Bovine herpesvirus 4 ORF73 is dispensable for virus growth in vitro, but is essential for virus persistence in vivo

M. Thirion,1 B. Machiels,1 F. Farnir,2 G. Donofrio,3 L. Gillet,1 B. Dewals1 and A. Vanderplasschen1

Correspondence
A. Vanderplasschen
A.vdplasschen@ulg.ac.be

Received 28 April 2010
Accepted 24 June 2010

ORF73 orthologues encoded by different rhadinoviruses have been studied extensively. These studies revealed that the ORF73 expression product (pORF73) is a multifunctional protein essential for latency that enables episome tethering to mitotic chromosomes and modulates cellular pathways implicated in growth and survival of latently infected cells. Comparison of pORF73 orthologues encoded by rhadinoviruses reveals important variations in amino acid sequence length and composition. Bovine herpesvirus 4 (BoHV-4) encodes by far the shortest ORF73 orthologue, with a size equivalent to only 22 % of that of the largest orthologues. The present study focused on determining whether BoHV-4 ORF73 is a bona fide gene and investigating whether it is essential for latency, as established for larger ORF73 orthologues. Our results demonstrate that BoHV-4 ORF73 is transcribed as immediate-early polycistronic mRNA together with ORF71. Using a BoHV-4 bacterial artificial chromosome clone, we produced a strain deleted for ORF73 and a revertant strain. Deletion of BoHV-4 ORF73 did not affect the capacity of the virus to replicate in vitro, but it prevented latent infection in vivo using a rabbit model. Interestingly, the strain deleted for ORF73 induced an anti-BoHV-4 humoral immune response comparable to that elicited by the wild type and revertant recombinants. Together, these results demonstrate that, despite its relatively small size, BoHV-4 ORF73 is a functional homologue of larger rhadinovirus ORF73 orthologues, and highlight the potential of ORF73 deletion for the development of BoHV-4 as a vector in vaccinology.

INTRODUCTION

Gammaherpesviruses, like all viruses in the family Herpesviridae, exhibit two distinct phases in their life cycle: lytic replication, characterized by a transcription programme in which immediate-early (IE), early (E) and late (L) genes are expressed successively; and latency, characterized by the maintenance of the viral genome as a non-integrated episome and the expression of a limited number of viral genes and microRNAs (Cai et al., 2005; Roizman & Pellett, 2007; Sarid et al., 1998; Weck et al., 1999). Upon reactivation, latency shifts to lytic replication.

The gammaherpesvirus human herpesvirus 8 (HHV-8, also termed KSHV for Kaposi’s sarcoma-associated herpesvirus), is the prototype of the genus Rhadinovirus. During latency, HHV-8 expresses three main ORFs: ORF71, ORF72 and ORF73 (Sarid et al., 1998, 1999). ORF71 and ORF72 encode viral homologues of cellular FLIP (Thome et al., 1997) and D cyclin (Li et al., 1997), respectively. ORF71 and ORF72 are expressed together with ORF73 as a polycistronic mRNA (Dittmer et al., 1998; Talbot et al., 1999). Whilst some rhadinoviruses encode orthologues of ORF71 and/or ORF72, all rhadinoviruses encode an ORF73 orthologue. For example, bovine herpesvirus 4 (BoHV-4) encodes orthologues of ORF71 and ORF73, whereas ORF72 is absent (Zimmermann et al., 2001).

ORF73 orthologues encoded by different rhadinoviruses have been studied extensively. These studies revealed that the expression product of ORF73 (pORF73) is a multifunctional protein essential for latency (Calderwood et al., 2005; Fowler et al., 2003; Renne et al., 2001). The best-characterized
pORF73 is the latency-associated nuclear antigen 1 (LANA-1) of HHV-8. LANA-1 associates with mitotic chromosomes to enable episome tethering during division of latently infected cells and its subsequent partitioning between daughter cells (Ballestas et al., 1999; Barbera et al., 2006; Ye et al., 2004). This crucial role in latency is reinforced by the ability of LANA-1 to modulate cellular pathways implicated in cell growth and survival by targeting transcriptional regulators or co-regulators such as p53, pRB, CBP and sin3A (Friborg et al., 1999; Krithivas et al., 2000; Lim et al., 2001; Radkov et al., 2000). Moreover, LANA-1 prevents reactivation from latency by downregulating the expression of ORF50, which encodes the replication and transcriptional activator (Lan et al., 2004, 2005; Lu et al., 2006).

Comparison of pORF73 orthologues encoded by rhadinoviruses reveals important variations in amino acid sequence length and composition (Fig. 1). Alcelaphine herpesvirus 1, infecting wildebeest, and HHV-8 encode the largest pORF73 orthologues. HHV-8 pORF73 is a 1162 aa protein containing three regions: the N- and the C-terminal regions and a central region rich in acidic residues. The N- and C-terminal regions are both essential for episome maintenance and viral/cellular gene regulation (Ballestas et al., 1999; Krithivas et al., 2000; Lu et al., 2006). Within the genus Rhadinovirus, BoHV-4 encodes by far the shortest ORF73 orthologue, with a size equivalent to only 22% of that of LANA-1 (Fig. 1). BoHV-4 pORF73 consists of 253 aa. The first 138 aa and the last 102 aa are homologous to the LANA-1 N- and C-terminal regions, respectively.

BoHV-4 has been isolated throughout the world from healthy cattle, as well as those exhibiting a variety of diseases (Li et al., 2005; Markine-Goriaynoff et al., 2003). The African buffalo is also a natural host species of BoHV-4 (Dewals et al., 2006). Several features make BoHV-4 attractive as a viral expression vector and/or as a model for studying gammaherpesvirus biology (Donofrio et al., 2006; Gillet et al., 2005, 2009). Among these, BoHV-4 can establish lytic and latent infection in rabbit (Naeem et al., 1990; Osorio et al., 1982).

The present study focused on BoHV-4 ORF73, with the goal of determining whether BoHV-4 ORF73 is a bona fide gene and whether it is essential for latency, as established for larger ORF73 rhadinovirus orthologues. Our results demonstrate that BoHV-4 ORF73 is transcribed as IE polycistronic mRNA. Deletion of BoHV-4 ORF73 did not affect the

---

**Fig. 1.** Schematic representation of primary amino acid sequences of pORF73 orthologues encoded by rhadinoviruses. HHV-8 LANA-1 is shown at the top. This 1162 aa protein consists of three regions: (i) an N-terminal region (grey) containing a proline-rich (P-rich) domain; (ii) a central region (hatched) containing acidic repeat domains (D/E- and Q/E-rich domains) and a leucine zipper (L-zipper); and (iii) a C-terminal region (white). The N- and C-terminal regions are implicated in episome maintenance and viral/cellular gene regulation. AlHV-1, Alcelaphine herpesvirus 1; AtHV-3, ateline herpesvirus 3; CeHV-17, cercopithecine herpesvirus 17; MuHV-4, murid herpesvirus 4; OvHV-2, ovine herpesvirus 2; SaHV-2, saimiriine herpesvirus 2.
capacity of the virus to replicate in vitro, while it prevented latent infection in vivo. Altogether, the results of the present study suggest that, despite its relatively small size, BoHV-4 ORF73 is a functional homologue of larger rhadinovirus orthologues, and highlight the potential of ORF73 deletion for the development of BoHV-4 as a vector in vaccinology.

RESULTS AND DISCUSSION

BoHV-4 ORF73 is expressed as an IE gene
To determine whether BoHV-4 ORF73 is a bona fide gene, we assessed whether it is transcribed during BoHV-4 infection. RT-PCR was performed using first-strand cDNA made from BoHV-4-infected and mock-infected Madin–Darby bovine kidney (MDBK) cells (Fig. 2a). In contrast to mock-infected cells, cDNA from infected cells generated a 762 bp PCR product (Fig. 2a) corresponding to ORF73, as determined by sequencing (data not shown). When reverse transcriptase was omitted from the reactions, no PCR product was detected, indicating that it did not result from amplification of contaminant viral DNA. Together, these data demonstrated that ORF73 was transcribed during BoHV-4 replication in vitro. A similar conclusion was reached for ORF71 based on the data presented in Fig. 2(a).

Next, cycloheximide (CHX) and phosphonoacetic acid (PAA) were used to identify the ORF73 and ORF71 kinetic class of transcription. This experiment revealed that neither CHX nor PAA inhibited ORF73 and ORF71 expression, suggesting that both ORFs are expressed as IE genes. BoHV-4 ORF5 (Bo5) encoding the BoHV-4 major IE transcript, ORF21 and ORF22 were used as controls in this experiment; the results presented in Fig. 2(a) confirmed that they are IE, E and L genes, respectively. The absence of contaminant viral DNA in the mRNA preparations was confirmed by the absence of a PCR product when reverse transcriptase was omitted from the reactions (Fig. 2a). The expression of BoHV-4 ORF73 as an IE gene is consistent with earlier reports describing the expression of ORF73 orthologues encoded by rhadinoviruses early after infection (Ebrahimi et al., 2003; Martinez-Guzman et al., 2003; Sarid et al., 1999).

BoHV-4 ORF73 is expressed as polycistronic mRNA encompassing ORF71
To characterize ORF73 transcript(s), Northern blot analysis was performed on total RNA extracted from BoHV-4-infected and mock-infected MDBK cells using full-length ORF73 as the probe (Fig. 2b). A weak band of approximately 2.5 kb was detected as early as 4 h post-infection (p.i.) (Fig. 2b). At 8 h p.i., four bands were detected with approximate sizes of 2.0, 2.5, 3.0 and 4.5 kb, respectively (Fig. 2b). One additional band of approximately 7.5 kb was detected at 24 h p.i. (Fig. 2b). These results suggest that ORF73 expression involves transcripts of different sizes. No signal was detected in RNA extracted from mock-infected cells (Fig. 2b).

Recently, a study devoted to ORF71 revealed that it is expressed as a polycistronic RNA encompassing at least ORF73 (B. Machiels, personal communication). That study also identified the polyadenylation signal (AATAAA) of the transcript 104 bp downstream of the ORF71 stop codon. In the present study, RT-PCRs were performed on cytoplasmic RNA extracted from BoHV-4-infected MDBK cells to further characterize polycistronic RNA molecules encoding ORF71 and ORF73 (Fig. 2c, d). PCR products corresponding to ORF71–73 (1454 bp), the ORF73 end of ORF75 (1759 bp), and the ORF71 end of ORF75 (2453 bp) were amplified (Fig. 2d). None of the PCRs involving primers hybridizing upstream of primer 75end-rev generated a product (Fig. 2d), whereas all reactions generated the expected products when performed on control viral DNA (data not shown). The absence of contaminant viral DNA was confirmed by the absence of PCR product when reverse transcriptase was omitted from the reactions (data not shown). Together, these data suggest that ORF71 and ORF73 are expressed as a polycistronic mRNA. These results are consistent with previous reports describing the expression of ORF73 on polycistronic mRNA molecules (Dittmer et al., 1998; Talbot et al., 1999). At least two mRNAs were identified here using the combined Northern blot/RT-PCR approach: one encompassing both ORF71 and ORF73, and the second encompassing ORF71, ORF73 and part of ORF75 (Fig. 2b, d). Longer RNAs were identified using Northern blotting but not by RT-PCR. These molecules could represent pre-mRNA or could reflect an upstream 5′ start site. Supporting the latter hypothesis, 5′-RACE analysis done for the murine gammaherpesvirus-68 (MHV-68) ORF73 revealed a 5′ start extending at least in the terminal repeats and possibly even into the unique left end of the genome (Coleman et al., 2005).

Production and characterization of BoHV-4 ORF73 recombinants
To investigate the importance of ORF73 in virus replication in vitro and the biology of the infection in vivo, we produced a BoHV-4 strain deleted for ORF73 and a derived revertant strain (see Supplementary Fig. S1, available in JGV Online). These recombinants were produced using a bacterial artificial chromosome (BAC) clone of the BoHV-4 genome and prokaryotic recombination technologies (see Methods). The V. test BAC G plasmid was used as the parental plasmid to generate the V. test BAC G ΔORF73 plasmid in which the entire ORF73 was replaced by a galactokinase (galK) cassette, as described in Methods. Then, a revertant plasmid was generated. Infectious viruses were efficiently reconstituted by transfection of BAC plasmids into permissive cells. Finally, reconstituted infectious viruses were propagated in embryonic bovine lung stably expressing Cre recombinase (EBL-NLS-Cre) cells to generate BAC-excised strains (Supplementary Fig. S1). The molecular structures of the recombinant strains produced were confirmed by a
combined HindIII restriction endonuclease and Southern blot approach (Fig. 3) and by sequencing of the regions used to target recombination (data not shown). Moreover, the absence of a polar effect of ORF73 deletion on the expression of ORF71 was demonstrated by RT-PCR. All recombinants tested expressed ORF71 comparably (data

**Fig. 2.** (a) Determination of the ORF73 kinetic class of transcription. MDBK cells were mock-infected or infected with the BoHV-4 V. test strain, in the absence or presence of cycloheximide (CHX) or phosphonoacetic acid (PAA). At 24 h post-infection (p.i.), expression of IE Bo5, E ORF21, L ORF22, ORF71 and ORF73 was studied using an RT-PCR approach, as described in Methods. mRNA and cDNA represent PCR products generated when reverse transcriptase was omitted from the reactions, and RT-PCR products, respectively. (b) Northern blot analysis was performed on total RNA harvested from mock-infected and infected MDBK cells 4, 8 and 24 h p.i. with the BoHV-4 V. test strain. ORF73 was used as probe. To optimize the visualization of the bands in the 24 h p.i. sample, the corresponding line was exposed for a shorter period (right column). Weak and intense bands identified on the blot are marked with open arrowheads and solid arrowheads, respectively. (c, d) Characterization of ORF73 transcripts by RT-PCR. Cytoplasmic RNA extracted 24 h p.i. was analysed by RT-PCR using the pairs of primers described in (c). For each pair of primers, the size of the expected amplicon is indicated. The results of the PCR are presented in (d). PCR results suggesting the polycistronic nature of the ORF73 transcript are marked with asterisks.
ORF73 deletion does not affect virus growth in vitro

To address the role of ORF73 in BoHV-4 growth in vitro, V. test BAC G, V. test BAC G ΔORF73 and V. test BAC G ORF73 revertant excised strains were compared using multi-step growth and plaque size assays (Supplementary Fig. S2). Corresponding non-excised strains were also compared (data not shown). All viruses tested exhibited similar growth curves (P ≤ 0.05) and comparable plaque sizes. Taken together, these results indicate that ORF73 deletion does not affect BoHV-4 replication in vitro.

ORF73 deletion affects virus persistence in vitro in a ‘latency-like’ model

Bovine macrophage (BOMAC) cells have been shown to support a BoHV-4 non-replicative persistent infection mimicking latency (Donofrio & van Santen, 2001). Using this ‘latency-like’ model, we investigated whether ORF73 deletion affects the persistence of BoHV-4 infection in BOMAC cells. BOMAC cells were infected with the V. test BAC G, V. test BAC G ΔORF73, or V. test BAC G ORF73 revertant strains, and virus persistence was monitored over time by flow-cytometric quantification of EGFP-expressing cells (Fig. 4a). Independently of the recombinants, about 90% of the cells were positive for EGFP expression on day 2 p.i. For all three recombinants tested, the percentage of EGFP-positive cells decreased gradually over time. However, whilst comparable data were obtained for V. test BAC G and V. test BAC G ORF73 revertant strains, a faster decay of positive cells was observed for cells infected with the V. test BAC G ΔORF73 strain (Fig. 4a). For example, on day 10 p.i., whilst V. test BAC G- and V. test BAC G ORF73 revertant-infected cultures contained approximately 50% EGFP-positive cells, the percentage of EGFP-positive cells in BOMAC cells infected with V. test BAC G ΔORF73 strain was close to 0.

At least two hypotheses that are not mutually exclusive could explain the faster decay of EGFP-positive cells in V. test BAC G ΔORF73-infected culture compared with V. test BAC G and V. test BAC G ORF73. First, it is likely
that the deletion of ORF73 impaired the persistence of the viral episome through cell division, as demonstrated for several rhadinoviruses. However, further experiments are required to control that the observed reduction of EGFP-expressing cells correlates with a decay of circular viral genome in the culture. Second, the absence of ORF73 could have induced lytic replication (by release of the inhibition on ORF50) of BoHV-4 in BOMAC cells and consequently eradication of infected cells from the culture. For testing the latter hypothesis, BOMAC cells were infected with V. test BAC G, V. test BAC G ΔORF73 or V. test BAC G ORF73 revertant strains, then analysed at 48 h p.i. by flow cytometry for expression of the structural protein gB. The data presented in Fig. 4(b) demonstrate that, independent of the virus strain used for the infection, no expression of BoHV-4 gB could be detected in infected BOMAC cells (Fig. 4b, right column). In contrast, infection of MDBK cells used as a positive control led to a large proportion of gB-expressing cells (Fig. 4b, left column). All recombinants tested led to comparable percentages of gB-expressing cells. Together, these results support the hypothesis that BoHV-4 ORF73 could play a role in latency and justified the completion of in vivo experiments.

BoHV-4 ORF73 is essential for virus persistence in vivo

To investigate the importance of BoHV-4 ORF73 during latency in vivo, we used the rabbit model. Rabbits were inoculated with the V. test BAC G, V. test BAC G ΔORF73 or V. test BAC G ORF73 revertant excised strains (Fig. 5a, b). Regardless of inoculation group, all rabbits remained healthy for the course of the experiment, and none showed clinical symptoms or lesions. In addition, no lesion was observed at necropsy 113 days p.i. To address virus persistence, real-time PCR was performed on DNA extracted from PBMCs over time and from the spleen 113 days p.i. (Fig. 5a). After an eclipse phase of 12 days, viral copies ranging from 10^1 to 10^2 viral copies per 10^5 copies of β-globin were detected in PBMCs from all rabbits infected with the V. test BAC G or V. test BAC G ORF73 revertant excised strains (Fig. 5a). Interestingly, no rabbits infected with the V. test BAC G ΔORF73 excised strain had detectable viral genome in PBMCs throughout the experiment (Fig. 5a). Real-time PCR performed on the spleen confirmed the essential role of ORF73 for virus persistence in vivo. Indeed, whilst rabbits infected with the V. test BAC G or V. test BAC G ORF73 revertant excised strains contained at least 10^3 viral copies per 10^5 cellular β-globin copies, none of the rabbits infected with the V. test BAC G ΔORF73 excised strain exhibited a detectable amount of viral genome (Fig. 5a). To confirm these real-time PCR results, virus isolations were attempted by co-cultivation of spleen cells with permissive cells as described in Methods. Viral plaques were observed for the three rabbits of the group infected with the V. test BAC G excised strain and for two of the three rabbits infected with the V. test BAC G ORF73 revertant excised strain (data not shown).
contrast, no virus was isolated from spleen cells of rabbits infected with the V. test BAC G ΔORF73 excised strain. Virus-isolation assays performed with the same sources of spleen cells, but frozen–thawed before the assay, did not lead to virus isolation (data not shown). Taken together, these results suggested that BoHV-4 ORF73 is essential for virus latency. However, the BAC cloning strategy used here for production of the recombinants does not control for any step from virus reconstitution onwards. Also, one cannot exclude that the deletion of the entire ORF73 could have disrupted neighbouring virus functions. Although the disruption of other virus functions could in theory have

**Fig. 5.** Rabbit infection with BoHV-4 ORF73 recombinants. Groups consisting of three rabbits (R1, R2 and R3) were mock-infected or infected with V. test BAC G, V. test BAC G ΔORF73 or V. test BAC G ORF73 revertant excised strains. (a) Real-time PCR relative quantification of the BoHV-4 genome. DNA was extracted from PBMCs at the different times p.i. and from the spleen 113 days p.i. Data are expressed as the number of BoHV-4 ORF8 gene copies per 10⁵ rabbit β-globin gene copies for each rabbit. ■, R1; ○, R2; □, R3. (b) Rabbit anti-BoHV-4 humoral response. Sera were collected at different times p.i. and the titre of anti-BoHV-4 antibodies was estimated by ELISA. Assays were performed in triplicate for each rabbit. Each value represents the mean ± SD of the data obtained for the three rabbits from each group. ○, Mock-infected; ■, V. test BAC G excised strain; ▲, V. test BAC G ΔORF73 excised strain; ×, V. test BAC G ORF73 revertant excised strain.
explained the phenotype observed for the ORF73 BoHV-4 recombinant, this phenotype was consistent with less dramatic mutations of MHV-68 ORF73 (Fowler et al., 2003; Moorman et al., 2003).

Finally, the humoral immune response induced by the ORF73-deleted strain was compared with those observed with the wild-type parental and the revertant strains (Fig. 5b). Independently of the virus strain used for the inoculation, anti-BoHV-4 antibodies were detected as early as 6 days p.i. All infected rabbits exhibited comparable humoral immune responses. The statistical test used to analyse the data suggested that, for some time points, rabbits infected with the V. test BAC G ORF73 excised strain exhibited a higher response \((P \leq 0.05)\) (Fig. 5b) than those infected with the V. test BAC G or V. test BAC G ORF73 revertant excised strains; however, this statistical difference was not observed when Student’s \(t\)-test was used. Regardless, these data demonstrate that the immune response raised against the ORF73-deleted recombinant is at least comparable to the responses induced by the wild-type and revertant strains. The absence of response in the mock-infected group confirmed the absence of cross-contamination between the groups (Fig. 5b).

The results demonstrate that, despite its relatively small size, BoHV-4 ORF73 is a functional homologue of larger rhadinovirus ORF73 orthologues and is essential for latency \textit{in vivo}. The relatively small size of BoHV-4 ORF73 reflects mainly the lack of an internal repeat unit. In larger orthologues, this internal repeat mediates a cis-acting evasion of MHC class I-restricted antigen presentation. Interestingly, the ORF73 encoded by MHV-68 also lacks the internal repeat, but retains the evasion mechanism (Bennett et al., 2005). Further studies are required to determine whether BoHV-4 ORF73 has also retained an evasion mechanism.

Several features make BoHV-4 a good candidate as a gene-delivery vector, and previous studies have yielded evidence that BoHV-4 can be used successfully to transduce expression of foreign proteins (Donofrio et al., 2003; Moorman et al., 2003).

In conclusion, the results demonstrate that, despite its relatively small size, BoHV-4 ORF73 is a functional homologue of larger rhadinovirus ORF73 orthologues that is essential for latency \textit{in vivo}. The BoHV-4 ORF73-deleted strain may be a good candidate for the development of BoHV-4 as a vector in vaccinology.

**METHODS**

**Computational analysis.** Protein sequences were analysed with the CLUSTAL_X sequence alignment program (Thompson et al., 1997).

**Cells and viruses.** The MDBK cell line (ATCC CCL-22) was cultured in minimum essential medium (MEM; Invitrogen) containing 5% FCS (Harlan). Embryonic bovine lung (EBL; DSMZ ACC192), EBL stably expressing the Cre recombinase (EBL-NLS-Cre) (Gillet et al., 2005) and bovine macrophage (BOMAC) (Stabel & Stabel, 1995) cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) containing 10% FCS. The BoHV-4 V. test strain and the V. test BAC G-derived BAC clone have been described elsewhere (Gillet et al., 2005).

**RT-PCR, determination of kinetic class of transcription.** For the determination of kinetic class of transcription, 100 µg cycloheximide (CHX) ml\(^{-1}\) (Sigma) or 300 µg phosphonoacetic acid (PAA) ml\(^{-1}\) (Sigma) were added to the culture medium at the time of infection to inhibit \textit{de novo} protein synthesis or viral DNA polymerase, respectively. At 24 h p.i. of MDBK cells (m.o.i. of 1), cytoplasmic RNA was extracted using an RNeasy Mini kit (Qiagen) with on-column DNase I digestion. Reverse transcription and PCRs were performed using the SuperScript III One-step RT-PCR System with a Platinum \textit{Taq} DNA polymerase kit (Invitrogen). Gene-specific primers (Supplementary Table S1, available in JGV Online) were used to detect viral mRNAs. The absence of DNA template was verified by replacing the reverse transcriptase/\textit{Taq} mix with \textit{Taq} DNA polymerase only.

**Northern blotting.** MDBK cells were mock-infected or infected with the BoHV-4 V. test strain at an m.o.i. of 4. After an incubation period of 90 min, cells were washed with PBS and then overlaid with MEM containing 5% FCS. Cells were harvested at 4, 8 and 24 h p.i. RNA was then purified from the cells and analysed by Northern blotting according to standard protocols (Sambrook & Russell, 2001). The ORF73 was used as the probe for detecting the RNAs encoding ORF73.

**Production of BoHV-4 ORF73 recombinant strains.** BoHV-4 recombinants were produced using BAC cloning and prokaryotic recombinant technologies as described previously (Gillet et al., 2005). The V. test BAC G plasmid was used as the parental plasmid (Gillet et al., 2005). The V. test BAC G ORF73 and the V. test BAC G ORF73 revertant plasmids were produced using two-step \(galK\) positive/negative selection in bacteria (Warming et al., 2005; Supplementary Fig. S1). The first recombination process (\(galK\)-positive selection) consisted of replacing ORF73 with the \(galK\) gene, resulting in the V. test BAC G ORF73 plasmid. Recombination was achieved using the H1-\(galK-H2\) cassette (Supplementary Fig. S1) and consisted of the \(galK\) gene flanked by 60 bp sequences corresponding to the ORF73 flanking regions. This cassette was produced by PCR using the \(pgalK\) vector (Warming et al., 2005) as the template and
galK-H2endfw and galK-H1startrev as the forward and reverse primers, respectively (Supplementary Table S1). The second recombination process (galK-negative selection) consisted of restoring ORF73 to generate a revertant plasmid. Recombination was achieved using the H1-ORF73-H2 cassette (Supplementary Fig. S1), consisting of ORF73 flanked by 60 bp sequences corresponding to the ORF73-flanking regions. This cassette was produced by PCR using the BoHV-4 V. test genome as the template and H2endfw and H1startrev as the forward and reverse primers, respectively (Supplementary Table S1). Reconstitution of infectious virus from BAC plasmids was obtained by transfection into EBL cells. To excise the BAC cassette, reconstituted viruses were propagated in EBL-NLS-Cre cells expressing Cre recombinase to generate the corresponding excised strain (Supplementary Fig. S1).

Virus purification. Virus grown on MDBK cells was semi-purified as described previously (Vanderplasschen et al., 1993). Briefly, after removal of the cell debris, the virus present in the cell supernatant was pelleted by ultracentrifugation at 100 000 g through a 30% (w/v) sucrose cushion.

Southern blotting. Southern blot analysis was performed as described previously using ORF73 and the galK cassette as probes (Costes et al., 2009).

Plaque-size assay. MDBK cells were infected at an m.o.i. of 0.5. After an incubation period of 90 min, cells were washed with PBS and then overlaid with MEM containing 5% FCS and 0.6% (w/v) carboxymethylcellulose (CMC; Sigma) to obtain isolated plaques, as described previously (Vanderplasschen et al., 1993). At successive intervals after infection, plaques were stained by indirect immunofluorescent staining (see below) and observed by epifluorescence microscopy.

Multi-step growth curves. Triplicate cultures of MDBK cells were infected at an m.o.i. of 0.5. After an incubation period of 90 min, cells were washed with PBS and then overlaid with MEM containing 5% FCS. Supernatants of infected cultures were harvested at successive intervals after infection, and the amount of infectious virus was determined by plaque assay on MDBK cells as described previously (Vanderplasschen et al., 1993).

Episome-persistence assay. BOMAC cells were infected at an m.o.i. of 0.5. After an incubation period of 90 min, cells were washed with PBS and then overlaid with MEM containing 5% FCS. Supernatants of infected cultures were harvested at successive intervals after infection, and the amount of infectious virus was determined by plaque assay on MDBK cells as described previously (Vanderplasschen et al., 1993).

Animals. Specific-pathogen-free New Zealand White rabbits were housed individually throughout this study. Four groups were used, each with three rabbits. Animals from group 1 were mock-infected intravenously with PBS. Animals from groups 2, 3 and 4 were inoculated intravenously with 10⁸ p.f.u. V. test BAC G excised, V. test BAC G ORF73 excised and V. test BAC G ORF73 revertant excised strains, respectively. At the end of the experiment, rabbits were euthanized and a necropsy examination was performed, during which the spleen was collected. This animal study was accredited by the local ethics committee of the University of Liège (Belgium).

Isolation of PBMCs and preparation of spleen-cell suspension. Blood samples were collected and PBMCs were separated by a Ficoll-Paque Plus (GE Healthcare) density gradient as described previously (Dewals et al., 2008). Immediately after euthanasia, the spleen was removed and half of it was homogenized using a tissue grinder (VWR), passed through a stainless-steel sieve and washed in FCS-free MEM before further analyses.

Viral genome detection by real-time PCR. DNA was purified from the spleen and PBMCs using a QIAamp DNA Mini kit (Qiagen). Real-time PCR was performed as described elsewhere (Boudry et al., 2007). A 103 bp fragment corresponding to BoHV-4 ORF8 was amplified with the forward primer 8startfw and the reverse primer 8midlerev (Supplementary Table S1) in the presence of the fluorescent probe 5'-FAM-AACACGTCAAACGAAGCCATC-CACGTG-TAMRA-3'. Purified BoHV-4 V. test genome was used to establish standard curves. PCR amplifications and fluorescence reactions were carried out in an iCycler system (Bio-Rad) under the following conditions: initial activation of the Taq polymerase (Bio-Rad) at 94 °C for 5 min, followed by 50 cycles at 94 °C for 1 min, 51 °C for 30 s and 72 °C for 1 min. For cellular quantitative amplification, a 178 bp fragment of the rabbit β-globin gene was used (GenBank accession no. V00882) and amplified as described previously (Boudry et al., 2007).

Infectious-centre assay. Virus detection in the spleen-cell suspension was assayed by infectious-centre assay as follows. A total of 5 × 10⁶ MDBK cells grown in six-well cluster dishes (Becton Dickinson) were co-cultured for 4 days at 37 °C with 5 × 10⁵, 5 × 10⁴ or 5 × 10³ spleen cells in MEM containing 5% FCS, 0.6% CMC and 5 × 10⁻⁵ M β-mercaptoethanol (Merck). Cells were then fixed and stained for indirect immunofluorescent detection of viral antigen as described below.

Quantification of anti-BoHV-4 antibodies by ELISA. Nunc Maxisorp ELISA plates (Nalge Nunc) were coated for 18 h at 37 °C with 0.1% Tween 20 (v/v)-disrupted BoHV-4 virions (2 × 10⁶ p.f.u. per well), blocked in PBS containing 0.1% Tween 20 (v/v) and 3% BSA (w/v), and incubated with rabbit serum [diluted 1:300 in PBS containing 0.1% Tween 20 (v/v) and 3% BSA (w/v)]. Bound antibodies were detected with alkaline phosphatase-conjugated goat anti-rabbit IgG polyclonal antibody (Sigma). Washings with PBS containing 0.1% Tween 20 (v/v) and 3% BSA (w/v). Nitrophenylphosphate (Sigma) was used as substrate and A₄₀⁵ was read using a Benchmark ELISA plate reader (Thermo).

Indirect immunofluorescent staining. Indirect immunofluorescent staining was performed as described previously (Gillet et al., 2005). Mouse mAb 35 (diluted 1:100) raised against the BoHV-4 glycoprotein complex gp6–gp10–gp17 and Alexa Fluor 633 goat anti-mouse IgG (H+L) (2 μg ml⁻¹; Invitrogen) were used as primary and secondary antibodies, respectively.

Microscopy analysis. Samples were examined using an epifluorescence microscope TE2000 S Nikon and a Leica DC300F CCD camera system.

Flow cytometry. Flow-cytometry acquisitions and analyses were performed using a three-laser Becton Dickinson fluorescence-activated cell sorter (FACSARia).

Statistical analysis. The linear model y = μ + group + time + experiment + error was used to analyse viral multi-step growth curves and rabbit anti-BoHV-4 humoral responses. For the multi-step growth curve comparison, the experiment factor was not significant and was therefore not included in the model. For analysis of anti-BoHV-4 humoral responses, correlation between successive measurements made on the same animal was taken into account using compound symmetry structure. An ANOVA-2 test was used to determine the significance of the data obtained with the episome-persistence assay (P<0.0005).
ACKNOWLEDGEMENTS

M.T. is a Research Fellow of the Belgian Fonds pour la formation à la Recherche dans l’Industrie et dans l’Agriculture. L.G. and B.D. are research associate and postdoctoral researcher, respectively, of the Fonds de la Recherche Scientifique – Fonds National Belge de la Recherche Scientifique (FRS – FNRS). The authors are grateful to Cédric Delforge and Dominique Ziant for excellent technical assistance. This work was supported by grants from the FRS – FNRS (9.4564.04) and the University of Liège (Fonds spéciaux GE-03/14).

REFERENCES


