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Short Communication

Mutagenesis of a bovine herpesvirus type 1 genome cloned as an infectious bacterial artificial chromosome: analysis of glycoprotein E and G double deletion mutants

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The genome of bovine herpesvirus type 1 Schönböken was cloned as a bacterial artificial chromosome (BAC) by inserting mini F plasmid sequences into the glycoprotein (g) E gene. The resulting BAC clone, pBHV-1ΔgE, was transfected into bovine kidney cells and viable gE-negative BHV-1 (BHV-1ΔgE) was recovered. By RecE/T mutagenesis in *Escherichia coli*, the gG open reading frame was deleted from pBHV-1ΔgE. From the mutated BAC, double negative BHV-1ΔgE-gG was reconstituted and its growth properties were compared to those of rescuant viruses in which the gE gene was restored (BHV-1rev, BHV-1ΔgG). The mutant viruses did not exhibit markedly lowered virus titres. Plaque sizes of BHV-1ΔgE, BHV-1ΔgE-gG and BHV-1ΔgG, however, were reduced by 19 to 55 % compared to parental strain Schönböken or BHV-1rev. Our results suggested that gE and gG function independently from each other in cell-to-cell spread, because an additive effect on plaque formation was observed in the gE/gG double deletion mutant.

Bovine herpesvirus type 1 (BHV-1) is a major cause of bovine respiratory and genital tract disease (Gibbs & Rweyemamu, 1977). BHV-1 is classified as an *Alphaherpesvirus* and with its close relatives varicella-zoster virus (VZV), equine herpesvirus type 1 (EHV-1) and pseudorabies virus (PRV) forms the genus *Varicellovirus* (van Regenmortel *et al.*, 2000). BHV-1 encodes at least 10 glycoproteins, which have moderate to high homology with those of the prototype *Alphaherpesvirus* herpes simplex virus type 1 (HSV-1). Four of the glycoproteins are encoded by the unique-short (U_S) region of the genome, and sequence analyses of several *Alphaherpesvirinae* have suggested that glycoprotein (g) D, gG, as well as gE and gI have evolved by gene duplication (McGeoch, 1990; Schwyzer & Ackermann, 1996). Glycoprotein D is essentially involved in virus entry and direct spread of infectivity from an infected cell to neighbouring uninfected cells (direct cell-to-cell spread, *ctcs*) (Fehler *et al.*, 1992). By serial passage in cultured cells, however, gD-negative BHV-1 can undergo genomic changes that finally lead to gD-independent entry and *ctcs* (Schröder *et al.*, 1997). BHV-1 gG is a secreted glycosaminoglycan (Keil *et al.*, 1996), which is nonessential for virus growth (Nakamichi *et al.*, 2000), but it is required for efficient *ctcs* in bovine kidney cells. It was speculated that gG functions in maintaining the cell-to-cell junctional adhesion during BHV-1-infection (Nakamichi *et al.*, 2000). Furthermore, it appeared that expression of BHV-1 gG is required for correct localization of gE at the lateral boundaries of cell junctions, and that the absence of gG influences gE or gE–gI accumulation and results in a less efficient *ctcs* (Nakamichi *et al.*, 2002). BHV-1 gE and gI form a non-covalently linked dimer in infected cells and the virion envelope (Whitbeck *et al.*, 1996). The analysis of mutant viruses lacking gE and/or gI has demonstrated that the complex is involved in *ctcs* but does not play a role in virus entry, although large amounts of the dimer are packaged into the viral envelope (Rebordosa *et al.*, 1996; Yoshitake *et al.*, 1997). A recombinant BHV-1 with deletion of the genes encoding gG, gE and US2 was tested *in vivo* as a vaccine strain, but not further characterized *in vitro* (Belknap *et al.*, 1999). Only recently it was reported that deletion of gE in the Australian BHV-1 subtype 2 strain V155 cloned as a bacterial artificial chromosome (BAC) resulted in a gE-negative virus that produced high virus titres, but did not form visible plaques under an agarose overlay (Mahony *et al.*, 2002). Our study aimed at characterizing a BHV-1 mutant lacking two U_S glycoproteins, namely gE and gG, alone or in combination. Because *ctcs* investigated using plaque size assays was demonstrated to be the most significant phenotype of all gE or gG deletion mutants, special emphasis was put on answering the question whether an additive effect on virus spread in cultured bovine cells was observed if two glycoproteins involved in *ctcs* are absent from BHV-1-infected cells.

The mutant viruses were syngeneic and based on wild-type

BHV-1, subtype 2 strain Schönböken (Matheka & Straub, 1972; Engelhardt & Keil, 1996), which was cloned as a BAC. Using this clone it could be demonstrated that gG and gE or the gE–gI complex probably act in different steps of *ctcs*, because additive effects were observed after deletion of both gE and gG, and because deletion of gE resulted in a much greater reduction of virus plaque sizes than a deletion of gG.

BHV-1 strain Schönböken was propagated as described previously in Madin–Darby bovine kidney (MDBK, ATCC CCL-22), cells, which were grown in Dulbecco's modified essential medium (DMEM) supplemented with 10 % foetal calf serum (FCS) (Engelhardt & Keil, 1996). To obtain a BHV-1 BAC, viral DNA was prepared from infected cells

and co-transfected with recombinant plasmid pΔgE-pHA2 by the calcium phosphate precipitation method exactly as described by Rudolph *et al.* (2002). Recombinant plasmid pΔgE-pHA2 was generated by cloning of PCR fragments flanking the gE deletion (Fig. 1A) and insertion of the *Pac*I fragment of plasmid pHA2 (Adler *et al.*, 2000) into plasmid pTZ18R. The resulting construct contains an F origin of replication, the enhanced green fluorescent protein (GFP) open reading frame (ORF), and the *Escherichia coli* (*E. coli*) guanosine phosphoribosyl transferase (gpt) gene for selection in mammalian cells using mycophenolic acid, xanthine and hypoxanthine (Schumacher *et al.*, 2000; Adler *et al.*, 2000; Rudolph & Osterrieder, 2002). Progeny fluorescing virus resulting from the co-transfections of viral and
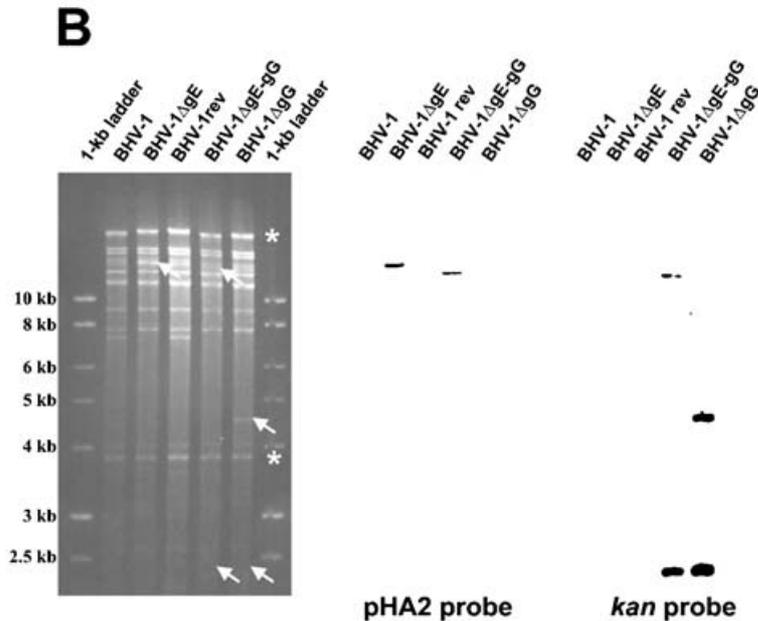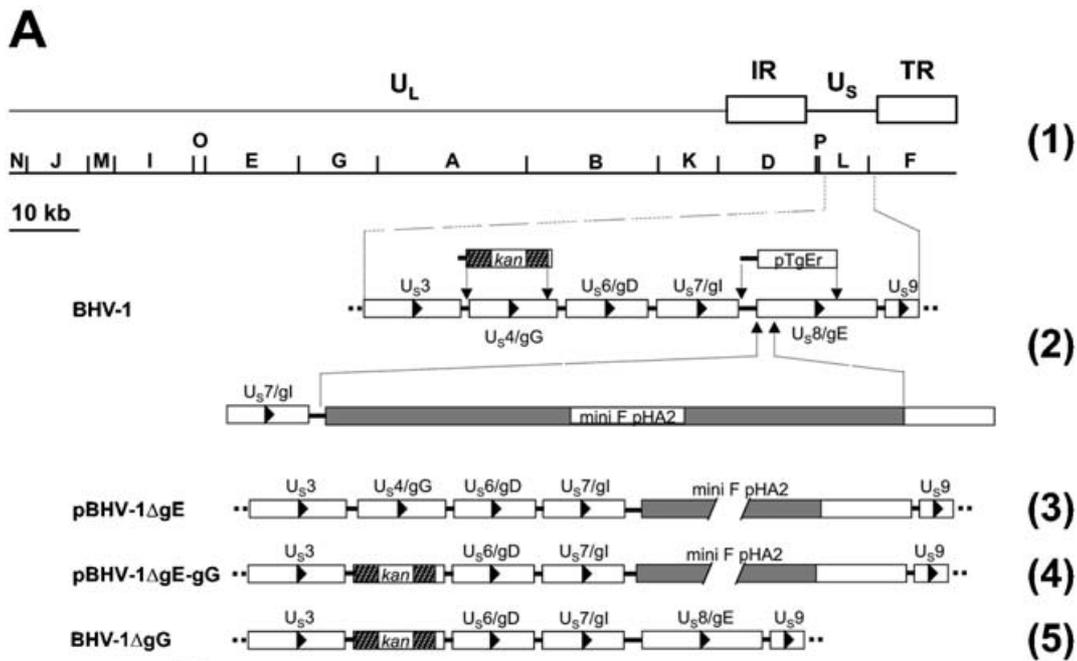
pΔgE-pHA2 DNA was purified to homogeneity and checked for the presence of pHA2 sequences by Southern blot analysis (data not shown). Virus DNA was prepared 6 h after infection and used to electroporate *E. coli* DH10B cells, which were spread on chloramphenicol (cam)-containing agar plates (Schumacher *et al.*, 2000; Rudolph & Osterrieder, 2002). Resistant colonies were grown in liquid medium and DNA was prepared by affinity chromatography (Qiagen). After primary testing of several BAC-containing colonies, one clone was chosen for further analysis and termed pBHV-1ΔgE. Restriction enzyme digestion and Southern blotting using pHA2 as a probe demonstrated that pBHV-1ΔgE exhibited the expected banding pattern and that mini F plasmid sequences had been inserted instead of a portion of the gE gene (Fig. 1B). Subsequently, bovine kidney cells [PT11; RIE11; collection of cell lines in veterinary medicine at the Federal Research Centre for Virus Diseases of Animals (CCLV), Insel Riems] were transfected with 1 µg of pBHV-1ΔgE DNA isolated from *E. coli*. GFP-expressing cells which developed into BHV-1-specific plaques (Fig. 2) were already seen 24 h after transfection. These results confirmed that the

BHV-1 genome was successfully cloned as an infectious BAC in *E. coli*.

The next step was deletion of the gG ORF from pBHV-1ΔgE using RecE/T cloning (Rudolph & Osterrieder, 2002; Tischer *et al.*, 2002). The kanamycin-resistance gene (*kan*) was amplified from plasmid pACYC177 (MBI Fermentas) using the primers listed in Table 1. The primers contained 50 bp homology arms bordering the sequence to be deleted and about 20 bp with homology to the *kan* gene for specific amplification. The PCR product (300 ng) was electroporated into DH10B cells containing pBHV-1ΔgE and pKD46 (Datsenko & Wanner, 2000) exactly as previously described and kanamycin-resistant colonies were isolated. BAC DNA was prepared, transfected into PT11 cells, and subjected to restriction enzyme digestion and Southern blot analyses (Fig. 1B). These analyses demonstrated that the gG ORF was successfully deleted from pBHV-1ΔgE by RecE/T cloning.

To perform functional studies using the generated mutants, pBHV-1ΔgE and pBHV-1ΔgE-gG lacking the gG ORF were transfected into PT11 cells and the resulting viruses were
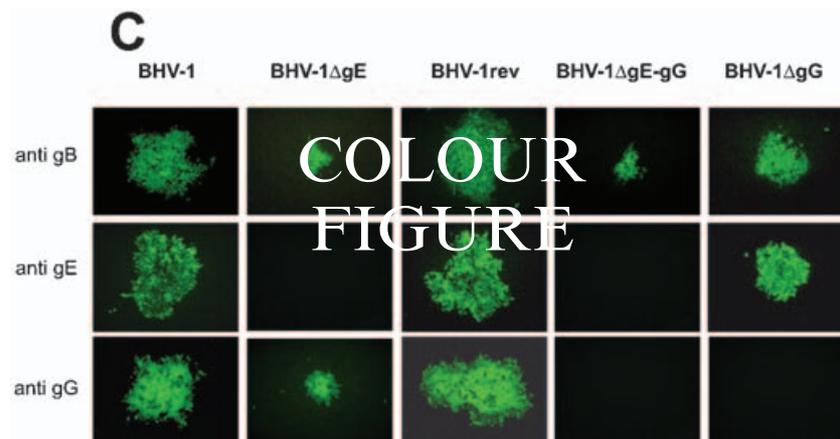
Fig. 1. (A). Schematic illustration of the cloning procedure to introduce mini F vector sequences into the BHV-1 Schönböken genome and of the deletion of the gG gene from cloned BHV-1 DNA (pBHV-1ΔgE). The organization of the approximately 136 kbp BHV-1 genome and the *Hin*dIII restriction map (1), the U$_S$ region and ORFs located therein (2) are shown. Two 1·4 kbp fragments in the gE gene region were amplified by PCR and cloned into plasmid pTZ18R. The 8·2 kbp mini F plasmid was released from recombinant plasmid pHA2 (Adler *et al.*, 2000), inserted between the two flanking regions, resulting in plasmid pΔgE-pHA2, which was used to introduce the mini F plasmid into the BHV-1 genome by homologous recombination (3). Glycoprotein G-encoding sequences were replaced by the kanamycin-resistance gene in pBHV-1ΔgE DNA by RecE/T cloning using plasmid pKD46 in *E. coli* strain DH10B (Rudolph & Osterrieder, 2002) (4). Recombinant plasmid pTgEr for repairing the gE deletion was generated by PCR amplification of a 1·0 kbp product and cloning into vector TopoTA2.1 (Invitrogen) (2). Recombination with plasmid pTgEr resulted in revertant genomes BHV-1rev (not shown) and BHV-1ΔgG (5). (B) Viral DNA isolated from MDBK cells infected with parental strain Schönböken (BHV-1), BHV-1ΔgE, the double deletion mutant BHV-1ΔgE-gG, as well as the revertant viruses BHV-1ΔgG and BHV-1rev was digested with *Hin*dIII and separated by 1·0 % agarose gel electrophoresis. An ethidium bromide-stained gel of the separated fragments is shown (left panel). Cleaved DNA was transferred to nylon membranes and sheets were incubated with a digoxigenin-labelled pHA2-specific probe or a kanamycin-resistance gene-specific probe. A DNA molecular mass marker (1 kb DNA ladder, Promega) was used as a size standard. Fragments in mutant virus genomes specifically recognized by the probes are indicated by arrows. Nonviral DNA fragments are marked with asterisks. (C) Immunofluorescence analysis of plaques induced by BHV-1rev, BHV-1ΔgE, BHV-1ΔgG and BHV-1ΔgE-gG in comparison to the parental strain Schönböken (BHV-1). Virus plaques were stained using IIF at 72 h post-infection with mAbs specific for gB, gE or gG, respectively. Bound antibodies were detected with anti-mouse ALEXA488 (Molecular Probes). Magnification, 200 ×.

termed BHV-1ΔgE and BHV-1ΔgE-gG, respectively (Fig. 1A). At the same time, rescuant viruses, in which the gE gene was restored, were generated by co-transfecting the cloned DNAs with a cloned PCR fragment (plasmid pTgEr) from strain Schönböken containing part of the gE ORF (Fig. 1A), and the resulting viruses were termed BHV-1rev (gE revertant of BHV-1ΔgE) and BHV-1ΔgG (gE revertant from the double deletion mutant BHV-1ΔgE-gG). After reconstitution of the mutant viruses, expression of gE and gG by the mutant viruses was tested by indirect immuno-fluorescence (IIF) using monoclonal antibodies (mAb) against the glycoproteins gB (mAb 72/14/6, G. M. Keil and others, unpublished data), gE and gG (mAb d3 and mAb BHV1gG, kindly provided by C. Schelp, Dr Bommeli AG, Switzerland; Fig. 1C). As expected, the parental strain Schönböken expressed high levels of gE, gG and gB. In contrast, BHV-1ΔgE reconstituted from pBHV-1ΔgE was unable to express gE, whereas expression of gG or gB remained unaffected. In the double deletion mutant BHV-1ΔgE-gG, generated by RecE/T cloning in *E. coli*, expression of neither gE nor gG was detected (Fig. 1C). The revertant viruses also exhibited the expected reaction pattern. Whereas BHV-1rev expressed gB, gE and gG at levels indistinguishable from those of parental strain Schönböken, expression of gE but not gG was detected in BHV-1ΔgG (Fig. 1C). From the results of the IIF testing we concluded that the generated mutant viruses exhibited the expected phenotypes.

In the next series of experiments, the growth properties of the generated mutant viruses were compared to those of the parental strain Schönböken. Single-step growth kinetics were determined on MDBK cells by infecting the cells at an m.o.i. of 1 for 1 h. Input virus was then inactivated by low-pH treatment using a citrate buffer (Highlander *et al.*, 1987), and at the indicated times after infection, intra- and extracellular virus titres were determined. It could be shown that both intra- and extracellular virus titres remained virtually unaffected by deletion of gE, gG or both gE and gG from BHV-1 (Fig. 2A), because no marked differences between the titres induced by the various viruses at any time post-infection could be detected. These results strongly suggested that the deleted U_S glycoproteins did not have a prominent effect on secondary envelopment or virus egress, even if a deletion of gE and gG was also introduced into the BHV-1 genome. Previous studies had suggested that both gE and gG are involved in direct spread of infectivity from an infected to a neighbouring uninfected cell and that mislocalization of gE is responsible for impaired *ctcs* of gG deletion mutants (Nakamichi *et al.*, 2000). To examine the effect of a gE/gG double deletion on BHV-1 *ctcs*, MDBK cells were seeded in six-well plates (Nunc), and 200 p.f.u. of the virus mutants was used to infect 1×10^6 cells. At 2 days after infection under a 0·25 % methylcellulose overlay (Neubauer *et al.*, 1997), plaque diameters of at least 150 plaques of each virus were determined and mean diameters and standard errors were calculated. Values for parental strain Schönböken were set to 100 % and the plaque diameters observed for the mutant viruses were expressed relative to this value. It could be shown that deletion of gE resulted in a 45 % reduction in plaque diameters, whereas the single deletion of gG did not result in reductions of plaque diameters exceeding 19 % (Fig. 2B). Simultaneous deletion of gE and gG resulted in virus plaques exhibiting a 55 % reduction in diameters (Fig. 2B). These findings strongly suggested that gE and gG, which are both involved in direct *ctcs*, act in different and non-overlapping steps of this membrane fusion process.

The salient findings presented in this communication are that the entire genome of the wild-type BHV-1 strain Schönböken was cloned as an infectious BAC and that the

Table 1. Primers used for the generation of the BHV-1 BAC and mutagenesis

Restriction enzyme sites are given in lower-case bold letters, while sequences in italic letters indicate additional bases that are not present in the BHV-1 sequence. The underlined sequences in the *kan* primers indicate sequences from pACYC177 used to amplify the kanamycin-resistance gene, whereas sequences in bold and in italics represent gG sequences allowing homologous recombination for recE/T-mediated deletion of the gG gene. The primer pair gE-rev-a/gE-rev-b was used to amplify a fragment spanning the gE deletion generated after insertion of the BAC plasmid and to construct repair plasmid pTgEr.

Primer	Sequence (5′→3′)	Fragment/plasmid generated
gE-1a	*TACC***gaattc**TGTTGCTCTGGATGGTGGTGCT	1.4-kb pΔgE-pHA2
gE-1b	*ATAT***ggtaccttaattaa**ACCCTCTCGCGTGCGCCGTTCC	1.4-kb pΔgE-pHA2
gE-2a	*TATA***ttaattaa**ACCGCGAGTGCTTCACCGACGT	1.4-kb pΔgE-pHA2
gE-2b	*TCAT***tctaga**CGGGTCCCTAAACCAAACTTTGAA	1.4-kb pΔgE-pHA2
gG-*kan*-a	***GAGCGAACGCGAGCGAACGCGAGCGCAAGCGCGAGCACAAGACTGCGATC*** <u>CGATTTATTCAACAAAGCCACG</u>	*kan* for gG deletion
gG-*kan*-b	***GTAAACGCGGGGCAGCGGGGTCGGGGCGGCGTGGGGTGCCGGCGATCAGA*** <u>GCCAGTGTTACAACCAATTAACC</u>	*kan* for gG deletion
gE-rev-a	GATCTCGCTCGCGTGTGTCTTGG	pTgEr
gE-rev-b	GGTGCCTCGGGGTGGAAGATG	pTgEr

A

B
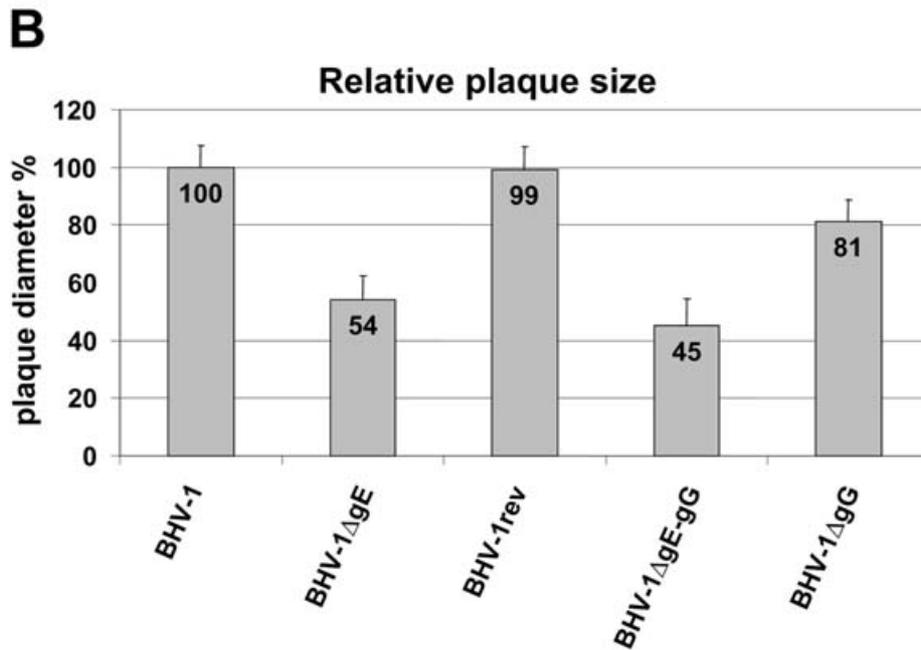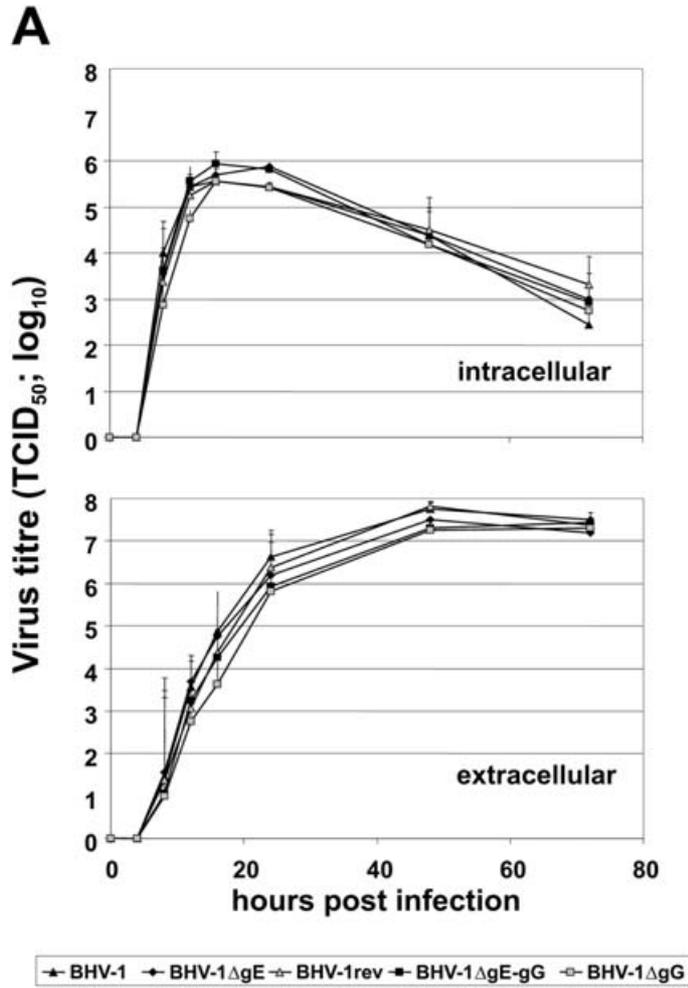
absence of both gE and gG had an additive effect on direct *ctcs* of this *Alphaherpesvirus*. Construction of a BAC was a good basis for these experiments, because adaptation of double mutants, which is frequently observed by cell culture isolation of mutant herpesviruses, is unlikely using this novel technology. Previous studies had suggested that gE and gG function in *ctcs* and it was speculated that both glycoproteins may interact or act even synergistically in this membrane fusion event, because distribution of gE in infected cells was altered in the absence of gG expression (Nakamichi *et al.*, 2002). The results presented here using gE and gG single or double deletion mutants, which were based on an infectious BHV-1 clone and included the analysis of relevant revertant viruses, clearly demonstrated that the absence of gG did not over-proportionally influence the growth properties of a gE-negative BHV-1 mutant. In addition, the observed additive effect strongly argues against the possibility that the reduced *ctcs* capabilities of gG-negative BHV-1 are caused by mislocalization of gE (Nakamichi *et al.*, 2002). Whereas gE was confirmed as a major player in BHV-1 *ctcs*, gG appears to play a minor role in a step of *ctcs* that is clearly independent from that mediated by gE. It is important to note that no significant reductions in intra- or extracellular virus titres were observed in any of the generated BHV-1 mutants, although virus plaque sizes were significantly reduced in the case of gE- and gE/gG-negative BHV-1 (>50 %). Mahony *et al.* (2002) speculated that genetic differences between BHV-1 subtypes 1 and 2 are the reason for the absence of virus plaque formation, which they observed after deletion of gE. Since the BHV-1 BAC reported here was also derived from a subtype 2 strain (Schönböken; Matheka & Straub, 1972), and plaque formation was clearly evident using methylcellulose overlays, other reasons may be responsible for these differing observations. Strain-specific properties, an influence of TK deletion on virus growth (Mahony *et al.*, 2002) or technical details, e.g. the use of different overlays for plaque size determinations, may be the reason for the differing interpretations of an effect of deletion of gE.

Taken together, our observations strongly support the hypothesis that *Alphaherpesvirus* secondary envelopment and egress on the one hand, and direct cell-to-cell spread on the other, are independent from each other, although fusion of membranes containing viral proteins is required for both processes. Future work will concentrate on the systematic analysis of BHV-1 tegument and membrane (glyco)proteins to contribute to the elucidation of the general principles of *Alphaherpesvirus* egress and direct cell-to-cell spread.

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Fig. 2. Single-step growth kinetics (A) and plaque sizes (B) of BHV-1rev, BHV-1ΔgE, BHV-1ΔgE-gG and BHV-1ΔgG compared with parental strain Schönböken (BHV-1). For virus growth kinetics, MDBK cells were infected with the various viruses at an m.o.i. of 1 for 1 h at 37 °C. Non-penetrated virus was inactivated by low-pH treatment. Infected cells were washed and overlaid using cell culture medium. At the indicated times after infection, intracellular and extracellular virus titres were determined by titration on MDBK cells and are given as TCID$_{50}$ ml^{-1}. For plaque size determinations, MDBK cells in six-well plates were infected with 200 p.f.u. per well and overlaid with 0.25 % methylcellulose for 48 h. The average diameter of plaques formed by BHV-1 Schönböken was set to 100 %. Bars indicate standard errors.

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