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Complementation Analysis of Pseudorabies Virus gE and gI Mutants in Retinal Ganglion Cell Neurotropism

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Pseudorabies virus glycoproteins gE and gI are required to infect some, but not all, regions of the rodent central nervous system after peripheral injection. After infection of the retina, pseudorabies virus mutants lacking either gE or gI can subsequently infect neural centers involved in the control of circadian function but cannot infect visual circuits mediating visual perception or the reflex movement of the eyes. In this study, we used genetic complementation to test the hypothesis that gE and gI are required for entry into the specific retinal ganglion cells that project to visual centers. These data strongly suggest that gE and gI must function after the viruses enter primary neurons in the retina.

Pseudorabies virus (PRV) is highly neurotropic in a variety of animals, including swine, its natural host (28). It is clear from analyses with rodent models that PRV invasion of the central nervous system occurs in an ordered fashion in which the virus passes through synaptically linked neurons (2, 4, 7, 14, 18, 21–23, 25, 26). Nevertheless, the molecular mechanisms involved in the infection and specific transport of PRV through neural circuits are not well understood. To gain insight into these mechanisms, our laboratories have focused on the infection and transport of PRV in the rat visual system. Retinal ganglion neurons project through the optic nerves and terminate in a number of functionally distinct regions of the central neuraxis. PRV infects and replicates in these primary neurons after introduction into the vitreous body of the eye and is subsequently transported by anterograde axonal mechanisms to retinorecipient neurons in cytoarchitecturally defined regions of the central nervous system (4). We have characterized the infectivity of several PRV mutants in this system and have defined two specific PRV genes required to infect a subset of retinorecipient neurons (3, 4, 27). This is a novel neurotropism phenotype, since these PRV mutants can infect neural centers involved in circadian rhythm regulation but not neurons involved in visual perception or reflex eye movement. The two genes are the so-called nonessential genes encoding gE and gI. (We use the common nomenclature for alphaherpesvirus glycoproteins based on the herpes simplex virus system agreed to at the 1993 International Herpesvirus Workshop. For this report, gI [eye] replaces gp63 and gE replaces gI [one].)

After PRV infection of the retina, gE and gI are required for infection of the dorsal geniculate nuclei and the optic tectum but not for infection of the suprachiasmatic nucleus or the intergeniculate leaflet (3, 27). Infection of these central visual centers is dependent upon the virus invading and replicating in ganglion cells in the retina, traveling anterogradely through axons in the optic nerve, and passing transneuronally to infect and replicate within retinorecipient neurons in the diencephalon and midbrain. In contrast, neural circuitry involved in the photic modulation of circadian function remains susceptible to productive infection by viral mutants lacking these glycoproteins (2, 3, 27). PRV gE and gI form a heterooligomer (most likely a heterodimer) which has been proposed to be the unit of function (27, 30). Previously, we speculated that these glycoproteins were required for virion entry into all functionally distinct classes of retinal ganglion cells (4). However, we also noted that the restricted neurotropism exhibited by these mutants could be due to a defect in transport or release of virus at retinorecipient centers (3, 27). In the present study, we examined this question by a set of complementation experiments.

The methods are described in our previous publications (2–4, 27). Briefly, viruses were propagated on PK15 cells, titers were determined, and viruses were stored frozen at −80°C prior to injection. PRV strain Becker (PRV-Be) is the parental strain for all the PRV mutants used in this analysis. PRV91 and PRV98 are isogenic strains of PRV-Be with defined deletions of the gE and gI genes, respectively (26). PRV99 is PRV-Be lacking both gE and gI (27). In the coinfection studies (n = 13), approximately 10^5 PFU each of PRV91 and PRV98 was injected in a volume of 2 μl into the vitreous body of the right eye. In parallel control studies (n = 50 [27]), other animals were injected with an equivalent volume of PRV-Be, PRV91, PRV98, or PRV99. Additional controls included injection of the two mutants into separate eyes (n = 2), injection of one mutant into the eye and the other into the wall of the stomach (n = 2), and intravitreal injection of a 1:1 mixture of PRV91 and PRV99 (n = 2). Animals were sacrificed 50 to 100 h following inoculation, and the distribution of infected neurons throughout the neuraxis was assessed by using a rabbit polyclonal antiserum generated against acetone-inactivated PRV and the avidin-biotin modification of Sternberger's immunoperoxidase procedure (24). The details of this method as applied in our laboratories have been published previously (4).

Mixed infection by gE and gI null mutants can help determine if both gE and gI are necessary for viral entry into retinal ganglion cells projecting to the dorsal geniculate nucleus and tectum or for a subsequent step after virion entry into these retinal ganglion cells. While PRV91 encodes only gI and PRV98 encodes only gE, a cell infected with both mutants will produce gE and gI capable of forming a functional complex (27). Moreover, these dually infected cells should also produce phenotypically mixed viruses with both gE and gI in their

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TABLE 1. Virus infection of circadian centers and visual centers[a]

Virus	Presence of viral antigen in:		Comments
	Suprachiasmatic nucleus and intergeniculate leaflet (circadian centers)	Optic tectum and dorsal geniculate nucleus (visual centers)	
PRV-Be	+	+	Parental virus
PRV91	+	−	PRV-Be with gE deletion
PRV98	+	−	PRV-Be with gI deletion
PRV99	+	−	PRV-Be lacking gE and gI
PRV91-PRV98	+	+	1:1 mixed infection
PRV91-PRV99	+	−	1:1 mixed infection

[a] The methods of infection and the genotypes of the viral mutants have been described (2–4, 27). The 1:1 mixed infections consisted of a 2-μl volume containing a total of 2×10^5 PFU (1×10^5 PFU of each virus) injected into the posterior chamber of the eye. +, significant transneuronal infection of the particular region as demonstrated by immunohistochemical localization of PRV as shown in Fig. 1. −, no obvious infection of the particular region as demonstrated by immunolocalization of PRV (Fig. 1). The results show that gI and gE are not required for initial entry into retinal ganglion cells.

envelopes. Thus, if gE and gI were required for entry into specific cells in the retina, no complementation (formation of a functional complex) would be possible in these cells. If, however, the defect occurred after entry, we might expect to see complementation after mixed infection.

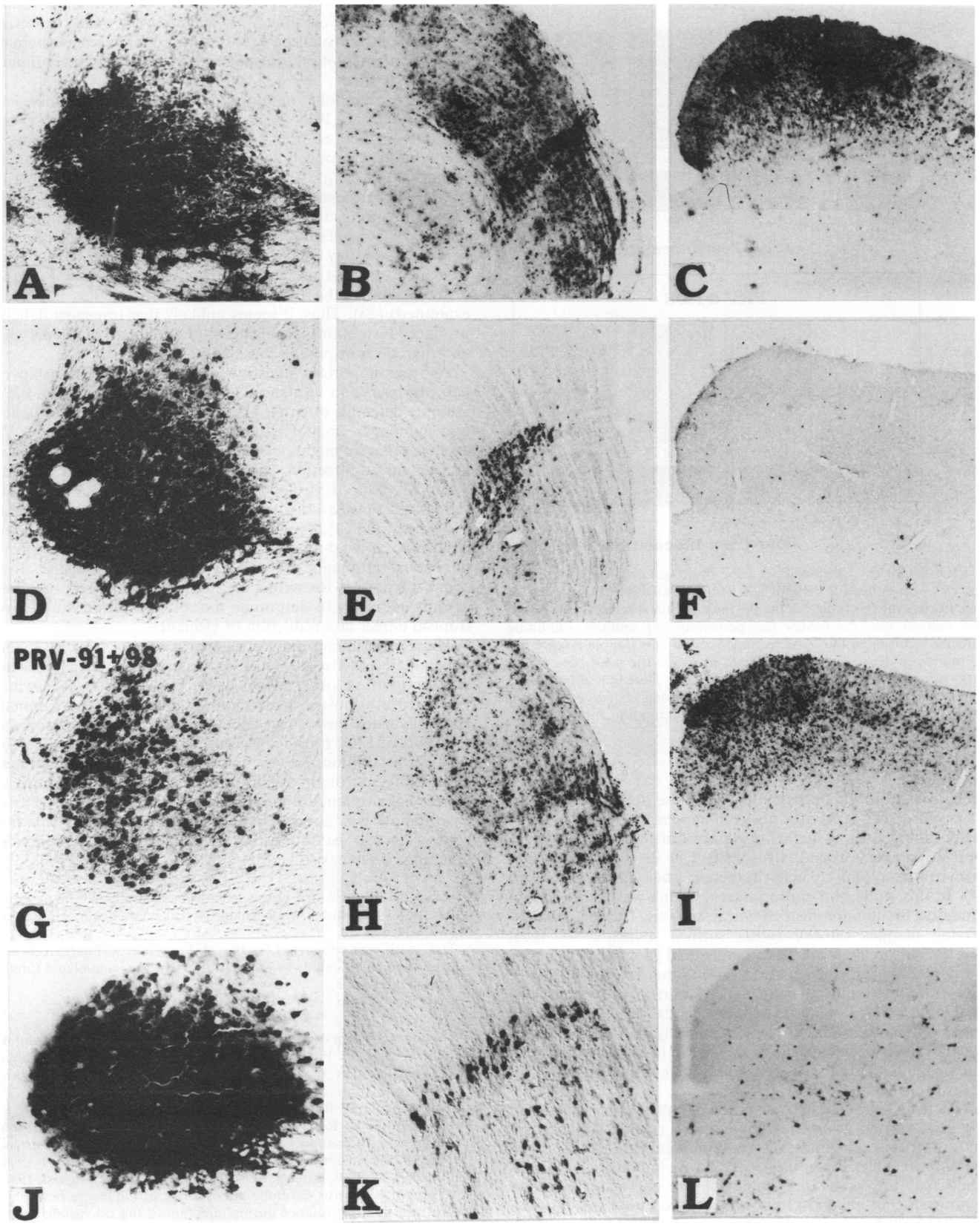
Previously, our only assay for functional complementation was complex formation and increased efficiency of export (27). In this study, the assay for complementation relied on the striking phenotype characterized by the failure of PRV91 or PRV98 to infect neurons in the dorsal geniculate nucleus or optic tectum after retinal infection. If the hypothesis that both gE and gI are required on a single virus particle for entry into this functionally distinct subset of ganglion cells is correct, then a 1:1 mixture of PRV91 and PRV98 should not be able to infect the optic tectum or dorsal geniculate nucleus (no complementation). However, if entry were not defective, and instead the gE and gI mutants could not carry out a later step in the primary neurons, then mixed infection might result in complementation allowing subsequent infection of neurons in these areas. Positive complementation would be strong evidence that gE and gI are not required for initial entry into retinal ganglion cells.

A summary of our results is outlined in Table 1, and photomicrographs of representative coronal sections through retinorecipient regions of the neuraxis stained for PRV antigen are shown in Fig. 1. The results are clear: mixed infection of PRV91 and PRV98 gave complementation, as indicated by the robust infection of the dorsal geniculate nucleus and optic tectum that was virtually indistinguishable from wild-type PRV-Be infection (compare Fig. 1A through C and G through I) and clearly different from that of either PRV91 or PRV98 alone (Fig. 1D through F and J through L). Significantly, no complementation was observed in control studies (data not shown) following (i) injection of one eye with PRV91 and the other eye with PRV98, (ii) injection of PRV98 into the eye and infection of the dorsal motor vagal complex in the brain stem via injection of PRV into the wall of the stomach, or (iii) injection of a 1:1 mixture of PRV91 and PRV99 (deletion of both gE and gI) into the vitreous body.

Our interpretation of these results is that gE and gI are not required for entry into the primary neurons but are necessary for subsequent anterograde transneuronal infection of retinorecipient neurons. There are a number of sites at which the absence of these proteins could limit the subsequent transneuronal passage of virus. Perhaps gE and gI or the gE-gI complex is required for anterograde transport of newly replicated intracellular virions or the gE-gI complex participates in the identification of the postsynaptic membrane at synapses. Mettenleiter and colleagues have suggested that gE is involved in release of PRV from infected tissue culture cells (16, 29). It is also possible that gI and gE are required for entry into second-order neurons in the dorsal geniculate nucleus or tectum following virion release from retinal afferents. We do not know if the proteins function as part of the intracellular virion or independently as a separate protein complex. Experiments to test these ideas are in progress.

We considered an alternative hypothesis in which our results would still be consistent with an entry defect. If the mutant viruses could first infect the permissive retinal ganglion cells projecting to the circadian rhythm centers, these cells could release complemented progeny locally in the retina and these viruses could then infect the ganglion cells projecting to other visual centers. We believe that this hypothesis is unlikely for the following reasons. First, the kinetics of appearance of complemented virus in the visual centers is essentially identical to that of the parental virus infection (Fig. 2). Introduction of a second infection cycle as required by the alternative hypothesis would be expected to result in delayed kinetics of virus appearance in the optic tectum. Second, our previous studies of the infectivity of this circuitry (2, 4, 27) have demonstrated that circadian centers become infected approximately 24 h after infection of the dorsal geniculate nucleus and tectum. If this reflects slower infection or replication in the retinal ganglion cell, then the kinetics of appearance of comple-

FIG. 1. Differential patterns of infectivity in the rat visual system after single and mixed infections of PRV gE and gI null mutants. Coronal sections of brains from intraocularly infected animals were prepared for immunoreactivity as described in the text and Table 1, footnote *a*. All photos were taken at the same magnifications and settings. Viral antigen was detected in the suprachiasmatic nucleus (left column), geniculate complex (center column), and optic tectum (right column) by polyvalent serum Rb134 made against inactivated PRV virions. Each row shows results for one infected animal; animals were infected with PRV-Be (A to C), PRV91 (D to F), a 1:1 mixture of PRV91 and PRV98 (G to I), and PRV98 (J to L). PRV-Be infects all the retinorecipient regions while PRV91 and PRV98 infect only the circadian centers (suprachiasmatic nucleus and intergeniculate leaflet). Note that the mixed infection (G to I) pattern is similar to that found for the wild-type PRV-Be infection (A to C). The scattered positive cells seen in panels F and L reflect transneuronal passage of virus through neurons of the oculomotor and Edinger-Westphal nuclei, which were infected by leakage of virus from the posterior chamber of the eye into the orbit (4).


A
B
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PRV-91+98
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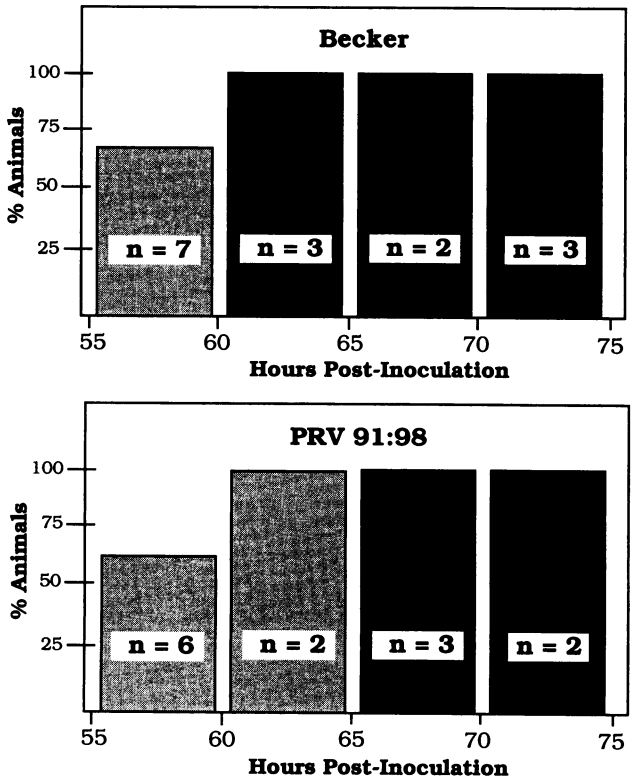

FIG. 2. Kinetics of infection of the optic tectum after injection of the eye with either PRV-Be or a 1:1 mixture of PRV91 and PRV98. The horizontal axis indicates the postinoculation survival interval, and the vertical axis designates the percentage of animals exhibiting infected neurons in the optic tectum. Shaded bars denote a scattered to moderate density of infected neurons, while the solid bars reflect large numbers of infected neurons (cf. Fig. 1C). Note that although the numbers of infected neurons differ slightly among the two experimental groups, there was no difference in the temporal appearance of infected neurons in the optic tectum.

mented virus in the optic tectum would be delayed by at least 24 h. Third, wild-type virus does not appear to spread focally in retinal infections, as might be expected if nonspecific release were occurring. Instead, virus spread in the retina occurred primarily in columns of cells predicted to be synaptically linked (4). In this study, the entire infected retina was removed and mounted for immunohistochemical analysis. Rather than observing plaques on the retina surface indicative of focal cell-to-cell spread, we observed individual ganglion cells which were infected and well separated from other infected cells (4). Therefore, the temporal course and pattern of central nervous system infection as well as the lack of focal cell-to-cell spread in the retina are incompatible with an early infection of retinal ganglion cells projecting to circadian centers that would produce complemented virus for subsequent retinal infection of neurons projecting to visual centers.

It is formally possible that some recombination occurred in the primary neuron in the retina, giving rise to wild-type gE and gI viruses, and that these recombinants could then have gone on to infect the visual circuits. It is important to understand that this possibility would still be consistent with the hypothesis that gE and gI are not required for entry into the primary neuron, since both viruses have to enter one cell to recombine. Even so, recombinants are predicted to be rare because the recombination event required to create a wild-type virus must occur within less than 113 bp of homology present between the two deletions (27). Because we see such extensive infection of the visual centers after mixed infection, we believe that complementation and not recombination is the primary event.

There are a number of important unanswered questions. For example, why are PRV gE and gI not required for transsynaptic infection of all neurons? We have observed no requirement for these gene products for infection of neural centers innervating the viscera (20) and the tongue (data not shown), and they are not necessary for these mutant strains to infect visual circuits involving circadian function. However, in recent experiments, we have observed that injection of gE mutants into the heart leads to only restrictive infection of a subset of the brain stem neurons that are infected by identical injection of the wild-type virus (the role of gI in this system has not been examined) (23). Thus, it seems unlikely that transport deficits produced by mutants lacking gE or gI are due to problems with anterograde transport in these cells.

Another important question concerns the relationship of neurotropism and virulence. It is well established that PRV mutants defective in either gE or gI exhibit reduced virulence in a number of animal systems (1, 6, 10, 13, 15, 17, 19). We expected that we might observe complementation for virulence in our mixed-infection experiments since we had already observed reduced virulence for PRV91 and PRV98 in our retina infection paradigm compared with that of the parental PRV-Be strain (3). We have not observed enough animals to determine if wild-type virulence is restored in the mixed infections, although preliminary observations suggest that complementation for virulence is only partial at best. Further work is necessary to determine the relationship of the neurotropism defect and reduction of virulence.

These experiments may have general relevance for PRV and the alphaherpesviruses. While our experiments were done with rats, supporting observations have been made with swine, PRV's natural host. For example, Jacobs et al. and Kimman and colleagues have suggested that gE is important for the transport of PRV through the porcine central nervous system (6, 10). In addition, Kovacs and Mettenleiter have suggested that gE plays a role in organ tropism (11). Since alphaherpesviruses have retained homologs of gE and gI (5, 8, 9, 12) yet have unique host specificity, tropism, and patterns of pathogenesis, it will be interesting to determine what common functions, if any, exist.

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