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Review

Molecular and cellular biology of Borna disease virus infection

Keizo Tomonaga *, Takeshi Kobayashi, Kazuyoshi Ikuta

Department of Virology, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565-0871, Japan

Abstract

Borna disease virus (BDV) is a noncytolytic, neurotropic RNA virus that causes neurobehavioral disorders in a wide variety of warm-blooded animals. Recent evidence has revealed that BDV uses a unique strategy in its transcription and replication and directly affects cellular functions of infected central nervous systems. BDV research will provide new insights not only into the biology of neurotropic RNA virus but also into neuropsychiatry.

Keywords: Borna disease virus; Persistent infection; Neurobehavioral diseases

1. Introduction

Borna disease (BD) is a severe, frequently fatal, neurological disease characterized by a progressive, nonpurulent encephalomyelitis in horses and sheep. Although BD was originally described only in the endemic areas including southern Germany and Switzerland, BD horses have been recently found in Japan [1–3]. The causative agent, Borna disease virus (BDV), is a nonsegmented, negative-sense, single-strand (NNS) RNA virus that belongs to the *Mononegavirales* [4]. BDV is highly neurotropic and has a noncytolytic strategy for replication in cells. Despite the similarity in genome organization to other members of this order, BDV replicates and transcribes in the nucleus of infected cells. While nuclear replication and transcription are also found in nucleorhabdovirus (NNS RNA virus of plants), BDV is the only known animal NNS virus employing this property. Based on its unique features, BDV has been classified into a new family, *Bornaviridae*.

BDV can experimentally infect many vertebrates from rabbits and monkeys to chickens, and causes neurological symptoms. Epidemiological studies have demonstrated that natural infection of BDV has been found in a wide variety of hosts. Symptomatic natural infection of BDV with fatal outcome has been reported in cats, cattle, dogs and rabbits [2]. BDV infection was also demonstrated by serological study in ostriches with a paresis. Furthermore, asymptomatic natural infection in various animal species has been documented worldwide. Studies indicate that the host range of BDV probably includes all warm-blooded animals [2].

In the past two decades, research interest in BDV in humans has increased, because it has been claimed that neurological and emotional disturbances exhibited by BDV-infected animals resemble those in humans often to a remarkable degree. It was speculated that BDV infection might be related to some neuropsychiatric disorders such as affective disorders or psychosis. In 1985, the first serological evidence for BDV infection in humans was reported. Up to present, numerous studies have documented that BDV can infect humans. Furthermore, isolation of human BDV from the peripheral blood or brain of neuropsychiatric patients has been reported from several countries [5–7]. These studies strongly indicated that BDV is a human pathogen and raised the question of a possible link between BDV and certain human mental disorders. However, some scientists believe there is a substantial risk of contamination of human samples by laboratory BDV strains, making the establishment of any relationship between human BDV and psychiatric disorders highly controversial.

The study of BD and BDV has a broad impact on diverse areas of biology and medicine, notably on molecular genetics of RNA viruses, neurobiology, biological psychiatry, and public health. In this review, we discuss recent findings concerning the molecular and cellular biology of BDV.

2. Molecular biology of BDV

2.1. Genomic organization

The cloning and sequencing of two BDV isolates from horses has demonstrated that the genome of BDV is about 8.9 kb in length with complementary termini similar to

* Corresponding author: Tel.: +81-6-6879-8308; fax: +81-6-6879-8310.
E-mail address: tomonaga@biken.osaka-u.ac.jp (K. Tomonaga).

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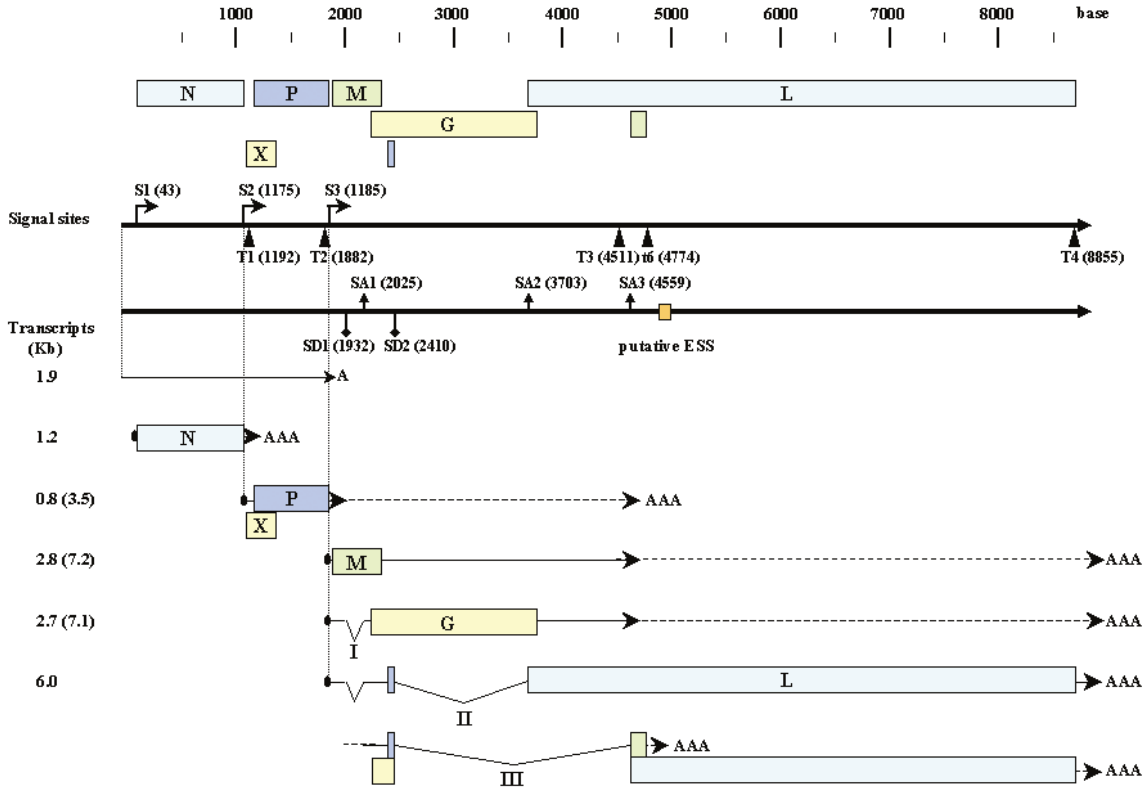

Fig. 1. Genomic organization and transcription map of BDV. N, nucleoprotein p38/p40N; X, protein X p10; P, phosphoprotein p24/p16; M, matrix protein gp18; G, envelope glycoprotein gp94; L, polymerase protein p190; S1-S3, transcription initiation sites; T1-T4 and t6, polyadenylation/termination sites; SA1-SA3, splice acceptor sites, SD1 and SD2, splice donor sites; ESS, exon splicing suppressor. The positions of the sites are given for the antigenome in parentheses. Positions of the introns (I, II, III) are also indicated.

those of other NNS RNA viruses (Fig. 1) [8,9]. The BDV antigenome consists of at least six open reading frames (ORFs). The ORFs encode nucleoprotein p40/p38 (N), phosphoprotein p24/p16 (P), matrix protein gp18 (M), envelope protein gp94 (G), and a predicted RNA-dependent RNA polymerase p190 (L) in 5' to 3' order. In addition, a small ORF X, which overlaps the P ORF, was identified as encoding a protein p10 (X) (Fig. 1).

The 3' terminal sequence of the BDV genome had a high content of adenine and uridine residues and is likely to contain promoters that are necessary for both transcription and replication of the virus, while the 5' terminal genomic sequence must contain promoter only for the replication, which functions as an antigenomic sense. Similar to other families of *Mononegavirales*, comparison of the 3' and 5' termini of BDV genomic RNA revealed complementarity [8,9].

The genomes of *Mononegavirales* contain signal sequences that regulate transcriptional initiation and polyadenylation/termination (Pt) by the viral polymerase in the noncoding intergenic regions within the genomes. Molecular biological analysis of BDV transcripts clearly demonstrated that BDV has at least three uracil-rich initiation (S1, S2 and S3) and five Pt signals (T1, T2, T3, T4 and t6). The initiation signals contain a semi-conserved U-rich motif that is not found in the other NNS RNA virus initiation signals, while Pt motifs in the BDV genome are similar to those in other NNS RNA viruses (Fig. 1). Intriguingly, the BDV genome has unusual positioning of the signal sequences at the gene junctions (Fig. 1). The signal sequences of BDV cannot be clearly divided into transcription termination signal, intergenic and transcription initiation signal regions. The transcription initiation signal (S2) for the second transcription unit (0.8-kb RNA) is located 18 nucleotides upstream of the Pt site (T1) of the first transcription unit (1.2-kb RNA) (Fig. 1). Furthermore, the second Pt signal (T2) is fully contained within the third initiation signal (S3). A similar organization is also found in other NNS RNA viruses, such as respiratory syncytial virus (RSV), where the start signal for the polymerase (L) gene is located 68 nucleotides upstream of the Pt signal of the M2 gene [10]. In RSV, to access the L start signal, the polymerase must first transcribe the M2 gene in its entirety and arrive at the M2 Pt signal [11]. Then, it seems to backtrack to the L start signal. In addition, it has been also demonstrated that approximately 90% of the RSV L gene

transcripts terminate at the M2 Pt signal to produce short mRNAs, and the synthesis of full-length L mRNA thus depends on transcriptional readthrough [11]. In BDV, it has not been determined whether the overlap of transcriptional signals has significant impacts on the transcription of the respective genes.

Unlike other NNS RNA viruses, BDV employs the RNA splicing machinery for gene expression. The genome contains two splice donor (SD1 and SD2) and three splice acceptor (SA1, SA2 and SA3) sites (Fig. 1) [12–15]. The sequence motifs of the splice sites are consistent with the mammalian splice site consensus sequences, and splicing yields at least three introns (intron I, II and III) in the BDV genome (Fig. 1).

Genome stability is another striking characteristic of the BDV genome. Two complete genomic sequences of BDV from horses showed more than 95% homology [4]. The sequence variations between human BDV isolates previously reported were also limited on the degrees of 3-4% at the nucleotide level [5–7,16]. Furthermore, partial sequences obtained from different BDV-infected animal species in the field, as well as from human cases of BDV infection, are also very similar to each other. In fact, a human BDV isolate from the brain of a schizophrenic patient obtained at autopsy in Japan showed a high degree of homology in the complete genomic sequence with the horse-derived isolates (unpublished data). Recently, an isolate the sequence of which differs from all other BDV previously reported has been demonstrated in an Austrian horse [17]. The strain differs from all other strains by more than 15% at the nucleotide level. This isolate might provide a better understanding of the evolution and extremely high degree of genetic stability of BDV in the field. We have to determine whether this subtype of BDV exists in other countries or animals throughout the world.

2.2. BDV proteins

2.2.1. Nucleoprotein

The first ORF of the BDV antigenome encodes the viral nucleoprotein, N. Previous studies indicated that N protein is critical for nuclear targeting of the BDV ribonucleoprotein (RNP) complex [18], because N binds to other viral proteins and is probably an essential component of the viral RNPs, although interaction between N and the viral genome has not yet been directly demonstrated. The BDV N protein has two isoforms with molecular weight of 40 and 38 kDa (p40N and p38N), of which the translation start sites are separated by a 13-amino acid stretch [19]. Although functional differences between the roles of p40N and p38N in the viral life cycle are unclear, p38N lacks the nuclear localization signal (NLS), which is located in the N-terminal of p40N, P_3**KRRL**VDDA$_{11}$ [18]. Both N proteins are, however, found in the nucleus of infected cells, suggesting that p38N travels into the nucleus mediated by interaction by other viral proteins.

Recently, we demonstrated that BDV N protein also has nuclear export activity, which is mediated by an internal nuclear export signal (NES) with the canonical leucine-rich motif, **L**$_{128}$TE**L**E**I**SS**I**FSHCC$_{141}$ [20]. Interestingly, the region of N NES overlaps the site of interaction with P protein. The nuclear export activity of a p38N is blocked by coexpression of P protein. These results suggested that BDV N protein must play a critical role in the nucleocytoplasmic transport of BDV RNP complexes in combination with other viral proteins such as P. A possible role of N protein in nucleocytoplasmic shuttling of viral RNPs is discussed below in detail.

2.2.2. X protein

The smallest transcript of BDV, as mRNA of 0.8 kb, is bicistronic and encodes ORFs for 10-kDa, X and P proteins. The ORF of X starts 49 nucleotides upstream from P ORF and overlaps with the 71 N-terminal amino acid of P protein in a different frame. Anti-X antibody demonstrated that the X protein is located in the nucleus in infected cells but shows cytoplasmic localization in the absence of other viral proteins. As the X protein has been shown to bind directly or indirectly to P and N [21,22], these viral proteins would promote nuclear targeting of X in infected cells. The function of the BDV X protein is presently unknown. However, interaction with other viral proteins indicated that X might play a role as cofactor for the viral polymerase. The N-terminal of X was recently shown to be critical for interaction with P and to contain a consensus leucine-rich sequence, D$_4$**L**R**L**T**LL**E**L**VRR**L**N$_{17}$, which is very similar to NES found in other viral proteins such as Rev of HIV-1 and Rex of HTLV-1. The nuclear export activity has not, however, been clearly determined in the region of the X protein [22]. These reports demonstrated that the nuclear export activity of the X protein is inefficient in transiently transfected cells and also showed that the mutant X protein lacking the NES-like sequence shows a subcellular distribution similar to the wild-type X protein. We have recently shown that X protein is phosphorylated in infected cells as well as in cells transfected with X cDNA (unpublished data). Therefore, some modification of the protein such as phosphorylation may be involved in the full activity of nuclear export of the BDV X protein.

2.2.3. Phosphoprotein

The second transcription unit (0.8-kb mRNA) of BDV also encodes a 24-kDa phosphoprotein, P. The phosphoproteins of other NNS RNA viruses are essential cofactors for viral polymerase. Although there is no direct evidence concerning the function of the BDV P protein in infected cells, it is postulated that the BDV P protein has similar roles to other NNS RNA viral phosphoproteins in the life cycle of the virus. The BDV P protein has nuclear localization activity, which is mediated by a bipartite NLS within the sequence. The NLS contains unique proline-rich motifs in both amino, **P**$_{29}$R**P**RKI**P**R$_{36}$, and carboxyl,

P$_{181}$**P**RIY**P**QL**P**SA**P**T$_{193}$, termini [23]. Mutational analysis of the signals revealed that the proline residues are critical for nuclear localization activity of the P protein [23]. Furthermore, previous studies demonstrated that P interacts with itself, N and X proteins and colocalizes with the proteins in the nuclei of infected cells.

We have recently shown that the 0.8-kb mRNA also produces a 16-kDa protein, named P', in addition to P and X proteins [24].P' was detected by anti-P monoclonal antibody and was shown to exist in BDV-infected cell lines as well as infected animal brains. Transient expression analysis of mutated cDNA clones encoding the BDV 0.8-kb mRNA revealed that the 16-kDa protein is initiated at the second AUG codon on the same ORF of P protein. These observations demonstrated the presence of three functional AUG codons in the smallest mRNA of BDV and also suggested that a leaky scanning mechanism is involved in translational initiation at downstream AUG codons of the bicistronic mRNA of BDV [24]. Furthermore, P' was found in the BDV-specific nuclear foci and was associated with the other viral proteins in BDV-infected cells. Interestingly, the P' protein lacks two possible phosphorylation sites for protein kinase Cε (PKCε) (Ser$_{26}$ and Ser$_{28}$) but not the sites for casein kinase II (CKII) (Ser$_{70}$ and Ser$_{86}$) [25], raising a possibility that P' might have different roles from P in infected cells.

2.2.4. Matrix protein

The BDV M is a predicted matrix protein of M_r 18,000 (gp18), which translated from the transcripts initiated at the S3 site (Fig. 1). In general, matrix proteins of NNS RNA viruses are associated with the inner layer of the plasma membranes and play roles in virion assembly and budding. Although M proteins in other *Mononegavirales* are not known to be glycosylated, lectin binding and endoglycosidase sensitivity assays indicated that the BDV M protein is likely to be an unusual complex-type N-linked glycoprotein [26]. Very recently, however, a contrary finding has been reported. Kraus et al. carefully demonstrated that BDV M is not glycosylated and located at the inner layer of the viral membrane, as is the case of homologous proteins of other members of NNS RNA viruses [27]. Previous studies indicated that the BDV M protein forms stable tetramers in vivo and has strong hydrophobic sequences, which might be involved in membrane spanning of this protein [28]. It has been demonstrated that anti-sera against the M protein have neutralizing activity and that BDV infection in cultured cells is prevented in the presence of the M protein [26,29,30]. These observations suggested that BDV M might be present at the surface of the virion envelope. On the other hand, however, it is also possible that neutralizing activity of the anti-sera may be directed against M-coated nucleocapsid complexes, which could be transmitted to adjacent cells in a cell-to-cell manner, rather than complete virions [31]. The cell-to-cell spread of infectious particles was also reported in other NNS RNA viruses such as measles virus [32].

In rhabdovirus, it was reported that the M protein is associated with viral RNPs and inhibits viral transcription [33]. Although there is no direct evidence about interaction between BDV RNPs and the M protein, localization of the M in the nucleolus might suggest a possible role of the BDV M protein in viral replication in infected cells.

2.2.5. Envelope protein

The transcripts started at the S3 transcription initiation site also included an ORF predicted to encode polypeptide of 56 kDa, G. Sequencing analysis and immune-electron microscopy study suggested that this protein is a BDV envelope glycoprotein [34]. Although BDV G may be expressed from unspliced transcripts by leaky ribosomal scanning, in vitro translation analysis demonstrated that intron I-spliced transcripts are more efficient to express G protein and that a 13-amino acid minicistron, which is produced by the intron I-splicing, enhanced the translation of G ORF [35]. The 56-kDa protein yields 94-kDa N-linked glycoprotein (gp94) with extensive high mannose- and/or hybrid-type oligosaccharide modifications. The unglycosylated and glycosylated proteins are proteolytically cleaved by the subtilisin-like protease furin and produce a molecule of 43 kDa (gp43), which corresponds to its C terminus [36,37]. Endoglycosidase studies demonstrated that the glycosylation process is required for stabilization of the protein. Subcellular localization studies demonstrated that gp94 accumulates in the endoplasmic reticulum, whereas gp43 reaches the cell surface [36]. Furthermore, both products were found to be associated with infectious virions. The presence of neutralization epitopes on the G protein and its capacity to interfere with infectivity suggested that the G protein is important for viral entry [36,38]. Previous study demonstrated that the terminal carbohydrate residues in the G protein are essential for the neutralization of the virion [30]. These features suggested that BDV gp94 is involved in attachment to the cell surface receptor, whereas furin-cleaved gp43 is involved in pH-dependent fusion after internalization of the virion by endocytosis [37,39]. Very recently, it has been demonstrated that a pseudotype vesicular stomatitis virus expressing the N terminus of the BDV G protein was neutralized by BDV-specific antiserum [40]. This observation suggested that the N terminus of the BDV G is sufficient for receptor recognition and virus entry into the cells.

2.2.6. Polymerase protein

From its position and sequence homology with other NNS RNA viral polymerases, the most 3' ORF in the BDV antigenome is predicted to encode the viral RNA-dependent RNA polymerase, L. Sequence analysis demonstrated that the putative catalytic domains found in the L proteins of NNS RNA viruses were well conserved in BDV L [8,9]. Expression of the L protein from the third transcription units

is dependent on a splicing event that fuses a small upstream ORF that overlaps with the 5' end of the G ORF [41] (Fig. 1). The protein is detected by serum antibodies from infected rats and is present in the nucleus, where it colocalizes with P [41]. BDV L was also shown to be phosphorylated by cellular kinases and to interact with the P protein. The intracellular movement or functional mechanism of action of BDV L has not yet been determined. Detailed analysis of the L protein is necessary for a better understanding of the biology of BDV and for generation of a reverse-genetic system for this virus.

2.3. Replication of BDV

2.3.1. Replication/Transcription

The BDV has unique properties in replication and transcription of its genome; BDV is the only known animal NNS RNA virus that replicates and transcribes in the nucleus of infected cells, whereas the other animal viruses of this group undergo their life cycle in the cell cytoplasm. It has been proposed that the nucleolus is the site of transcription and replication of BDV RNP complexes [31].

Polyadenylation and capping structures are found in the transcripts of BDV but not in the genome and antigenome RNAs (Fig. 1) [8,9]. It is unlikely that the polymerase of BDV employs a primer for RNA initiation. The BDV polymerase recognizes transcription start signals within the genome. The start signals contain a unique motif that is not found in other NNS RNA viruses. In contrast, Pt motifs of BDV are very similar to those in other *Mononegavirales*. The molar abundance of each BDV transcript in infected cells is considered to resemble the 3'-5' declining transcriptional gradient of other NNS viruses. It is also possible, however, that the transcriptional abundance of each transcript of BDV might be affected by the unusual positioning of the transcription signals within the genome as mentioned above. BDV produces several polycistronic transcripts. Among six different proteins of BDV, only N is translated from monocistronic mRNA, while transcripts of other viral proteins are polycistronic (Fig. 1). Transcriptional readthrough of the Pt signal is also a unique strategy for BDV transcription. The readthrough of T3 and t6 signals within the genome is essential for the expression of the L protein. Recently it has been demonstrated that readthrough of t6 may have an initial role in regulation of intron III splicing [15].

In other *Mononegavirales*, the ratio or abundance of each viral protein in the cells regulates the switch from transcription to production of full-length viral antigenome or genome [42]. Although there is no direct evidence regarding how BDV regulates the switch mechanism, it is also possible that the molecular ratio of viral proteins such as N and P in the nucleus of infected cells is involved in the mechanism.

2.3.2. Splicing

Splicing of viral pre-mRNAs is one of the most efficient strategies to produce several different proteins from small viral genomes. BDV employs the RNA splicing machinery for gene expression and the genome contains at least three introns: intron I, nucleotide (nt) 1932–2025; intron II, nt 2410–3703; intron III, nt 2410-4559 (Fig. 1) [12–15]. Transcripts that retain intron I serve as messages for expression of the M protein of BDV, and those that retain intron II serve as messages for expression of the envelope glycoprotein. Transcripts that lack both introns serve as messages for expression of the polymerase protein of BDV. In addition, it is likely that intron III-spliced RNAs produce novel proteins of BDV, although the proteins have not yet been identified in infected cells. The intron III-spliced RNAs could form predicted ORFs in all three frames. Frame 1 generated the largest ORF, which encodes the first 58 amino acids of the G protein fused to the L protein lacking one quarter of the N-terminus. Frames 2 and 3 generated ORFs encoding putative products of 75 and eight amino acids, respectively [13,15]. Interestingly, introns II and III shared the 5' splice site, indicating the existence of alternative splicing in BDV pre-mRNAs. Recently, we demonstrated that the alternative splicing of introns II and III in BDV is regulated by an alternative Pt and *cis*-acting exon splicing suppressor (ESS) element within the L gene that contains similar motifs with other viral and cellular ESSs (Fig. 1) [15]. The pre-mRNAs that are terminated at the t6 signal might splice the intron III sequence efficiently, because the pre-mRNAs could not carry the ESS region in their sequences. Further evidence including in vivo study will need to clarify the detailed role of the alternative splicing of BDV in the replication in infected cells.

2.3.3. Nucleocytoplasmic transport

BDV replicates and transcribes in the nucleus of infected cells, and therefore, nuclear import and export of the viral genome are critical for the viral life cycle. Recent studies have suggested that the nuclear transport activity of BDV is mediated by the major viral antigens such as N and P that contain NLS and/or NES within their sequences [18,20,23]. The NLS- and NES-containing proteins can directly or indirectly bind to the viral nucleic acids and travel between the cytoplasm and nucleus through the nuclear pore complex. Although the results obtained from transfection assays may not exactly reflect events occurring during infection, it is possible that the RNPs of BDV could actively enter the nucleus by the function of NLSs in N and P proteins and that NES in the N protein could contribute to leaving the nucleus after replication.

BDV must employ a switch mechanism that changes the direction of nuclear transport of the viral RNPs dependent on the viral life cycle in infected cells. The nucleocytoplasmic shuttling proteins such as N could play an essential role in the switch mechanism. The mechanism of N may be mediated by interaction with the P protein and production of

an NLS-lacking N isoform, p38N. Interestingly, the NES of N exactly overlaps one of the P-binding sites [20]. It is therefore possible that the nuclear export activity of BDV N is blocked by direct binding of P to the N NES. Recent studies have also revealed that binding between BDV P and X proteins is mediated via a putative-NES region within the X protein [21,22]. These observations suggested that P might act as a nuclear retention factor of the N and X proteins by binding directly to the NES.

Recently, we proposed a model of nucleocytoplasmic trafficking of BDV RNP, in which relative levels of p40N, p38N and P in the nucleus may play a key role for determining the direction of BDV RNP movement (Fig. 2) [20]. Increased levels of P could mediate retention of the viral RNPs in the nucleus by the masking of the NES of N during the nuclear replication stage (Fig. 2). On the other hand, a lower concentration of P in the nucleus can increase free NES, mediating nuclear export of N-containing RNP complexes (Fig. 2). In addition, p38N might play an important role in the nuclear export of the viral RNPs. The p38N should be imported into the nucleus with other viral proteins as complexes. The presence of the NLS-lacking p38N in the N multimer can increase the relative number of NES when compared with the NLS. An increased number of NES in the N complex would enhance nuclear export of the viral RNPs for maturation or assembly of the progeny virions (Fig. 2). At present, however, there is little evidence supporting significant changes in the ratio between N and P during the viral life cycle [43]. Further experiments using quantitative techniques will be necessary to understand the nucleocytoplasmic trafficking of BDV RNPs in detail.

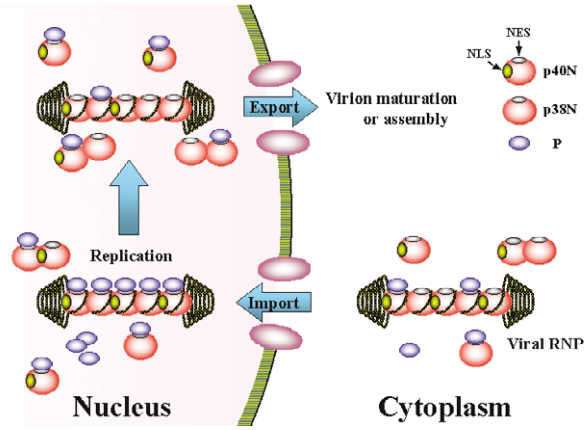

Fig. 2. Nucleocytoplasmic shuttling of BDV RNP. BDV genomic RNA is associated with multimer of p40N and p38N. BDV-p40N and P actively import viral RNPs from cytoplasm to nucleus via their NLSs (nuclear localization signals). BDV-p40/p38N also contains NES (nuclear export signal), which overlaps the binding site to P. Direct interaction between N NES and P in the nucleus might block nuclear export of BDV RNPs during the replication of BDV. Molecular ratio or interaction among these proteins in the nucleus seems to be important for determination of the direction of viral RNPs.

3. Cellular biology of BDV

3.1. Cell tropism of BDV

3.1.1. In vitro

The BDV has a strikingly broad cell tropism in cell culture. The first culture of BDV in cell lines was demonstrated after cocultivation with primary culture from BDV-infected rabbit hippocampal cells. BDV can infect most of the cell lines derived from various organs and tissues of different species from humans to birds and establish persistent infection in the cultured cells.

A unique biological feature of BDV is its noncytopathic replication in cultured cells. The virus replicates in the cells without causing any cytopathic effects. There are no apparent differences between BDV-infected and uninfected cells. Furthermore, BDV easily establishes persistent infection after several passages in cultured cell lines. To date, persistent infection of BDV has been demonstrated in numerous cell lines such as MDCK, MDBK, Vero, CrFK, C6, OL, PC12, SK-N-SH and 293. These persistently infected cell lines contributed to the characterization of virus-specific antigens, as well as diagnostic investigations of BDV using sensitive immunoassays. The nucleus-dependent antigen is a typical picture of BDV infection in cultured cells. In the infected cells, BDV-specific foci, dust-like antigen spots, are always observed in the nucleus. Expression of recombinant viral proteins in transfected cells cannot produce such foci in the nucleus, suggesting that they might represent replication complexes of viral RNPs. Experiments using in situ hybridization proposed, however, that the nucleolus is the site of BDV transcription and replication [31]. Therefore, it may be possible that the BDV-specific foci outside the nucleolus are branch places for viral RNPs that are not synthesizing viral RNAs. Another important feature of BDV in vitro is that it is strongly cell-associated and produces extremely low levels of infectious virus per cell, although the cells express high levels of BDV RNAs and proteins. In addition, it is difficult to conclusively identify the viral particles in infected cultured cells. It remains to be determined how BDV produces infectious particles and propagates between the cells in culture.

3.1.2. In vivo

One of the most prominent features of BDV infection is the heavy inflammatory reaction in the central nervous system (CNS). In naturally infected animals, the most severe inflammation is found in the olfactory bulb, gyrus, caudate nucleus and the hippocampus [1,2]. The mesencphalon, central gray matter, substantia nigra and hypothalamus are also affected. Only moderate changes occur in the spinal cord. Histological analysis reveals severe nonpurulent meningoencephalitis with massive perivascular and parenchymal infiltration in naturally infected animals. The perivascular cuffings consist mainly of monocytes, T lym

phocytes ($CD4^+$ and $CD8^+$) and to a lesser extent plasma cells. Despite the severe encephalitis, the nerve cells are morphologically intact even in the areas surrounded by inflammatory cells. Furthermore, degeneration of neurons is rare in naturally infected hosts, although astrocytosis is commonly observed in the areas of inflammation.

Viral-specific antigens are present in the nucleus and/or the cytoplasm of neurons, as well as astrocytes and oligodendrocytes. The BDV-specific inclusion bodies (Joest-Degen body), which occur most frequently in the large neurons such as the pyramidal cells of the hippocampus, are found in the nucleus.

The best-characterized animal model of BDV infection is the rat. In experimentally infected rats, the histopathology of the CNS is dependent on the immune status of the host at the time of inoculation, the genetic background and the route of infection [44–47]. Inoculation of immunocompetent adult rats results in marked immune-mediated meningoencephalitis consistent with the classical BD observed in naturally infected horses and sheep. BDV spreads by axonal and transneuronal transmission. In addition to neurons, BDV antigens have also been demonstrated in astrocytes, oligodendrocytes, Schwann cells and ependymal cells. In the process of persistent infection in adult rats, degeneration of the neurons is observed. In contrast, immunoincompetent rats such as neonates show a tolerant infection without signs of BD or encephalitis [46,47]. The neonatal infection causes significant alterations in development of the CNS. Developmental injury in the cerebellum and hippocampus has been widely reported in newborn rat models. These reports provided evidence for direct effects of BDV infection on cellular functions in the absence of immunopathological degeneration. Our recent study using neonatal Mongolian gerbils also supported the direct effects of BDV on neuronal functions. The infected newborn gerbils cause fatal neurological diseases without any neuroanatomical abnormalities or neuronal cell losses [48]. Furthermore, gerbils treated with immuno-suppressive agent also showed severe neurobehavioral diseases by the infection, indicating that BDV induces immune-independent neuropathogenesis in the brains.

In persistently infected animals, BDV appears in various extraneural tissues and organs, which seem to be infected via peripheral axons. Furthermore, BDV RNAs are also detected in peripheral blood cells of both naturally and experimentally infected hosts including humans by using sensitive RT-PCR techniques.

3.2. Association with cellular factors

BDV causes persistent infection in neuronal and nonneuronal cells in the CNS. In vivo studies of naturally and experimentally infected animals suggested that BDV can directly affect cellular functions, as severe neurobehavioral disease or neurodevelopmental damage are induced in BDV-infected animals without encephalitis or other immune-mediated cell disturbances. Furthermore, the CNS of the BDV-infected neonatal rats shows a progressive decrease of synaptic density and plasticity, as well as alteration of the expression levels of cytokines, neurotrophic factors, neurotransmitters and their receptors [47,49,50]. These observations indicated that BDV is likely to modify the microenvironment of infected cells by association with cellular factors and thus cause CNS disturbance. There have been some previous reports that BDV infection changes the cellular microenvironment. Very recently, interaction between BDV and a glutamate receptor, kainite 1, in the CNS was postulated from the distribution pattern of BDV antigen in infected rat brains [50]. This report proposed that the neuropathological observations such as neuronal degeneration in the dentate gyrus in persistently infected animals resulted from a neurotransmitter imbalance caused by multiple interactions between neurotransmitter systems and BDV at the synapses. Hans et al. reported that persistent infection of BDV constitutively activated the pathway of mitogen-activated protein kinase (MAPK) but efficiently blocked translocation of activated extracellular signal-regulated kinase to the nucleus in PC12 cells [51]. This study suggested that the absence of neuronal differentiation of BDV-infected PC12 cells treated with nerve growth factor is due to the aberrant activation of MAPK in the cells. Another study has also demonstrated that BDV infection sustained activation of Raf/MEK/ERK signaling cascade in several different cell lines [52]. In addition, this group demonstrated inhibition of BDV spread in cultured cell by the MAPK inhibitor U0126, suggesting that activity of the cascade might be essential for efficient transmission of the virus in cells. Since this kinase must be involved in the differentiation or development of neuronal cells in the CNS, it should be determined whether BDV infection can directly stimulate the signaling pathway in cells.

Besides neurons, astrocytes are the main target of BDV in the CNS. A recent study revealed that acute BDV infection directly causes marked inhibition of glutamate uptake by feline primary cortical astrocytes [53], which is essential to maintenance of homeostasis of the function of the neurons. This study suggested that the disruption of the astrocyte's ability to prevent glutamate-mediated neurotoxicity could contribute to the neuropathogenesis of BDV in the absence of immuno-pathology-related brain damage.

To date, there has been no evidence of direct interactions between BDV-specific proteins and cellular factors. Recently, however, we have shown that the P protein of BDV specifically binds a neurite outgrowth factor, amphoterin, in vivo [54]. Amphoterin is a 30-kDa heparin-binding protein, of which expression gradually decreases during brain development. Previous studies revealed that amphoterin directly mediated neurite outgrowth in neural cell lines and primary rat brain cells by interaction with its cellular receptor, RAGE (receptor for advanced glycation end products) [55]. It has been revealed that RAGE-mediated cell process

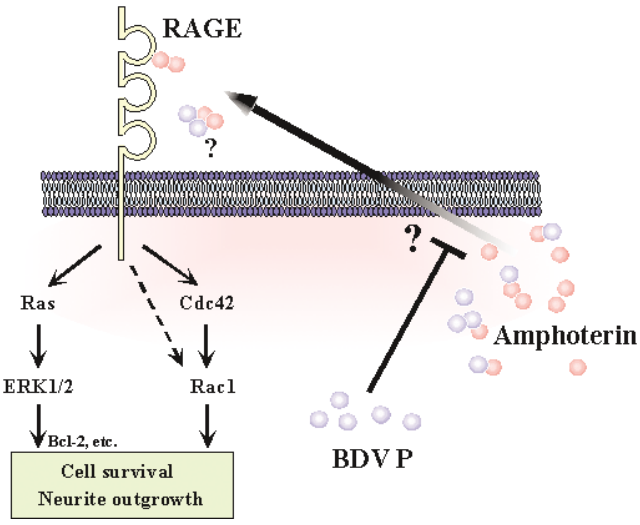

Fig. 3. A possible role of the BDV P protein for amphoterin functions in neuronal cells. Amphoterin is secreted from the cell surface and binds to the ectodomain of RAGE. Ligation of RAGE by amphoterin induces cell signaling for cell survival or neurite outgrowth [55]. BDV P may directly inhibit secretion of amphoterin from the cells by the binding and affect the function of amphoterin in the cells. The P might be secreted from the cell surface as a complex with amphoterin and prevent ligation between RAGE and amphoterin.

extension depends on the Rho family GTPases Cdc42 and Rac but not on Rho (Fig. 3) [55]. Recently, Huttunen et al. demonstrated that activation of RAGE by amphoterin promotes cell survival through increased expression of the anti-apoptotic protein Bcl-2, which is regulated via the Ras/ERK signaling pathway (Fig. 3) [56]. Furthermore, it has been reported that amphoterin is important for neuron–glial cell interaction. The degree of neuron–glia contact in the peripheral nervous system is correlated with expression level of amphoterin in glial cells. These studies indicated that amphoterin plays an important function in neurite outgrowth of neural cells, as well as in network formation of neuronal cells in the developing or injured brain. We showed that infection by BDV, as well as purified P protein in the culture medium, significantly decreased neurite outgrowth of cells grown on laminin, indicating the functional inhibition of amphoterin by interaction with the P protein. Immunohistochemical analysis revealed decreased levels of the amphoterin protein at the leading edges of BDV-infected cells. Moreover, the expression of RAGE was not significantly activated in BDV-infected cells [54]. These results suggested that the secretion of amphoterin from the cell surface or the interaction of amphoterin with RAGE is directly inhibited by binding of the P protein (Fig. 3). This observation suggested a possibility that the developmental damage or disturbance of neuronal cells observed in neonatally BDV-infected rats may be directly due to inhibition of amphoterin functions by the P protein in cells.

4. Future directions

In the past two decades, BDV research has impacted various research fields, not only biology of neurotropic RNA viruses but also neuropsychiatry. Although several discoveries regarding this unusual pathogen have been made, some major issues with broad consequences for biology and medicine remain to be resolved. Despite numerous reports of BDV infection in humans, problems such as variety of seroprevalence among different laboratories, low levels of antibody titers, and sequence similarities between human BDV isolates and horse-derived laboratory strains all raised the fundamental questions of whether BDV infects humans and truly contributes to neuropsychiatric disorders. Although the broad potential host range of BDV suggests that humans are targets for infection, the sources and routes of human infection are not clear. Furthermore, the size of the population of animals affected by neurological diseases caused by BDV infection outside the classical endemic areas is not yet known. Detailed epidemiology and surveillance with reliable techniques should be performed in domestic animals around the world.

A more thorough understanding of the molecular and cellular biology of BDV is needed to explain the neuropathogenesis of this virus. The functions of each viral protein, as well as the mechanism of persistent infection by the virus in the nucleus have not been fully determined. Determination of the regulation of viral gene expression in the nucleus will further our understanding of the unique cell tropism of BDV in the CNS. The generation of infectious molecular clones of BDV using a reverse-genetic system would be a breakthrough approach in this research field.

BDV is highly neurotropic, noncytolytic and induces long-lasting persistent infection both in vivo and in vitro. Since large amounts of the viral proteins are detected in infected cells, determination of the detailed effects of viral proteins or cellular functions will also provide insight into the mechanism by which CNS infection of BDV can result in neurodevelopmental damage and neurobehavioral disorders in the host.

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