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Herpes Simplex Virus Type 1 dUTPase Mutants Are Attenuated for Neurovirulence, Neuroinvasiveness, and Reactivation from Latency

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Herpes simplex virus type 1 (HSV-1) encodes a dUTPase which has been shown to be dispensable for normal viral replication in cultured cells (S. J. Caradonna and Y. Cheng, J. Biol. Chem. 256:9834–9837, 1981; F. B. Fisher and V. G. Preston, Virology 148:190–197, 1986). However, the importance of this enzyme in vivo has not been determined. In this report, HSV-1 strain 17 syn$^+$ and two isogenic engineered dUTPase-negative mutants were characterized in the mouse model. Both mutants replicated with wild-type kinetics and achieved wild-type titers in cultured cells. The mutants were 10-fold less neurovirulent than 17 syn$^+$ following intracranial inoculation and more than 1,000-fold less virulent following footpad inoculation. The dUTPase$^-$ mutants replicated with wild-type kinetics in the footpad and entered and replicated efficiently in the peripheral nervous system of the mouse. However, their replication in the central nervous system was significantly reduced. The dUTPase$^-$ strains established latent infections but displayed a greatly reduced reactivation frequency in vivo. Neurovirulence, neuroinvasiveness, and reactivation frequency were all restored by recombination with wild-type dUTPase sequences. These results have important implications with regard to anti-herpesvirus therapeutic strategies.

Following infection at the body surface, herpes simplex virus (HSV) is transported through axons to nerve cell bodies in the innervating sensory ganglia. In most cases acute viral replication ceases and latent infections are established in neurons. Rarely, significant replication of HSV in the peripheral nervous system (PNS) following primary infection or reactivation from latency leads to infection of the central nervous system (CNS) and severe disease, including fatal encephalitis (reviewed in reference 12).

The viral gene products which contribute to the neurotropism, neuroinvasiveness, and neurovirulence of HSV are largely unknown, but several lines of evidence demonstrate that viral enzymes which alter the cellular environment to promote viral DNA replication play a central role. The HSV type 1 (HSV-1)-encoded thymidine kinase (13) and ribonucleotide reductase (4) have both been shown to be required for maximal neurovirulence. An additional locus of neurovirulence has been mapped to the genomic region which encodes the viral dUTPase (37).

In cultured cells, HSV-1 dUTPase is not required for viral replication, presumably because a cellular counterpart is present in sufficient quantity (1, 14, 27, 41). While infection with wild-type (wt) HSV-1 results in the down regulation of host cell dUTPase function (27), infection with dUTPase$^-$ mutants does not (21, 41). However, the host cell dUTPase activity is cell cycle associated, with maximal levels detected during S phase (5, 10, 15, 20). In nondividing cells such as neurons, this function presumably would not be supplied in *trans*. The dUMP generated by the dUTPase activity is utilized for the de novo synthesis of dTTP (9, 24, 26). In neurons the amount of available dTTP may be limiting for viral genome replication. In addition, the reduction of available dUTP has been shown to limit the misincorporation of uracil residues in DNA, which can lead to point mutations or strand breakage (3, 16, 17, 26).

In this report we examine the role of the HSV-1 dUTPase in viral interaction with the mouse nervous system. Two different dUTPase-negative mutants were significantly less neurovirulent and neuroinvasive than their parent strain, 17 syn$^+$. The mutants retained the capacity to enter and replicate efficiently in the PNS and establish latent infections, but they reactivated with reduced frequency in vivo following hyperthermia induction. Therefore, the HSV-1 dUTPase is an important determinant of neurovirulence, neuroinvasiveness, and establishment of and/or reactivation from latency.

MATERIALS AND METHODS

Cells and viruses. Rabbit skin cells (RSC) and primary mouse embryo cells (MEC) were cultured as previously described (38). Virus strains used were derived from the wt HSV-1 strain 17 syn$^+$, which was obtained from J. Subak-Sharpe of the Medical Research Council Virology Unit in Glasgow, Scotland. The derivation and history of 17 syn$^+$ have been previously described (38). The dUTPase$^-$ insertion mutant, dUT$^-$1218, was provided by V. Preston. This virus carries a 12-bp in-frame insertion which converted the *Asp*718 site at bp 107305 to a *Hin*dIII site. In their report, Fisher and Preston (14) fully characterized this mutant in cultured cells, demonstrating that dUT$^-$1218 replicated as well as its parent, 17 syn$^+$. That study and one completed by Williams demonstrated that this virus induced no detectable dUTPase activity in infected cultured cells (14, 41).

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 (38). Base pair numbering, restriction endonuclease maps, and restriction fragment names are based upon the complete sequence of

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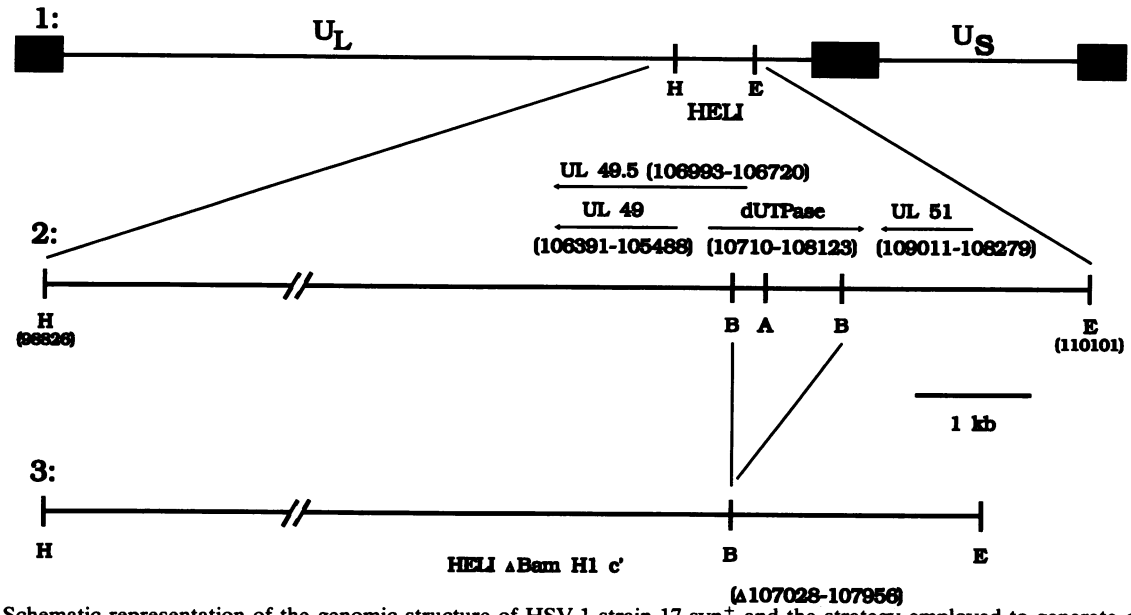


FIG. 1. Schematic representation of the genomic structure of HSV-1 strain 17 syn$^+$ and the strategy employed to generate dUTPase$^-$ strains. (Line 1) Schematic of the 152-kb HSV-1 genome. The thick boxes are representative of the terminal and internal repeat elements of the unique long (U_L) and unique short (U_S) regions of the HSV-1 genome. The vertical lines designate the ends of the *Hin*dIII-*Eco*RI L-I fragment. (Line 2) Expanded view of the *Hin*dIII-*Eco*RI L-I restriction fragment (HELI; bp 98826 to 110101). The transcripts encoding the predicted ORFs of interest (UL49, UL49.5, dUTPase, and UL51) are indicated by arrows. The base pairs spanned by each ORF are denoted parenthetically. Selected restriction sites are indicated by vertical lines (B, *Bam*HI; A, *Asp*718; H, *Hin*dIII; E, *Eco*RI) (29). (Line 3) Depiction of the altered HELI, in which the *Bam*HI c' fragment was deleted. The dUTPase$^-$ mutants, 17B1 and 17B2, were generated by cotransfection of 17 syn$^+$ genomic DNA and this cloned fragment.

HSV-1 strain 17 syn$^+$ compiled by McGeoch and colleagues (29).

Production of recombinant virus strains and nucleic acid analysis. A portion of the 17 syn$^+$ dUTPase coding region was removed from the *Hin*dIII-*Eco*RI fragment L-I (HELI, bp 98826 to 110101) by deleting the *Bam*HI c' fragment (bp 107028 to 107956). The method of generating recombinant virus strains by transfection of genomic viral DNA and cloned fragments has been previously described (34, 35). Transfection of subconfluent RSC monolayers by using Ca_2PO_4 was performed by using the modified, cloned HELI fragment and an empirically optimized amount of genomic 17 syn$^+$ DNA. The strategy employed is depicted in Fig. 1.

Recombinant viruses carrying the deletion (loss of the 928-bp *Bam*HI c' fragment) were identified by Southern blot restriction fragment length polymorphism analysis as previously described (38). Restriction enzymes were purchased from Gibco/Bethesda Research Laboratories (Gaithersburg, Md.). Digestions were completed by using the recommended conditions of the manufacturer. Viruses in which the dUTPase gene was restored were generated by homologous recombination with the unaltered HELI in the case of 17B1 and 17B2, or with a 6.3-kb subfragment of HELI (bp 103811 to 110101) for the restoration of dUT$^-$1218.

Northern (RNA) blots. Total RNA was isolated from infected RSC cultures 12 h postinfection (p.i.) by using RNAzol as described by the manufacturer (Cinna/Biotecx, Friendswood, Tex.). Total RNA (10 μg) was denatured by glyoxalation, separated in 1% agarose, and then electrophoretically transferred to nylon-supported nitrocellulose (GeneScreen; DuPont/New England Nuclear) by using the recommended conditions of the manufacturer. Following an overnight prehybridization in 50% deionized formamide–1% sodium dodecyl sulfate (SDS)–1 M NaCl–10% dextran sulfate–200 μg of herring sperm DNA per ml at 42°C, ^{32}P-labeled probes (5 × 10^6 cpm) were hybridized to the Northern blot for an additional 12 to 24 h at 42°C. Labeled probes (10^8 dpm/μg) were generated by random hexamer priming by using the suggested protocol of Boehringer Mannheim (Indianapolis, Ind.). The blots were then washed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1% SDS at 55°C and used to generate autoradiograms. X-Omat-AR films (Eastman Kodak, Rochester, N.Y.) were exposed to the blots with an intensifying screen for 1 to 3 days depending on the blot. The blots were also exposed in a Molecular Dynamics PhosphorImager and analyzed with ImageQuant software (Molecular Dynamics) for quantitation. Size markers were purchased from GIBCO/Bethesda Research Laboratories.

dUTPase enzyme assay. dUTPase activity was determined by using a modification of the method of Williams (41). Briefly, RSC monolayers (approximately 5 × 10^7 cells) were infected at 1 PFU per cell. At 18 h p.i., cells were harvested by scraping them into 1 ml of 10 mM Tris (pH 8.0)–1 mM $MgCl_2$–20% glycerol–2 mM β-mercaptoethanol–0.2 mM phenylmethylsulfonyl fluoride. The cell extract was sonicated by four 15-s pulses. Protein concentrations were determined by using the BioRad colorometric assay (Richmond, Calif.). The reaction mixtures (total volume, 100 μl) contained 50 mM Tris (pH 8.0), 2 mM β-mercaptoethanol, 1 mM $MgCl_2$, 0.1% bovine serum albumin, 2 mM *p*-nitrophenylphosphate, 0.03 mM H^3-dUTP (21 Ci/mmol; Amersham), and 25 μg of protein from the infected-cell extract. The reaction was allowed to proceed for 30 min at 37°C and was terminated by spotting 50 μl of the reaction mixture onto DE81 circles (Whatman). The circles were processed exactly

as previously described (42). Mock-infected and 17 syn$^+$-infected RSC extracts were used as standard controls in all experiments.

Mice. Male, outbred, Swiss Webster mice (Charles River), 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.

Quantitation of neurovirulence, neuroinvasiveness, and latency. Neurovirulence was quantified by PFU per lethal dose 50% (PFU/LD_{50}) ratios following intracranial inoculation of mice as previously described (36). Following inoculations, mice were monitored for 21 days and scored for death by encephalitis. PFU/LD_{50} ratios were calculated by using the method of endpoint estimation as described by Reed and Muench (30). Neuroinvasiveness was quantified by PFU/LD_{50} ratios following footpad inoculation as previously described (33). Animals were monitored for 21 days for hind-limb paralysis and death by encephalitis. PFU/LD_{50} ratios were calculated as described above.

The ability of the selected viral strains to establish a latent infection was qualitatively examined by explant cocultivation. Latently infected dorsal root ganglia (DRG) were explanted 30 days p.i. from mice infected with 5×10^3 PFU of 17 syn$^+$ or 10^8 PFU of 17B1. Explanted DRG were cultured on RSC monolayers. These cultures were observed daily over 3 weeks for viral cytopathic effect. When cytopathic effect was observed, culture media was removed and plated onto fresh RSC monolayers as a source of viral DNA. Viral DNA was obtained from these monolayers as previously detailed and analyzed by Southern blot to confirm genomic structures (35).

Induced in vivo reactivation. The recently developed hyperthermia-based method to induce reactivation in mice in vivo (31) was employed to assess the induced reactivation phenotype of the dUTPase$^-$ mutants. Mice were inoculated on the hind footpads with 100 PFU of the specified virus and maintained for 30 days. Animals were then subjected to hyperthermia as previously described (31). At 24 h posttreatment, the DRGs were removed from sacrificed animals, snap frozen, homogenized, and plated on RSC monolayers to detect infectious virus. Viral DNA from positive cultures was analyzed by Southern blot to confirm genomic structure as described above.

RESULTS

The dUTPase-negative viruses. The genomic region of HSV-1 that encodes the dUTPase is depicted in Fig. 1. Two strategies were employed to eliminate viral dUTPase activity by mutation of this gene. Previously, an in-frame insertion of a 12-bp *Hin*dIII linker at the *Asp*718 site (bp 107305) had been shown to destroy dUTPase activity (14, 41). An isolate of 17 syn$^+$ containing this mutation, dUT$^-$1218, was kindly provided by V. Preston. The derivation of dUT$^-$1218 has been described (14). Because of the nature of this mutation, it was still possible that undetectable levels of dUTPase activity were produced by dUT$^-$1218. Therefore, viral strains carrying deletions of the dUTPase coding region were generated. Figure 1 illustrates the strategy employed to remove the 5′ end of the dUTPase coding region (UL50, bp 107010 to 108123) from the cloned HELI. Recombination of this altered HELI into the wt strain 17 syn$^+$ resulted in the removal of the 928-bp *Bam*HI c′ fragment (bp 107028 to 107956) forming an open reading frame (ORF) containing the first 6 amino acids of dUTPase followed by 66 amino acids encoded by a different reading frame. Similar mutants have been recently characterized in cell culture (1, 27).

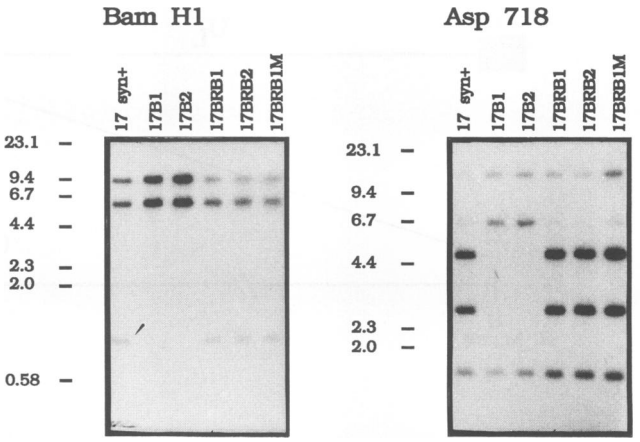


FIG. 2. Southern blot analysis of 17 syn$^+$ and derived viruses. Following restriction enzyme digestion with *Bam*HI or *Asp*718, the viral DNA fragments were separated in 0.8% agarose and transferred to nitrocellulose as previously described (38). ^{32}P-labeled HELI was hybridized to the blots to demonstrate the engineered mutation in the recombinants. The sample source is indicated above the blot. Migration of DNA size markers is denoted to the left of the panel. In the *Bam*HI blot, the *Bam*HI c′ fragment is denoted in the 17 syn$^+$ lane. In the *Asp*718 blot, the loss of the *Asp*718 site at bp 107305 in the dUTPase mutants resulted in the fusion of the *Asp*-718 U and O fragments which are present in the lanes containing 17 syn$^+$ and rescued virus DNA.

Plaque isolates which contained the *Bam*HI c′ deletion were selected by Southern blot restriction fragment length polymorphism analysis using radiolabeled HELI as a probe. To control for the possibility of unselected second site mutations, two independent isolates from separate cotransfection cultures were characterized. As shown in Fig. 2, both isolates, designated 17B1 and 17B2 (for 17 syn$^+$ *Bam*HI c′ deletions 1 and 2, respectively), contained the specific deletion as indicated by the loss of the 928-bp *Bam*HI fragment c′ (*Bam*HI digest). Digestion with *Asp*718 generated the expected, novel 6,549-bp fragment which resulted from the fusion of *Asp*718 fragments U and O.

Viruses in which the *Bam*HI c′ fragment was restored were generated by cotransfection of the unaltered HELI fragment and 17B1 or 17B2 viral genomic DNA as described above. Two restored viruses designated 17BRB1 and 17BRB2 were selected by Southern blot analysis of plaque isolates. As shown in Fig. 2, both of these viruses regained the 928-bp *Bam*HI c′ fragment as well as the proper *Asp*718 restriction pattern. The dUTPase gene was restored to dUT$^-$1218 by cotransfection of genomic DNA and a subfragment of the unaltered HELI spanning bp 103811 to 110101. Plaque isolates were again screened by Southern blot analysis for restoration of the *Asp*718 site at bp 107305. Two independently derived restored isolates, 1218R4C and 1218R4F, were selected and biologically characterized. Comprehensive restriction endonuclease and Southern blot analyses did not reveal any additional alterations in the viruses generated in this study (data not shown). However, subsequent studies revealed that a second mutation had occurred in 17B2, and therefore this virus was not included in further studies.

An additional restored virus, designated 17BRB1M, was isolated following selection in the mouse nervous system as

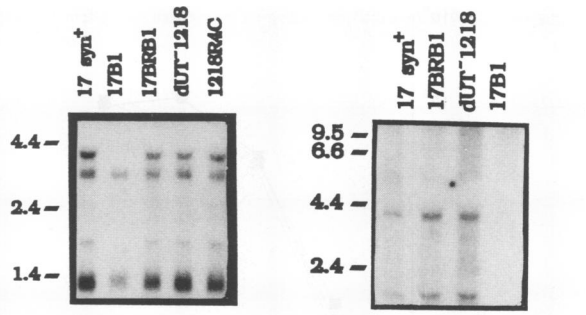
has been described (38). Briefly, the unselected, amplified cotransfection cultures which resulted in the isolation of 17BRB1 were used to inoculate four mice on the hind footpads. These animals were observed for the development of encephalitis. Three of the four animals displayed CNS disease signs within 8 days of inoculation. The brains from these animals were harvested, homogenized, and plated onto RSC monolayers. A total of 10 plaque isolates from the brain homogenates were screened by Southern analysis. Each isolate demonstrated the restored restriction pattern. One isolate, 17BRB1M, was further purified and biologically characterized.

To ensure that restoration of neuroinvasiveness to 17B1 was specifically due to repair of the dUTPase gene (rescue of *Bam*HI c'), four independent cotransfections of 17B1 and the HELI regions flanking *Bam*HI c' were performed. Infection of 13 mice with these cotransfection cultures failed to select any viruses capable of reaching the brain from the footpad. No CNS disease signs were observed in any of the mice. Therefore, the sequences of clone HELI which reside outside of *Bam*HI c' were not capable of restoring neuroinvasiveness to this mutant.

Transcriptional analysis of the dUTPase locus. Deletion of the *Bam*HI c' fragment from 17 syn$^+$ was designed to affect only the dUTPase gene, leaving the neighboring UL49 and UL51 loci intact. The deletion did not affect UL51 (bp 109011 to 108279), since the mRNA which encodes this ORF terminates at bp 108281, 325 bp from the right end of the deletion (18) (Fig. 1). UL49 (bp 106391 to 105488) is also unaffected by this deletion; however, because of the proximity of its transcriptional start site (bp 106550, 478 bp from the left end of the *Bam*HI c' deletion) (18), the deletion may have affected the UL49 promoter.

A previous report on transcription of this region in the HSV-1 strain KOS suggested that a possible ORF exists between UL49 and UL50 (18). In a recent report, it was demonstrated that this ORF is translated during infection of cultured cells, and it was designated UL49.5 (2). Although the deletion of *Bam*HI c' does not eliminate any of the UL49.5 coding region (bp 106993 to 106720), it does eliminate the entire promoter and the transcriptional start site of the UL49.5 mRNAs, and thus 17B1 is potentially a double mutant.

The effect of the engineered mutations on the transcription of UL49 and UL49.5 was determined by Northern blot analysis. RNA for Northern analysis was isolated from infected RSC cultures 12 h p.i. Two representative blots are shown in Fig. 3. The blot on the left demonstrates that all the viruses analyzed produced the two transcripts encoding the UL49 ORF (1.3 and 3.2 kb) (18). This blot was also hybridized to a probe specific for the 1.3-kb HSV-1 thymidine kinase mRNA to control for variability in the viral RNA loaded in each lane. Following both hybridizations, the band intensities were quantified in a Molecular Dynamics PhosphorImager and analyzed with ImageQuant software (Molecular Dynamics). The resulting data were used to generate the table presented in Fig. 3. The ratio of the UL49 mRNAs to the thymidine kinase-specific RNA for each of the dUTPase mutants was found to be equivalent to the ratios seen in 17 syn$^+$. It can be therefore concluded that neither dUTPase mutation affected transcription of UL49. As shown in the right-hand blot, a probe which was specific for the UL49.5 mRNAs did not hybridize to any mRNAs produced by 17B1. Therefore, 17B1 is indeed a double mutant, and these results demonstrate that UL49.5 is not essential for viral replication in cultured cells. The insertion



Ratio of the Indicated Transcript to TK

Virus	UL49		UL49.5	
	1.3 kb	3.2 kb	1.8 kb	3.8 kb
17 syn$^+$	1.9	0.71	0.29	0.72
17B1	1.8	0.86	-	-
17BRB1	2.2	0.73	0.24	0.79
dUT$^-$1218	2.2	0.88	0.28	0.70
1218R4C	1.7	0.42	0.22	0.62

FIG. 3. Northern analysis of transcription through the dUTPase region of 17 syn$^+$ and the derived viruses. Total RNA was isolated from infected RSC cultures 12 h p.i., separated in 1.2% agarose, and electrophoretically transferred to nylon-supported nitrocellulose as described in Materials and Methods. Specific mRNAs were detected by hybridization to ^{32}P-labeled probes. Migration of RNA size markers is denoted at the left of the panels. The left blot was hybridized to a 768-bp fragment (bp 105825 to 106593) spanning a region shared by UL49 and UL49.5. Following autoradiography, this blot was stripped and hybridized to a 539-bp fragment (bp 47053 to 47592) specific for the thymidine kinase mRNA. For both probes, the blot was scanned by using a Molecular Dynamics PhosphorImager for quantitation. The blot on the right was hybridized to a 243-bp fragment (bp 106596 to 106839) specific for the UL49.5 mRNAs. The table indicates the ratio of the indicated UL49- or UL49.5-specific mRNA to the corresponding amount of thymidine kinase-specific mRNA to control for viral RNA loading.

dUTPase mutant, dUT$^-$1218, clearly produced the UL49.5 RNAs (1.8 and 3.8 kb) (18) at levels equivalent to that of 17 syn$^+$. As expected, the restored 17BRB1 and 1218R4C produced wt levels of UL49 and UL49.5 mRNAs. The 3.2-kb UL49 mRNA was underexpressed in 1218R4C, but as will be described below, this isolate displays wt phenotypes in all the properties tested. These data demonstrated that neither the deletion nor the insertion grossly affected the transcription of the neighboring genes.

In vitro characterization of 17B1. Replication kinetic analysis in primary MEC confirmed that no general replication defect was present in 17B1. MEC cultures were infected at 0.001 PFU per cell to reflect the conditions of in vivo infection, where cells greatly outnumber virus. 17B1 and its dUTPase-restored derivatives replicated with wt kinetics, attaining titers of approximately 10^5 PFU/ml by 72 h p.i. (Fig. 4). These results clearly demonstrated that the replication of these viruses was indistinguishable from that of 17 syn$^+$ in cultured murine cells, in agreement with results reported previously for other cell culture systems (1, 14, 27, 41).

The assay to detect dUTPase activity developed by Williams (41) was employed to establish the level of the dUTPase activity induced by the viruses generated in this

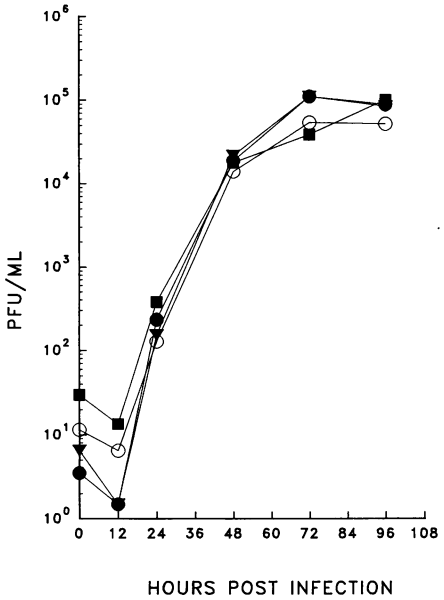


FIG. 4. Replication kinetic analysis in MEC cultures. Triplicate cultures of MEC were infected with the indicated viruses at a multiplicity of infection of 0.001. At the indicated times p.i., the cultures were harvested in triplicate, processed by three successive freeze-thawing cycles, and finally analyzed for infectious virus. ▼, 17 syn$^+$; ○, 17B1; ●, 17BRB1; ■, 17BRB1M.

study. Crude protein extracts of infected or mock-infected RSC were assayed for their ability to hydrolyze H^3-dUTP. Table 1 shows the average of three determinations of dUTPase activity relative to that of mock-infected RSC. As expected, 17B1 did not induce detectable dUTPase activity in RSC monolayers. The dUTPase$^+$ viruses (wt and restored) showed a 2.5- to 3-fold induction of dUTPase activity, consistent with previous observations (6).

Neurovirulence of the dUTPase mutants. As a measure of the capacity to replicate in and destroy neural tissue, averaged PFU/LD_{50} ratios were generated following direct intracranial inoculations of mice. In all cases at least two and sometimes three experiments were performed. Apparent CNS disease signs (hunched posture, seizures, and ruffled fur) in mice inoculated with the dUTPase$^-$ viruses were delayed by 24 to 48 h compared to the same signs in mice inoculated with 17 syn$^+$ (data not shown). Consistent with these observations, both dUTPase$^-$ mutants yielded average PFU/LD_{50} ratios 10-fold reduced from that of 17 syn$^+$ (Table 2).

Restoration of the dUTPase locus resulted in viruses with wt virulence (17BRB1, 17BRB1M, 1218R4C, and 1218R4F [Table 2]), indicating that the reduced neurovirulence observed was due to the engineered perturbations. Furthermore, the onset and severity of CNS disease signs following inoculation with the restored viruses were indistinguishable from those for the wt 17 syn$^+$ (data now shown). It is significant that both 17B1 and dUT$^-$1218 demonstrated a similar reduction in neurovirulence, indicating that the lack of UL49.5 does not measurably contribute to the reduced neurovirulence phenotype of 17B1.

TABLE 1. Induced dUTPase activity

Virus	dUTPase activity[a]
Mock-infected RSC	1
17 syn$^+$	3.11 ± 0.15
17B1	1.09 ± 0.12
17BRB1	2.50 ± 0.11
17BRB1M	2.80 ± 0.22

[a] The data presented represent the average dUTPase activity ± the standard deviation induced in cell extracts in three independent trials. The numbers represent the fold induction of dUTPase activity over mock-infected RSC cultures.

TABLE 2. Virulence characteristics of the viruses

Virus	PFU/LD_{50}	
	Intracranial[a]	Peripheral[b]
17 syn$^+$	8.3	2.9×10^3
dUT$^-$1218	3×10^2	3.5×10^6
1218R4C	1	1.2×10^3
1218R4F	4	5.5×10^3
17B1	3.3×10^2	$>6.6 \times 10^6$
17BRB1	2.7	8.5×10^4
17BRB1M	7	3.5×10^4

[a] Mice were inoculated in the left brain hemisphere with serial 10-fold dilutions of the indicated virus stocks (5 mice per dilution). PFU/LD_{50} ratios were determined by the method of Reed and Muench.

[b] Mice were inoculated with serial 10-fold dilutions on both abraded hind footpads as previously described (33). The infected animals were observed for 21 days p.i. LD_{50} estimations were calculated as described above.

Neuroinvasiveness of dUTPase 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 12). In this study the ability of each virus to invade the CNS from a peripheral site of inoculation was examined as a measure of neuroinvasiveness. To determine the contribution of the viral dUTPase in this process, mice were inoculated on both abraded hind footpads and examined for 21 days for fatal encephalitis as described in Materials and Methods. The data from these experiments are summarized as averaged PFU/LD_{50} ratios in Table 2.

An inoculum of as little as 100 PFU of wt 17 syn$^+$ consistently caused hind-limb paralysis in at least 1 of 5 mice. This inoculum led to encephalitis and death by 7 to 11 days p.i. in a subset of the animals (data not shown). The average PFU/LD_{50} ratio for 17 syn$^+$ following peripheral inoculation was 2.9×10^3 (Table 2). In contrast, 17B1 did not induce any paralysis or encephalitis even at the highest titers inoculated (6.6×10^6 PFU [Table 2]) and is therefore three to four orders of magnitude reduced in its ability to invade the nervous system by this assay. The insertion mutant, dUT$^-$1218, yielded PFU/LD_{50} ratios averaging 3.5×10^6 (Table 2) and was therefore at least three orders of magnitude less neuroinvasive than its parent 17 syn$^+$. Restoration of the dUTPase gene resulted in wt levels of neuroinvasiveness in the rescued dUT$^-$1218 isolates (1218R4C and 1218R4F [Table 2]). Neuroinvasiveness was also restored to 17B1 following rescue of the dUTPase mutation; however, 17BRB1 and 17BRB1M were both 10-fold less neuroinvasive than 17 syn$^+$ (Table 2).

In vivo replication kinetic analysis in murine tissues. Viral replication kinetic analysis in mouse tissue was employed to determine the anatomical location of the restriction of the dUTPase$^-$ mutants. Mice were inoculated on both rear footpads, and tissues following the natural course of viral infection from the footpad to the brain were analyzed for

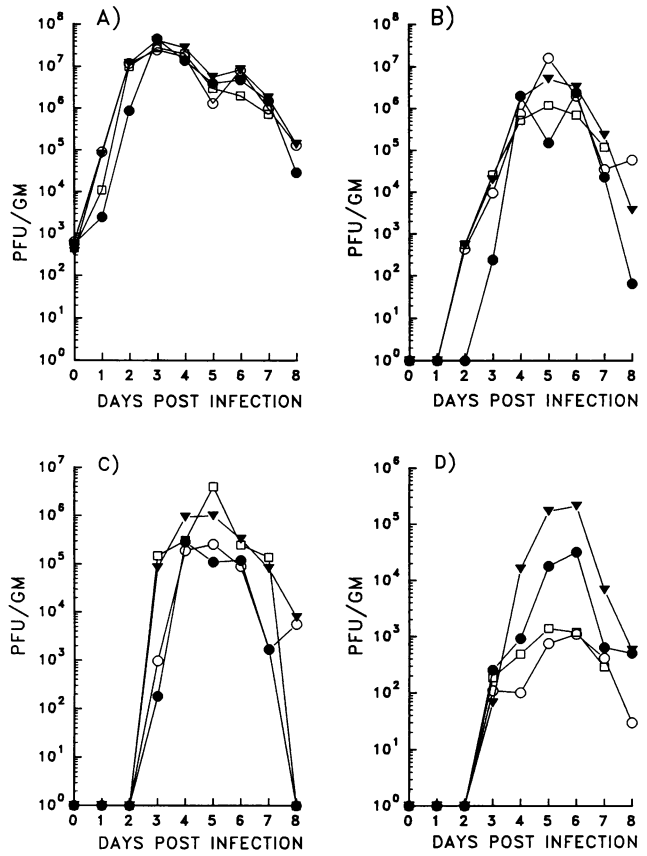


FIG. 5. Replication kinetics in mouse tissues in vivo. Mice were infected on both hind footpads with 5×10^4 PFU of the indicated viruses. At the indicated times p.i., two mice infected with each virus were sacrificed, and tissues were then collected and stored at −80°C. Tissues were homogenized in cell culture medium and, following clarification by centrifugation, were assayed for virus content. The results for footpad (A), sciatic nerve (B), DRG (C), and spinal cord (D) are presented as PFU/gram of tissue. ▼, 17 syn$^+$; □, dUT$^-$1218; ○, 17B1; ●, 17BRB1.

infectious virus at 24-h intervals. The results are presented graphically in Fig. 5.

All the viruses examined, including dUT$^-$1218 and 17B1, reached equivalent peak titers in the footpad 3 days p.i. (Fig. 5, panel A) and replicated equivalently in the sciatic nerve and DRG. Thus, HSV-1 dUTPase and UL49.5 are not required for efficient replication in the mouse PNS (panels B and C). Restricted replication of both of the dUTPase$^-$ mutants was first apparent in the mouse CNS (spinal cord), where peak titers generated by the mutants were two to three orders of magnitude reduced from 17 syn$^+$ (panel D). The restored 17BRB1 reached titers equivalent to those attained by 17 syn$^+$ in the mouse CNS, indicating that rescue of this locus restores the ability of the virus to replicate in the CNS (Fig. 5, panel D) and to cause disease (Table 2).

The results demonstrate that both dUTPase$^-$ mutants replicated with wt kinetics in nonneural tissues and in the PNS but were defective in entry into or replication in the CNS. It was not possible to distinguish between these two possibilities. Immunohistochemical staining for HSV-1 antigens in acutely infected DRG revealed viral protein production in many different cell types, including neurons, a result consistent with the findings of others (7). No difference was detected between DRG infected with 17B1, with dUT$^-$1218, or with 17 syn$^+$ (data not shown). However, it is possible that infectious virion production within the neurons was selectively reduced, leading to reduced viral entry into the CNS. Conversely, the dUTPase$^-$ isolates may have replicated efficiently in the DRG neurons and spread to the CNS neurons, where replication was severely restricted.

TABLE 3. In vivo reactivation following hyperthermia

Virus	In vivo reactivation frequency[a]
17 syn$^+$	14/17 (82)
dUT$^-$1218	1/10 (10)
1218R4C	8/12 (67)
1218R4F	9/12 (75)
17B1	6/29 (21)
17BRB1	7/10 (70)

[a] In vivo reactivation frequency [number of animals with reactivation/total number of animals (percent reactivation)] was scored by the recovery of infectious virus from the pooled DRG from individual latently infected animals 24 h posthyperthermia.

Establishment of and reactivation from latency. To determine whether 17B1 could establish latent infections, the DRG from animals (n = 8) latently infected with 17B1 were examined by standard explant cocultivation. These animals had been inoculated with the highest titer stock available (6.6×10^6 PFU/60 μl). The explanted DRG of animals (n = 10) latently infected with the wt, 17 syn$^+$ (infected with 1 LD_{50} or 3×10^3 PFU), were employed as controls for normal latency and reactivation by explant cocultivation.

The results showed that 17B1 was capable of establishing latent infections, as virus reactivated from the DRG of all eight animals by 13 days after explant cocultivation. By 7 days after explant cocultivation, 100% of the DRG from animals latently infected with 17 syn$^+$ had reactivated. In contrast, only 50% of the cocultivation cultures from animals infected with 17B1 were positive at this time, suggesting that 17B1 either established fewer latent infections, reactivated with delayed kinetics, or was slowed in its spread to the RSC monolayers.

The recently developed hyperthermia model of induced reactivation (31) was employed to examine the ability of both of the dUTPase$^-$ mutants to reactivate in vivo. Groups of mice were inoculated on the hind footpads with 100 PFU of one of the six viruses, maintained for 30 days, and then subjected to transient hyperthermia as previously described (31). The results are presented in Table 3.

The frequency of reactivation of 17 syn$^+$ in these studies was consistent with a previous report for this virus (31). Interestingly, both dUTPase$^-$ mutants demonstrated a significantly reduced ability to reactivate in vivo by this method. 17BRB1, 1218R4C, and 1218R4F reactivated as frequently as 17 syn$^+$. In other experiments using mice inoculated with as much as 6.6×10^6 PFU of 17B1 or 3.5×10^5 PFU of dUT$^-$1218, reactivations were still observed less often than in mice inoculated with 100 PFU of 17 syn$^+$ (data not shown). Therefore, a functional HSV-1 dUTPase gene product is necessary for efficient establishment of, and/or reactivation from, latency.

DISCUSSION

Analysis of wt HSV-1 strain 17 syn$^+$ and two derived dUTPase-negative mutants allowed an assessment of the

role of this enzyme in infection of murine neural tissues. dUT$^-$1218 contains an insertion of four codons in the dUTPase reading frame and does not induce any detectable viral dUTPase activity in infected-cell cultures (14, 41). 17B1 has a deletion of 928 bp in the dUTPase ORF and encodes only the first six amino acids of the dUTPase. This deletion also removed the entire promoter and start site of the UL49.5 transcript (2, 18). No transcription of the UL49.5 gene was detected in cells infected with this mutant. Although a previous report suggested that UL49.5 may be an essential gene product (2), it was found that 17B1 replicated normally in cell culture.

Both mutants were less neurovirulent than 17 syn$^+$ following intracranial inoculation and were ≥1,000-fold less neuroinvasive following infection of the footpad. The only observed phenotypic difference between the two dUTPase$^-$ mutants was in the PFU/LD_{50} ratios generated following footpad inoculation. 17B1 did not induce any disease signs in mice inoculated with maximal titer. In contrast, dUT$^-$1218 was lethal following inoculation of ~10^6 PFU and induced hind-limb paralysis in mice inoculated with 10^4 PFU (data not shown). There are several possible explanations for this difference. First, because of the nature of the in-frame insertion mutation in dUT$^-$1218, this mutant may be leaky. However, as described by McGeoch, the insertion in dUT$^-$1218 is within a conserved region which may be involved in glycoside binding and phosphate transfer (28). As has been noted, several groups have been unable to detect any HSV-1-specific dUTPase activity following infection of cells with this virus (14, 41). Second, the loss of the UL49.5 gene product may slightly affect the ability of 17B1 to invade and replicate in the CNS. Finally, 17B1 may carry an unselected mutation that slightly alters its phenotype. In support of this third possibility, restoration of the dUTPase locus to 17B1 resulted in two isolates that were not fully restored for neuroinvasiveness, suggesting that a second mutation, outside of the rescuing DNA fragment, was inadvertently selected.

While deleterious to the host, the capacity to replicate in neural tissues may be required for the efficient establishment of and/or reactivation from latency. Mutants in the viral thymidine kinase and ribonucleotide reductase both show a decreased capacity to replicate in neural tissues, and such mutants have been shown to establish latency less efficiently (22, 23, 25, 32). In contrast, both dUTPase$^-$ mutants analyzed in this study replicated with wt kinetics in the murine PNS, and 17B1 reactivated from latently infected ganglia in 100% of the animals tested by explant cocultivation experiments. As noted in Results, the kinetics of reactivation of 17B1 were delayed as compared to those for the wt 17 syn$^+$. Such a delay has been suggested to indicate a reduced ability to reactivate from latency in vivo (39).

Hyperthermic treatment of latently infected mice demonstrated that the in vivo reactivation frequencies of both dUTPase$^-$ mutants were significantly reduced compared with those of the parental 17 syn$^+$. This is in agreement with reports which demonstrate that nonneuroinvasive isolates reactivate in vivo much less frequently than neuroinvasive strains (19, 31). Whether these findings are the result of inefficient establishment of latency or of the inability of noninvasive strains to replicate to detectable titers following induction of reactivation has not yet been determined.

Restoration of the dUTPase gene to both 17B1 and dUT$^-$1218 demonstrated that the attenuated phenotypes of these mutants were due primarily to the loss of this viral enzyme. Restoration of dUTPase to dUT$^-$1218 resulted in fully wt viruses. Restored viruses of 17B1 were wt for neurovirulence and reactivation from latency and only slightly reduced in neuroinvasiveness. None of these properties could be restored to 17B1 with 10 kb of genomic sequence from this region which lacked the dUTPase ORF.

These results suggest that engineered mutation of the viral dUTPase gene could be exploited in live viral vaccine strategies. Vaccine strains lacking this gene would be less likely to cause postvaccination encephalitis. In addition, this enzyme might serve as a target for antiviral chemotherapy. Compounds which show high selectivity for inactivating the viral enzyme have been described (21, 40). While these compounds are not therapeutically useful, they suggest that useful compounds might be produced. If a key role of this enzyme in vivo is to increase available dTTP pools in neurons, such compounds would work additively or synergistically with currently used antiviral agents such as acyclovir. Design of toxic compounds selectively activated by the viral enzyme might also be possible (8). Such compounds would selectively kill virus-infected cells. Further, as many diverse viruses encode dUTPase activities (11, 28, 29, 43, 44), such enzymes may serve as a general target for antiviral agents.

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