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ICP34.5 Mutants of Herpes Simplex Virus Type 1 Strain 17syn+ Are Attenuated for Neurovirulence in Mice and for Replication in Confluent Primary Mouse Embryo Cell Cultures

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In a recent report, the neurovirulence of herpes simplex virus type 1 (HSV-1) was mapped to the ICP34.5 gene (J. Chou, E. R. Kern, R. J. Whitley, and B. Roizman, Science 250:1262–1266, 1990). In this report, specific mutations within ICP34.5 were constructed in HSV-1 strain 17syn+ to determine the effects of these mutations in a fully neurovirulent isolate. It was found that termination of the ICP34.5 gene after the N-terminal 30 amino acids resulted in a mutant, 17termA, which was 25- to 90-fold reduced in neurovirulence. This reduction of neurovirulence was associated with restricted replication of the mutant virus in mouse brain. The reduced replication phenotype was also evident in the trigeminal and dorsal root ganglia following inoculation at the periphery. 17termA was capable of replicating with wild-type kinetics in mouse footpads, and therefore the restriction seen in neural tissues was not due to a generalized replication defect in mouse cells. Significantly, replication of the mutant was also restricted in the mouse cornea in vivo and in confluent primary mouse embryo cells and mouse 10T1/2 cells in vitro. However, 17termA replicated with much greater efficiency in subconfluent mouse embryo cells, suggesting that the physiological state of the cell may be an important factor for productive replication of this mutant. Restoration of the ICP34.5 gene to the mutant resulted in a virus which displayed wild-type neurovirulence and replication kinetics in all cells and tissues tested.

Herpes simplex virus type 1 (HSV-1), which is ubiquitous in the human population, is the leading cause of acute sporadic fatal viral encephalitis in the United States and responsible for over 200,000 cases of blindness per year (8, 11). It has been demonstrated that virus isolates vary widely in their capacity to induce such severe disease states in experimental animals (6). The molecular basis for this variation is largely unknown, but the viral genes which contribute to efficient replication in select tissues in vivo are of fundamental importance.

Several lines of evidence demonstrate that a locus associated with neurovirulence of HSV-1 maps to sequences in the long internal and terminal repeat sequences. Viruses containing mutations which map to this region have been reported to be as much as 1 million-fold reduced for neurovirulence when assayed by intracranial inoculation of mice (18, 31, 32, 38). Only one HSV-1 open reading frame (designated ICP34.5 [1] or RL1 [7]) within the neurovirulence locus defined by these mutants has so far been identified. This genomic region also contains *cis*-acting sequences important for cleavage and packaging of the HSV genome and for the regulation of the immediate-early transactivator protein ICP0 (3, 5, 16–18).

Recently, Chou et al. (2) reported that in HSV-1 strain F, premature termination of the ICP34.5 protein resulted in a mutant (designated R4009) which replicated 4-fold less well than the parental strain F in Vero cell cultures and was reduced for neurovirulence in mice by more than 100,000-fold compared with strain F (PFU/50% lethal dose [LD_{50}] > 10^7). However, the relative contribution of the ICP34.5 gene product to neurovirulence is still in question, as HSV-1 F yields a PFU/LD_{50} ratio 10^3-fold higher than that of other wild-type HSV-1 strains in adult mice (6), and the genetic background which contains a mutation may greatly influence the phenotype observed.

Here we report the results of analysis of two isogenic ICP34.5 mutants of the neurovirulent HSV-1 strain 17syn+ (PFU/LD_{50} ≤ 10). Deletion of eight codons of ICP34.5 centered around amino acid 30 had no detectable effect on neurovirulence or viral replication. Introduction of stop codons at this site, exactly the same mutation as was engineered into R4009, resulted in a 25- to 90-fold reduction in neurovirulence for mice (PFU/LD_{50} = 250 to 900). This phenotype could be attributed to a diminished capacity to replicate in brain tissue. The replication defect displayed by this mutant was not restricted solely to the nervous system but was also evident on the surface of the eye in vivo and in primary mouse embryo cells (MEC) in culture under certain conditions. However, the mutant replicated efficiently in other tissues in vivo and in subconfluent MEC cultures in vitro. When the wild-type ICP34.5 sequence was recombined into the premature termination mutant, wild-type levels of both neurovirulence and replicative capacity were restored. The implications of these results with regard to the pathogenesis of herpetic infections are discussed.

MATERIALS AND METHODS

Mice. Four-week-old male Swiss Webster mice (Charles River) were used for these studies. Animals were maintained in American Association for Laboratory Animal Care-approved areas.

Cells. Rabbit skin cells were cultured in Eagle's minimal essential medium (MEM) supplemented with 5% newborn calf serum and antibiotics (250 U of penicillin per ml and 250 μg of streptomycin per ml) in a 5% CO_2 atmosphere at 37°C.

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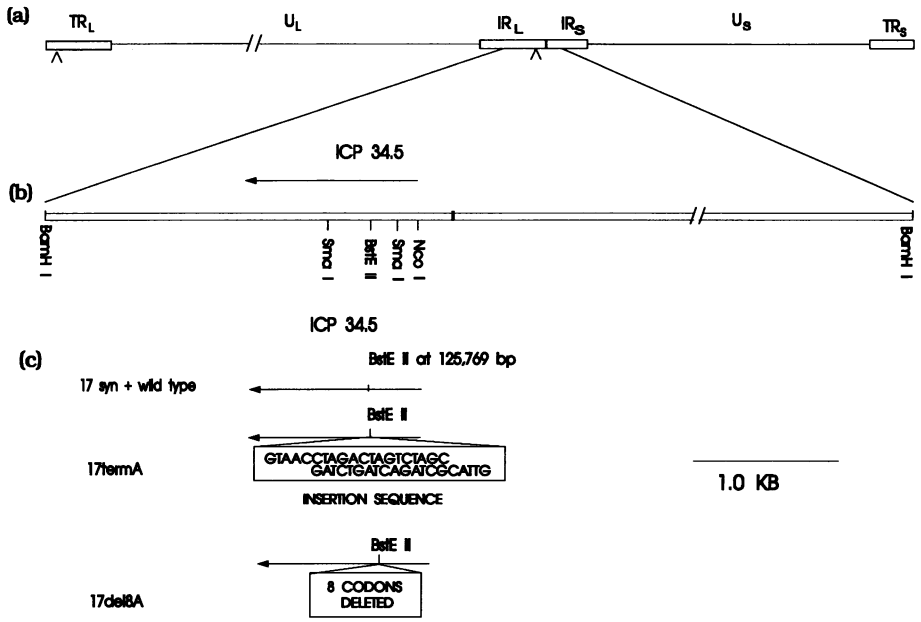

FIG. 1. Schematic diagram of the HSV-1 strain 17syn+ genome. (a) HSV-1 genome in the prototypic P-isomer arrangement. Unique long (U_L), unique short (U_S), terminal repeat long (TR_L), internal repeat long (IR_L), internal repeat short (IR_S), and terminal repeat short (TR_S) regions are shown. Arrowheads indicate locations of ICP34.5. (b) *Bam*HI S+Q region of the genome. Location and direction of ICP34.5 and the primary latency-associated transcript (LAT) sequence are indicated. The relevant restriction sites used for construction of the mutations are indicated with corresponding base pair numbers (4). (c) Mutations constructed within virus isolates 17termA and 17del8A.

Primary MEC cultures were prepared from 16-day Swiss Webster mouse embryos as described previously (9, 36) and cultured in MEM supplemented with 10% fetal calf serum under the same conditions. Murine C3H/10T1/2 cells (ATCC CCL 226) were cultured in Dulbecco's MEM supplemented with 10% fetal calf serum and same antibiotics and under the same incubation conditions. Media and serum were purchased from GIBCO/BRL (Gaithersburg, Md.).

Virus. HSV-1 strain 17syn+ was obtained from J. H. Subak-Sharpe, Medical Research Council Virology Unit, Glasgow, Scotland (38). Restriction endonuclease fragment names and base pair designations are based on the sequence of this strain as compiled by McGeoch and colleagues (7, 21, 22). Viral stocks were generated by routine passage in rabbit skin cell cultures as described (36).

Construction of virus mutants. The three isogenic virus mutants used in these experiments were constructed from the parental strain 17syn+. Methods for constructing recombinants are described elsewhere (33, 35, 37, 38). Enzymes were purchased from GIBCO/BRL and used as instructed by the manufacturer. Briefly, the 17syn+ *Bam*HI fragment S+Q (bp 123459 to 129396) was cloned in pUC19. The plasmid was linearized at the unique *Bst*EII site (bp 125769 in the virus genome) and subjected to limited BAL 31 nuclease digestion as described previously (26). The digested ends were filled with the Klenow fragment of DNA polymerase I and ligated with T4 ligase. Clones which contained deletions were initially identified by restriction fragment length polymorphism (RFLP) analysis on 6% polyacrylamide gels. Sequencing by the Sanger method (27) was used to identify a clone containing a deletion of eight codons (codons 28 to 35). Unit-length 17syn+ DNA was cotransfected with the altered *Bam*HI S+Q fragment, and a recombinant virus containing the deletion in both copies of the ICP34.5 gene was identified by RFLP Southern blot analysis, using a nick-translated ^{32}P-labeled *Bam*HI S+Q fragment probe as previously described (35). Following three rounds of plaque purification, the resulting mutant virus was designated 17del8A (Fig. 1).

To construct mutant 17termA, a 20-bp linker (GTAACCTA GACTAGTCTAGC and its complementary sequence GTTA CGCTAGACTAGTCTAG) was inserted into the *Bst*EII site (bp 125769) within the *Bam*HI S+Q fragment. The oligonucleotide sequences were synthesized on an Applied Biosystems 380B DNA synthesizer. This is the same linker sequence as used to generate mutant R4009 in the strain F background (2) and results in the premature termination of the ICP34.5 protein. The termination is predicted to occur after the initial 30 amino acids. This clone was then used in cotransfection experiments as described above, and viral isolates containing the mutation in both copies of the ICP34.5 gene were identified by RFLP Southern blot analysis for the *Spe*I sites introduced by the linker. The restored virus 17termAR was generated by cotransfections of 17termA viral DNA with wild-type 17syn+ *Bam*HI S+Q sequence (38). Viruses in which both copies of the ICP34.5 gene were repaired were identified by RFLP Southern blot analysis for the loss of the *Spe*I sites. All mutant viral genomes were further analyzed by Southern blot analysis with a variety of labeled probes spanning the majority of the HSV-1 genome following digestion with three restriction endonucleases (*Bam*HI, *Eco*RI, and *Asp*718) as previously described in detail (35). No unexpected genome perturbations were detected (data not shown). The genomic structures of viruses 17del8A and 17termA are shown schematically in Fig. 1.

Extract preparation and Western immunoblot analysis for detection of ICP34.5. Extracts of rabbit skin cells infected with HSV-1 at a multiplicity of infection (MOI) of 10 were prepared by homogenization in 10 mM Tris (pH 7.6) containing 0.4% Nonidet P-40. Nuclei and insoluble material were removed by centrifugation, and extracts were boiled in sodium dodecyl sulfate, loaded onto 10% polyacrylamide gels, electrophore-

sed, and transferred to nitrocellulose as recently described (28). The uniformity of transfer was evaluated by staining the nitrocellulose with Ponceau S, which was subsequently removed by washing in phosphate-buffered saline. Western blot analysis of the transferred proteins was carried out in a 10-well blotting chamber (Hoefer Scientific), using a three-step biotin-avidin-alkaline phosphatase system. The primary antibody (78.3; a generous gift of A. MacLean and M. Brown, MRC Virology Unit, Glasgow, Scotland) was a total serum from a rabbit hyperimmunized with a peptide consisting of 10 repeats of the sequence PAT, a repeat which is predicted to be present 5 times in the ICP34.5 protein of strain 17syn+ and 10 times in that of strain F (20). The primary antibody was used at a 1:400 dilution. The secondary antibody, biotinylated goat anti-rabbit immunoglobulin G (Vector, Burlingame, Calif.) was diluted 1:500, and the alkaline phosphatase conjugate was diluted 1:400. Blots were developed with the chromogenic substrate Immuno Select (GIBCO-BRL) as specified by the manufacturer. To demonstrate specificity, 100 μl of the primary antiserum was preincubated with 10 μg of the peptide for 30 min at 37°C.

Neurovirulence assays. Mice (five per dilution) were inoculated intracranially in the left brain hemisphere (36) with serial 10-fold dilutions ranging from 10^7 to 10^0 PFU of virus in 0.03 ml of MEM. Animals were maintained for 21 days and scored for death by encephalitis. PFU/LD_{50} ratios were calculated by the method of Karber as described in reference 15.

Replication kinetics. Single-step and multistep replication kinetics experiments were performed at 37°C on confluent or subconfluent primary MEC or 10T1/2 cell cultures as previously described (36). In single-step experiments, the MOI was 10 PFU per cell; for multistep experiments, the MOI was 0.01 (MEC) or 0.005 (10T1/2 cells) PFU per cell.

Mice (three per time point) were inoculated intracranially with 5×10^5 PFU in a 0.03-ml volume. At indicated times postinfection (p.i.), three mice infected with each virus were sacrificed, and brains were collected and stored at −80°C. Brain tissues were pooled, homogenized as 10% suspensions in MEM, clarified at 5,000 × *g* for 5 min, and assayed for virus titer on rabbit skin cell monolayers (36).

Virus replication was assayed following footpad inoculation of mice with 10^6 PFU in 0.05 ml as previously described (32). At indicated times p.i., mice were sacrificed (three mice per time point), and hind feet and dorsal root ganglia were collected and stored at −80°C. Virus content was then assayed by standard techniques (36).

Virus replication following eye inoculation was assayed as previously described (36). Mice were inoculated bilaterally following corneal scarification with 10^6 PFU in 15 μl per eye. Animals were sacrificed at various times p.i. (three mice per time point), and eyes and trigeminal ganglia were collected, stored at −80°C, and assayed for virus content as described above.

RESULTS

Construction of specific ICP34.5 mutants. Deletions within the ICP34.5 gene may disrupt the packaging and recombination signals of HSV-1 (2, 5, 18, 31, 39) as well as having the potential to perturb the promoter of the required gene ICP0 (16, 17). The ICP34.5 mutations in this study were designed to minimally perturb the viral genome. A clone of the 17syn+ *Bam*HI S+Q fragment was mutated at the *Bst*EII site at bp 125769 on the HSV-1 genome. One mutation was engineered by limited BAL 31 deletion mutagenesis, resulting in a 24-bp deletion centered around the *Bst*EII site and the loss of eight

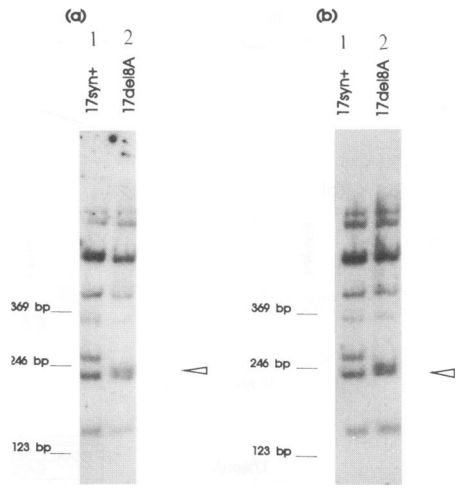

FIG. 2. Genomic structure of 17del8A. (a) Viral DNAs were isolated, digested with *Bam*HI and *Sma*I, separated by electrophoresis on 6% polyacrylamide gels, transferred to nitrocellulose by capillary blotting, and probed with ^{32}P-labeled *Bam*HI S+Q as described in the Materials and Methods. The expected deletion of 24 bp resides within a 260-bp *Sma*I fragment (arrowhead). The wild-type fragment is 284 bp (21). (b) The same analysis was performed on virus recovered from the brains of infected mice as described in the text.

codons from the ICP34.5 open reading frame. It was reasoned that such an in-frame mutation might not disrupt ICP34.5 function, and such a mutant could serve as a control for any secondary effects that mutations at this site could have on neighboring genes or *cis*-acting signals. A second mutation was made by introducing a 20-bp linker (GTAACCTAGACTA GTCTAGCC) into the *Bst*EII site. This is precisely the same mutation as engineered into the strain F mutant R4009 and results in the introduction of stop codons in all three frames of both DNA strands at this site. The exact nature of the mutations introduced into the *Bam*HI S+Q fragment was confirmed by DNA sequence analysis (data not shown). The design of each mutation is shown schematically in Fig. 1. Recombinant viruses which contained the mutations in both copies of the ICP34.5 gene were produced by cotransfection of genomic DNA from HSV-1 strain 17syn+ and the mutated *Bam*HI S+Q fragments. Mutant isolates were identified by Southern blot RFLP analysis and plaque purified. Figure 2 depicts the results from a Southern blot of the deletion mutant isolates. Lanes 1 shows the hybridization of 17syn+ sequences, with a wild-type *Sma*I band at 284 bp. Lanes 2 shows mutant 17del8A, with the 24-bp deletion at the *Bst*EII site within this *Sma*I fragment causing a band shift from 284 to 260 bp. The mutation is present in both copies of the long repeat sequences, since there is no wild-type 284-bp band detectable. As can also be seen, all other restriction fragments present comigrate with bands in the 17syn+ lane. No other perturbations in the mutant viral genomes were detected (data not shown).

Figure 3 shows the results of genomic analysis of 17termA. Lane 1 shows the pattern obtained with 17syn+ DNA. The *Bam*HI S+Q band migrates at 5.8 kb, with the Q and S bands migrating as a doublet at 3.3 and 2.5 kb. Lane 2 contains the mutant 17termA sample. The expected shift of the S+Q band from 5.8 kb to a doublet at 3.5 and 3.3 kb, representing the added *Spe*I sites, is indicative of the stop codon insert. The Q band comigrates at 3.3 kb, with the S band truncated to 2.3 kb.

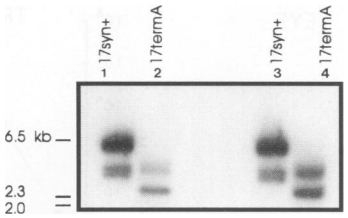

FIG. 3. Genomic structure of 17termA. Viral DNAs were isolated, digested with *Bam*HI and *Spe*I, electrophoresed, transferred, and probed with ^{32}P-labeled *Bam*HI S+Q as described in Materials and Methods. Lane 2 reveals the added *Spe*I site within the insertion sequence. Sizes of markers (lambda phage DNA *Hin*dIII digests) are indicated at the left. In lanes 3 and 4, the same analysis was performed on viral DNA recovered from infected mouse brains as described in Materials and Methods.

Since no wild-type 17syn+ bands were present in this lane, the mutation was present in both copies of ICP34.5. Cotransfections were performed with mutant genomic DNA and wild-type 17syn+ *Bam*HI S+Q to restore the ICP34.5 gene, and wild-type viruses were identified by Southern blot analysis as described above and plaque purified. The resulting isolate 17termAR contained an intact ICP34.5 gene at both loci, and its restriction endonuclease fragment pattern was indistinguishable from that of 17syn+ (data not shown).

17del8A and 17termA were tested for the ability to replicate in rabbit skin cell cultures. Each of these isolates replicated with wild-type kinetics and yielded wild-type titers of 10^8 PFU/ml, and therefore no replication defects were detectable (data not shown).

Detection of ICP34.5 in infected cell lysates. Immunoblot analysis was performed to determine whether 17termA was indeed a truncation mutant of ICP34.5. A polyclonal antiserum raised against 10 repeats of the peptide sequence PAT was used to detect ICP34.5 in the cytoplasmic fraction of infected rabbit skin cells. A previous report demonstrated that a rabbit serum raised against this peptide was specific for the ICP34.5 gene product (1).

As shown in Fig. 4, this antiserum detected a protein with an apparent molecular size of 41 kDa in lysates from cells infected with HSV-1 strain F (lane A). The apparent molecular size of

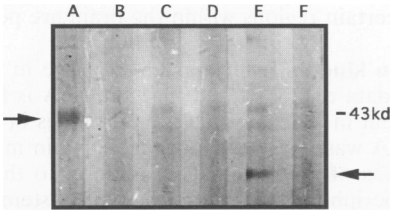

FIG. 4. Detection of ICP34.5 by Western immunoblot analysis of infected rabbit skin cell extracts. Protein extracts were prepared, electrophoresed, transferred, and analyzed for the presence of the ICP34.5 antipeptide antibody as described in Materials and Methods. The migration of molecular weight standards (GIBCO-BRL) is indicated at the right. Lane A, HSV-1 strain F extract. The position of ICP34.5 is indicated by the arrow at the left. Lane B, HSV-1 strain F extract reacted with antiserum preadsorbed with peptide. Lane C, 17termA extract. Lane D, 17termA extract with preadsorbed primary antibody. Lane E, 17termAR extract. The position of the ICP34.5 band is indicated by the arrow at the right. The decreased mobility of this protein compared with that of strain F is discussed in the text. Lane F, 17termAR extract reacted with preadsorbed antiserum.

TABLE 1. Neurovirulence of ICP34.5 mutants[a]

Virus isolate	PFU/LD_{50} ratio	
	Expt 1	Expt 2
17syn+	9.8 ± 5.2	ND
17termA	948 ± 300	244 ± 90
17del8A	3.7 ± 2.0	1.3 ± 0.7
17termAR	3.3 ± 1.6	ND

[a] Mice were inoculated intracranially in the left brain hemisphere (36) with serial 10-fold dilutions ranging from 10^7 to 10^0 PFU of virus in 0.03 ml of MEM. Animals were maintained for 21 days and scored for death by encephalitis. Ratios were calculated by the method of Karber as described in reference 15. ND, not determined.

this protein is in close agreement with the 43 kDa obtained in a previous report (1). This protein was not detected in mock-infected extracts or infected cell extracts incubated with preimmune serum (not shown). Preincubation of the serum with peptide $(PAT)_{10}$ eliminated this reactivity (lane B). No specific protein band was detected in 17termA extracts regardless of whether the serum was preincubated with peptide (lanes C and D). A band of 39 kDa was specifically detected in the 17termAR extract, and as above, this reactivity was eliminated by incubation with peptide (lanes E and F, respectively). On similar blots, a protein of the same apparent molecular size was detected in extracts from cells infected with 17syn+. The faster migration of this protein is consistent with the molecular size predicted from the DNA sequence of strain 17syn+ and most likely reflects the fact that only 5 repeats of the PAT repetitive amino acid sequence are present, compared with 10 in strain F (1, 7, 20). These data indicate that the insertion sequence resulted in truncation of the ICP34.5 protein after the first 30 amino acid residues as predicted, since no ICP34.5 protein could be detected in extracts infected with 17termA. It is unlikely that a truncated form of the protein was produced, as there are no additional methionine codons in the open reading frame (7, 20).

Neurovirulence phenotypes of the ICP34.5 mutants. The neurovirulence of the mutants was quantified by PFU/LD_{50} ratios generated in intracranially inoculated mice. The parent strain 17syn+ served as a positive control. 17del8A yielded ratios that were indistinguishable from wild-type ratios (Table 1). Thus, deletion of these eight amino acids from the ICP34.5 reading frame did not affect the neurovirulence phenotype. In addition, this finding demonstrated that this region of the genome can be perturbed minimally without adversely affecting the transcription of neighboring essential genes or the efficient cleavage and packaging of the viral genome.

In contrast to the more than 100,000-fold reduction in neurovirulence reported for a premature termination mutant of ICP34.5 in strain F, this same mutation in strain 17syn+ background resulted in a 25- to 90-fold increase in the PFU/LD_{50} ratio (Table 1). It should be noted, however, that a few mice consistently survived inoculation of 10^4 PFU of 17termA; this is not the case with 17syn+, inoculation of 10^2 PFU of which is sufficient to kill 100% of the mice. In addition, mice inoculated with 17termA survived longer than those infected with equivalent titers of 17syn+ (data not shown). The fact that the mutation in ICP34.5 was responsible for the decrease in neurovirulence observed was confirmed by analysis of the rescued virus 17termAR. This virus produced a PFU/LD_{50} ratio indistinguishable from that of 17syn+ (Table 1), and the kinetics of death of the animals were the same (data not shown).

The reason for the difference in neurovirulence phenotype

between 17termA and R4009 is not yet known, but one possibility was that our mutant viral stocks were contaminated with virus which carried at least one intact copy of the ICP34.5 gene. It has been shown that replication in mouse brain tissue is a powerful selective pressure for virulent virus isolates (38), and such agents could amplify in vivo, resulting in a lethal infection. To test for this possibility, virus was recovered from the brain tissue of moribund mice inoculated with low titers of 17termA or 17del8 10 to 13 days p.i. Such isolates would be highly enriched for wild-type virus if the mutant stock were contaminated with low levels of 17syn+ (38). The genomic DNA of the recovered virus was compared with that of wild-type DNA by Southern blot RFLP analysis. No reversions or rearrangements to wild-type sequence were detected (17del8A [Fig. 2b]; 17termA [Fig. 3]). Clearly, the neurovirulence displayed by these mutants could not be attributed to contamination with wild-type virus.

It was not possible to determine a PFU/LD_{50} ratio for 17termA following inoculation of peripheral tissues of the mouse. No mice died after inoculation of full-strength viral stock on either the footpad or the eye. Furthermore, the mice did not display any signs of central nervous system disease (hunched posture, roughened fur, ataxia, or convulsions). Therefore, the PFU/LD_{50} ratio of 17termA was $>6 \times 10^7$ after footpad inoculation, compared with a ratio of $\sim 10^3$ for strain 17syn+ (32).

Replication kinetics in vivo. It is possible that the nonneuroinvasive and reduced neurovirulence phenotypes of 17termA are the result of a generalized replication defect in any mouse cell. Viral replication kinetic analysis in mouse tissue was used to determine the anatomical location of the restriction of the ICP34.5 termination mutant. Mice were inoculated either on both rear footpads or in both eyes as described above, and peripheral and ganglionic tissues were analyzed for infectious virus at 24-h intervals. The results are presented graphically in Fig. 5.

In the mouse footpad, 17termA replicated with kinetics indistinguishable from that of 17syn+. Both isolates reached titers of $>10^7$ PFU/g by day 3 p.i. Therefore, no generalized replication defect was evident in this tissue. In contrast, the mutant was severely restricted at the level of the dorsal root ganglia. Infectious virus was not detected until day 5 p.i., and the amount present was reduced by 3 orders of magnitude compared with the parent strain. Whether the virus detected was produced in neurons or other cell types within the ganglia was not determined.

In the eye, mutant 17termA did not replicate efficiently. The titer recovered was reduced by 3 orders of magnitude 3 days p.i., and virus could no longer be recovered from the eye after day 3. In contrast, 17syn+ was recovered through day 7. Within the trigeminal ganglia, both 17termA and 17syn+ were detected on day 1 p.i., although the yield of 17termA was reduced by 1 log. By day 3, 17termA was reduced by 3 logs compared with 17syn+. 17termA was not recovered from the trigeminal ganglia after day 3, although 17syn+ was recovered through day 7 p.i.

For acute viral replication kinetics in brain, mice were inoculated intracranially with 5×10^5 PFU and assayed for virus yields in brain tissue over 5 days (Fig. 6). Two orders of magnitude less virus was recovered from mice inoculated with 17termA 48 h p.i. There are no time points for 17syn+ after 48 h, since no mice in this group survived beyond this point at this inoculation titer. While 17termA continued to replicate in the brain past this time and is lethal at this inoculation titer, the replication kinetics suggest that the pathology induced may be quite different from that of a wild-type strain. Further analysis will be required to determine whether only a subpopulation of the cells or certain regions within the brain are permissive for this virus.

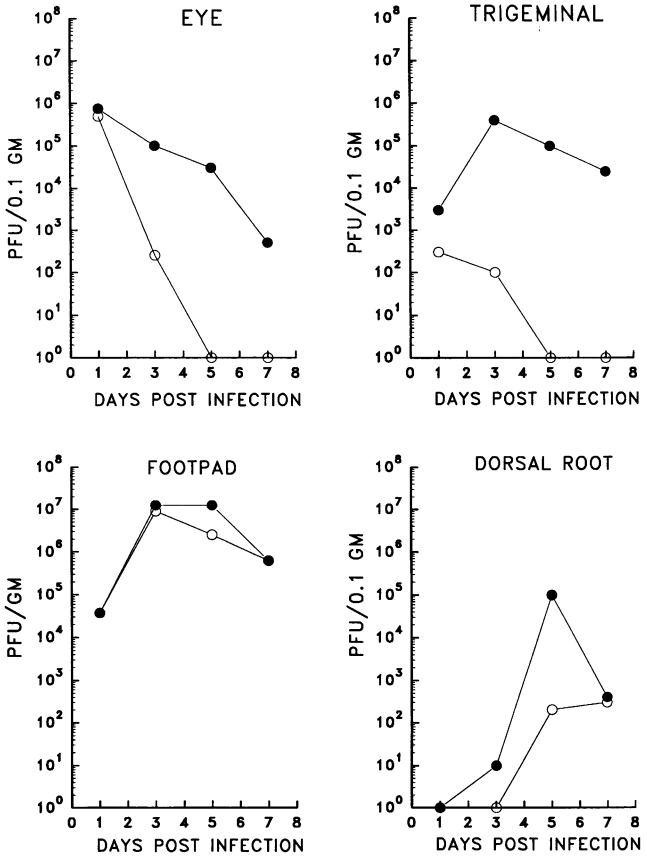

FIG. 5. Virus replication kinetics in vivo. Mice were inoculated by corneal scarification of both eyes with 10^5 PFU per eye. At the indicated times p.i., three mice infected with each virus were sacrificed, and the eyes and trigeminal ganglia were removed and stored at −80°C. The tissues were pooled, homogenized, clarified, and then assayed for virus titers as described in Materials and Methods. For virus replication in the mouse footpad and dorsal root ganglia, mice were inoculated by footpad abrasion on both hind feet with 10^5 PFU per foot. Infected tissues were pooled and processed as described above at the indicated time points p.i. ●, 17syn+; ○, 17termA.

Replication kinetics in primary MEC. The in vivo replication kinetic data demonstrated that 17termA is fully replication competent in at least some mouse tissues in vivo. Therefore, 17termA was not replication defective in mouse cells in general. It was of interest that in addition to the restriction seen in the peripheral and central nervous system tissues, this mutant also failed to replicate efficiently in the eye. Cells on the eye surface are a largely quiescent population (19), as are several cell types in the ganglia and brain (10, 23). A possible explanation for these observations is that a factor(s) present in actively dividing cells can complement the ICP34.5 termination mutant phenotype. To test this possibility, replication kinetics were examined in primary cultures of MEC under confluent (largely quiescent) and subconfluent (actively dividing) conditions.

Multistep kinetics (MOI = 0.01 PFU per cell) were carried out in subconfluent primary MEC cultures (about 40% confluence at infection). As shown in Fig. 7, 17termA replicated

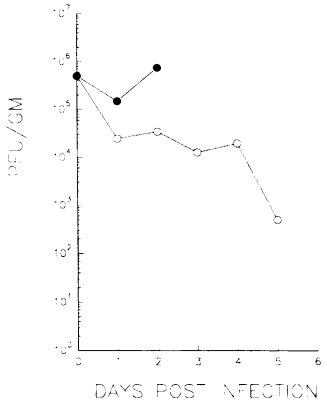

FIG. 6. Virus replication kinetics in mouse brain. Mice were inoculated intracranially with 5×10^5 PFU. At the indicated time points p.i., three mice infected with each virus were sacrificed, and the entire brains were removed and stored at −80°C until assayed. The brains were pooled and homogenized as a 10% suspension in cell culture media and assayed for virus content as described in Materials and Methods. ●, 17syn+; ○, 17termA.

efficiently in these cultures. Viral yields of 17termA were reduced by about 10-fold compared with 17syn+ levels, but the rate of virus replication was the same. In contrast, the replication of mutant 17termA was significantly restricted in confluent MEC cultures. By 24 h, yields of this mutant were reduced by 1 log compared with wild-type levels; by 48 h, yields were reduced 100-fold. Thus, the yield of 17termA in these cultures was <1 PFU per cell, whereas the yield of 17syn+ was about 100 PFU per cell. Taken together, these results suggest that a function present in actively dividing MEC was able to compensate, at least partially, for the replication restriction seen with this mutant within confluent primary MEC cultures.

Confluent mouse 10T1/2 cells are restrictive for replication of 17termA. Primary MEC cultures contain many diverse cell types, and it is possible that these vary in their permissivity for replication of the ICP34.5 mutant (9). Therefore, the replication of 17termA was examined in a defined cell line. Mouse 10T1/2 cells were chosen because they are very contact inhibited (24). Multistep kinetics were carried out in these cultures at an MOI of 0.005 PFU per cell. Strains 17syn+, 17del8A, and 17termAR were used as controls. As seen in Fig. 7, the replication of 17termA was severely restricted in these cells. Less than 100 PFU/ml was recovered at the peak of 3 days p.i. Virus production in these cultures was therefore less than 0.001 PFU per cell. In contrast, all three control viruses demonstrated similar kinetics of replication and reached 10^6 to 10^7 PFU/ml (~100 PFU per cell) by 3 days p.i.

The restriction seen in these cultures was evident even at a very high MOI. Confluent cultures of 10T1/2 cells were infected with 50 PFU per cell and assayed over a 30-h period. 17termA produced $<10^4$ PFU/ml (burst size, <0.1 PFU per cell). In contrast, the control viruses rapidly reached titers of $>10^7$ PFU/ml, with a burst size of >100 PFU per cell (data not shown). Taken together with data presented above, these results demonstrate that ICP34.5 null mutants are restricted in a variety of cell types and not solely in neuronal cells as has been suggested (2, 4). Furthermore, they suggest that the physiological state of the cell may be important in overcoming the restriction of replication of ICP34.5 mutants.

DISCUSSION

While several lines of evidence have shown that functions important for the neurovirulence phenotype of HSV-1 and HSV-2 map to the long terminal repeat of the HSV genome (2, 18, 31, 35, 38), the relative contributions of *cis*-acting signals and open reading frame (ICP34.5 RL1) encoded in this region are still unclear. In this study, we confirm the results reported by Chou et al. that the ICP34.5 gene plays a role in HSV-1 neurovirulence (2), although clearly this gene does not play as predominant a role in strain 17syn+ as was suggested for strain F (2, 4).

In addition, we have extended these findings by demonstrating that the phenotype is not displayed solely in neurons as suggested (4) but also in other cell types both in vivo and in vitro. Strain 17syn+ and two isogenic mutants designed to affect only the ICP34.5 reading frame were analyzed. Mutant 17del8A contains a 24-bp deletion in both copies of the ICP34.5 gene centered around the *Bst*EII site at 595 and 125769 bp on the HSV-1 genome. This deletion resulted in the loss of amino acids 28 to 35 from the protein. 17del8A was fully neurovirulent and replicated with wild-type kinetics in all cells and tissues tested. Therefore, a small deletion at this location

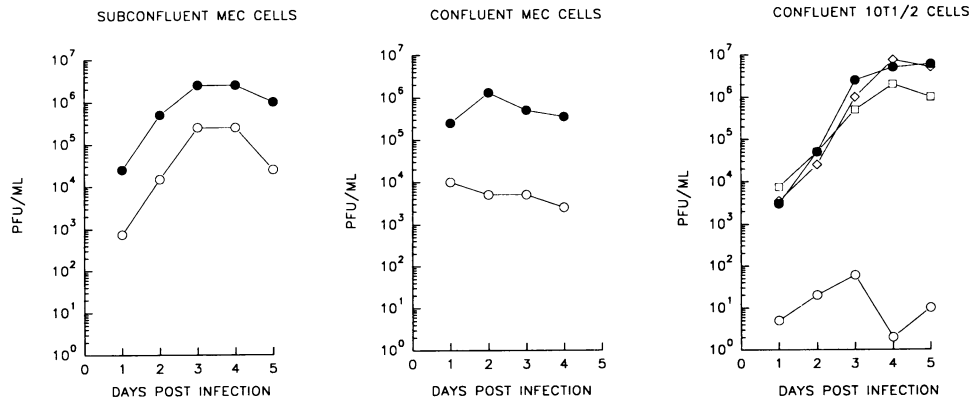

FIG. 7. Virus replication kinetics. Primary MEC cultures in 35-mm-diameter tissue culture dishes were infected at an MOI of 0.01 PFU per cell. At the indicated time points, duplicate cultures were harvested and stored at −80°C until assayed for virus titers. 10T1/2 cell cultures were maintained under the same conditions but infected at an MOI of 0.005 PFU per cell. ●, 17syn+; ○, 17termA; □, 17del8A; ◇, 17termAR.

did not adversely effect the expression of neighboring genes, or the function of the packaging signals, to a detectable extent.

Mutant 17termA contains an insertion of 20 bp in both copies of the ICP34.5 gene at the *Bst*EII sites. This is the same mutation as was recently reported in mutant R4009 in the HSV-1 strain F background and results in the premature termination of ICP34.5 after the amino-terminal 30 amino acids (2). It was found that 17termA was 25- to 90-fold reduced in neurovirulence, as assayed by intracranial inoculation of mice (PFU/LD_{50} = 250 to 900). Restoration of the ICP34.5 gene, as determined both by genomic structure and production of the protein, resulted in isolate 17termAR, which was fully neurovirulent and wild type in all other characteristics tested, indicating that the mutant phenotype was due to the ICP34.5 disruption specifically. In contrast, R4009 was reported to be completely nonneurovirulent ($PFU/LD_{50} > 10^7$) (2). Thus, 17termA is at least 10,000-fold more neurovirulent than R4009.

The reason for the difference in neurovirulence phenotype between 17termA and R4009 is not yet known, but there are several possibilities. In this study, neurovirulence was assayed in adult outbred Swiss Webster mice, while the studies with R4009 were performed in weanling BALB/c mice (2). However, it is unlikely that this accounts for the difference seen, as BALB/c mice are more susceptible to HSV infection than are Swiss Webster mice (6, 36), and weanling mice are considerably more susceptible than adult mice (34). Unknown second-site mutations in the strain F-based mutant may have contributed to its avirulent phenotype. Such mutations may be present in strain F itself or may have been introduced during the construction of R4009. This latter possibility was not tested by repair of the ICP34.5 gene to determine whether this restored virulence to the mutant (2). Evidence exists that strain F is itself less virulent than strain 17syn+ in mice following intracranial inoculation. The PFU/LD_{50} ratio of strain F in 4-week-old BALB/c mice inoculated intracranially is $>10^4$. In contrast, several other wild-type laboratory HSV-1 isolates, including 17syn+, and all early-passage clinical isolates tested yielded ratios of ~10 (6). It therefore seems likely that the neurovirulence displayed by ICP34.5 mutants is the result of a combination of factors and depends on the genetic background in which the mutation is expressed.

The phenotype of R4009 is similar to that of deletion mutations reported in this locus, including 1716 on the strain 17syn+ background and R3616 on the strain F background. The boundaries of the deletions reported to date also include the direct repeat 1 of the cleavage and packaging signals, upstream regulatory regions of the ICP0 gene promoter, or both (2, 5, 16–18). ICP0 is required for efficient replication of the virus, and reduced levels of this critical regulator may affect the course of infection (12, 25, 29). It is perhaps significant that, where tested, these mutants replicated less well than their parent strains in any cell type, including the cells in which they were produced (2).

17termA was replication competent in actively dividing primary MEC (mostly fibroblasts [9]) but more severely restricted in confluent MEC. This mutant also failed to replicate efficiently in confluent mesodermally derived mouse 10T1/2 cells following infection even at an MOI as high as 50 PFU per cell. The failure of the mutant to efficiently replicate in such cultures is likely due to the mutation introduced into the ICP34.5 gene, as restoration of this gene resulted in isolate 17termAR, which displayed wild-type replication kinetics both in vitro and in vivo. Whether this restriction to replication of the mutant is the result of shutoff of protein synthesis following viral DNA replication as was seen in R4009-infected SK-N- SH neuroblastoma cells (4) has not yet been determined. These results suggest that the physiological state of the cell is an important factor in permissivity for ICP34.5 mutants. Further experimentation should reveal whether permissivity is associated with a specific stage in the cell cycle or with cell cycling time.

Despite the quantitative difference in neurovirulence between the mutants, it is clear that the replication of ICP34.5 null mutants is restricted in the central nervous system. In addition, 17termA was completely avirulent following footpad inoculation with a PFU/LD_{50} of $>10^8$, compared with a PFU/LD_{50} of $\sim10^3$ for strain 17syn+ (32). While the mutant replicated with wild-type kinetics in the footpad, this reduction in neuroinvasiveness could be attributed to a severe restriction in replication at the level of the dorsal root ganglia. Clearly then, this locus is required for efficient replication within both the central nervous system and, perhaps more biologically relevant, the peripheral nervous system.

Chou and Roizman have proposed that the role of the ICP34.5 protein is to permit virus replication in sensory neurons by preventing apoptosis (4). Our findings demonstrate the requirement for this protein for productive infection of cells at the body surface, particularly the cornea. This result along with results obtained in cultured cells demonstrates that the effect of ICP34.5 is not neuron specific and suggests a more generalized function for ICP34.5. This protein may increase the number and/or types of cells at the body surface which can be productively infected by allowing replication of the virus in quiescent cell types. This could lead to an increased transmission rate and increase the chance of infection of neurons and subsequent establishment of latency. This gene product may be particularly important for infections of the eye leading to recurrent keratitis and blindness. It has recently been demonstrated that viral replication occurs in sensory neurons during reactivation from latency in vivo (28). A potential function of ICP34.5 may be to permit replication in neurons during a reactivation event leading to recurrent disease and transmission of the virus to new hosts.

McGeoch and Barnett (20) reported that 63 amino acids near the carboxy terminus of RL1 (ICP34.5) share a high amino acid identity with the mouse gene myD116 (14). Recently, an open reading frame in African swine fever virus with significant sequence identity to the same region of myD116 and ICP34.5 was described (30). The function of myD116 is not known, but its expression is induced in a myeloid leukemia cell line upon differentiation mediated by interleukin 6 (13). It is of interest that African swine fever virus replicates in mature macrophages (30). It is possible that ICP34.5 serves as an agonist or antagonist of a cellular factor such as myD116. The presence or absence of such a cellular factor may compensate for the replication restriction of ICP34.5 mutants within some cells but not in quiescent cells at the body surface or in the peripheral and central nervous systems.

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