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Neurovirulence of glycoprotein C(gC)-deleted bovine herpesvirus type-5 (BHV-5) and BHV-5 expressing BHV-1 gC in a rabbit seizure model

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Herpesvirus glycoprotein C (gC) is one of the major virus attachment proteins. Bovine herpesvirus type 1 (BHV-1) causes respiratory and genital diseases in cattle, whereas BHV-5 causes acute meningoencephalitis in calves. The gC gene sequence of these two viruses are substantially different. To determine the contribution of the BHV-5 glycoprotein gC (gC5) to the neuropathogenesis of BHV-5, we have constructed two BHV-5 recombinants: gC-deleted BHV-5 (BHV-5gCΔ) and BHV-5 expressing BHV1 gC (BHV-5gC1). Neurovirulence properties of these viruses were analyzed using a rabbit seizure model that distinguishes BHV-1 and -5 based on their differential neuropathogeneses. Intranasal inoculations of BHV-5gCΔ and BHV-5gC1 viruses produced neurological signs in 30% and 40% of the infected rabbits, respectively. Immuno-histochemistry results showed that the number of infected neurons was 2 – 4-fold less with the gC-deleted BHV-5 than with the wild-type BHV-5. The gC-deleted BHV-5 did not invade the hippocampus but invaded additional sites not invaded by wild-type BHV-5. Similarly, the BHV-5gC1 virus failed to invade the hippocampus, but it did not invade the additional sites. Virus isolation results suggest that these recombinants replicate less efficiently in the brain than the wild-type and gC-revertant viruses. However, compared to the gC-deleted BHV-5, the gC-exchanged BHV-5gC1 replicated better within the CNS. These results indicate that gC regulates BHV-5 neurotropism in some areas of the olfactory pathway. Additionally, gC is important for BHV-5 neurovirulence in the olfactory pathway but it is not essential. *Journal of NeuroVirology* (2000) **6,** 284 – 295.

Keywords: glycoprotein C; neurovirulence; BHV-5

Introduction

Bovine herpesvirus type 5 (BHV-5) is a neurovirulent α-herpesvirus that causes fatal encephalitis in calves (Belknap *et al*, 1994; D'Offay *et al*, 1993). Non-neurovirulent bovine herpesvirus type 1 (BHV-1) is associated with abortions and respiratory infections (subtype 1.1) and genital infections (subtype 1.2) in cattle (Wyler *et al*, 1989). Both BHV-1 and BHV-5 are neurotropic viruses that establish latency in the trigeminal ganglion (TG) following intranasal and/or conjunctival inoculation (Ashbaugh *et al*, 1997; Rock *et al*, 1986). Although BHV-1 and BHV-5 share 85% DNA homology, they differ in their ability to cause neurological disease in calves (Belknap *et al*, 1994). In a rabbit seizure model, non-neurovirulent BHV-1.1 and neurovirulent BHV-5 infections displayed differential neuropathogeneses (Chowdhury *et al*, 1997). Following intranasal inoculation, BHV-5 invades the brain via the olfactory pathway resulting in acute neurological disease in rabbits comparable to that in calves (Belknap *et al*, 1994). Viral antigen and neuronal damage are present in the affected rabbit brains within areas connected through the olfactory pathway (Lee *et al*, 1999). These areas include the olfactory bulb, anterior olfactory nucleus, piriform/entorhinal cortex, frontal/cingulate cortex, hippocampus/dentate gyrus, amygdala, dorsal raphe and locus coeruleus. Although these rabbits have a few infected neurons in the TG, further invasion of the virus to the pontine and spinal trigeminal nuclei

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(second order neurons in the trigeminal pathway) within the pons and medulla, respectively, does not occur (Lee *et al*, 1999). In contrast, BHV-1 does not invade the central nervous system (CNS) of inoculated rabbits; however, infected neurons are detected in the TG (Lee *et al*, 1999).

Herpesvirus envelope glycoproteins play important roles in pathogenicity by mediating entry of the virion into the host cell, maturation of the virus, cell-to-cell spread of virus, and virus release (Card *et al*, 1992; Dingwell *et al*, 1994, 1995; Enquist *et al*, 1999; Mettenleiter *et al*, 1987; Rajcnai *et al*, 1990; Yuhasz and Stevens, 1993). Alpha-herpesviruses, including BHV-1, encode at least 11 envelope glycoproteins (Roizman and Sears, 1996), and all of these likely have counterparts in BHV-5. Primary attachment of BHV-1, equine herpesvirus type 1 (EHV-1), herpes simplex virus (HSV), and pseudo-rabiesvirus (PRV) to target cells is mediated by glycoprotein C (gC) binding to cell surface glycosamine glycans (Herold *et al*, 1991; Liang *et al*, 1991; Karger and Mettenleiter, 1993; Mettenleiter *et al*, 1990; Okazaki *et al*, 1991). This binding is sensitive to treatment with heparin or heparinase (Liang *et al*, 1993; Mettenleiter, 1994; Okazaki *et al*, 1991, 1994; WuDunn and Spear, 1989). In the case of HSV and BHV-1, the binding of the virion to cell surface proteoglycan is mediated additionally by gB (Byrne *et al*, 1995; Herold *et al*, 1994; Li *et al*, 1995). BHV-1 gC is also important for efficient viral replication *in vitro* and *in vivo* in cattle (Liang *et al*, 1991, 1992). Like HSV and PRV, BHV-1 gC binds complement component C3b and prevents neutralization of the virus by complement (Eisenberg *et al*, 1987; Huemer *et al*, 1993; Kubota *et al*, 1997; Mettenleiter, 1994; Schreurs *et al*, 1988). Thus, gC helps the virus to circumvent an important defence system of the host and allows immune evasion early in infection, before antibodies develop (Friedman *et al*, 1996; Harris *et al*, 1990; Huemer *et al*, 1993; Lubinski *et al*, 1998).

Despite the involvement of gC in the early steps of infection, efficient viral replication, and immune evasion, the gCs of HSV-1, PRV, EHV-1 and BHV-1 are dispensable for *in vitro* virus growth (Karger *et al*, 1995; Liang *et al*, 1992; Osterrieder, 1999; Robbins *et al*, 1986; Whealy *et al*, 1988). In their natural hosts, gC-negative PRV and BHV-1 remain virulent (Kritas *et al*, 1994a,b; Liang *et al*, 1992). Therefore, gC is not the only virulence determinant in α-herpes viruses, and some gC functions may be compensated for by other glycoproteins (Banfield *et al*, 1995; Herold *et al*, 1994; Kritas *et al*, 1994a,b; Mettenleiter *et al*, 1987, 1988).

The nucleotide and predicted amino acid sequences of BHV-1 gC (gC1) and BHV-5 gC (gC5) differ significantly at the N-terminal third of the protein (Chowdhury, 1995). The biological significance of these differences is unknown. Therefore, we investigated the contribution of gC5 to neuroinvasion and neurovirulence of BHV-5. Two gC-recombinant viruses, gC-deleted BHV-5 and gC-exchanged BHV-5 (carrying BHV-1 gC) were constructed and evaluated for neuropathogenicity in a rabbit seizure model (Chowdhury *et al*, 1997). The systemic spread of the various mutant viruses within the CNS was evaluated at different post-inoculation survival times by immunohistochemistry, histopathology, and virus isolation.

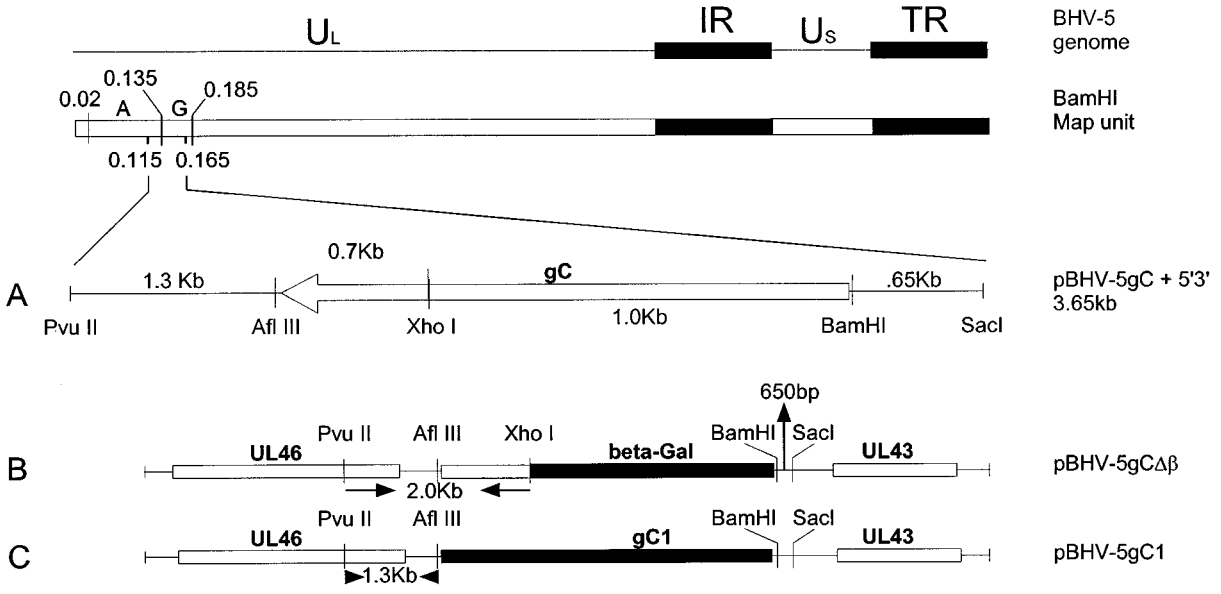


Figure 1 BHV-5 genomic structure and schematic maps of gC recombinant plasmids. The genomic organization of BHV-5 depicted on top consists of unique long (U_L) and short (U_S) regions and two repeat regions (I_R) and (T_R). The region encompassing the gC gene (*Sac*I/*Pvu*II) is enlarged (**A**). The arrow represents the BHV-5 gC ORF, and the direction of transcription. (**B**, **C**) illustrate the regions of interest showing the schematic structures of the plasmids pBHV-5gCΔ*β* and pBHV-5gC1, respectively.

Results

Construction and analysis of BHV-5 gC-deleted, BHV-5 gC-revertant, and gC-exchanged viruses

The DNAs from two gC-deleted recombinant BHV-5 (BHV-5gCΔ1 and 2) and wild-type BHV-5 were analyzed by Southern blot hybridization (Figure 2). The absence of the sequence specified by the 1 Kb *Bam*HI/*Xho*I fragment and the presence of the β-gal gene sequences in the gC-deleted recombinant isolate but not in wild-type BHV-5 demonstrated that the intended recombination in these isolates had taken place in a site-specific manner. Additionally, the 97 kD BHV-5 gC protein was absent in the gC-deleted BHV-5 (Figure 3A lanes 2 and 4; Figure 3D lanes 3 and 4) but was present in the wild-type parent (Figure 3A,D lanes 6 and 2, respectively) and BHV-5 gC-revertant viruses (Figure 3D lane 5). Interestingly, the revertant virus produced approximately twofold less gC (Figure 3D lane 5) than the wild-type BHV-5 (Figure 3D lane 2).

Expression of BHV-1 gC in the gC-exchanged BHV-5 was verified by immunoblotting with BHV-1 gC-specific MAb F2 (Chowdhury, 1995). The MAb F2 reacted with the 97 kD BHV-1 gC expressed by wild-type BHV-1 (Figure 3B lane 3) and BHV-5gC1 (Figure 3B lane 5) but not with BHV-5 gC (Figure 3B lane 6).

In vitro *growth characterization of recombinant viruses in MDBK cells*

One-step growth experiments were conducted to compare the growth kinetics of BHV-5 gCΔ and BHV-5 gC1 viruses to those of the parental BHV-5 strain TX-89. The virus growth curve (Figure 4) demonstrates that the time course and yield of infectious progeny of BHV-5 gCΔ differed from those of wild-type and gC-exchanged BHV-5. Whereas titers of the wild-type BHV-5 and BHV-

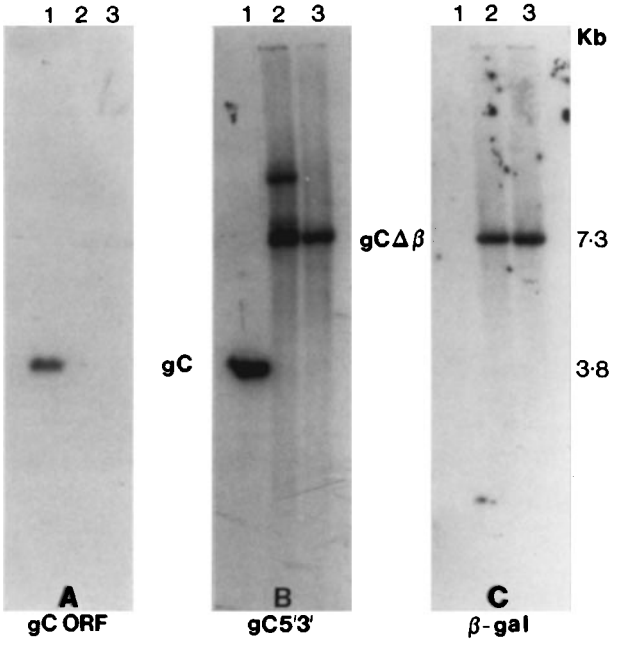


Figure 2 Southern blot analysis of gC-deleted recombinant BHV-5 virus DNA. The DNA from wild-type strain TX89 (lane 1) and from the two recombinants BHV-5gCΔ1 and BHV-5 CΔ2 (lanes 2 and 3, respectively) were digested with *Pst*1 and *Eco*RI restriction enzymes, separated by agarose gel electrophoresis, and blotted to nylon membranes. (**A**–**C**) were probed with a *Bam*HI/*Xho*I fragment containing gC ORF coding (Figure 1A), gC ORF and adjacent flanking sequences (*Sac*I/*Afl*III; Figure 1A), and β-gal sequences, respectively. Note that the recombinant virus DNA bands hybridized in **B** and **C** (marked as gCΔβ) are 3.5 kb larger (as a result of insertion of 4.5 Kb β-gal sequence at the gC locus and deletion of 1 kb gC-specific sequence).

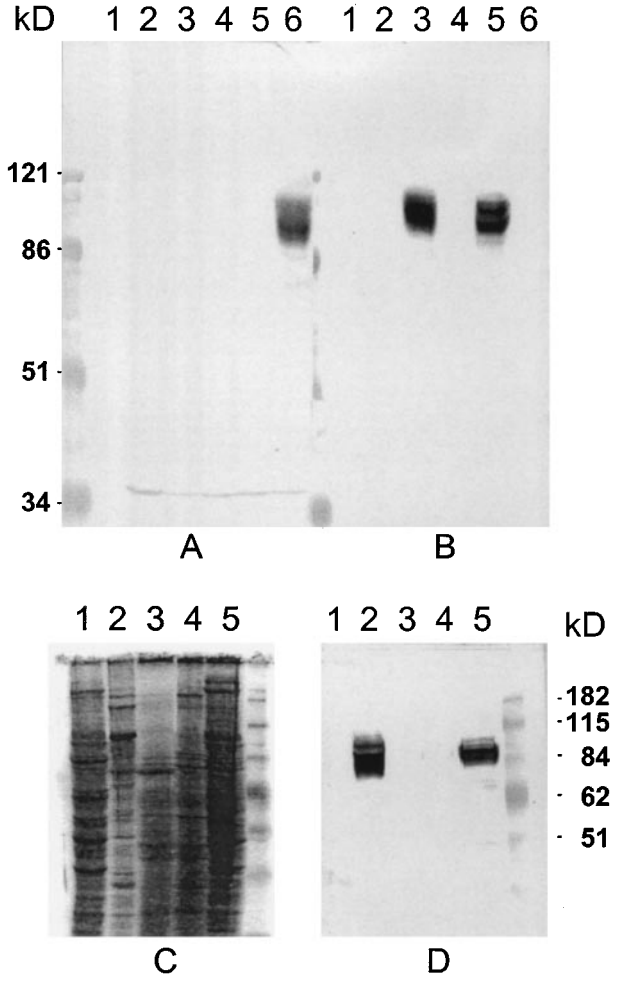


Figure 3 Immunoblotting analysis of BHV-5 gC-deleted and gC-revertant viruses. (**A**, **B**) containing Madin-Darby bovine kidney (MDBK) cell lysates (lane 1), BHV-5gCΔ1 (lane 2), BHV-1 Cooper strain (lane 3), BHV-5gCΔ2 (lane 4), BHV-5gC1 (lane 5) and BHV-5 wild-type strain TX89 (lane 6) were immunoblotted with BHV-5 and BHV-1 gC-specific MAb 8B1 and F2 (Chowdhury, 1995), respectively. (**C**) is a Coomassie blue-stained SDS–PAGE gel containing mock-infected MDBK cell lysates (lane 1), BHV-5 wild-type strain TX89 (lane 2), BHV-5gCΔ1 (lane 3), BHV-5gCΔ2 (lane 4) and BHV-5gC-revertant (lane 5). (**D**) is the immunoblot of **C** with BHV-5 gC-specific MAb 8B1.

5gC1 at different time points were similar, the amount of BHV-5 gCΔ virus produced was reduced 10-fold or more throughout the course of the infection.

Neutralization of gC-deleted BHV-5 by complement alone

To determine the role of BHV-5 gC in protecting virus from antibody-independent complement neutralization, BHV-5 gC-deleted and BHV-5 gC-revertant viruses were incubated with complement-inactivated and untreated fetal bovine and rabbit sera. When complement was inactivated, no obvious reduction occurred in the number of plaques for both the BHV-5 gC-deleted and BHV-5 gC-revertant viruses. In contrast, when these viruses were incubated with untreated sera, a 40–50-fold reduction occurred in the number of plaques for the gC-deleted virus (data not shown). Thus, in the absence of gC, complement markedly neutralized BHV-5.

Pathogenicity of recombinant viruses in rabbits

gC-deleted BHV-5 is neuroinvasive but has reduced neurovirulence and altered neurotropism compared to the gC-rescued or wild-type viruses Five out of 18 rabbits (28%) infected with BHV-5 gCΔ recombinant virus had detectable neurological signs. The remaining 13 rabbits did not develop neurological signs by 13 d.p.i. Virus was isolated from the olfactory bulb (20 p.f.u./g) without further passage in only one rabbit. After passage, virus was isolated from the olfactory bulb (one rabbit), anterior cortex (five rabbits), posterior cortex (two rabbits), and mid brain (two rabbits). Virus was not isolated from the brains of any other rabbit infected with gC-deleted BHV-5 (Table 1). In the nasal mucosa, the gC-deleted virus grew with 10–20-fold less yield when compared with the wild-type (data not shown).

Spread of the gC-deleted BHV-5 in the CNS following intranasal infection was evaluated by immunostaining and compared with that of the wild-type BHV-5 (Figures 5 and 6, and Table 2). Virus-specific antigen was detected in the olfactory bulb by 4–6 d.p.i. (Figure 5). By 8–10 d.p.i., infected neurons were detected in the anterior

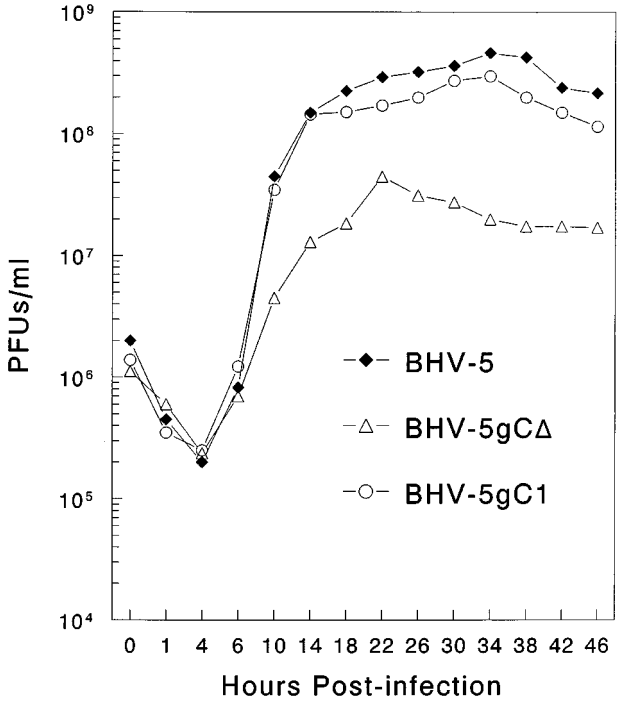


Figure 4 One-step growth curve of BHV-5gCΔ2, BHV-5gC1, and their parent wild-type BHV-5 strain TX89 in MDBK cells. Confluent MDBK cells were infected with viruses at an m.o.i. of 5 p.f.u. After 1 h adsorption at 4°C, residual input viruses were removed. The cultures were washed three times with PBS, and 5 ml of medium were added into each flask before further incubation (37°C). At indicated time intervals, replicate cultures were frozen. Virus yields were determined by titration on MDBK cells. Each data point represents the average of duplicate samples obtained from separate infections.

Table 1 Summary of clinical signs, histopathology findings and virus location

Virus	*Animal number*	*Neurological signs***	*Histopathology*	*Virus isolation from brain*				
				Olfactory bulb	*Anterior cortex*	*Posterior cortex*	*Mid brain+ diencephalon*	*Pons+ medulla*
BHV-5gC revertant	8	Severe (1)			+++ (3)	++ (3)		
		Mild (6)	NT†	+ (3)	++ (1)	+ (1)	+ (2)	+ (1)
BHV-5gC deleted	18	Severe (4)		+* (1)				
		Mild (1)	●●‡	+ (1)	+* (5)	+* (2)	+* (2)	–
				– (16)	– (13)			
BHV-5gC1	13	Severe (2)			+* (3)	+* (2)	+* (2)	
		Mild (3)	●●	+ (2)	+ (2)	+ (2)	+ (2)	+ (2)
				– (11)	– (8)	++ (1)	++ (1)	

*Virus was recovered after repassage of the infected cells. **Numbers in parenthesis indicate number of animals showing clinical signs or brain segments positive and/or negative for the virus isolation. –, No virus detected; +, 10–100 p.f.u./g of tissue; ++, 150–2000 p.f.u./g of tissue. +++, 2100 to 20 000 p.f.u./g of tissue. Histopathology: ●●, diffuse to moderate meningo-encephalitis in the piriform and entorhinal cortices, diffuse lymphocytic infiltration in the piriform and entorhinal cortices, diffuse microgliosis throughout the cortex, perivascular cuffing in the superficial cortex and neuronal degeneration in the cortical pyramidal layers (laminae 2 and 3; NT, not tested; † compared with the previous results (Chowdhury *et al*, 1997); ‡increased gliosis and lymphocytic infiltration.

Table 2 Summary of viral spread in the brain and TG after intranasal inoculation

	Presence and location of viral antigen												
Virus	*OB*	*AON/LOT*	*Pir*	*Hippo*	*Amyg*	*CG*	*NC*	*LC*	*DR*	*LDT*	*VT*	*SN/C*	*TG*
BHV-5 wild-type	++	++++	++++	+++	+++	+++	−	+	++	+	−	−	+*
BHV5Gc deleted	++	++	++++	+*	++	++	++	+	++*	−	++*	+	+*
BHV-5gC1	++*	++	+++	−	+	++	−	−	+	−	−	−	+*

−, no labeling, *less than half of the grading range, + 1−25, ++ 30−150, +++ 160−500, ++++ >500 labeled neurons per field at 5× magnification. OB, olfactory bulb; AON, anterior olfactory nucleus; LOT, lateral olfactory tubercle; Pir, piriform cortex; Hippo, hippocampus; Amyg, amygdala; CG, cingulate cortex; NC, nucleus caudatus; LC, locus coeruleus; DR, dorsal raphe; LDT, lateral dorsal tegmentum; VT, ventral tegmentum; SN/C, substantia nigra compacta; TG, trigeminal ganglion.

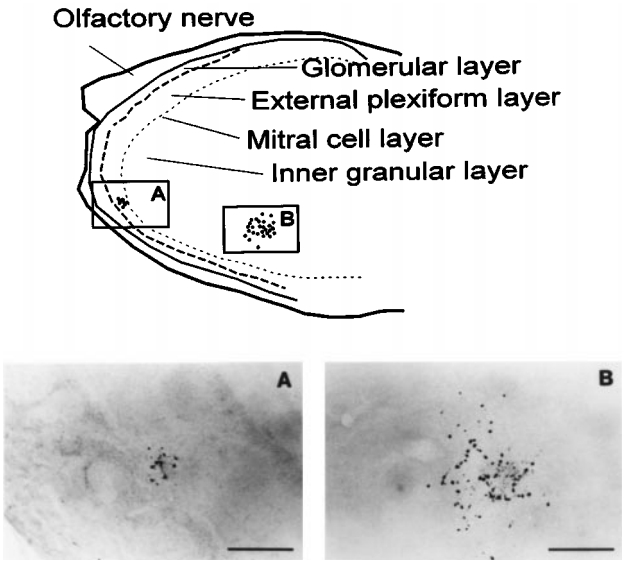


Figure 5 Localization of gC-deleted BHV-5 antigen in the main olfactory bulb at 4−6 d.p.i. Line drawing of the olfactory bulb (sectioned in sagittal plane). Frames indicate areas shown in micrographs, indicating with dots the immunostained neurons. Immunostained cells had dense black precipitate over the cell nuclei. (**A**) At 4 d.p.i., immunostained neurons occurred in the glomerular layer, the external plexiform, mitral cell layer, and inner granular neurons. Detail of boxed area under higher power magnification. (**B**) At 6 d.p.i., immunostained neurons occurred in the inner granular neurons. Scale bars: 200 μm.

olfactory nucleus (>500/field), piroform cortex (>500/field), amygdala (50−100/field), hippocampus (5−10 or none/field), cingulate cortex (50−100/field), caudate nucleus (50−100/field), locus coeruleus (5−10/field), dorsal raphe (30−60/field), substantia nigra compacta (25−50/field), ventral tegmentum (10−20/field) (Figure 6), and TG (2−10/field). The pontine and spinal nuclei (2nd order neurons in the trigeminal pathway) at the pons and medulla did not contain infected neurons (data not shown).

Seven out of eight (87%) of rabbits infected with BHV-5 gC-revertant virus had variable neurological signs (Table 1). With the exception of one rabbit that showed seizures, the neurological signs were less severe than the wild-type, but notably more than those elicited by the gC-deleted virus. The amount of virus isolated from the brain was greater compared to the gC-deleted virus (Table 1).

Rabbits infected with the wild-type BHV-5 had 2−4-fold more immunostained neurons in the olfactory bulb, anterior olfactory nucleus, piriform cortex, amygdala, cingulate cortex, and dorsal raphe compared to the gC-deleted virus. In the hippocampus and dentate gyrus, approximately 500−1000-fold more neurons were labeled. The wild-type BHV-5 did not invade the caudate nucleus, substantia nigra, and ventral tegmentum (Table 2).

gC-exchanged BHV-5 does not show a significant increase in neurovirulence and with the exception of hippocampus, spreads to all the neuronal sites affected by the wild-type BHV-5 Five of 13 rabbits (40%) infected with BHV-5C1 had detectable neurological signs by 13 d.p.i. Virus was isolated without passage from the olfactory bulbs (two rabbits), anterior cortices (two rabbits), pons/medulla (two rabbits), posterior cortices (three rabbits) and midbrain+diancephalon blocks (three rabbits). After passage, virus was isolated additionally from the anterior cortices (three rabbits), posterior cortices (two rabbits) and midbrain/diencephalon blocks (two rabbits) (Table 1). In the nasal mucosa, gC-exchanged BHV-5 grew with very similar yield when compared with the wild-type BHV-5 (data not shown).

By 6 d.p.i., immunostained neurons were present in the olfactory bulb in numbers comparable to those in rabbits infected with gC-deleted BHV-5. By 8 and 10 d.p.i., virus infected neurons were present in the anterior olfactory nucleus (150−300/field), piriform cortex (400−500/field), amygdala (10−20/field), cingulate cortex (50−100/field), and dorsal raphe (10−20/field). The hippocampus, dentate gyrus caudate nucleus, substantia nigra compacta, and ventral tegmentum were not infected (Table 2). Thus, with the exception of the hippocampus and dentate gyrus, BHV-5gC1 invaded similar sites as the wild-type.

Histopathology Histopathology of the rabbit brains infected with gC-deleted and gC-exchanged BHV-5 was qualitatively similar. Each group showed

variable degrees of neuronal necrosis and degeneration within regions of viral invasion. Neuropil in these areas frequently was rarefied, and mild to moderate gliosis occurred. Variable-sized cuffs of lymphocytes were present around blood vessels in the affected regions. Similarly, lymphocytic infiltrates were present within the adjacent meninges. The gliosis and lymphocytic infiltration seemed to be quantitatively greater in rabbits infected with the gC-deleted virus (Figure 7B) compared to those infected with the gC-exchanged and wild-type BHV-5 viruses (Figure 7 panels A and C, respectively). Gliosis and lymphocytic infiltration in the gC-exchanged and wild-type BHV-5-infected rabbits were similar.

Discussion

Following intranasal infection of rabbits, BHV-5 invades the CNS via the olfactory pathway (Chowdhury *et al*, 1997; Lee *et al*, 1999). Results of this study indicate that the gC-deleted virus is neuroinvasive but show altered neurotropism. Additionally, the virus replicated within the CNS with reduced yield compared to the wild-type. Therefore, gC is important for neurotropism and full neurovirulence potential of the virus but is not essential for BHV-5 neuroinvasiveness and neurovirulence.

Differences were observed in the neurotropism and neurovirulence of the gC-deleted and wild-type viruses. The gC-deleted BHV-5 virus invaded 3rd, 4th, and/or 5th order neurons in the olfactory pathway, but fewer neurons in these areas were infected compared with wild-type BHV-5 (Lee *et al*, 1999). Only a few or no hippocampal neurons were invaded; in contrast, these neurons are consistently invaded by wild-type BHV-5. Additionally, the gC-deleted virus invaded the caudate nucleus, substantia nigra, and ventral tegmentum, anatomical regions usually not invaded by wild-type BHV-5 (Lee *et al*, 1999).

Spread of the gC-deleted virus to the caudate nucleus could be due to anterograde transport of the virus from the piriform and entorhinal cortices, regions that send afferent imputs to the stratum globus pallidus and caudate putamen (Zahn and Heimer, 1987; Zahn *et al*, 1987). Subsequently, the virus could spread retrogradely to the substantia nigra and ventral tegmentum, both of which have efferent connections to the caudate nucleus (Zahn and Heimer, 1987; Zahn *et al*, 1987). Although abundant virus was detected in the anterior olfactory nucleus (AON) and piriform/entorhinal cortices (regions connected to the hippocampus and dentate gyrus), the neurons within the hippocampus/dentate gyrus were not infected. Similarly, areas in the CNS connected to the piriform cortex (e.g., the medial septum, the lateral hypothalamus and the diagonal band of Borca) were not invaded by the gC-deleted virus. This failure to infect neurons in the hippocampus and the selective invasion of additional sites could be due to altered neurotropism of the gC-deleted virus within the olfactory pathway. The neurotropism could be altered by specific interactions of other viral proteins, such as gB, which replaces the attachment function of gC in its absence (Byrne *et al*, 1995; Herold *et al*, 1994; Li *et al*, 1995).

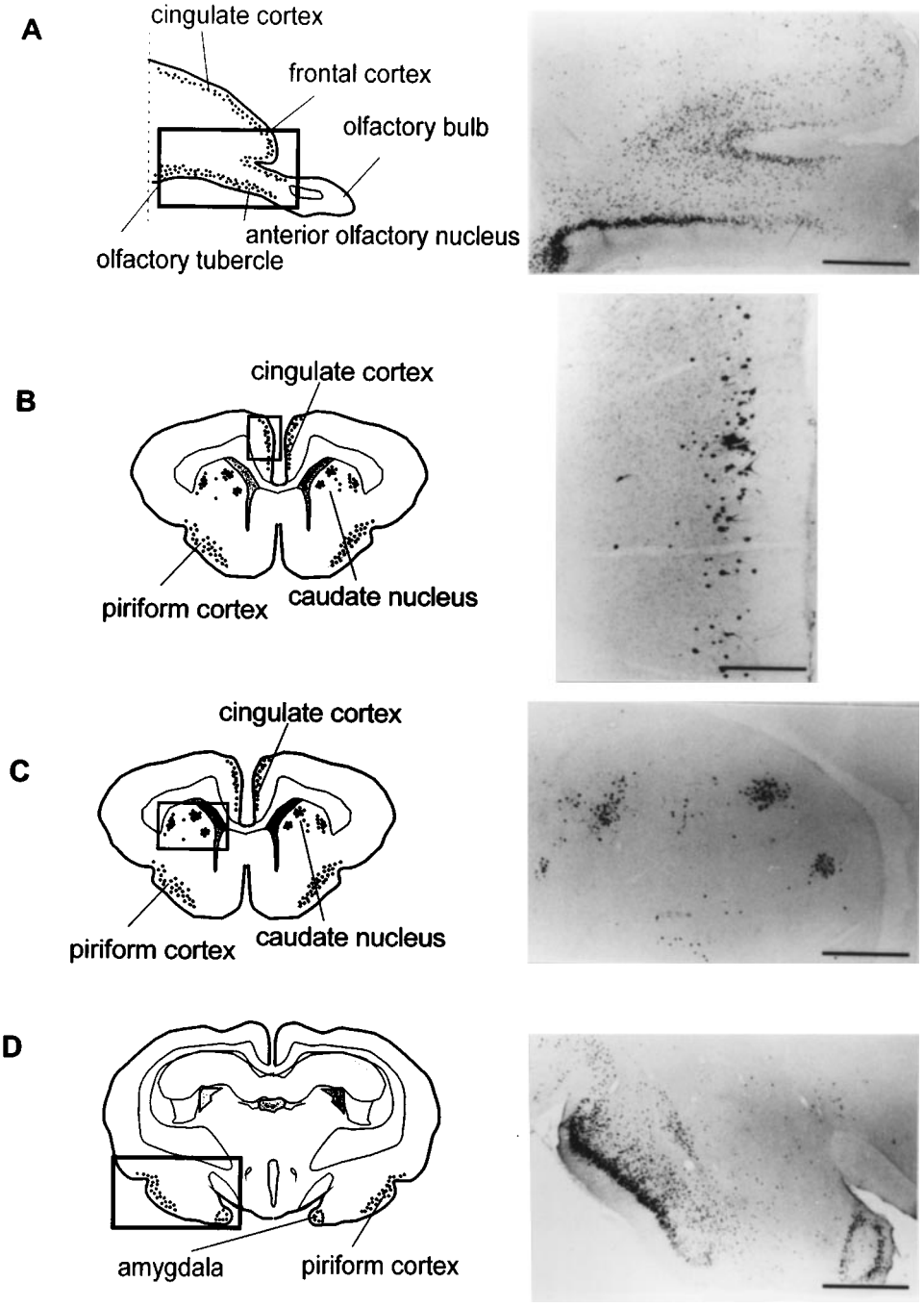
In vitro characteristics of BHV-5gC1 were similar to those of wild-type BHV-5, indicating that BHV-1 gC can complement BHV-5 gC. However, *in vivo* characteristics of the virus indicated that gC1 did not complement gC5 with respect to hippocampal distribution of infected neurons. Alternatively, BHV-5 expressing gC1 may differ in its neurotropism in the olfactory pathway with respect to the hippocampus.

For the gC-deleted and gC-exchanged BHV-5, 28% and 40% of infected rabbits, respectively, had neurological signs. These signs were slightly more severe in the gC-exchanged-infected rabbits. In contrast, 70–80% of rabbits infected with the BHV-5 gC-revertant virus had neurological signs. Whereas higher amounts of virus were isolated from rabbits infected with gC-rescued BHV-5, only small amounts of virus were isolated from rabbits infected with gC-deleted BHV-5. Slightly more virus was isolated from rabbits infected with BHV-5gC1 than from those infected with gC-deleted BHV-5. Thus, the neurovirulence of the BHV-5gC1 virus was slightly greater than that of the gC-deleted virus but less than that of the wild-type BHV-5. A recent study found that gC1 and gC5 differ in their receptor binding properties; a 10-fold higher concentration of heparin was required to block BHV-5 and BHV-1 expressing gC5. However, gC5 alone did not transfer the neurovirulence properties of BHV-5 to BHV-1 (Liman *et al*, 1998, personal communication). Collectively, these results indicate that with the exception of the hippocampus and dentate gyrus, BHV-5 gC is not required for viral entry and subsequent spread within higher order neurons of the olfactory pathway. However, it is beneficial for efficient virus replication in the brain, a feature that could not be complemented fully by gC1.

Although the severity of neurological disease was greater in rabbits infected with BHV-5 gC-revertant virus and they yielded more virus than those infected with the gC-deleted virus, the revertant virus was less neurovirulent and yielded less virus than wild-type BHV-5 (Chowdhury *et al*, 1997; Lee *et al*, 1999). The lower virus yield and neurovirulence may have been due to the reduced amount of gC produced by the revertant virus (Figure 3D). Further studies are needed to address this question.

Histologic lesions in rabbits infected with gC-deleted and gC-exchanged BHV-5 viruses were similar to wild-type BHV-5. However, when rabbits

in each group were viewed collectively, there was a slight collective increase in gliosis and lymphocytic infiltration in rabbits infected with gC-deleted virus compared to those infected with wild-type and BHV-5gC1 virus. These differences may reflect individual animal variation; alternatively, they could indicate a slightly more aggressive host response to the gC-deleted virus compared to the gC-containing viruses. One possible explanation for the increased host reaction could be a slower rate of host cell penetration and spread that would provide greater opportunity for exposure of the host to viral antigens. Another possibility is an increased virus neutralization and lysis of virus-infected cells by antibody-dependent and antibody-independent complement pathways. This function has been mapped to gC and its homologs in HSV-1 and -2, PRV, and BHV-1, all of which can bind to complement component C3b (Friedman *et al*, 1996; Harris *et al*, 1990; Huemer *et al*, 1993; Lubinski *et al*, 1998). Thus, in the absence of gC, mutant α-herpesviruses are susceptible to rapid



complement-mediated virus neutralization and host cell lysis (Friedman *et al*, 1996; Lubinski *et al*, 1998). Together, a greater opportunity for exposure of the host to viral antigens and increased complement activity could result in increased host response and viral clearance. These assumptions

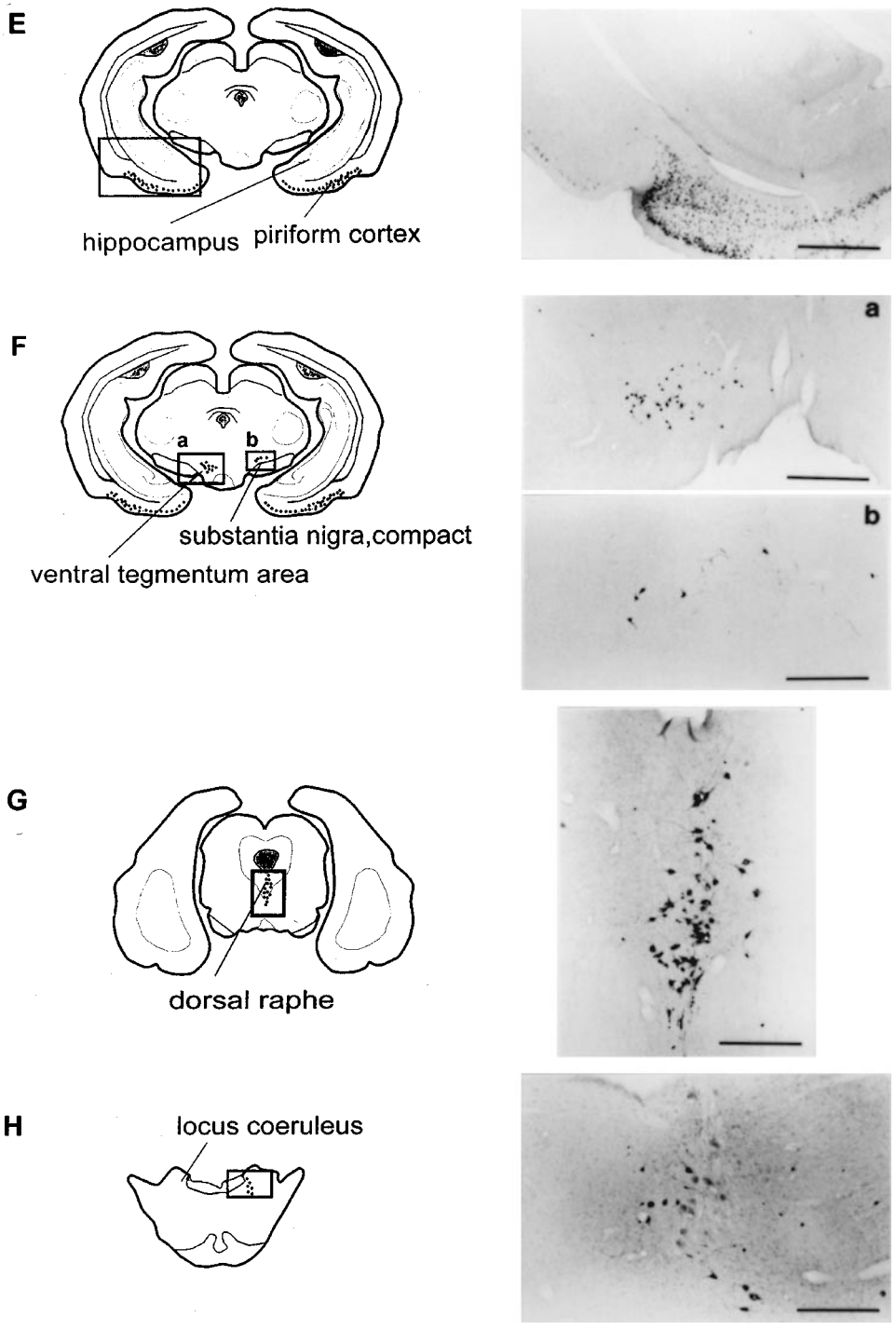


Figure 6 Localization of gc-deleted BHV-5 antigen in the rabbit brain at 8 d.p.i. Schematic representation of the rabbit brain depicting the CNS distribution of BHV-5-positive neurons with black dots indicating infected areas. Frames indicate areas shown in bright-field photo-micrographs where immunostained neurons had dense black precipitate over the nuclei. (**A**) Lateral olfactory tubercle (LOT), anterior olfactory nucleus (AON) and frontal cortex; (**B**) cingulate cortex; (**C**) caudate nucleus; (**D**) piriform cortex and amygdala; (**E**) piriform cortex and hippocampus; (**F**) substantia nigra compact and ventral tegmentum area; (**G**) dorsal raphe and (**H**) locus coeruleus. Scale bars: 1600 μm (**A**, **D**, **E**), 800 μm (**C**, **F** part a) 400 μm (**B**, **F** part b, **G**, **H**).

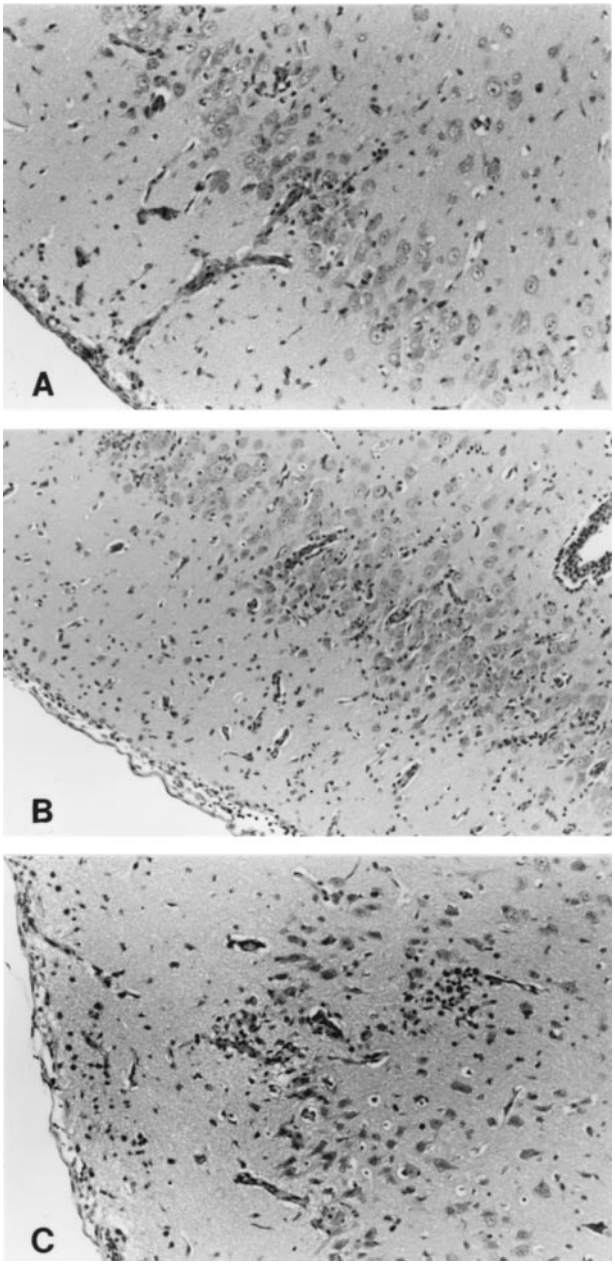


Figure 7 Representative histopathology of the ventral cortex near the level of the hippocampus from rabbits infected with the BHV-5gC1 (**A**), gC-deleted BHV-5 (**B**), and the wild-type BHV-5 (**C**) viruses. Note that gliosis and lymphocytic infiltrates were slightly increased in rabbits infected with the gC-deleted virus (**B**). Hematoxylin and eosin, ×60.

correlate well with the virus isolation results for the gC-deleted BHV-5.

We conclude that gC regulates BHV-5 neurotropism in some areas of the olfactory pathway. Additionally, gc is important for the expression of BHV-5 full neurovirulence potential, but it is not essential. Recently, we reported that BHV-5 gE plays a significant role in neuroinvasiveness and neurovirulence of BHV-5 (Chowdhury *et al*, 2000). However, gE5 alone did not transfer the neurovirulence properties of BHV-5 to BHV-1. It is possible that BHV-5 gC in concert with other viral proteins, such as gE, may contribute to the differential neuropathogenesis of BHV-5. Herpesviruses containing deletions of both gE and gC are severely attenuated compared to those with a single gene deletion (Kudelova *et al*, 1991). Therefore, both the gC and gE genes may be necessary for optimal viral neuroinvasiveness and neurovirulence.

Materials and methods

Virus strains and cell lines

The BHV-1 Cooper (Colorado-1) strain (American Type Culture Collection; Rockville, MD, USA) and BHV-5 TX-89 strain (D'Offay *et al*, 1993) were used in this study. The two viruses were propagated and titrated in Madin-Darby bovine kidney (MDBK) cells grown in Dulbecco modified Eagle's medium (DMEM) supplemented with heat-inactivated 10% fetal bovine serum.

Construction of BHV-5 gC deletion/β-gal insertion plasmid

The BHV-5 gC ORF maps between .125 and .135 on the BHV-5 genome within the *Bam*HI 'A' fragment (Figure 1). The 5′ gC upstream sequences are contained within the *Bam*HI 'G' fragment (map units .135–.185). To assemble the BHV-5 gC gene and its flanking sequence, first a 4.2 Kb *Bam*HI–*Kpn*I subfragment (map units .135–.165) of the *Bam*HI 'G' fragment was cloned into the *Bam*HI–*Kpn*I sites of pUC19. The resulting plasmid was designated pBHV5BmKp 4.2. The pBHV5gC5′ plasmid containing 650 bp 5′ gC flanking sequences was constructed by deleting most of the insert sequences in pBHV5BmKp 4.2 with the enzyme *Sac*I (*Sst*I) collapse. The entire BHV-5 gC-coding and its 3′ downstream sequences contained within the 3 Kb *Bam*HI/*Pvu*II (blunt) (map units .135–.115) was purified and cloned into the *Bam*HI/*hinc*II-digested pBHV5gC5′ clone. The resulting clone was designated pBHV5gC+5′3′ (Figure 1A). To delete the BHV-5 gC ORF sequences the pBHV5gC+5′3′ plasmid was digested with enzymes *Bam*HI and *Xho*I and blunt ended with klenow; then the larger fragment was gel purified and ligated to the 4.5 Kb *Pst*I fragment (blunt ended with T4 DNA polymerase) of pCMV*β* (Clonetech, Palo Alto, CA, USA) containing the HCMV IE promoter regulated *β*-gal gene. This resulted in deletion of the 1.01 kb *Bam*HI/*Xho*I fragment of the pBHV5gC+5′3′ plasmid containing the region coding for the first 300 aa of BHV-5 gC and insertion of the *β*-gal gene cassette in the resulting plasmid pBHV5gCΔ*β* (Figure 1B). The *β*-gal gene cassette is flanked by the BHV-5-specific 650 bp upstream sequences (containing 5′ gC regulatory sequenfes) and 2.0 kb downstream sequences (containing sequences downstream to

the BHV-5gC termination codon) required for recombination with viral DNA.

Construction of BHV-5gC1 exchange plasmid (containing BHV-1 gC ORF coding region)
To exchange the BHV-5gC coding region with the BHV-1 gC coding region, the entire BHV-5gC-coding region that is contained with the 1.7-Kb *Bam*HI/*Afl*II fragment (Chowdhury, 1996) was released. The larger fragment was then gel purified, and ligated to the 1.7 kb *Bam*HI/*Afl*II fragment containing the whole BHV-1 gC ORF (Fitzpatrick *et al*, 1989). The resulting plasmid pBHV5gC1 (verified by restriction mapping and sequencing) contains the identical gC upstream and downstream sequences as in pBHV5gC+5′3′, but their gC coding region is exchanged with the BHV-1 gC ORF coding sequences (Figure 1C).

Generation of recombinant viruses

gC-deleted βgal expressing BHV-5 (BHV-5gCΔ) To obtain a gC-deleted BHV-5 recombinant virus, linearized pBHV5gCΔ*β* plasmid DNA (Figure 1B) and full-length wild-type BHV-5 virus DNA were cotransfected in MDBK cells using lypofectamine (Gibco BRL, Life Technologies, Inc., Grand Island, NY, USA) as described earlier (Chowdhury *et al*, 1999). Recombinant viruses expressing *β*-galactosidase were plaque purified three times by screening for blue plaque under a Bluo-Gal (Gibco BRL, Life Technologies, Inc.) overlay as described previously (Chowdhury, 1996). Two recombinant viruses (BHV-5gCΔ 1 and 2) were characterized further by Southern blot analysis using gC ORF coding, gC flanking, and *β*-gal sequence regions as probes and confirmed by Western blot analysis with anti-BHV-5gC-specific MAb 8B1 (Chowdhury, 1995). BHV-5gCΔ refers to the BHV-5gCΔ2 recombinant virus which was used in the experiments reported here.

gC-exchanged BHV-5 (BHV-5gC1) recombinant
To generate the gC-exchanged recombinant BHV-5 expressing BHV-1 gC, linearized pBHV5gC1 (Figure 1C) and full-length BHV-5gCΔ DNA were cotransfected using the methods described above. Recombinant viruses with the BHV-1gC ORF coding sequences incorporated and the *β*-gal gene deleted were identified as white plaques after screening with Bluo-Gal. These recombinant viruses were plaque purified and analyzed by Western blot with BHV-1 gC-specific MAb F2 (Chowdhury, 1995) for the expression of BHV1gC.

gC revertant BHV-5 To generate the BHV-5 revertant, the plasmid pBHV5gC+5′3′ (Figure 1A) was linearized and cotransfected with full-length recombinant BHV-5 gC-deleted (BHV-5 gCΔ) DNA using the methods described above. The revertant virus, in which the gC deletion was rescued, was identified as white plaques after screening with Bluo-Gal. This recombinant virus was plaque purified and verified by Western blot analysis with the anti-BHV5gC-specific MAb 8B1.

Virus growth curve experiment
One-step virus growth experiments were conducted, as described earlier (Chowdhury, 1996), to compare the growth kinetics of BHV-5gCΔ and BHV-5gC1 to those of wild-type BHV-5. A series of replicate cultures of MDBK cells were infected separately with 5 p.f.u. per cell. Infected cultures were harvested at successive intervals postinfection, and virus stocks were prepared for use in virus titration assays.

Complement-mediated and antibody-independent virus neutralization test
The wild-type and gC-deleted BHV-5 viruses were incubated (1 h at 37°C) with complement inactivated (30 min at 56°C) and untreated normal fetal bovine and rabbit sera that contained no detectable antibodies against BHV-1 and -5. The ability of complement alone to neutralize the virus with and without its gC (wild-type and gC-deleted viruses, respectively) was tested by plaque reduction assay as described previously (Chowdhury *et al*, 1986).

Animal experiments, tissue processing, and immunohistochemistry
Four-week-old New Zealand White rabbits weighing 500–600 g (Myrtles Rabbitry, Thomson Station, TN, USA) were maintained in laboratory isolation cages in our vivarium with food and water freely available throughout the experiments. All procedures conducted in the rabbits were approved by the Kansas State University Animal Care and Use Committee.

Virus isolation and histopathology (experiments 1–3) A rabbit seizure model (Chowdhury *et al*, 1997) was used to determine the neurovirulence properties of gC-deleted BHV-5 (expt. 1), gC-revertant BHV-5 (expt. 2), and BHV-5gC1 (expt. 3). Eighteen, 13, and eight rabbits were infected with gC-deleted BHV-5, BHV-5gC1, and gC-rescued BHV-5, respectively. Inoculation with 2×10^7 p.f.u./0.5 ml/nostril of the respective virus into the paranasal sinuses was conducted as described earlier (Chowdhury *et al*, 1997). Following inoculation, rabbits were observed four times daily for neurological symptoms. Nasal swabs were obtained to determine the efficiency of virus replication in the nasal/olfactory epithelium (Chowdhury *et al*, 1997). A rabbit was sacrificed when it had neurological symptoms or by 14 days post infection (d.p.i.). The brain was removed and divided mid-sagittally; the left half of the brain and the TG were used for virus isolation, and the right half was processed for

histopathological examination (Chowdhury *et al*, 1997). All cultures that lacked cytopathic effects were repassaged to confirm the absence of the virus.

Immunohistochemical processing (experiments 4–5) To determine the neural spread of gC-deleted BHV-5 (expt. 4) and BHV-5gC1 (expt. 5), 12 rabbits were inoculated intranasally with each virus as described above. Rabbits were euthanized on days 2, 4, 6, 8, 10, and 14 d.p.i. or when neurological signs were present. Two rabbits were euthanized at each time period and perfused transcardially by 10% buffered neutral formalin. The brain and TG were used for immunohistochemistry as previously described (Lee *et al*, 1999).

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