A Novel Borna Disease Virus Vector System That Stably Expresses Foreign Proteins from an Intercistronic Noncoding Region[▽]

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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 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 nonpropagating rBDV, ΔGLLP/M, which lacks the envelope glycoprotein (G) and a splicing intron within the polymerase gene (L), by the transcomplementation system with either transient or stable expression of the G gene. 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.

Borna disease virus (BDV) belongs to the *Bornaviridae* family within the nonsegmented, 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 an efficient and stable gene delivery system to the central nervous system (CNS).

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

In addition to the insertion sites of foreign gene cassettes in the genome, the flexibility of the virus genome to modify the efficiency of viral replication, as well as host cell tropism, also could be important in establishing a more effective and useful

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virus vector system. In many virus vectors, propagation-defective and pseudotyped viruses have been developed by the modification of viral genomes and deletion or mutation of viral genes (4, 5, 13, 35). In BDV, some point mutations, including in the phosphoprotein (P), X, and RNA polymerase (L) genes, have been shown to modify the efficiency of virus replication in cultured cells and animal brains (1, 21). For example, two amino acid substitutions, L1116R and N1398D, in the L gene (designated the L_{RD} mutation) are known to enhance significantly the polymerase activity as well as the replication kinetics of rBDV (1). However, there have been only a few studies on the modification of the viral genome to develop an efficient and safe BDV vector system.

In this study, we established a novel BDV vector, BDV P/M, which harbors an extra transcription unit in the short intercistronic noncoding region of the genome between the P and matrix (M) genes (P/M). BDV P/M was shown to stably express several reporter genes, including GFP, DsRed, and luciferase (Luc), in cultured cells without attenuation of their expression. Furthermore, we developed a nonpropagating rBDV, Δ GLLP/M, which lacks an envelope glycoprotein (G) gene and a splicing intron within the L gene, by the transcomplementation of the G protein. Interestingly, rBDV Δ GLLP/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 the BDV G protein may be essential for the release of infectious particles and the generation of pseudotyped BDV virions harboring the vesicular stomatitis virus (VSV) G protein by using the rBDV Δ GLLP/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 BDV strain He/80, was inserted into the XhoI and NotI sites of pCAG-HRSV3. A plasmid containing an extra transcription cassette, pFct-BDV P/M, was generated by insertion of transcription initiation (S3) and termination (T2) signal sequences in the P/M region with BstBI and PacI sites (Fig. 1A). The plasmid harboring the expression cassette in the 5' end of the genome, pFct-BDV 5', also was constructed using the BstBI and PacI restriction enzymes. The L_{RD} mutation (1) was introduced into the pFct-BDV P/M plasmid by PCR-based mutagenesis. To generate foreign gene expression vectors, reporter genes, including GFP, DsRed, luciferase, and LacZ, were amplified from pGFP-N1 (Clontech), pDsRed-monomer-C1 (Clontech), pGL3-promoter vector (Promega), and pcDNA 5/TO/lacZ (Invitrogen), respectively, and inserted into pFct-BDV P/M or pFct-BDV Δ GLLP/M vector with BstBI and PacI sites.

The pFct-BDV Δ GFP/M-GFP, in which two ATG codons (nucleotides [nt] 2236 and 2248) in the G open reading frame (ORF) were changed to ACG, was constructed from pFct-BDV P/M-GFP by PCR mutagenesis (see Fig. 3A). The intron II-deleted, L ORF-linearized BDV vector, pFct-BDV Δ GLLP/M-GFP, was developed by deleting the intron II sequence (nt 2410 to 3703) from pFct-BDV Δ GFP/M-GFP using a PCR amplification method (see Fig. 3A).

The expression vector pBDV-G was generated by subcloning the G ORF of strain He/80 into the KpnI and SmaI sites of plasmid pCXN2. For efficient expression of BDV G in transfected cells, silent mutations were introduced at the splicing donor (SD) and acceptor (SA) sequences in the G gene. The cDNA of VSV G was amplified from pVPack-VSV-G (Stratagene) and subcloned into the EcoRI and EcoRV sites of the pCXN2 vector to yield pVSV-G. The rabies virus (RaV) G expression plasmid, pRaV-G, was constructed by subcloning the Nishigahara strain G, which was kindly given by Naoto Ito (Gifu University, Japan), into the KpnI and EcoRV sites of the pCXN2 vector. Expression plasmids of chimeric G proteins of VSV and RaV, in which the CT regions were exchanged with the CT of BDV G, were constructed by PCR mutagenesis. Detailed information about the primers and PCR procedures used to generate these plasmids is available from the authors. Nucleotide sequences of the recombinant plasmids were confirmed by DNA sequencing.

Virus rescue. 293T cells were seeded in 35-mm dishes and transfected with 2 μ g of BDV vector plasmid, 0.25 μ g of pCA-N (34), 0.025 μ g of pCXN2-P (34), and 0.25 μ g of pCA-L (34) using Lipofectamine 2000 (Invitrogen). To rescue rBDV Δ GFP/M GFP and Δ GLLP/M-GFP, 0.1 μ g of pBDV-G was transfected together with the helper plasmids expressing BDV N, P, and L genes into 293T cells. At 3 days posttransfection, the cells were detached using trypsin and seeded onto 10-cm dishes. One day after the first passage, approximately 1×10^5 Vero or Vero-BG cells were cocultured with the transfected 293T cells. The cultures were split every 3 days, and the rescue efficacy of recombinant viruses was evaluated by fluorescence microscopy using an anti-BDV N monoclonal antibody (MAb) or by monitoring the expression of GFP. The nonpropagating rBDVs were rescued by transient transfection with expression plasmids encoding BDV, VSV, and RaV G and chimeric G proteins between these viruses. Vero cells stably infected with rBDV Δ GLLP/M-GFP in 35-mm dishes were transfected with 3 μ g of the G expression construct using Lipofectamine 2000 (Invitrogen), and the virus particles were collected from the culture supernatant or from sonicated cells at 48 h posttransfection.

Virus infection. Vero or Vero-BG cells were infected with the amounts of rBDV stock indicated in Results and in the figure legends at 37°C. After absorption for 1 h, the cells were washed with phosphate-buffered saline (PBS) and passaged within 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 1×10^5 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 brain or cross sections of the brain 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. (optimal cutting temperature) compound. Cryostat sections (4 μ m) 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 guidelines 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% CaCl₂, and 0.05 M Tris-HCl, pH 7.5) for 5 min at 37°C. 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 4°C and then with the secondary antibodies with PBS for 1 h at 37°C. The cells were counterstained with 4',6'-diamidino-2-phenylindole (DAPI).

Indirect immunofluorescence assay (IFA). Cells were fixed with 4% paraformaldehyde and permeabilized by PBS with 0.4% Triton-X. After permeabilization, the cells were incubated with the primary antibody, anti-BDV N MAb, for 1 h at 37°C. Subsequently, the cells were washed three times with PBS and incubated with secondary antibody, goat anti-mouse IgG antibody conjugated with Cy3 (Jackson Immuno Research), or goat anti-rabbit IgG antibody conjugated with Alexa Fluor 488 (Invitrogen) for 1 h at 37°C. The cells were counterstained with DAPI. After further washing with PBS, the cells were examined with a Ti-E inverted microscope with a C1 confocal laser scanning system (Nikon).

Luciferase and LacZ reporter assays. Vero cells were infected with rBDV P/M-luciferase in 16-mm culture dishes. At 24, 48, and 72 h postinfection, cells were lysed and subjected to a luciferase assay with Luciferase Cell Culture Lysis Reagent (Promega), according to the manufacturer's recommendations. The activity of β -galactosidase from the rBDV Δ GLLP/M-LacZ-infected Vero-BG cells was analyzed by a β -galactosidase staining kit (PanVera), according to the manufacturer's recommendations.

FIG. 1. Efficient expression of GFP from an intergenic P/M region of a BDV vector. (A) Schematic representation of a BDV vector construct harboring a GFP gene in an intergenic region between the P and M genes. The genetic organization of BDV and the sequences surrounding the inserted transcription cassette are shown. The additional transcription start and termination signals (S3 and T2) and restriction enzyme sites (BstBI and PacI) are indicated. The lowercase letters indicate the sequence of the inserted transcription cassette. (B) Expression of GFP in rBDV P/M-GFP-infected Vero cells. The cell nuclei are stained by DAPI (blue). Scale bar, 10 μ m. (C) Growth kinetics of wt and GFP-expressing rBDVs in Vero cells. The viral growth rate was monitored by IFA using anti-N MAb. Symbols: filled circle, rBDV wt; filled square, rBDV P/M-GFP; open triangle, rBDV 5'GFP. (D) Comparison of the levels of GFP expression between rBDV P/M-GFP and rBDV 5'GFP in Vero cells. The rBDV-infected Vero cells were detected by anti-N MAb (red) and GFP fluorescence (green). Scale bar, 20 μ m. (E and F) The expression levels of GFP protein and mRNA were determined by Western (E) and Northern (F) blot analyses. The antibodies (E) and riboprobes (F) used in the analyses are indicated to the left of the panels. (G and H) The BDV-P/M vector has a great capacity for insertion of various foreign genes of different lengths. The Vero cells were infected with cell-free rBDV P/M-Luc at an MOI of 1.0 (G). Luciferase activity was detected in the infected cells as early as 72 h after infection. Symbols: filled diamond, rBDV P/M-Luc; open square, rBDV P/M-GFP. RLU, relative luciferase units. (H) Stable expression of DsRed in Vero cells infected with rBDV P/M-DsRed at 20 days postinoculation. Scale bar, 10 μ m.

Western blot analysis. Vero cells infected with rBDV were lysed, and the proteins were separated by 12% SDS-PAGE, followed by transfer to Immobilon-P transfer membranes (Millipore). Membranes were then blocked with 5% skim milk in PBS and incubated with the primary antibodies (anti-BDV N and P MAbs and anti-BDV M and G polyclonal antibodies [pAb]). After three washes with 0.05% Tween 20 in PBS, horseradish peroxidase-conjugated secondary antibodies (Jackson Immuno Research) were applied for 1 h at 37°C. The membrane was washed three times, and bound antibodies were detected using an ECL Western blotting system (Amersham Pharmacia Biotech).

Northern blot analysis. Total RNA was extracted with TRIzol reagent (Invitrogen) from cells infected with rBDV. Aliquots of 1.0 μ g of total RNA were electrophoresed through a 1.0% agarose gel containing 2.2% formaldehyde and transferred onto nylon membranes (Roche) with 20 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0). After UV cross-linking, the membrane was prehybridized in hybridization solution for 30 min at 65°C. This step was followed by hybridization overnight with a digoxigenin (DIG)-labeled RNA

probe. The hybridized probe was detected with an alkaline phosphatase-conjugated anti-DIG Fab fragment (Roche).

RESULTS

Efficient expression of foreign genes from an intergenic region between the P and M genes of BDV. A previous study by Schneider et al. showed that replication-competent rBDV was not able to be rescued when an extra transcription unit was inserted into the P/M region of the BDV genome (26). To examine further the availability of the P/M region for expression of a foreign gene, we inserted a GFP cassette into the site as indicated in Fig. 1A. The nucleotide sequences of the cas-

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 infected with rBDV 5'GFP at 2 weeks postinoculation. GFP expression was monitored by fluorescence stereomicroscopy. (B) GFP fluorescence levels were compared between rats infected with rBDV P/M-GFP and 5'GFP in the cerebrum cortex and cerebellum at 2 months postinoculation. Cryosections of brain samples were reacted with anti-N Mab. Merged images are also shown with nuclear DAPI staining (blue). Scale bar, 50 μ m. (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. Scale bar, 100 μ m. (D) GFP signals in the brain of an 8-month-old mouse neonatally inoculated with rBDV P/M-GFP. Stereomicroscopic images of the sagittal section and the cryostat section of the brain are shown. Scale bar, 50 μ m.

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

At first, we infected Vero cells with cell-free rBDV at a multiplicity of infection (MOI) of 0.01, and the growth kinetics of rBDV P/M-GFP were compared to those of wild-type (wt) recombinant virus and rBDV 5'GFP. As shown in Fig. 1C, the growth kinetics of both GFP-expressing viruses seemed to be slightly retarded compared to the kinetics of wt rBDV, but they were propagated efficiently and reached over 90% infection by 3 weeks postinfection. We also performed IFA to investigate the expression of GFP in Vero cells. As shown in Fig. 1D, rBDV P/M-GFP induced an apparently stronger GFP signal than rBDV 5'GFP, while the expression levels of N were comparable among the various recombinant viruses. The Western

and Northern blot analyses confirmed higher expression of GFP from the P/M region than from the 5' end of the genome (Fig. 1E and F).

In addition to the GFP gene (720 bp), we could rescue rBDV P/M viruses harboring DsRed (678 bp) and luciferase (1,653 bp) in the P/M region, and we verified the stable expression of these genes in infected cells. Both the rescue efficiencies and growth kinetics of rBDV P/M-DsRed and -Luc were quite comparable to those of rBDV 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 1.0×10^3 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 fluorescent 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 the 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 in those infected with 5'GFP virus (Fig. 2B). Furthermore, the stable expression of GFP from the rBDV P/M-GFP virus was demonstrated in mouse brains (Fig. 2C). The neurons in the hippocampus region of infected mice showed 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 a foreign gene in the brain.

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 reverse transcription-PCR (RT-PCR) whether the sequences of the newly introduced transcriptional cassette are stable during the replication of the rBDV 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 the infection of mouse brains (data not shown), suggesting that the additional transcriptional unit and its surrounding sequences are fairly stable during the multiplication of the rBDV 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 a 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, Δ GP/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, Δ GLLP/M (Fig. 3A), with the idea that the deletion of the intron in L mRNA may not only lead to the efficient production of L during the replication but also increase the capacity for insertion of foreign genes into the genome. 293T cells were transfected with the resultant plasmid, pFct-BDV Δ GP/M-GFP or pFct-BDV Δ GLLP/M-GFP, together with three helper plasmids (for N, P, and L expression) and the G expression plasmid, and then cocultured with Vero cells stably expressing BDV G (Vero-BG). At first, the rescue efficiencies were compared between the P/M-GFP, Δ GP/M-GFP, and Δ GLLP/M-GFP viruses at 12 days after cocultivation. As shown in Fig. 3B, the cells transfected with pFct-BDV Δ GLLP/M-GFP yielded a much higher level of recombinant virus than those transfected with pFct-BDV Δ GP/M-GFP. Furthermore, although the numbers of transfected plasmids, as well as the cells used for the rescue of recombinant viruses, were different between Δ GLLP/M-GFP and P/M-GFP viruses, the rescue efficiency of the L-linearized construct was comparable to or more efficient than that of the wt P/M-GFP

FIG. 3. Construction and efficiency of rescue of the G-deficient BDV vectors. (A) Schematic representations of the Δ GP/M-GFP and Δ GLLP/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) is also shown. (B) Comparison of rBDV rescue efficiencies between Δ GP/M-GFP, Δ GLLP/M-GFP, and P/M-GFP vectors. The numbers of GFP-positive foci were counted by fluorescence microscopy at 12 days after cocultivation. The data of Δ GP/M-GFP and Δ GLLP/M-GFP viruses represent triplicate experiments.

vector, suggesting that the linearized, intronless L gene enables efficient replication of rBDV.

To examine the cell-free infectivity and stability of the Δ GLLP/M virus, the cell-free virus of rBDV Δ GLLP/M-GFP-infected Vero-BG cells was inoculated into either Vero-BG or parental Vero cells at an MOI of 0.01, and the growth kinetics of the viruses were monitored by IFA. Although the infection rate of the Δ GLLP/M-GFP virus gradually increased in Vero-BG cells, the parental Vero cells did not support the propagation of the Δ GLLP/M-GFP virus (Fig. 4A). Interestingly, however, the infection by rBDV Δ GLLP/M-GFP was stably maintained during the observation period of 24 days (Fig. 4A and B). The Vero cells persistently infected with Δ GLLP/M-GFP virus exhibited the nuclear dot structure specific for BDV replication. (Fig. 4B, insets). Western blotting confirmed the lack of G gene expression in rBDV Δ GLLP/M-GFP-infected Vero cells (Fig. 4C). The persistent infection of Δ GLLP/M-GFP virus without propagation was also observed in human-derived oligodendrogloma (OL) cells (Fig. 4D). On the other hand, we investigated whether the lack of G expression and the deletion of an intron within the L gene increased the capacity of Δ GLLP/M vector to insert a foreign gene into the P/M region. As shown in Fig. 4E, the rBDV Δ GLLP/M virus harboring the LacZ gene (3,060 bp) was successfully rescued and established persistent infection in Vero-BG cells. All of these observations demonstrate that G is essential for virus propagation but not for the maintenance of persistent infection of BDV and suggest that rBDV Δ GLLP/M is a

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 was evaluated by fluorescence 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) Fluorescent 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. Scale bar, 50 μm (10 μm in insets). (C) Western blot analysis of ΔGLLP/M-GFP-infected Vero cells. 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 against GFP (monoclonal). (D) Fluorescent microscopic images of OL cells persistently infected with rBDV ΔGLLP/M-GFP. 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. Scale bar, 20 μm. (E) Stable expression of LacZ in Vero-BG cells infected with rBDV ΔGLLP/M-LacZ. Scale bar, 100 μm.

nonpropagating virus, which may provide safe, stable, and high-capacity foreign gene delivery *in vivo*.

The ΔGLLP/M vector shows that the carboxyl-terminal region in the BDV G protein is essential for release of infectious particles. In many virus vector systems, pseudotyping of the virus envelope protein may be an effective strategy to expand host cell tropism. Furthermore, improvement of the efficacy of virus particle release from producer cells could be necessary to generate a high-titer virus stock. Therefore, understanding the mechanisms of the release of BDV infectious particles, as well as the envelopment by BDV G, may be crucial for the development of further applications of the BDV P/M vector system. As a first step toward these goals, therefore, we tried to determine the region of BDV G essential for the release of infectious particles by using the ΔGLLP/M-GFP system. Previous studies clearly revealed that the CT region of the mononegavirus G glycoprotein may play a key role in assembly of infectious virus particles in concert with M (11, 12, 28). We thus generated a series of alanine substitution mutants in the CT region of BDV G (Fig. 5B) and transfected the expression plasmids into a Vero cell line persistently infected with ΔGLLP/M-GFP virus, Vero-ΔGLLGFP. 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 replaced by alanines, 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.

Finally, we 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, infectivity was not detected in the supernatants of either VSV G- or RaV G-transfected cells. Interestingly, however, the chimeric VSV G, but not the chimeric RaV G, in which the CT regions are replaced with the CT of BDV G (Fig. 5C), was shown to produce infectious particles in the culture supernatant of 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 of 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.

DISCUSSION

In this study, we showed that foreign genes can be stably expressed from an intercistronic region of the BDV genome in

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 numbering of BDV G is indicated. SP, signal peptide; TM, transmembrane domain; CT, cytoplasmic tail. (B) Diagram of a series of alanine substitution mutants of the CT region. The infectious titers in the supernatants of rBDV Δ GLLP/M-GFP-infected Vero cells transfected with each construct were monitored by focus-forming assay at 48 h after infection of the cells. The titers are shown to the right. ND, not detected. (C) Schematic representation of VSV G, RaV G, and chimeric G constructs. VG-Bct, VSV G protein harboring the CT region of BDV G (Bct); RV-Bct, RaV G protein harboring Bct. (D and E) The infectious titers in the supernatants of the Vero- Δ GLLP/M cells transfected with G expression plasmids. The titers were monitored by focus-forming assay at 48 h after inoculation in Vero cells. ND, not detected. (E) Comparison of the infectious titers of BDV G, VG-Bct, and RV-Bct viruses. Data represent triplicate experiments.

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

Recently, Ackermann et al. reported that GFP expression from the rBDV 5'GFP construct is reduced markedly in mouse brains from 2 months postinfection, probably because of mutations in the termination/initiation signals upstream of the inserted GFP gene (2). This observation suggests that expres-

sion of an additional gene from the 5' end of the BDV genome may have deleterious effects on virus replication, leading to attenuation of transcription of the inserted gene. The additional transcription initiation signal downstream of the L gene may affect the efficiency of the termination of transcription of L. Alternatively, the additional sequences may disrupt structures at the end of the BDV genome, such as the ITR and panhandle structures, important for the initiation of RNA replication and formation of the 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 over an observation period of at least 8 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 foreign genes of various lengths, at least below 3,060 bp, in the Δ GLLP/M vector. All of these observations indicated that the 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 reasons. 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 1,293 nt for expression of the downstream L gene (7, 31, 32). The G precursor protein, GP, plays a key role in BDV entry into susceptible cells, and the GP1, the N-terminal subunit of GP, is known to be required for receptor recognition and virus entry (14, 15, 16, 19, 22). Previous studies suggested that the expression and correct processing of BDV GP are necessary for BDV dissemination in primary cultures of neurons and that neutralizing antibodies against BDV GP completely inhibited virus spread (3). These observations suggested that the enveloped viral particles, not nonenveloped viral RNPs, are crucial for dissemination of BDV between cultured cells (3), as in the case of other mononegaviruses, such as RaV (10). On the other hand, in an independent system, it was reported that cell-to-cell spread of BDV requires neither the expression of the primary receptor nor the processing of BDV GP (6). These observations showed the complexity of the roles of G in BDV infectivity and propagation in neurons. In this study, we mutated the first two methionines and an intron (intron II) for the L gene in the G ORF of the P/M-GFP vector to generate rBDV lacking the G envelope protein, Δ GLLP/M virus. The

transiently enveloped rBDV from G-expressing Vero cells showed cell-free infectivity and established persistent infection of Vero cells in the absence of G expression. Interestingly, the infection seemed to be maintained without spreading in the cultured cells, suggesting that BDV G is required not for the establishment of persistent infection in a single cell but, rather, for cell-to-cell spread. Our result revealed that persistent infection by the Δ GLLP/M-GFP virus can be maintained for at least 2 months without losing the expression of GFP (data not shown), demonstrating that the Δ GLLP/M system may provide a promising BDV vector for *in vivo* use with safe and persistent foreign gene expression in the infected cells.

The Δ GLLP/M virus provided an intriguing finding that intron II of the L gene may negatively regulate the expression of L polymerase because deletion of intron II markedly enhances the efficiency of rescue of the rBDV compared with the Δ GP/M virus. In addition, the L-linearized construct seemed to be more efficient than the wt P/M-GFP vector for rescue of rBDV, despite the transcomplementation of G in the rescue system. This result also demonstrated that the linearization of the L gene does not have any adverse effect on the replication of rBDV. Previous studies revealed that splice donor 2 (SD2; nt 2410) can produce two introns, introns II and III, in the BDV genome using alternative splicing machinery, and these generate the mature L mRNA and predicted small ORFs, respectively (30). Because the SD2 sequence was mutated in the Δ GLLP/M vector, alternative splicing using intron III could also be defective in the recombinant viruses. Although control of the alternative splicing between introns II and III during BDV replication has not been elucidated yet, skipping over the splicing reactions of L transcripts might be likely to increase the polymerase activity, leading to high efficiency of rBDV rescue. Along with the L_{RD} mutation, the regulation of L splicing may be a key to improving the recovery rate of rBDV in the reverse genetics system.

Our analysis using rBDV Δ GLLP/M-GFP-infected cells also provided an excellent model for studying the release of infectious BDV and virion formation in infected cells. Previous studies using other mononegaviruses revealed that the M and G proteins may be critical for assembly or budding of infectious virions in concert (11, 12, 28). In this study, we demonstrated that the CT region of BDV G may be essential for the release of infectious particles using cells persistently infected with rBDV Δ GLLP/M-GFP. Our results showed that an 8-amino-acid stretch, ⁴⁹⁴RRRRLGRW⁵⁰¹, in the CT region may play a critical role in the 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- Δ GLLGFP cells (Fig. 5D). This result suggested that the function of the CT region of BDV G may be at least a necessary, if not sufficient, prerequisite 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 efficiencies of formation of pseudotyped virions according to the source of the G protein may be a key to understanding 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- Δ GLLGFP cells by the transcomplementation assays because the amount of infectious particles released into the supernatants was still small even in the supernatant of cells transfected with wt BDV G. Therefore, further studies using more detailed recombinant constructs from VSV, RaV, and BDV G proteins will be required to confirm the release mechanism and the efficient production of the pseudotyped virions in the Δ GLLP/M system, and such experiments are now under way in our laboratory. The pseudotyped BDV may not only extend the application of BDV vectors to many cell types and tissues other than the CNS but also contribute to the development of low invasive or nonpathological rBDV lacking the immunogenicity of the envelope protein.

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

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