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A novel Borna disease virus vector system that stably expresses foreign proteins from an intercistronic noncoding region

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Running Title: Establishment of a novel BDV vector

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Abstract

Borna disease virus (BDV), a nonsegmented, negative-strand RNA virus, infects a wide variety of mammalian species and readily establishes a long-lasting, persistent infection in brain cells. Therefore, this virus could be a promising candidate as a novel RNA virus vector enabling stable gene expression in the central nervous system (CNS). Previous studies demonstrated that the 5' untranslated region of the genome is the only site for insertion and expression unit of a foreign gene. In this study, we established a novel BDV vector, in which an additional transcription cassette has been inserted into an intercistronic noncoding region between the viral phosphoprotein (P) and matrix (M) genes. The recombinant BDV (rBDV) carrying green fluorescent protein (GFP) between the P and M genes, rBDV P/M-GFP, expressed GFP efficiently in cultured cells and rodent brains for a long-period of time without attenuation. Furthermore, we generated a non-propagating rBDV, ΔGLLP/M, which lacks the envelope glycoprotein (G) and a splicing intron within the polymerase gene (L), by the trans-complementation system with either transient or stable expression of the G. Interestingly, rBDV ΔGLLP/M established a persistent infection in cultured cells with stable expression of GFP in the absence of the expression of G. Using persistently infected rBDV ΔGLLP/M-infected cells, we determined the amino acid region in the cytoplasmic tail (CT) of BDV G important for the release of infectious rBDV particles and also demonstrated that the CT region may be critical for the generation of pseudotyped rBDV having vesicular stomatitis virus G protein. Our results revealed that the newly established BDV vector constitutes an alternative tool, not only for stable expression of foreign genes in the CNS but also for understanding the mechanism of the release of enveloped virions.

Introduction

Borna disease virus (BDV) belongs to the *Bornaviridae* family within the non-segmented, negative-strand RNA viruses and is characterized by highly neurotropic and noncytopathic infection (18, 31). Previous studies revealed that BDV infects a wide variety of mammalian species, suggesting that its host range probably includes all warm-blooded animals. The most striking feature of BDV is that it readily establishes a long-lasting persistent infection in the cell nucleus (9, 31). BDV establishes a stable infection without causing apparent cell damage, even in brain cells (8), making this the only animal RNA virus capable of intranuclear parasitism. These features of BDV suggest that this virus could be a promising candidate for efficient and stable gene delivery system to the central nervous system (CNS).

Recent developments of the reverse genetics system of BDV revealed that the virus has the capacity to express foreign genes from the 5' end of the genome (26). Recombinant BDV (rBDV) with green fluorescence protein (GFP) near the 5' end of the genome (rBDV 5'GFP) successfully infected, and was propagated in, cultured cells. This study suggested that the 5' end of the BDV genome might be the only site for insertion of the GFP expression cassette, because rBDV could not be rescued after introduction of the cassette into other regions of the genome (26). On the other hand, a recent study using the rBDV 5'GFP revealed that the termination signal upstream of the GFP gene is modified by insertion of additional A residues, resulting in downregulation of GFP expression within several weeks of infection of rat brains (2). This observation suggested that although the 5' end of the BDV genome is capable of the expression of a foreign gene, an additional transcriptional cassette in this region may affect the replication of BDV adversely, probably because of destruction of specific genome-end

67 structures, such as the inverted terminal repeat (ITR) and panhandle structure, both of which
68 could be important for the transcription/replication processes of the viral polymerase (24, 25,
69 27). Furthermore, because there is a clear polarity of initiation of transcription from the 3' end
70 to the 5' end of the genome in mononegaviruses (17, 23, 29), a transcription unit at the 5' end
71 of the genome may be unsuitable for efficient expression of foreign genes. Considering the
72 complexities of genome construction and the transcription mechanism of BDV, it may be
73 conceivable that BDV has a lower capacity than other mononegaviruses for the insertion of an
74 additional expression unit in the genome.

75 As well as the site of insertion of a foreign gene cassette in the genome, the flexibility
76 of the virus genome to retain the capacity to replicate efficiently, as well as host cell tropism,
77 also could be important in establishing a more effective and useful virus vector system. In
78 many virus vectors, propagation-defective and pseudotyped viruses have been developed by
79 modifying viral genomes and deletion or mutation of viral genes (4, 5, 13, 35). In BDV, some
80 point mutations, including in the phosphoprotein (P), X and RNA polymerase (L) genes, have
81 been shown to modify the efficiency of virus replication in cultured cells and animal brains (1,
82 21). For example, two amino acid substitutions, L1116R and N1398D, in the L gene are
83 known to enhance significantly the polymerase activity, as well as the replication kinetics, of
84 rBDV (1). However, there have been only few studies on the modification of the viral genome
85 to develop the efficient and safe BDV vector system.

86 In this study, we established a novel BDV vector, BDV P/M, which harbors an extra
87 transcription unit in the short intercistronic noncoding region of the genome between the P
88 and matrix (M) genes, P/M. BDV P/M was shown to stably express several reporter genes,

including GFP, DsRed and luciferase, in cultured cells without attenuation of their expression. Furthermore, we developed a non-propagating rBDV, ΔGLLP/M, which lacks an envelope glycoprotein (G) gene and a splicing intron within the L gene, by the trans-complementation of the G. Interestingly, rBDV ΔGLLP/M-GFP was able to establish persistent infection in a single cell without the expression of G. We also show that the cytoplasmic tail (CT) of BDV G may be essential for the release of infectious particles and the generation of pseudotyped BDV virions having vesicular stomatitis virus (VSV) G protein, by using the rBDV ΔGLLP/M virus system. Our results show that the BDV P/M vectors may constitute a new system, not only for efficient and safe gene delivery in vivo, but also for understanding the mechanism of envelope assembly and virion formation of BDV, which also could be important for improving the utility of BDV as a virus vector.

Materials and Methods

Cells. Vero cells and Vero cells stably expressing BDV G (Vero-BG) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% fetal bovine serum (FBS). The OL cell line, derived from a human oligodendroglioma, was cultured in DMEM-high glucose (4.5%) supplemented with 5% FBS. HEK 293T cells were cultured in DMEM supplemented with 10% FBS.

Plasmid constructions. The RNA polymerase II (Pol II)-driven BDV minigenome plasmid, pCAG-HRSV3, has been described previously (34). To generate a plasmid expressing the BDV full-length antigenome RNA, pFct-BDV, a cloned full-length cDNA of

111 BDV strain He/80 was inserted into the *Xho* I and *Not* I sites of pCAG-HRSV3. A plasmid
112 containing an extra transcription cassette, pFct-BDV P/M, was generated by insertion of
113 transcription initiation (S3) and termination (T2) signal sequences in the P/M region with
114 *Bst*B I and *Pac* I sites (Fig. 1A). The plasmid harboring the expression cassette in the 5' end
115 of the genome, pFct-BDV 5', also was constructed using the *Bst*B I and *Pac* I restriction
116 enzymes. The L_{RD} mutation (1) was introduced into the pFct-BDV P/M plasmid by
117 PCR-based mutagenesis. To generate foreign gene expression vectors, reporter genes,
118 including GFP, DsRed, luciferase and LacZ were amplified from peGFP-N1 (Clontech),
119 pDsRed-Monomer-C1 (Clontech), pGL3-Promoter Vector (Promega), and pcDNA 5/TO/lacZ
120 (Invitrogen) and inserted into pFct-BDV P/M or pFct-BDV ΔGLLP/M vector with *Bst*B I and
121 *Pac* I sites.

122 The pFct-BDV ΔGP/M-GFP, in which two ATG codons (nt 2236 and 2248) in the G
123 ORF were changed to ACG, was constructed from pFct-BDV P/M GFP by PCR mutagenesis
124 (Fig. 3A). The intron II-deleted, L ORF-linearized BDV vector, pFct-BDV ΔGLLP/M GFP,
125 was developed by deleting the intron II sequence (nt 2410 to 3703) from pFct-BDV ΔGP/M
126 GFP using a PCR amplification method (Fig. 3A).

127 The expression vector, pBDV-G, was generated by subcloning the G ORF of strain
128 He/80 into the *Kpn* I and *Sma* I sites of plasmid pCXN2. For efficient expression of BDV G in
129 transfected cells, silent mutations were introduced at the splicing donor (SD) and acceptor
130 (SA) sequences in the G gene. The cDNA of VSV G was amplified from pVPack-VSV-G
131 (Stratagene) and subcloned into the *Eco*R I and *Eco*R V sites of pCXN2 vector to yield
132 pVSV-G. The rabies virus (RaV) G expression plasmid, pRaV-G, was constructed by

133 subcloning of the Nishigahara strain G, which was kindly gifted by Naoto Ito (Gifu University,
134 Japan), into the *Kpn* I and *Eco*R V sites of pCXN2 vector. Expression plasmids of chimeric G
135 proteins of VSV and RaV, in which the CT regions were exchanged with that of BDV G, were
136 constructed by PCR mutagenesis. Detailed information about the primers and PCR procedures
137 used to generate these plasmids is available from the authors. Nucleotide sequences of the
138 recombinant plasmids were confirmed by DNA sequencing.
139
140 **Virus rescue**. 293T cells were seeded in 35 mm dishes and transfected with 2 μg of BDV
141 vector plasmid, 0.25 μg of pCA-N (34), 0.025 μg of pCXN2-P (34) and 0.25 μg of pCA-L
142 (34) using Lipofectamine^{TM}2000 (Invitrogen). To rescue rBDV ΔGP/M GFP and ΔGLLP/M
143 GFP, 0.1 μg of pBDV-G was transfected together with the helper plasmids expressing BDV N,
144 P and L into 293T cells. Three days posttransfection, the cells were detached using trypsin and
145 seeded onto 10 cm dishes. One day after the first passage, approximately 1×10^5 Vero or
146 Vero-BG cells were cocultured with the transfected 293T cells. The cultures were split every 3
147 days and the rescue efficacy of recombinant viruses was evaluated by fluorescence
148 microscopy using an anti-BDV N monoclonal antibody (mAb) or by monitoring the
149 expression of GFP. The non-propagating rBDVs were rescued by transient transfection with
150 expression plasmids encoding BDV, VSV and RaV G and chimeric G protein between these
151 viruses. Vero cells stably infected with rBDV ΔGLLP/M-GFP in 35 mm dishes were
152 transfected with 3 μg of the G expression construct using Lipofectamine^{TM}2000 (Invitrogen)
153 and the virus particles were collected from the culture supernatant or from sonicated cells at
154 48 h posttransfection.

Virus infection. Vero or Vero-BG cells were infected with the amounts of rBDV stock indicated at 37ºC. After absorption for 1 h, the cells were washed with PBS (-) and passaged with the appropriate interval of a few days. Virus propagation was detected by indirect immunofluorescence or GFP assay.

C57BL/6J mice and Lewis rats (Oriental Yeast Co. Ltd., Shiga, Japan) were inoculated intracranially with 1×10^3 focus forming units (FFU) of rBDV stock per animal within 24 h of birth. The infected animals were sacrificed at the appropriate month after inoculation and the brains were collected for analysis. To assess GFP expression in the brain, the whole or cross sections of the brains were rinsed with PBS (-) and examined under a Leica WILD M10 fluorescence stereomicroscope (Leica Microsystems, Wetzlar, Germany). Brain samples were then fixed in 4% paraformaldehyde, cryopreserved in 25% sucrose, and frozen in Tissue-Tek O.C.T. compound. Cryostat sections (4 μm) were examined under a fluorescence microscope. Sections also were examined by immunohistochemical analysis after staining with anti-BDV N mAb. All animal experimentation conformed to the guide for the care and use of laboratory animals of the Research Institute for Microbial Diseases, Osaka University.

Histological analysis. Frozen sections were incubated with trypsin solution (0.1% trypsin, 0.1% $CaCl_2$, and 0.05 M Tris-HCl, pH 7.5) for 5 min at 37ºC. The sections were then permeabilized by PBS (-) with 0.05% Triton-X and blocked with 10% normal goat serum in PBS (-) for 30 min at room temperature. The sections were incubated with primary antibody with 5% normal goat serum in PBS (-) overnight at 4ºC and then with the secondary

177 antibodies with PBS (-) for 1 hour at 37ºC. The cells were counterstained with
178 4',6'-diamidino-2-phenylindole (DAPI).
179
180 **Indirect immunofluorescence assay (IFA).** Cells were fixed with 4% paraformaldehyde
181 and permeabilized by PBS (-) with 0.4% Triton-X. After permeabilization, the cells were
182 incubated with the primary antibody, anti-BDV N mAb, for 1 h at 37ºC. Subsequently, the
183 cells were washed three times with PBS (-) and incubated with secondary antibody, goat
184 anti-mouse IgG antibody conjugated with Cy3 (Jackson Immuno Research) or goat anti-rabbit
185 IgG antibody conjugated with Alexa Fluor 488 (Invitrogen) for 1 h at 37ºC. The cells were
186 counterstained with DAPI. After further washing with PBS (-), the cells were examined with
187 an inverted microscope Ti-E with a confocal laser scanning system C1 (Nikon).
188
189 **Luciferase and LacZ reporter assays.** Vero cells were infected with rBDV
190 P/M-luciferase in 16 mm culture dishes. At 24, 48 and 72 h postinfection, cells were lysed and
191 subjected to luciferase assay with Luciferase Cell Culture Lysis Reagent (Promega),
192 according to the manufacturer's recommendations. The activity of β-Galactosidase from the
193 rBDV ΔGLLP/M-LacZ infected Vero-BG cells was analyzed by β-Galactosidase Staining Kit
194 (PanVera), according to the manufacture's recommendations.
195
196 **Western blot analysis.** Vero cells infected with rBDV were lysed and the proteins
197 separated with 12% SDS-PAGE, followed by transfer to Immobilon-P Transfer Membrane
198 (Millipore). Membranes were then blocked with 5% skimmed milk in PBS (-) and incubated

199 with the primary antibodies (anti-BDV N and P mAb and anti-BDV M and G polyclonal
200 antibodies [pAb]. After three washes with 0.05% Tween-20 in PBS (-), horseradish
201 peroxidase-conjugated secondary antibodies (Jackson Immuno Research) were applied for 1 h
202 at 37ºC. The membrane was washed three times and bound antibodies were detected using the
203 ECL Western Blotting System (Amersham Pharmacia Biotech).
204
205 **Northern blot analysis.** Total RNA was extracted from cells infected with rBDV with
206 TRIzol reagent (Invitrogen). Aliquots of 1.0 μg of total RNA were electrophoresed through a
207 1.0% agarose gel containing 2.2% formaldehyde and transferred onto Nylon Membranes
208 (Roche) with 20 × SSC (1 × SSC = 150 mM NaCl plus 15 mM sodium citrate, pH 7.0). After
209 UV cross-linking, the membrane was prehybridized in hybridization solution for 30 min at
210 65ºC. This step was followed by hybridization overnight with a digoxigenin (DIG)-labeled
211 RNA probe. The hybridized probe was detected with an alkaline phosphatase-conjugated
212 anti-DIG Fab fragment (Roche)
213
214 **Results**
215 **Efficient expression of foreign genes from an intercistronic region between the P and M**
216 **genes of BDV.** A previous study by Schneider et al. (2007) showed that
217 replication-competent rBDV was not able to be rescued when an extra transcription unit was
218 inserted into the P/M region of the BDV genome (26). To examine further the availability of
219 the P/M region for expression of a foreign gene, we inserted a GFP cassette into the site as
220 indicated in Fig. 1A. The nucleotide sequences of the cassette could be slightly different from

221 those reported previously, at least in terms of the restriction enzyme sites for cloning of the
222 cassette, whilst both cassettes use the same T2 and S3 sequences for additional transcription
223 termination and initiation signals, respectively (Fig. 1A). We transfected the resultant plasmid,
224 pFct-BDV P/M-GFP, which is driven by the Pol II promoter, into 293T cells along with helper
225 plasmids (see Materials and Methods). A few weeks after cocultivation with Vero cells, rBDV
226 harboring the GFP gene in the P/M region, termed rBDV P/M-GFP, was rescued successfully
227 (Fig. 1B). Consistent with previous studies (26), the L1116R and N1398D mutations in the L
228 gene (L_{RD}) enhanced the efficiency of rescue of the rBDV P/M-GFP virus (data not shown)
229 and, therefore, we used the L_{RD} mutant plasmid in the backbone of BDV P/M vectors for
230 further experiments.

231 At first, we infected Vero cells with cell-free rBDVs at a multiplicity of infection (MOI)
232 of 0.01 and the growth kinetics of rBDV P/M-GFP were compared to wild-type (wt)
233 recombinant virus and rBDV 5'GFP. As shown in Fig. 1C, the growth kinetics of both
234 GFP-expressing viruses seemed to be slightly retarded compared to wt rBDV but they were
235 propagated efficiently and reached over 90% infection by 3 weeks postinfection. We also
236 performed IFA to investigate the expression of GFP in Vero cells. As shown in Fig. 1D, rBDV
237 P/M-GFP induced an apparently stronger GFP signal than rBDV 5'GFP, while the expression
238 of N is comparable among the various recombinant viruses. The western and northern blot
239 analyses confirmed higher expression of GFP from the P/M region than the 5' end of the
240 genome (Figs. 1E and F).

241 In addition to the GFP gene (720 bp), we could rescue rBDV P/M viruses harboring
242 DsRed (678 bp) and luciferase (1653 bp) in the P/M region and verified the stable expressions

of these genes in infected cells. Both the rescue efficiency and growth kinetics of rBDV P/M-DsRed and -Luc were quite comparable to that of P/M-GFP virus (data not shown). Using rBDV P/M-Luc, we could monitor the transcription of BDV in infected cells at a very early stage of infection. The luciferase activity was clearly detected in Vero cells as early as 72 h postinfection (Fig. 1G), at which time BDV replication had not been demonstrated by other methods. Furthermore, efficient expression of DsRed was also demonstrated in Vero cells infected with the rBDV (Fig. 1H). The signal intensity of DsRed appeared to be overall equal to that of GFP, suggesting that the P/M region has the capacity to harbor various genes without attenuating viral replication.

Stable expression of GFP in rBDV P/M-GFP-infected rodent brains. To investigate whether rBDV P/M can efficiently and stably express a foreign gene in animal brains, we inoculated 1.0 x 10^3 FFU of rBDV P/M-GFP and 5'GFP viruses intracranially into neonatal Lewis rats and C57BL/6J mice and GFP expression was monitored by fluorescence stereomicroscopic and immunohistochemical analyses. As shown in Fig. 2A, a GFP signal was detected in the injected hemisphere of the rBDV P/M-GFP-infected rat brain and was much brighter than that of the 5'GFP at 2 weeks postinoculation. The expression of GFP was clearly visible in the neurons of the cerebrum cortex and cerebellum at 2 months after inoculation (Fig. 2B). Although the N-positive neurons were detected equally in the brain regions after infection by rBDV P/M-GFP and 5'GFP viruses, GFP fluorescence seemed to be much greater in the neurons infected with rBDV P/M-GFP than with 5'GFP virus (Fig. 2B). Furthermore, the stable expression of GFP from the P/M-GFP virus was demonstrated in

mouse brains (Fig. 2C). The neurons in the hippocampus region of infected mice showed a strong GFP fluorescence at 2-months postinoculation (Fig. 2C). Notably, the GFP signal in the brains of rBDV P/M-GFP-infected mice was observed and stable over an observation period of at least 8 months (Fig. 2D). These results indicated that rBDV P/M can establish a persistent infection, leading to the lasting expression of foreign gene in the brains.

Previous work using virus vectors derived from mononegaviruses revealed that insertion of an additional transcriptional unit into viral gene junctions causes mutations of the transcriptional initiation or termination sites that control the transcription of the inserted or neighboring genes (2, 33). Therefore, we next determined using RT-PCR whether the sequences of the newly introduced transcriptional cassette are stable during the replication of the P/M-GFP virus. No sequence alterations or deletions were found to have been introduced into the rBDV P/M-GFP genome at least 8 months after infection of mouse brains (data not shown), suggesting the additional transcriptional unit and its surrounding sequences are fairly stable during the multiplication of the P/M-GFP virus in the brains.

Generation of propagation-defective rBDV P/M viruses lacking the translation initiation codons for the G gene and an intron region within the L gene. To improve the BDV P/M vector system, we next tried to generate propagation-defective rBDV P/M. First, we changed two ATG codons (nt 2236 and 2248) in the G ORF to ACG to abrogate initiation of translation of the G protein, ΔGP/M (Fig. 3A). Furthermore, we deleted an intron II sequence within the L gene (nt 2410 to 3703) to create a linearized, intronless L gene, ΔGLLP/M (Fig. 3A), with the idea that the deletion of the intron in L mRNA may not only lead to the efficient

287 production of L during the replication but also increase the capacity for insertion of foreign
288 genes into the genome. 293T cells were transfected with the resultant plasmid, pFct-BDV
289 ΔGP/M-GFP or pFct-BDV ΔGLLP/M-GFP, together with three helper plasmids (N, P and L)
290 and the G expression plasmid, and then cocultured with Vero cells stably expressing BDV G,
291 Vero-BG. At first, the rescue efficiency was compared between P/M-GFP, ΔGP/M-GFP and
292 ΔGLLP/M-GFP viruses at 12 days after the cocultivation. As shown in Fig. 3B, the cells
293 transfected with pFct-BDV ΔGLLP/M-GFP yielded a much higher level of recombinant virus
294 than those with pFct-BDV ΔGP/M-GFP. Furthermore, although the numbers of transfected
295 plasmids, as well as the cells used for the rescue of recombinant viruses, were different
296 between ΔGLLP/M-GFP and P/M-GFP viruses, the rescue efficiency of L linearized construct
297 was comparable to or more efficient than that of wt P/M-GFP vector, suggesting that
298 linearized, intronless L gene enables efficient replication of rBDV.

299 To examine the cell-free infectivity and stability of the ΔGLLP/M virus, the cell-free
300 virus of rBDV ΔGLLP/M-GFP-infected Vero-BG cells were inoculated into either Vero-BG or
301 parental Vero cells at an MOI of 0.01, and the growth kinetics of the viruses were monitored
302 by IFA. Although the infection rate of ΔGLLP/M-GFP virus gradually increased in Vero-BG
303 cells, the parental Vero cells did not support the propagation of the ΔGLLP/M-GFP virus (Fig.
304 4A). Interestingly, however, the infection by rBDV ΔGLLP/M-GFP was stably maintained
305 during the observation period of 24 days (Figs. 4A and 4B). The Vero cells persistently
306 infected with ΔGLLP/M-GFP virus exhibited the nuclear dot structure specific for BDV
307 replication. (Fig. 4B, insets). Western blotting confirmed the lack of G expression in rBDV
308 ΔGLLP/M-GFP-infected Vero cells (Fig. 4C). The persistent infection of ΔGLLP/M-GFP

309 virus without propagation was also observed in human-derived oligodendroglioma OL cells
310 (Fig. 4D). On the other hand, we investigated whether the lack of G expression and the
311 deletion of an intron within L gene increase the capacity of ΔGLLP/M vector to insert foreign
312 gene into the P/M region. As shown in Fig. 4E, the rBDV ΔGLLP/M harboring LacZ gene
313 (3060 bp) was successfully rescued and established persistent infection in Vero-BG cells. All
314 these observations demonstrated that G is essential for virus propagation but not for the
315 maintenance of persistent infection of BDV and suggested that rBDV ΔGLLP/M is a
316 non-propagating virus, which may provide safe, stable and high-capacity foreign gene
317 delivery in vivo.
318
319 **The ΔGLLP/M vector shows that the carboxyl-terminal region in BDV G is essential for**
320 **release of infectious particles.** In many virus vector systems, pseudotyping of the virus
321 envelope protein may be an effective strategy to expand the host cell tropism. Furthermore,
322 improvement of the efficacy of virus particle release from producer cells could be necessary
323 to generate a high titer virus stock. Therefore, understanding the mechanisms of the release of
324 BDV infectious particles, as well as the envelopment by BDV G, may be crucial for the
325 development of further applications of the BDV P/M vector system. As a first step towards
326 these goals, therefore, we tried to determine the region of BDV G essential for the release of
327 infectious particles, using the ΔGLLP/M-GFP system. Previous studies clearly revealed that
328 the CT region of the mononegavirus G glycoprotein may play a key role in assembly of
329 infectious virus particles in concert with M (11, 12, 28). We thus generated a series of alanine
330 substitution mutants in the CT region of BDV G (Fig. 5B) and transfected the expression

plasmids into a ΔGLLP/M-GFP virus persistently infected Vero cell line, Vero-ΔGLLGFP, which was obtained by limiting dilution of Vero cells infected with ΔGLLP/M-GFP virus. At 48 h after transfection, the virus titers in the culture supernatants were evaluated in Vero cells. As shown in Fig. 5B, none of the mutants, except for the $QE^{502/503}A$ mutant, in which two residues at the end of the C terminus are substituted by alanine, could rescue the infectious viruses. This result indicated that at least the amino acids at positions 494 to 501 of BDV G are required for efficient release or infectious particle formation of BDV in the cells.

We finally investigated whether the CT region of BDV G is required for the generation of pseudotyped rBDV harboring other virus envelope proteins. To this end, we at first transfected G expression plasmids of BDV, VSV, and RaV into the Vero-ΔGLLGFP cells, and at 48 h after transfection the virus titers in the culture supernatants were evaluated. As shown in Fig. 5D, although the cells transfected with BDV G plasmid yielded the rBDV in the supernatant as shown above, the infectivity was not detected in the supernatants of both VSV and RaV G-transfected cells. Interestingly, however, the chimeric VSV G, but not RaV G, of which the CT region is exchanged with that of BDV G (Fig. 5C), was shown to produce infectious particles in the culture supernatant of the transfected Vero-ΔGLLGFP cells (Fig. 5E). These results suggested that the CT region of BDV G may enhance the efficiency of release or formation of pseudotyped rBDV using VSV G. All these data demonstrated that the ΔGLLP/M virus system may provide a useful tool not only for investigating the mechanisms of BDV particle formation and virion budding but also for generating the pseudotyped rBDV particles.

353 **Discussion**

354 In this study, we showed that foreign genes can be stably expressed from an intercistronic
355 region of the BDV genome in both cultured and animal brain cells. Together with the
356 previous report by Schneider et al (26), this suggests strongly that BDV may be a good vector
357 platform for gene delivery to the CNS. In this study, it was very surprising that our system
358 expressing several foreign genes from the P/M region enabled successful rescue of the rBDV,
359 despite the fact that a vector with the same genome construction did not yield recombinant
360 virus in a previous study (26). Although the factors accounting for this difference are not
361 obvious, it is highly likely that only a single nucleotide difference exerts the adverse effects
362 on the rescue of rBDV in the reverse genetics system. It will be of interest to compare the
363 sequence differences between these vectors to understand the genomic feature necessary for
364 BDV replication or propagation.

365 Recently, Ackermann et al. reported that GFP expression from the rBDV 5'GFP
366 construct is reduced markedly in mouse brains from two months postinfection, probably
367 because of mutations in the termination/initiation signals upstream of inserted GFP gene (2).
368 This observation suggests that expression of an additional gene from the 5' end of BDV
369 genome may have deleterious effects on virus replication, leading to attenuation of
370 transcription of the inserted gene. The additional transcription initiation signal downstream of
371 the L gene may affect the efficiency of the termination of transcription of L. Alternatively, the
372 additional sequences may disrupt structures at the end of the BDV genome, such as the ITR
373 and panhandle structures, important for the initiation of RNA replication and formation of the
374 viral ribonucleoprotein (RNP) (24, 25, 27). Furthermore, very recently, Martin et al. clearly

showed that specific sequences in close proximity to the 3’ ends of the viral genome and antigenomic RNA are required for the elongation of the genomic RNA after the realignment of the 3’ termini (20). As this process could be essential for the maintenance of the BDV-specific genome-end structure during replication, it may be possible that the long extra sequences in the 5’ end of the genome disrupt the realignment of the 3’ terminus of antigenomic RNA, resulting in reduced virus replication. On the other hand, we demonstrated that the P/M-GFP virus exhibited no attenuation of the extra transcription unit, even in mouse brains during our observation periods of at least eight months. Sequencing analysis revealed that no attenuating mutations are introduced in the sequences of the inserted transcriptional unit. Furthermore, the expression levels of both P and M appeared not to differ between rBDV P/M and wt viruses. In addition, we could demonstrate that the P/M region must have a considerable capacity for insertion of various length foreign genes, at least below 3060 bp in ΔGLLP/M vector. All these observations indicated that P/M region may be a more flexible, stable and suitable site for insertion of an extra transcription cassette in the BDV genome, suggesting that the BDV P/M system provides an efficient platform as a BDV vector.

The potential for pathogenesis is an important concern for many virus vectors and replication- and propagation-incompetent viral vectors have been created for safety reason. In this study, we tried to generate propagation-defective rBDV by deleting the G ORF, using the P/M vector system. The G ORF of BDV encodes an envelope glycoprotein, which overlaps with the upstream M ORF, and contains a long intron of a 1293 nt for expression of the downstream L gene (7, 31, 32). The G precursor protein, GP, plays a key role in BDV entry to susceptible cells and the GP1, the N-terminal subunit of GP, is known to be required for

397 receptor recognition and virus entry (14, 15, 16, 19, 22). Previous studies suggested that the
398 expression and correct processing of BDV GP are necessary for BDV dissemination in
399 primary cultures of neurons and that neutralizing antibodies against BDV GP completely
400 inhibited virus spread (3). These observations suggested that the enveloped viral particles, not
401 nonenveloped viral RNPs, are crucial for dissemination of BDV between cultured cells (3), as
402 in the cases of other mononegaviruses, such as RaV (10). On the other hand, in an
403 independent system, it was reported that cell-to-cell spread of BDV does not require the
404 expression of the primary receptor, as well as the processing of BDV GP (6). These
405 observations showed the complexity of the roles of G in BDV infectivity and propagation in
406 neurons. In this study, we mutated the first two methionines and an intron (intron II) for the L
407 gene in the G ORF of the P/M-GFP vector to generate rBDV-lacking the G envelope protein,
408 ΔGLLP/M virus. The transiently enveloped-rBDV from G-expressing Vero cells showed
409 cell-free infectivity and established persistent infection of Vero cells in the absence of G
410 expression. Interestingly, the infection seemed to be maintained without spreading in the
411 cultured cells, suggesting that BDV G is not required for the establishment of persistent
412 infection in a single cell but rather for cell-to-cell spread. Our result revealed that persistent
413 infection by the ΔGLLP/M-GFP virus can be maintained for at least more than 2 months
414 without losing the expression of GFP (data not shown), demonstrating that the ΔGLLP/M
415 system may provide a promising BDV vector for in vivo use with safe and persistent foreign
416 gene expression in the infected cells.

417 The ΔGLLP/M virus provided an intriguing finding that intron II of the L gene may
418 negatively regulate the expression of L polymerase, because deletion of intron II markedly

419 enhances the efficiency of rescue of the rBDV when compared with the ΔGP/M virus. In
420 addition, the L-linearized construct seemed to be more efficient than the wt P/M-GFP vector
421 for rescue of rBDV, despite the trans-complementation of G in the rescue system. This result
422 also demonstrated that the linearization of L gene does not have any adversary effect on the
423 replication of rBDV. Previous studies revealed that the splice donor 2 (SD2; nt 2410) can
424 produce two introns, introns II and III, in the BDV genome using alternative splicing
425 machinery, which generate the mature L mRNA and predicted small ORFs, respectively (30).
426 Because the SD2 sequence was mutated in the ΔGLLP/M vector, alternative splicing using
427 intron III could be also defective in the recombinant viruses. Although control of the
428 alternative splicing between introns II and III during BDV replication has not been elucidated
429 yet, skipping over the splicing reactions of L transcripts might be likely to increase the
430 polymerase activity, leading to high efficiency of rBDV rescue. Along with the L_{RD} mutation,
431 the regulation of L splicing may be a key to improve the recovery rate of rBDV in the reverse
432 genetics system.

433 Our analysis using rBDV ΔGLLP/M-GFP-infected cells also provided an excellent
434 model for studying the release of infectious BDV and the virion formation in infected cells.
435 Previous studies using other mononegaviruses revealed that M and G proteins may be critical
436 for the assembly or budding of infectious virions in concert (11, 12, 28). In this study, we
437 demonstrated that the CT region of BDV G may be essential for the release of infectious
438 particles, using the rBDV ΔGLLP/M-GFP persistently infected cells. Our result showed that
439 an 8-amino acid stretch, ^{494}RRRRLGRW^{501}, in the CT region may play a critical role in the
440 production of infectious virions. Understanding the interaction between M and G of BDV

may provide valuable insights into, not only the improvement of the efficiency of infectious particle releases from BDV-infected cells, but also the generation of pseudotyped viruses using other virus envelope proteins. In fact, we could show that the chimeric VSV G protein harboring the CT region of BDV G facilitates the production of infectious particles in the supernatant of Vero-ΔGLLGFP cells (Fig. 5D). This result suggested that the function of the CT region of BDV G may be at least a necessary prerequisite, if not sufficient, to generate pseudotyped virions using the envelope proteins of other viruses. On the other hand, we could not obtain evidence that the chimeric RaV G having the BDV CT region produces infectious virions in the supernatant of the transfected cells. This may be due to the differences of the intracellular localization of the maturation and/or the budding of G proteins between BDV and RaV. Alternatively, in RaV infection the specific interaction of G with other viral components might be necessary for efficient virion assembly and release. The difference in efficiency of formation of pseudotyped virions by the source of the G protein may be a key to understand the life cycle of BDV, especially assembly and budding of virions.

In this study, we could not demonstrate the detailed characteristics of the infectious virions released from the Vero-ΔGLLGFP cells by the trans-complementation assays, because the amount of infectious particles released into the supernatants were still low even in the supernatant of cells transfected with wt BDV G. Therefore, further studies using more detailed recombinant constructs between VSV, RaV and BDV G will be required to confirm the release mechanism and the efficient production of the pseudotyped virions in the ΔGLLP/M system, and such experiments are now underway in our laboratory. The pseudotyped BDV may not only extend the application of BDV vectors to many cell types and tissues other than

463 the CNS but also contribute to the development of low- or non-pathological rBDV lacking the
464 immunogenicity of the envelope protein.

465 We investigated the potential of BDV as a virus vector using a novel BDV vector
466 system. Our results showed that the newly established BDV P/M vector may provide a good
467 system not only for efficient and safe gene delivery in vivo but also for basic research
468 regarding BDV replication and virion formation. Further improvements of the BDV P/M
469 vector system, including the reduction of possible pathogenesis and expansion of the capacity
470 for insertion of foreign genes, are necessary to establish a useful virus vector and are now in
471 progress.

472

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580 **Figure legends**

581

582 **Fig. 1. Efficient expression of GFP from an intercistronic P/M region of a BDV vector.**
583 (A) Schematic representation of a BDV vector construct harboring a GFP gene in an
584 intercistronic region between the P and M genes. The genetic organization of BDV and the
585 sequences surrounding the inserted transcription cassette are shown. The additional
586 transcription start and termination signals (S3 and T2) and restriction enzyme sites (*Bst* BI
587 and *Pac* I) are indicated. (B) Expression of GFP in rBDV P/M-GFP-infected Vero cells. The
588 cell nuclei are stained by DPAI (blue). Bar, 10 μm. (C) Growth kinetics of wt and
589 GFP-expressing rBDVs in Vero cells. The viral growth rate was monitored by IFA using
590 anti-N mAb. Symbols; rBDV wt (closed circle), rBDV P/M-GFP (closed square), rBDV
591 5'GFP (open triangle). (D) Comparison of the levels of GFP expression between rBDV
592 P/M-GFP and rBDV 5'GFP in Vero cells. The rBDV-infected Vero cells were detected by
593 anti-N mAb (red) and GFP fluorescence (green). Bar, 20 μm. (E) and (F) The expression
594 levels of GFP protein and mRNA were determined by western (E) and northern (F) blot
595 analyses, respectively. The antibodies (E) and riboprobes (F) used in the analyses are
596 indicated to the left. (G and H) The BDV-P/M vector has a great capacity for insertion of
597 various foreign genes of different lengths. (G) The Vero cells were infected with cell-free
598 rBDV P/M-Luc at an MOI of 1.0. The luciferase activity was detected in the infected cells as
599 early as 72 h after infection. Symbols; rBDV P/M-Luc (closed diamond), rBDV P/M-GFP
600 (open square). RLU, relative luciferase unit. (H) Stable expression of DsRed in the Vero cells
601 infected with rBDV P/M-DsRed at 20 days postinoculation. Bar, 10 μm.

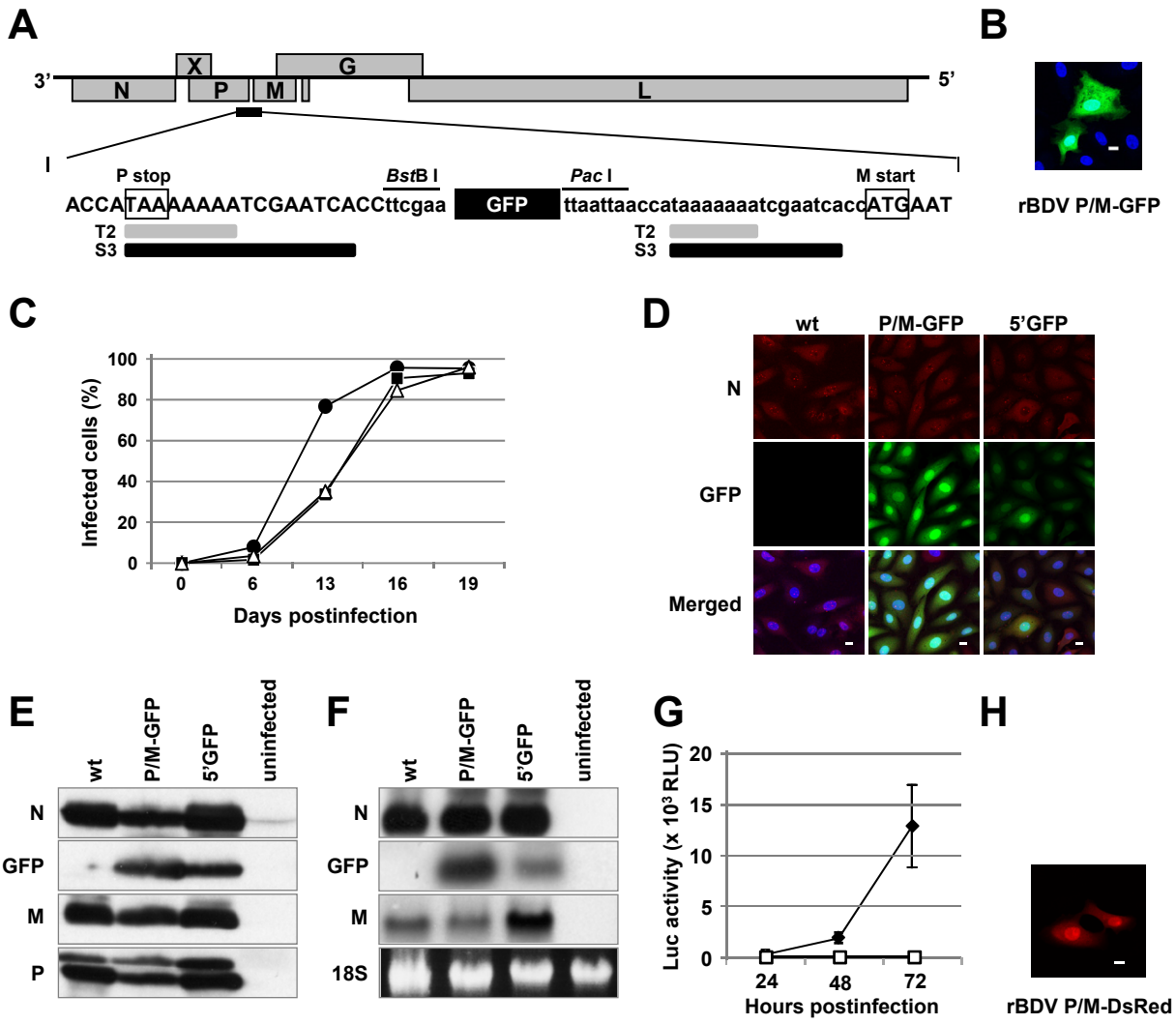
Fig. 2. rBDV P/M stably expresses GFP in rodent brains. (A) The rat brains infected with rBDV P/M-GFP exhibited a brighter GFP signal than those with rBDV 5'GFP at 2 weeks postinoculation. The GFP expression was monitored by fluorescence stereomicroscopy. (B) The GFP fluorescence was compared between rats infected with rBDV P/M-GFP and 5'GFP in the cerebrum cortex and cerebellum at 2 months postinoculation. The cryosections of the brain samples were reacted with anti-N mAb. Merged images also are shown with nuclear DAPI staining (blue). Bar, 50 μm. (C) A representative stereomicroscopic image of the cross section of the brain of 2-month-old C57BL/6J mice neonatally infected with rBDV P/M-GFP. Immunohistochemical staining of the cryostat sections was examined by fluorescence microscopy following staining with an antibody specific for BDV N. Bar, 100 μm. (D) GFP signals in the brain of an 8-month-old mouse neonatally inoculated with rBDV P/M-GFP. The stereomicroscopic image of the sagittal section and the cryostat section of the brain are shown. Bar, 50 μm.

Fig. 3. Construction and efficiency of rescue of the G-deficient BDV vectors. (A) Schematic representations of the ΔGP/M-GFP and ΔGLLP/M-GFP vectors. Arrows indicate the sites of the first two methionine codons that were mutated in the G-deficient vectors. The region of intron II (nt 2410 to 3703) also is shown. (B) Comparison of rBDV rescue efficiency between ΔGP/M-GFP, ΔGLLP/M-GFP and P/M-GFP vectors. The numbers of GFP-positive focuses were counted by fluorescence microscopy at 12 days after cocultivation. The data of ΔGP/M-GFP and ΔGLLP/M-GFP viruses represent triplicate experiments.

Fig. 4. The G-deficient rBDV establishes persistent infection in cultured cells. (A) Growth kinetics of ΔGLLP/M-GFP virus in Vero-BG and Vero cells. The cell-free virus was inoculated onto the cells at an MOI of 0.01. Infected cells were split every 3 days and GFP expression evaluated by fluorescent microscopy. The filled squares and open triangles indicate the infection rates of rBDVs in Vero-BG (left vertical axis) and Vero (right vertical axis) cells, respectively. (B) The fluorescence microscopic images of rBDV ΔGLLP/M-GFP-infected Vero-BG (left panel) and Vero (right panel) cells at 12 days postinfection. Cells were counterstained with DAPI. The cells were immunostained with anti-N mAb (red; insets). Merged images are shown. Bar, 50 μm (10 μm in insets). (C) Western blot analysis of ΔGLLP/M-GFP-infected Vero cells. The cloned Vero cells persistently infected with rBDV ΔGLLP/M-GFP were analyzed by western blotting using specific antibodies against BDV G (polyclonal) and N (monoclonal) and GFP (monoclonal). (D) The fluorescence microscopic images of rBDV ΔGLLP/M-GFP persistently infected OL cells. After the infection, the rBDV-infected cells were cloned by limiting dilution. The cells were immunostained with anti-N mAb (red). Cells were counterstained with DAPI. Merged images are shown. Bar, 20 μm. (E) Stable expression of LacZ in the Vero-BG cells infected with rBDV ΔGLLP/M-LacZ. Bar, 100 μm.

Fig. 5. Identification of the carboxyl-terminal region in the BDV G cytoplasmic tail necessary for the release of infectious particles. (A) Schematic representation of BDV G. The amino acid number of BDV G is indicated. SP, signal peptide; TM, transmembrane

646 domain; CT, cytoplasmic tail. (B) Diagram of a series of alanine substitution mutants of CT
647 region. The infectious titers in the supernatants of rBDV ΔGLLP/M-GFP-infected Vero cells
648 transfected with each construct were monitored by focus-forming assay at 48 h after infection
649 of the cells. The titers are shown to the right. N.D, not detected. (C) Schematic representation
650 of VSV, RaV G, and chimeric G constructs. Bct, CT region of BDV G. (D and E) The
651 infectious titers in the supernatants of the Vero-ΔGLLGFP cells transfected with G expression
652 plasmids. The titers were monitored by focus-forming assay at 48 h after inoculation in Vero
653 cells. ND, not detected. (E) VG-Bct and RV-Bct indicate chimeric VSV and RaV G constructs
654 of which CT region are exchanged with that of BDV G, respectively. Data represent triplicate
655 experiments.
656
657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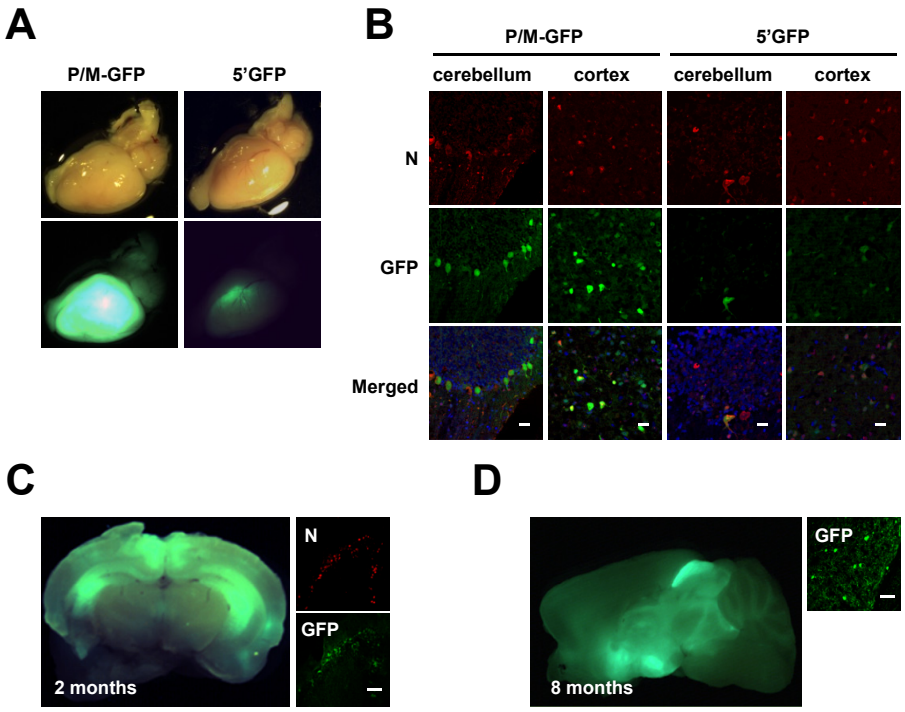
3'
X
G
N
P
M
L
5'
I
P stop
BstB I
Pac I
M start
ACCATAAAAAAATCGAATCACCttcgaa
GFP
ttaattaaccataaaaaaatcgaatcaccATGAAT
T2
S3
T2
S3
B
rBDV P/M-GFP
C
Infected cells (%)
100
80
60
40
20
0
0
6
13
16
19
Days postinfection
D
wt
P/M-GFP
5'GFP
N
GFP
Merged
E
wt
P/M-GFP
5'GFP
uninfected
N
GFP
M
P
F
wt
P/M-GFP
5'GFP
uninfected
N
GFP
M
18S
G
Luc activity (x 10³ RLU)
20
15
10
5
0
24
48
72
Hours postinfection
H
rBDV P/M-DsRed

Fig. 1


A
P/M-GFP
5'GFP
B
P/M-GFP
5'GFP
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cortex
cerebellum
cortex
N
GFP
Merged
C
N
GFP
2 months
D
GFP
8 months

Fig. 2

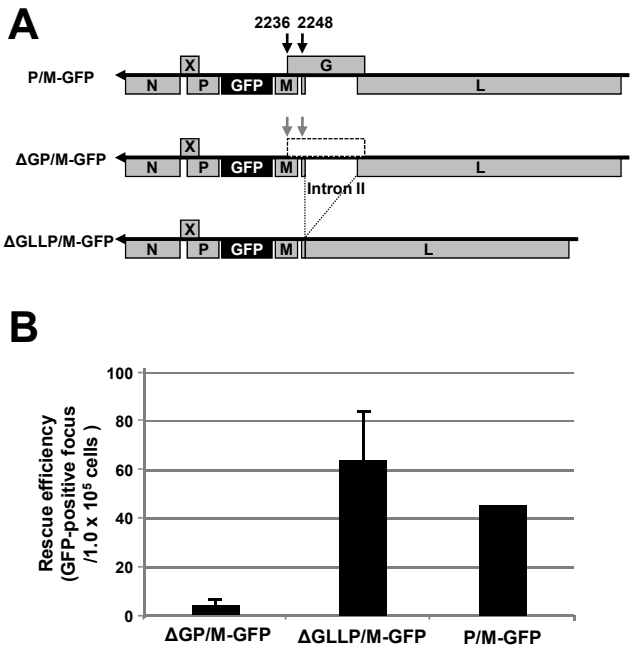
A
2236 2248
P/M-GFP
X
G
N
P
GFP
M
L
ΔGP/M-GFP
Intron II
ΔGLLP/M-GFP
B
Rescue efficiency
(GFP-positive focus
/1.0 x 10⁵ cells)
100
80
60
40
20
0
ΔGP/M-GFP
ΔGLLP/M-GFP
P/M-GFP

Fig. 3

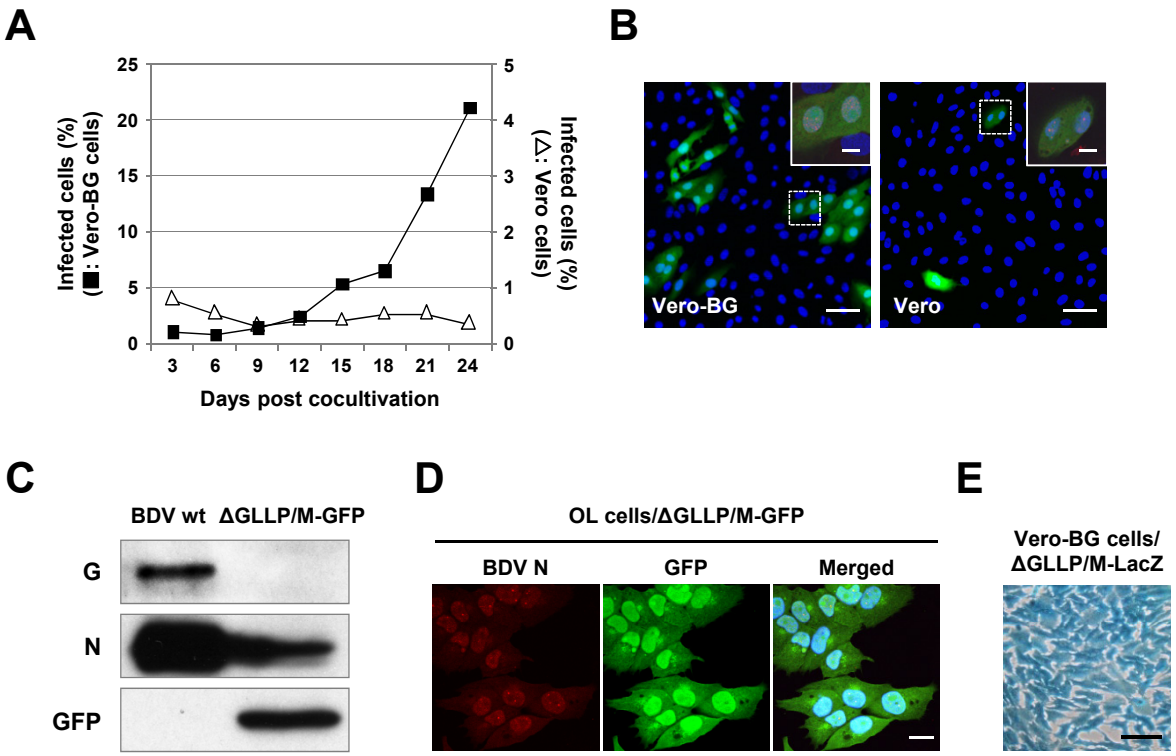
A
B
C
D
E
Infected cells (%)
(■: Vero-BG cells)
Infected cells (%)
(△: Vero cells)
Days post cocultivation
Vero-BG
Vero
BDV wt
ΔGLLP/M-GFP
G
N
GFP
OL cells/ΔGLLP/M-GFP
BDV N
GFP
Merged
Vero-BG cells/
ΔGLLP/M-LacZ

Fig. 4

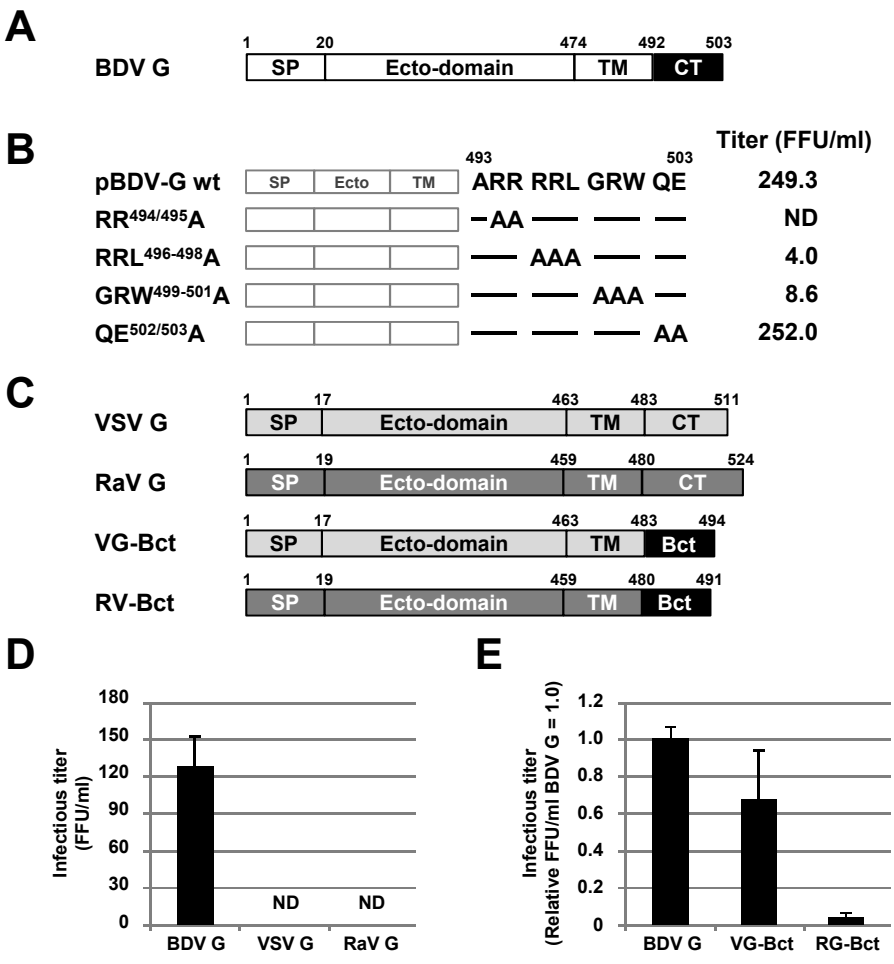
A
BDV G
1
20
474
492
503
SP
Ecto-domain
TM
CT
B
Titer (FFU/ml)
493
503
pBDV-G wt
SP
Ecto
TM
ARR RRL GRW QE
249.3
RR494/495A
AA
ND
RRL496-498A
AAA
4.0
GRW499-501A
AAA
8.6
QE502/503A
AA
252.0
C
VSV G
1
17
463
483
511
SP
Ecto-domain
TM
CT
RaV G
1
19
459
480
524
SP
Ecto-domain
TM
CT
VG-Bct
1
17
463
483
494
SP
Ecto-domain
TM
Bct
RV-Bct
1
19
459
480
491
SP
Ecto-domain
TM
Bct
D
Infectious titer (FFU/ml)
180
150
120
90
60
30
0
ND
ND
BDV G
VSV G
RaV G
E
Infectious titer (Relative FFU/ml BDV G = 1.0)
1.2
1.0
0.8
0.6
0.4
0.2
0
BDV G
VG-Bct
RG-Bct

Fig. 5