

1 Bovine herpesvirus 4 modulates its β -1,6-*N*-acetylglucosaminyltransferase activity through alternative
2 splicing
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15 **Running title:** BoHV-4 glycosyltransferase alternative splicing

16 **Keywords:** Alternative splicing / BoHV-4 / Core 2 *O*-glycan / Gammaherpesvirus / Viral C2GnT-M

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24 **Text:** 5599 words **Abstract:** 245 words **Importance:** 144words

25 **Abstract**

26 Carbohydrates play major roles in host-virus interactions. It is therefore not surprising that,
27 during co-evolution with their hosts, viruses have developed sophisticated mechanisms to hijack to their
28 profit different pathways of glycan synthesis. Thus, the Bo17 gene of Bovine herpesvirus 4 (BoHV-4)
29 encodes a homologue of the cellular Core 2 β -1,6-*N*-acetylglucosaminyltransferase-mucin type (C2GnT-
30 M) which is a key player for the synthesis of complex O-glycans. Surprisingly, we show in this study that,
31 as opposed to what is observed for the cellular enzyme, two different messenger RNAs are encoded by
32 the Bo17 gene of all available BoHV-4 strains. While the first one corresponds to the entire coding
33 sequence of the Bo17 gene, the second results from the splicing of a 138 bp intron encoding critical
34 residues of the enzyme. Antibodies generated against the Bo17 C-terminus showed that the two forms
35 of Bo17 are expressed in BoHV-4 infected cells, but enzymatic assays revealed that the spliced form is
36 not active. In order to reveal the function of these two forms, we then generated recombinant strains
37 expressing only the long or the short form of Bo17. Although we did not highlight replication differences
38 between these strains, glycomic analyses and lectin neutralization assays confirmed that the splicing of
39 the Bo17 gene gives the potential to BoHV-4 to fine tune the global level of Core 2 branching activity in
40 the infected cell. Altogether, these results suggest the existence of new mechanisms to regulate the
41 activity of glycosyltransferases from the Golgi apparatus.

42

43 **Importance**

44 Viruses are masters of adaptation that hijack cellular pathways to allow their growth. Glycans
45 play a central role in many biological processes and several studies have highlighted mechanisms by
46 which viruses can affect glycosylation. Glycan synthesis is a non-template process regulated by the
47 availability of key glycosyltransferases. Interestingly, Bovine herpesvirus 4 encodes one of such enzymes
48 which is a key enzyme for the synthesis of complex O-glycans. In this study, we show that, in contrast to

49 cellular homologues, this virus has evolved to alternatively express two proteins from this gene. While
50 the first one is enzymatically active, the second results from the alternative splicing of the region
51 encoding the catalytic site of the enzyme. We postulate that this regulatory mechanism could allow the
52 virus to modulate the synthesis of some particular glycans in function of the location and/or of the
53 moment of the infection.

54 Introduction

55 A lot of the key molecules involved in the innate and adaptive immune response are
56 glycoproteins (1, 2). These carbohydrates are classified by the nature of their linkage to the protein as
57 either *N*-glycans or *O*-glycans. Both *N*- and *O*-glycans initiate various biological functions through
58 interaction with receptors. Among *O*-glycans, mucin-type-*O*-glycans can either be linear or branched (3).
59 The central biosynthetic pathway used to synthesize complex-type *O*-glycans is through Core 2 branching
60 (4). Core 2 branched *O*-glycan are synthesized by Core 2 β -1,6-*N*-acetylglucosaminyltransferases
61 (Core2 β 1,6GnT) (5) and are involved in important immune mechanisms such as T- and B-cell homing (6)
62 or T-lymphocytes differentiation (7).

63 For millions of years, viruses have been co-evolving with their hosts. During this co-evolution
64 process, viruses had to deal with the physiology of the host in order to overcome its barriers and defense
65 mechanisms by mimicking or hijacking host biological processes in their favor. A growing list of studies
66 has highlighted the importance of the glycome in the virus lifecycle (8) and the ability of some viruses to
67 manipulate the cellular and viral glycome (9). Viruses may modify glycomes by different mechanisms: (i)
68 by regulating the expression of host glycosyltransferases or glycosidases and/or (ii) by acquiring
69 mutations that affect glycosylation sites or glycan binding specificities and/or (iii) by encoding their own
70 glycosyltransferases (10) or glycosidases (11). Very few viruses infecting vertebrates are known to
71 encode glycosyltransferases (10, 12), one of these viruses is Bovine herpesvirus 4 (BoHV-4) (13).

72 BoHV-4 is a gammaherpesvirus which has been isolated throughout the world from healthy
73 cattle as well as from those exhibiting a variety of diseases (14). While BoHV-4 genome has a reduced set
74 of open reading frames (ORFs) homologous to cellular genes (15, 16), its Bo17 gene encodes a functional
75 homologue of the cellular Core2 β 1,6GnT type M (C2GnT-M) (13) that has been acquired from a recent
76 ancestor of the african buffalo (17). This gene is non-essential for virus replication despite contributing to
77 post-translational modification of virion proteins (18).

78 In the present study, we pursued the characterization of the BoHV-4 Bo17 gene. Surprisingly, we
79 show that Bo17 undergoes an alternative splicing that generates two different products of expression. In
80 contrast to the full-length form of the protein, the shortened spliced form is devoid of enzymatic activity
81 and confers to BoHV-4 the potential to fine tune the Core 2 branching activity of the infected cell.
82

83 **Materials and Methods**

84 **Cells and viruses.** Madin-Darby bovine kidney (MDBK, ATCC CCL-22), Embryonic Bovine Lung cells (EBL,
85 German Collection of Microorganisms and Cell Culture [DSMZ] ACC192), BOMAC (19), Embryonic Bovine
86 Trachea (EBTr, ATCC CCL-44), Bovine Turbinate (BT, CRL-1390), Bovine mammary epithelial cells (Mac-T)
87 (20), Georgia Bovine kidney (GBK) (21) and Chinese Hamster Ovary (CHO, ATCC CCL-61) (22) cells were
88 cultured in Dulbecco's modified Eagle Medium (DMEM) (Invitrogen) containing 10% fetal calf serum
89 (FCS), 2% Penicillin/Streptomycin (Invitrogen) and 1% non-Essential amino acids (Invitrogen). Moreover,
90 a primary cell culture of Bovine Bronchial Epithelial (BBE) cells, obtained as previously described (23),
91 was also used. The BoHV-4 V. test strain has been initially isolated from a case of orchitis (24). LVR 140,
92 MOVAR 33/63, DN599, 66-P-347, 108, 130 and Buf. strains of BoHV-4 representative of the BoHV-4
93 species isolated throughout the world and from different animal species were used in this study (18).

94

95 **RT-PCR.** Cytoplasmic RNA was isolated from mock infected and BoHV-4 infected cells 24h after infection
96 (MOI of 2 PFU/cell). Contaminating DNA was removed by DNase treatment. cDNA was produced by using
97 Transcriptor High Fidelity cDNA Synthesis Kit (Roche Applied Science) with poly d(T) primer. The cDNA
98 products were amplified by PCR with *Taq* polymerase (New England Biolabs) using primers specific for
99 Bo17. The forward primer 5'-ATGAAGATGGCTGGG-3' and the reverse primer 5'-
100 TCAAAGTTCAGTCCCATAGAT-3' were used to amplify the entire coding sequence of Bo17 (primers ATG-
101 STOP). The same PCR were performed directly on cytoplasmic RNA to verify the absence of contaminant
102 DNA.

103

104 **Antibodies.** Anti-Bo17 422-441 polyclonal-mono-specific antibodies were produced as follows. On day 0,
105 equal volumes of diluted KLH-peptide (amino acids 422 to 441 of the BoHV-4 V. test Bo17 protein)
106 (1 mg/mL) and Freund's complete adjuvant were emulsified and injected subcutaneously into the rabbits

107 at three different sites (200µg/rabbit). On day 14, 28, 42, 56 and 70, each rabbit was immunized again
108 with 100 µg of KLH-peptide (1 mg/mL) emulsified with incomplete Freund's adjuvant. Serum was
109 collected on day 77. Specificity of the immune sera against the Bo17 422-441 peptide was tested by
110 ELISA as described previously (25). Briefly, Nunc Maxisorp ELISA plates (Nalge Nunc, N.Y.) were coated
111 (18 h, 4°C) with unfused Bo17 422-441 peptides, blocked in PBS/0.1% Tween-20/1% BSA, and incubated
112 with sera. Bound antibodies were detected with horseradish peroxidase-conjugated goat anti-rabbit IgG
113 pAb (Sigma). The plates were washed ×5 in PBS/0.1% Tween-20 after each step. The detection substrate
114 was nitrophenylphosphatase (Sigma). Absorbance was read at 405nm using a Benchmark ELISA plate
115 reader (Thermo). For detection of gp180 protein (encoded by the Bo10 gene) on western blotting, we
116 used a rabbit monospecific polyserum raised against the C-term end of the protein (anti-Bo10-c15) (26).
117 For some western blots, we used serum of a rabbit infected intravenously with 10⁸ PFU of the BoHV-4 V.
118 test strain and collected 63 days post-inoculation (27).

119

120 **Western Blot.** Infected cells or purified virions were lysed and denatured by heating (95°C, 5 min) in SDS-
121 PAGE sample buffer (31,25 mM Tris-HCl pH 6.8, 1% (w/v) SDS, 12,5% (w/v) glycerol, 0.005% (w/v)
122 Bromophenol Blue, 2.5% (v/v) 2-mercaptoethanol). Proteins were resolved by electrophoresis on Mini-
123 PROTEAN TGX (Tris-Glycine eXtended) precast 4-15% resolving gels (Bio-Rad) in SDS-PAGE running buffer
124 (25 mM Tris-Base, 192 mM glycine, 0.1% (w/v) SDS) and transferred to polyvinylidene difluoride
125 membranes (Immobilon-P transfer membrane, 0.45 µM pore size, Millipore). The membranes were
126 blocked with 3% non-fat milk in PBS/0.1% Tween-20, and then incubated with anti-Bo17-422-441, anti-
127 Bo10-c15 rabbit antibodies or anti BoHV-4 rabbit polyserum in the same buffer. Bound antibodies were
128 detected with horseradish peroxidase-conjugated goat anti-rabbit IgG pAb (Dako Corporation), followed
129 by washing in PBS/0.1% Tween-20, development with ECL substrate (GEHealthcare) and exposure to X-
130 ray film.

131

132 **In vitro enzymatic assay.** To measure the activity of both forms of pBo17, soluble recombinant forms
133 were generated as previously described (13). The sequences of pBo17 and pBo17 spliced without their
134 transmembrane region were amplified by primers "Bo17 soluble sense" 5'-
135 **CCGGATCCCCTCAGGTTTAAATGTGATGTAG**-3' corresponding to the BamHI site (in bold) and nucleotides
136 99 to 121 of Bo17 (in italics) and "Bo17 soluble Rev" 5'-CCATGCATTCAAAGTTCAGTCCCATAGATGG-3'. The
137 fragments obtained were T/A cloned into the pGEM-T Easy vector (Promega Corporation), and then
138 digested with BamHI and SpeI and inserted into the pcDNA 3.A opened with BamHI and XbaI. The pcDNA
139 3.A is a pcDNA3.1-derived vector harboring a sequence encoding a signal peptide and the IgG binding
140 domain of protein A (28). Enzymatic assays were performed as described previously (28). Briefly, these
141 enzyme assays were carried out with pcDNA3-A pBo17, pcDNA3-A pBo17 Spliced or pcDNA3-A h-C2GnT-
142 M that were transiently transfected into CHO cells. The soluble chimeric enzymes were then absorbed to
143 IgG-Sepharose from the concentrated cell culture supernatant and used as enzyme source for the assays.
144 As acceptors, Gal β 1 $\rightarrow$ 3GalNAc α $\rightarrow$ p-nitrophenol and GlcNAc β 1 $\rightarrow$ 3GalNAc α $\rightarrow$ p-nitrophenol were used
145 for assaying C2GnT and C4GnT activities, respectively.
146 Gal β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 6Man α -1 $\rightarrow$ 6Man- β $\rightarrow$ octyl and GlcNAc β 1 $\rightarrow$ 3Gal β -
147 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 6Man α -1 $\rightarrow$ Man β 1 $\rightarrow$ octyl were used for assaying IGnT activity. Because addition of N-
148 acetylglucosamine occurs at the underlined galactose residues, the two acceptors serve for centrally
149 acting IGnT (cIGnT) and predistally acting IGnT (dIGnT), respectively. CHO cells transfected with pcDNA3-
150 A vector were used as a negative control. The radioactivity obtained with the negative control was
151 subtracted from the values obtained in experiments with the enzymes. When detected, the activities
152 were expressed relatively to C2GnT activity, which was defined as 100 %.

153

154 **Production of BoHV-4 Bo17 mutant strains.** The BoHV-4 V. test strain deleted for Bo17 ORF (Bo17 Del)
155 and the corresponding revertant strain (Bo17 Rev) have been described previously (18). The recombinant
156 strains Bo17 MUDIR and Bo17 Spliced were derived from a cloned BoHV-4 BAC (29). Firstly, the Bo17
157 MUDIR sequence was generated by site-directed mutagenesis as follows. Two mutations were inserted
158 in the 5' splice site and one mutation in the 3' splice site. Briefly, a first PCR with sense primer "HindIII
159 sense.native" 5'-GAAGA**AAGCTTT**GCCCGGGAC-3', containing a native HindIII restriction site in bold, and a
160 reverse primer "Mudir XhoI" 5'-GG**CTCGAG**AGGCCACAAAATAGGCATTCCC**G**GTGAAC-3', containing a
161 native XhoI restriction site in bold and in which a mutation in the sequence was incorporated in bold
162 underlined, allowed to obtain a mutation in the 3' splicing site. This fragment was cloned into the vector
163 pGEM-T Easy / EcoRI I (18) instead of the WT sequence by HindIII and XhoI restriction sites. To introduce
164 two other mutations at the 5' splicing site, a second PCR was performed with the sense primer "HindIII
165 sense.native" and the reverse primer "Mudir KpnI" 5'-
166 GCGGTACCTCAGACTCCATACTGTT**TTTGC**CTTCAAC-3' containing a KpnI restriction site in bold and two
167 mutations in the sequence in bold underlined. This second fragment was then HindIII/KpnI restricted and
168 cloned into the corresponding sites cloned in pGEM-T Easy/EcoRI I already mutated for the 3' splicing
169 site. We obtained a pGEM-T Easy with the Bo17 MUDIR sequence. The Bo17 SPLICED sequence was
170 directly generated from the spliced product obtained by amplification of Bo17 with the ATG-STOP
171 primers on cDNA from BoHV-4 infected cells (Figure 1A). The spliced band was then HindIII/XhoI
172 restricted and cloned into the corresponding sites of the pGEM-T Easy/EcoRI I. Mutagenesis of WT V. test
173 BAC G plasmid was performed by a two-step mutagenesis procedure in bacteria using the shuttle
174 plasmid pST76KSR (29). Plasmids used to induce homologous recombination were constructed as
175 follows. Each construct was subcloned as a HindIII/XhoI fragment into the same sites of the pST76K-SR
176 shuttle vector, and recombined into the BoHV-4 BAC (29). We also isolated, by the same way, revertants
177 of the Bo17 MUDIR and Bo17 Spliced BACs in which the Bo17 locus was restored to its WT form. The

178 correct construction of each mutant was confirmed by DNA sequencing. Reconstitution of infectious
179 virus from BAC plasmids was obtained by transfection in MDBK cells.

180

181 **Southern blot.** Southern blot analysis (30) of viral DNA digested with EcoRI was performed with probe
182 corresponding to nucleotides 26–774 of the V. test Bo17 ORF.

183

184 **Growth curves.** The growth kinetics of mutant and revertant viruses were compared to that of WT as
185 described (31). Cell cultures were infected at a multiplicity of 0.01 (multistep assay). After 1h of
186 adsorption, the cells were washed then overlaid with DMEM containing 10 % FCS. Supernatants of
187 infected cultures and infected cells were harvested at successive intervals and the amount of infectious
188 virus was determined by plaque assay on MDBK cells. Plaques were visualized after immunostaining with
189 Mab35 recognizing gB (27).

190

191 **Virus purification.** BoHV-4 virions grown on MDBK cells were purified as described (32). Briefly, after
192 removal of the cell debris by low-speed centrifugation (1,000 x *g*, 10 min), virions present in the infected
193 cell supernatant were harvested by ultracentrifugation (100,000 x *g*, 2 h) through a 30% (w/v) sucrose
194 cushion, then centrifuged through two successive 20-50% (w/v) potassium tartrate gradients in PBS
195 (100,000 x *g*, 2 h). Virions were finally washed and concentrated in PBS (100,000 x *g*, 2 h).

196

197 **O-glycomic profile analysis.** MDBK cells (3×10^6 cells) were infected with Bo17 MUDIR, Bo17 MUDIR Rev,
198 Bo17 Spliced and Bo17 Spliced Rev strains at a MOI of 1 PFU per cell. 36h post-infection, cells were
199 harvested, lysed in PBS containing 0.5% (v/v) SDS and treated as described previously (33). Briefly, all
200 samples were subjected to homogenization in an extraction buffer (25 mM Tris, 150 mM NaCl, 5mM
201 EDTA and 1% 3-[3-cholamidopropyl dimethyl-ammonio]-1-propane sulfonate, CHAPS at pH 7.4). After

202 reduction and carboxymethylation, samples were sequentially digested with trypsin (Sigma-Aldrich) and
203 PNGase F (Roche). N-glycans were separated from peptides and/or glycopeptides on Sep-Pak cartridges
204 (Waters, Hertfordshire, United Kingdom), and O-glycans were released from the latter by reductive
205 elimination. After purification on Dowex 50W-X8 (H) 50–100 mesh columns (Sigma-Aldrich), the O-
206 glycans samples were permethylated and then further purified with Sep-Pak cartridges. O-glycans were
207 eluted in aqueous acetonitrile fractions and then lyophilized. Permethylated samples were dissolved in
208 10 µl of methanol, and 1 µl of the dissolved sample was premixed with 1 µl of matrix (20 mg/mL 2,5-
209 dihydroxy-benzoic acid in 70% v/v aqueous methanol) and spotted onto a target plate. Matrix-assisted
210 laser desorption ionisation (MALDI) MS and tandem MS (MS-MS) data were acquired using a 4800
211 MALDI-TOF/TOF mass spectrometer (Applied Biosystems) in positive ion mode. The collision energy was
212 set to 1 kV, and argon was used as collision gas. The data obtained were viewed and processed using
213 Data Explorer 4.9 (AB Sciex UK Limited). Comparison of the relative abundance of Core 2 and Core 1 O-
214 glycan (ratio Core2/Core 1) was done by comparing the total of relative intensity of the Core 2 peaks and
215 the total of relative intensity of the Core 1 peaks, the maximum peak being defined as 100%. A ratio
216 Core2/Core1 was obtained for each replicate and mean values were finally compared together between
217 samples.

218

219 **Details of Experimental Data Utilized to Define Glycan Structures.** (i) All O-glycans are assumed to have
220 reducing end GalNAc based on known biosynthetic pathways. (ii) Monosaccharide compositions in terms
221 of numbers of Hex, HexNAc etc is derived from MALDI-MS in the +ve ion mode of molecular ions of
222 reductively eliminated permethylated species which were assigned manually. (iii) MALDI-TOF/TOF
223 MS/MS fragmentation of the following molecular ions: m/z 534, 779, 895, 983, 1187, 1256, 1344, 1548
224 and 1705. Fragment ions were identified manually and with the assistance of the Glycoworkbench tool
225 (Version 1.2). Details provided were guided by MIRAGE (34).

226

227 **Lectin neutralization assay.** *Artocarpus integrifolia* lectin (Jacalin, recognizing Core 1 structures) was
228 purchased from Vector Laboratories. Briefly, virus was incubated (2h, 37°C) with jacalin at various
229 concentrations and the amount of infectious lectin-treated virus was determined by plaque assay on
230 MDBK cells.

231 Results

232 **Bo17 mRNA undergoes alternative splicing.** The initial DNA sequence analysis of the BoHV-4
233 genome demonstrated that the Bo17 gene is highly homologous to the cellular C2GnT-M (13). Then, RT-
234 PCR experiments identified a product of expression corresponding to the full-length coding sequence of
235 the gene and encoding a functional β 1,6-GnT forming Core 2, Core 4 and I structures (13). Expression of
236 this active Bo17 product was confirmed for all available BoHV-4 strains by RT-PCR and subsequent
237 enzymatic activity assay (18).

238 To investigate deeper the transcription of this Bo17 message during BoHV-4 cycle, we repeated
239 RT-PCR on mRNA from BoHV-4 infected MDBK cells with another set of primers located at both ends of
240 the Bo17 open reading frame (ATG-STOP primers). As previously described, a product corresponding to
241 the expected size of a full-length Bo17 mRNA, was observed. Surprisingly, a second band was also visible
242 (Figure 1A). DNA sequencing revealed that this band corresponded to the BoHV-4 Bo17 gene sequence
243 but displayed a disappearance of 138 bp (Figure 1B) suggesting that Bo17 mRNA could undergo
244 alternative splicing. We therefore inspected both ends of the 138 bp deleted sequence for potential
245 splice donor and acceptor sites. By comparing consensus splice donor and acceptor sequences (35) with
246 these Bo17 sequences, we observed that 5' GT and 3' AG as well as other important nucleotides were
247 present at both ends of the 138 bp deleted sequence (Figure 1B). Finally, as the splicing patterns for a
248 single gene in different cells are quite different, we tested Bo17 splicing in cells of different origins
249 including kidney (MDBK, GBK), mammary epithelium (MAC-T), respiratory epithelium (EBL, EBTr, BT, BBE)
250 and macrophage (BOMAC). Interestingly, Bo17 splicing was observed in all tested cells (Figure 1C).
251 Altogether, these results show that BoHV-4 Bo17 mRNA undergoes alternative splicing in cells of various
252 origins, at least for the V. test strain.

253

254 **Bo17 splicing is conserved among BoHV-4 strains but not in its cellular counterparts.** To
255 estimate the conservation of these splice donor and acceptor sites, we compared sequences of eight
256 contemporary BoHV-4 strains representative of different phylogenetic clades (17). This comparison
257 revealed that both the splice donor and the splice acceptor sites are conserved in these BoHV-4 strains
258 (Figure 2A). Moreover, RT-PCR on mRNA from MDBK cells infected with these eight different strains
259 confirmed that two expression products are encoded by the Bo17 genes of all tested strains (Figure 2B).
260 In contrast, sequence analysis of cellular C2GnT-M from different animal species showed that none of
261 these sequences displayed both of these consensus regions (Figure 2A). It has to be noted that the
262 weaker band observed in the mock infected cells corresponds to the cellular C2GnT-M. Indeed, the
263 BoHV-4 Bo17 gene displays 94.6% identity with the gene encoding bovine C2GnT-M and the primers
264 used to amplify the entire sequence of Bo17 have only one mismatch (at base 10 of forward primer) with
265 the cellular C2GnT-M sequence. It has to be noted that, as expected, this gene does not undergo
266 alternative splicing of its mRNA. However, based on this homology, it was not possible to set up a
267 quantitative RT-PCR assay in order to estimate the amounts of the different transcripts.

268
269 **BoHV-4 Bo17 gene encodes two proteins.** In order to test if both of these two mRNA products
270 encode proteins, Bo17-specific polyclonal antibodies were generated by immunizing rabbits with a
271 keyhole limpet hemocyanin (KLH)-conjugated peptide (VLQCLEEYLRHKAIYGTEL) corresponding to the
272 shared C-terminal end of the predicted Bo17 gene products (amino acids 422-441) (Figure 3A).
273 Immunization against the peptide was confirmed by ELISA (Figure 3B). To identify proteins recognized by
274 the anti-Bo17-422-441 serum, western blot was performed on wild-type (WT), Bo17 Del or Bo17 Rev
275 infected MDBK cells. In BoHV-4 WT and Bo17 Rev samples, proteins of apparent molecular mass (MM) of
276 52 kDa and 45 kDa were detected under reducing conditions (Figure 3C). No such proteins were detected
277 in cells infected by the BoHV-4 Bo17 Del strain. The predicted MM of the full-length Bo17 gene product is

278 50.8 kDa (estimated by the Compute pI/MW tool of the ExPASy server), so this result suggests that the
279 full-length Bo17 mRNA encodes the observed 52 kDa protein, hereafter called pBo17 full-length, and
280 that the spliced Bo17 mRNA encodes a 45 kDa protein, hereafter called pBo17 Spliced.

281

282 **The spliced form of pBo17 is not enzymatically active.** The impact of the splicing of pBo17 on its
283 enzymatic activity was firstly assessed by aligning the sequences of different mammalian C2GnT-L and
284 C2GnT-M with the sequence of pBo17 full-length and pBo17 Spliced (Figure 4A). While, five of the six
285 amino acids found to be involved in direct acceptor substrate interactions (36) are conserved in pBo17
286 full-length, the splicing of Bo17 affects three (E243, K251 and R254, green asterisks in Figure 4A) of these
287 five amino acids (identified by green and magenta asterisks in Figure 4A) playing a role in acceptor
288 substrate. Especially, K251 and R254 residues were found to make key hydrogen bonds and van der
289 Waals interaction with the bound disaccharide acceptor (37). Thus, alternative splicing removes key
290 amino acids involved in acceptor substrate binding site and could therefore affect the activity of pBo17.
291 The 3D structure of pBo17 (Figure 4B) was then estimated on the basis of the X-ray crystal structure of
292 the murine C2GnT-L (36) in order to better visualize the spliced region (in red) and the three amino acids
293 involved in catalytic activity that disappear after splicing (identified in green).

294 Finally, we tested the activity of the pBo17 Spliced protein by an *in vitro* enzymatic assay. As
295 described previously, the full-length form of pBo17 was shown to have Core 2, Core 4 and I branching
296 activities (13) as does its cellular counterpart (28). In contrast, the chimeric pBo17 spliced protein did not
297 exhibit Core 2, Core 4 or I activities (Figure 4C).

298

299 **Generation of the Bo17 MUDIR and Spliced BoHV-4 mutants.** A BoHV-4 Bo17 knockout strain
300 has previously been described (18). To unravel the function of both the spliced and the unspliced Bo17
301 expression products, we generated two additional Bo17 mutant viruses. In the Bo17 MUDIR mutant, we

302 punctually mutated the Bo17 splicing donor site (T to C) and the Bo17 splice acceptor site (A to C) in
303 order to only express the unspliced form (pBo17 full-length) without affecting its amino acids sequence.
304 On the opposite, the Bo17 Spliced strain was generated in order to only encode the Bo17 spliced
305 sequence (Figure 5A). Enzymatic restriction of viral DNA and subsequent Southern blotting (Figure 5B)
306 confirmed the expected genomic structures of the two mutants and of the revertants of both these
307 strains. The expected mutations were further confirmed by DNA sequencing (data not shown). As
308 expected, RT-PCR analysis showed that the Bo17 MUDIR and the Bo17 Spliced strains express the
309 unspliced and the spliced messenger RNA respectively (Figure 5C). Finally, to confirm that the two Bo17
310 encoded proteins observed in BoHV-4 WT infected cells are encoded by the two Bo17 derived mRNA, we
311 performed western blots on lysates of cells infected with these different Bo17 mutants. Immunoblotting
312 with the anti-Bo17-422-441 rabbit polyserum confirmed that the Bo17 MUDIR viral strain only expresses
313 pBo17 full-length and that the Bo17 Spliced strain only expresses pBo17 spliced while WT and revertant
314 strains expressed both (Figure 5D). As expected, the Bo17 Del strain expressed none of the Bo17
315 encoded proteins.

316

317 **Effect of Bo17 mRNA splicing on BoHV-4 replication *in vitro*.** We previously showed that the
318 Bo17 knockout strain does not display any growth deficit *in vitro*. However, sole expression of any of the
319 two Bo17 encoded protein could influence BoHV-4 growth. To address this question, multistep growth
320 assays were performed on MDBK, EBTr and BT cells with the different Bo17 mutants. None of the Bo17
321 mutants displayed any *in vitro* growth deficit in any of these different cell lines (Figure 6 A-C).

322

323 **Effect of BoHV-4 infections on Core 2 O-glycans contents of infected cells.** To identify the
324 influence of the expression of the different forms of pBo17 on the glycome of infected cells, we
325 characterized the structures of O-glycans isolated from cells Mock-infected or infected with the Bo17

326 Del, Bo17 MUDIR, Bo17 MUDIR Rev, Bo17 Spliced and Bo17 Spliced Rev strains of BoHV-4. Firstly, MDBK
327 cells were infected or not with the different Bo17 mutants at a MOI of 1 for 36h. Then, these infected
328 cells were processed through a glycomic workflow. Consistent and reproducible data were obtained
329 from two repeats of experiments. Assignment of Core 1 or Core 2 structures were based on MS/MS
330 fragmentation patterns (see supplementary Fig. 1). In comparison with the Mock- and Bo17 Del-infected
331 samples, the results reveal an increase in Core 2 *O*-glycan structures detected in Bo17 MUDIR, MUDIR
332 Rev and Spliced Rev infected cells (Figure 7A-F). For instance, the peaks at m/z 1187.5 and 1548.7 have a
333 higher relative abundance in those cells than in Mock- or Bo17 Del-infected cells. In order to quantify
334 these differences, the Core2/Core1 ratios were calculated (Figure 7G). While the mean Core2/Core1
335 ratios were 0.075 and 0.07 in Mock- and Bo17 Del-infected cells respectively, the mean ratios observed
336 in Bo17 MUDIR, MUDIR Rev and Spliced Rev infected cells were significantly higher, indicating that there
337 are proportionally more Core 2 *O*-glycan structures in those cells. In contrast, the mean Core2/Core1
338 ratio measured in Bo17 Spliced-infected cells did not differ significantly from those of Mock- and Bo17
339 Del-infected cells (Figure 7G). Altogether, these results show that the expression of either the full-length
340 pBo17 or the inactive spliced pBo17 determines the global Core 2 branching activity of BoHV-4 infected
341 cells.

342

343 **Effect of Bo17 splicing on BoHV-4 virions *O*-glycosylation.** It has previously been shown that
344 Bo17 expression contributes to post-translational modifications of BoHV-4 structural proteins (18).
345 Sequence analysis using NetOGlyc 4.0 Server (38) revealed that in the BoHV-4 strain, most of the
346 potential virions *O*-glycans (>98%) are located on gp180 (~70%) and on the glycoprotein B (gB) (~28%).
347 This was confirmed by mass spectrometry analysis (32). Moreover, we showed that *O*-glycans associated
348 with gp180 account for most of the sensitivity to neutralization by the *O*-glycans specific lectin, jacalin
349 (26). We therefore investigated how Bo17 expression and splicing affects glycosylation of gp180 in

350 virions. Briefly, we purified virions of the different strains on tartrate gradients. Then, we subjected these
351 virion preparations to a western blotting analysis with a polyserum recognizing gp180 (39). Interestingly,
352 while we observed a clear band at approximately 180 kDa in WT, Bo17 Rev, Bo17 MUDIR, Bo17 MUDIR
353 Rev and Bo17 Spliced Rev virions samples, the apparent molecular mass of the protein was reduced in
354 the Bo17 Del and the Bo17 Spliced samples (Figure 8A).

355 Lectin neutralization assay is another strategy to highlight the type of *O*-glycans present at the
356 virion surface of the different mutant strains. As described previously (26), we used the lectin from
357 jackfruit seeds (jacalin). This lectin binds to a GalNAc α 1-peptide, in which C6-OH of α GalNAc is free (i.e.,
358 Core1) whereas it cannot recognize a GalNAc α 1-peptide with a substitution at the C6 position (i.e.,
359 Core2) (40). Jacalin can block entry of another gammaherpesvirus, Murid herpesvirus 4, by steric
360 hindrance (41) especially as the amount of Core 1 structures is important at the surface of the virion.
361 Therefore, we tested the capacity of Jacalin to inhibit MDBK infection by the different mutant viruses.
362 Although the infection by WT, Bo17 MUDIR and the revertant strains was moderately inhibited by
363 jacalin, this neutralization was significantly stronger for the Bo17 Del strain and even more for the Bo17
364 Spliced strain (Figure 8B). Altogether, these results show that Bo17 expression and splicing may affect
365 the structure of *O*-glycans on BoHV-4 virions and in particular on the glycoprotein gp180.

366

367 **Discussion**

368 During co-evolution with their hosts, viruses have developed sophisticated mechanisms in order
369 to hijack some important cellular function to promote infection. Deciphering these interactions could not
370 only allow a better understanding of the virus biology but could also reveal unknown cellular pathways
371 as previously shown (42). Polysaccharides play central roles in many important biological processes. It is
372 therefore not surprising that several viruses have exploited glycosylation to their benefit (9).

373 Protein O-glycosylation involves the addition of monosaccharides in a stepwise process requiring
374 no glycan template. Thus, each unique oligosaccharide structure reflects the sequential order in which
375 multiple enzymes act in glycan synthesis. Control of the synthesis of specific carbohydrate structures is
376 therefore achieved by regulating the expression of the enzymes required for its construction. In many
377 cases, this is accomplished by controlling the expression of key enzymes. Interestingly, BoHV-4 encodes a
378 functional homologue of the cellular C2GnT-M (13) which is a central enzyme for the synthesis of
379 complex-type O-glycans (4). Many biologically significant oligosaccharide structures are constructed on
380 this branch, including some important cell recognition and adhesion molecules involved in immune
381 response (3, 5, 43). Expression of this enzyme is therefore likely important in the BoHV-4 cycle.

382 In this study we showed that, in contrast to its cellular counterparts, BoHV-4 modulates its
383 C2GnT-M activity through alternative splicing (Figures 1-4) with changes of the glycome composition at
384 the global cellular level (Figure 7) but also at the viral level (Figure 8). The splicing of Bo17 was observed
385 in cells of various origins including kidney (MDBK, GBK), mammary epithelium (MAC-T), respiratory
386 epithelium (EBTr, BT, EBL and BBE) and macrophage (BOMAC) suggesting that *in vivo* expression of the
387 spliced form is very likely. Interestingly, we showed that the consensus sites for splicing are conserved
388 across all the available BoHV-4 strains (Figure 2), but not in the cellular homologues from different
389 species among which the C2GnT-M gene of *Syncerus caffer*, which is phylogenetically the closest (44).
390 This therefore indicates a strong selection pressure for the capacity of Bo17 splicing. Interestingly,

391 Markine-Goriaynoff et al. pointed that, in comparison with cellular homologs, the molecular clock of the
392 viral sequences increased (44). In contrast, among the viral sequences, the rate of evolution was similar
393 to what is observed among the cellular sequences. They hypothesized that this could either reflect a
394 relaxation of the constraint to preserve the protein sequence or, more interestingly, a positive selection
395 for critical changes in the sequence after gene acquisition. Acquisition of the capacity to undergo splicing
396 is certainly one of these changes. A central question about this mechanism is why BoHV-4 uses
397 alternative splicing of Bo17 gene instead of regulating the expression of an active form of pBo17? One of
398 the reasons could be that the pBo17 spliced protein, though enzymatically inactive, has its own function.

399 For many glycosyltransferases, the information that instructs Golgi localization is located within a
400 relatively short sequence of amino acids in the N-termini of these proteins comprising: the cytoplasmic
401 tail, the transmembrane spanning region, and the stem region (CTS) (45, 46). Moreover, it has been
402 observed that glycosyltransferases form homo- or hetero-oligomers that are too large to be incorporated
403 in recycling vesicles (47). As pBo17 spliced CTS is identical to the CTS of the full-length form (Figure 1B)
404 and highly homologous to that of the cellular C2GnT-M (13), pBo17 spliced could replace its active
405 counterparts in Golgi glycosyltransferases rafts. Expression of the spliced inactive form could therefore
406 have dominant-negative effect. As C2GnT-M is a central player of complex O-glycan biosynthesis,
407 alternative splicing of Bo17 would allow the virus to affect either positively or negatively the kind of
408 carbohydrate produced by the cell. This effect could be regulated over time or in some cellular contexts.
409 Unfortunately, our glycomic analysis was not able to highlight such an effect, probably due to the very
410 low content of Core 2 O-glycan structures in mock-infected MDBK cells (Figure 7). However, this
411 hypothesis might explain why the sensitivity of virions to neutralization by jacalin, which recognizes Core
412 1 structures, was the highest for the Bo17 spliced strain. Indeed, Bo17 spliced virions were more
413 sensitive to jacalin neutralization than Bo17 Del virions (Figure 8B) suggesting therefore that the
414 expression of the inactive form of the Bo17 enzyme could turn off the C2GnT activity of the cell. This will

415 have to be tested in the future in other experimental conditions. Indeed, this property could be
416 particularly important at the time scale of a virus as a replicative cycle takes less than twenty hours a
417 period which is similar to the half-life of most Golgi proteins (48).

418 Modulation of Core 2 structures could have several implications in the BoHV-4 lifecycle. Thus, we
419 have shown that pBo17 activity affects gp180 *O*-glycans (Figure 8A). We recently showed that gp180 and
420 its glycans provide part of a glycan shield for otherwise vulnerable viral epitopes on other viral
421 glycoproteins among which the glycoprotein L (26). However, we did not observe any difference of
422 sensitivity to antibody neutralization between the strains described in this study (data not shown). One
423 reason could be that the pBo17 enzymatic activity affects mainly the kind of *O*-glycans at the virion
424 surface but not their presence or not. Key neutralizing epitopes would therefore remain hidden.
425 Interestingly, while the dense shield of glycans that decorate different viruses were believed to be
426 refractory to antibody recognition, several recent reports showed that it can be recognized by rare
427 neutralizing antibodies (49-53). Bo17 alternative splicing could therefore provide some antigenic
428 variation of these protecting carbohydrates. The effect of Bo17 splicing on antibody neutralization could
429 therefore only be observed over time, for example when the virus reactivates from an immune host to
430 allow transmission, or at the scale of a population. Interestingly, recent studies have highlighted
431 remarkable variability between individual antibody responses with respect to their reactivity against
432 glycopeptides of EBV and HSV-2 (54, 55). The capacity of transmission of the different viral strains used
433 in this study should therefore be compared. However, herpesvirus transmission models are difficult to
434 set up (56) and none is available for BoHV-4.

435 BoHV-4 has been shown to persist in CD11b cells of infected animals (57). Expression of Core 2
436 structures has been shown to regulate monocytes extravasation and migration of monocytes derived-DC
437 to lymph nodes (58-60). BoHV-4 could therefore regulate extravasation of infected monocytes and
438 migration of these cells in tissues or towards draining lymph nodes by modulating glycan structures at

439 the infected cell surface. Interestingly, another gammaherpesvirus has been shown to hijack myeloid
440 cells for host colonization (61, 62) and therefore sophisticated mechanisms of control of those cells are
441 possible. Further studies will be required to test these hypotheses.

442 Altogether, our results showed that the BoHV-4 Bo17 gene sequence evolved to allow alternative
443 splicing and expression of a spliced inactive protein. To our knowledge, a similar phenomenon has never
444 been observed for any other Golgi glycosyltransferase. Only one similar phenomenon has been observed
445 for human UDP-glucuronosyltransferase enzymes (UGTs) and particularly for the UGT1A gene (63).
446 Indeed, shorter UGT1A isoforms are deficient in glucuronic acid transferase activity and function as
447 negative regulators of enzyme activity through protein-protein interaction. As Golgi localization and
448 activity of some glycosyltransferases is also mediated by enzyme oligomerization, we propose that
449 pBo17 spliced expression could allow BoHV-4 to regulate C2GnT-M activity of the infected cells. This
450 hypothesis will have to be tested in the future.

451 In conclusion, the present study of the BoHV-4 Bo17 gene revealed that, similarly to what has
452 been observed for enzymes of other families, inactive isoforms of Golgi glycosyltransferases might be
453 expressed after mRNA alternative splicing. This new regulatory mechanism could have implication in our
454 understanding of BoHV-4 lifecycle but also more generally of cellular biology.

455 **Acknowledgements**

456 This work was supported by the following grants: starting grant (D-09/11), grant C-12/68 and GLYVIR ARC
457 of the University of Liège and "credit de recherche" n° J.0146.13 of the F.R.S. – FNRS. A.D. and S.M.H. are
458 funded by the Biotechnology and Biological Sciences Research Council (BB/K016164/1 and
459 BB/F008309/1) and M.F. is funded by NIH grant UO1 CA168925. AD is a Wellcome Trust Senior
460 Investigator. C.L. and B.M. are Postdoctoral Researchers of the “Fonds de la Recherche Scientifique -
461 Fonds National Belge de la Recherche Scientifique” (F.R.S. - FNRS). The authors thank Prof. F. Farnir for
462 helpful discussion.

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641

642

643 **Figure legends**

644 **Figure 1. Bo17 mRNA undergoes alternative splicing.** (A) RT-PCR analysis of BoHV-4 Bo17 reading frame.
645 MDBK cells were infected with BoHV-4 V.test strain (MOI of 0.5 PFU/cell). 24 h p.i., expression of Bo17
646 was studied using primers amplifying the entire Bo17 coding sequence (primers ATG-STOP, 1323 bp). The
647 same PCR was performed directly on cytoplasmic RNA to verify the absence of contaminant DNA. Sizes in
648 bp are indicated on the left. (B) The two PCR products obtained by RT-PCR were purified and sequenced.
649 Then alignments were performed using CLUSTAL W. The first sequence corresponds to the entire coding
650 sequence of the Bo17 gene. The second sequence displayed a disappearance of 138 bp potentially
651 generated by splicing. The grey boxes indicate the putative splice donor and acceptor sites. The
652 sequence in red indicates the intron. (C) MDBK, EBL, BOMAC, EBTr, BT, BBE, MAC-T and GBK cells were
653 infected with BoHV-4 V.test strain (MOI of 0.5 PFU/cell). 24 h p.i., expression of Bo17 was studied using
654 primers amplifying the entire Bo17 coding sequence (primers ATG-STOP, 1323 bp). The same PCR was
655 performed directly on cytoplasmic RNA to verify the absence of contaminant DNA (data is only shown for
656 the MDBK sample). Sizes in bp are indicated on the left.

657
658 **Figure 2. The Bo17 gene displays consensus splice sites that are conserved among BoHV-4 strains but**
659 **not in their cellular counterparts.** (A) The consensus sites for Bo17 splicing (GT and AG in boxes) are
660 conserved in all the BoHV-4 strains that were available. Sequences of cellular homologous β -1,6-N-
661 acetylglucosaminyltransferases from different species were aligned with Bo17 sequences of BoHV-4
662 strains. The cellular sequences do not present the consensus sites for splicing. Nucleotides that are not
663 conserved are highlighted in red. (B) MDBK cells were infected with different strains of BoHV-4 (MOI of
664 0.5 PFU/cell). 24 h p.i., expression of Bo17 was studied by RT-PCR using primers amplifying the entire
665 Bo17 coding sequence (primers ATG-STOP, 1323 bp). The same PCR were performed directly on
666 cytoplasmic RNA to verify the absence of contaminant DNA (data not shown). The weak band observed

667 in the mock-infected sample corresponds to the expression of the cellular C2GnT-M. Sizes in bp are
668 indicated on the left.

669

670 **Figure 3. Two proteins are encoded by the BoHV-4 Bo17 gene.** (A) Amino acids comparisons of the
671 spliced and unspliced Bo17 products. The grey box indicates the predicted transmembrane region of
672 both Bo17 expression products. Putative N-glycosylation sites are highlighted with asterisks. Amino acids
673 422-441 used to immunized rabbits are highlighted in a black box. (B) Immunization of rabbits with the
674 Bo17-422-441 peptide elicited antibody response against the peptide. The anti-peptide antibody
675 responses were determined for the two immunized rabbits by anti-Bo17 422-441 ELISA as described in
676 Materials and Methods. (C) Detection of specific BoHV-4 proteins by the anti-Bo17-422-441 serum.
677 MDBK cells were infected with the WT V. test, the Bo17 Del or the Bo17 Rev strain of BoHV-4 (1
678 PFU/cell). 48h later, cells were scraped off the plate, and western blotting was carried out as described in
679 Materials and Methods. The position of molecular mass (MM) standard is shown on the left of the gel.
680 The open and filled triangles indicate the two proteins encoded by the BoHV-4 Bo17 gene.

681

682 **Figure 4. The spliced form of the pBo17 enzyme is inactive.** (A) Multiple sequence alignment of β -1,6-*N*-
683 acetylglucosaminyltransferases involved in core 2 *O*-glycan biosynthesis. Residues that are absolutely
684 conserved among murine C2GnT-L, human C2GnT-L, human C2GnT-M, bovine C2GnT-M, pBo17 and
685 pBo17 Spliced sequences are highlighted in grey. The murine C2GnT-L secondary structure (36) is shown
686 above alignment and is colored to highlight the putative stem region (grey) and the catalytic domain
687 (cyan). In the catalytic domain, the region absent of pBo17 Spliced is highlighted in red. Residues forming
688 direct interactions with the acceptor substrate are marked with an asterisk. Green asterisks show
689 residues that disappear with the splicing and purple asterisks show those that are not affected by the
690 splicing. (B) The 3D structure of pBo17 was generated by SWISS-MODEL and visualized with ICM-Browser

691 according to colors described above. (C) Soluble chimeric forms of h-C2GnT-M, pBo17 and pBo17 Spliced
692 were assayed for C2GnT (C2), C4GnT (C4), cIGnT (cl) and dIGnT (dl) activities as described in the Material
693 and Methods. The soluble form of pBo17 full-length has Core 2, Core 4 and I branching activities whereas
694 the pBo17 spliced form has none of these activities.

695

696 **Figure 5. Generation of the Bo17 MUDIR and Spliced BoHV-4 mutants.** (A) Schematic representation of
697 the strategies followed to produce the recombinant BoHV-4 strains. The recombinant strains Bo17
698 MUDIR and Bo17 Spliced were derived from a cloned BoHV-4 BAC by the aid of pST76KSR plasmid.
699 Firstly, the Bo17 MUDIR sequence was generated by site directed mutagenesis. Two mutations were
700 inserted in the 5' splice site and one mutation in the 3' splice site (the two consensus sites are
701 highlighted by grey boxes). The mutated nucleotides are highlighted in red. The Bo17 Spliced sequence
702 was directly generated from the spliced product obtained by amplification with Bo17 ATG-STOP primers
703 on cDNA from BoHV-4 infected cells. The Bo17 MUDIR mutant only encodes the long form (enzymatically
704 active) of pBo17 with no amino acid substitution. The Bo17 Spliced mutant only encodes the spliced form
705 of pBo17 (inactive). (B) Verification of the molecular structure. The DNA of the different BoHV-4 strains
706 were analyzed by EcoRI restriction (left panel) and further tested by Southern blot using probe
707 corresponding to nucleotides 26–774 of the V. test Bo17 ORF (right panel). White triangle and white star
708 indicate the restriction fragments containing the eGFP ORF and the BAC cassette, respectively. Black
709 triangle shows the restriction fragment that contains Bo17 Spliced. Marker sizes in kbp are indicated on
710 the left. (C) RT-PCR analysis of BoHV-4 Bo17 expression by the different mutants. MDBK cells were
711 infected with different mutants (MOI of 0.5 PFU/cell). 24 h post-infection, expression of Bo17 was
712 studied using primers specific for Bo17 sequence (primers ATG-STOP, 1323 bp). Only the entire transcript
713 or the spliced transcript was detected for the recombinant Bo17 MUDIR virus and Bo17 Spliced virus,
714 respectively. Reverse transcriptase (RT) reactions were executed (+) or omitted (-) to verify the absence

715 of contaminant DNA. The weak band observed in the Bo17 Del sample corresponds to the expression of
716 the cellular C2GnT-M. Sizes in bp are indicated on the left. **(D)** Detection of specific BoHV-4 protein by
717 the anti-Bo17-422-441 serum. MDBK cells were infected with the different BoHV-4 strains as indicated (1
718 PFU/cell). Forty-eight hours later, cells were scraped off the plate, and western blotting was carried out
719 as described in Materials and Methods. The position of molecular mass (MM) standard is shown on the
720 left of the gel. The open and filled triangles indicate the bands containing respectively pBo17 full-length
721 and pBo17 spliced.

722

723 **Figure 6. Replication of BoHV-4 MUDIR and Spliced strains *in vitro*.** MDBK (A), EBTr (B) or BT (C) cells
724 grown in 6-well cluster dishes were infected at an MOI of 0.05 in a multistep assay as described in
725 Materials and Methods with different BoHV-4 strains. Supernatant of infected cultures and infected cells
726 were harvested at different times (days) post-infection, and the amount of infectious virus was
727 determined by plaque assay on MDBK cells. Time zero post-infection is re-titration of the inoculums to
728 ensure that similar amounts of virus were put on the cells. Plaques were visualized by
729 immunofluorescence staining as described in Materials and Methods.

730

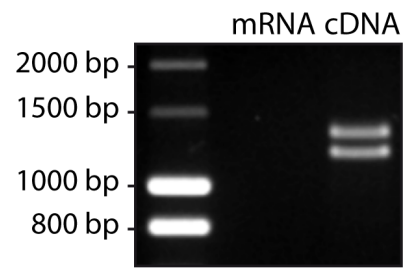
731 **Figure 7. MALDI-TOF-MS profile of O-glycans from MDBK infected cells with BoHV-4 mutant strains.**
732 **(A-F)** We determined the glycomic profiles of reduced and permethylated O-glycans detected in MDBK
733 infected cells with Bo17 MUDIR (A), Bo17 MUDIR Rev (B), Bo17 Spliced (C) and Bo17 Spliced Rev (D)
734 BoHV-4 mutants. Structural assignments were based on MS and MS/MS data and knowledge of O-glycan
735 biosynthetic pathways. These data are representative of two independent experiments. **(G)** The relative
736 intensity of Core 1 and Core 2 peaks were calculated relative to the Core 1 peak at m/z 895 defined as
737 100 %. Then, the ratio Core 2/Core 1 corresponds to the total of relative intensities of the Core 2 peaks
738 divided by the total of relative intensities of the Core 1 peaks. These ratios were calculated for each

739 replicate of each sample. The data presented are the average $\pm$ SDs for 2 measurements and were
740 analyzed by Welch's t-test relatively to the mean value obtained for the mock-infected sample, ** $p <$
741 0.01, * $p < 0.05$, ns: non significant.

742

743 **Figure 8. Effect of Bo17 splicing on BoHV-4 glycosylation.** (A) Purified virions were subjected to western
744 blotting with a rabbit monospecific polyserum raised against the gp180 glycoprotein (anti-Bo10-c15
745 serum) or whole BoHV-4 virions. The glycoprotein gp180 has a lower molecular mass for the viruses
746 devoid of the enzyme Bo17 (Bo17 Del) as well as for the viruses expressing an inactive form of pBo17
747 (Spliced). The position of a MM standard (in kDa) is shown on the left. (B) Sensitivity of different BoHV-4
748 mutants to Jacalin mediated neutralization. BoHV-4 mutant strains were incubated (2h, 37°C) with
749 increasing amounts of Jacalin (Core 1 O-glycan specific lectin) and then assayed for infectivity on MDBK
750 cells. BoHV-4 titers are expressed relative to virus without lectin. The data presented are the average $\pm$
751 SEMs for 3 measurements and were analyzed by 2 way ANOVA and Bonferroni posttests, *** $p < 0.001$.

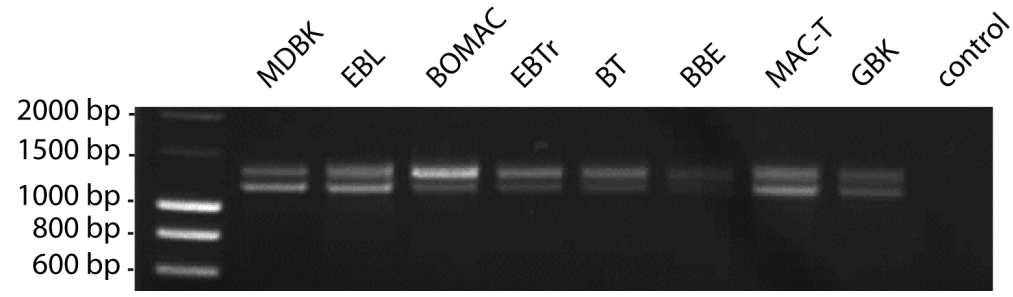
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B

Bo17	ATG	AAG	ATG	GCT	GGG	TGG	AAG	AAG	AAG	CTT	TGC	CCG	GGA	CAT	CAT	CTG	TGG	GCC	CTG	GGC	TGC	TAT	ATG	CTG	CTG	GCC	GTT	GTT	TCT	CTG
Unknown	ATG	AAG	ATG	GCT	GGG	TGG	AAG	AAG	AAG	CTT	TGC	CCG	GGA	CAT	CAT	CTG	TGG	GCC	CTG	GGC	TGC	TAT	ATG	CTG	CTG	GCC	GTT	GTT	TCT	CTG
Bo17	AGA	CTT	TCC	CTC	AGG	TTT	AAA	TGT	GAT	GTA	GAT	TCC	TTG	GAT	CTG	GAG	TCC	AGG	GAC	TTC	CAG	AGT	CAG	CAC	TGT	AGG	GAC	ATG	TTG	TAC
Unknown	AGA	CTT	TCC	CTC	AGG	TTT	AAA	TGT	GAT	GTA	GAT	TCC	TTG	GAT	CTG	GAG	TCC	AGG	GAC	TTC	CAG	AGT	CAG	CAC	TGT	AGG	GAC	ATG	TTG	TAC
Bo17	AAT	AGC	CTG	AAG	CTG	CCA	GCA	AAG	AGA	TCC	ATC	AAC	TGT	TCT	GGG	ATC	ACG	CGA	GGG	GAC	CAG	GAA	GCA	GTG	GTC	CAG	GCC	CTC	CTG	GAC
Unknown	AAT	AGC	CTG	AAG	CTG	CCA	GCA	AAG	AGA	TCC	ATC	AAC	TGT	TCT	GGG	ATC	ACG	CGA	GGG	GAC	CAG	GAA	GCA	GTG	GTC	CAG	GCC	CTC	CTG	GAC
Bo17	AAC	CTG	GAA	GTG	AAG	AAG	AAG	CGG	CCG	CCT	CTC	ACT	GAC	ACC	TAT	TAC	CTC	AAC	ATA	ACC	AGG	GAC	TGT	GAG	CGC	TTC	AAG	GCC	CAA	AGG
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Bo17	AAG	TTC	ATA	CAG	TTC	CCA	CTG	AGT	AAA	GAA	GAG	CTA	GAC	TTC	CCC	ATT	GCC	TAC	TCG	ATG	GTG	GTC	CAT	GAG	AAG	ATT	GAG	AAC	TTT	GAA
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Bo17	CGG	CTG	CTG	CGA	GCC	GTG	TAT	GCC	CCT	CAG	AAC	ATA	TAC	TGT	GTC	CAC	GTG	GAT	GTG	AAG	TCC	CCA	GAG	ACT	TTC	AAA	GAG	GCG	GTC	AAG
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Bo17	GCC	ATT	ATT	TCC	TGC	TTC	CCC	AAT	GTC	TTC	ATG	GCC	AGT	AAG	TTG	GTC	CCG	GTG	GTT	TAT	GCC	TCC	TGG	TCC	AGA	GTG	CAA	GCT	GAC	CTG
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Bo17	AAC	TGT	ATG	GAA	GAC	TTG	CTC	CAG	AGC	TCA	GTG	TCA	TGG	AAG	TAC	TTA	CTG	AAT	ACA	TGC	GGG	ACA	GAC	TTT	CCC	ATA	AAG	ACC	AAT	GCC
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Bo17	GAG	ATG	GTC	CTG	GCC	CTC	AAG	ATG	TTG	AAG	GGT	AAG	AAC	AGT	ATG	GAG	TCT	GAG	GTA	CCT	TCT	GAG	TCC	AAA	AAA	AAT	CGC	TGG	AAA	TAC
Unknown	GAG	ATG	GTC	CTG	GCC	CTC	AAG	ATG	TTG	AAG	GGT	AAG	AAC	AGT	ATG	GAG	TCT	GAG	GTA	CCT	TCT	GAG	TCC	AAA	AAA	AAT	CGC	TGG	AAA	TAC
Bo17	CGC	TAT	GAG	GTG	ACA	GAC	ACA	CTG	TAC	CCT	ACC	AGC	AAG	ATA	AAA	GAC	CCT	CGC	CGT	GAT	AAT	TTA	CCC	ATG	TTC	ACA	GGG	AAT	GCC	TAT
Unknown	CGC	TAT	GAG	GTG	ACA	GAC	ACA	CTG	TAC	CCT	ACC	AGC	AAG	ATA	AAA	GAC	CCT	CGC	CGT	GAT	AAT	TTA	CCC	ATG	TTC	ACA	GGG	AAT	GCC	TAT
Bo17	TTT	GTG	GCC	TCT	CGA	GCC	TTT	GTC	CAA	CAT	GTC	TTA	GAC	AAC	CCT	AAA	TCC	CAA	ATA	CTG	GTT	GAA	TGG	GTG	AAA	GAC	ACA	TAT	AGC	CCC
Unknown	TTT	GTG	GCC	TCT	CGA	GCC	TTT	GTC	CAA	CAT	GTC	TTA	GAC	AAC	CCT	AAA	TCC	CAA	ATA	CTG	GTT	GAA	TGG	GTG	AAA	GAC	ACA	TAT	AGC	CCC
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Unknown	GAC	GAA	CAC	CTC	TGG	GCC	ACC	CTT	CAG	CGT	GCT	CCG	TGG	ATG	CCT	GGC	TCT	GTT	CCT	AGC	CAC	CCA	AAG	TAT	CAC	ATC	TCA	GAC	ATG	ACT
Bo17	GCC	ATC	GCC	AGG	CTC	GTC	AAG	TGG	CAG	TAC	CAC	GAG	GGA	GAT	GTC	AGC	ATG	GGG	CGC	CCT	TAT	GCA	CCC	TGC	TCT	GGA	ATC	CAT	CGG	AGG
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Bo17	GCC	ATC	TGC	ATT	TAC	GGG	GCC	GGG	GAC	CTG	TAC	TGG	ATT	CTC	CAG	AAC	CAT	CAC	CTC	TTG	GCA	AAC	AAG	TTT	GAC	CCG	AGG	GTG	GAT	GAT
Unknown	GCC	ATC	TGC	ATT	TAC	GGG	GCC	GGG	GAC	CTG	TAC	TGG	ATT	CTC	CAG	AAC	CAT	CAC	CTC	TTG	GCA	AAC	AAG	TTT	GAC	CCG	AGG	GTG	GAT	GAT
Bo17	AAC	GTC	CTG	CAG	TGC	TTA	GAA	GAG	TAC	CTA	CGT	CAT	AAG	GCC	ATC	TAT	GGG	ACT	GAA	CTT	TGA	1323								
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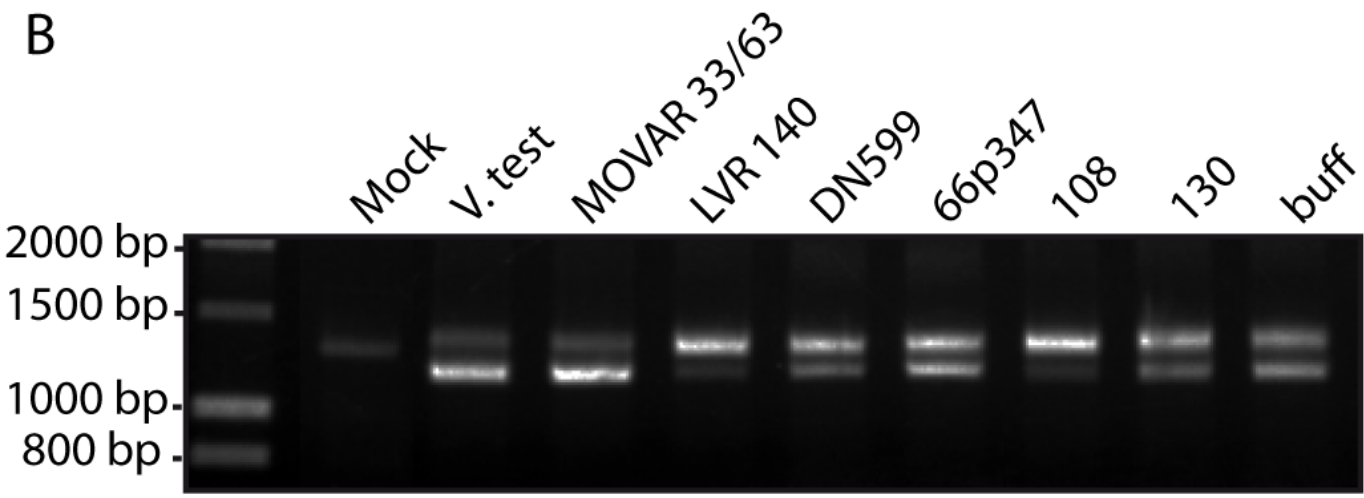
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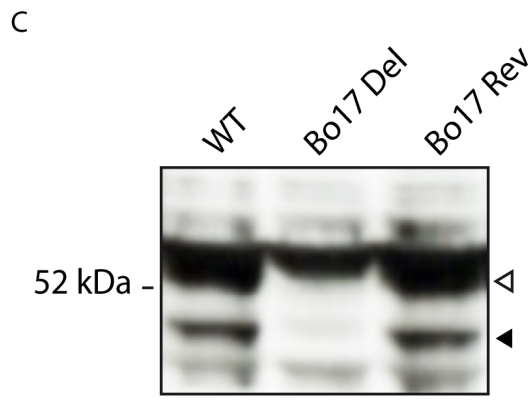
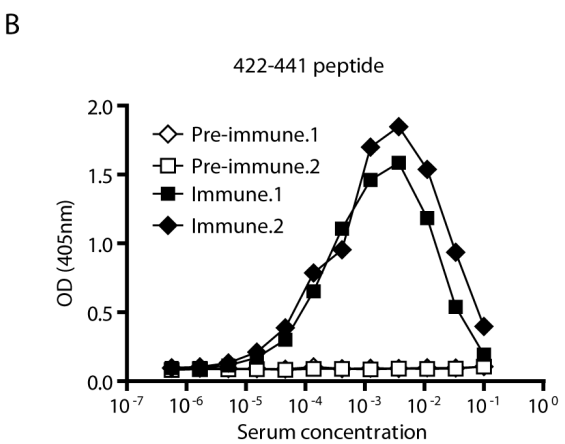
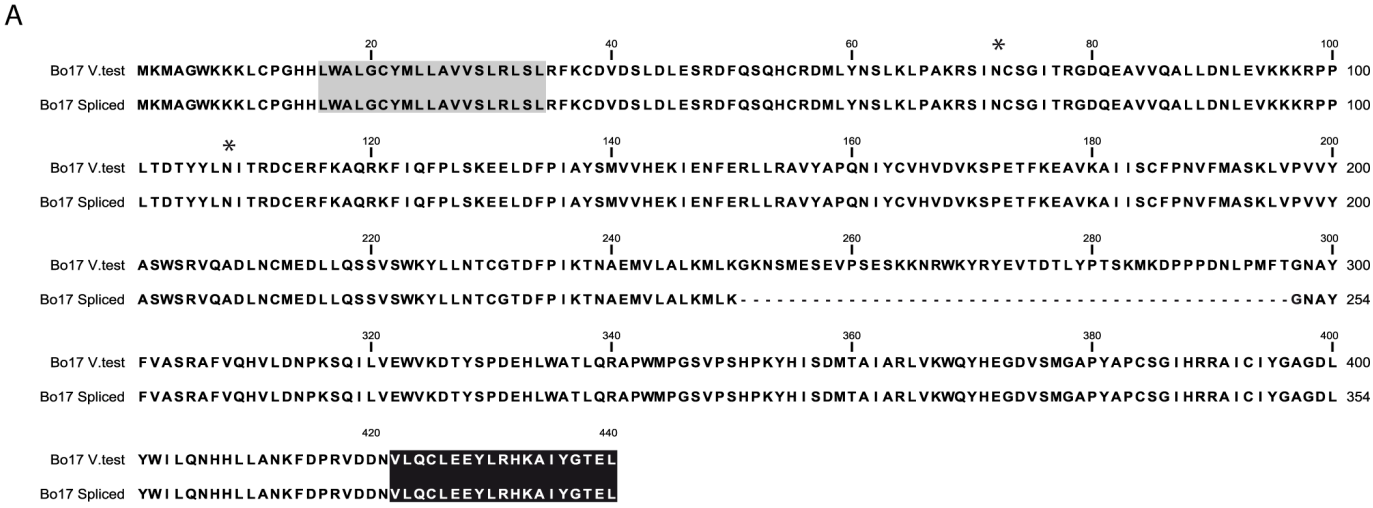


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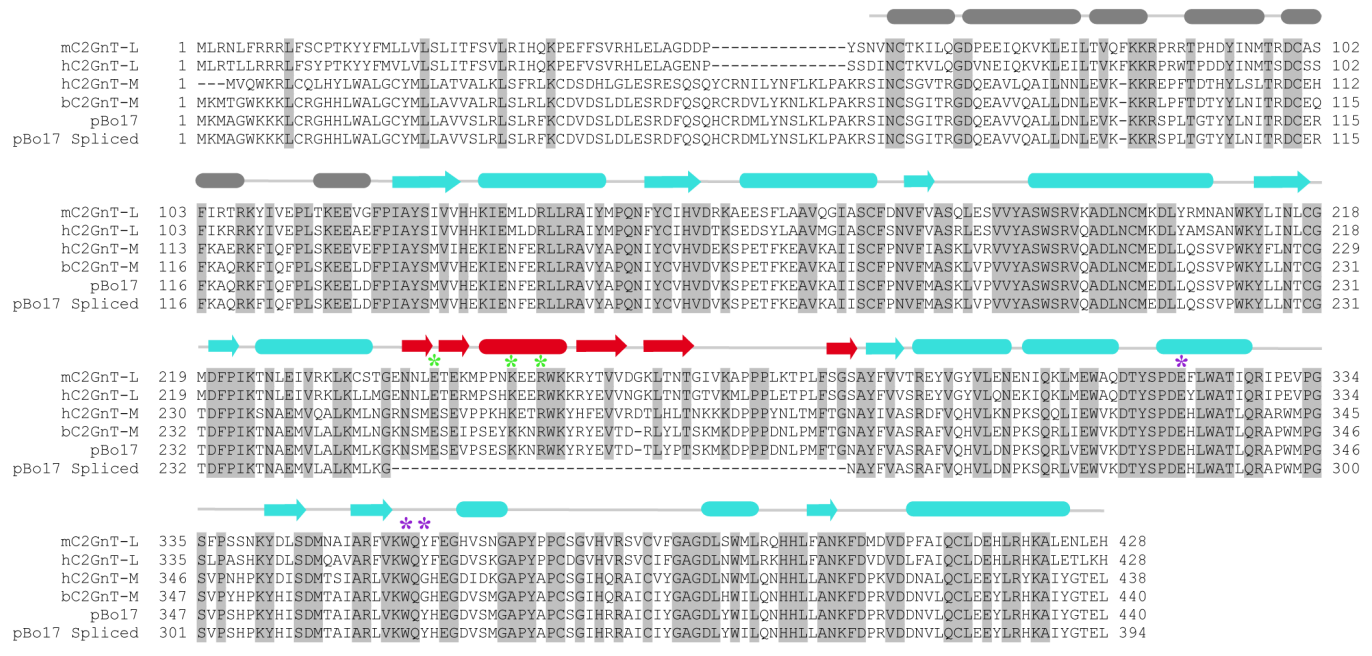
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Buff	TTG	AA T	GGT	AAG	AAC	-----	TGT	TCA	CAG	G AA	ATG	
Giraffa camelopardalis	TTG	AA T	GG G	AAG	AAC	-----	TGT	TCA	C GG	GGA	ATG	
Cervus elaphus	TTG	AA T	GG G	AAG	AAC	-----	TGT	TCA	C AG	GGA	ATG	
Ovis aries	TTG	AA T	GG G	AAG	AAC	-----	TGT	TCA	C T G	GGA	ATG	
Syncerus caffer	TTG	AA T	GG G	AAG	AAC	-----	TGT	TCA	C AG	GGA	ATG	
Bos grunniens	TTG	AA T	GG G	AAG	AAC	-----	TGT	TCA	C GG	GGA	ATG	
Bos taurus	TTG	AA T	GG G	AAG	AAC	-----	TGT	TCA	C GG	GGA	ATG	
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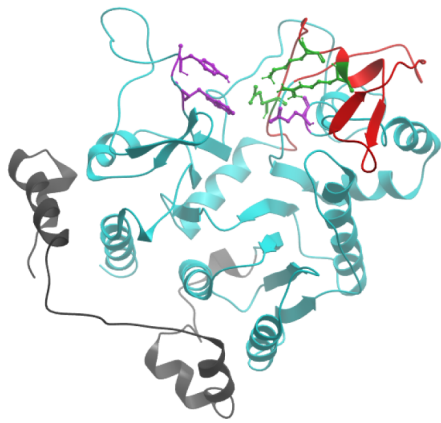




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