The core 2 \(\beta-1,6-N\)-acetylglucosaminytransferase-M encoded by bovine herpesvirus 4 is not essential for virus replication despite contributing to post-translational modifications of structural proteins

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The Bo17 gene of bovine herpesvirus 4 (BoHV-4) is the only virus gene known to date that encodes a homologue of the cellular core 2 \(\beta-1,6-N\)-acetylglucosaminytransferase-mucine type (C2GnT-M). Recently, our phylogenetic study revealed that the Bo17 gene has been acquired from an ancestor of the African buffalo around 1–5 million years ago. Despite this recent origin, the Bo17 sequence has spread to fixation in the virus population possibly by natural selection. Supporting the latter hypothesis, it has been shown by our group for the V. test strain that Bo17 is expressed during BoHV-4 replication \textit{in vitro}, and that Bo17 expression product (pBo17) has all three enzymic activities exhibited by cellular C2GnT-M, i.e. core 2, core 4 and I branching activities. In the present study, firstly it was investigated whether encoding a functional C2GnT-M is a general property of BoHV-4 strains. Analysis of nine representative strains of the BoHV-4 species revealed that all of them express the Bo17 gene and the associated core 2 branching activity during virus replication \textit{in vitro}. Secondly, in order to investigate the roles of Bo17, its kinetic class of expression was analysed and a deleted recombinant strain was produced. These experiments revealed that Bo17 is expressed as an early gene which is not essential for virus replication \textit{in vitro}. However, comparison of the structural proteins, produced by the wild-type, the revertant and the deleted viruses, by 2D gels demonstrated that pBo17 contributes to the post-translational modifications of structural proteins. Possible roles of Bo17 \textit{in vivo} are discussed.

INTRODUCTION

Bovine herpesvirus 4 (BoHV-4) is a gammaherpesvirus which has been isolated throughout the world from healthy cattle, as well as those exhibiting a variety of diseases (Thiry et al., 1992). Isolates of BoHV-4 have also been recovered from other ruminant species such as American bison (\textit{Bison bison}) (Todd & Storz, 1983), African buffalo (\textit{Syncerus caffer}) (Rossiter et al., 1989) and sheep (Van Opdenbosch et al., 1986). Sporadic isolations were reported in lion (Egelhof, 1991), cat (Fabricant et al., 1971) and owl monkey (\textit{Aotus trivirgatus}) (Barahona et al., 1973). BoHV-4 has a B-type genome structure consisting of a long unique region (LUR) flanked by polyrepetitive DNA (prDNA) elements (Fig. 1a). The entire BoHV-4 genome sequence has recently been published (Zimmermann et al., 2001). This sequence confirmed that BoHV-4 is a member of the \textit{Rhadinovirus} genus and revealed that, in comparison to the other members of this genus, BoHV-4 has a reduced set of ORFs homologous to cellular genes. However, the Bo17 gene of BoHV-4 is the only virus gene known to date that encodes a homologue of the cellular core 2 \(\beta-1,6-N\)-acetylglucosaminytransferase-mucine type (C2GnT-M) (Vanderplasschen et al., 2000).

C2GnT-M is a member of the \(\beta-1,6-N\)-acetylglucosaminyltransferase (\(\beta1,6\)GnT) gene family. \(\beta1,6\)GnTs are involved in the synthesis of (GlcNAc/\(\beta1\rightarrow6\))Gal(NAc) linkages and
play crucial roles in glycan synthesis (Fukuda, 1994; Schachter, 1994). Three activities have been described in the β1,6GnT gene family based on the acceptor substrate: the synthesis of the O-glycan core 2 (Galβ1→3(GlcNAcβ1→6)GalNAc) and core 4 (GlcNAcβ1→3(GlcNAcβ1→6)GalNAc) structures, and the synthesis of the I structure (GlcNAcβ1→3(GlcNAcβ1→6)Gal). Among the β1,6GnTs described to date, C2GnT-M is the only enzyme able to form all three enzymic activities described in this family, i.e. core 2, core 4 and I structures.

The core 2 structure is produced by core 2 β1,6GnT (C2GnT) activity using core 1 (Galβ1→3GalNAc) as an acceptor substrate (Fukuda et al., 1986; Piller et al., 1988). In many cells, the latter is the major constituent of O-glycans.
Roles of Bo17 in the biology of BoHV-4 infection

(Fukuda, 1994). The biological importance of the core 2 structure is explained by the various ligand carbohydrates that can be formed in core 2 branched oligosaccharides. For example, sialyl Le\(^\alpha\) and sulfated sialyl Le\(^\alpha\) present in core 2 branched oligosaccharides have been shown to be preferential ligands for P- and L-selectin (Lowe, 1994; Hemmerich et al., 1995; Wilkins et al., 1996; Bistrup et al., 1999; Hiroaka et al., 1999; Fukuda, 2002). The core 4 \(\beta 1,6\)GnT (C4GnT) activity generates core 4 from core 3 (GlcNAc\(\beta 1\rightarrow 3\)GalNAc). Core 4 is mainly expressed in mucin-producing tissues (Schachter & Brockhausen, 1992).

For example, sialyl Lex and sulfated sialyl Lex present in erythrocytes and also occurs on many mucins (Fukuda, 1994). Expression of cellular \(\beta 1,6\)GnT products changes during development, immunodeficiency and oncogenesis. During T-cell development, the synthesis of the core 2 branch is highly regulated. For example, quiescent T cells in the peripheral blood express simpler O-glycans, while core 2 branched O-glycans appear when T cells are activated by mitogens (Piller et al., 1988). Immunodeficiencies, such as AIDS (Lefebvre et al., 1994) and Wiskott–Aldrich syndrome (Higgins et al., 1991; Piller et al., 1991) are associated with aberrant expression of core 2 O-glycans on T cells. Changes in C2GnT expression have also been observed during oncogenesis. An increase in expression of core 2 branched oligosaccharides by cancer cells has been reported in leukaemia, colonic carcinoma and cells transfected with T24Hras (Brockhausen et al., 1991; Saitoh et al., 1991; Yang et al., 1994; Vavasseur et al., 1995).

Recently, our phylogenetic study revealed that the BoHV-4 Bo17 gene has been acquired from an ancestor of the African buffalo around 1.5 million years ago, implying that cattle subsequently acquired BoHV-4 by cross-species transmission (Markine-Goriaynoff et al., 2003). This observation makes Bo17 the most recent known example of herpesvirus gene acquisitions, followed by the virus interleukin 10 genes of Epstein–Barr virus (human herpesvirus 4, HHV-4) and equine herpesvirus 2 (EHV-2). In the context of herpesvirus evolution, the acquisition of the Bo17 gene 1.5 million years ago should be considered as a recent event. Indeed, several observations have demonstrated that the evolution of herpesviruses is a relatively slow process. For example, it has been estimated that the actual divergence existing between the closely related human simplex virus 1 (human herpesvirus 1, HHV-1) and 2 (human herpesvirus 2, HHV-2) reflects eight million years of evolution (Dolan et al., 1998). However, despite the recent origin of the Bo17 gene, the survey of 34 BoHV-4 strains isolated from different continents and from different animal species revealed that they all encode Bo17 ORF, suggesting that the gene has spread to fixation in the virus population, possibly by natural selection. Moreover, analysis of the ratio of nonsynonymous to synonymous substitutions revealed a strong constraint on nonsynonymous changes since the transfer of the cellular gene to the viral genome. Taken together, these data suggest that Bo17 encodes a functional C2GnT-M that is important for BoHV-4. Consistent with this hypothesis, the Bo17 expression product (pBo17) of the BoHV-4 \(\gamma\) test strain was shown to have conserved all three enzymic activities exhibited by cellular C2GnT-M, i.e. core 2, core 4, and \(1\) branching activities (Vanderplaschen et al., 2000).

In the present study, we are pursuing our investigation on the roles of Bo17 in the biology of BoHV-4 infection. As the Bo17 gene has been acquired very recently during evolution, we first investigated whether encoding a functional C2GnT-M is a general property of BoHV-4 strains. Secondly, the kinetic class of expression of the Bo17 gene was determined and a BoHV-4 strain deleted for this gene has been produced. This study demonstrates that encoding a functional C2GnT-M is a general property of BoHV-4 strains, and that Bo17 is expressed as an early gene that is not essential for virus replication \(\textit{in vitro}\) despite contributing to post-translational modifications of structural proteins. Possible roles of Bo17 \(\textit{in vivo}\) are discussed.

**Fig. 1.** Production of a recombinant BoHV-4 strain deleted for Bo17. Using the BoHV-4 \(\gamma\) test strain as parental strain, a recombinant BoHV-4 strain deleted for Bo17 and a derived revertant strain were produced by homologous recombination. (a) Construction of the recombination cassettes. The EcoRI restriction map of the entire BoHV-4 \(\gamma\) test strain is shown at the top. The EcoRI I fragment containing the Bo17 gene was first cloned into the pGEM-T Easy vector resulting in pGEM-T–EcoRI I. Most of the Bo17 ORF was then replaced by an EGFP expression cassette as described in Methods, resulting in pGEM-T–EcoRI I-delBo17. (b) Production of the recombinant strains. The delBo17 \(\gamma\) test strain was produced by recombination between the genome of the wild-type \(\gamma\) test strain and the linearized pGEM-T–EcoRI I-delBo17 vector. A revertant strain (revBo17 \(\gamma\) test) was then produced by recombination between the genome of the delBo17 \(\gamma\) test strain and the linearized pGEM-T–EcoRI I. (c) Characterization of BoHV-4 recombinants by a combined restriction endonuclease and Southern blotting approach. The DNA of the parental strain \(\gamma\) test and the derived recombinant strains delBo17 \(\gamma\) test and revBo17 \(\gamma\) test were analysed by EcoRI restriction (left panel) and further tested by Southern blot using probes corresponding to nucleotides 31–910 of the \(\gamma\) test Bo17 ORF (middle panel) or EGFP ORF (right panel). White and open arrows indicate the restriction fragments containing Bo17 and EGFP ORFs, respectively. Marker sizes (MS) in kbp are indicated on the left.

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METHODS

Cells and virus. Madin–Darby bovine kidney (MDBK, ATCC CCL-22), embryonic bovine trachea (EBTr, ATCC CCL-44) and embryonic bovine lung cells (EBL, German collection of microorganisms and cell cultures DSMZ ACC192) were cultured in Minimum Essential Medium (Gibco-BRL) containing 10% fetal calf serum (FCS). Nine strains of BoHV-4 representative of the BoHV-4 species isolated throughout the world and from different animal species were used in this study (listed in Table 1).

RT-PCR. Cytoplasmic RNA was isolated from mock-infected and BoHV-4 infected MDBK cells 24 h after infection (m.o.i. of 10 p.f.u. per cell) using an RNeasy Mini Kit (Qiagen), and then further purified using High Pure RNA Isolation Kit (Roche). Reverse transcriptase (RT) reactions were performed on 150 ng of RNA using First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech). The 5′-NotI-d(T)18-3′ primer provided in the kit was used for these reactions. Finally, cDNA products were amplified by PCR using primers specific for Bo17 or bcl-xL sequences. Bo17 was amplified using the forward primer 5′-GATAAATCCACGTCAGGATCCCAATTTACGCGTTAAGATACATTG-3′ and the reverse primer 5′-TCAGATCTGAGTGTCAGCTTAAGATACATTG-3′ and the reverse primer 5′-TCAGATCTGAGTGTCAGCTTAAGATACATTG-3′ corresponding to nucleotides 868–890 and nucleotides 1323–1303 of the ORF, respectively (GenBank accession no. AF231105) (Vanderplasschen et al., 2000). Cellular bcl-xL was amplified with the forward primer 5′-TTGGACAATCCGCGTGTTG-3′ and the reverse primer 5′-GTAGATTGATGAGGTCAAGTG-3′ designed for the human sequence (GenBank accession nos Z23115, L20121, Z23116 L20122). These bcl-xL primers allowed simultaneous amplification of bcl-xL (765 bp) and bcl-xL (576 bp).

Antibodies. The mouse monoclonal antibody (mAb) 1G10 (PharMingen) reacts with human leukosialin independently of the deletion of the BoHV-4 glycoprotein complex gp6/gp10/gp17 (Vanderplasschen et al., 1995). The mAb 35 is raised against the BoHV-4 V. test strain and the V. test Bo17 ORF or EGFP ORF. The mAb 35 is IgG1 and IgG2b, respectively.

Vectors. The pcDSRs-leukosialin vector encodes human leukosialin (Yeh et al., 1999). The pEGFP-C1 (Clontech) vector encodes an enhanced green fluorescent protein (EGFP) eukaryotic expression cassette.

Indirect immunofluorescent staining. Cells were fixed in PBS containing 4% (w/v) paraformaldehyde for 10 min on ice and then 20 min at 20°C. In order to ensure permeabilization, the staining (incubation and washes) was performed in PBS containing 10% (v/v) FCS and 0.2% (w/v) saponin (PBSFS) (Sigma). The mAbs T305 (1/100), 1G10 (1/100), and 35 (1/1000) were used as primary antibodies at the indicated dilutions. The mAbs 35 and 1G10 were detected with Alexa Fluor 488 goat anti-mouse IgG (H+L) (Alexa488–GAM, 1/200, Molecular Probes) or Alexa Fluor 568 rabbit anti-mouse IgG (H+L) (Alexa568–RAM, 1/200, Molecular Probes). The mAb T305 was detected with R-phycocerythrin goat anti-mouse IgG1 antibodies (PE–GAMlgG1, 1/250, Southern Biotechnology Associates).

Confocal microscopy analysis. Confocal microscopy analysis was performed with a TCS SP confocal microscope (Leica) as described previously (Vanderplasschen et al., 2000).

Production of a BoHV-4 Bo17 deleted strain. A BoHV-4 V. test strain partially deleted for the Bo17 ORF was produced by homologous recombination as described elsewhere (Meyer et al., 1998). The recombination cassette was produced as follows (Fig. 1a). Firstly, the restriction fragment EcoRI I (containing Bo17) of the V. test strain genome was cloned into the pGEM-T–EcoRI I vector (Promega) resulting in pGEM-T–EcoRI I. Secondly, an EGFP expression cassette was produced by PCR using the forward primer 5′-GAGATCTCCGATCACTTGCACTAGG-3′ corresponding to HindIII site and nucleotides 4729–0023 of the pC1-EGFP vector (Clontech) (GenBank accession no. U55763) and the reverse primer 5′-GGCTCGAGTATCCGCTGGATCCGATCACTTGCACTAGG-3′ corresponding to XhoI site and nucleotides 1652–1629 of pC1-EGFP. A modified version of the pC1-EGFP vector in which most of the multiple cloning site had been deleted by digestion with BglII and BamHI was used as template. Thirdly, the EGFP cassette released by HindIII and XhoI digestion was cloned into pGEM-T–EcoRI I treated with the same enzymes, thus resulting in pGEM-T–EcoRI I-delBo17 in which nucleotides 31–910 of the Bo17 ORF are replaced by the EGFP expression cassette. The latter construction was used to generate the delBo17 V. test strain by homologous recombination (Meyer et al., 1998) (Fig. 1b). Finally, a revertant strain (revBo17 V. test) was produced based on the same approach using the pGEM-T–EcoRI I vector as recombination cassette.

Southern blot. Southern blot analysis of viral DNA digested with EcoRI was performed as described previously (Markine-Goriaynoff et al., 2003) using probes corresponding to nucleotides 31–910 of the V. test Bo17 ORF or EGFP ORF.

Growth curves. Single-step and multi-step virus growth experiments were conducted to compare the growth kinetics of mutant and revertant viruses to those of the parent virus. A series of triplicate cultures of EBL cells was infected at a m.o.i. of 5 for single-step and 0.05 for multi-step assays. After 1 h of adsorption, the cells were washed, then overlaid with MEM containing 5% FCS. Supernatant of infected cultures and infected cells were harvested at

<table>
<thead>
<tr>
<th>Strain(s)*</th>
<th>Host species</th>
<th>Country of isolation</th>
<th>Reference source</th>
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<tbody>
<tr>
<td>V. test</td>
<td>Cattle (Bos taurus)</td>
<td>Belgium</td>
<td>Thiry et al. (1981)</td>
</tr>
<tr>
<td>LVR 140</td>
<td>Cattle (Bos taurus)</td>
<td>Belgium</td>
<td>Wellemans et al. (1983)</td>
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<td>Hungary</td>
<td>Bartha et al. (1966)</td>
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<tr>
<td>DN599</td>
<td>Cattle (Bos taurus)</td>
<td>United States</td>
<td>Mohanty et al. (1971)</td>
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<td>66-p-347</td>
<td>American bison (Bison bison)</td>
<td>United States</td>
<td>Todd &amp; Storz (1983)</td>
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<td>M40</td>
<td>Zebu (Bos indicus)</td>
<td>India</td>
<td>Moreno-Lopez et al. (1989)</td>
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*The BoHV-4 strains used in this study were isolated in different countries and from various host species.
successive intervals after infection, and the amount of infectious virus was determined by plaque assay on MBDK cells as described previously (Vanderplasschen et al., 1993).

**Plaque size.** EBL cells grown on coverslips were infected with BoHV-4 and then overlaid with MEM containing 5% FCS and 0-6% (v/v) carboxymethylcellulose (CMC) (Sigma) in order to obtain isolated plaques as described elsewhere (Vanderplasschen et al., 1993). At successive intervals after infection, plaques were stained by indirect immunofluorescent staining using mAb 35 and Alexa568-GAM as described above. Pictures of plaques were captured with a CCD camera system (DC 300F, IMSi, version V1.20, Leica). Plaque area were then determined with the AnalySIS 3.2 software (Soft Imaging System).

**Determination of Bo17 kinetic class of transcription.** These experiments were performed as described elsewhere (van Santen, 1991; Lomonte, 1997). Briefly, subconfluent monolayers of MBDK cells were infected with BoHV-4 V. test strain at an m.o.i. of 1 p.f.u. per cell. At the time of infection, cycloheximide (CHX) (100 μg ml⁻¹) or phosphononoic acid (PAA) (300 μg ml⁻¹) were added to the culture medium to inhibit de novo protein synthesis or DNA polymerase, respectively. Eight hours after infection, cytoplasmic RNA was extracted, purified and treated as described above for RT-PCR. The cDNA products were amplified by PCR using primers specific for Bo5 encoding BoHV-4 major immediate early (IE) transcript (van Santen, 1991), ORF21 encoding thymidine kinase expressed as a late (E) gene (Kit, 1985), ORF22 encoding glycoprotein H (gH) expressed as a late (L) gene (Lomonte, 1997) and Bo17. The cDNA fragment encoding Bo5 (868 bp resulting from splicing of the 1140 bp ORF) was amplified using the forward primer 5'-GCTAGAAGAAAATGGCCAGTAAAG-3' (corresponding to the sequence upstream of the starting codon and the 13 first nucleotides of the ORF) and the reverse primer 5'-CATGTCCTGAGTGGGTCTATG-3' (corresponding to the last 22 nucleotides of the ORF) (GenBank accession no. M60043) (van Santen, 1991). The cDNA fragment encoding ORF21 (499 bp) was amplified using the forward primer 5'-CATGGAGAGGGGCTAGAGG-3' and the reverse primer 5'-CTTACACACAGACTCTTTGC-3' corresponding to nucleotides 841–860 and 1318–1339 of the ORF, respectively (GenBank accession no. S49773) (Lomonte et al., 1992). The cDNA fragment encoding ORF22 (564 bp) was amplified using the forward primer 5'-CCGCGTTGAAACAAGTTCT-3' (corresponding to nucleotides 1563–1582 of the ORF) and the reverse primer 5'-GTCAGAGAACATATGTAACATC-3' (corresponding to the last 19 nucleotides of the ORF and the downstream sequence) (GenBank accession no. Z79633) (Lomonte et al., 1997). The cDNA fragment encoding Bo17 (456 bp) was amplified as described above.

**Statistical analysis.** Student’s t-test was used to test for the significance of the results (P<0.01).

**Virus purification.** BoHV-4 grown on EBL cells was 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 centrifugation through a 30% (v/v) sucrose cushion, then centrifuged through two successive 20–50% (v/v) potassium tartrate gradients.

**2-Dimensional gel electrophoresis (2-DE).** 2-DE was performed essentially as described elsewhere (Rabilloud et al., 1997). Purified viruses were treated with PlusOne 2-D Clean-Up Kit (Amersham Pharmacia Biotech) before solubilization in an isoelectric focusing buffer containing 7 M urea, 2 M thiourea, 4% (v/v) CHAPS, 0-5% (v/v) Triton X-100, 20 mM DTT, 0-5% (v/v) carrier ampholytes pH 3-5-10 (Amersham Pharmacia Biotech) and trace amounts of bromophenol blue. Immobilized pH gradient (IPG) strips (Amersham Pharmacia Biotech) spanning the appropriate pH range were rehydrated by incubation overnight (at room temperature) in the solubilized protein samples using the Immobiline Drystrip Reswelling Tray (Amersham Pharmacia Biotech). Isoelectric focusing was performed in the Multiphor II (Amersham Pharmacia Biotech) system at 20°C using the Pharmacia EPS 3500 XL power supply in gradient mode according to the manufacturer’s procedure. Prior to the second dimension SDS-PAGE, the IPG strips were equilibrated twice for 15 min in buffer containing 6 M urea, 30% (v/v) glycerol, 50 mM Tris–HCl (pH 8.8), 2% (w/v) SDS, 2-5 mg DTT ml⁻¹ and traces of bromophenol blue. In the second equilibration step, DTT was omitted and substituted with 45 mg iodoacetamide ml⁻¹. Electrophoresis in the second dimension was carried out in the Protean II XI (Bio-Rad) apparatus at 20°C and using a 12-5% polyacrylamide gel. The power program consisted of two phases: 5 mA per gel for 2 h, followed by 8 mA per gel until the tracing dye reached the bottom of the gel. Proteins were visualized by silver staining essentially as described elsewhere (Oakley et al., 1980). The reagents dithiothreitol, SDS and Triton X-100 were purchased from Sigma, ammonium persulphate and TEMED (N,N,N’,N’-tetramethylethlenediamine) from Bio-Rad, and other chemicals were supplied by Merck.

**RESULTS**

**Expression of Bo17 in the BoHV-4 species.** In our recent phylogenetic study (Markine-Goraiyoff et al., 2003), we surveyed 34 BoHV-4 strains isolated from different continents and from different animal species, and found that all possess the Bo17 sequence. This observation suggests that Bo17 has spread to fixation in the virus population despite its recent acquisition. While this may have occurred through random genetic drift, the observation of constraint on nonsynonymous substitutions in Bo17 suggests that the presence of the gene is advantageous, and thus that the spread was driven by natural selection. Supporting this hypothesis, it has been shown, in the BoHV-4 V. test strain, that Bo17 is transcribed during virus replication and that its translation product directs the expression of core 2 in infected cells (Vanderplasschen et al., 2000).

In the present study, we investigated whether expressing Bo17 and its associated core 2 branching activity is a general property in the BoHV-4 species. With that goal in mind, nine strains representative of the BoHV-4 species were selected (see Table 1). The expression of Bo17 was first investigated by RT-PCR approach as described in Methods. PCRs were performed on first strand cDNA made from infected or mock-infected MBDK cells. Cells infected with the nine strains gave rise to the expected 456 bp Bo17 PCR product (Fig. 2b). When reverse transcriptase was omitted from the reactions, the products seen in infected cells were not detected, indicating that the latter did not result from amplification of contaminant viral DNA (Fig. 2a). In contrast to infected cells, no Bo17 PCR product was generated from mock-infected MBDK cells. Cells infected with the nine strains gave rise to the expected 456 bp Bo17 PCR product (Fig. 2b). When reverse transcriptase was omitted from the reaction further
supporting the absence of contaminant DNA in the RNA preparations (Fig. 2a). Taken together, these results suggest that expressing Bo17 is a general property of BoHV-4 strains.

Knowing that all strains tested express Bo17 during virus replication, we investigated whether its translation product has conserved the core 2 branching activity in all strains. To test this hypothesis, EBTr cells (which are core 2 negative) were transfected with the pcDSRz-leukosialin vector, expressing human leukosialin, then infected with the nine BoHV-4 strains. Finally, the cells were subjected to immunofluorescent staining as described in the legend of Fig. 3. Similar results were obtained with the nine strains. As an example, the results generated with the DN599 strain are presented in Fig. 3. Staining with mAb 1G10 (which reacts with human leukosialin independently of the expression of core 2 branched oligosaccharides on the target protein) revealed that both mock-infected and infected EBTr cells expressed human leukosialin (Fig. 3a, e). Staining with mAb T305, which reacts only with human leukosialin expressing core 2 branched oligosaccharides, revealed positive cells in BoHV-4 infected cells (Fig. 3g) but not in mock-infected cells (Fig. 3c). Moreover, the double immunofluorescent staining performed with mAb T305 and mAb35 (which reacts with the BoHV-4 glycoprotein complex gp6/gp10/gp17) revealed that all core 2 expressing cells were infected by BoHV-4 (compare Fig. 3f with 3g).

Effect of Bo17 deletion on BoHV-4 replication in vitro

The results presented above suggest that since the acquisition of the Bo17 gene, a strong constraint has been applied on this gene to maintain its expression during the virus replication cycle and to conserve the core 2 branching activity of its translation product. All together, these data suggest that Bo17 plays an important function in the biology of BoHV-4 infection. In order to address the importance of Bo17 in virus replication in vitro, a BoHV-4 strain deleted for Bo17 and a revertant strain were produced. The strategy used is described in Methods (Fig. 1). The BoHV-4 V. test strain was used as parental strain to generate the delBo17 V. test in which most of the Bo17 ORF has been replaced by an EGFP expressing cassette (Fig. 1a, b). Finally, a revertant strain was also generated. The molecular structures of the recombinant strains produced were confirmed by a combined restriction endonuclease and Southern blot approach (Fig. 1c) and by sequencing the regions used to target recombination (data not shown). All approaches confirmed that the recombinants produced had the correct molecular structure. Moreover, RT-PCR experiments and core 2 branching activity assays performed with the three viruses (V. test, delBo17 V. test and revBo17 V. test) revealed that the expression of Bo17 and core 2 branching activity were abolished in delBo17 V. test infected cells, but restored in revBo17 V. test infected cells (data not shown).

In order to investigate the role of Bo17 in BoHV-4 growth in vitro, one-step and multi-step growth assays were performed (Fig. 4). Ideally, those assays should be performed in a cell line which supports BoHV-4 replication and which is negative for core 2, core 4 and I branching activities (the activities mediated by pBo17 or by cellular C2GnT-M). To date, only cell lines negative for core 2 branching activity

![Fig. 2. Expression of Bo17 by BoHV-4 strains. MDBK cells were mock-infected or infected with nine different BoHV-4 strains isolated from different countries and from various host species (see Table 1). Twenty-four hours after infection, the expression of Bo17 and cellular bcl-X<sub>L/S</sub> was analysed by a RT-PCR approach as described in Methods. (a) PCR products generated when the RT was omitted from the reactions. (b) RT-PCR products. MS in bp are indicated on the left.](image-url)
have been described. The EBtr cells used above are negative for the expression of core 2 structure and support BoHV-4 replication. However, those cells are primary culture and cannot be expanded enough for the purposes of the present experiment. Consequently, EBL cells were used. EBL cultures contain 99% core 2 negative cells (data not shown).

First, we compared replication of wild-type V. test, delBo17 V. test and revBo17 V. test in a single round of replication (Fig. 4a, b). There was no difference in virus replication. Similar results were obtained in multi-step growth assays (Fig. 4c, d). Furthermore, the plaque sizes were similar for the mutant, revertant, and wild-type viruses (Fig. 5). Taken together, these results indicate that Bo17 is not essential for BoHV-4 replication in vitro and suggest that Bo17 exerts its biological functions in vivo.

**Determination of Bo17 kinetic class of transcription**

Several hypotheses that are not mutually exclusive could be made concerning the role of Bo17 in the biology of BoHV-4 infection in vivo (see discussion). All these hypotheses postulate that pBo17 is involved in post-translational modifications of viral and/or cellular glycoproteins, and consequently, implicate that Bo17 is expressed at the latest as an E gene. To test this hypothesis, CHX and PAA were used to identify Bo17 kinetic class of transcription (Fig. 6). This experiment revealed that Bo17 expression is prevented by CHX but not by PAA treatments suggesting that Bo17 is expressed at the latest as an E gene. Bo5, ORF21 and ORF22 were used as controls in this experiment; the results presented in Fig. 6 confirmed that they are IE, E and L genes, respectively. The absence

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**Fig. 3.** Expression of core 2 branching activity in BoHV-4 infected cells. Subconfluent monolayers of EBTr cells (which are negative for the expression of core 2 structure) grown on coverslips were transfected with pcDSRα-leukosialin using FuGENE 6 transfection reagent (Roche). Twenty-four hours after transfection, the cells were mock-infected (a–d) or infected (e–h) with BoHV-4 at a m.o.i. of 10. Twenty-four hours after infection, the cells were treated for indirect immunofluorescent staining as described in Methods: mAb 1G10 reacting with leukosialin (a, e), mAb 35 reacting with BoHV-4 glycoprotein complex gp6/gp10/gp17 (b, f) and mAb T305 raised against leukosialin expressing core 2 oligosaccharides (c, g) were used as primary antibodies and were revealed by Alexa488–GAM, Alexa488–GAM and PE–GAMIgG1, respectively. For simultaneous immunofluorescent detection of BoHV-4 and expression of core 2 structure (b–d and f–h), the cells were successively incubated with mAb 35, Alexa488–GAM, mAb T305 and PE–GAMIgG1. Sets of three horizontal panels (b–d and f–h) represent analyses of the same cells; 3b and f, and c and g, are analyses for Alexa488 and PE fluorescent emissions, respectively; 3d and h show the merged Alexa488 and PE signals. The side of each panel corresponds to 75 µm of the specimen. This experiment has been performed with the nine BoHV-4 strains listed in Table 1. Similar results were obtained with all strains tested, as an example, the results generated with the DN599 strain are presented in this figure.
of contaminant viral DNA in the mRNA preparations was confirmed by the absence of a PCR product when the reverse transcriptase was omitted from the reactions. Furthermore, the estimated molecular size of the Bo5 RT-PCR product revealed that it derived from the amplification of cDNA (868 bp) rather than from the viral genome (1140 bp) (data not shown).

**Bo17 contributes to post-translational modification of structural proteins**

To detect potential post-translational modifications of structural proteins by pBo17, preparations of purified virions from wild-type virus, the deletion mutant and the revertant were analysed by 2-DE. IPG strips spanning the pH ranges from 3 to 10, 4 to 7 or 6 to 11 were used. Differences between the deletion mutant and the other viruses were most clearly seen when using pH range 4 to 7 for the first dimension, as shown in Fig. 7. To facilitate comparison between the gels some of the spots present in all gels were arbitrarily numbered 1 to 27. Several spots were detectable in the deletion mutant only, or present at markedly increased intensity in this virus. These spots are labelled with circles in Fig 7(b). Most of them are in series of two to four spots with similar molecular mass, and some of them appear as additional spots within existing series. Although these proteins have not yet been identified, comparison with 2-DE patterns of herpes simplex virus proteins strongly suggests that such series are glycoproteins (Palfreyman et al., 1983; Sathananthan et al., 1996). Two single spots designated A and B (Fig. 7b) were unique to the deleted virus and were apparently unrelated to any of the series. Finally, close to the edges of the polyacrylamide gel, two clusters of spots identified by arrowheads were specific to the deleted virus (Fig. 7b). These clusters were absent in wild-type virus and the revertant (Fig. 7a, c) but could be replaced by the cluster of spots identified by an arrow in Fig. 7(a, c). All together, these data demonstrate that pBo17 contributes to post-translational modifications of structural proteins.

**DISCUSSION**

Bovine herpesvirus 4 is the only virus known to date that encodes a homologue of the cellular C2GnT-M (Vanderplasschen et al., 2000). Recently, our phylogenetic study revealed that the BoHV-4 Bo17 ORF has been acquired from an ancestor of the African buffalo around...
Despite this recent origin, the survey of 34 BoHV-4 strains isolated throughout the world suggested that the Bo17 sequence has spread to fixation in the virus population possibly by natural selection. Supporting the latter hypothesis, we showed, for the V. test strain, that Bo17 is expressed during BoHV-4 replication in vitro, and that Bo17 expression product has all three enzymic activities exhibited by cellular C2GnT-M, i.e., core 2, core 4, and I branching activities (Vanderplasschen et al., 2000). In the present study, we first investigated whether encoding a functional C2GnT-M is a general property of BoHV-4 strains. Analysis of nine representative strains of the BoHV-4 species revealed that all of them express the Bo17 gene and the associated core 2 branching activity during virus replication in vitro. Secondly, in order to investigate the roles of Bo17, we analysed its kinetics of expression and produced a deleted recombinant strain. These experiments revealed that Bo17 is expressed as an early gene that is not essential for virus replication in vitro. However, comparison of the structural proteins produced by the wild-type and the deleted viruses by 2-DE demonstrated that pBo17 contributes to the post-translational modifications of structural proteins. Although the present study did not demonstrate that these modifications consist in glycosylation of glycoproteins, the latter assumption is supported by pBo17 enzymic activities and by the 2-DE patterns of the proteins affected by Bo17 deletion (Fig. 7).

Several observations suggest that the Bo17 gene has spread to fixation in the virus population by natural selection rather than by genetic drift. Firstly, the analysis of the ratio of nonsynonymous to synonymous substitutions in the Bo17 gene of nine representative strains of the virus species revealed a strong constraint on synonymous changes since the transfer of the cellular gene to the viral genome (Markine-Goriaynoff et al., 2003). Secondly, the data presented in the present study demonstrated that these nine strains express Bo17 during virus replication and direct the synthesis of core 2 branched oligosaccharides in BoHV-4 infected cells. Thirdly, the 2-DE analysis performed in this study demonstrated that pBo17 contributes to post-translational modifications of BoHV-4 structural proteins. Taken together, these observations support the hypothesis that Bo17 encodes a function that is somehow important for BoHV-4.

The deletion of Bo17 had no effect on BoHV-4 replication in vitro, this observation is consistent with the recent acquisition of Bo17. Indeed, our phylogenetic study demonstrated 1-5 million years ago (Markine-Goriaynoff et al., 2003). Despite this recent origin, the survey of 34 BoHV-4 strains isolated throughout the world suggested that the Bo17 sequence has spread to fixation in the virus population possibly by natural selection. Supporting the latter hypothesis, we showed, for the V. test strain, that Bo17 is expressed during BoHV-4 replication in vitro, and that Bo17 expression product has all three enzymic activities exhibited by cellular C2GnT-M, i.e., core 2, core 4, and I branching activities (Vanderplasschen et al., 2000). In the present study, we first investigated whether encoding a functional C2GnT-M is a general property of BoHV-4 strains. Analysis of nine representative strains of the BoHV-4 species revealed that all of them express the Bo17 gene and the associated core 2 branching activity during virus replication in vitro. Secondly, in order to investigate the roles of Bo17, we analysed its kinetics of expression and produced a deleted recombinant strain. These experiments revealed that Bo17 is expressed as an early gene that is not essential for virus replication in vitro. However, comparison of the structural proteins produced by the wild-type and the deleted viruses by 2-DE demonstrated that pBo17 contributes to the post-translational modifications of structural proteins. Although the present study did not demonstrate that these modifications consist in glycosylation of glycoproteins, the latter assumption is supported by pBo17 enzymic activities and by the 2-DE patterns of the proteins affected by Bo17 deletion (Fig. 7).

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Fig. 7. Effects of Bo17 deletion on BoHV-4 structural proteins. Wild-type V. test (a), delBo17 V. test (b) and revBo17 V. test (c) virions grown in EBL cells were purified and subjected to 2-DE analysis as described in Methods. Eighteen cm IPG strips spanning the pH range from 4 to 7 and 12.5% polyacrylamide gels were used for the first and second dimensions, respectively. Proteins were visualized by silver staining. Some selected spots present in all gels were arbitrarily numbered from 1 to 27. Spots labelled with circles in panel (b) are either seen in the deletion mutant only, or present in this virus at markedly increased intensity as compared to the other viruses. Additional differences between the deletion mutant and the other viruses are detected at the edges of the gels and are identified with arrows and arrowheads. Positions of molecular mass markers are shown on the left of the gels.
that Bo17 was acquired around 1.5 million years ago (Markine-Goriaynoff et al., 2003). This observation makes Bo17 the most recent known example of herpesvirus gene acquisitions; clearly, the Bo17 biological function could not be essential for virus replication before then. The fact that a virus gene is not essential for virus replication in vitro does not preclude that this gene is important in vivo. Myxoma virus (MYXV) is one of the very few viruses known to date for encoding a glycosyltransferase (Jackson et al., 1999). Interestingly, the MST3N gene of MYXV was shown to encode a functional 2,3-sialyltransferase which is not essential for virus replication in vitro but enhances virulence in vivo by a still unknown mechanism (Jackson et al., 1999).

The biological functions of cellular β1,6GnTs have been shown to rely on both qualitative and quantitative regulation of their expression (Fukuda, 2002). Consequently, the effects of pBo17 activity expression in vivo should vary based on the cell type infected; unless the infection occurs in cells which do not express β1,6GnT activities or unless BoHV-4 infection completely down regulates the expression of host cell β1,6GnT activities. The latter hypothesis is unlikely because BoHV-4 does not affect host cell protein expression (Augsburger & Metzler, 1989). However, several hypotheses that are not mutually exclusive could be made concerning the role of Bo17 in the biology of BoHV-4 infection. The results presented in this study demonstrate that pBo17 contributes to post-translational modifications of BoHV-4 structural proteins. In vivo those modifications could affect the tropism of the virion and/or its sensitivity to antibody and/or complement neutralization. Supporting the latter hypothesis, it has been shown that the resistance of Sindbis virus virions to complement is affected by the composition of the glycosaminoglycans, and notably the level of sialylation expressed on the cells in which the virus was grown (Hirsch et al., 1983; Meri & Pangburn, 1990).

In addition to its effect on structural proteins, it is attractive to speculate that pBo17 could also affect the glycosylation of viral and/or cellular glycoproteins exposed on the plasma membrane of the infected cells, thus affecting the biological properties of the infected cell. Based on the biological functions described for cellular β1,6GnTs (Brockhausen, 1999; Tsuboi & Fukuda, 2001; Fukuda, 2002), several hypotheses could be made. Firstly, as proposed for the virion, it is possible that the post-translational modifications directed by pBo17 affect the sensitivity of the infected cells to antibody and/or complement lysis. Secondly, pBo17 expression could modify the interactions between infected cells and cells of the immune system, thus protecting the infected cells from the cellular immune response. Consistent with this, several studies have demonstrated that increasing the level of C2GnT activity, and of the resulting core 2 branched oligosaccharides, significantly decreases interactions between the expressing cells and cells of the immune system (Fukuda & Tsuboi, 1999). Thirdly, after primary infection BoHV-4 replicates in mucosal cells, then spreads to mononuclear blood cells, where it can establish latency, and eventually replicates after reactivation (Thiry et al., 1992). Interestingly, in contrast to mucosal cells, resting mononuclear blood cells do not express C2GnT activity (Yeh et al., 1999). However, upon activation they express core 2 branching activity which contributes to the exposure of sialyl Lewis X structures acting as ligand for selectins at the cell surface and eventually to the exit of the expressing cells from the bloodstream. It is attractive to speculate that through expression of Bo17, BoHV-4 could direct the tropism of the infected cells to secondary sites of replication and/or reexcretion. Further experiments are required to identify BoHV-4 and cellular proteins subjected to pBo17 enzymic activities and to determine the effect of these post-translational modifications on the biological properties of the virion and the infected cell.

In conclusion, this study demonstrates that expressing a functional C2GnT-M is a general property of BoHV-4 strains that is not essential for virus replication in vitro despite contributing to post-translational modifications of structural proteins. Further studies are required to determine the biological functions of this unique viral gene in the biology of BoHV-4. These studies will provide a synergy between the fields of virology and glycobiology, and should reveal the evolutionary advantage conferred to a virus expressing β1,6GnT activities. Eventually, those studies should also facilitate the understanding of the biological functions of cellular β1,6GnTs in physiological and pathological conditions.

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