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Short communication

Bovine herpesvirus type 1 (BoHV-1) anterograde neuronal transport from trigeminal ganglia to nose and eye requires glycoprotein E

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The requirement of bovine herpesvirus type 1 (BoHV-1) envelope protein gE (Us8 homolog) for establishment of latency and reactivation in trigeminal ganglia (TG) was examined. Although BHV-1 gE-rescued and gE-deleted viruses were isolated from nasal or ocular swabs during primary infection, only the gE-rescued virus was isolated following dexamethasone-induced reactivation. Furthermore, gC protein expression, which requires viral DNA replication for its expression, was detected in TG of calves infected with either virus following reactivation. These studies suggest that gE is required for anterograde transport of BoHV-1 from neuronal cell bodies in the TG to their nerve processes. *Journal of NeuroVirology* (2009) **15,** 196–201.

Keywords: anterograde transport; BoHV-1; glycoprotein E; reactivation from latency

Bovine herpesvirus type 1 (BoHV-1) is an alphaherpesvirus that causes abortions, respiratory infections, and genital infections in cattle (Wyler *et al*, 1989; Tikoo *et al*, 1995). During primary infection, BoHV-1 replicates in the nasal and ocular epithelium, and then capsids together with the tegument enter the sensory nerve endings of the ophthalmic and maxillary branches of the trigeminal nerve located in the nasopharynx and eye. Subsequently, virion particles are transported retrogradely to cell bodies in the trigeminal ganglia (TG) where the virus establishes lifelong latent infection by maintaining episomal form of the viral genomic DNA after a limited replication cycle (Jones, 2003). During latent infection, BoHV-1 latency-related (LR) transcript and the proteins encoded by LR gene can be detected in the TG; however, virus DNA replication and productive infection do not occur (Jiang *et al*, 1998; Jones, 1998, 2003). Periodic reactivation from BoHV-1 latency can lead to nasal and ocular virus shedding. Following reactivation from latency, productive viral gene expression is readily detectable in TG sensory neurons (Jones and Chowdhury, 2007) and infectious virus particles are transported anterogradely from the cell bodies in TG to axon termini in the nasopharynx and eye where they infect epithelial cells, resulting in virus replication and virus shedding (Jones, 2003; Butchi *et al*, 2007). Therefore, the retrograde and anterograde transport mechanisms are crucial for the transmission and maintenance of BoHV-1 in the susceptible cattle population.

Earlier reports on gE (Us8)-deleted BoHV-1 shedding following dexamethasone-induced reactivation are not consistent. In several cases, virus is isolated from nasal and ocular swabs following reactivation (Lemaire *et al*, 2001; Dispas *et al*, 2003), in other cases; the latent virus fails to reactivate and is not shed in the nasal and ocular secretions (Kaashoek

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et al, 1996, 1998). Considering that the Us9 gene is immediately downstream of gE and is required for anterograde viral transport (Butchi *et al*, 2007), deletion of gE coding sequences if not carefully done could have an effect on Us9 gene expression. The previous studies did not consider the status of Us9 expression while analyzing virus isolation following reactivation. Therefore, the present study was undertaken to compare reactivation from latency of a gE-deleted BoHV-1 with an intact Us9 gene in cattle.

To generate a BoHV-1 gE-deleted recombinant virus, first a gE deletion plasmid pBHV-1 gEΔ EGFP (enhanced green fluorescence protein) in which EGFP cassette is flanked by gE upstream and downstream sequences was constructed (Figure 1A).

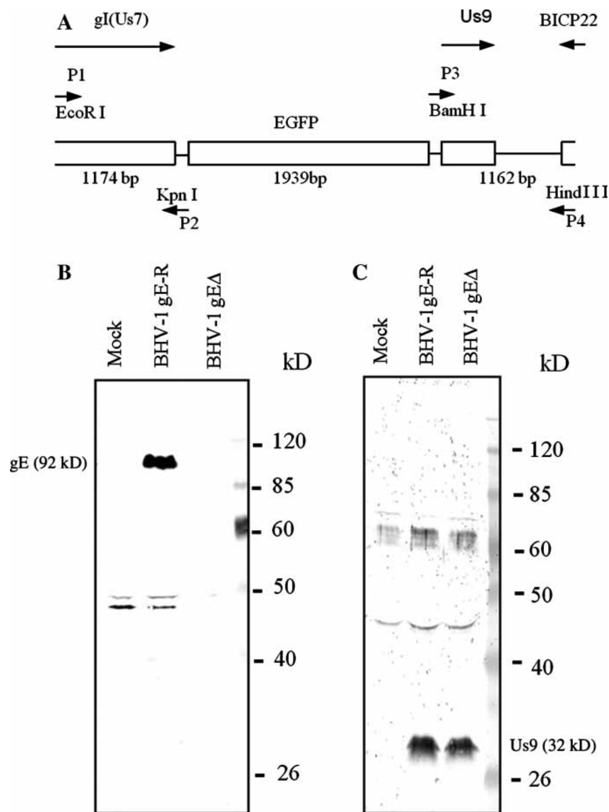

Figure 1 (**A**) Schematic illustration of the construction of gE (Us8 homolog) deletion plasmid pBHV-1 gEΔ-EGFP. Primer pairs P1/P2 (incorporating EcoRI/KpnI) and P3/P4 (incorporating BamHI/HindIII) were used to amplify gE upstream (gE/gI [Us7 homolog] intergenic region and partial gI sequence) and gE downstream (Us9 and partial bICP22 [Us1 homolog]) flanking sequences. (**B**) Characterization of gE-deleted BoHV-1 (BoHV-1gEΔ) and gE-rescued BoHV-1 (BoHV-1gE-R) by immunoblotting with BoHV-1 gE–specific antibody (Chowdhury *et al*, 2000). (**C**) Immunoprecipitation/immunoblotting analysis of Us9 in BoHV-1gEΔ virus–infected cell lysates. Immunoprecipitates (with goat Us9-specifc antibody) were separated by SDS-PAGE and the Western blotting was performed with rabbit Us9-specific antibodies (Chowdhury *et al*, 2006).

Briefly, gE upstream (1174-bp) and downstream (1162-bp) flanking sequences were amplified by long polymerase chain reaction (PCR) using a XL-Long PCR kit (Applied Biosystems, Foster City, CA) as described previously (Chowdhury *et al*, 2006). These PCR-generated fragments introduced EcoRI/KpnI and BamHI/HindIII sites in the gE upstream and gE dowstream sequences, respectively, which were used for cloning and assembling them into pGEM3Z vector (Promega, Madison, WI). In the resulting clone, pBHV-1 gEΔ, the entire gE (Us8) open reading frame (ORF) is deleted and the KpnI and BamHI sites of multiple cloning sites of pGEM3Z are flanked by gE upstream and downstream sequences, respectively. After verifying the nucleotide sequence, an EGFP gene cassette was inserted into the KpnI/BamHI sites of pBHV-1 gEΔ. Briefly, a 1939-bp fragment with KpnI/BamHI enzyme sites at the 5′ and 3′ ends, respectively, was amplified by PCR using primer pairs P5-P6 (Table 1) and plasmid pEGFP/C1-collapse DNA as a template, as described previously (Chowdhury *et al*, 2006), and inserted into the KpnI/BamHI sites of the plasmid pBHV-1 gEΔ, resulting in pBHV-1gEΔ EGFP (Figure 1).

To generate a gE (Us8) ORF-deleted BoHV-1, linearized pBHV-1gEΔ EGFP and full-length wild-type BoHV-1 DNA were cotransfected in Madin-Darby bovine kidney (MDBK) cells using Lipofectamine (Gibco BRL, Life Technologies, Grand Island, NY) as described previously (Chowdhury *et al*, 1999). Recombinant viruses expressing EGFP were plaque purified three times and further verified by immunoblotting with BoHV-1 gE-specific and BoHV-1 Us9-specific antibodies (Figure 1B and C) as described previously (Al-Mubarak and Chowdhury, 2004). To generate a BoHV-1 gE-rescued virus, plasmid clone containing the entire Us (Unique Short) segment of BoHV-1 genomic DNA was linearized and cotransfected with full length recombinant BoHV-1 gE-deleted viral DNA. A gE-rescued virus producing nonfluorescent plaques was isolated by plaque purification three times and further verified by immunoblotting with gE-specific and Us9-specific antibodies (Figure 1B and C). As expected, the gE-deleted virus expressed wild-type levels of Us9 (Figure 1C) but no gE (Figure 1B), and the rescued virus expressed wt levels of gE and Us9. As expected, in infected MDBK cells overlaid with 1.6% carboxymetheyl cellulose, the gE-deleted virus produced smaller plaques relative to the gE-rescued and wild-type BHV-1 (data not shown). Smaller plaques by the gE-deleted virus is due to the defect in the cell-to-cell spread of the virus and the phenomenon is well documented for alpha herpesviruses including BHV-1 (Enquist *et al*, 1999; Chowdhury *et al,* 1999, 2000).

To determine the in vivo infectivity, pathogenicity and latency/reactivation properties of the two viruses, 4-month-old calves were sedated with xylazine (1 mg/kg of body weight) and infected by intranasal and intraocular instillation of 1×10^7

Table 1 List of primers used for PCR reactions

Primer	Name	Sequence
gE upstream	Forward-P1	GCTGCAGACC**GAATTC**GCCTCCTGCCC GCG
	Reverse-P2	CCAAATGCCCTTTT**GGTACC**CTCTCGCGTG CGC
gE downstream	Forward-P3	GCGCGACTCAAGT**GGATCC**TCCGCTAGGCG
	Reverse-P4	CGCCGGCC**AAGCTT**TCGCCAGCGAGTTTAGG
GFP[a]	Forward-P5	CGAACTGA**GGTACC**TACAGCGTGAGC
	Reverse-P6	CGCGTTA**GGATCC**ATTGATGAGTTTGG

[a] Green fluorescence protein gene.
Bold letters indicate restriction sites.

plaque-forming units (PFU) (per each nostril or eye) (Butchi *et al*, 2007). All calves used for this study were cross-bred, weighed approximately 200 kg, and were seronegative for BoHV-1 and BVDV. All procedures involving virus amplification or virus isolation were performed in cultures of bovine kidney cells (MDBK) free of pestivirus or any other adventitious virus or agent. Animal handling and sample collections were performed in accordance with the American Association of Laboratory Animal Care guidelines.

In the first animal experiment, two calves in each group were either infected with BoHV-1 gE-deleted virus or BoHV-1 gE-rescued virus as described earlier (Butchi *et al*, 2007). At 46 days post infection (46 d.p.i.), dexamethasone (dex) (0.5 mg/Kg body weight) injections were given to reactivate the latent viruses as described earlier (Winkler *et al*, 2000; Butchi *et al*, 2007). These calves were sacrificed at 30 days post reactivation (30 dex) and their TG were divided into two halves. One half was frozen and the other half was fixed in phosphate buffered 10% formalin for further analysis.

In the second animal experiment, 12 calves were infected. Six calves in each group were either infected with BoHV-1 gE-deleted virus or with BoHV-1 gE-rescued virus using the same route and dose as described above. From each group, two calves were euthanized at 4 d.p.i. (primary infection), at 38 d.p.i. (latent infection). The remaining two calves in each group were treated with dex as described above at 46 d.p.i. for reactivation of the latent virus and euthanized at 4 days post dex (50 d.p.i.). Following euthanasia, their TG samples were processed as described above. The duration and amount of virus shed in the nose and eye during primary infection, latency, and after reactivation from latency was determined by plaque assays on swabs collected at different times post infection as described earlier (Butchi *et al*, 2007). The results show that during primary infection, nasal and ocular virus shedding of gE-deleted virus was detected for only 4 d.p.i. (Figure 2A and B). However, virus shedding in animals infected with gE-rescued BoHV-1 lasted for 10 d.p.i. in the eye and 17 d.p.i. in the nose (Figure 2A and B). Because the BoHV-1 gE protein does not have a viral Fc receptor function (Whitbeck *et al*, 1996), a relatively shorter period of gE-deleted virus shedding is not due to the lack of BoHV-1 gE Fc receptor function. We predict that in the absence of the cell-to-cell spread function of gE, the gE-deleted virus is eliminated earlier from the respiratory and ocular epithelial tissues by the host immune response.

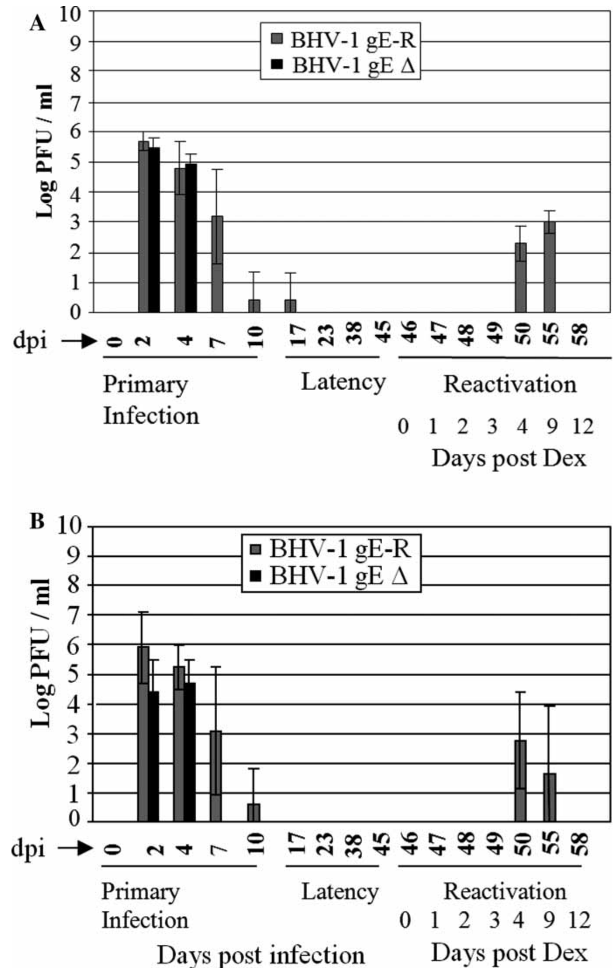

Figure 2 Isolation and quantitation of virus present in nasal (**A**) and ocular (**B**) secretions during acute, latent, and postreactivation stages of infection. Virus was isolated from nasal and ocular secretions of calves infected with BoHV-1 gE-R and BoHV-1 gEΔ viruses at different times post infection is shown. Additionally, virus isolation following reactivation is shown as days post dexamethasone treatment up to day 12 post dexamethasone treatment.

During latency (23 to 46 d.p.i.), as expected, infectious virus was not detected in ocular or nasal swabs of calves infected with either virus (Figure 2A and B), which was consistent with previous reports (Jones, 2003). Following reactivation from latency, the BoHV-1 gE-rescued virus was recovered from nasal or ocular swabs up to 9 days post reactivation (Figure 2A and B). In contrast, the gE-deleted virus was not detected in ocular or nasal swabs for at least 21 days after dex treatment (data shown in Figure 2A and B are up to 12 days post dex). TGs collected during primary infection (at 4 d.p.i.), during latency (at 38 d.p.i.), and after reactivation (at 4 days post dexamethasone treatment) were processed for virus isolation and glycoprotein C (gC) expression by immunohistochemistry as described earlier (Smith *et al*, 1989; Butchi *et al*, 2007). Infectious virus was detected in TG homogenates during primary infection for both viruses. These results indicated that both viruses (gE-deleted and gE-rescued) penetrated the sensory nerve endings efficiently and were transported from the initial infection sites in the nose and eye retrogradely to TG where they replicated, briefly, and produced infectious virus. These results are consistent with earlier reports using a gE-deleted BHV-5 or PRV recombinant virus (Yang *et al*, 1999; Enquist *et al*, 1999; Chowdhury *et al*, 2000; Enquist, 2002). During latency and after reactivation, infectious virus was not isolated from TG homogenates or TG explants. Because productive replication of virus does not take place during latent infection (Jones, 2003), negative virus isolation results from the TG homogenates during latent infection was expected. Following dex treatment, infectious virus was not detected in TG homogenates or TG explants regardless of which virus was used to infect calves. As shown in Figure 2A and B, gE(Us8)-rescued virus was isolated at 4 days post reactivation from ocular or nasal swabs of latently infected calves. In some instances, virus was not detected in TG homogenates or explants from the same calf that was shedding virus in the nose and eye. These results are consistent with the notion that virus isolation from latently infected TG explants and homogenates is not always positive during reactivation (Jones, 2003).

Paraffinized formalin-fixed TG sections from BoHV-1 gE-rescued and BoHV-1 gE(Us8)-deleted virus–infected calves sacrificed, at 4 d.p.i. (primary infection), 38 d.p.i. (latency), and 4 days post dexamethasone treatment (reactivation) were analyzed by immunohistochemistry using gC-specific monoclonal antibody (Mab) F2 essentially as described by Smith *et al* (1989) with minor modifications. The tissues were treated with proteinase K (50 μg/ml in TE pH 8.0; Sigma, catalog number P6556) for 30 min at 37°C to retrieve the antigens prior to incubation with Mab F2. Results shown in Figure 3A show that both during acute primary infection and following reactivation, TG infected with gE-rescued and gE-deleted viruses expressed gC, a $\gamma 2$ protein (Arsenakis *et al*, 1988). However, the percentage of gC+ neurons was lower following dex treatment of calves infected with the gE-deleted virus or the gE-rescued virus (Figure 3A). As expected, during latency, gC+ neurons were not detected regardless of the virus used to infect calves. Because gC is a late $\gamma 2$ gene, which requires ongoing DNA replication for its expression (Arsenakis *et al*, 1988), detection of the gC protein in TG neurons of calves latently infected with the gE-deleted virus suggested that dex initiated viral DNA replication and lytic gene expression.

Serum samples were collected prior to and after dexamethasone treatment in both calf studies. Based on the serum neutralization titers (Figure 3A), the primary immune response against the gE-deleted virus was approximately 10-fold lower relative to the gE-rescued virus–infected calves. This may have been due to the fact that the gE-deleted virus replication during acute primary infection lasted only 4 d.p.i., whereas the gE-rescued virus replicated for 10 or 17 days in the eye or nose, respectively. Additionally, it is possible that lower neutralizing titers against the gE-deleted virus is a result of fewer infected cells at the primary infection site due to defective cell-to-cell spread of the virus.

Following dexamethasone treatment, a secondary antibody response only occurred in calves infected with gE-rescued viruses (Figure 3A), which correlated with virus shedding in nasal or ocular swabs. Earlier studies (Rock *et al*, 1992; Lovato *et al*, 2003; Jones and Chowdhury, 2007) predicted that a secondary immune response following reactivation of the latent virus required virus replication at peripheral sites. Our results are in agreement with these previous predictions, and there is a correlation between detecting infectious virus in the nasal or ocular swabs during reactivation from latency and a secondary antibody response.

In conclusion, the results of virus isolation from three different anatomical sites (TG, nose, or eye), and the immunohistochemistry data indicated that gE was not necessary for retrograde transport to TG during acute infection and the establishment of latency was not altered by the lack of gE expression. However, the gE-deleted virus showed a defect in reactivation property in the nose and cornea. Similar results were also obtained for Us9-deleted BoHV-1 (Butchi *et al*, 2007). Because expression of Us9 in the gE-deleted BoHV-1 was unaffected, the anterograde spread defect of the mutant virus must be due to the deletion of gE. Although it is difficult to determine in calves, axonal transport of viral glycoproteins gB, gC, and gD were defective in rabbits olfactory neurons in culture infected with gE-deleted BHV-1 (S. I. Chowdhury, unpublished data). Recent reports in PRV and HSV-1 also indicated that alphaherpesvirus gE is required for efficient axonal localization of structural proteins,

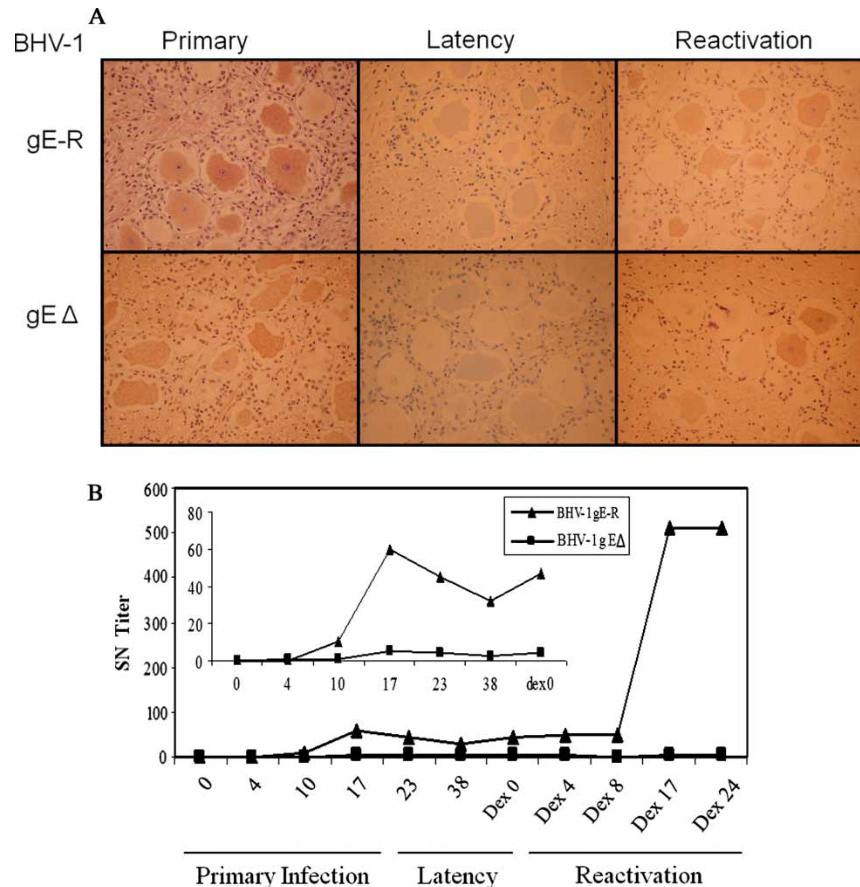

Figure 3 (**A**) BoHV-1 gC–specific immunoperoxidase staining of TGs showing positive staining in animals infected with BoHV-1gE-R) and BoHV-1 gEΔ viruses during primary infection and reactivation but not during latency. Counterstaining was either performed with Meyers hematoxylin (primary and reactivation) or with 0.5% methyl green (latency). (**B**) Serum neutralizing antibody titers in calves during primary, latent, and postreactivation stages of infection. Plaque reduction tests was performed with constant amount of virus (100 PFU) and serial twofold dilutions of serum as described earlier (Chowdhury *et al*, 1998). The inset panel is shown to illustrate the antibody titers during acute primary infection more clearly.

including capsids, tegument, and membrane glycoproteins (Ch'ng and Enquist, 2005; Wang *et al*, 2005). Taken together the results of gC expression in the TG, we believe that both the gE-rescued and the gE mutant viruses reactivated in the TGs of latently infected animal; however, in the absence of gE, the virus axonal transport and subsequent nasal and ocular shedding could not occur.

As noted earlier, in some instances, gE-deleted virus is isolated from nasal and ocular swabs following reactivation (Lemaire *et al*, 2001; Dispas *et al*, 2003). It is known that BoHV-1 can also establish a quiescent infection in non-neuronal sites such as in tonsils (Winkler *et al*, 2000; Perez *et al*, 2005). Although the exact mechanism of latency-reactivation in tonsil is not well understood, occasional isolation of gE-deleted virus following reactivation from latency could be from non-neural sites such as tonsil, which does not require anterograde axonal transport.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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