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Neuron-Specific Restriction of a Herpes Simplex Virus Recombinant Maps to the UL5 Gene

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We have previously shown that, when compared with either parent, a herpes simplex virus type 1/herpes simplex virus type 2 intertypic recombinant (R13-1) is attenuated by 10,000-fold with respect to neurovirulence in mice. Despite this, after intracranial inoculation, R13-1 replicated to titers of 10^5 PFU per brain. We present evidence that the restriction is specific for replication in neurons and have taken a three-step approach in determining the basis of the attenuation by (i) characterizing cellular tropism of the virus in both central and peripheral nervous systems, (ii) defining where in the viral replication cycle the restriction is manifest, and (iii) identifying the genetic basis of the restriction through marker rescue analysis. Following inoculation into the animal, R13-1 viral antigens predominate in nonneuronal cells, and the block to replication in neurons was found to be beyond the level of adsorption and penetration. Despite the restricted replication within neurons, the virus established a latent infection in spinal ganglia and could be reactivated by in vitro cocultivation of the ganglia. In studies carried out in cell culture, R13-1 was found to replicate normally in mouse embryo fibroblasts and primary mouse glial cells but was restricted by 1,000-fold in primary mouse neurons and PC12 cells. R13-1 appeared to produce normal levels of early RNA in these cells, but production of DNA and late RNA was less than that of the wild type. Marker rescue analysis localized the fragment responsible for restoring neurovirulence to UL5, a component of the origin-binding complex implicated in replication of the viral genome. Our results with this virus, with a cell-specific restriction, suggest that a neuron-specific component is involved in viral replication.

A definition of how viruses replicate preferentially in certain organs and tissues is central to an understanding of viral disease. With respect to herpes simplex viruses (HSVs) specifically, the nervous system plays a critical pathogenetic role, and the central nervous system (CNS) is a significant target organ. In recent years, we and others have identified viral genes specifically involved in accessing the CNS (neuroinvasive genes) and in causing disease after the virus is in this organ (neurovirulence genes).

In all studies, mutant strains of HSV have proven invaluable, not only in defining differences between neuroinvasion and neurovirulence but also in facilitating molecular dissection of the genetic basis for these properties. For example, noninvasive mutants have highlighted the importance of viral glycoproteins facilitating spread through the nervous system (11, 28). Mutants in several viral genes have defined neurovirulence, and in general these mutants replicate well in dividing cells but are restricted in postmitotic cells (particularly neurons) of the nervous system. Viruses of this group contain mutations in the thymidine kinase (TK) (6, 8), ICP34.5 (3), dUTPase (2, 21), or US3 genes (19, 20), and all replicate poorly in the CNS.

We previously showed that, following intracranial inoculation, an intertypic recombinant of HSV (R13-1) replicates to high titers in the CNSs of mice. However, when compared with the wild type, it is attenuated by some 10,000-fold in its capacity to kill the animals (12). In this communication, we characterize properties of the mutant which define the basis for this lack of concordance between viral replication and capacity to induce disease.

In initial experiments, the virus was shown to be nonneuroinvasive, and this property was used to define cellular tropism in vivo. Following footpad inoculation, the virus was restricted within spinal ganglia, and analyses of acutely infected ganglia revealed that although viral antigen was easily detected in supporting cells, it could be detected in only a few neurons. This result led to the hypothesis that restricted replication within neurons was responsible for the lack of neurovirulence seen earlier. Studies with cell cultures indicated that R13-1 was restricted in primary neurons but replicated to near-wild-type levels in primary glial cell preparations. The virus also replicated very poorly in PC12 cells and exhibited restricted DNA synthesis in these cells. To identify the viral mutation involved, marker rescue experiments with HSV type 1 (HSV-1) DNA fragments were performed, and UL5, a gene encoding a component of the primase-helicase complex, was shown to confer neurovirulence to R13-1 (which has HSV-2 sequences at this locus). The recombinants also replicated normally in PC12 cells, indicating that the UL5 locus in R13-1 is responsible for both the restricted replication and the lack of neurovirulence.

This mutant, with the UL5 gene of HSV-2 replacing the UL5 gene of HSV-1, defines a new type of neurovirulence mutant in which the property of replication in the CNS is not directly linked to capacity to induce disease. Since the virus is able to replicate in glial cells, significant replication in these cells is not critical for survival of the animal. Finally, since the involved gene is specifically concerned with viral DNA replication, it is postulated that a host component which interacts with the helicase-primase complex is required for this process and that

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this component is present in an altered form or quantity in neurons.

MATERIALS AND METHODS

Cells and virus. The viruses used in this study were the standard HSV-1 laboratory strain 17*syn*$^+$ and the recombinant R13-1, both of which have been described previously (12). Rabbit skin (RS) cells, employed to prepare and titrate virus stocks and for transfection experiments, were prepared and passaged by standard methods (25, 26). The RS cells were propagated in minimal essential medium supplemented with 5% calf serum, 250 U of penicillin, 250 μg of streptomycin per ml, 2.5 μg of amphotericin B per ml and 292 μg of L-glutamine per ml. Rat-2 cells were obtained from the American Type Culture Collection and were propagated in minimal essential medium supplemented with 10% fetal bovine serum (FBS), 250 U of penicillin, 250 μg of streptomycin per ml, 2.5 μg of amphotericin B per ml, and 292 μg of L-glutamine per ml. PC12 (rat pheochromocytoma) cells were obtained from the American Type Culture Collection and were propagated in RPMI 1640 supplemented with 10% horse serum, 5% FBS, 250 U of penicillin, 250 μg of streptomycin per ml, 2.5 μg of amphotericin B per ml, and 292 μg of L-glutamine per ml. For experiments in which PC12 cells were differentiated, 50 ng of mouse salivary gland nerve growth factor (Sigma, St. Louis, Mo.) per ml was added to the culture medium 4 days prior to infection, and tissue culture dishes were pretreated with poly-L-lysine (10). Primary mouse CNS neurons were prepared from 12- to 14-day Swiss-Webster embryos (9) and were propagated in serum-free medium (Dulbecco modified Eagle medium–nutrient mixture F-12 Ham [1:1] [Sigma], 3 g of glucose per liter, 2.5 mg of insulin per ml, 20 μg of progesterone per ml, 1 μg of nerve growth factor per ml, 15 μM sodium selenite, 100 μg of transferrin per ml). Briefly, brains were removed and placed in serum-supplemented medium (Dulbecco modified Eagle medium–F-12; 3 g of glucose per liter, 10% FBS). The meninges and blood vessels were removed by dissection, and the tissue was minced into 0.5-mm^3 pieces and dissociated by vigorous aspiration through a 5-ml pipette several times. The suspension was centrifuged at 700 × *g* for 5 min at 4°C, and the pellet was resuspended in 5 ml of medium. The cells were counted and plated at a density of 2×10^5 cells per ml in serum-supplemented medium into 12-well cluster dishes that had been poly-L-lysine treated. These dishes were incubated for 30 min at 37°C. At the end of the incubation period, the medium was removed, and 2 ml of serum-free medium was added to each well. For primary glial cells, the above procedure was modified by using newborn mouse embryos, and medium with D-valine was substituted for that with L-valine.

Infections of mice. For neurovirulence determinations, groups of six mice were inoculated in the left cerebral hemisphere with various dilutions of virus in a final volume of 30 μl, and PFU/50% lethal dose (LD_{50}) were calculated by the formula of Reed and Muench (22). For tracer studies, mice were infected by scarification of both rear footpads, and at the indicated times groups of four mice were killed. Feet, sciatic nerve, dorsal roots, spinal ganglia, spinal cord, and brains were dissected, frozen in liquid nitrogen, and titered for infectious virus as previously described (26).

In order to test for the relative abilities of latently infected dorsal root ganglia to reactivate, mice were infected with 5×10^3 PFU of either 17*syn*$^+$ or R13-1 on both rear footpads, and 21 days after infection, spinal ganglia (L4 and L5) from each of eight mice per virus were pooled separately (four ganglia) and cultured on RS cells. Cultures were maintained in minimal essential medium supplemented with 10% FBS and fed every other day. The cultures were examined daily and were scored positive for reactivation on the basis of the appearance of cytopathic effects of the monolayer.

Immunofluorescence. For detection of viral antigen, spinal ganglia were removed from mice following perfusion with phosphate-buffered saline (PBS; 0.1 M $NaPO_4$ [pH 7.2], 0.875 g of NaCl per liter) and 4% paraformaldehyde fixative (4% paraformaldehyde, 0.1 M $NaPO_4$ [pH 7.2], 0.875 g of NaCl per liter) (16). The ganglia were postfixed in 4% paraformaldehyde fixative for 2 h and equilibrated with 30% sucrose (in PBS) for 4 h. The ganglia were then frozen in liquid nitrogen, and 6-μm sections were cut onto poly-L-lysine-treated slides. Sections were blocked with 3% normal goat serum (NGS) in PBS for 30 min. The sections were then stained with fluorescein isothiocyanate-conjugated rabbit anti-HSV antisera (Dako Corp., Carpinteria, Calif.) at a dilution of 1:250 in PBS plus 1% NGS for 1 h at room temperature, washed 5 times with PBS plus 1% NGS, mounted with 1 mg of *p*-phenylenediamine per ml in 90% glycerol (13), and viewed by indirect immunofluorescence.

Transfections and marker rescue analysis. Subcloning of the region of the genome implicated in the neurovirulence phenotype was performed by standard methods (1). Cotransfections of plasmid DNA with full-length R13-1 DNA were performed with Lipofection reagent (Life Sciences, Gaithersburg, Md.) on 80% confluent monolayers of RS cells in 60-mm-diameter dishes. All transfections were set up in duplicate and typically involved 1 μg of viral DNA with an amount of plasmid DNA yielding a molar ratio of 1:10. Transfected cultures were harvested after 96 h by scraping cells into the media, and the suspension was assayed for virulence as previously described (12, 26). Briefly, 0.03 ml of the transfection cell suspension was inoculated into the brains of two mice. When the mice died, brains were removed and homogenized as a 10% (wt/vol) solution in complete media. The homogenate was clarified by centrifugation (3,000 × *g* for 5 min at 4°C), and 0.03 ml of this homogenate was inoculated into each of six mice. If two or more of these mice died, brains were removed and virus was isolated and purified by plaque purification. Plaques were picked into 96-well dishes containing RS cells. After all cells were killed, the cells were blotted to nylon membranes (Hybond-N; Amersham, Arlington Heights, Ill.) with a dot blot minifold, and the membranes were treated with 1.5 M NaCl–0.1 M NaOH–0.2 M Tris (pH 7.5)–2× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7]). The blots were then hybridized with one of two oligonucleotides specific for the HSV-1 UL5 gene, either 5′ CAC GTC GAG CTG TTG TTC GTC C 3′ or 5′ AGG CCC CCG CTC CTA CAC GCC TTG CT 3′, by tetramethylammonium chloride hybridization methodology at 55°C (1). Duplicate blots of each screening were hybridized so that recombinants hybridizing with both probes could be selected. HSV-1 and HSV-2 controls were included on each blot so that the specificities of the tetramethylammonium chloride hybridizations could be assessed.

Quantitation of the amounts of virus reaching the spinal ganglia at early times after infection. After infection of the footpad, four mice at each interval were killed at 3, 4, and 5 h, times at which we know that viral DNA can first be detected in the spinal ganglia (24) but which are prior to the onset of DNA replication. Controls included mice killed at time zero. In all cases, ganglia (L4 and L5) were dissected from the mice, snap-frozen in liquid nitrogen, pulverized with a sterile pestle, and resuspended in 200 μl of lysis buffer (100 mM NaCl, 10 mM Tris [pH 8.0], 0.5% sodium dodecyl sulfate [SDS], 0.1 mg

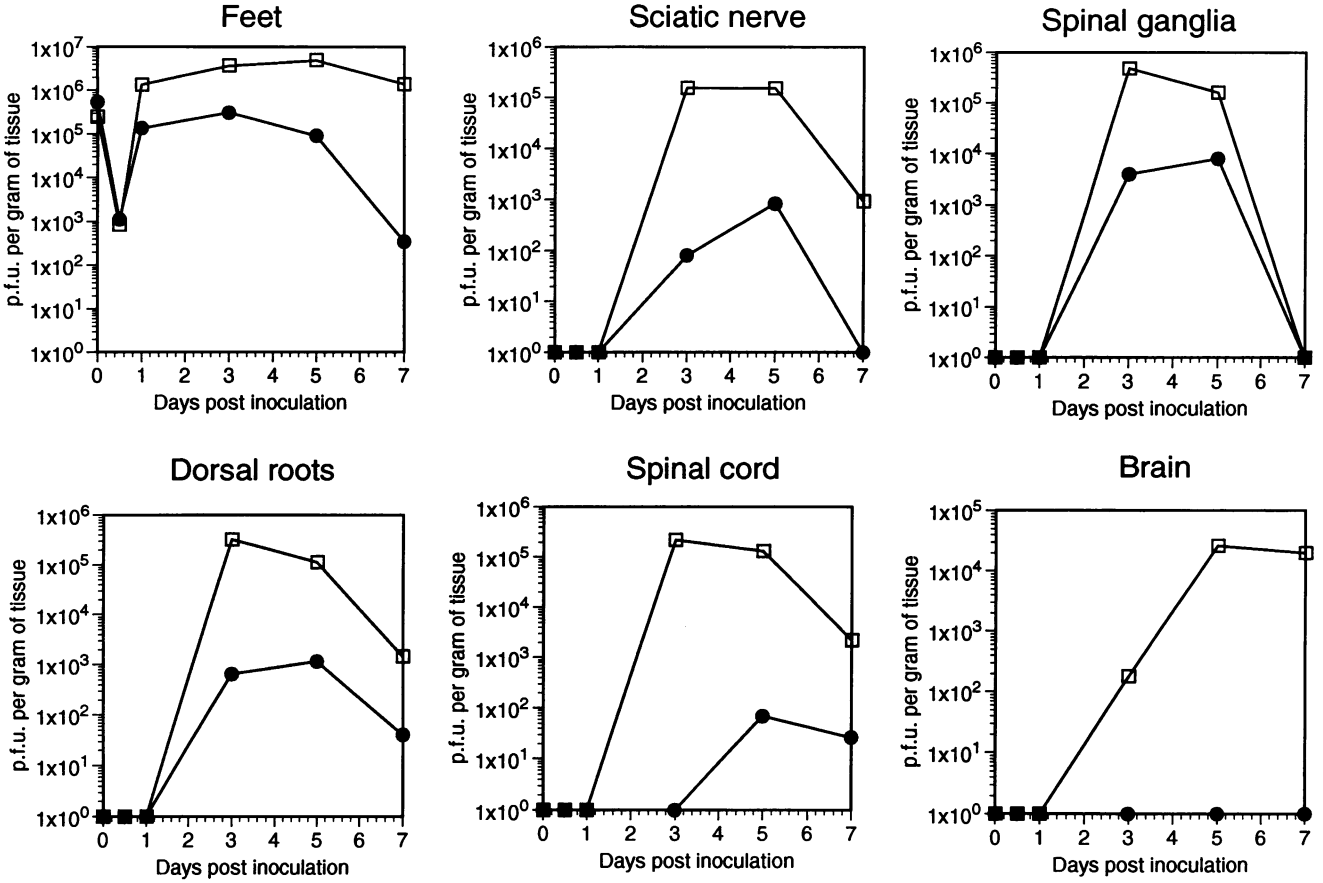


FIG. 1. Yields of 17*syn*$^+$ and R13-1 viruses in various neuronal tissues following footpad inoculation of mice. Six-week-old female Swiss-Webster mice were inoculated on both rear footpads with 10^6 PFU (total) of either 17*syn*$^+$ or R13-1. At the times indicated, groups of four mice were killed, and the feet, sciatic nerves, spinal ganglia, dorsal roots, spinal cords, and brains were dissected and pooled. The tissues were homogenized as 10% (wt/vol) suspensions and titrated for infectious virus. □, 17*syn*$^+$; ●, R13-1.

of proteinase K per ml). Samples were then incubated for 3 h at 55°C, extracted once with phenol-chloroform (1:1) and once with chloroform-isoamyl alcohol (24:1), ethanol precipitated, and resuspended in Tris-EDTA. Oligonucleotide primers specific for sequences within the DNA polymerase gene of HSV were used to amplify a 92-bp fragment by PCR. The reaction mixtures included 0.5 μg of each sample of ganglionic DNA; a 0.5 μM concentration of each primer (primer 1, 5′ CAT CAC

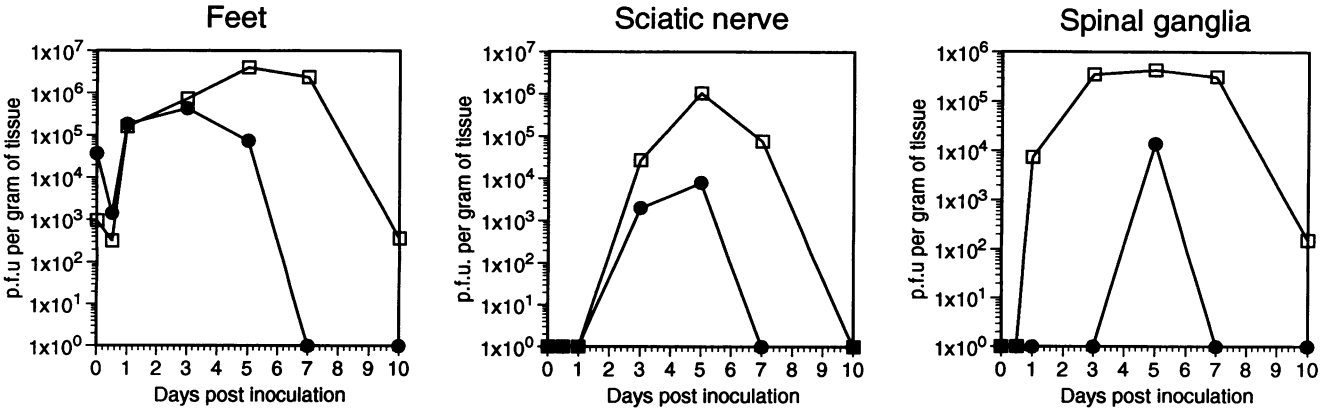
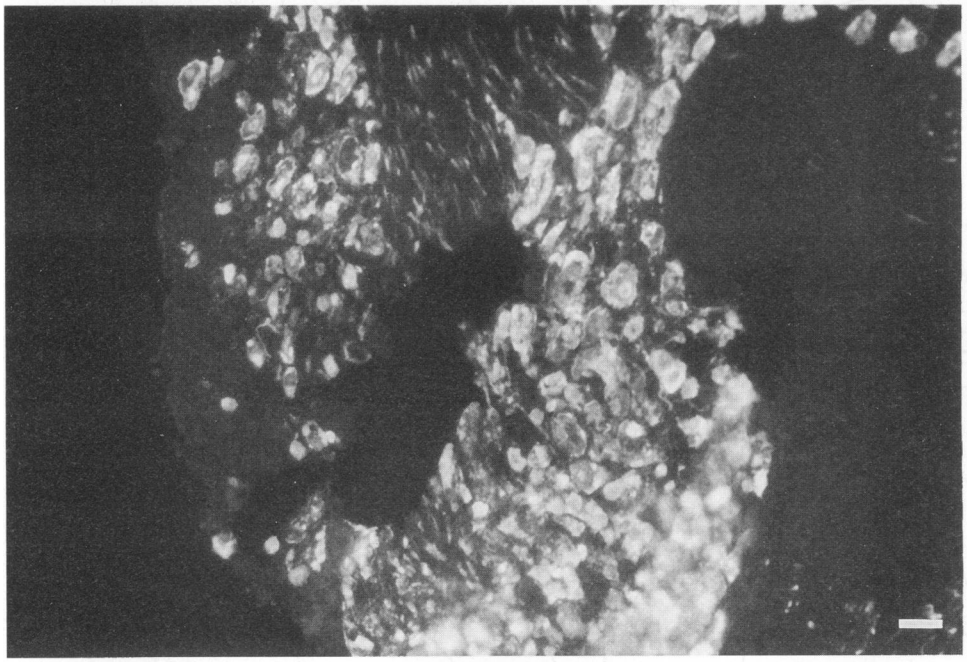


FIG. 2. Yields of 17*syn*$^+$ and R13-1 viruses in various neural tissues following footpad inoculation of mice with differing dosages. The dose of virus used to infect feet was adjusted so that an equivalent amount of each virus was delivered to spinal ganglia. Thus, to compensate for the reduced yield of R13-1 observed in feet (Fig. 1), 10-fold-less 17*syn*$^+$ was used. Viral yields from feet, spinal ganglia, and spinal cord were determined as for Fig. 1. □, 17*syn*$^+$; ●, R13-1.

A.



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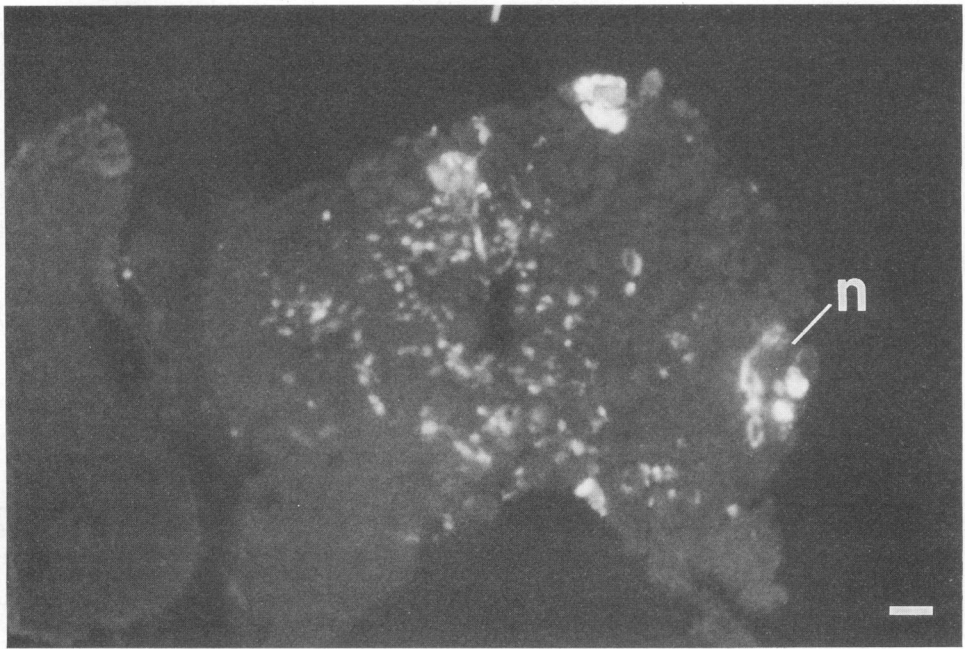


FIG. 3. 17*syn*$^+$ and R13-1 viral antigens in infected murine spinal ganglia. Mice were infected on both rear footpads, and 4 days postinfection spinal ganglia were removed and fixed as described in Materials and Methods. The ganglia were frozen and cryosectioned into 6-μm sections. These sections were incubated with a fluorescein isothiocyanate-conjugated anti-HSV rabbit antiserum and viewed by indirect immunofluorescence. (A) 17*syn*$^+$-infected ganglia; (B) R13-1-infected ganglia. (B) A large neuron (indicated by n) that is not expressing viral antigens is surrounded by supporting cells that are. Bar, 100 μm.

CGA CCC GGA GAG GGA 3′; primer 2, 5′ GGG CCA GGC GCT TGT TGG TGT 3′); 67 mM Tris (pH 8.8); 16.6 mM ammonium sulfate; 6.7 mM $MgCl_2$; 0.17 mg of bovine serum albumin per ml; a 1.25 mM concentration of each of dGTP, dCTP, dATP, and dTTP; 1 μCi of [^{32}P]dATP (~6,000 Ci/mmol); and 2.5 U of *Taq* polymerase (Perkin-Elmer). The thermal cycles used for the PCR of the samples were 94, 68, and 72°C, with the first cycle having a duration of 3 min at each temperature; an additional 30 cycles were for 1 min at each temperature. A standard curve was included to provide a basis for quantitation. Following PCR, the samples were extracted with chloroform-isoamyl alcohol (24:1), ethanol precipitated, and resuspended in loading buffer, and one half of each sample was resolved on a 7.5% polyacrylamide gel. Following electrophoresis for 2.5 h at 150 V, the gel was dried and exposed to film. The extent of hybridization was determined by scanning the blot on a PhosphorImager and then integrating the product bands with ImageQuant software (Molecular Dynamics, Sunnyvale, Calif.).

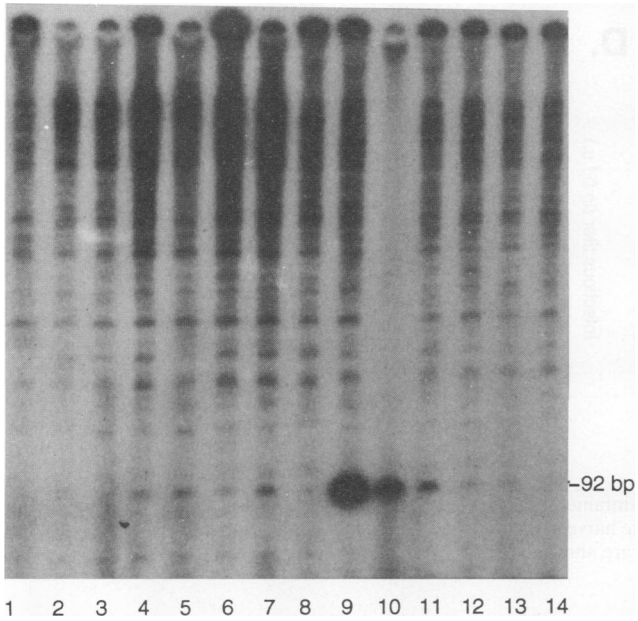


FIG. 4. Quantitative PCR analysis of HSV DNA reaching spinal ganglia 3, 4, and 5 h after footpad infection. Mice were inoculated with 10^7 PFU of either R13-1 or 17*syn*$^+$. At the times indicated below, spinal ganglia were removed (4 mice at each time point), DNA was extracted, and samples were analyzed by PCR as described in Materials and Methods. Primers specific for a 92-bp fragment within the DNA polymerase gene of HSV in conjunction with [^{32}P]dATP were used to identify the HSV DNA in the samples. Lane 1, ganglia taken at time zero; lanes 2 and 3, 17*syn*$^+$ and R13-1 at 3 h postinfection; lanes 4 and 5, 17*syn*$^+$ and R13-1 at 4 h postinfection; lanes 6 and 7, 17*syn*$^+$ and R13-1 at 5 h postinfection. Lanes 8 through 14, standard curve in which 0, 1.6, 0.16, 0.016, 0.0016, 0.00016, and 0.000016 pg, respectively, of plasmid DNA containing the target sequences for the PCR primers were added to samples containing 0.5 μg of DNA from uninfected ganglia. The gel was scanned with a PhosphorImager, and the area under the peaks was integrated with ImageQuant software. The number of genome equivalents present in each set of ganglia was calculated after the sample values were plotted against the standard curve. The samples in the standard curve correspond to 0, 16,000, 1,600, 160, 16, 1.6, and 0.16 viral genomes (lanes 8 to 14, respectively). Genomes in the test samples are calculated to be 0, 687, 718, 750, 1,312, 562, and 1,750 in lanes 1 to 7, respectively.

RNA and DNA analyses. Viral DNA accumulation in cultured cells was determined by infection of cells in 60-mm-diameter dishes, and at various times postinfection the cells were harvested by being scraped into the media. The cell suspension was then centrifuged at 800 × *g*, and the pellet was resuspended in lysis buffer (50 mM Tris [pH 8.0], 1% SDS; 1 mg of proteinase K per ml). After 3 h of incubation at 55°C, the samples were extracted once with phenol and once with phenol-chloroform. Dilutions of the resulting samples were blotted onto a nylon membrane as described above. These dot blots were then hybridized with a ^{32}P probe spanning the *Hin*dIII A fragment of the viral genome, and the relative intensities of the hybridization signal were quantitated with a PhosphorImager (Molecular Dynamics).

RNA was extracted from 60-mm-diameter dishes following lysis of the monolayers with a solution of 4 M guanidinium isothiocyanate; 0.5% sodium *N*-lauryl sarcosine, 25 mM sodium citrate (pH 7.0), 0.1 M 2-mercaptoethanol, and 0.1% antifoam A. The lysed cell suspension was then sheared by trituration with a 22-gauge needle and applied to a CsCl cushion (5.7 M CsCl, 0.1 M EDTA [pH 7.5]), and the RNA was pelleted by centrifugation at 44,000 rpm in a Beckman SW55 rotor for 12 h. The pellets were resuspended in Tris-EDTA and blotted to nylon membranes as described above. Replicate blots were then hybridized with probes specific for the early message, TK (internal *Xba*I-*Hin*dIII fragment); the late message, glycoprotein C (internal *Eco*RI-*Xba*I fragment), or the immediate-early message, ICP4 (internal 3.2-kb *Hin*cII fragment).

RESULTS

R13-1 is restricted in sensory neurons. Previous studies had demonstrated that R13-1 neurovirulence is attenuated 10,000-fold compared with that in wild-type virus (12). In order to address the pathogenesis of R13-1 in a more manipulatable system, we assumed that the virus would also be restricted in sensory neurons and examined infected sensory ganglia. In this system the cell types are easier to distinguish and the virus can be introduced with no trauma to the tissues to be studied. Equivalent amounts of R13-1 and HSV-1 strain 17*syn*$^+$ were inoculated onto the rear footpads of mice, and at various intervals the amounts of virus in relevant tissues were determined. As Fig. 1 indicates, at the initial site of replication there is approximately 10-fold-less R13-1 than 17*syn*$^+$ at 24 h after inoculation. In spinal ganglia, differences in relative yields between the two viruses increase, with the yield of R13-1 being some 100-fold-less than that of the wild type. At the next major site of viral replication, the spinal cord, the restriction is even greater, with no R13-1 being detected until day 5 and the difference in titers being 10,000-fold. Finally, in the case of

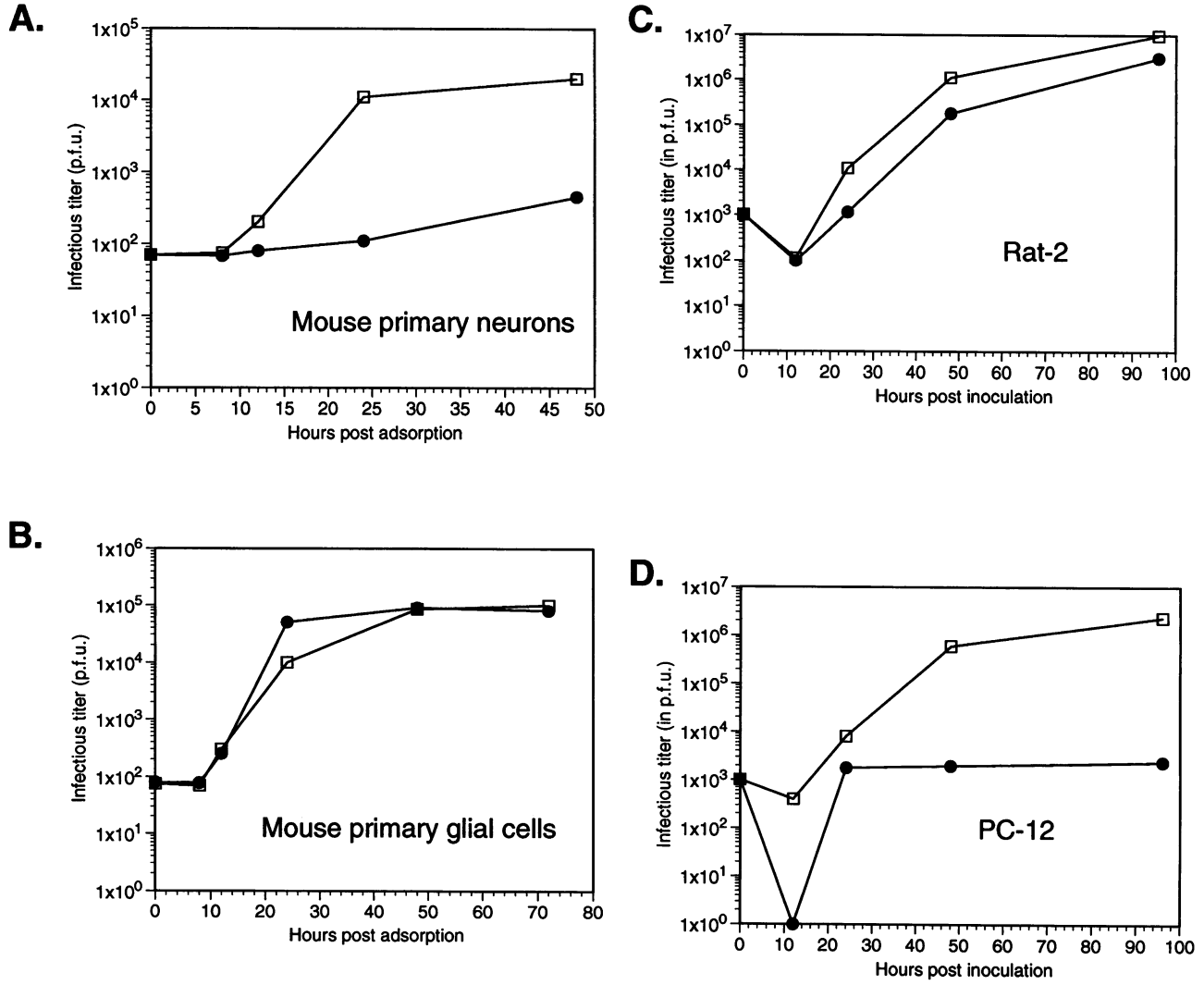


FIG. 5. Replication cycles of 17*syn*$^+$ and R13-1 viruses in neural cells maintained in vitro. Cultures were infected at a multiplicity of infection of 0.1 PFU with 17*syn*$^+$ (□) or R13-1 (●), and at the times indicated cells were harvested and titrated for virus. Replication cycles in mouse primary neuron (A), primary glial cultures (B), Rat-2 cells (C), and PC12 cells (D) are shown.

R13-1, no virus could be detected in the brain, and the amounts of R13-1 found in the sciatic nerve and dorsal roots were also greatly reduced. From these results, it is clear that R13-1 is severely restricted with respect to spread through the nervous system.

In dorsal root ganglia R13-1 antigen is detected predominantly in nonneuronal cells. To determine the basis for the restriction observed in the spinal ganglia, we examined, by immunofluorescence methods, the expression of viral antigens in spinal ganglia. In order to evaluate the antigen production resulting from similar amounts of each virus reaching spinal ganglia from the feet, the dose of R13-1 was adjusted to compensate for the slightly reduced yields observed in the feet (Fig. 1). In this manner, an amount of R13-1 identical to that of 17*syn*$^+$ could be delivered to the spinal ganglia just prior to the time when the antigen production in the ganglia was to be observed (4 days postinfection). With this modification, viral antigen production and distribution within different cell types in the ganglia could be compared. As shown in Fig. 2, the adjusted dose results in a comparable amount of virus being produced in the primary infection of feet for the first 3 days after infection. As is also evident from this figure, the restriction of virus yield in spinal ganglia is dramatic; compared with that in 17*syn*$^+$, over 50-fold-less virus is detectable at day 5 postinfection, while no virus is seen at earlier times. It should be noted that while the adjusted dose of virus compensates for a decrease in virus in the feet until day 3 in these tissues, R13-1 decreases more rapidly than does 17*syn*$^+$ on subsequent days. One probable contributing phenomenon to these kinetics is seeding of the foot at later times by virus produced in the spinal ganglia, with 17*syn*$^+$ supplying more virus to the feet over these intervals. In the immunohistochemical experiments (Fig. 3), a significant number of neurons can be seen to be producing viral antigens in 17*syn*$^+$-infected ganglia. However, in the case of R13-1, only a few neurons could be seen to express viral antigens, and most antigens are found in supporting cells surrounding the neurons. These results suggest that the virus is restricted in replication within dorsal root ganglion (DRG) neurons. Finally, it should be noted that while the virus is seen to be restricted in replication and antigen production in

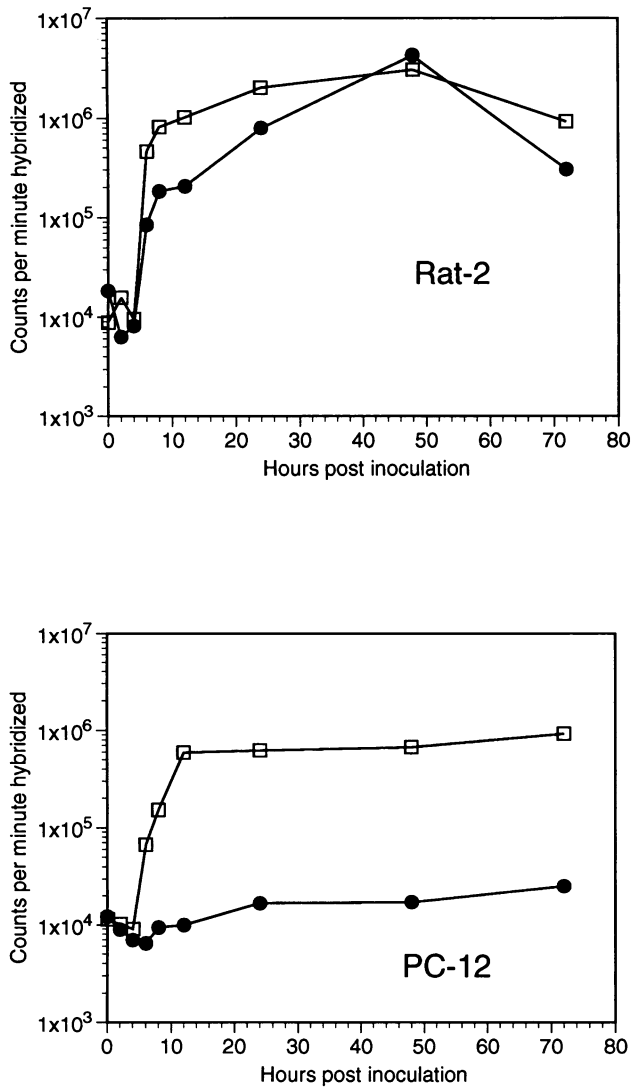


FIG. 6. Accumulation of 17*syn*$^+$ or R13-1 viral DNA in Rat-2 and PC12 cells. Rat-2 or PC12 cells were infected at a multiplicity of infection of 0.1 with either 17*syn*$^+$ (□) or R13-1 (●), and at the indicated times the cells were harvested and DNA dot blots were prepared as described in Materials and Methods. The blots were hybridized with a probe specific for a common region of the viral genome, and the relative intensity of hybridization signal was quantitated with a PhosphorImager.

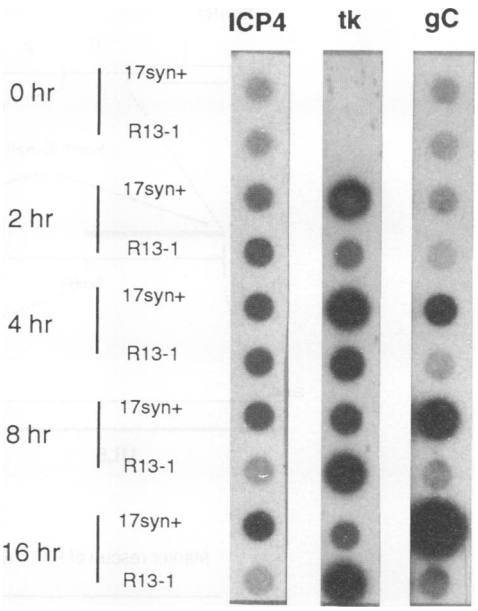
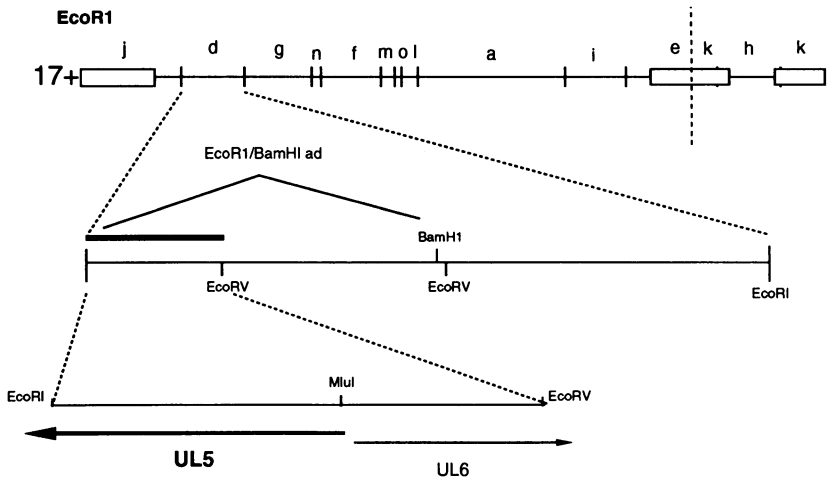


FIG. 7. Comparison of immediate-early, early, and late viral message synthesis in PC12 cells infected with 17*syn*$^+$ or R13-1 viruses. Cells were infected with 17*syn*$^+$ and R13-1 at a multiplicity of infection of 10 PFU and then harvested at intervals postinfection, and RNA was extracted. This RNA was blotted with a dot blot minifold and probed with probes to the ICP4, TK, and gC genes. Representative values determined by PhosphorImager analysis are as follows. ICP4 at 2 h: 17*syn*$^+$, 11,898, and R13-1, 19,641; ICP4 at 4 h: 17*syn*$^+$, 16,960, and R13-1, 19,672; TK at 2 h: 17*syn*$^+$, 45,365, and R13-1, 16,823; TK at 4 h: 17*syn*$^+$, 62,309, and R13-1, 40,463; TK at 8 h: 17*syn*$^+$, 39,519, and R13-1, 59,086; gC at 8 h: 17*syn*$^+$, 102,392, and R13-1, 6,982; and gC at 16 h: 17*syn*$^+$, 866,625, and R13-1, 9,753.

neurons, it establishes latent infection in these cells. In vitro cocultivation experiments in which ganglia from mice latently infected with either 17*syn*$^+$ or R13-1 were induced to reactivate demonstrate that pooled L4 and L5 (four ganglia) spinal ganglia from each of eight mice reactivated. The times in culture until virus-specific cytopathic effects was observed ranged from 5 to 8 days (average, 6 days) for each virus.

R13-1 infects sensory nerve termini and is transported to ganglia as efficiently as 17*syn*$^+$. In order to determine whether there was an initial restriction in the ability of R13-1 to infect nerve termini in the feet and then be transported to the somas in the spinal ganglia, we infected mice with 10^7 PFU of either R13-1 or 17*syn*$^+$. At intervals ganglia were removed, extracted, and subjected to PCR in order to detect viral DNA, as described in Materials and Methods. As shown in Fig. 4, at 3 h after infection, 2,900 counts are detected in ganglia infected with 17*syn*$^+$ and 3,025 counts are detected in those infected with R13-1. The amount of viral DNA increases at 4 and 5 h, respectively, with slightly more DNA being detected in the samples containing R13-1. In other experiments, if the levels were not equivalent, there was always a small increase in the amount of R13-1 DNA detected. The reason for this is not clear, but it could be related to differences in particle/PFU ratios between the two viruses. In any case, we conclude that R13-1 DNA enters somas as efficiently as that from 17*syn*$^+$.

R13-1 is restricted in replication in primary neurons but not glial cells. The previous experiments indicated that virus replication was selectively restricted in neurons. To investigate this phenomenon in more detail, we studied infection of primary neurons and supporting cells in vitro. Viral growth curves comparing 17*syn*$^+$ and R13-1 in the two cell types are shown in Fig. 5A and B. There was a significant difference in the ability of R13-1 to replicate in neuronal cells, with some 1,200-fold-less virus being produced than in the case of 17*syn*$^+$. The replication of R13-1 in glial cells, however, was almost identical to that of 17*syn*$^+$. As was observed with spinal ganglia, in cultured cells R13-1 exhibits a restriction specific to neurons. In an attempt to determine if R13-1 was restricted in any established lines (which would make biochemical analysis easier), a number of established lines, including mouse and human neuroblastomas, were studied. In most cell lines examined (as represented by Rat-2 cells in Fig. 5C), R13-1 replicated normally. In undifferentiated PC12 cells, however, as



Marker rescue of R13-1with cloned subfragments of the 17+ EcoR1 d fragment

EcoR1 d sub-fragments	Exp A	Exp B
---	0/6	0/6
EcoR1 D	4/6	3/6
EcoRV/EcoRV	1/6	0/6
EcoR1/EcoRV (UL5+UL6)	3/6	3/6
EcoR1/MluI (UL5)	2/6	4/6
MluI/EcoRV (UL6)	0/6	0/6

FIG. 8. Marker rescue strategy for generation of virulent R13-1 recombinants. Shown at the top is an *Eco*RI map of the HSV-1 strain 17*syn*$^+$ genome with an expanded view of the *Eco*RI D fragment directly below it. This fragment was previously shown to be sufficient to restore virulence to R13-1 (12). In this study, subfragments of this region were subcloned and assayed after intracranial inoculation for their ability to restore virulence to R13-1 following *co*transfection. The subfragments assayed are shown in the table, and the number of animals killed compared with the number of animals inoculated is presented for two separate experiments.

shown in Fig. 5D, the degree of restriction was comparable to that seen in primary neurons, with only slightly more virus detected at late times than at times immediately following adsorption. Since PC12 cells can be induced to differentiate and stop dividing following nerve growth factor treatment, it seemed possible that differentiated PC12 cells would be even more restrictive to R13-1 replication than the undifferentiated cells; however, the restriction observed in differentiated PC12 cells was no greater than that observed in the undifferentiated PC12 cells (data not shown). It should be noted that in most permissive cell lines examined, R13-1 exhibits slightly reduced yields (two- to fivefold) compared with 17*syn*$^+$, and this may mirror the slightly reduced yields observed earlier for mouse feet (Fig. 1).

A number of replication-deficient mutants, such as those with lesions in the TK gene, demonstrate restricted replication on cells that are not actively dividing, presumably because the virus is limited by the availability of nucleotides. When yields of R13-1 from serum-starved and actively dividing RS cells were compared, they were equivalent (data not shown).

Restriction of R13-1 is prior to or at the level of viral DNA replication. PC12 cells were used to investigate the point in the replication cycle when R13-1 is restricted. For comparison, Rat-2 cells (fibroblasts) were used, since PC12 is a rat-derived line. Viral DNA was isolated from infected cells at several time points and analyzed by dot blot analysis as described in Materials and Methods. As shown in Fig. 6, the amount of viral DNA detected in R13-1-infected PC12 cells is approximately 100-fold less than in Rat-2 cells, indicating that the restriction of R13-1 was at or before the level of DNA replication. The ability to detect input viral DNA by this method demonstrates that the block occurs after the virus enters the cell. In order to determine whether any viral transcripts were made by the R13-1-infected cells, RNA was purified and dot blots were hybridized with probes specific for different classes of viral mRNA. As can be seen in Fig. 7, while R13-1 produces TK message, ICP4, and glycoprotein C (gC) RNA in Rat-2 cells, no gC RNA is detectable in PC12 cells. The fact that the levels of the R13-1 early (TK) message in PC12 cells are similar to those of 17*syn*$^+$ indicates that early events in the infection proceed normally, while the inability to detect gC (late) message in these cells indicates that the block in the infective cycle occurs prior to the initiation of late events. This latter finding is in concordance with the lack of DNA synthesis in PC12 cells.

Marker rescue analysis identifies UL5 as the gene conferring neurovirulence to R13-1. Previously, the region implicated in virulence had been localized to the 9-kb *Eco*RI-*Bam*HI

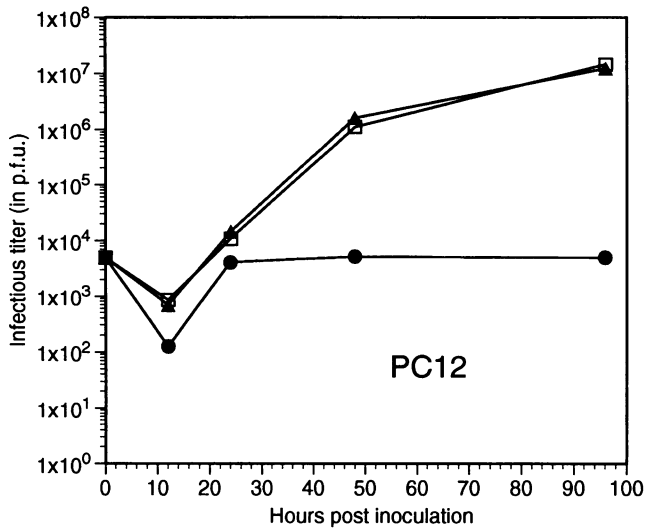


FIG. 9. Replication cycles of the UL5 rescuant, 17*syn*$^+$, and R13-1 viruses in PC12 cells. Cultures were infected at a multiplicity of infection of 0.1 PFU with the rescuant (▲), 17*syn*$^+$ (□), or R13-1 (●), and at the times indicated cells were harvested and virus was titrated.

ad fragment in the left-hand region of the genome (12). This region was subcloned, and four *Eco*RV subclones which spanned the fragment were transfected and assayed for their abilities to restore virulence. The results of these experiments are summarized in Fig. 8 where it can be seen that the left-hand *Eco*RI-*Eco*RV fragment (open reading frames UL5 and UL6) restored the virulent phenotype. The fragment was further subcloned to an *Eco*RI-*Mlu*I fragment (containing only UL5) and a *Mlu*I fragment (containing UL6). These were transfected and assayed, and the results indicated that the UL5 ORF was sufficient to restore virulence. UL5 is a component of the helicase-primase complex of HSV, is essential for DNA replication, and interacts with two other virally encoded components in order to form this complex (4). In the case of R13-1, this gene has been replaced with the homologous type 2 sequence, thus forming a chimeric complex with the other virally encoded subunits of the origin-binding complex. It should be noted that in R13-1, the origin-binding complex would be predicted to be composed of UL5 and UL8 from HSV-2 and UL52 from HSV-1 (12). The predicted amino acid changes between type 1 and type 2 UL5 genes and their relationship to these results are presented in the next section.

Rescued viruses are virulent in mice. Two plaque-purified isolates from separate transfection experiments were plaque purified from the brains of mice that died from encephalitis in the marker rescue experiments. As described in Materials and Methods, these viruses were screened with labeled oligonucleotides specific for the type 1 UL5 gene. To assay for virulence, the viruses were then inoculated intracranially into mice. In this test, the two recombinants demonstrated LD_{50}s of 1×2.5 and 1×10 PFU, while R13-1 killed at 5×10^4 PFU. In addition, in order to ensure that in vivo passage of the transfections did not select for viruses with additional mutations elsewhere in the genome that restored virulence, two additional recombinants were selected directly from separate transfection experiments, again with the oligonucleotide probes specific for HSV-1 UL5. These isolates were shown to have LD_{50}s of 10 and 8 PFU, respectively.

Rescued virus shows normal replication in neurons and PC12 cells. Since the marker rescue experiments demonstrated that the HSV-1 UL5 fragment was sufficient to restore the virulence to R13-1, replication in PC12 cells was studied. As shown in Fig. 9, the replication kinetics in PC12 cells of one rescuant isolated in cell culture are identical to those of wild-type virus. It should also be noted that this virus replicates in a manner similar to that of the wild type in Rat-2 cells, and immunofluorescence analyses of infected ganglia indicate that it and the wild type express similar amounts of viral antigen and that the cellular distribution of these products is identical. That is, unlike R13-1, significant numbers of neurons demonstrate antigens (data not shown).

DISCUSSION

This study has exploited the HSV intertypic recombinant R13-1 to determine the genetic basis of a mutation that confers on this virus a novel phenotype—the ability to replicate to high titers in the CNS without being virulent. Our analysis has shown that the replacement of UL5 from HSV-1 with its homolog in HSV-2 renders the recombinant virus defective for replication in neurons without affecting replication in other cells. Several unique biological properties of this mutant provide insight into certain aspects of virus-neuron interactions, and these are discussed below.

A number of replication-deficient mutants are restricted for replication in nervous tissues, and prototypes of one major class are represented by those with alterations in TK and UTPase genes. These genes have been implicated in the regulation of nucleotide pools, and the mutations presumably interfere with supplemental nucleotide pools needed for HSV replication in cells of the nervous system that are not actively dividing. R13-1 does not fall into this class because the physiologic state of the cell does not relate to restriction of R13-1. R13-1 replicates well in the CNS and in serum-starved cells, while TK$^-$ mutants are restricted under these conditions. In addition, R13-1 is restricted in both primary neurons and in PC12 cells; the latter represents an established cell line which actively divides. From these considerations, we suggest that the mutation in R13-1 defines a replication-minus genotype specific for neurons that is characterized by high-titer replication in other cells of the CNS and attenuation in neurovirulence. This suggests that there are at least two classes of neurovirulence mutations: one in genes which regulate nucleotide pools, and one at or near the level of DNA polymerase activity. In the latter regard, although involved cells have not been identified, HSV-1 mutants with lesions in the polymerase gene have been shown to be avirulent when inoculated into the CNS (7). The relevant implication is that mutants deficient in nucleotide pool metabolism act at a general level by interfering with the replication of the virus in almost all cells of the CNS that are not actively dividing. The class represented by R13-1, and possibly the polymerase mutants, would represent a class that exhibits a more specific restriction within neurons that is linked to a difference in the interaction of the viral replication components with those supplied by the neuron.

Neuron-specific influences on herpesvirus replication have also been suggested by Kosz-Vnenchak et al. (14). In their experiments, immediate-early and early gene expression was stimulated by viral DNA replication in neurons, a phenomenon not observed in other cell types. Although translational effects have not been ruled out, our results are compatible with these findings, since when compared with 17*syn*$^+$, ganglionic neurons infected with R13-1 possessed decreased levels of immediate-early and early proteins.

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                                        ________I________ +                    +
HSV-1   MAAAGGERQLDGQKPGPPHLQQPGDRPAVPGRAEAFLNFTSMHGVQPILKRIRELSQQQLDGAQVPHLQWFR   72
HSV-2   MAAAGGERQLDGQKPGPPHLQQPGDRPAVPGRAEAFLNFTSMHGVQPILKRIRELSQQQLDGAQVPHLQWFR   72

                                  +     + +                +       +   +#    # #
HSV-1   DVAALESPAGLPLREFPFAVYLITGNAGSGKSTCVQTINEVLDCVVTGATRIAAQNMYAKLSGAFLSRPINT  144
HSV-2   DVAALESPAGLPLREFPFAVYLITGNAGSGKSTCVQTLNEVLDCVVTGATRIAAQNMYVKLSGAFLSRPINT  144

                                  +     + +                +       +   +#    # #
HSV-1   IFHEFGFRGNHVQAQLGQYPYTLTSNPASLEDLQRRDLTYYWEVILDLTKRALAASGGEELRNEFRALAALE  216
HSV-2   IFHEFGFRGNHVQAQLGQHPYTLASSPASLEDLQRRDLTYYWEVILDITKRALAAHGGEDARNEFHALTALE  216

        +               ++            +____II_____     #                #     ____
HSV-1   RTLGLGQGALTRLAPATHGALPAFTRSNVIVIDEAGLLGRHLLTAVVYCWWMINALYHTPQYAARLRPVLVC  288
HSV-2   QTLGLGQGALTRLASVTHGALPAFTRSNIIVIDEAGLLGRHLLTTVVYCWWMINALYHTPQYAGRLRPVLVC  288

        ____III_____                                  +   +      ____IV_____
HSV-1   VGSPTQTASLESTFEHQKLRCSVRQSENVLTYLICNRTLREYARLSYSWAIFINNKRCVEHEFGNLMKVLEY  360
HSV-2   VGSPTQTASLESTFEHQKLRCSVRQSENVLTYLICNRTLREYTRLSHSWAIFINNKRCVEHEFGNLMKVLEY  360

                          +
HSV-1   GLPITEEHMQFVDRFVVPENYITNPANLPGWTRLFSSHKEVSAYMAKLHAYLKVTREGEFVVFTLPVLTFVS  432
HSV-2   GLPITEEHMQFVDRFVVPESYITNPANLPGWTRLFSSHKEVSAYMAKLHAYLKVTREGEFVVFTLPVLTFVS  432

                    +  #  +   +                      +                +       +
HSV-1   VKEFDEYRRLTHQPGLTIEKWLTANASRITNYSQSQDQDAGHMRCEVHSKQQLVVARNDVTYVLNSQIAVTA  504
HSV-2   VKEFDEYRRLTQQPTLTMEKWITANASRITNYSQSQDQDAGHVRCEVHSKQQLVVARNDITYVLNSQVAVTA  504

            +    +    #                                + +   +                  +
HSV-1   RLRKLVFGFSGTFRAFEAVLRDDSFVKTQGETSVEFAYRFLSRLIFSGLISFYNFLQRPGLDATQRTLAYAR  576
HSV-2   RLRKMVFGFDGTFRTFEAVLRDDSFVKTQGETSVEFAYRFLSRLMFGGLIHFYNFLQRPGLDATQRTLAYGR  576

        +            ++## ++#####+######      +  +
HSV-1   MGELTAEILSLRPKSSGVPTQASVMADAGAPGERAFDFKQLGPRDGGPDDFPDDDLDVIFAGLDEQQLDVFY  648
HSV-2   LGELTAELLSLRRDAAGASATRAADTSDRSPGERAFNFKHLGPRDGGPDDFPDDDLDVIFAGLDEQQLDVFY  648

           ##+          #  #          #+  +     +  #                +
HSV-1   CHYTPGEPETTAAVHTQFALLKRAFLGRFRILQELFGEAFEVAPFSTYVDNVIFRGCEMLTGSPRGGLMSVA  720
HSV-2   CHYALEEPETTAAVHAQFGLLKRAFLGRYLILRELFGEVFESAPFSTYVDNVIFRGCELLTGSPRGGLMSVA  720

                      #      +          +   +         +
HSV-1   LQTDNYTLMGYTYARVFAFADELRRRHATANVAELLEESPLPYVVLRDQHGFMSVVNTNISEFVESIDSTEL  792
HSV-2   LQTDNYTLMGYTYTRVFAFAEELRRRHATAGVAEFLEESPLPYIVLRDQHGFMSVVNTNISEFVESIDSTEL  792

                              _________V_________          ____VI____      +
HSV-1   AMAINADYGISSKLAMTITRSQGLSLDKVAICFTPGNLRLNSAYVAMSRTTSSEFLRMNLNPLRERHERDDV  864
HSV-2   AMAINADYGISSKLAMTITRSQGLSLDKVAICFTPGNLRLNSAYVAMSRTTSSEFLHMNLNPLRERHERDDV  864


HSV-1   ISEHILSALRDPNVVIVY      882
HSV-2   ISEHILSALRDPNVVIVY      882
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FIG. 10. Comparison of the predicted amino acid sequences of the UL5 genes of HSV-1 and HSV-2. The predicted amino acid sequence of UL5 was translated from the nucleic acid sequence of HSV-1 (18) and HSV-2 (17) and is represented in one-letter code. Conservative amino acid differences are indicated by a +, and nonconservative differences are shown by a #. The six major highly conserved helicase motifs (30) are indicated by a line above the sequence.

Our study indicates that UL5 plays a specific role facilitating viral replication in neurons. We postulate that replacement of UL5 with its HSV-2 counterpart results in the formation of an altered helicase-primase complex. UL5 normally interacts with two other viral proteins, UL8 and UL52, in order to form this complex (4, 29), and in the case of R13-1, the HSV-2 component would form a chimeric complex that is functional for viral DNA replication in most cell types. However, it is possible that the helicase-primase complex is more elaborate and also contains one or more cellular proteins. If this were the case, in neurons the protein could either be absent or possess properties unique to this cell type. The nature of the phenotype observed in R13-1 suggests the existence of a cellular factor that is a component of the origin-binding complex required for replication. As examples, this could be a polymerase or an accessory factor.

As to a potential domain in UL5 which may be involved, alignment of the predicted amino acid sequences of UL5 from HSV-1 (17*syn*$^+$) and HSV-2 (HG52) shows a high degree of conservation (Fig. 10). Of particular interest in this comparison are the six conserved helicase domains that have been reported for the HSV-1 UL5 gene and that were shown to have essential roles in the function of the UL5 protein (30). There is only one predicted amino acid change between HSV-1 and HSV-2 within these motifs, and this is a conservative replacement of a valine for an isoleucine in the first residue of motif II. From this comparison, it seems unlikely that changes in the activity of the critical helicase domains are responsible for the

restriction exhibited by R13-1. Instead, the comparison focuses attention on the amino acid substitutions which lie between the conserved motifs. While the majority of these substitutions are conservative changes, there is a region of divergence between residues 589 and 607 where 17 of the 18 residues are different between the two strains. Of potential significance is the observation that 12 of these 18 residues are nonconservative changes and result in an overall change in the predicted net charge of the region of −1.4 to −4.8. This shift is more suggestive of alpha-helix formation than of the beta-sheet structure predicted from the 17*syn*$^+$ sequence. These changes could result in differences in the interactions of the HSV-2 UL5 component with the other components of the helicase-primase complex either by disrupting the contact sites of protein-protein interactions or by changing the tertiary structure of the protein. We would suggest that studies of the chimeric complex associated with R13-1 might prove useful for defining interactions of components of the primase-helicase complex.

With respect to more general features of herpetic disease, an important component of HSV pathogenesis is illustrated by studies of R13-1. R13-1 does not replicate well in neurons but is unaltered in capacity to replicate in glial cells. Clearly, viral replication in neurons is a requirement for the development of clinically apparent neurologic disease, including encephalitis.

Finally, the ability of this recombinant to replicate in non-neuronal cells suggests its potential application as a vector for the introduction of genes into the CNS. As mentioned earlier, even though this virus does not replicate well in neurons, we know that it establishes latent infections efficiently. This result was predictable, since we and others have demonstrated that viral gene expression and DNA replication are not required to establish latent infection (5, 15, 23, 27). The lack of virulence exhibited by this mutant, coupled with the fact that it replicates to high titers in the CNS, would allow the virus to establish latent infections relatively efficiently. This eliminates a problem associated with many current nonreplicating HSV-based vectors. In those systems, latent infection is limited to the neurons initially infected by input virus.

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