The Characteristic Site-specific Reactivation Phenotypes of HSV-1 and HSV-2 Depend upon the Latency-associated Transcript Region

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Summary

After replication at sites of initial inoculation, herpes simplex virus type 1 and 2 (HSV-1 and HSV-2) establish lifelong latent infections of the sensory and autonomic neurons of the ganglia serving those sites. Periodically, the virus reactivates from these neurons, and travels centrifugally along the neuronal axon to cause recurrent epithelial infection. The major clinically observed difference between infections with herpes simplex virus type 1 and type 2 is the anatomic site specificity of recurrence. HSV-1 reactivates most efficiently and frequently from trigeminal ganglia, causing recurrent ocular and oral-facial lesions, while HSV-2 reactivates primarily from sacral ganglia causing recurrent genital lesions. An intertypic recombinant virus was constructed and evaluated in animal models of recurrent ocular and genital herpes. Substitution of a 2.8-kbp region from the HSV-1 latency-associated transcript (LAT) for native HSV-2 sequences caused HSV-2 to reactivate with an HSV-1 phenotype in both animal models. The HSV-2 phenotype was restored by replacing the mutated sequences with wild-type HSV-2 LAT-region sequences. These sequences or their products must act specifically in the cellular environments of trigeminal and sacral neurons to promote the reactivation patterns characteristic of each virus.

Materials and Methods

Cells and Viruses. Vero cells were obtained from American Type Culture Collection (ATCC, Rockville, MD), and maintained in 1:1 minimum essential medium/medium 199, (Quality Biologicals, Gaithersburg, MD) with 10% heat-inactivated fetal bovine serum (Quality Biologicals, Gaithersburg, MD) and 1% GASP (a mixture of L-glutamine, aureomycin, streptomycin, and...
penicillin, Quality Biologicals, Gaithersburg, MD). Herpes simplex virus type 2, strain 333, was obtained from Gary Hayward (Johns Hopkins University, Baltimore, MD). Herpes simplex virus type 1, strain 17syn+, was obtained from Dr. John Hay (SUNY-Buffalo, Buffalo NY). Virus stocks were grown in Vero cells, and plaque titered in duplicate before inoculation of animals.

**Mutant Virus Construction.** Sequences homologous to the XhoI site in the HSV-2 LAT region were introduced via site-directed mutagenesis into an Avr2-AluI clone of the HSV-1 LAT region sequences. This mutation was verified by DNA sequencing. For the mutant virus, the 2,820 bp HSV-1 strain 17syn+ NotI-XhoI fragment was inserted between NotI and XhoI sites of a clone spanning the HSV-2 strain 333 SphI-BamH1 fragment shown in Fig. 1. This DNA was used to construct the mutant virus by homologous recombination with HSV-2 strain 333 DNA as described (18). After identification of mutant virus, plaque purification was performed until Southern hybridization identified no evidence of contamination with the parent. At this point, two additional plaque purifications were performed to yield a stock of HSV-2 333/LAT1. This procedure was repeated, using wild-type SphI-BamH1 DNA and 333/LAT1 DNA to produce the rescuant, HSV-2 333/LAT1R. Additional Southern hybridizations using 32p-radiolabeled probes were performed to yield a stock of HSV-2 333/LAT1. This procedure was repeated, using wild-type SphI-BamH1 DNA and 333/LAT1 DNA to produce the rescuant, HSV-2 333/LAT1R. Additional Southern hybridizations using 32p-radiolabeled probes were performed to validate the correctness of each virus stock.

**Animal Studies.** All animal experiments were performed in AAALAC-certified facilities. New Zealand white rabbits weighing 2,000–2,500 g were infected with each virus in three independent experiments. Scarified (in one experiment) or unscarified guinea pigs were performed by investigators masked as to inoculation. After inoculation, all manipulations and observations were performed by investigators who were masked as to inoculating virus. 5–6 wk after inoculation, transcorneal iontophoresis of 0.01% epinephrine (0.8 mA for 8 min, once a day for three consecutive days) was used to attempt induction of ocular viral shedding from unscared eyes. No eyes shed virus for any of the 3 d before iontophoresis. Eye swabs were taken daily for 7–9 d after the first iontophoresis. Swabs were cultivated on primary rabbit kidney (PRK) cells, and determined to be positive if cytopathic effect consistent with HSV infection was observed. Two independent experiments were performed in guinea pigs. Female Hartley guinea pigs (Charles River Breeding Laboratories, Wilmington MA) weighing 400–525 g were inoculated with 10^5.7 PFU of each virus on day 0 by rupture of the vaginal closure membrane with a moistened calcium alginate tipped swab and instillation of 0.1 cc virus. Lesion severity was scored daily (on a scale from 0–4) until resolution of the acute infection. Disease severity was calculated as the area under the lesion score-day curve, for the duration of the acute infection. These same guinea pigs were observed daily for recurrence frequency from days 15–63 after inoculation. All observations of the guinea pigs were performed by investigators masked as to inoculating virus. Guinea pigs that were not evaluable for the entire observation period (either primary or recurrence phase) were excluded from analysis.

**Cocultivation and Quantitative Polymerase Chain Reaction.** Trigeminal ganglia were removed from rabbits after completion of the epinephrine iontophoresis experiments, removed from the outer

![Figure 1](image-url)
sheath, separated into two or three pieces, and placed in tissue culture with PKR cells. Cultures were observed daily for evidence of cytopathic effect, and reported as positive on the first day that HSV cytopathic effect was evident.

Ganglionic DNA was extracted from trigeminal ganglia of three rabbits latently infected with each virus and from three uninfected rabbits. PCR controls consisted of 100 ng of DNA extracted from an uninfected rabbit trigeminal ganglion spiked with 10^1 to 10^6 copies of HSV-2 DNA in 10-fold increments. In each reaction, 100 ng (quantified spectrophotometrically) of DNA was subjected to coamplification with two primer pairs by polymerase chain reaction (94°C X 1 min, 55°C X 1 min, 72°C X 2 min) for 30 cycles. The primers were GAACACGGGCCTGATGTGTG and CAATGCGTAGCCGAGAAAAGAG (which are specific for a 181-bp product in the HSV-2 glycoprotein C region), and CCATTCTTACCTCACATCGG and TCGCTCCTGGAAGATGTGAGTGTG (which are specific for a 134-bp product in rabbit glyceraldehyde-3-phosphate dehydrogenase). The products were alkaline denatured and subjected to slot blotting on Nytran membranes (Schleicher & Schuell, Keene, NH) using a 32P-radiolabeled probes derived either by end-labeling an oligonucleotide internal to the rabbit G3PDH product (TGTTC-CAGTATGGTCCACC) or by random priming of a 1-kbp gel-pure Bgl2-BstE2 fragment from within HSV-2 gC. Signals were quantified using a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA). For each sample, the ratio of the HSV-2/G3PDH signals was calculated and compared with scores obtained using the spiked controls. The approximate number of copies of HSV DNA in the three ganglia latently infected with each virus was calculated by linear extrapolation between the spiked control scores directly above and below those for each sample. Controls using DNA from the three uninfected rabbits and using no template (oligonucleotide primers only) gave rise to no signal attributable to HSV.

Figure 2. Southern hybridization of DNA extracted from HSV-2 333 (lanes 1, 4, and 7), HSV-2 333/LAT1 (lanes 2, 5, and 8), and HSV-2 333/LAT1R (lanes 3, 6, and 9). DNA was cleaved with Sphl, and subjected to Southern hybridization with an HSV-2 Sphl-BamH1 probe (lanes 1-3), an HSV-2 sequence-specific Notl-Sall probe (lanes 4-6), or an HSV-1 sequence-specific Sphl-Sphl probe (lanes 7-9). Marker sizes (in kbp) are shown to the left of the gel.

Results

Mutant Virus Construction and Evaluation. To test our hypothesis, we introduced an alteration into HSV-2 strain 333 which substituted the LAT region from HSV-1 strain 17syn+ for native HSV-2 LAT sequences, and designated this virus HSV-2 333/LAT1 (Fig. 1). The substituted sequences start upstream of the LAT promoter and include the LAT promoter, primary LAT 5’ end sequences, and most LAT intron sequences (excluding only 160 bases from the 3’ end of the LAT intron). A resuant HSV-2 333/LAT1R was constructed by replacing the substituted HSV-1 sequences in HSV-2 333/LAT1 with parent HSV-2 sequences.

The construction of the mutant and resuant viruses relative to the sequences of the parent virus was verified by Southern hybridization. Purified DNA extracted from HSV-2 333, HSV-2 333/LAT1, and HSV-2 333/LAT1R was cleaved with the restriction endonuclease Sphl and probed with radiolabeled DNA fragments specific for the HSV-2 and HSV-1 LAT regions. In DNA extracted from wild-type and resuant virus, a probe spanning the HSV-2 Sphl-BamH1 region (Fig. 2, lanes 1-3) detected a predicted 4,083-bp fragment within the long repeat, and a doublet of 3,073 and 2,829 bp representing fragments that span Spfl sites present within the unique-long segment on either side of the HSV-2 genomic repeats and the first Spfl site within the repeats. Smaller (<200 bp) Spfl fragments are not visualized on this gel. Changes in the location of the first Spfl site within the repeats led to detection of the predicted fragments of 4,169 bp for the larger band, and 2,236 and 1,992 bp for the doublet in the mutant virus. An internal HSV-1 derived 786-bp Spfl fragment in the mutant lacks homology with the HSV-2 sequences in the probe, and is not detected. A probe spanning the HSV-2 Notl-Sall region (lanes 4-6), which is specific for HSV-2 sequences, detected the appropriate doublets in wild-type and resuant virus, and showed no homology with mutant virus sequences. A probe spanning the HSV-1 786 bp Spfl-Spfl region (lanes 7-9) detected only the 786-bp Spfl fragment in the mutant virus, and showed no homology with wild-type or resuant sequences. Additional Southern hybridizations with BamH1, EcoRV, Notl, SalI, and XhoI digests of virus DNA also yielded the predicted results when hybridized with these probes (data not shown).

Assessment of LAT Substitution In Vivo in Rabbit Eyes. The rabbit ocular model of HSV-1 infection mimics natural human infection with HSV-1. The virus causes acute ocular infections, establishes latency in trigeminal ganglia, and can be induced to reactivate by appropriate stimuli (14). Also similar to human infections, HSV-2 does not reactivate well in vivo from rabbit trigeminal ganglia.

After corneal inoculation with HSV-2 333, HSV-2 333/LAT1, HSV-2 333/LAT1R, or HSV-1 17syn+, all rabbits exhibited comparable acute epithelial HSV keratitis during
postinoculation (PI) days 3–6. All tested eyes had cleared and showed no epithelial defect by 21 d after inoculation. We enumerated ocular recurrences in three independent experiments of latently infected rabbits subjected to ocular iontophoresis of epinephrine (summarized in Table 1). The procedure efficiently induced reactivation from rabbit eyes inoculated with HSV-1 strain 17 syn+; 71% of eyes and 30% of swabs yielded virus after iontophoresis. As expected, wild-type HSV-2 333 did not reactivate well from the latently infected rabbits (10% of eyes, 1% of swabs). The rescan 333/LAT1R behaved similarly to wild-type HSV-2 333. However, the mutant 333/LAT1 reactivated almost as efficiently as HSV-1 did (56% of eyes, 21% of swabs). All positive swabs were verified to contain the expected, wild-type HSV-2 333 did not reactivate well from the procedure efficiently induced reactivation from rabbit eyes postinoculation (PI) days 3–6. All tested eyes had cleared and reactivates spontaneously to cause recurrent lesions in guinea pigs, establishes latency in sacral genital herpes. As is the case in humans, HSV causes acute and recurrent infections as assessed by the area under the lesion-score curve.

### Table 1. Induced Reactivation (via Ocular Iontophoresis of Epinephrine) of HSV Mutants from Latently Infected Rabbits

| Virus | No. of rabbits* | Positive eyes/total eyes | Positive swabs/total swabs |
|-------|-----------------|--------------------------|---------------------------|
| HSV-1 17 syn+ | 7               | 10/14 (71%)              | 30/101 (30%)             |
| HSV-2 333    | 6               | 1/10 (10%)               | 1/80 (1%)                |
| HSV-2/LAT1   | 6               | 5/9 (56%)                | 14/68 (21%)              |
| HSV-2/LAT1R  | 10              | 2/19 (11%)               | 3/152 (2%)               |

*Cumulative data from three independent experiments.

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*Cumulative data from three independent experiments.

### Table 2. Acute and Spontaneous Recurrent Infections of HSV Mutants in Guinea Pigs

| Virus | Primary (d1–14) genital skin disease severity | Days with recurrent genital skin lesions (d15–63) |
|-------|---------------------------------------------|-----------------------------------------------|
|       | mean ± SE (no. of animals)                  | mean ± SE (no. of animals)                    |
| HSV-1 17syn+ | 4.3 ± 0.8 (10)                            | 0.9 ± 0.6 (10)                                |
| HSV-2 333    | 5.3 ± 1.1 (9)                              | 4.1 ± 1.6 (9)                                 |
| HSV-2/LAT1   | 5.2 ± 0.8 (13)                             | 1.5 ± 0.5 (11)                                |
| HSV-2/LAT1R  | 4.8 ± 0.5 (14)                             | 5.9 ± 1.0 (14)                                |

*Cumulative data from two independent experiments.

The rescuant 333/LAT1R behaved similarly to wild-type HSV-2 333/LAT1. HSV-2 333/LAT1 recurred with a frequency comparable between 17syn+ vs. HSV-2 333/LAT1 and HSV-2 333 vs. HSV-2 333/LAT1R.

#### Table 3. Virus recovery from latently infected rabbit trigeminal ganglia by cocultivation and detection of viral DNA by quantitative PCR

| Virus | No. positive/total ganglia | Average days to positive | Range |
|-------|---------------------------|--------------------------|-------|
| HSV-1 17syn+ | 1/1                      | 11                        | 11    |
| HSV-2 333    | 4/4                      | 14                        | 10–24 |
| HSV-2/LAT1   | 2/2                      | 21                        | 17, 24|
| HSV-2/LAT1R  | 5/5                      | 16                        | 12–17 |

*Mean copy number in three ganglia latently infected with each virus.
Discussion

The present study demonstrates that site-specific virus reactivation depends upon LAT-region sequences. In the context of either HSV-1 or HSV-2, the HSV-1 LAT region is both necessary and sufficient for the HSV-1 site-specific reactivation phenotype from sacral and trigeminal ganglia. The HSV-2 LAT region is necessary for the HSV-2 phenotype of efficient reactivation from sacral ganglia, although a potential requirement for additional, as-yet unidentified HSV-2-specific viral factors is not strictly ruled out by these experiments. The precise mechanism by which the LAT region expresses this phenotype is unclear. It is conceivable, although unlikely, that the site-specific reactivation phenotype could be attributable to the small exchange of 3' ICP0 sequences between HSV-1 and HSV-2 in the mutant virus. However, the mutation did not shift the ICP0 reading frame, and there are only minor differences in ICP0 3' amino acid sequences between HSV-1 and HSV-2. We were unable to identify any phenotypic differences between the viruses which would be expected to be associated with alterations in ICP0 function (ICP0 appeared normal on Northern hybridizations of productively infected Vero cells [data not shown]; one-step growth, acute infections, and recovery by cocultivation of the viruses were normal).

The HSV-1 and HSV-2 LAT promoters have very similar sequences, and in this experiment did not differ substantially in their ability to direct latent transcription of LAT. Moreover, previous studies have indicated that differences in the quantities of LATs transcribed by HSV during latency are not associated with differences in recurrence phenotypes (25, 26). Thus, LAT promoter sequences are also unlikely to be directly responsible for site-specific reactivation, although we cannot exclude differences in expression during reactivation which are not evident during latency. Because the reactivation phenotype was independent of the ability to transcribe latent LAT RNA, the LAT-associated phenotype is probably not attributable to an antisense mechanism (e.g., with ICP0). The LAT phenotype also appears unlikely to be attributable to LAT sequences transcribed under the control of the LAT promoter, but downstream of the substituted Notl-Xhol fragment. Theoretically, HSV reactivation could be controlled by another as yet unidentified transcript under the control of the LAT promoter.

There are no well-conserved potential open reading frames between HSV-1 and HSV-2 in the LAT sequences we substituted. Experiments in which viruses with mutations disrupting potential open reading frames within the HSV-1 LAT intron were evaluated in rabbits and mice, indicated that these theoretical open reading frames do not play a role in virus reactivation (27, 28). Thus, it appears unlikely that a protein encoded by LAT is responsible for its effect on reactivation.

These results imply that the LAT region confers the phenotype of efficient site-specific reactivation, which is the major clinically relevant phenotypic difference between HSV-1 and HSV-2. Site-specificity of reactivation appears to be most dependent on differences in ability to reactivate from the ganglion, rather than differences in access to the ganglion. Consistent with previous reports of LAT mutants, we identified no effect of our mutation on the ability of the virus to replicate acutely or to establish or maintain latency in either animal model. Thus, products of this genomic region (possibly the LAT RNAs themselves) must act in a specific manner within the cellular milieu of either trigeminal or sacral ganglia. This experiment definitively assigns to the LAT region an active and critical role in virus reactivation from latency. Additional studies of chimeric viruses, for example, in which HSV-2 LAT sequences are substituted for wild-type HSV-1 sequences or in which smaller regions are substituted between HSV-1 and HSV-2 will yield further insight into the role of the LAT in site-specific reactivation. Further study of interactions between cellular constituents and the LAT DNA and RNA sequences should yield a fundamental understanding of the mechanisms both of anatomic site-specific reactivation, and of herpes simplex virus reactivation in general.
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