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Altered Pathogenesis in Herpes Simplex Virus Type 1 Infection due to a Syncytial Mutation Mapping to the Carboxy Terminus of Glycoprotein B

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A syncytial (*syn*) variant of herpes simplex virus type 1 strain 17 syn$^+$ was selected by serial passage in heparin, a glycosaminoglycan which potently inhibits herpes simplex virus infectivity. This virus, 17 hep syn, is sixfold more heparin resistant than its parent. By using marker transfer techniques, its *syn* phenotype, but not heparin resistance, was mapped first to the *Bam*HI G fragment (0.343 to 0.415 map units) and then to a 670-bp *Kpn*I-*Pst*I subclone (0.345 to 0.351 map units) encoding the carboxy terminus of glycoprotein B (gB). Three cloned syncytial recombinants were generated from cotransfections of 17 syn$^+$ with either 17 hep syn *Bam*HI-G or the 670-bp subclone. After footpad inoculation of mice, 17 hep syn was as virulent as its parent, despite reaching lower titers in feet, sciatic nerves, dorsal root ganglia, spinal cords, and brains. Animals infected with 17 hep syn or the gB recombinant viruses developed a unique pattern of disease that was strikingly different than that seen with wild-type virus: severe inflammation and edema of the inoculated limb and death without antecedent paralysis. Histopathologic examination revealed limitation of spinal involvement by 17 hep syn to the dorsal aspect of the cord and decreased virus-induced damage in the central nervous system. The genetically unrelated *syn* variant MP, in contrast, was avirulent and did not cause severe local inflammation. After intracerebral inoculation, 17 hep syn was highly virulent and replicated to high titers in the brain. Yet, unlike the parental virus, it resulted in an altered distribution of herpes simplex virus antigens, which were limited to the ependymal and subependymal regions surrounding the lateral ventricles. Despite their syncytial phenotype and pathogenic properties, the recombinant viruses, unlike 17 hep syn, were not heparin resistant. We conclude that a transferable alteration in the 670-bp carboxy-terminal portion of the glycoprotein gB gene of 17 hep syn results in both its syncytial phenotype and the unique pattern of disease that it causes but does not result in heparin resistance. These observations provide direct biological evidence for an important role for herpes simplex virus gB in pathogenic events both at the peripheral site of infection and within the nervous system.

The glycosaminoglycan heparin is a potent inhibitor of herpes simplex virus (HSV) infectivity in vitro. The effect is observed only if heparin is present during the adsorption period, suggesting that it neutralizes extracellular virus (8, 17, 26, 29). HSV type 2 (HSV-2) has been reported to be more heparin resistant than HSV-1 in that its cytopathic effects on cell monolayers progress more rapidly than those of HSV-1 when heparin is present (12, 18). Recently, Wu-Dunn and Spear reported that heparan sulfate, a molecule closely resembling heparin and a ubiquitous component of the mammalian cell surface, is involved in the initial virus-cell interaction, perhaps as part of a viral receptor complex (31).

Because HSV-2 isolates are more neuroinvasive (16, 18) and heparin resistant, we sought to determine whether these properties are related. In experiments designed to isolate heparin-resistant mutants of HSV, we found that passage of an HSV-1 strain 17 syn$^+$ virus stock in heparin resulted in selection for a syncytial (*syn*) mutant capable of forming giant polykaryocytes on infected monolayers. This mutant, named 17 hep syn, is more heparin resistant than either its parent viral stock or HSV-2. In addition, 17 hep syn is virulent and results in a remarkable pattern of disease in mice with marked local inflammation at the inoculation site and death without paralysis. In this paper, we describe the biologic and pathogenic properties of this virus and genetically map both its *syn* mutation and the altered pathogenesis of infection, but not heparin resistance, to the carboxy-terminal portion of glycoprotein B (gB), demonstrating that gB is an important determinant of the pattern of viral disease in the living host.

MATERIALS AND METHODS

Cells and viruses. Rabbit skin (RS) cells were maintained in Eagle minimal essential media (MEM) supplemented with penicillin, streptomycin, amphotericin, and 5% calf serum. Freshly prepared mouse embryo fibroblasts (MEF) and human foreskin fibroblasts were maintained in MEM supplemented with 10% fetal calf serum. HEp-2 cells, African green monkey kidney (Vero) cells, and baby hamster kidney cells were maintained in MEM supplemented with 10% calf serum. Laboratory HSV strains used include 17 syn$^+$ (HSV-1), HG52 (HSV-2) (25), and the syncytial HSV-1 strain MP (9). The origin of 17 hep syn is described in Results.

Viral passages for heparin selection. Virus was passaged in just subconfluent RS cells in 25-cm^2 flasks at a multiplicity of infection of 0.1 with or without added heparin (100 U/ml; Elkins-Sinn, Inc., Cherry Hill, N.J.) or 0.3% human immune serum globulin (Miles Inc., Cutter Biologicals, West Haven,

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Conn.) in medium. When 100% cytopathic effect was observed, 1 ml of this culture was added to 4 ml of medium containing the appropriate additive, and this mixture was used to infect a fresh monolayer. Serial passages were continued in this manner until the desired passage number was reached.

Plaque assays and plaque purification. Viral titers were determined by plaque assay of 10-fold serial dilutions of samples overlaid with medium containing 0.3% human immune serum globulin. Plaque purification of virus was performed three successive times with limiting dilutions of virus inoculated onto RS cell monolayers as for plaque assays.

Heparin resistance assay. A sample containing 10^5 PFU of virus was placed in 1.5 ml of medium with or without heparin (100 U/ml) and incubated for 1 h in a 37°C water bath. Tenfold serial dilutions were made, and titers were determined simultaneously. The results are reported as heparin resistance or the percentage of surviving virus: (PFU in heparinized sample)/(PFU in control unheparinized sample) × 100.

In vitro replication kinetics. MEFs were infected at a multiplicity of infection of 0.0001 PFU per cell at both 31 and 38.5°C. Cells and overlying media from replicate samples were collected daily, and titers were determined.

Acyclovir sensitivity. RS cells were infected at a multiplicity of infection of 0.0002 PFU per cell and overlaid with media with acyclovir sodium (Burroughs Wellcome, Research Triangle Park, N.C.) at 0, 1, 10, and 100 μmol/liter. Results are reported as the concentration of acyclovir necessary to decrease the number of plaques by 50%.

Murine pathogenesis studies. Five-week-old, male Swiss-Webster mice (Simonsen, Gilroy, Calif., and Charles River Labs, Raleigh, N.C.) were inoculated with virus via the hind footpad or intraperitoneal route. In the acyclovir treatment experiments, 1.5 mg of acyclovir sodium per ml was added to the drinking water from days 1 to 10 postinoculation. Four-week-old mice were used for intracerebral inoculations. The PFU/50% lethal dose (LD_{50}) ratios were calculated by the method of Reed and Muench (20). After footpad inoculation with one LD_{50}, as determined for each virus, replication kinetics in vivo were analyzed by sacrificing mice at various time points and removing the feet, sciatic nerves, dorsal root ganglia, spinal cords, and brains. Tissues from two or three animals were pooled at each time point and homogenized, and titers were determined in triplicate on RS cells. After intracerebral inoculation with 10 PFU of each virus, brains were removed from two to three animals at each time point, and titers were determined in triplicate. Differences in mean viral titers at each time point were analyzed by the Student *t* test. In vivo growth curve experiments were repeated once. In addition, tissues were removed for histopathologic studies at various time points after footpad and intracerebral inoculation. Tissue was placed in phosphate-buffered Formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Immunoperoxidase staining of HSV antigens was performed with rabbit immunoglobulin to HSV-1 strain MacIntyre (DAKO Corp., Santa Barbara, Calif.) diluted 1:100 as the primary antibody and a commercial avidin-biotin peroxidase kit (Vectastain ABC reagent kit; Vector Labs, Burlingame, Calif.). Sections were counterstained with hematoxylin.

Molecular studies. Viral DNA was isolated from infected RS cells by using ultracentrifugation in sodium iodide gradients (30) followed by extensive dialysis against 10 mM Tris (pH 8.0)–0.1 mM EDTA.

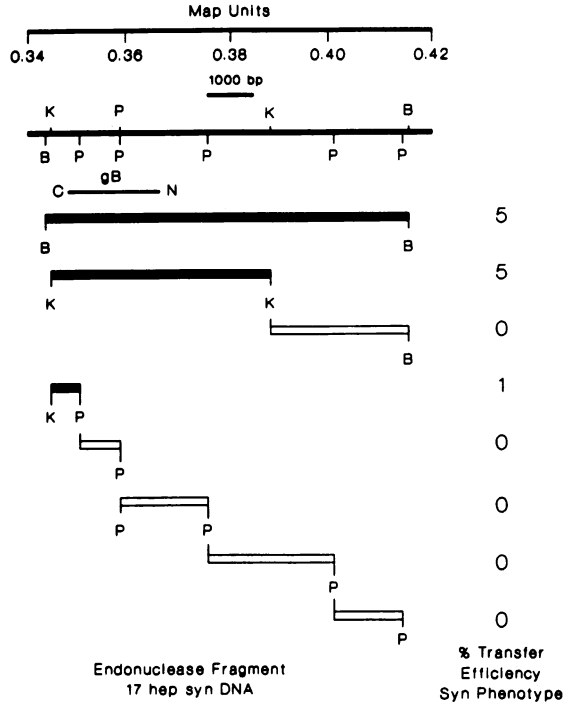

FIG. 1. Schematic illustration of the physical locations of the restriction endonuclease fragments of 17 hep syn DNA used in the studies. Cloned (■) and eluted (□) fragments are shown. The physical location of the gene encoding gB with amino (N) and carboxy (C) termini is also shown. Restriction sites: B, *Bam*HI; K, *Kpn*I; P, *Pst*I. At least 500 plaques were examined in each transfection experiment to determine the transfer efficiency of the *syn* phenotype, which is indicated to the right of each DNA region.

Restriction endonucleases were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.), New England BioLabs Inc. (Beverly, Mass.), or Boehringer Mannheim Biochemicals (Indianapolis, Ind.) and used as specified by the manufacturers. Digested DNA was examined electrophoretically by separation in 0.6 to 0.8% agarose gels (10). Nomenclature of the fragments was as described earlier for the prototype conformation of viral DNA (21).

For transfections, unit-length 17 syn^+ DNA was mixed with a 5- to 20-fold molar excess of either restriction endonuclease-cleaved fragments of genomic 17 hep syn DNA or cloned 17 hep syn DNA fragments, cotransfected in 60-mm tissue culture dishes with calcium phosphate in *N*-2-hydroxethylpiperazine-*N'*-2-ethanesulfonic acid buffer, and subjected to osmotic shock (28). When complete cytopathic effect was evident, progeny virus was harvested and the percentage of *syn* phenotype was determined by examining plaque morphology on fresh RS cells.

The 17 hep syn fragments used for localizing the *syn* phenotype were cloned into pUC18 (14) and used to transform *Escherichia coli* DH5. Plasmids were propogated, DNA was isolated, and the inserts were identified by standard methods (10). The specific 17 hep syn fragments used in this study are detailed in Fig. 1. Gene map units (m.u.) and restriction endonuclease sites were derived from the published sequence of the long unique region of HSV-1 strain 17 syn^+ (13).

RESULTS

Isolation of 17 hep syn. After five serial passages of HSV-1 strain 17 syn$^+$ in the presence of heparin, we noted 5% *syn* plaques. No *syn* plaques were seen after passages of 17 syn$^+$ without heparin or when HSV-2 strain HG52 was passaged in heparin. When the original unpassaged 17 syn$^+$ stock employed was examined, we found 0.5% *syn* plaques. In contrast, no *syn* plaques were observed in other unpassaged 17 syn$^+$ stocks in our laboratory, and heparin passage of these stocks did not yield *syn* variants. To test whether the selection was simply due to neutralization by heparin of extracellular virus and an increased ability of the *syn* variant to spread from cell to cell, we performed passages of the same *syn*-containing 17 syn$^+$ stock in the presence of neutralizing antibody (0.3% human immune serum globulin). In contrast to our findings with heparin, there was no enrichment for the *syn* mutant after five serial passages in the presence of neutralizing antibody.

The heparin-selected *syn* mutant (named 17 hep syn) was plaque purified three times; it had a stable *syn* phenotype through multiple passages in RS cells. It formed syncytia on all cell types tested, including primary human foreskin fibroblasts, HEp-2 cells, primary mouse embryo fibroblasts, Vero cells, and baby hamster kidney cells. Restriction endonuclease digests with *Bgl*II, *Eco*RI, *Hin*dIII, and *Kpn*I revealed no significant differences between 17 syn$^+$ and 17 hep syn (Fig. 2).

Genetic mapping of the syncytial locus of 17 hep syn and production of recombinant viruses. We then performed marker transfer experiments by cotransfections (see Materials and Methods) with unit-length 17 syn$^+$ DNA and various eluted *Bam*HI restriction fragments of 17 hep syn DNA. The highest transfer efficiency (5%) of the *syn* phenotype was seen with the *Bam*HI G fragment (0.343 to 0.415 m.u.) (Fig. 1). A low transfer frequency (1%) was seen with eluted *Bam*HI-F and -H and was felt likely due to contaminating *Bam*HI-G present in the eluted fragments. For this reason, cloned 17 hep syn fragments were employed next. Only the cloned *Bam*HI-G was able to transfer the *syn* phenotype. To more finely map the *syn* locus, cloned *Bam*HI-G of 17 hep syn was digested with *Kpn*I and *Pst*I and subcloned. Only the subcloned 4,700-bp *Kpn*I N fragment (0.345 to 0.388 m.u.) and the subcloned 670-bp *Kpn*I-*Pst*I fragment (0.345 to 0.351 m.u.) transferred the *syn* phenotype (Fig. 1).

For further studies (below), three *syn* recombinant viruses were derived either from the cotransfection of cloned *Bam*HI-G or the cloned 670-bp *Kpn*I-*Pst*I fragment of 17 hep syn with unit-length 17 syn$^+$ DNA. Recombinant viruses were selected based on their syncytial phenotype, plaque purified three times, and designated R1 (from cotransfection with the 17 hep syn *Bam*HI G fragment) and R2 and R3 (from cotransfections with the 670-bp 17 hep syn *Kpn*I-*Pst*I fragment).

Heparin resistance. In the heparin resistance assay, 17 hep syn was sixfold more resistant to heparin than its parent, 17 syn$^+$, and threefold more resistant than HSV-2 strain HG52 (Table 1). To test whether heparin selectability is a property shared by another virus with a *syn* phenotype, we made a virus mixture containing 10^5 PFU of 17 syn$^+$ and 0.5% of the HSV-1 *syn* mutant MP. After five serial passages in heparin as described previously, MP was enriched 50-fold, accounting for 25% of the total virus. In heparin resistance assays, MP was also heparin resistant, with values similar to those of 17 hep syn (Table 1). In contrast, the 17 syn$^+$ × 17 hep syn

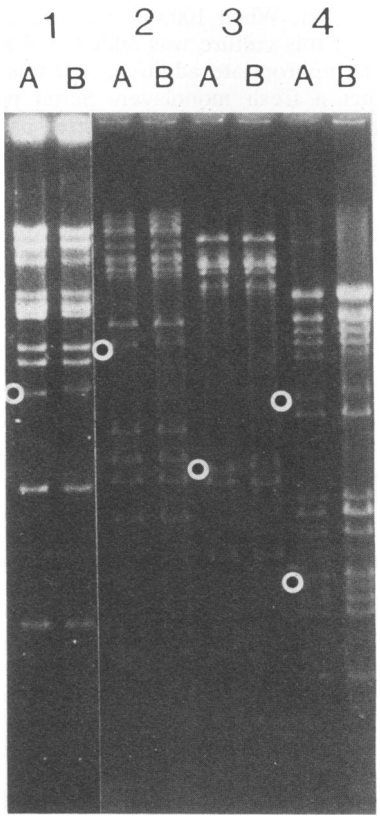

FIG. 2. Restriction endonuclease digests of 17 hep syn (A) and 17 syn$^+$ (B) DNA with *Hin*dIII (lanes 1), *Bgl*II (lanes 2), *Eco*RI (lanes 3), and *Kpn*I (lanes 4). The circles to the left of each digest pair indicate the *Hin*dIII M, *Bgl*II L, *Eco*RI K, and *Kpn*I K and R fragments of the prototype conformation of viral DNA (21). These fragments lie in the terminal repeat portion of the HSV genome, and the increased mobility of the 17 hep syn fragments probably corresponds to a loss of *a* sequences in 17 hep syn. Such minor variations are common among stocks of HSV.

*Bam*HI-G (R1) or *Kpn*I-*Pst*I (R2 and R3) recombinant viruses were not heparin resistant despite their syncytial phenotype.

Pathogenesis of infection and in vivo viral replication. To compare the pathogenic properties of 17 hep syn with its wild-type parent, 17 syn$^+$, we inoculated mice on one hind footpad with either virus. 17 hep syn was as virulent as its parent, with a PFU/LD_{50} ratio of 10^6 to 10^7 (Table 2). In all instances, we recovered virus from the brains of animals that

TABLE 1. Heparin resistance assay results

Virus	% Surviving virus[a] (mean ± SD)
17 syn$^+$	0.1 ± 0
HG52	0.2 ± 0
17 hep syn	0.6 ± 0.13
MP	0.4 ± 0.11
R1	0.07 ± 0.02
R2	0.06 ± 0.04
R3	0.07 ± 0.01

[a] (PFU in heparinized sample)/(PFU in control unheparinized sample) × 100.

TABLE 2. PFU/LD_{50} ratios in mice

Inoculation route and expt	PFU/LD_{50} ratio	
	17 syn$^+$	17 hep syn
Footpad		
1	$10^{6.8}$	$10^{5.8}$
2	$10^{6.6}$	$10^{7.0}$
3	$10^{6.9}$	$10^{6.9}$
Intraperitoneal		
1	$10^{2.0}$	$10^{4.0}$
2	$10^{1.0}$	$10^{2.6}$

died after inoculation with 17 hep syn. We noted, however, that 17 hep syn-inoculated mice developed a strikingly different clinical pattern of disease than did those infected with 17 syn$^+$. The inoculated limb became severely inflamed, edematous, and excoriated (Fig. 3), and the mice died without antecedent paralysis of the hindlimbs. The 17 syn$^+$ × 17 hep syn recombinant viruses R1 through R3 caused a pattern of disease identical to that caused by 17 hep syn after footpad inoculation. In contrast, the genetically unrelated HSV-1 syncytial variant MP did not result in hindlimb edema, inflammation, or death.

We next compared in vivo replication kinetics after footpad inoculation with 10^6 PFU, the LD_{50} for both viruses. For nearly all time points, 17 hep syn attained significantly lower titers than 17 syn$^+$ in all tissues tested (Fig. 4); the differences were reproducible on repeat experiments (data not shown). In stained sections of spinal cord, brain stem (midpons), and cerebral cortex, less virus-induced damage was seen after inoculation with 17 hep syn, and the corresponding immunoperoxidase stained sections showed less viral antigen deposition (Fig. 5). 17 hep syn was unlike its wild-type parent in that its antigen deposition and pathology in the spinal cord were limited to the dorsal aspect and did not spread to the ventral horns and motor neurons (Fig. 5D). We found no evidence of hematogenous dissemination of virus; cultures of hearts, lungs, livers, and kidneys were always negative.

Because of the unique clinical changes noted in the hindlimb after footpad inoculation with 17 hep syn (Fig. 3A), we examined stained sections of the affected area. Unlike the pathology seen with wild-type virus, marked edema and acute inflammation of the dermis and underlying soft tissue were evident by day 5 postinoculation (Fig. 3B), and tissue Gram staining often revealed gram-positive cocci in the areas of inflammation (data not shown). However, all manifestations of disease after footpad inoculation of 17 hep syn were entirely prevented by antiviral treatment of the animals with acyclovir (see Materials and Methods). To test an alternate route of infection that would eliminate the marked superficial hindlimb inflammation and possible superinfection with bacteria, mice were inoculated intraperitoneally. By this route, 17 hep syn remained virulent, but 10- to 100-fold less so than its parent, 17 syn$^+$ (Table 2).

To allow analysis of central nervous system (CNS) disease

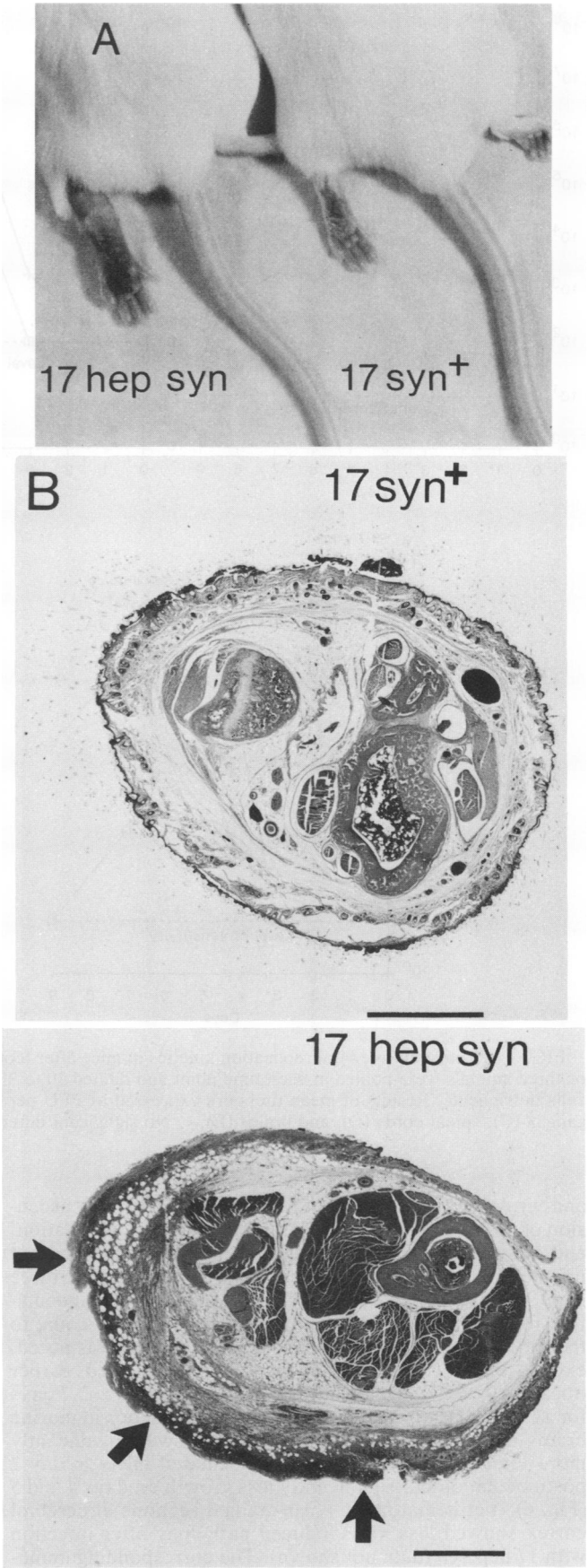

FIG. 3. (A) The mouse hindlimb 5 days after inoculation with 17 syn$^+$ or 17 hep syn. (B) Histopathology of cross-sections through the hindlimb after footpad infection with 17 syn$^+$ or 17 hep syn. Arrows indicate marked skin and soft tissue inflammation and edema seen in 17 hep syn-inoculated limbs. Bars, 1 mm.

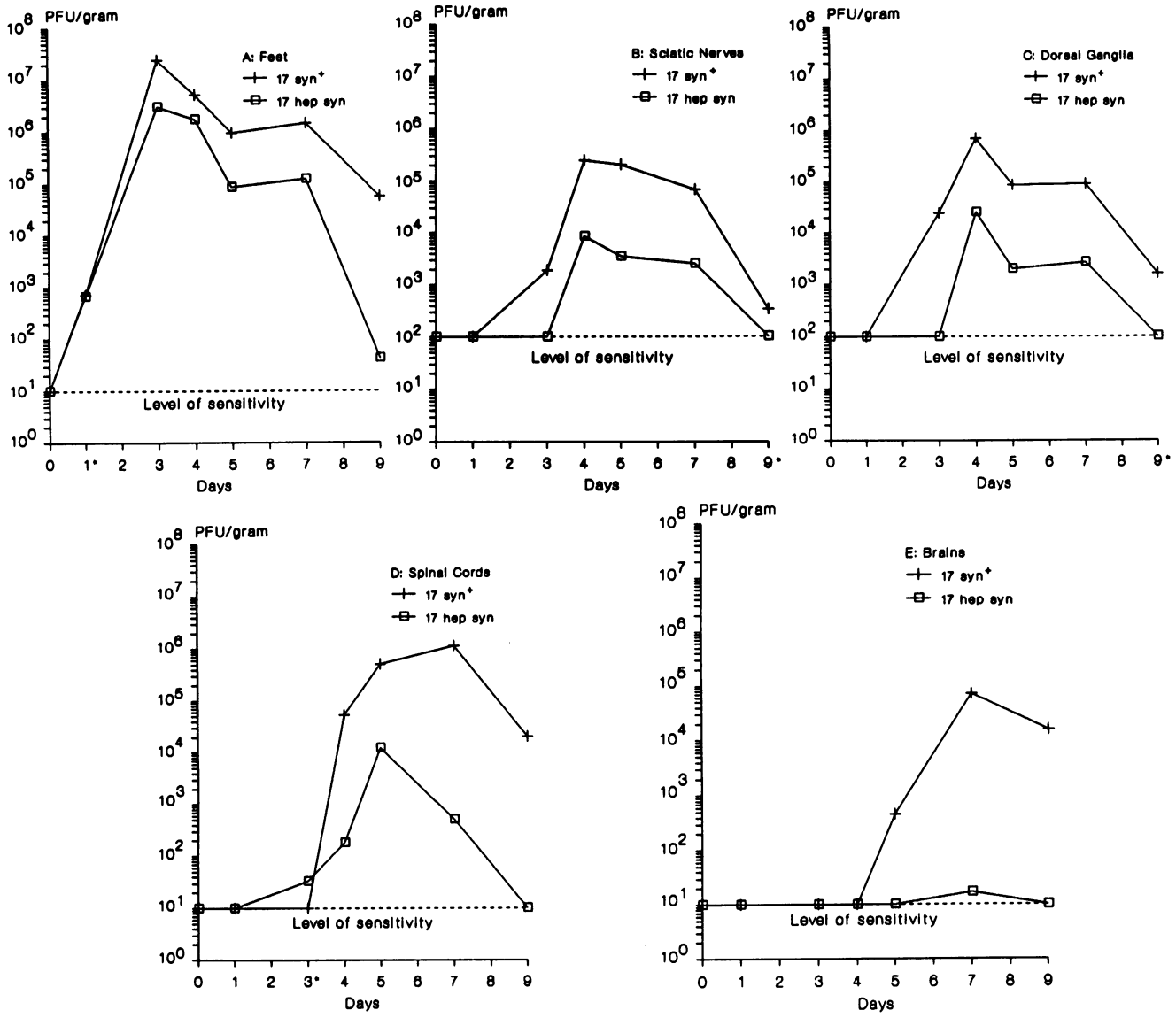

FIG. 4. Representative viral replication kinetics in mice after footpad inoculation of 10^6 PFU of 17 syn$^+$ or 17 hep syn. Tissues from two or three animals were pooled at each time point and diluted 10 to 25% (wt/vol) in tissue culture medium, and titers were determined on RS cells in triplicate. Results of mean titers are expressed as PFU per gram of tissue and shown from feet (A), sciatic nerves (B), dorsal root ganglia (C), spinal cords (D), and brains (E). *, No significant difference in mean viral titers (see Materials and Methods).

and virulence independent of factors that might limit ascension of the virus within the CNS after peripheral inoculation, and independent from the local footpad infection, we next infected mice by direct intracerebral inoculation. In three experiments, 17 hep syn was fully virulent by this inoculation route, with a PFU/LD_{50} ratio of less than 10, equal to that of 17 syn$^+$. No difference in clinical disease was noted, except that 17 hep syn-inoculated animals died earlier (mean, 3.9 ± 1.7 days postinoculation versus 4.7 ± 2.7 days for 17 syn$^+$ [$P = 0.2$]). In vivo replication kinetics in murine brains after inoculation with 10 PFU showed significantly more viral growth in 17 hep syn-inoculated mice on day 2 postinoculation but significantly less growth on days 4 and 5 (Fig. 6). Yet hematoxylin-eosin-stained sections of cerebral cortex showed less virus-induced pathology after infection with 17 hep syn (data not shown). The corresponding immunoperoxidase-stained sections showed a markedly altered distribution of HSV antigens, with 17 hep syn localizing primarily to the ependymal and subependymal regions surrounding the lateral ventricles (Fig. 7).

In vitro replication of 17 hep syn. To determine whether the decreased in vivo replication of 17 hep syn observed in the mouse CNS after footpad inoculation was due to a more general problem in viral replication, we studied the replication of 17 hep syn in vitro in mouse embryo fibroblasts at a multiplicity of infection of 0.0001 PFU per cell. 17 hep syn replicated as well as 17 syn$^+$ at both 31 and 38.5°C (Fig. 8). Thus, 17 hep syn is not temperature sensitive and is not defective for replication in mouse cells. Finally, because some HSV-1 *syn* mutants are thymidine kinase deficient (23), we tested the acyclovir susceptibility of 17 hep syn by using a plaque reduction assay. Both 17 hep syn and its parent

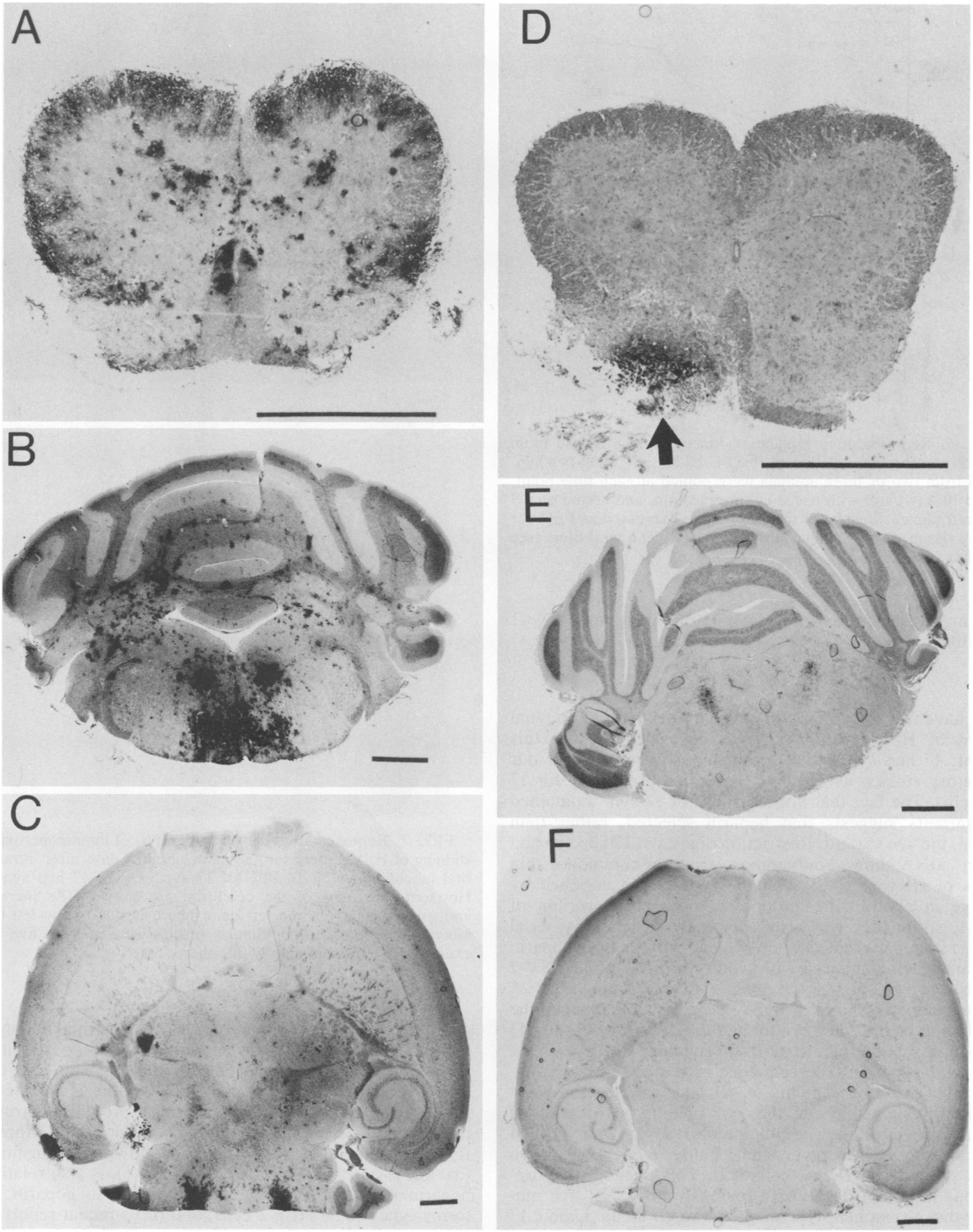

FIG. 5. Representative photomicrographs of immunoperoxidase staining of HSV antigens in the CNS of a mouse 7 days after footpad inoculation of 17 syn$^+$ (A, B, C) or 17 hep syn (D, E, F). Tissues shown are transverse sections of the lumbosacral spinal cord (A, D), transverse sections of the cerebellum and brain stem at the midpontine level (B, E), and horizontal sections of the cerebrum (C, F). Bars, 1 mm. Note restriction of viral antigen to the dorsal spinal cord in 17 hep syn infection (arrow in panel D). Similar results were seen in five mice examined after inoculation with either virus.

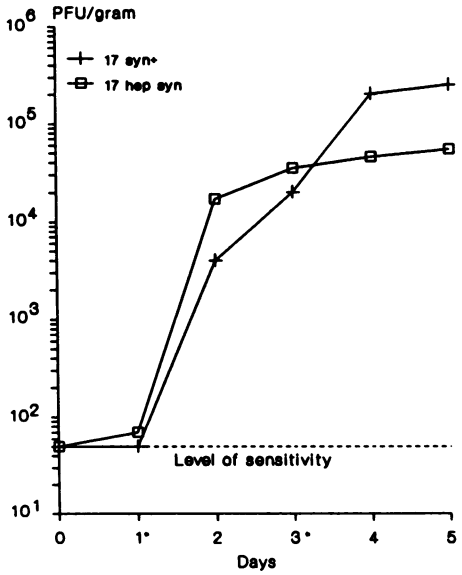

FIG. 6. Representative replication kinetics in the mouse brain after intracerebral inoculation of 10 PFU of 17 syn$^+$ or 17 hep syn. Brains from two or three animals were pooled at each time point, diluted 10% (wt/vol) with tissue culture medium, and titered on RS cells in triplicate. Results of mean titers are expressed as PFU per gram of tissue. *, No significant difference in mean viral titers (see Materials and Methods).

were highly sensitive to acyclovir (50% infective dose, ≤1 μmol/liter).

DISCUSSION

We have found that heparin passage selects for a syncytial variant of HSV-1 strain 17 syn$^+$. We believe that this mutant, 17 hep syn, arose spontaneously from one of our laboratory stocks, as has been described previously for 17 syn$^+$ (15). The fact that not all stocks of 17 syn$^+$ contained *syn* variants suggests that heparin selected, rather than caused, the *syn* variant. Restriction digests of 17 hep syn and 17 syn$^+$ DNA showed only minor variations common within stocks of HSV (Fig. 2). 17 hep syn was not enriched by passage in neutralizing antibody. Another *syn* variant of HSV-1, strain MP, was also selectively enriched by serial passage in heparin, and 17 hep syn and MP are both heparin resistant when compared with wild-type HSV-1 and HSV-2 strains.

Using cloned 17 hep syn DNA, we genetically mapped the *syn* phenotype of 17 hep syn to the *Bam*HI G fragment (0.343 to 0.415 m.u.) and then to a 670-bp subclone (0.345 to 0.351 m.u.) of *Bam*HI-G. The sequence in the 670-bp clone encodes the carboxy terminus of gB and contains the point mutation responsible for the *syn* phenotype of *ts*B5, a temperature-sensitive *syn* mutant of HSV-1 strain HFEM (2–4). We found, however, that, unlike *ts*B5, 17 hep syn forms syncytia on HEp-2 cells (22). This phenotypic property has not been described previously in HSV-1 *syn* mutants whose *syn* phenotype maps to the gB locus. Unlike 17 hep syn and MP, the syncytial recombinants R1 through R3 generated with 17 hep syn *Bam*HI-G or its 670-bp *Kpn*I-*Pst*I subclone were not heparin resistant, nor did they form syncytia on HEp-2 cells.

We thus believe that the properties of heparin resistance and syncytium formation on HEp-2 cells are controlled by another locus that is altered in 17 hep syn but not in the cloned recombinant viruses R1 through R3. In particular, it is possible that these properties are due to the absence of or alterations in glycoprotein gC. This hypothesis is based on the following evidence: (i) preliminary protein electrophoretic data indicating that 17 hep syn, but not the R1 through R3 recombinants, lacks gC (data not shown); (ii) our finding that HSV-1 strain MP, which lacks gC (6), is also relatively heparin resistant, is selected by passage in heparin, and forms syncytia on HEp-2 cells; and (iii) a recent report that glycoprotein gIII of pseudorabies virus, the homolog of HSV gC, binds to heparin (32).

Most important, we observed unique pathogenic properties that differ markedly from those of the parental virus after inoculation of mice with either 17 hep syn or the gB recombinant virus R1, R2, or R3. Footpad inoculation re-

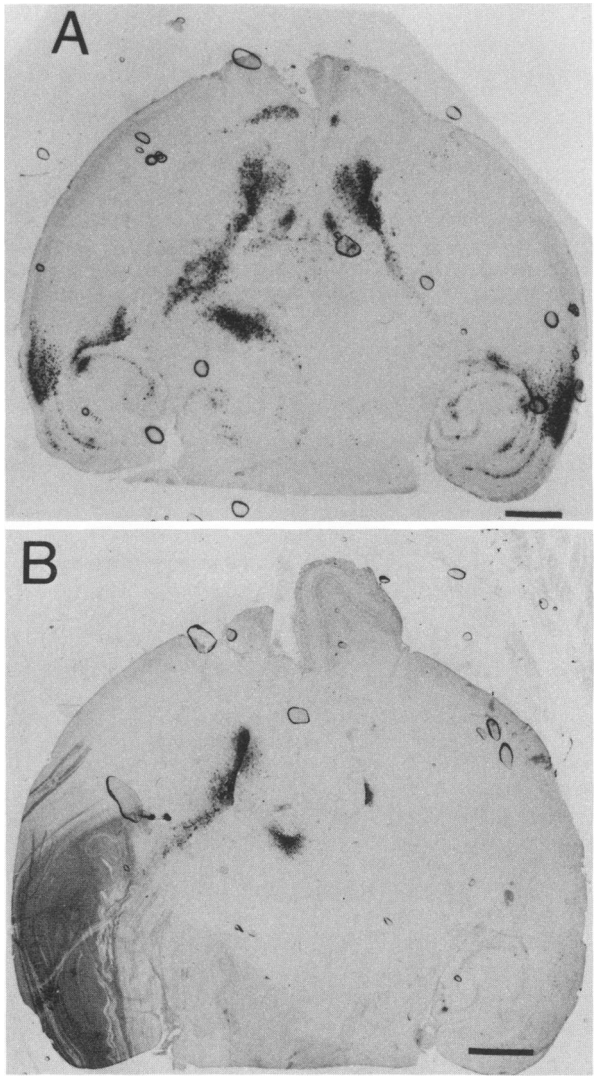

FIG. 7. Representative photomicrographs of immunoperoxidase staining of HSV antigens in the mouse brain 4 days after intracerebral inoculation with 10 PFU of 17 syn$^+$ (A) or 17 hep syn (B). Horizontal sections of the cerebrum are shown. The increased antigen deposition in the left hemisphere is due to the left-sided inoculation. Bars, 1 mm. Similar results were seen in five mice examined after inoculation with either virus.

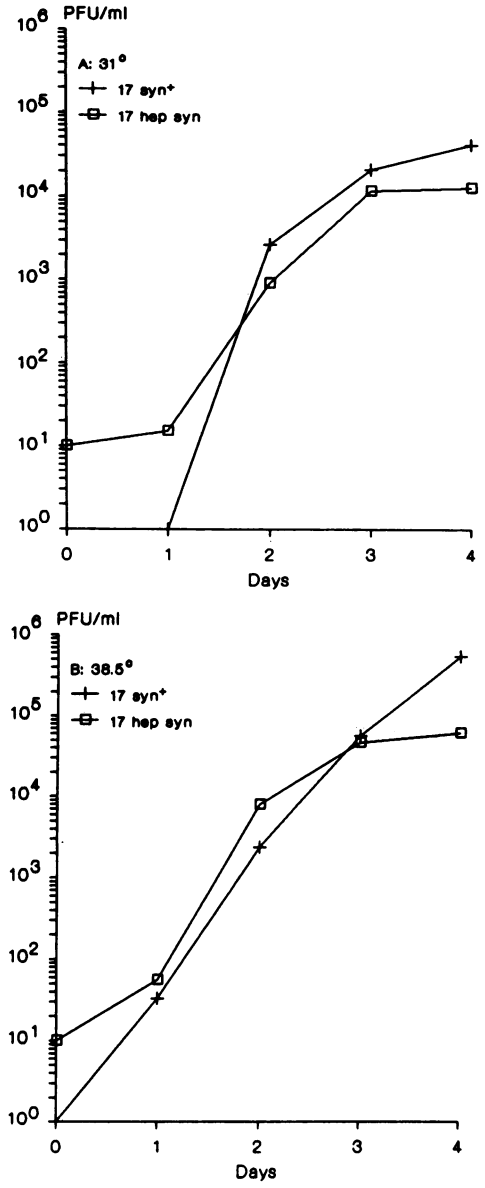

FIG. 8. In vitro replication kinetics of 17 syn$^+$ and 17 hep syn at 31°C (A) or 38.5°C (B) in mouse embryo fibroblasts after inoculation of 0.0001 PFU/cell.

sults in severe inflammation of the hindlimb but no paralysis before death. Spinal cord pathology is specifically limited to the dorsal cord ipsilateral to the inoculation site. Despite lower CNS titers of virus, 17 hep syn was as virulent as its parent, 17 syn$^+$. Although bacterial superinfection of the inflamed hindlimb may play a role in the death of 17 hep syn-inoculated mice, we always found virus in the brain at death. Furthermore, antiviral treatment with acyclovir completely prevents disease, whereas antibiotic treatment does not (data not shown). Also, after intraperitoneal inoculation, 17 hep syn is virulent, albeit less so than 17 syn$^+$. After direct intracerebral inoculation, 17 hep syn is highly neurovirulent and results in the rapid death of infected animals, yet a marked alteration in neuropathologic findings is again noted. In 17 hep syn infection, in contrast to wild-type virus infection, antigen deposition is limited to the ependymal and subependymal regions surrounding the lateral ventricles. We hypothesize that the severe local inflammation provoked by 17 hep syn may allow invasion of the CNS despite its lesser replicative capabilities in vivo and that, once the CNS is invaded, its marked neurovirulence then results in lethal infection.

The lack of paralysis after footpad inoculation with 17 hep syn was also observed by Dix et al. with other HSV-1 *syn* variants (5). Therefore, this may be a general property related to the *syn* phenotype and could be explained at a neuropathological level by our immunohistological finding showing limitation of virus to the dorsal spinal cord. Unlike the *syn* strains that Dix et al. studied, however, 17 hep syn does kill mice. The marked hindlimb inflammation we noted has not, to our knowledge, been previously described. Hind footpad inoculation with the recombinant viruses R1 through R3 also results in marked footpad inflammation and a pattern of disease identical to that seen with 17 hep syn. This is not, however, a property of all HSV-1 *syn* variants, strain MP, whose *syn* locus maps elsewhere in the genome (19), does not produce this effect. In addition, our laboratory has found that transfer to 17 syn$^+$ of the HSV-1 ANG syncytial mutation, which we have also mapped to within gB, also results in marked footpad inflammation after infection with the recombinant viruses (unpublished data). We therefore conclude that this type of hindlimb inflammation is due to a transferable alteration in gB, and we hypothesize that the altered gB could evoke tissue damage either directly or by enhancing the host inflammatory response.

We found 17 hep syn to be fully virulent after intracerebral inoculation, which is consistent with data concerning other *syn* mutants (5). Given that viral replication kinetics of 17 hep syn do not differ significantly from those of the wild-type virus in the brain, we found the decrease both in the deposition of HSV antigens and in brain pathology surprising. The neurovirulence observed could be related to the altered localization of infection noted or due to alterations in brain cell and/or organ function, not detectable histologically, perhaps at the level of the cell membrane. Interestingly, Thompson and Stevens (27) reported a similar distribution of HSV antigens after intracerebral inoculation with the intertypic recombinant HSV strain RE6. However, RE6 is unable to replicate in the mouse brain and is totally avirulent (27), whereas 17 hep syn, despite similar CNS localization, replicates well and is highly neurovirulent.

In conclusion, from studies of 17 hep syn and the recombinant viruses R1 through R3 we have determined that the genetic information both for their syncytial phenotype and their unique pathogenic properties is contained within the *Bam*HI G fragment, specifically the 670-bp *Kpn*I-*Pst*I fragment encoding the carboxy terminus of gB. The glycoprotein gB has previously been shown to be essential for viral infectivity (24) and to play a critical role in the penetration and fusion of cells in vitro (11). In addition, gB is an important immunogen in the living host (1, 7). Our studies now provide direct support for an important role for gB in vivo as a determinant of the pathogenesis of HSV infection not only early in infection in peripheral tissues but also within the nervous system itself.

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REFERENCES

1. **Blacklaws, B. A., A. A. Nash, and G. Darby.** 1987. Specificity of the immune response of mice to herpes simplex virus glycoproteins B and D constitutively expressed on L cell lines. J. Gen. Virol. **68**:1103–1114.
2. **Bzik, D. J., B. A. Fox, N. A. DeLuca, and S. Person.** 1984. Nucleotide sequence specifying the glycoprotein gene, gB, of herpes simplex virus type 1. Virology **133**:301–314.
3. **Bzik, D. J., B. A. Fox, N. A. DeLuca, and S. Person.** 1984. Nucleotide sequence of a region of the herpes simplex virus typel gB glycoprotein gene: mutations affecting rate of virus entry and cell fusion. Virology **137**:185–190.
4. **DeLuca, N., D. J. Bzik, V. C. Bond, S. Person, and W. Snipes.** 1982. Nucleotide sequences of herpes simplex virus type 1 (HSV-1) affecting virus entry, cell fusion and production of glycoprotein gB (VP7). Virology **122**:411–423.
5. **Dix, R. D., R. R. McKendall, and J. R. Baringer.** 1983. Comparative neurovirulence of herpes simplex virus type 1 strains after peripheral or intracerebral inoculation of BALB/c mice. Infect. Immun. **40**:103–112.
6. **Draper, K. G., R. H. Costa, G. T.-Y. Lee, P. G. Spear, and E. K. Wagner.** 1984. Molecular basis of the glycoprotein-C-negative phenotype of herpes simplex virus type 1 macroplaque strain. J. Virol. **51**:578–585.
7. **Eberle, R., and R. J. Courtney.** 1980. Preparation and characterization of specific antisera to individual glycoprotein antigens comprising the major glycoprotein region of herpes simplex virus type 1. J. Virol. **35**:902–917.
8. **Hochberg, E., and Y. Becker.** 1968. Adsorption, penetration and uncoating of herpes simplex virus. J. Gen. Virol. **2**:231–241.
9. **Hoggan, D. M., and B. Roizman.** 1959. The isolation and properties of a variant of herpes simplex producing multinucleated giant cells in monolayer cultures in the presence of antibody. Am. J. Hyg. **70**:208–219.
10. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
11. **Manservigi, R., P. G. Spear, and A. Buchon.** 1977. Cell fusion induced by herpes simplex virus is promoted and suppressed by different viral glycoproteins. Proc. Natl. Acad. Sci. USA **74**: 3913–3917.
12. **Marks-Hellman, S., and M. Ho.** 1976. Use of biological characteristics to type herpesvirus hominis types 1 and 2 in diagnostic laboratories. J. Clin. Microbiol. **3**:277–280.
13. **McGeoch, D. J., M. A. Dalrymple, A. J. Davison, A. Dolan, M. C. Frame, D. McNab, L. J. Perry, and J. E. Scott.** 1988. The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. J. Gen. Virol. **69**:1531–1574.
14. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. **101**:20–78.
15. **Moira Brown, S., D. A. Ritchie, and J. H. Subak-Sharpe.** 1973. Genetic studies with herpes simplex type 1. The isolation of temperature-sensitive mutants, their arrangement into complementation groups and recombination analysis leading to a linkage map. J. Gen. Virol. **18**:329–346.
16. **Nahmias, A. J., W. R. Dowdle, J. H. Kramer, C. F. Luce, and S. C. Mansour.** 1969. Antibodies to herpesvirus homonis types 1 and 2 in the rabbit. J. Immunol. **102**:956–962.
17. **Nahmias, A. J., and S. Kibrick.** 1964. Inhibitory effect of heparin on herpes simplex virus. J. Bacteriol. **87**:1060–1066.
18. **Plummer, G., J. L. Waner, and C. P. Bowling.** 1968. Comparative studies of type 1 and type 2 "herpes simplex" viruses. Br. J. Exp. Pathol. **49**:202–208.
19. **Pogue-Guile, K. L., and P. G. Spear.** 1987. The single base pair substitution responsible for the syn phenotype of herpes simplex virus type 1, strain MP. Virology **157**:67–74.
20. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating 50% endpoints. Am. J. Hyg. **27**:493–497.
21. **Roizman, B.** 1979. The structure and isomerization of herpes simplex virus genomes. Cell **16**:481–494.
22. **Ruyechan, W. T., L. S. Morse, D. M. Knipe, and B. Roizman.** 1979. Molecular genetics of herpes simplex virus. II. Mapping of the major viral glycoproteins and of the genetic loci specifying the social behavior of infected cells. J. Virol. **29**:677–697.
23. **Sanders, P. G., N. M. Wilkie, and A. J. Davison.** 1982. Thymidine kinase deletion mutants of herpes simplex virus type 1. J. Gen. Virol. **63**:277–295.
24. **Sarmiento, M., M. Haffey, and P. G. Spear.** 1979. Membrane proteins specified by herpes simplex viruses. III. Role of glycoprotein VP7 (B_2) in virion infectivity. J. Virol. **29**:1149–1158.
25. **Subak-Sharpe, J. H., S. M. Brown, D. A. Ritchie, M. C. Timbury, J. C. M. Macnab, H. S. Marsden, and J. Hay.** 1974. Genetic and biochemical studies with herpesvirus. Cold Spring Harbor Symp. Quant. Biol. **39**:717–730.
26. **Takemoto, K. K., and P. Fabisch.** 1964. Inhibition of herpes virus by natural and synthetic acid polysaccharides. Proc. Soc. Exp. Biol. Med. **116**:140–144.
27. **Thompson, R. L., and J. G. Stevens.** 1983. Biological characterization of a herpes simplex virus intertypic recombinant which is completely and specifically nonneurovirulent. Virology **131**: 171–179.
28. **Thompson, R. L., E. K. Wagner, and J. G. Stevens.** 1983. Physical location of a herpes simplex virus type-1 gene function(s) specifically associated with a 10 million-fold increase in HSV neurovirulence. Virology **131**:180–192.
29. **Vaheri, A., and K. Cantell.** 1963. The effect of heparin on herpes simplex virus. Virology **21**:661–662.
30. **Walboomers, J. M. M., and J. T. Schegget.** 1976. A new method for the isolation of herpes simplex virus type 2 DNA. Virology **74**:256–258.
31. **WuDunn, D., and P. G. Spear.** 1989. Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. J. Virol. **63**:52–58.
32. **Zuckerman, F., L. Zsak, L. Reilly, N. Sugg, and T. Ben-Porat.** 1989. Early interactions of pseudorabies virus with host cells: functions of glycoprotein gIII. J. Virol. **63**:3323–3329.