Evidence that the Herpes Simplex Virus Type 1 Uracil DNA Glycosylase Is Required for Efficient Viral Replication and Latency in the Murine Nervous System

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Herpes simplex virus (HSV) encodes a uracil DNA glycosylase (UNG; UL2), which has been shown to be dispensable for normal replication of HSV-1 in cultured cells (J. Mullaney, H. W. Moss, and D. J. McGeoch, *J. Gen. Virol.* 70:449–454, 1989). In adult neurons, UNG activity is undetectable (F. Focher, P. Mazzarello, A. Verri, U. Hubscher, and S. Spadari, *Mutat. Res.* 237:65–73, 1990), suggesting that the HSV-1 UNG may play an important role in viral replication in neurons acutely and/or following reactivation. To examine the contribution of the HSV-1 UNG *in vivo*, two independent strain 17 Syn⁺ Ung[−] mutants, designated uB1 and uB2, were examined in a mouse model of herpetic disease. Following direct intracranial inoculation, both mutants exhibited a 10-fold reduction in neurovirulence compared with the parental strain 17 Syn⁺. Inoculations by a peripheral route demonstrated that the Ung[−] mutants were at least 100,000-fold less neuroinvasive than 17 Syn⁺. Replication kinetics *in vivo* demonstrated that uB1 and uB2 replicated less well in both the mouse peripheral and central nervous systems. Latency was established by both of the mutants in 100% of the animals examined. Following transient hyperthermia, however, the frequency of reactivation of the mutants *in vivo* was dramatically reduced. Restoration of the UNG locus resulted in full neurovirulence, neuroinvasiveness, and the ability to reactivate *in vivo*. These findings suggest that the HSV-1 UNG plays an important role during acute viral replication *in vivo* and possibly in the reactivation process.

Herpes simplex virus (HSV) replicates within sensory neurons both acutely (17) and following reactivation of latent infections (19, 20). In terminally differentiated, nondividing cells, such as neurons, conditions are not supportive of efficient DNA synthesis. Such cells produce only minimal levels of enzymes involved in DNA replication (4, 23, 33) and contain limited deoxynucleoside triphosphate (dNTP) pools sufficient for only a few minutes of DNA synthesis (9, 16). To overcome this adverse environment, HSV encodes a thymidine kinase (TK), ribonucleotide reductase, and dUTPase which increase dNTP pool sizes (reviewed in reference 17). These viral enzymes are dispensable for replication in cultured cells, but null mutants at these loci are less virulent than wild-type (wt) strains and replicate less well in the nervous system (3, 7, 8, 14).

HSV also encodes one enzyme involved in the repair of DNA, the uracil DNA glycosylase (UNG) (12, 31). The viral enzymes involved in DNA replication fidelity have yet to be examined but may also contribute to virulence and viral replication *in vivo*. The HSV-1-encoded UNG excises uracil residues from DNA, producing an apyrimic site which can then be repaired by cellular functions (10). Like its cellular counterpart, the HSV-1 UNG functions to reduce the point mutations and strand breakage that are associated with the presence of uracil in DNA (10). The HSV-1 UNG may also help to maintain regulatory signals present in DNA sequences which can be altered by the presence of uracil (5, 28). For example, the presence of uracil in the HSV unique short origin of replication (OriS) has been demonstrated to alter the interaction of the origin-binding protein with its target sequence (5).

In cultured cells, the HSV-1 UNG activity is dispensable for viral replication (12), presumably because adequate cellular activity is present. The cellular UNG activity, however, is cell cycle associated, with maximal activity detected during DNA synthesis, and is not detectable in adult neural tissues (4), suggesting that this enzyme might be required for efficient replication in the nervous system.

The introduction of uracil residues into nascent DNA can be caused by misincorporation of dUTP for dTTP by the viral DNA polymerase (5, 13) or by the spontaneous deamination of cytosine residues, which would result in a potentially mutagenic base pair mismatch with a guanosine residue (reviewed in references 10 and 29). During latent infection of the neuron, the viral genome may accumulate a large uracil load due to cytosine deaminations. Such a uracil burden may directly affect the ability of the virus to efficiently replicate following reactivation, by altering the function of key viral sequences such as the OriS (5).

To examine the role of the HSV-1 UNG in viral interaction with the mouse nervous system, two independently derived engineered Ung[−] mutants were generated and characterized. Virulence assays demonstrated that both of the mutants were less neurovirulent than the parental strain, 17 Syn⁺, and were 100,000-fold less neuroinvasive following inoculation at a peripheral site. Both mutants retained the capacity to replicate in mouse cells *in vivo* and to enter the host nervous system but were reduced in the ability to replicate in murine peripheral nervous system (PNS) and central nervous system (CNS) tissues. Latent infections were established by the Ung[−] mutants in 100% of the animals examined, but both isolates reactivated with greatly reduced frequency *in vivo* following transient hyperthermia. Assessment of the latent viral DNA burden by quantitative PCR (QPCR) demonstrated a reduction in the number of copies of the Ung[−] mutant viral

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genomes. Two isolates in which the UNG gene was restored demonstrated wt virulence and reactivation frequencies, confirming that the observed mutant phenotypes were due to the engineered perturbations. These results strongly support the hypothesis that HSV-1 UNG activity is necessary for efficient viral replication in neural tissues and would therefore make a valid target for antiviral chemotherapies.

MATERIALS AND METHODS

Cells and viruses. Rabbit skin cells (RSC) were cultured as previously described (27). NIH 3T3 cells were obtained from the American Type Culture Collection (CRL 6442) and were cultured as recommended. Virus strains used were derived from the wt HSV-1 strain 17 Syn⁺, which was obtained from J. Subak-Sharpe, Medical Research Council Virology Unit, Glasgow, Scotland. The derivation and history of 17 Syn⁺ have been described previously (27). Virus stocks were prepared from infected RSC monolayers. Methods for viral titrations as well as in vitro and in vivo replication kinetics have been described elsewhere (27). Base pair numbering, restriction endonuclease maps, and restriction fragment names are based on the complete sequence of HSV-1 strain 17 Syn⁺ compiled by McGeoch and colleagues (11).

Production of recombinant virus strains and nucleic acid analysis. The HSV-1 UNG open reading frame (ORF; UL2, bp 9886 to 10888) was disrupted by insertion of a ~4-kb *Xba*I insert that was prepared from plasmid pFJ3 (a kind gift of D. McGeoch) (12). This fragment encodes the β -galactosidase (β -Gal) gene under the control of the simian virus 40 early promoter as described previously (12). The β -Gal gene was inserted into the unique *Xba*I site within the UNG ORF at bp 10636 and then recombined into the 17 Syn⁺ genome by cotransfection with LipofectACE (GIBCO/BRL, Gaithersburg, Md.) as recommended by the manufacturer.

Recombinant viruses were identified by the formation of a blue precipitate following incubation with the chromagen 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal; Fisher). Blue plaques were purified to homogeneity by limiting dilution. To control for the possibility of unselected, second-site mutations, two independent isolates from separate cotransfection cultures were isolated and designated uB1 and uB2 (Ung⁻ blue 1 and 2). The UNG gene was restored to each mutant by homologous recombination with wt sequences spanning bp 8662 to 11820. Following plaque purification, the rescued isolates were designated uB1R and uB2R (uB1 and uB2 restored).

The genomic structures of all the viruses were confirmed by Southern blot restriction fragment length polymorphism analysis as previously described (27). Briefly, viral genomic DNA that had been digested by the indicated restriction enzymes was size separated in 0.8% agarose and Southern blotted as described previously (22). The blots were hybridized to ³²P-labeled probes at 42°C for 4 to 6 h. After washing, the blots were exposed to X-Omat-AR films (Eastman Kodak, Rochester, N.Y.) for 6 to 24 h, depending on the blot. When necessary, the blots were stripped in boiling 1% sodium dodecyl sulfate and reprobed. Restriction enzymes were purchased from GIBCO/BRL. Digestions were completed by using the conditions recommended by the manufacturer.

Expression of UL1 and UL3. RSC and NIH 3T3 monolayers on 22-mm-diameter coverslips were infected with the selected viruses at a multiplicity of infection (MOI) of 10 PFU per cell. Mock-infected monolayers served as negative controls. At various times postinfection (p.i.), the infected monolayers were fixed for 5 min in -20°C methanol and then rinsed extensively

in phosphate-buffered saline (PBS). Following blocking for 30 min in 10% normal goat serum (GIBCO/BRL), the primary antibody (specific for either gL or the UL3 product) was applied to the monolayers and allowed to incubate overnight in a humid chamber at room temperature. The coverslips were rinsed in PBS, briefly incubated with 10% normal goat serum, and then treated with a 1:50 dilution of goat anti-rabbit antibody conjugated to fluorescein isothiocyanate (Kirkegaard & Perry Laboratories) for 30 min in the dark. Following a final rinse in H₂O, the coverslips were mounted on slides and visualized with a Zeiss Axiophot microscope system equipped with epifluorescence optics. Representative photomicrographs (40 $\times$ or 63 $\times$ power) were generated by using T-Max 400 film (Kodak) and are indicative of essentially all cells on the coverslips.

Two primary antisera were used. The first antiserum was generated in rabbits to the HSV-1 UL1 (gL) gene product and was kindly provided by David Johnson (18). The second antiserum was raised in rabbits to the HSV-2 UL3 gene product. This antiserum was a generous gift of Sal Caradonna, who previously reported its use for immunofluorescent recognition of the HSV-1 UL3 gene product (31). Both sera were applied at a dilution of 1:500 in 10% normal goat serum.

Quantitation of neurovirulence, neuroinvasiveness, and latency. Male outbred Swiss Webster mice (Charles River and Sassco), 4 to 5 weeks of age, were used throughout these studies. Animals were housed in American Association for Laboratory Animal Care-approved quarters with unlimited access to food and water.

Neurovirulence, or the capacity of the viruses to replicate in murine brain and induce fatal encephalitis, was quantified by PFU/lethal dose 50% (LD₅₀) ratios following intracranial inoculation of 10-fold serial dilutions of the indicated virus as described previously (26). Groups of five mice were inoculated with 30 μ l in the left brain hemisphere, monitored for 21 days, and scored for death. Neuroinvasiveness was quantified by PFU/LD₅₀ ratios following inoculation of the abraded hind footpads as previously described (24). Animals inoculated in this fashion were also monitored for 21 days. PFU/LD₅₀ ratios were calculated by the method of endpoint estimation as described by Reed and Muench (15).

The ability of the selected viral strains to establish a latent infection was qualitatively examined by explant-cocultivation. Latently infected trigeminal ganglia (TG) were explanted at least 30 days p.i. from mice infected on both scarified corneas with ~10⁶ PFU of 17 Syn⁺, uB1, or uB2. Explanted TG were cultured in minimal essential medium supplemented with 10% fetal calf serum for 3 days, at which time the TG and media were plated on RSC monolayers and observed daily for up to 2 weeks for viral cytopathic effect. The genomic structures of virus from selected reactivations were confirmed by Southern blotting.

Latent viral DNA burden. QPCR was performed by the method of Katz et al. (8). Mice were infected as detailed for the explant-cocultivation experiments and included animals infected with ~10⁶ PFU of uB1R and uB2R. At least 30 days p.i., the TG pairs were harvested and DNA was isolated with G Nome DNA isolation reagents (Bio 101, La Jolla, Calif.) as recommended by the manufacturer. The DNA preparations were subjected to an additional RNase A treatment (100 μ g/ml at 37°C for 2 h), followed by ethanol precipitation in the presence of 2.5 M ammonium acetate prior to QPCR. Each DNA preparation was quantified in a Beckman model 2400 spectrophotometer and examined by gel electrophoresis to assess the quality.

QPCR was completed essentially as described previously (8).

FIG. 1. Schematic representation of the HSV-1 UNG locus in strain 17 Syn⁺ and the strategy used to generate the Ung⁻ mutants uB1 and uB2. The top line shows a schematic representation of the 152-kb HSV-1 genome. The solid boxes represent the terminal and internal repeat elements of the HSV-1 genome. The second line shows expansion of the left end of the genome beginning at the *Bam*HI site (B) at bp 2907 and extending to the *Asp* 718 site (A) at bp 16269. Restriction enzyme sites of interest are indicated as E (*Eco*RI) and X (*Xba*I). The hatched boxes represent the ORFs described in the text. An arrowhead indicates the position of the first codon. The asterisk indicates a second possible initiation site for the UL3 ORF. Transcripts mapped in this region by Singh and Wagner (20) are depicted by arrowheads. The estimated sizes of the RNAs and their kinetic classes are also indicated. Transcript sizes observed on Northern blots prepared in this study are indicated parenthetically. Finally, the position of the β -Gal gene insertion is indicated. The bottom line shows a schematic of the Ung⁻ virus genome, with the insertion that disrupts the UNG gene indicated. SV40 PRO, simian virus 40 promoter; pA, polyadenylation site.

Samples of ganglionic DNA (100 ng) and, as standards, 100 ng of mouse liver DNA spiked with known concentrations of HSV DNA (ranging from 20,000 to 2 copies of the HSV genome) were mixed with 50 pmol of each of two primer pairs. One primer set was specific for the HSV-1 TK gene, and a second set was specific for the single-copy mouse adipsin gene exactly as described previously (8, 19). PCR was performed for 30 cycles of 1 min at 94°C, 2 min at 55°C, and 1 min at 72°C, with a final extension of 7 min at 72°C. The products were analyzed by separation in 6% polyacrylamide gels and then transferred to nylon-supported nitrocellulose filters (Bio-Dyne B; GIBCO/BRL) and UV cross-linked. The filters were prehybridized and then hybridized to one of two oligonucleotides that had been end labeled with ³²P, using T4 polynucleotide kinase (GIBCO/BRL). One probe was specific for HSV TK, and the other was specific for mouse adipsin as described previously (8). The blots were washed and then exposed to X-Omat-AR film to generate autoradiograms and scanned in a Molecular Dynamics PhosphorImager for quantification, using ImageQuant software (Molecular Dynamics).

Induced in vivo reactivation. Latently infected mice were subjected to transient hyperthermia as described previously (20) to assess the induced reactivation phenotype of the Ung⁻ mutants in vivo. In this assay, the mice were inoculated on both scarified cornea with the indicated virus as detailed above and maintained for at least 30 days. Animals were then subjected to hyperthermia as described previously (20). At 24 h posttreatment, TG pairs were removed from sacrificed animals, homogenized, and plated on RSC monolayers to detect infectious virus. Viral DNA from selected positive cultures was analyzed by Southern blotting to confirm genomic structure.

RESULTS

Construction of the Ung⁻ viruses. The genomic region of HSV-1 that encodes the UNG is depicted in Fig. 1 along with the strategy used to generate the Ung⁻ viruses. The UNG ORF (bp 9886 to 10888) in 17 Syn⁺ was disrupted by insertion of an ~4-kb *Xba*I fragment encoding the β -Gal gene under the control of the simian virus 40 promoter. This mutation was described by Mullaney and colleagues in an HSV-1 17 Syn⁺-derived strain, designated *in1601* (12). In their report, Mullaney and colleagues demonstrated that strain *in1601* did not induce any detectable UNG activity, indicating that this mutation eliminated a functional UNG ORF from the 17 Syn⁺ genome.

The genomic structures of all of the recombinant viruses were examined by Southern blot restriction fragment length polymorphism analysis. A representative blot that was successively hybridized to a probe specific for the UL2 locus and then a β -Gal probe is presented in Fig. 2. Following hybridization to the UL2 locus probe (HSV bp 8662 to 11820), the lanes loaded with *Asp* 718-digested uB1 and uB2 DNA demonstrated the expected 9,202- and 7,524-bp fragments (Fig. 2A, left). These bands resulted from the addition of an *Asp* 718 site within the β -Gal gene and the inserted 4 kb of DNA (Fig. 1). The wt UNG locus does not contain an *Asp* 718 site; therefore, the single 12,610-bp *Asp* 718 fragment is present in the 17 Syn⁺ lane.

Digestion of uB1 and uB2 DNA with *Eco*RI resulted in the presence of three bands (1,957, 16,223, and 21,384 bp) following hybridization to the UL2 locus probe (Fig. 2A, right), again due to the additional 4 kb of DNA and a novel *Eco*RI site within the β -Gal gene (Fig. 1). The wt pattern demonstrates

FIG. 2. Southern blot analysis of 17 Syn⁺ and the derived viruses. (A) Following restriction enzyme digestion with *Asp* 718 or *Eco*RI, the viral DNA fragments were separated in 0.8% agarose and transferred to nitrocellulose as previously described (21). A ³²P-labeled probe spanning bp 8662 to 11820 was hybridized to the blots to demonstrate the genomic structures of the viruses in this region. Sample sources are indicated above the blot. Migration of the DNA size markers (in kilobases) is shown at the right. This blot was stripped and then hybridized to a ³²P-labeled β -Gal-specific probe to demonstrate the incorporation of the mutation in uB1 and uB2 (B).

two *Eco*RI fragments which include the long terminal repeat ends of the genome or *Eco*RI fragments j and c (12,593 and 17,754 bp). Following stripping, hybridization of the same blot with a β -Gal-specific probe demonstrated the expected bands following digestion with either *Eco*RI or *Asp* 718 (Fig. 2B). These bands are not present in the wt 17 Syn⁺ lanes.

Viruses in which the UNG locus was restored were generated by cotransfection of wt sequences spanning bp 8662 to 11820 and uB1 or uB2 viral genomic DNA. Two restored viruses, designated uB1R and uB2R, were initially selected by the inability to convert X-Gal to the blue reaction product. Their genomic structures were confirmed by Southern blot analysis of purified plaque isolates. As shown in Fig. 2, both uB1R and uB2R regained the wt restriction pattern following digestion with *Asp* 718 or *Eco*RI (Fig. 2A). As expected, neither uB1R nor uB2R demonstrated any β -Gal-specific sequences following hybridization with the β -Gal probe (Fig. 2B).

Analysis of the ability of the UNG mutants to replicate in NIH 3T3 cells following infection at a low MOI (0.01 PFU per cell) and a high MOI (5 PFU per cell) demonstrated that both uB1 and uB2 were fully replication competent, reaching peak titers equivalent to those of the parental 17 Syn⁺ strain (Fig. 3). These findings are consistent with the report of Mullaney and colleagues demonstrating that the Ung⁻ mutant *in1601* replicated efficiently in BHK C13 cells (12) and confirm that the HSV-1 UNG is dispensable for efficient replication in cultured cells.

Expression of the UL1 and UL3 gene products. Insertion of the β -Gal gene cassette into the 17 Syn⁺ UL2 ORF at bp

10636 was designed to affect only the UNG gene, leaving the neighboring UL1 and UL3 ORFs intact. The UL1 ORF (bp 9339 to 10011) encodes gL, which is required for replication in cultured cells (6, 18). The UL3 ORF (bp 10959 to 11664) was predicted by sequence analysis and was demonstrated to be dispensable for viral replication (1). During the characterization of uB1 and uB2, transcription mapping of this region was completed in HSV-1 and shared prior to publication by Singh and Wagner (21), greatly aiding these experiments. Their results demonstrated a complex pattern of transcription, which is summarized in Fig. 1. The transcription map of the UL1-UL3 region of HSV-2 was reported previously by Worrad and Caradonna (31) and demonstrated a similar pattern.

Transcription mapping of the region demonstrated that gL (UL1) could be translated from two colinear transcripts of 2.6 and 1.8 kb. The 1.8-kb RNA utilizes the first rightward poly(A) signal (bp 10945), which also is utilized by the 1.4-kb mRNA encoding the UNG gene (21). The 2.6-kb RNA reads through this poly(A) signal, terminating in downstream sequences that occur after the UL3 ORF (bp 11717). Interestingly, Singh and Wagner concluded that no unique transcript capable of encoding the entire UL3 ORF was produced (21). However, a transcript of ~600 bp was mapped to the 3' end of the UL3 ORF, as depicted in Fig. 1. Recently, a protein product which shares ~74% homology with the predicted HSV-1 UL3 product was identified for the HSV-2 UL3 ORF (32). In this report, a 1-kb mRNA analogous to the 600-bp RNA reported by Singh and Wagner in HSV-1 (21) was mapped to the UL3 ORF (31, 32).

To determine if uB1 and uB2 produced gL and UL3 transcripts, Northern blot analysis of RNA isolated from infected RSC cultures 6 h p.i. was performed. Hybridization of a UL1-UL3-specific probe (bp 10154 to 11335) to 17 Syn⁺, uB1R, and uB2R Northern-blotted RNAs demonstrated the transcription pattern described by Singh and Wagner (Fig. 1), with the exception of the reported 600-bp RNA that appeared to be between 900 and 1000 bp on several Northern blots and hybridized to a UL3-specific probe (data not shown). Both uB1 and uB2 also produced this 900-bp UL3-specific transcript at levels equivalent to wt levels (data not shown).

As expected, both uB1 and uB2 were found to have an altered transcription pattern for the remaining RNAs (Fig. 1). No 1.4-, 1.8-, or 2.6-kb transcripts were expected as a result of the insertion of the 4 kb of β -Gal sequences. Instead, an increase of 4 kb in sequence at bp 10636 and the addition of the simian virus 40 polyadenylation signal present in the β -Gal construct predict an increase in the size of the 1.8- and 2.6-kb transcripts to ~5.4 kb and an increase in the size of the 1.4-kb transcript to ~5 kb. These predicted bands were evident in uB1 and uB2 RNA samples following hybridization to either a UL1-UL3 region- or β -Gal-specific probe (data not shown).

To control for the variability in the viral RNA concentrations in each sample, the blot also was hybridized to a probe specific for the 1.3-kb HSV-1 TK mRNA (11). Following normalization for loading, all three RNA species in the UL1-UL3 region in uB1 and uB2 were detected at higher levels compared with the corresponding wt RNAs. Addition of the β -Gal sequences could have augmented transcription or message stability of this region. Alternately, the significant difference in the sizes of three of the four transcripts in uB1 and uB2 may have altered their efficiency of transfer to the membranes.

The presence of the expected uB1 and uB2 RNAs did not demonstrate that gL and the UL3 gene product were produced. To directly demonstrate the presence of gL and the UL3 protein, infected RSC or NIH 3T3 cell monolayers were

FIG. 3. Replication kinetic analysis in NIH 3T3 cell cultures. Triplicate cultures of NIH 3T3 cells were infected with the indicated viruses at an MOI of 0.01 PFU per cell (A) or 5 PFU per cell (B). At the indicated times p.i., the cultures were harvested in triplicate, processed by three successive freeze-thawing cycles, and analyzed for infectious viral content on RSC monolayers. ♦, 17 Syn⁺; ○, uB1; ●, uB1R; □, uB2; ■, uB2R.

immunostained, at various times p.i., with antisera specific for these proteins. Representative photomicrographs of infected RSC monolayers are presented in Fig. 4. Immunostaining for gL or UL3 on cells infected with uB1 or uB2 were indistinguishable from cells infected with 17 Syn⁺ or the restored viruses (Fig. 4A and C versus Fig. 4C and D). Mock-infected monolayers also were immunostained and demonstrated minimal background fluorescence (data not shown).

In these studies, several times p.i. were examined to determine if the β -Gal cassette altered the course of expression of the gL or UL3 protein. No obvious difference between Ung⁻ mutant- or wt-infected RSC or NIH 3T3 monolayers could be detected, suggesting that the engineered mutation of the UL2 locus did not alter dramatically the expression of either the UL1 or UL3 ORF. As expected, staining for the gL protein was strongest at later times p.i. (for example, 8 h p.i.; Fig. 4A and B). Worrall and Caradonna demonstrated previously that the UL3 protein was detected easily at 3 h p.i. in CV-1 cells infected with HSV-1 strain KOS (31). In these studies, peak staining for UL3 in cells infected with 17 Syn⁺ or the other viruses was observed approximately 5 h p.i. (Fig. 4C and D). These results demonstrate that the engineered mutation in uB1 and uB2 did not overtly perturb the expression of either the UL3 or gL protein and suggest that the phenotypes observed for uB1 and uB2 are due primarily to the loss of UNG activity.

Neurovirulence of the Ung⁻ mutants. As an initial measure of the capacity to replicate in neural tissue, averaged PFU/

LD₅₀ ratios were generated following direct intracranial inoculations of mice. If the HSV-1 UNG activity was important for replication in neural tissues, it was expected that the Ung⁻ mutants would demonstrate a reduced ability to cause a fatal encephalitis in animals and an increase in the PFU/LD₅₀ ratios would be observed. Consistent with this prediction, the results of at least two experiments for each virus demonstrated that the Ung⁻ mutants yielded average PFU/LD₅₀ ratios 10-fold greater than the parental 17 Syn⁺ ratios (Table 1). Interestingly, no difference in the time to death or the onset of CNS disease signs was observed in animals infected with the Ung⁻ mutants or 17 Syn⁺. Restoration of the UNG locus resulted in viruses with wt virulence (uB1R and uB2R; Table 1), confirming that the reduced neurovirulence observed was due to the engineered perturbations and suggesting that the HSV-1 UNG may play an important role in viral replication in the CNS.

Neuroinvasiveness of Ung⁻ mutants. Infection of a human host with HSV-1 occurs at body surfaces where the virus replicates, spreads to the PNS, and in some cases invades the CNS (reviewed in reference 2). To determine the contribution of the viral UNG in this process, mice were inoculated on both abraded hind footpads and monitored for 21 days for fatal encephalitis. The data from these experiments are summarized as averaged PFU/LD₅₀ ratios in Table 1.

An inoculum of as little as 100 PFU of wt 17 Syn⁺ consistently caused hind limb paralysis in at least 20% of the mice (14). This inoculum led to encephalitis and death by 7 to 11 days p.i. in a subset of the animals (data not shown). The

FIG. 4. In situ detection of gL and UL3 proteins by immunofluorescence. RSC monolayers on 22-mm-square coverslips were infected with 5 PFU of uB1 (A and C) or uB1R (B and D) per cell. Infected monolayers were fixed at 9 h p.i. (A and B) or 5 h p.i. (C and D) and then stained with an antiserum to gL (A and B) or to the UL3 product (C and D). Mock-infected monolayers had negligible staining (not shown). Results similar to those presented were obtained following staining of monolayers infected with 17 Syn⁺, uB2, or uB2R (not shown).

average PFU/LD₅₀ ratio for 17 Syn⁺ following peripheral inoculation has been established as $\sim 3 \times 10^3$ (Table 1) (14, 25). In contrast, uB1 and uB2 did not induce any paralysis or encephalitis even at the highest titers inoculated (2×10^7 and

6×10^8 PFU, respectively; Table 1) and are therefore 4 to 5 orders of magnitude reduced in their ability to induce signs of severe disease by this assay. Restoration of the UNG gene resulted in wt levels of neuroinvasiveness in the rescued isolates, again confirming that the observed phenotypes were due to the engineered mutation (uB1R and uB2R; Table 1).

In vivo replication kinetic analysis in murine tissues. Viral replication kinetic analysis in mouse tissue was used to assess the efficiency of replication of the Ung⁻ mutants in various murine tissues. After inoculation of mice on both rear footpads, tissues following the natural course of viral infection from the footpad to the spine were analyzed for infectious virus at 24-h intervals. The results are presented graphically in Fig. 5.

Both of the mutants demonstrated a slight decrease in replication efficiency in the footpad, attaining peak titers ~ 10 -fold lower than 17 Syn⁺ titers (Fig. 5A). With the exception of uB1, the peak titers generated by all the viruses in the sciatic nerve were equivalent, suggesting that no defect in entry into the PNS was present (Fig. 5B and C). In the sciatic nerve, uB1 demonstrated kinetics which were equivalent to those of the other viruses, suggesting that the reduced peak

TABLE 1. Virulence properties of the viruses

Virus	PFU/LD ₅₀	
	Intracranial ^a	Peripheral ^b
17 Syn ⁺	8	3×10^3
uB1	112	$>2 \times 10^7$
uB2	60	$>6 \times 10^8$
uB1R	3	6×10^3
uB2R	6	9×10^3

^a Outbred Swiss Webster 4- to 5-week-old male mice were inoculated in the left brain hemisphere with serial 10-fold dilutions of the indicated virus stocks (five mice per dilution). Following observation for 21 days, PFU/LD₅₀ ratios were estimated by the method of Reed and Muench (15). The data represent averages of two to three experiments.

^b Groups of mice were inoculated with serial 10-fold dilutions on both abraded hind footpads and were observed for 21 days. PFU/LD₅₀ estimations were calculated as described above, and the data represent averages of two to three experiments.

FIG. 5. Replication kinetics in mouse tissues in vivo. Mice were infected on both hind footpads with 5×10^4 PFU of the indicated viruses. At the indicated times p.i., two mice per virus were sacrificed. Collected tissues were stored at -80°C and then homogenized in cell culture medium. Following clarification by centrifugation, the homogenates were assayed for infectious virus content. The results for footpad, sciatic nerve, dorsal root ganglia (DRG), and spine are presented as PFU per gram of tissue. $\blacklozenge$, 17 Syn⁺; $\circ$, uB1; $\bullet$, uB1R; $\square$, uB2; $\blacksquare$, uB2R.

titer attained on day 5 was most likely due to decreased titers in the footpad.

Restricted replication of both of the Ung⁻ mutants was apparent in the mouse PNS and CNS (dorsal root ganglia and spinal cord), where titers generated by the mutants were consistently 1 to 2 orders of magnitude lower than 17 Syn⁺ titers (Fig. 5C and D). Both uB1 and uB2 reached titers equivalent to wt titers in the dorsal root ganglia ($\sim 10^6$ PFU/g at day 3), suggesting efficient entry into the PNS; however, neither virus replicated as well as 17 Syn⁺, generating titers 10- to 100-fold lower on days 5 and 7 p.i. (Fig. 5C).

In the spine (CNS), similar results were observed; both of the mutants replicated less well than 17 Syn⁺, with peak titers on day 5 at least 100-fold lower than wt titers (Fig. 5D). This reduction may represent reduced replication at this site, reduced entry of virus as a result of the restriction observed in the PNS, or a combination of these possibilities. Both uB1R and uB2R reached titers equivalent to those attained by 17 Syn⁺ in the mouse PNS and CNS, indicating that repair of this locus restores the ability of the virus to replicate in both the PNS and CNS (Fig. 5C and D) and to cause disease (Table 1).

TABLE 2. Latency and reactivation properties of the viruses

Virus	Reactivation	
	In vitro ^a (no. of animals positive/ no. examined [%])	In vivo ^b (no. of animals from which virus was recovered/ no. tested [%])
17 Syn ⁺	23/23 (100)	14/17 (82) 12/18 (67)
uB1	15/15 (100)	4/15 (27)
uB2	15/15 (100)	2/9 (22)
uB1R	ND	8/10 (80)
uB2R	ND	5/7 (71)

^a Assayed by explant-cocultivation of latently infected TG pairs on RSC monolayers. Animals had been inoculated on both scarified corneas with $\sim 10^6$ PFU at least 30 days prior. ND, not determined.

^b Determined by recovered virus from TG pairs taken from animals 24 h postinduction of reactivation by transient hyperthermia (19). The TG pairs were homogenized, clarified, and then plated on RSC monolayers, which were observed for plaque formation. The animals were infected as described above.

Establishment of and reactivation from latency. To determine if Ung⁻ viruses could establish latent infections, TG pairs from animals (15 per virus) latently infected with uB1 or uB2 were examined by explant-cocultivation. These animals had been inoculated on both eyes with $\sim 10^6$ PFU at least 30 days prior. The explanted TG of animals ($n = 10$) latently infected with wt 17 Syn⁺ (infected with 5×10^5 PFU) were used as controls for normal latency and reactivation by explant-cocultivation. Despite the observed reduction in replication efficiency in the mouse PNS (Fig. 5C), uB1 and uB2 were fully capable of establishing latent infections, as virus reactivated from the TG of all of the 15 animals examined (Table 2).

Qualitatively, the Ung⁻ mutants were able to establish latent infections in murine TG as efficiently as wt 17 Syn⁺. To more quantitatively address the ability of the Ung⁻ mutants to establish latency, the latent viral DNA burden in TG pairs of animals infected with the Ung⁻ viruses was assessed. The QPCR method of Katz et al. (8) was used, as this method allowed detection of a minimal number of viral genome equivalents and a more sensitive estimation of viral genome copies than other protocols. The QPCR was completed essentially as described by Katz et al. (8).

To quantify the number of viral genomes present in each sample, a set of standard samples of 100 ng of mouse liver DNA spiked with 10-fold dilutions of known concentrations of HSV DNA was included in the experiment. The HSV DNA standards ranged from 20,000 to 2 copies in these studies. All of the products were analyzed by polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose, and hybridized first to a TK-specific probe and then, following stripping, to an adipsin-specific probe. As reported by Katz and colleagues (8), a relatively linear relationship between the signal intensity of the hybridized TK product and the HSV copy number was observed on a log-log scale. QPCR was consistently able to detect as few as 2 HSV genome equivalents in 100 ng of mouse DNA; however, accurate estimation of 2 to 20 genome equivalents was complicated by reduced linearity between the signal intensity in the standards and HSV copy number.

To control for variability in the amount of DNA in each sample as well as PCR efficiency, each sample was normalized to the average adipsin product quantity. On the basis of normalized counts in the product bands, the results indicated that the TG pairs latently infected with the Ung⁻ mutants on average had twofold less TK product signal than 17 Syn⁺ or either of the restored isolates (Fig. 6 and Table 3). As has been

FIG. 6. Quantification of the latent viral DNA burden in murine TG pairs. QPCR was completed as described by Katz et al. (8). Briefly, 100-ng samples of DNA isolated from latently infected animals (five per virus) or uninfected animals (negative controls) were subjected to PCR in the presence of two primer pairs. One pair was specific for the HSV-1 TK locus, and the other was specific for the single-copy mouse adipsin gene. As standards (STD) for quantitation, 100-ng samples of uninfected mouse DNA were spiked with 10-fold dilutions of HSV-1 DNA ranging from 20,000 to 2 copies and also were subjected to QPCR (lanes 3 to 7 on the STD blot). The PCR products were analyzed by PAGE, blotted, and hybridized with one of two ^{32}P -labeled probes specific for either the TK or adipsin PCR product. The blots were then analyzed in a Molecular Dynamics PhosphorImager, and the data are compiled in Table 3. The blots were also used to generate the autoradiograms shown.

TABLE 3. Quantitation of HSV DNA burden in latently infected TG pairs by QPCR

Virus	Median no. of:		Range of counts
	Counts ^a	No. of molecules/ TG pair ^b	
17 Syn ⁺	127,549	1×10^6	113,231–135,335
uB1	47,782	9×10^4	36,018–139,019
uB1R	135,377	3×10^6	95,106–259,024
uB2	46,895	9×10^4	30,014–114,005
uB2R	117,643	9×10^5	52,160–182,788

^a The PhosphorImager scan of the blots was used to determine the number of counts within each TK and adipsin band; the averaged 100-ng adipsin product quantity served to standardize all values. The average number of normalized counts in the TK product band is presented for each virus (five TG pairs per virus).

^b Determined by comparison with the set of standards in which a 10-fold dilution series of known concentrations of HSV was mixed with 100 ng of mouse DNA, which allowed estimation of the number of HSV copies per cell equivalent. The number of HSV molecules present in a TG pair was calculated on the basis of an estimated 10^7 cells in a TG pair.

previously observed (8, 19), a great deal of variability in the amount of viral DNA was evident in each group of latently infected TG pairs (five per virus); therefore, the median rather than average values were used as estimates of typical counts and genome equivalents. The range of normalized counts observed is also presented (Table 3).

To estimate the number of copies of viral DNA present in a TG pair, it was assumed that the average TG pair contained $\sim 10^7$ cells, based on recovery of $\sim 40 \mu\text{g}$ of DNA per TG pair. The results of genome equivalent estimations suggest that the typical latent infection by an Ung⁻ mutant results in $\sim 10^5$ copies of viral DNA per TG pair, while TG pairs infected with 17 Syn⁺ or either of the two restored isolates have $\sim 10^6$ (Table

3), or approximately a 10-fold difference. It should be noted, however, that for both uB1 and uB2, at least one of the TG pairs was found to contain levels of HSV-1 genomes equivalent to that observed for 17 Syn⁺ or the restored viruses.

Having established by two independent assays that the Ung⁻ viruses established latent infections in murine TG, we sought to assess the role of the HSV-1 UNG activity in reactivation *in vivo*, by using the hyperthermia model of induced reactivation (20). Groups of mice were inoculated as described for the establishment studies, maintained for at least 30 days, and then subjected to transient hyperthermia as described previously (20). The results are presented in Table 2.

Following transient hyperthermia of mice that were latently infected with 17 Syn⁺, virus is consistently recovered from between 60 and 80% of the animals at 24 h posttreatment (14, 20). The results of two control experiments with 17 Syn⁺ are consistent with these findings (Table 2). Both of the Ung⁻ mutants demonstrated a significantly reduced ability to reactivate *in vivo* by this method, with uB1 and uB2 virus recovered from only 22 to 27% of the animals following hyperthermia (Table 2). In contrast, the rescued viruses reactivated as frequently as 17 Syn⁺, confirming that the reduced reactivation phenotype of the Ung⁻ viruses was due to the engineered mutation.

DISCUSSION

The results presented in this study strongly support the hypothesis that the HSV-1 UNG plays an important role in viral replication *in vivo* and in particular in neural tissues. With the characterization of two independently derived, 17 Syn⁺-based Ung⁻ mutants, we have confirmed that the HSV-1 UNG is dispensable for replication in cultured cells as previously reported by Mullaney and colleagues (12) and have established that HSV-1 strains lacking UNG activity display reduced neurovirulence and neuroinvasiveness and reactivate with reduced frequency *in vivo*. These observed phenotypes were due to the engineered perturbation of the UNG gene and not due to unselected, secondary mutations, since rescue of the locus with a minimal wt fragment restored virulence and latency properties.

The presence of uracil in HSV-1 DNA may dramatically effect normal functioning of DNA sequences, as in the case of the OriS and the origin-binding protein (5). In addition, the presence of uracil in viral DNA may lead to unacceptably high levels of mutation, resulting in nonviable progeny. The characterization of uB1 and uB2 strongly supports an important role for the HSV-1 UNG activity *in vivo* both acutely and following reactivation from latency. Both of the Ung⁻ viruses replicated less well in neural tissues, as evidenced by virulence assays (Table 2) and direct replication kinetics in murine tissues (Fig. 5).

Transcriptional analysis of the UL1-UL3 region was reported during completion of these studies by Singh and Wagner for HSV-1 (21) and previously for HSV-2 by Worrall and Caradonna (31, 32). As indicated, transcription of this region is complex, with two of the transcripts encoding multiple ORFs (Fig. 1). The UNG ORF is encoded by the reported 1.4-kb mRNA (21), which is altered in uB1 and uB2 (data not shown). The UL1 ORF encodes gL, which is required for replication in cultured cells (6, 18) and is most likely translated from the 1.8- and/or 2.6-kb mRNAs.

Several lines of evidence suggest that the insertion introduced into UL2 ORF of mutants uB1 and uB2 did not adversely effect the expression of UL1, which encodes gL (6). Modified versions of the two 5'-coterminal mRNAs which

encode the ORF for UL1 as recently mapped by Singh and Wagner (21) were detected by Northern blot analysis. The sizes of these transcripts were as predicted by the engineered mutation, and the relative abundance of the transcripts was not reduced compared with that of the mRNA encoding TK (data not shown). In addition, no obvious difference was detected between cells infected with uB1, uB2, or the restored viruses following immunofluorescence using gL-specific antisera (Fig. 4A and B). Finally, gL is an essential protein required for viral replication in cultured cells (6, 18). No general replication defect was detected in the mutants, and therefore the phenotypes for uB1 and uB2 cannot be due easily to the lack of gL.

It also is possible that the phenotypes observed were due wholly or in part to a perturbation of the expression of the UL3 gene product. We think this unlikely, since the only unique transcript that encodes UL3 was found in normal abundance (data not shown). It has been suggested that UL3 also might be translated from the third cistron present in the 2.6-kb mRNA (21). Because of the nature of the mutation engineered into uB1 and uB2, this third cistron does not exist in the modified message. However, we have generated viral mutants in which the UL3 ORF was specifically disrupted, and these viruses reactivate with normal frequency in mouse TG following hyperthermic stress (14a). Finally, the UL3 gene product was detected in uB1- and uB2-infected cells (Fig. 4C) by immunofluorescence using an anti-UL3 serum. The staining pattern observed was indistinguishable from that of cells infected with wt or restored viruses (Fig. 4D). Since immunofluorescence is not quantitative, the possibility remains that the level of UL3 protein produced by uB1 and uB2 was affected and thus contributed to the observed phenotypes. More subtle mutations of the UL2 and UL3 genes will be required to address this possibility.

The Ung⁻ viruses established latency in 100% of the animals tested (Table 2), but a reduced viral genome burden was observed in latently infected TG (Fig. 6). It is not yet clear whether the reduced amount of HSV DNA detected was the result of the establishment of fewer latent infections or the presence of fewer HSV genome copies per latently infected cell. The fact that a dramatic reduction in reactivation frequency in vivo was observed for both of the Ung⁻ viruses suggests that the reduced latent genome burden was biologically significant. Restoration of the UL2 region in uB1 and uB2 resulted in viral strains which were phenotypically wt for the establishment of and reactivation from latency. Several studies of UNG activity from various sources have demonstrated that the excision of uracil residues formed in DNA by spontaneous deamination of cytosine is the primary function of this enzyme (29). It has been suggested by McGeoch and colleagues that the HSV-1 UNG activity may be especially critical following extended periods of latency (12). Our results support the hypothesis that UNG activity may be involved in removal of uracil residues from key regulatory sequences which are crucial for response to induction stimuli.

Our results suggest that engineered mutation of the HSV UNG gene would be advantageous in the production of attenuated vaccine candidates. Mutants lacking this enzyme retained the ability to replicate at the body surface and would therefore be expected to induce a strong immune response. The reduced ability of such mutants to replicate in the PNS and CNS as well as their reduced capacity to reactivate from latency would limit the chance of severe postvaccination sequelae. Finally, the recent report of a specific inhibitor of the HSV-1 UNG enzyme (30) suggests that compounds which selectively inhibit the HSV-1 UNG activity might be devel-

oped. Such compounds might be useful for reducing the frequency of recurrent disease.

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