JOURNAL OF VIROLOGY, Aug. 1997, p. 5820–5827 Vol. 71, No. 8
0022-538X/97/$04.00+0

The gE and gI Homologs from Two Alphaherpesviruses Have Conserved and Divergent Neuroinvasive Properties

A. C. KNAPP, P. J. HUSAK, AND L. W. ENQUIST*

Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544

Received 13 February 1997/Accepted 25 April 1997

The membrane glycoproteins gE and gI are encoded by pseudorabies virus (PRV), a neurotropic, broad-host-range alphaherpesvirus of swine. PRV gE and gI are required for anterograde spread to a restricted set of retinorecipient neurons in the brain after infection of the rat retina. A related alphaherpesvirus, encoding gE and gI homologs, is called bovine herpesvirus 1.1 (BHV-1.1). BHV-1.1 is a respiratory pathogen of highly restricted host range and, in contrast to PRV, is unable to propagate in or cause disease in rodents. We have shown previously that the BHV-1.1 gE and gI proteins are capable of complementing the virulence functions of PRV gE and gI in a rodent model (A. C. Knapp and L. W. Enquist, J. Virol. 71:2731–2739, 1997). We examined the ability of the BHV-1.1 gE and gI homologs to direct circuit-specific invasion of the rat central nervous system by PRV. Both complete open reading frames were cloned into a PRV mutant lacking the PRV gE and gI genes. Recombinant viruses were analyzed for the ability to invade the rat brain after infection of the retina. Surprisingly, in a portion of the animals tested, the BHV-1.1 gE and gI proteins functioned autonomously to promote spread of PRV to a subset of retinorecipient regions of the brain. First, the presence of BHV-1.1 gI alone, but not PRV gI alone, promoted viral invasion of the optic tectum. Second, expression of BHV-1.1 gE alone facilitated PRV infection of a subset of neurons in the hippocampus not normally infected by PRV. When both BHV-1.1 proteins were expressed in a coinfection, all retinorecipient regions of the rat brain were infected. Therefore, depending on the viral source, homologs of gE and gI differentially affect spread between synaptically connected neurons in the rat.

The hallmark of the alphaherpesvirus family, which include pseudorabies virus (PRV) and bovine herpesvirus 1 (BHV-1), is their ability to parasitize the nervous system. Invasion of neuronal tissue is usually benign, typically resulting in the establishment of a latent infection in sensory ganglia of the peripheral nervous system (19). Reactivation results in the dissemination of the virus from the ganglion to the periphery of the host, presumably facilitating virus spread and maintenance in the host population. Under certain circumstances (e.g., infection of the very young or a different host), viral infection can lead to a central nervous system (CNS) infection, resulting in a devastating, often fatal encephalitis. In addition, if the host has reduced immunocompetence, alphaherpesviruses can exhibit uncontrolled dissemination to the periphery with severe medical complications (25). The molecular mechanisms that control virus spread are poorly understood. Two viral membrane glycoproteins, gE and gI, are widely conserved among alphaherpesviruses and are known to affect cell-cell spread in tissue culture and in animal model systems (1, 5, 15–17). gI and gE form a complex, which is considered to be the unit of function for anterograde spread in some, but not all, neurons (7). Viruses that express gE and gI exhibit increased virulence, but the relationship between virulence (ability to cause disease) and cell-cell spread is not clear. gI and gE may have separate functions in virulence because a gI null mutant is more virulent than a gE null mutant (10).

For herpes simplex virus and varicella-zoster virus, gE and gI may also defeat antibody-mediated immune defenses at the mucosal surfaces by acting as Fc receptors, but the role of this mechanism in primary infection is unclear (6, 27). The Fc receptor activities of herpes simplex virus and varicella-zoster virus gE and gI may be more relevant in controlling the reactivated virus at the mucosal surface in immunized hosts. The gE and gI homologs of other alphaherpesviruses have not been reported to have such Fc receptor activity (24).

While maintenance of gI and gE within the phylogenetically divergent alphaherpesvirus family corroborates their importance in the life cycle of the alphaherpesviruses, their functions have been difficult to ascertain because gE/gI mutants have few, tractable phenotypes in tissue culture of only certain cell lines (10). Some biochemical properties of gI and gE, including their ability to form a complex or to facilitate cell-cell spread in certain tissue culture cell lines, are consistent among family members, while others, including the ability to bind the Fc region of immunoglobulins, appear not to be a common feature of the gI and gE homologs (6, 7, 23, 24).

The swine pathogen PRV frequently causes a lethal encephalitis in piglets, which strictly depends on gI and gE (7). By contrast, one substrain of the close relative BHV-1, BHV-1.1, never invades the brain even though it encodes gE and gI homologs (22). Nevertheless, BHV-1.1 mutants lacking gE are much less virulent (11, 21). As both viruses establish latency in the trigeminal ganglia of their hosts, we sought to determine if the gI and gE homologs from this non-brain-invasive alphaherpesvirus could complement PRV gE/gI null mutants for the ability to invade the brain. We have previously cloned the BHV-1.1 gE and gI genes into a PRV mutant deleted for its own gE and gI genes (12). We have demonstrated that these recombinant viruses produce high levels of the BHV-1.1 proteins and complement the virulence defect of the PRV gE/gI-minus virus vector in the rat model (12).

The Enquist and Card laboratories have demonstrated that the PRV gI and gE genes are required for intraneuronal, anterograde transport within retinal ganglion cells that project to a functionally distinct subset of visual centers (the optic

* Corresponding author. Mailing address: Department of Molecular Biology, Princeton University, Princeton, NJ 08544. Phone: (609) 258-2415. Fax: (609) 258-1035. E-mail: Lenquist@molbiol.princeton.edu.

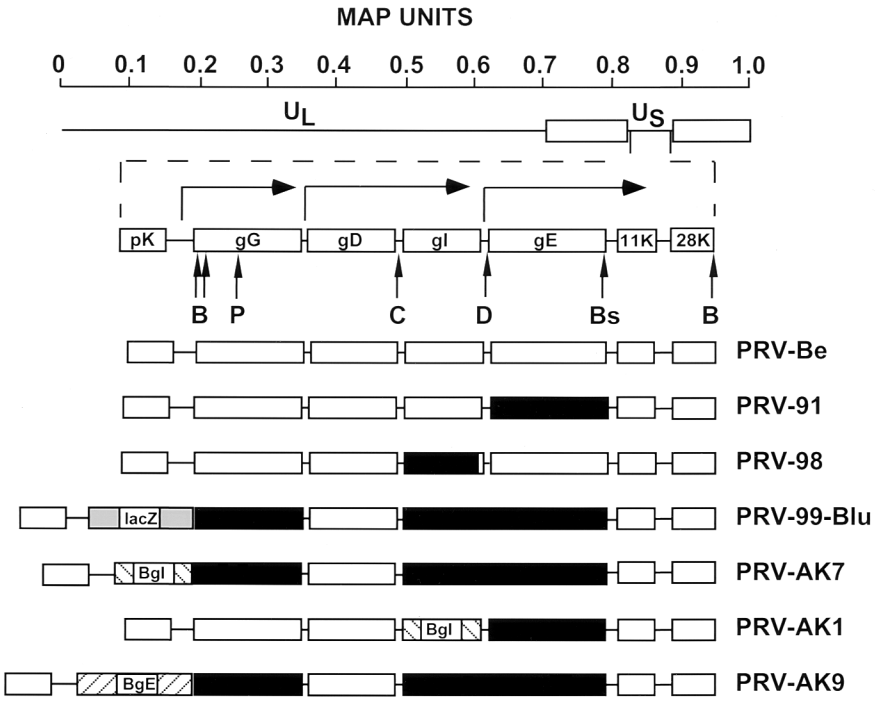

FIG. 1. PRV genome and map of relevant mutations. The PRV U_S region is indicated on the second line; the map units and relevant restriction sites that cut at or within the genes are indicated. The arrows show the direction of transcripts. Deletions in the wild-type strain PRV-Be are denoted by the black boxes, and the names of the mutants carrying these deletions are indicated. The insertion sites of the BHV-1.1 gI and gE open reading frames within the unique short region are depicted by the hatched boxes in the bottom three lines. B, *Bam*HI; P, *Pst*I; C, *Csp*I; D, *Dra*I; Bs, *Bsp*EI.

tectum and dorsal geniculate nucleus [DGN]) involved in visual perception and reflex eye movement in the rat brain (23). By contrast, gE and gI are not required for anterograde transport within retinal ganglion cells that project to circadian rhythm centers (23). This phenotype is called restricted neurotropism, as gE and gI are required to spread to some CNS centers and not others. We have demonstrated that this phenotype is not due to inability of PRV gE and gI null mutants to infect the ganglion cells that project to the optic tectum and DGN but rather is due to events after infection because coinfection with a PRV gE deletion virus and a PRV gI deletion virus (neither of which can infect the optic tectum or dorsal geniculate centers on its own) gave a robust infection of both (9). Consistent with this interpretation, the infected visual centers that result from such a coinfection contain only mutant viruses and not wild-type recombinants, suggesting that intracellular complementation had occurred (9b). In this report, we test the hypothesis that the gE and gI homologs from BHV-1.1 will complement PRV spread in the retinal infection paradigm.

MATERIALS AND METHODS

Virus strains and recombinants. All viruses were derived from PRV strain Becker (PRV-Be) and were propagated on PK15 cells following single plaque purification as described previously (8). Recombinant viruses used in this report (Fig. 1) were constructed and tested for the presence and expression of all relevant genes in the U_S region as described previously (12). The PRV strains PRV-98, PRV-91, and PRV-99-Blue are isogenic with wild-type PRV-Be and carry defined deletions in either gI (PRV-98), gE (PRV-91), or both (PRV-99). PRV-99-Blue expresses β-galactosidase in the PRV gG locus. The PRV-99 recombinants PRV-AK7 and PRV-AK1 express BHV-1.1 gI in the PRV gG and gI loci, respectively. The PRV-99 recombinant PRV-AK9 expresses BHV-1.1 gE in the PRV gG locus.

Antisera. Rabbit polyclonal antiserum Rb133 was prepared against acetone-inactivated PRV virions and recognizes PRV virus glycoproteins as well as capsid

TABLE 1. Summary of animal infections of retinorecipient areas of the rat CNS

Virus strain(s)	Virus genotype	No. of animals	Labeling[a]				
			SCN	Intergeniculate leaflet	DGN	Optic tectum	Hippocampus
PRV-91 + PRV-98	PRV gE^- + PRV gI^-	10	+++	+++	+++	+++	−
PRV-99-Blue	PRV $gG^-/gI^-/gE^-$	2	+++	+++	−	−	−
PRV-AK7 + PRV-AK9	PRV gG^-/BHV gI^+ + PRV gG^-/BHV gE^+	6	+++	+++	++ (3)	++ (5)	++ (1)[b]
PRV-AK-9 + PRV-91	PRV gG^-/BHV gE^+ + PRV gE^-	3	+++	+++	−	−	+++ (1)
PRV-AK1	PRV gI^-/BHV gI^+	2	+++	+++	−	++ (1)	−
PRV-AK7	PRV gG^-/BHV gI^+	5	+++	+++	−	++ (3)	−
PRV-AK9	PRV gG^-/BHV gE^+	6	+++	+++	−	−	+++ (3)

[a] +++, strong labeling in nucleus in all consecutive sections; ++, strong labeling in most sections; −, no labeling. Numbers in parentheses indicate numbers of animals with viral antigen in the indicated areas. All other areas were positive for viral antigen in 100% of the animals.

[b] Positive staining observed in one coinfection that did not result in spread to the optic tectum or the dorsal geniculate.

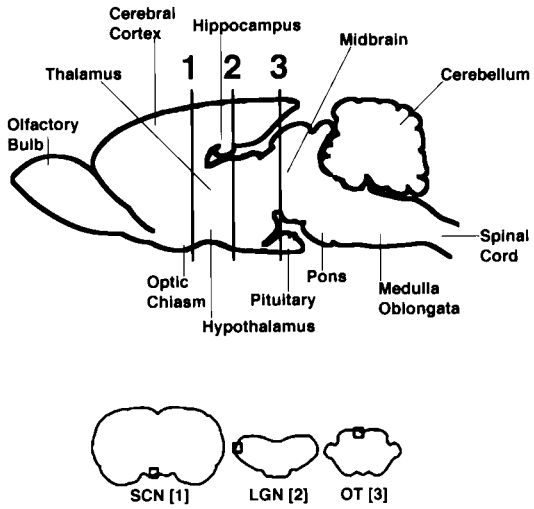

FIG. 2. Sagittal image of rat brain with coronal position of retinorecipient centers. Retinorecipient areas of the rat brain include the SCN (region 1), lateral geniculate nucleus (LGN; region 2), and optic tectum (OT; region 3). Viruses expressing gE and gI can spread to and infect central visual projection fields located in the forebrain, such as the hypothalamus (SCN) and the thalamus (DGN, IGL, and VGN). In addition, the most prominent midbrain target is the superior rostral colliculus (3), also called the optic tectum. Viruses lacking gE, gI, or both are able to infect only a subset of the retinorecipient regions, including the SCN and the IGL, but not the optic tectum or DGN. Note that the coronal brain slices from levels 2 and 3 are shown without cortex, which separates during sectioning.

proteins (3, 8). The polyvalent rabbit antiserum Rb1544 recognizes the glycosylated precursor of PRV gI but not the mature species (23). Rabbit polyvalent antisera against BHV-1.1 gI and gE are specific for BHV-1.1 gI and gE, respectively, and each recognizes both the precursor and mature species (24).

Animal experiments, tissue processing, and immunohistochemistry. Adult male Sprague-Dawley rats, weighing 200 to 300 g at the time of the experiment, were used. Food and water were freely available during the course of the experiment, and the photoperiod was standardized to 14 h of light/10 h of dark (light on at 0600). Experimental protocols were approved by the Animal Welfare Committee and were consistent with the regulations stipulated by the American Association for Accreditation of Laboratory Animal Care and those in the Animal Welfare Act (Public Law 99-198). The animals were confined to a biosafety level 2 facility, and the experiments were conducted with specific safeguards as described previously (3, 8).

For intravitreal injections, 1 to 2 μl of virus suspension was injected into the vitreous humor of the right eye of an anesthetized animal (Table 1 presents a summary of animal infections). All virus stocks were diluted to the same titer of 2×10^8 PFU/ml (as determined on PK15 cells) so that similar amounts of PFU were injected for all experiments. For the coinfection experiments, virus stocks of equal titers were mixed 1:1 (vol/vol) before inoculation. When symptoms of infection were overt (12), the animals were euthanized by being given an overdose of anesthesia; the animals were then perfused and dissected as described previously (8). Immunohistochemical analyses of coronal brain slices have been described elsewhere (3, 8). Tissues were taken for analysis at the approximate mean times to death for PRV-Be (72 ± 6 h), PRV-99 (104 ± 19 h), PRV-AK7 (89 ± 8 h), PRV-AK9 (92 ± 6 h), and PRV-AK7–PRV-AK9 coinfection (81.5 ± 6 h) (data taken from reference 12).

In the intravitreal infection paradigm, wild-type PRV infects and replicates in retinal ganglion cells in the eye and newly replicated virus is transported via anterograde mechanisms to axon terminals within the CNS (Fig. 2) (8). These terminals make synaptic contact with a variety of secondary neurons in anatomically defined regions, and the virus may cross at or near these synapses to infect the secondary neurons, depending on its gE/gI genotype.

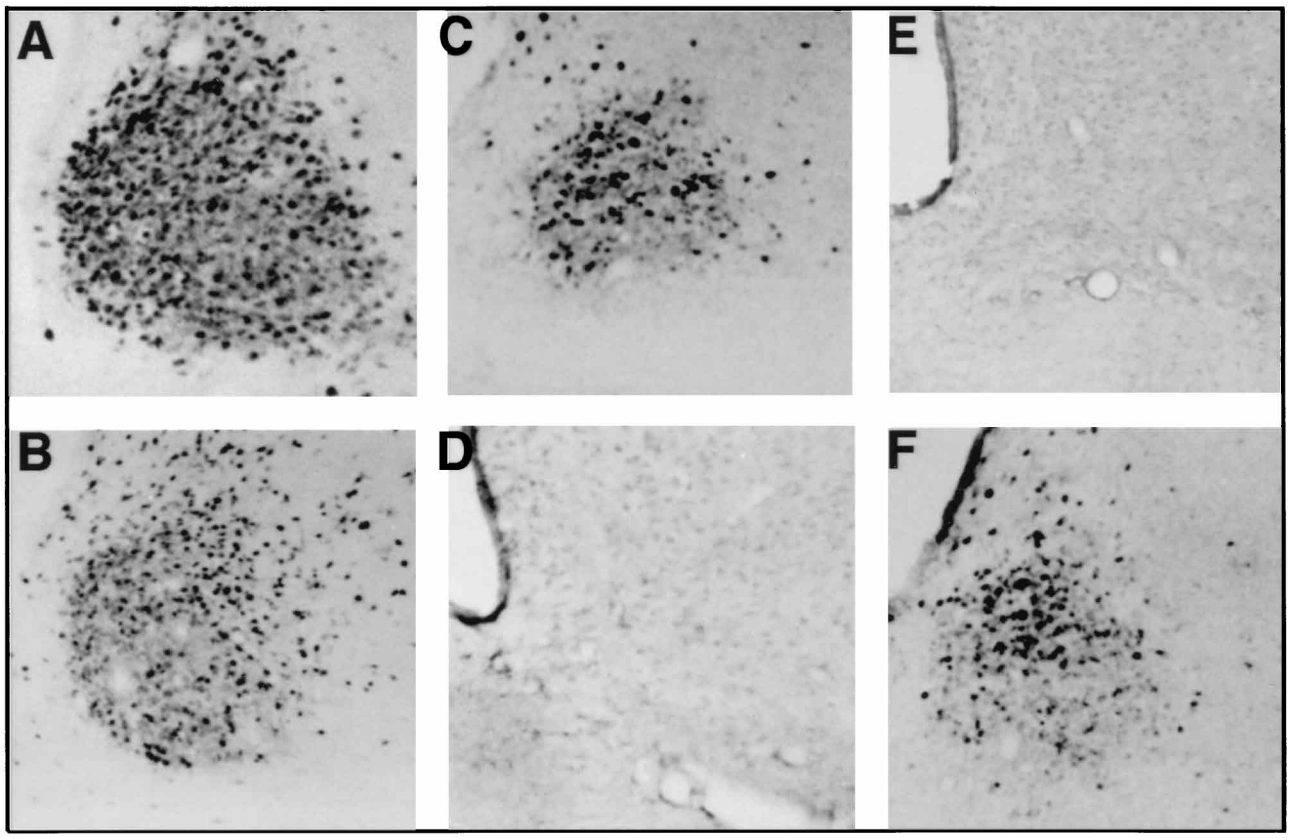

FIG. 3. BHV-1.1 gI and gE are expressed in the rodent brain. Coronal sections of brains from animals infected with PRV-AK7 (top row) and PRV-AK9 (bottom row) were analyzed by immunohistochemistry as described in Materials and Methods. All photos were taken at the same magnification and settings. Viral antigen was detected in the SCN with polyvalent antiserum Rb133 specific for PRV virion proteins (left column), polyvalent antiserum specific for BHV-1.1 gI (middle column), and polyvalent antiserum specific for BHV-1.1 gE (right column).

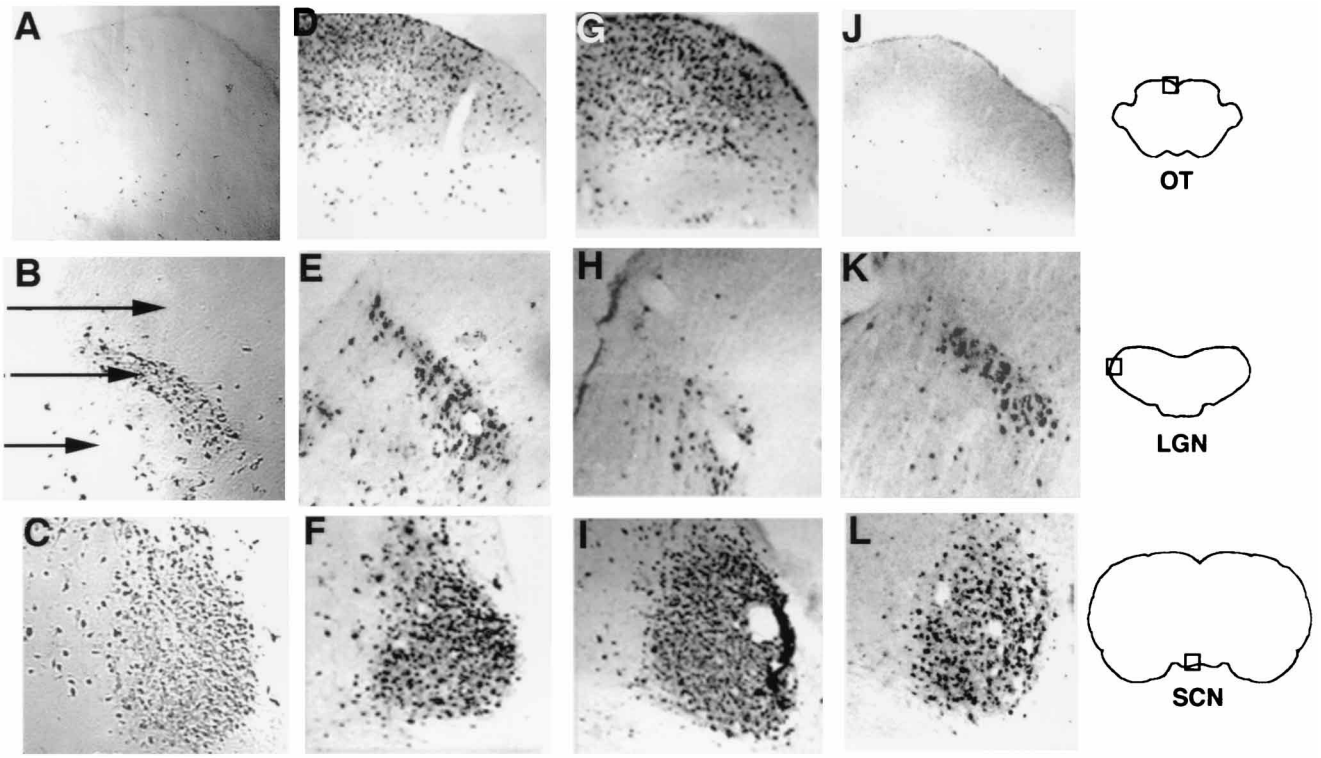

FIG. 4. BHV-1.1 gI partially restores spread to the visual centers. Coronal sections of brains were analyzed by immunohistochemistry, and images from similar regions were produced at the same magnification. Animals were injected intraocularly with PRV-AK9 (A to C), PRV-AK7 (D to F), PRV-AK1 (G to I), or PRV-AK99-Blue (J to L). Polyvalent antiserum Rb133 made against PRV virions reacted with viral antigen in the optic tectum (OT; top row), lateral geniculate nucleus (LGN; middle row), and SCN (bottom row). Each column presents data from the same infected animal. The BHV-1.1 gI-expressing PRV recombinants infected all circadian rhythm centers and only one of the main visual centers, the optic tectum (D to J), while PRV recombinants with BHV-1.1 gE (A to C) or *lacZ* (J to L) expressed from the same promoter infected only the circadian rhythm centers. The top arrow indicates the DGN, the middle arrow indicates the IGL, and the bottom arrow indicates the VCN.

RESULTS

The PRV recombinants express the appropriate BHV-1.1 protein in the rat brain. As BHV-1.1 has a very restricted host tropism and cannot infect rodents, it was of interest to test for BHV-1.1 homolog expression in the rat brain. Consecutive coronal brain sections were analyzed for PRV virion proteins and for BHV-1.1 gI and gE by immunohistochemistry. As a representative example, we show the immunostaining in the suprachiasmatic nucleus (SCN) achieved with antibodies specific for PRV virion proteins (Fig. 3A and B), BHV-1.1 gI (Fig. 3C and D), or BHV-1.1 gE (Fig. 3E and F) after intraocular injections with PRV-AK7 (top row) and PRV-AK9 (bottom row). The appearance of immunoreactivity for the BHV-1.1 gI or gE protein was specific, since BHV-1.1 gI or gE antigen was detected only in animals that were infected with mutant PRV-AK7 or PRV-AK9, respectively (Fig. 3C and F). In this experiment, we attempted to show that the BHV-1.1 glycoproteins gI and gE can be expressed in the PRV-99 mutant in vivo. We showed that the PRV recombinants PRV-AK7 and PRV-AK9, carrying BHV-1.1 gI and gE in PRV gG, are able to express the glycoproteins in the SCN of the rat brain.

BHV-1.1 gI expressed by gE/gI-minus PRV has novel neurotropic properties not found with the PRV gI homolog. PRV mutants lacking gI (PRV-98) or gE (PRV-91) have phenotypes identical to those of a gI/gE double mutant (PRV-99) in the retinal infection paradigm. All three mutants, when infected individually, infect the circadian centers but not the optic tectum and DGN (23). As expected, PRV-AK9 and PRV-99-Blue showed the phenotype of PRV-99, being unable to infect the optic tectum and the dorsal geniculate (Fig. 4A to C and J to L, respectively). However, we were surprised to find in three of five animals that the PRV-AK7 recombinant expressing BHV-1.1 gI was able to promote spread of PRV to the rodent optic tectum but not to the dorsal geniculate (Fig. 4D to F). The two remaining animals showed no infection of the optic tectum or the dorsal geniculate while infecting the circadian centers. In the three animals with optic tectum infection, superficial and deeper layers in the contralateral but not ipsilateral tectum were infected, indicating that the virus had reached the superior colliculus by direct anterograde transport from retinal ganglion cells through the optic tract. The presence of BHV-1.1 gI protein was confirmed by staining with polyclonal antiserum for BHV-1.1 gI (data not shown). This unexpected result was not due to high expression levels of BHV-1.1 gI under the strong PRV gG promoter, because similar results were obtained after the infection with PRV-AK1, a recombinant virus that expresses about 15 times less BHV-1.1 gI than PRV-AK7 (Fig. 4G to I) (12). One of two animals infected with PRV-AK1 showed a qualitatively similar staining pattern (Fig. 4G to I). These results were not due to the insertional inactivation of gG, because PRV-99-Blue (*Escherichia coli lacZ* inserted into the gG locus) never infected the optic tectum (Fig. 4J) (9a). At this time, we do not understand why this phenotype was not observed in all infected animals. Nevertheless, these experiments demonstrate that BHV-1.1 gI, in the absence of any gE homolog, confers unique neurotropic proper-

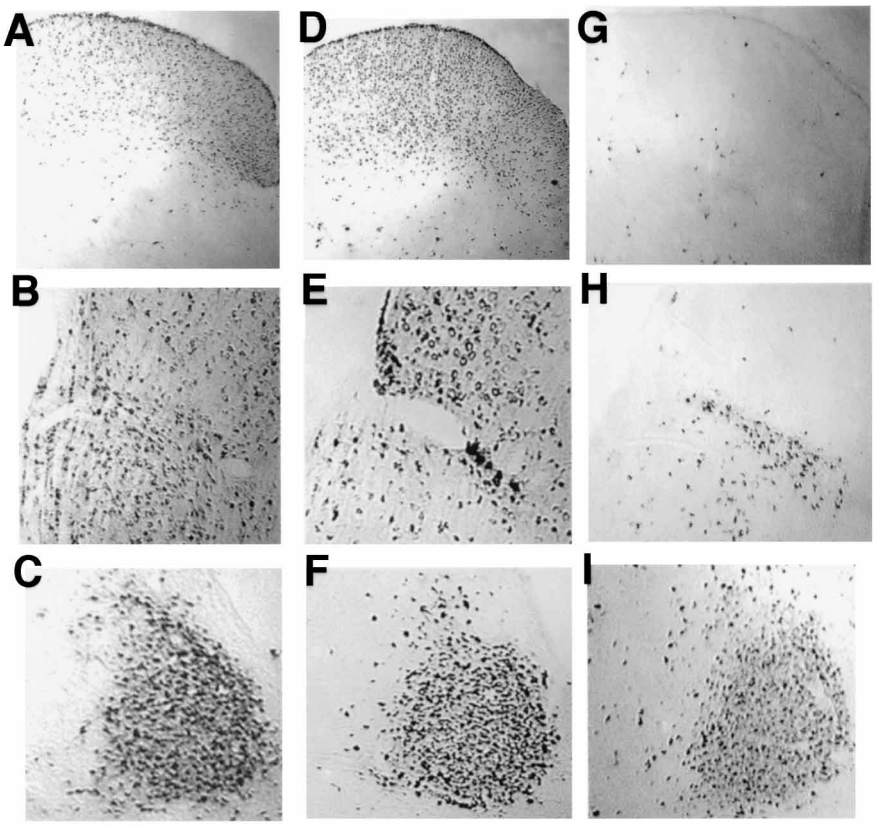

FIG. 5. Coinfection by PRV-AK7 and PRV-AK9 restores virus spread to all visual centers. Mixtures of the designated viruses were made as described in Materials and Methods. Animals were infected with 1 to 2 μl of each mixture, and virus spread to the retinorecipient areas of the brain was determined by immunohistochemical analysis as described in Materials and Methods. Results from three infected animals are shown: the control coinfection of PRV-98 with PRV-91, showing complementation and restoration of the wild-type phenotype (left column); PRV-AK7 with PRV-AK9, showing similar complementation (middle column); and PRV-AK9 with PRV-91, showing no complementation (right column).

ties to PRV not observed with expression of BHV-1.1 gE, PRV gE, or PRV gI alone.

Functional synergism between BHV-1.1 gI and gE. For PRV to infect the rat visual centers (the optic tectum and the DGN) after infection of the eye, both gE and gI must be expressed. As shown above, PRV recombinants expressing BHV-1.1 gI were able to infect a subset of the visual centers in the rat CNS, namely, the optic tectum but not the DGN. We asked if the partial complementation by BHV-1.1 gI could be enhanced by coinfection with PRV-AK9, a virus that expresses BHV-1.1 gE.

Control coinfections with the PRV gI- and gE-expressing mutants PRV-91 and PRV-98 resulted in full restoration of wild-type virus infection (PRV-Be) as described previously (Fig. 5A to C) (9). Similar results were obtained after coinfection with PRV-AK7 and PRV-AK9 (Fig. 5D to F). Notice that all retinorecipient regions were infected (Fig. 5D to F), which is in contrast to the limited-spread phenotype observed after the injection of only PRV-AK7 (Fig. 4E): the DGN now stained positive for viral antigen (Fig. 5E). Thus, coexpression of both proteins gave a phenotype identical to that of wild-type PRV.

We also investigated whether PRV gI could cooperate with BHV-1.1 gE in promoting infection of the visual brain centers. In this experiment, we coinfected the rat retina with a mixture of PRV-AK9, expressing BHV-1.1 gE, and PRV-91, expressing PRV gI. As shown in Fig. 5G to I, we observed no complementation and saw only the restricted spread of PRV to the circadian rhythm centers, characteristic of infection by each individual mutant. Thus, the gI homolog from PRV and gE homolog from BHV-1.1 cannot function together in vivo to promote spread to the rat visual centers. We have been unable to detect complex formation between BHV-1.1 gE and PRV gI when tissue culture cells were coinfected with PRV-AK9 and PRV-91, suggesting that these two proteins do not physically interact (data not shown).

BHV-1.1 gE facilitates spread of PRV to the hippocampus. Infection of the eye by PRV-AK9 was similar to infection by any PRV gE or gI deletion mutant in that only a subset of the retinorecipient areas of the brain were infected. However, in three of six infected animals, we noted one striking difference: PRV-AK9 gave a robust infection of pyramidal cells in the hippocampal formation. Representative coronal sections through the hippocampus from one such infection are shown in Fig. 6. Figure 6A is a Nissl stain of infected tissue highlighting the dentate gyrus (dark staining) for orientation; Fig. 6B shows the absence of viral antigen in this region after wild-type virus infection; Fig. 6C and the enlargement in Fig. 6D show the robust immunostaining of PRV-AK9-infected pyramidal cells in the CA1 and CA3 regions of Ammon's horn (20). As a control, we replaced the BHV-1.1 gE gene with wild-type DNA from PRV, restoring expression of gG, and found that the ability to infect the hippocampus was lost (data not shown), indicating that this phenotype is associated with the expression of BHV-1.1 gE.

Interestingly, we also observed hippocampal infection after coinfection with PRV-AK7 and PRV-AK9 in an animal that

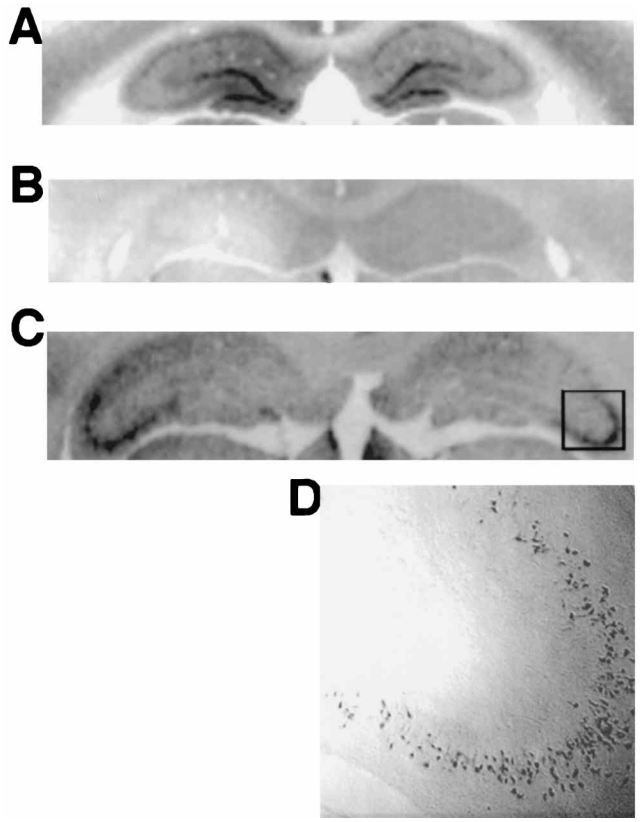

FIG. 6. BgE facilitates spread of PRV to the hippocampus. (A) Thionine (Nissl stain) staining of dentate gyrus in PRV-Be-infected brain. (B) Negative immunohistochemical reaction with RB133 serum (anti-PRV virion proteins) in the hippocampus of a PRV-Be-infected brain. (C) Positive immunohistochemical reaction with Rb133 serum in the hippocampus of a PRV-AK9 infected brain. (D) Magnification of PRV-positive area indicated in panel C in the hippocampus. Pyramidal cells in the CA1 and CA3 regions of Ammon's horn are specifically stained.

showed no infection of the optic tectum or dorsal geniculate (Table 1). We also saw infection of the hippocampus in one coinfection of PRV-AK9 and PRV-91. More infected animals must be analyzed before conclusions can be drawn, but it may be that infection of the optic tectum and the hippocampus are mutually exclusive when BHV-1.1 gE and gI, but not PRV gI, are present (Table 1).

DISCUSSION

The membrane glycoproteins of all alphaherpesviruses fall into two operationally defined classes: those required for infection of cells in tissue culture and those that are dispensable for efficient infection of cells in culture. The functions of the latter class are often revealed by experimental infection of animals. Accordingly, genetic analysis of function in nonessential glycoproteins has been difficult in vivo. An underlying assumption for our experiments is that conserved herpesvirus proteins retain similar functions that may be revealed in diverse animal models. In this study, we used a rodent model of neural circuit-specific infection to assess conservation of function of the gE and gI membrane proteins encoded by PRV and BHV-1.1. PRV is a neurotropic alphaherpesvirus whose natural host is the swine but is capable of infecting and causing disease in a wide variety of vertebrates including rodents and birds (26). By contrast, BHV-1.1 is a respiratory pathogen of highly restricted host range limited essentially to cattle (22). The gE and gI proteins encoded by PRV are required for efficient pathogenesis in swine, rodents, and day-old chicks (7). Similarly, BHV-1.1 mutants defective for gE have markedly reduced ability to cause disease in cattle (11).

Despite the differences between the two viruses in tropism and pathogenic spectrum, it was striking to us that PRV and BHV-1.1 gE mutants had similar reduced-pathogenesis phenotypes. In pursuing an explanation for this, we have demonstrated that despite differences in host range, the BHV-1.1 homologs were capable of complementing the virulence functions of PRV gE and gI in rats, supporting our contention that conserved proteins have conserved function independent of host and virus (12). As both PRV and BHV-1.1 establish a latent infection in sensory ganglia, we continued our analysis for complementation of function by determining if the BHV-1.1 gE and gI gene products can complement the specific neurotropism phenotypes of PRV gE/gI mutants.

In the natural host, PRV gE null mutants cannot spread to the olfactory bulb after infection of the olfactory epithelium, but such mutants retain the ability to infect various sensory ganglia where they can establish a latent infection (7). This restricted neurotropism phenotype appears to hold true in all animal models tested to date: PRV gE or gI null mutants can infect axon terminals and spread toward the cell body (defined as retrograde spread), but these same mutants, after infecting neuronal cell bodies, cannot spread toward axon terminals in many neurons (defective in anterograde spread). The Enquist and Card laboratories have developed a model to study restricted neurotropism and anterograde spread of herpesviruses involving infection of the rat retina (8). Spread from the retina into the brain occurs solely by anterograde transport, and in the rat, the regions of the brain where the axon terminals of retinal ganglion cells terminate are well known (3). Thus, wild-type PRV infects the retina and spreads by anterograde mechanisms to infect all known second-order neurons in the brain connected to the retina (so-called retinorecipient regions). By contrast, PRV gE and gI mutants are incapable of spreading to some retinorecipient regions. Like wild-type virus, PRV gE and gI mutants can spread efficiently by anterograde transport to the SCN and the intergeniculate leaflet (IGL) (circadian rhythm centers); they cannot spread to the optic tectum and dorsal lateral geniculate nuclei. This model is a sensitive indicator of function and was used to assess the ability of BHV-1.1 gE and gI genes to complement PRV gE and gI null mutants. As discussed below, we also assert that the ability to distinguish gE-dependent anterograde infection in some but not all circuits has relevance to natural primary and recurrent infections.

The experiments provided three unexpected results. First, the BHV-1.1 gI gene product alone can restore ability of PRV to infect the optic tectum but not the DGN. Second, the BHV-1.1 gE gene product alone enabled PRV to infect the hippocampus. Third, when both BHV-1.1 gE and gI were expressed in a coinfection, a robust infection of all visual centers was observed. The first two phenotypes are unique to the BHV-1.1 gE and gI genes and have never been observed for PRV mutants expressing either PRV gI or gE.

The first result, the ability of BHV-1.1 gI to promote infection of the optic tectum in the absence of a gE partner, was unexpected because a PRV mutant lacking gE (PRV-91) but expressing PRV gI cannot infect the optic tectum at all (23). Both PRV-AK1 and PRV-AK7 exhibited the same phenotype, indicating that it was not the particular construct or the level of BHV-1.1 gI that was responsible for the effect, as PRV-AK1 expressed at least 15-fold less BHV-1.1 gI compared to PRV-AK7 (12). Moreover, neither PRV-99-Blue (expressing *E. coli*

lacZ from the gG locus) nor PRV-AK9 (expressing BHV-1.1 gE from the gG locus) was able to spread to the optic tectum, indicating that gG played no role in the phenomenon. The novel spread phenotype promoted by BHV-1.1 gI suggests that the neurons projecting to the dorsal geniculate and the optic tectum are distinguishable, demonstrating that viral mutants can dissect neuronal circuitry.

The second novel finding was that PRV-AK9 (expresses BHV-1.1 gE) exhibited a novel gain-of-function phenotype: intravitreal infection with PRV-AK9 spread to the hippocampus in three of six animals (Table 1). The hippocampus is known to give rise to a dense bisynaptic projection to the medial hypothalamus that involves a synapse in the lateral septum (18). The pattern of infection that we observed in the hippocampal formation following intravitreal injection of PRV-AK9 may have been achieved by retrograde transsynaptic passage of virus through this circuitry. Consistent with this idea, we do observe infection of the lateral septum in every animal with a hippocampal infection (data not shown). Further work must be done following the temporal spread of PRV-AK9 after retinal infection to verify that the lateral septum becomes infected before the hippocampus. Other than the novel infection of the hippocampus, PRV-AK9 behaved as expected for a PRV mutant lacking gI but expressing gE; there was no infection of the optic tectum and dorsal geniculate but robust infection of the circadian centers. We also can see infection of the lateral septum in some animals infected with wild-type virus (data not shown). It is not clear at this time where wild-type PRV is blocked in infection of the projection system of the hippocampus to the retina and how this block is overcome by BHV-1.1 gE but not PRV gE. As BHV-1.1 has evolved to be a respiratory pathogen, we speculate that this novel rat brain infection pathway reflects the difference in ability of gE and gI gene products to affect virus spread between many different cell types. Perhaps the composition of synapses characterizing the retinal-hippocampal pathway share common features with cell-cell junctions in the respiratory tract.

The third novel observation was made when BHV-1.1 gE and gI were expressed together in a coinfection by PRV-AK9 and PRV-AK7 of the rat retina: several animals had a robust infection of all the visual centers (including the appropriate dorsal geniculate [three of six animals] and optic tectum [five of six animals]). However, as indicated in Table 1, the infection was quantitatively different from a wild-type PRV-Be infection that is usually seen over a more extensive area in the respective nuclei. We did not observe a hippocampus infection in these animals. However, we did observe a strong hippocampus infection in one animal that did not have any infected cells in the optic tectum or dorsal geniculate. If spread to the hippocampus and spread to the optic tectum are mutually exclusive, one possibility may be that BHV-1.1 gI inhibits the ability of gE to promote spread to the hippocampus by heterodimer formation and alternatively facilitates invasion of the visual centers. Hence, lack of optic tectum and geniculate infection following mixed PRV-91 (expresses PRV gI) and PRV-AK9 is consistent with a failure of PRV gI and BHV-1.1 gE to form a heterodimer (data not shown) and, in one case, infection of the hippocampus (Table 1; Fig. 5).

This work raises two general questions. First, what is the relationship of gE/gI-dependent cell-cell spread seen after tissue culture infection and gE/gI-dependent anterograde spread in neurons of living animals? Second, why would gE and gI be required for spread in some but not all anterograde circuits? We postulate that the answers to both questions lie in the mechanism of cell-cell spread promoted by gE and gI. It is likely that virus spread from cell to cell is directed to, and occurs at, unique junctions: epithelial junctions, sites of synaptic contact between neurons, or adhesion contacts between fibroblasts in tissue culture may be sites where gE and gI are required. Moreover, as these junctions differ between cell types, they may differ in their responses to gE and gI.

We suggest that in vivo, gE and gI may act at several stages of the herpesvirus life cycle to promote virus spread, both at the mucosal surface and after reactivation from bipolar sensory ganglion neurons. At the mucosal surface, infection by wild-type virus results in localized spread in the epithelial and surrounding cells promoted in part by gE and gI (2, 4, 16, 28). Although infection and the establishment of latency in sensory ganglia are independent of gE and gI expression (7, 13, 14, 21), gE and gI may selectively promote anterograde spread back to the mucosal surface at the exclusion of alternative spread to the CNS during reactivation.

In summary, the BHV-1.1 gE and gI genes cloned in gE/gI-minus PRV vectors were expressed at high levels in the rat brain and revealed novel properties that provided insight into herpesvirus pathogenesis. These studies suggest that gE and gI herpesvirus homologs possess analogous functions despite different host ranges and pathogenic niches of their parent viruses. Functional autonomy in the so-called nonessential genes like gE and gI implies conserved interactions with the host. This conclusion has been reached for the gC homologs where interaction with extracellular matrix proteins as well as host-specific complement components are well known (19). The molecular mechanisms by which gE and gI genes affect virulence and spread at the mucosal surface as well as in the nervous system are likely to be conserved among herpesviruses that encode them.

ACKNOWLEDGMENTS

We thank members of the Enquist lab for advice and encouragement. Bruce Banfield and Greg Smith provided constructive criticism and insight. We are grateful to Pat Card for detailed comments and assistance with visual system neuroanatomy and immunohistochemistry. Richard Miselis and Ming Yang provided assistance with neuroanatomy, immunohistochemistry, and advice on the animal surgeries.

This work was supported by NINDS grant R0133506.

REFERENCES

1. **Babic, N., T. C. Mettenleiter, G. Ugolini, A. Flamand, and P. Coulon.** 1994. Propagation of pseudorabies virus in the nervous system of the mouse after intranasal inoculation. Virology **204:**616–625.
2. **Balan, P., N. Davis-Poynter, S. Bell, H. Atkinson, H. Browne, and T. Minson.** 1994. An analysis of the in vitro and in vivo phenotypes of mutants of herpes simplex virus type 1 lacking glycoproteins gG, gE, gI or the putative gJ. J. Gen. Virol. **75:**1245–1258.
3. **Card, J. P.** 1990. Neurotropic properties of pseudorabies virus: uptake and transneuronal passage in the rat central nervous system. J. Neurosci. **10:** 1974–1994.
4. **Dingwell, K. S., C. R. Brunetti, R. L. Hendricks, Q. Tang, M. Tang, A. J. Rainbow, and D. C. Johnson.** 1994. Herpes simplex virus glycoproteins E and I facilitate cell-to-cell spread in vivo and across junctions of cultured cells. J. Virol. **68:**834–845.
5. **Dingwell, K. S., L. C. Doering, and D. C. Johnson.** 1995. Glycoproteins E and I facilitate neuron-to-neuron spread of herpes simplex virus. J. Virol. **69:** 7087–7098.
6. **Dubin, G., S. Basu, D. L. P. Mallory, M. Basu, R. Tal-Singer, and H. M. Friedman.** 1994. Characterization of domains of herpes simplex virus type 1 glycoprotein E involved in Fc binding activity for immunoglobulin G aggregates. J. Virol. **68:**2478–2486.
7. **Enquist, L. W.** 1994. Infection of the mammalian nervous system by pseudorabies virus (PRV). Semin. Virol. **5:**221–231.
8. **Enquist, L. W., and J. P. Card.** 1996. Pseudorabies virus: a tool for tracing neuronal connections, p. 334–348. *In* P. R. Lowenstein and L. W. Enquist (ed.), Protocols for gene transfer in neuroscience; towards gene therapy of neurological disorders. John Wiley & Sons, New York, N.Y.
9. **Enquist, L. W., J. Dubin, M. E. Whealy, and J. P. Card.** 1994. Complementation analysis of pseudorabies virus gE and gI mutants in retinal ganglion

cell neurotropism. J. Virol. **68:**5275–5279.
9a. **Husak, P.** Unpublished data.
9b. **Husak, P., and L. Enquist.** Unpublished data.
10. **Jacobs, L.** 1994. Glycoprotein E of pseudorabies virus and homologous proteins in other alphaherpesvirinae. Arch. Virol. **137:**209–228.
11. **Kaashoek, M. J., A. Moerman, J. Madic, F. A. M. Rijsewijk, J. Quak, A. L. J. Gielkens, and J. T. Van Oirschot.** 1994. A conventionally attenuated glycoprotein E-negative strain of bovine herpesvirus type 1 is an efficacious and safe vaccine. Vaccine **12:**439–444.
12. **Knapp, A. C., and L. W. Enquist.** 1997. Pseudorabies virus recombinants expressing functional virulence determinants gE and gI from bovine herpesvirus 1.1. J. Virol. **71:**2731–2739.
13. **Kritas, S. K., H. J. Nauwynck, and M. B. Pensaert.** 1995. Dissemination of wild-type and gC-, gE- and gI-deleted mutants of Aujeszky's disease virus in the maxillary nerve and trigeminal ganglion of pigs after intranasal inoculation. J. Gen. Virol. **76:**2063–2066.
14. **Kritas, S. K., M. B. Pensaert, and T. C. Mettenleiter.** 1994. Invasion and spread of single glycoprotein deleted mutants of Aujeszky's disease virus (ADV) in the trigeminal nervous pathway of pigs after intranasal inoculation. Vet. Microbiol. **40:**323–334.
15. **Kritas, S. K., M. B. Pensaert, and T. C. Mettenleiter.** 1994. Role of envelope glycoproteins gI, gp63 and gIII in the invasion and spread of Aujeszky's disease virus in the olfactory nervous pathway of the pig. J. Gen. Virol. **75:**2319–2327.
16. **Maidji, E., S. Tugizov, T. Jones, Z. Zhenwei, and L. Pereira.** 1996. Accessory human cytomegalovirus glycoprotein US9 in the unique short component of the viral genome promotes cell-to-cell transmission of virus in polarized epithelial cells. J. Virol. **70:**8402–8410.
17. **Mulder, W., J. Pol, T. Kimman, G. Kok, J. Priem, and B. Peeters.** 1996. Glycoprotein D-negative pseudorabies virus can spread transneuronally via direct neuron-to-neuron transmission in tis natural host, the pig, but not after additional inactivation of gE and gI. J. Virol. **70:**2191–2200.
18. **Risold, P. Y., and L. W. Swanson.** 1996. Structural evidence for functional domains in the rat hippocampus. Science **272:**1484–1486.
19. **Roizman, B., and A. E. Sears.** 1996. Herpes simplex viruses and their replication, p. 1043–1107. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Fundamental virology. Lippincott-Raven Publishers, Philadelphia, Pa.
20. **Swanson, L. W.** 1992. Brain maps: structure of the rat brain. Elsevier, Amsterdam, The Netherlands.
21. **Van Engelenburg, F. A. C., M. J. Kaashoek, J. T. van Oirschot, and F. A. M. Rijsewijk.** 1995. A glycoprotein E deletion mutant of bovine herpesvirus 1 infects the same limited number of tissues in calves as wild-type virus, but for a shorter period. J. Gen. Virol. **76:**2387–2392.
22. **Wentink, G. H., J. T. van Oirschot, and J. Verhoeff.** 1993. Risk of infection with bovine herpes virus 1 (BHV-1): a review. Vet. Q. **15:**30–33.
23. **Whealy, M. E., J. P. Card, A. K. Robbins, J. R. Dubin, H.-J. Rziha, and L. W. Enquist.** 1993. Specific pseudorabies virus infection of the rat visual system requires both gI and gp63 glycoproteins. J. Virol. **67:**3786–3797.
24. **Whitbeck, J. C., A. C. Knapp, L. W. Enquist, W. C. Lawrence, and L. J. Bello.** 1996. Synthesis, processing and oligomerization of bovine herpes virus type 1 gE and gI membrane proteins. J. Virol. **70:**7878–7884.
25. **Whitley, R. J., and M. Schlitt.** 1991. Encephalitis caused by herpesviruses, including B virus, p. 41–86. *In* W. M. Scheld, R. J. Whitley, and D. T. Durack (ed.), Infections of the central nervous system. Raven Press, New York, N.Y.
26. **Wittmann, G., and H.-J. Rziha.** 1989. Aujeszky's disease (pseudorabies) in pigs, p. 230–233. *In* G. Wittmann (ed.), Herpesvirus diseases of cattle, horses, and pigs. Kluwer Academic, Boston, Mass.
27. **Yao, Z., W. Jackson, B. Forghani, and C. Grose.** 1993. Varicella-zoster virus glycoprotein gpI/gpIV receptor: expression, complex formation, and antigenicity within the vaccinia virus-T7 RNA polymerase transfection system. J. Virol. **67:**305–314.
28. **Zuckermann, F. A., T. C. Mettenleiter, C. Schreurs, N. Sugg, and T. Ben-Porat.** 1988. Complex between glycoproteins gI and gp63 of pseudorabies virus: its effect on virus replication. J. Virol. **62:**4622–4626.