latent herpes simplex virus type 1 (HSV-1) infections within the nervous system have focused on the diploid genes encoding the latency-associated transcripts (LATs). The impaired explant reactivation of LAT variants from mouse trigeminal ganglia has implicated the LATs in the efficiency or speed of the reactivation process (D. A. Leib, C. L. Bogard, M. Kosz-Vnenchak, K. A. Hicks, D. M. Coen, D. M. Knipe, and P. A. Schaffer, J. Virol. 63:2893–2900, 1989; I. Steiner, J. G. Spivack, R. P. Lirette, S. M. Brown, A. R. MacLean, J. H. Subak-Sharpe, and N. W. Fraser, EMBO J. 8:505–511, 1989). However, it is not known how closely explant reactivation mimics the reactivation process in vivo. In the current study, a LAT variant (1704), parental strain (17+), and rescuant (1704R) were compared in vivo for reactivation of latent infection by iontophoresis in the rabbit eye model and in vitro by explant cocultivation of trigeminal ganglia from rabbits. Following iontrophoresis, 17+ and 1704R reactivated in vivo from 76 and 64% of rabbits, respectively, while 1704 reactivated only from 4% (1 of 25) of the animals. In explant reactivation experiments, 17+ and 1704R reactivated from 98 and 67% of rabbit trigeminal ganglia, while 1704 reactivated from only 28% of trigeminal ganglia. The mean time required for the appearance of reactivated 1704 in explant culture, 17 days, was significantly longer than for 17+ and 1704R, 8 to 9 days. Thus, the explant reactivation kinetics in rabbit trigeminal ganglia reflect the behavior of LAT variant 1704 in vivo in the rabbit eye model. These data support the role of the LATs in the reactivation process and support the hypothesis that explant reactivation is a suitable system for analyzing the biological behavior of HSV-1 variants with defined genetic alterations in the LAT gene.

After primary infection with herpes simplex virus (HSV), a latent state is established in neural (4) and perhaps also in nonneural tissue (16). Many herpetic diseases are the result of reactivation of latent virus; these can be life threatening in immunosuppressed individuals. The mechanisms of latency and reactivation have not been elucidated, although several experimental methods have been utilized to intentionally reactivate latent virus (6, 30, 31).

HSV type 1 (HSV-1) transcription during latency, as studied by in situ hybridization, is limited to a 10-kb region within the long repeat regions (Fig. 1) (1, 2, 5, 15, 21, 28, 29). Three herpesvirus-specific RNAs (latency-associated transcripts, LATs) are detectable by Northern blot (RNA) analysis and map to a 3-kb location within the region positive by in situ hybridization (21, 23, 29). Recently, Farrell et al. (3) have suggested that the 2.0-kb RNA is an intron from a larger transcript. The LATs are encoded by a diploid gene, overlap with the immediate early gene ICP0, and are transcribed in the opposite direction (21, 23, 29). The LATs are present in reduced amounts in acutely infected mice (24) and infected tissue culture cells (23) and may represent a new kinetic class of HSV-1 genes (25).

HSV-1 variants and mutants have been used to investigate the function and importance of the LATs during several stages of herpesvirus infection (10, 11, 13, 27). Through the studies of HSV-1 variants and mutants, it has been demonstrated that the LATs are not required for viral replication in tissue culture or in animal models or for the establishment or maintenance of a latent infection. These HSV-1 mutants, which do not express the LATs, establish latent infections in mouse peripheral sensory or trigeminal ganglia and reactivate following explant. HSV-1 strain 17+ variant 1704 (12, 14), which has a 3.5-kb deletion in the internal long repeat (IR_L) and adjacent unique region and a 0.9-kb deletion in the terminal long repeat (TR_L) (Fig. 1), does not express any detectable LATs during latency (27). In mice, variant 1704 established latency; however, following trigeminal ganglion explant, the time required for the LAT minus strain 1704 to reactivate was significantly greater than for parental strain 17+, implying that the deleted genetic information in 1704 modified the reactivation process in vitro and that the LATs may have a functional role during reactivation in vivo (27). An HSV-1 strain KOS-derived LAT deletion mutant, which lacks the LAT promoter, also reactivated inefficiently from explanted mouse trigeminal ganglia (13).

Many studies of HSV reactivation in explanted ganglia have been performed. This is clearly not a normal stimulus for reactivation of latent infections in humans and may not reflect the clinical situation. Rabbit and mouse eye models in which the latent virus is induced to reactivate in vivo by iontophoresis have been developed which may more accurately reflect the natural situation. However, there is great variation in the ability of wild-type strains to reactivate in these systems (6, 8). A LAT promoterless HSV-1 × HSV-2

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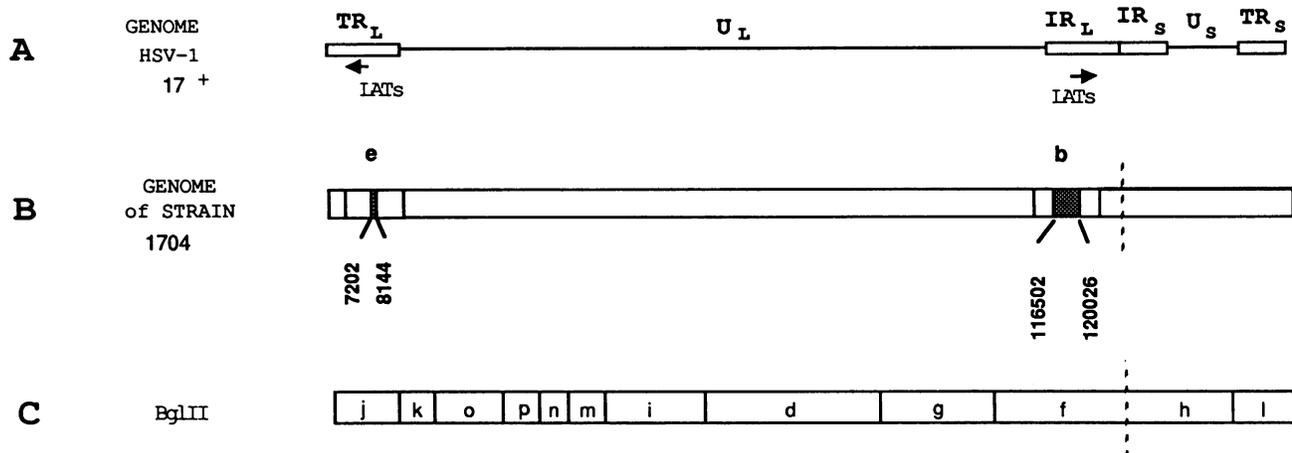† Present address: Hadassah University Hospital, Jerusalem, Israel.

FIG. 1. (A and B) Maps of HSV-1 genomes. (A) Parent strain 17$^+$ encoding two copies of LAT. One LAT gene is encoded in each of the long repeat (IR$_L$ and TR$_L$) regions. TR$_S$, short terminal repeat; IR$_S$, short internal repeat; U$_L$, long unique; U$_S$, short unique. (B) Deletion mutant 1704 derived from strain 17$^+$ has no intact LAT genes because of a 3.5-kb deletion in the IR$_L$ and adjacent unique region and a 0.9-kb deletion in the TR$_L$ (12) (no expression from either TR$_L$ or IR$_L$) (27). Shaded regions indicate the deletions in 1704. Nucleotide numbers (12) are from the sequence of strain 17$^+$ (17). (C) Map of restriction enzyme BglII. The joint fragments are as follows: a = f + h, c = f + l, b = j + h, and e = j + l.

recombinant reactivated poorly in the rabbit eye model (9). In the current work to investigate the role of the LATs during in vivo and in vitro reactivation in a rabbit eye model, we have used HSV-1 strain 17$^+$ (LAT$^+$), deletion mutant 1704 (LAT$^-$), and a rescuant, 1704R.

Deletion mutant 1704 has sequences deleted between nucleotides 7202 and 8144 of the TR$_L$ region (0.9 kb) and between nucleotides 116502 and 120026 of the IR$_L$ region (3.5 kb) (12). The rescuant was prepared with the BamHI b fragment of strain 17$^+$ and HSV-1 strain 1704 DNA. Figure 2 shows an autoradiograph of ^{32}P-labeled DNA from strains 17$^+$, 1704, and 1704R which demonstrates that 1704R is repaired in the lesions of 1704. The 0.9-kb deletion in the terminal repeat copy of the LAT results in a noticeable shift of the j fragment to the j* position (Fig. 2, lane 2). However, when the 0.9-kb deletion is present in the two large joint fragments b (j plus h) and e (j plus l), only a small shift is observed. The 3.5-kb deletion in the internal repeat copy of the LAT results in the shift of fragment f to coincide with fragments g. The joint fragment a (f plus h) shifts to the position of b, which has moved to the position of c (f plus l), and c has shifted to coincide with d, a unimolar fragment. The revertant 1704R, shown in lane 3, is identical to strain 17$^+$, shown in lane 1.

New Zealand White rabbits (3 to 3.5 kg) were inoculated in both eyes with 1.3 × 10^6 PFU/50 μl of HSV-1 strain 17$^+$, 1704, or 1704R. Acute ocular infection by these viruses was confirmed by virus isolation from eye swab samples collected on day 3 postinoculation and by clinical signs at day 5 postinoculation (30). Ocular disease for each virus was limited to conjunctivitis and occasional corneal epithelial involvement in the form of dendrites. No obvious differences in the severity of acute ocular disease were noted for the three viruses on day 5. Only eyes in which primary HSV infection was confirmed by virus isolation were iontophoresed at 4 weeks postinoculation. In vivo reactivation of latent HSV was induced by bilateral iontophoresis of 6-hydroxydopamine into the cornea, as previously described (22). Ocular shedding of virus was determined by swabbing the eyes daily for up to 16 days following iontophoresis (30)

and was most successful (virus isolated from tear samples) in animals infected with either 17$^+$ (19 of 25 [76%] infected in one or both eyes) or 1704R (7 of 11 [64%]), each with two copies of LATs (Table 1). In vivo reactivation of the LAT deletion mutant 1704 (no functional LAT copies) was significantly less (1 of 25 [4%]; $P < 0.001$ as determined by the chi-squared procedure and pairwise comparison). Restriction enzyme digestion and Southern blot analysis confirmed
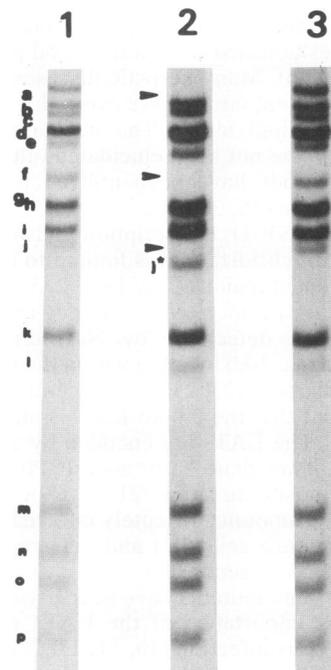

FIG. 2. Autoradiograph of in vivo ^{32}P-labeled HSV-1 DNA digested with BglII (18). Lane 1, strain 17$^+$; lane 2, 1704; lane 3, 1704R. Arrows show bands missing in 1704. j* indicates a truncated j fragment. Letters at left denote fragments.

TABLE 1. In vivo reactivation studies: isolation of HSV-1 from tear samples following intentional stimulation

Virus[a]	Reactivation by iontophoresis	
	No. of rabbits shedding virus/no. of rabbits tested (%)	No. of eyes shedding virus/no. of eyes tested (%)
17[+]	19/25 (76)	30/48 (63)
1704	1/25 (4)	1/35 (3)
1704R	7/11 (64)	10/22 (45)

[a] Chi-square pairwise comparison of eye data for each group is as follows: 17[+] versus 1704, $P < 0.0001$; 17[+] versus 1704R, not significant; 1704 versus 1704R, $P < 0.001$.

that the reactivated virus was indistinguishable from input virus for each strain (Fig. 3).

An in vitro explant cocultivation reactivation time course of HSV-1 strains 17[+], 1704, and 1704R is presented in Fig. 4. Upon sacrifice, the trigeminal ganglia were minced and cocultivated on monolayers of secondary rabbit kidney cells and examined daily for cytopathic effects for up to 28 days. The LAT minus strain, 1704, reactivated more slowly and less efficiently than the parental virus from explanted rabbit trigeminal ganglia, as has been demonstrated in mouse model systems (13, 28). The 17[+] strain was recovered most frequently from trigeminal ganglia (21 of 21 rabbits [100%]
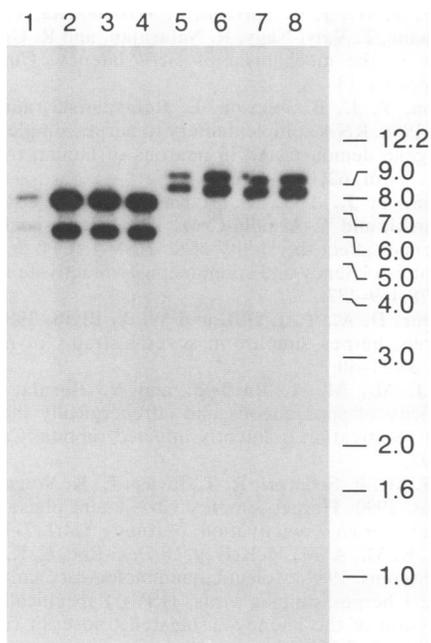

FIG. 3. DNA analysis of reactivated virus isolates. Both parental and reactivated virus isolates were used to infect CV-1 cells for isolation of virion DNA from the cytoplasmic fraction of the infected cells, as described by Pignatti et al. (18). BamHI restriction digests of parental 1704 (lane 1), reactivated 1704 (lanes 2, 3, and 4), parental 17[+] (lane 5), and reactivated 17[+] (lanes 6, 7, and 8) are shown. DNAs were resolved by agarose gel electrophoresis, transferred to nitrocellulose by Southern blotting, hybridized with ^{32}P-labeled, nick-translated HSV-1 (strain F) restriction fragment BamHI E (a gift of B. Roizman [19]), washed, and autoradiographed by standard procedures (20). Molecular sizes (in kilobases) are indicated at right.
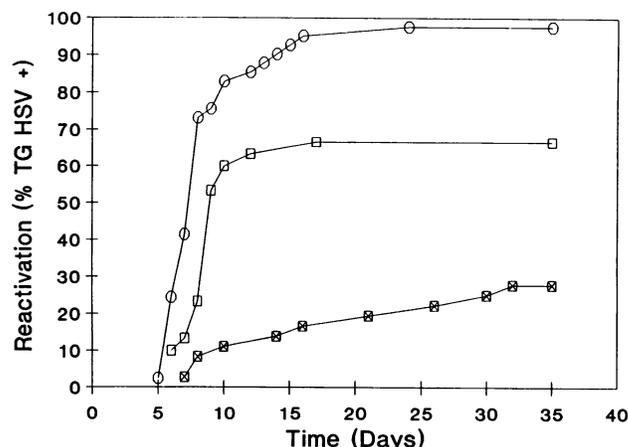

FIG. 4. Explant reactivation of latent virus. Trigeminal ganglia from rabbits inoculated with 17[+] (O), 1704 (⊠), and 1704R (□) were explanted onto rabbit kidney cell monolayers. Reactivated latent HSV induced cytopathic effects. Data are expressed as the percentage of trigeminal ganglia with reactivated latent HSV versus time of incubation.

infected in one or both trigeminal ganglia), while the 1704 strain was recovered least frequently (8 of 22 [36%]) and 1704R was recovered from 12 of 15 (80%) animals (Table 2). There appeared to be an inverse correlation between the incidence of isolating latent virus that had reactivated and the time required for reactivation; e.g., the 17[+] virus was reactivated most frequently (40 of 41 [98%] of trigeminal ganglia) in a mean time period of 8.6 days, 1704 was reactivated least frequently (10 of 36 [28%] of trigeminal ganglia) while requiring 17.2 days for reactivation, and 1704R required 9 days to be reactivated, which was almost identical to the parent 17[+] time of 8.6 days.

The advantages of the sensory ganglia/explant culture model are that reactivation is rapid and reproducible and the effect of treatments which might modify the reactivation process can be evaluated (26). The limitation of explant reactivation is that it is an artificial manipulation of latently infected tissue. It can be used to study some aspects of reactivation, in this case the effect of the LAT gene on the time of appearance of reactivated virus. Explant cocultivation will not be useful for studying every aspect of reactivation; for example, it may not reflect the subtle interplay of host immune or wound-healing responses with viral factors.

TABLE 2. In vitro reactivation studies: isolation of HSV-1 following explantation of trigeminal ganglia

Virus[a]	Cocultivation of TG[b]		Time required for appearance of CPE[c] (days)
	No. of rabbits with HSV-positive TG/no. of rabbits tested (%)	No. of HSV-positive TG/no. of TG tested (%)	
17[+]	21/21 (100)	40/41 (98)	8.6
1704	8/22 (36)	10/36 (28)	17.2
1704R	12/15 (80)	20/30 (67)	9.0

[a] Chi-square pairwise comparison of trigeminal ganglion data for each group is as follows: 17[+] versus 1704, $P < 0.0001$; 17[+] versus 1704R, $P < 0.001$; 1704 versus 1704R, $P < 0.002$.
[b] TG, trigeminal ganglia.
[c] CPE, cytopathic effects.

The greater efficiency of explant reactivation compared with in vivo reactivation may be due to a constant reactivation stimulus or the absence of immune mechanisms. A reactivation model that is perhaps more representative of natural HSV-1 reactivation is the rabbit eye model. Reactivation occurs spontaneously at a low frequency but can be induced in a significant proportion of stimulated animals. However, since several wild-type HSV-1 strains reactivate poorly in the rabbit eye model (6, 8), it also is not ideal for investigating the reactivation behavior of LAT variants.

The present study has focused on the role of LATs in acute, latent, and reactivated herpes virus infection. Deletions affecting the LATs in both the TR_L and the IR_L regions of the viral genome do not inhibit HSV-1 replication in cell cultures (14), mouse ocular tissue (27), or rabbit ocular tissue (8). Our studies with a rabbit eye model with HSV-1 strain 17^+, variant 1704 (which does not express the LATs), and rescuant 1704R provide data consistent with data for the mouse with the 1704 variant (27); i.e., the LATs are not required for HSV-1 replication or for the establishment or maintenance of latent infection but do stimulate the reactivation process.

Host species and strain differences are known to affect the ability of HSV-1 to establish latency and to spontaneously reactivate in vivo (7, 8). Recently, Gordon et al. (6) reported that in vivo reactivation studies do not necessarily correlate with in vitro reactivation studies. Spontaneous reactivation is thought not to occur readily in the mouse eye model, but it does occur with some regularity in the rabbit eye model, depending on the virus strain. Hill et al. (9), using a naturally occurring LAT$^-$ HSV-1 × HSV-2 recombinant (X10-13), demonstrated that the lack of LAT RNA did not alter the frequency or severity of spontaneous reactivation in the rabbit (which was infrequent for both viruses) but did affect induced reactivation. It is difficult to interpret the results with X10-13 for several reasons. (i) Since X10-13 is an HSV-1 × HSV-2 recombinant, there is no appropriate parental strain. (ii) It is not known whether the two HSV strains that contributed genetic material to X10-13 both reactivate in the rabbit eye model. This is important, because not all wild-type strains are reactivatable by iontophoresis (6, 8). (iii) Since the EcoRI j + k fragment used to construct the rescuant XC-20 is approximately 15 kb, the ability of XC-20 to reactivate in vivo may be the result of the acquisition of genes other than those encoding the LATs.

In the rabbit eye model, as in the mouse eye model (13, 27), the major latency-related phenotypic differences between the LAT$^+$ and LAT$^-$ viruses are (i) a less efficient reactivation of 1704 in vivo and (ii) a delay in the time required for the appearance of latent virus in explant-cocultivated trigeminal ganglia. The mean time required for the appearance of reactivated 1704 in vitro was approximately twice as long (17.2 days) as for 17^+ virus (8.6 days). The extended period required for explant reactivation and the low frequency of in vivo reactivation support an earlier suggestion that LATs modulate or facilitate explant reactivation (13, 27). HSV-1 variant 1704 expresses normal levels of the immediate early gene ICP0 (27), which suggests that the impaired reactivation of 1704 is not due to an effect on ICP0 expression. In addition to the LAT deletion, HSV-1 variant 1704 is also glycoprotein C negative (32). However, it has been shown that this glycoprotein C-negative phenotype is not related to the LAT gene or the impaired reactivation of 1704 (32).

From studies of HSV-1 LAT$^+$ and LAT$^-$ viruses in the rabbit model using both in vitro and in vivo reactivation

techniques, the data support and extend previous conclusions that (i) LAT expression is not essential for HSV-1 replication in vitro or in vivo and (ii) LAT expression is not required for the establishment or maintenance of latent virus infection within trigeminal ganglia. The current work strengthens the hypothesis that the LATs play an important role in the reactivation of latent HSV-1. Furthermore, the trigeminal ganglia explant reactivation model (rabbit or mouse) complements the in vivo model of iontophoretically induced reactivation and may be of significant value in evaluating the reactivation behavior of genetically engineered LAT variants and in the molecular dissection of LAT function.

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