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MOLECULAR BIOLOGY OF BORNA DISEASE VIRUS AND PERSISTENCE

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1. ABSTRACT

Borna disease virus (BDV) causes central nervous system (CNS) disease that is frequently manifested by behavioral abnormalities. Recent evidence indicates that the natural host range and geographic distribution of BDV is wider than originally thought BDV has been molecularly characterized as a non-segmented, negative single-stranded (NNS) RNA virus. Its genome (ca 8.9 kb), the smallest among known NNS RNA viruses, has an organization similar to that of other members of the order Mononegavirales. BDV has the property, unique among known animal NNS RNA viruses, of a nuclear site for the replication and transcription of its genome. The nucleocytoplasmic transport of BDV macromolecules is an essential component of the life cycle of BDV. An overlap of transcription units and transcriptive signals, overlap of ORFs, transcriptional readthrough and RNA splicing regulate expression of the BDV compact genome. The concurrent use of such diversity of strategies for the regulation of virus gene expression is unique among known NNS RNA viruses. Moreover, BDV appears to have also an unusual assembly process. Based on its unique genetic and biological features, BDV is considered to be the prototypic member of a new virus family, Bornaviridae, within the order Mononegavirales. Therefore, the investigation of the molecular biology of BDV may provide new insights about the biology of mononegaviruses, which include important human pathogens.

1. BIOLOGICAL CHARACTERISTICS:

1.1. Host range

Horses and sheep have been regarded as the main natural hosts of Borna disease virus (BDV). In these species BDV can cause BD, a frequently fatal neurologic disease manifested by behavioral abnormalities. However, more recent evidence indicates that the natural host range, geographic distribution and prevalence of BDV are wider than originally thought (1-4). Experimentally, BDV can infect phylogenetically distant species, from birds to rodents and non-human primates (1-3). Moreover, serological data and recent molecular epidemiological studies indicate that BDV can infect humans, and might be associated with certain neuropsychiatric disorders; however, BDV has not been implicated as a human pathogen yet (5, 6). Nevertheless, technical difficulties and limitations of the currently existing diagnostic tools for BDV, have led some investigators to question the worldwide distribution of BDV, and its relevance as a human virus (4). Improved knowledge of the immunobiology, as well as the molecular and cellular biology of BDV may facilitate the development of more sensitive and reliable assays for the epidemiologic investigations of BDV infection.

BDV infectivity and RNA have been detected in body secretions and excretions, suggesting that BDV could be transmitted through salivary, nasal and conjunctival secretions, and particularly urine and feces (7)**.** Infection may result from direct contact with these body fluids or by

exposure to contaminated water and food (3). Different BDV isolates can exhibit significant differences in their phenotypic expression. However, molecular epidemiological data have shown a remarkable sequence conservation, not only among BDV isolates within the same host species, but also among isolates from different animal species (1, 2, 5).

1.2. Tissue and cell tropism

BDV is non-cytolytic and highly neurotropic. Despite of its broad host range, in vivo BDV replicates preferentially in the central nervous system of the infected animal. Neurons and astrocytes are the primary cell targets of BDV, but oligodendrocytes and ependymal cells can be also infected (3). In addition, ectodermal derived cells become infected *in vivo* as a consequence of the centrifugal axonal spread of BDV (3). The virus can be also present in peripheral blood mononuclear cells (PBMC) from naturally and experimentally infected animals (1, 2, 7).

BDV can be grown in a variety of brain and non-brain derived established cell lines by cocultivation with infected brain cells, or by inoculation with brain homogenates from infected animals (3). In all known cases infected culture cells do not produce significant levels of extracellular cell-free virus, and have only very low cell-associated infectivity. Exposure of BDV-infected cells to a hypertonic buffer (250 mM $MgCl_2$) for 90 min at 37^0C can release up to 50 % of the total cell-associated infectivity (8), which provide a source of cell free virus. Homogenates from BDV infected cells and tissues, as well as cell free virus, can be quantified by using an immuno-focus assay.

2. VIRION MORPHOLOGY AND PHYSICAL CHARACTERISTICS

Electron microscopic studies of negative-stained cell-free BDV infectious particles have shown that they are of spherical morphology with a diameter ranging from 70 to 130 nm (9-11). These particles contain an internal electron-dense core (50 to 60 nm) and a limiting outer membrane envelope, which appears to be covered with spikes approximately 7.0 nm long (11). Partially purified BDV infectious particles have a buoyant density in CsCl of 1.16-1.22 g/cm^3, and in sucrose of 1.22 g/cm^3. Virus infectivity is rapidly lost by heat treatment at 56^0C, as well as at pH's below 5 and above 12, and by treatment with organic solvents, detergents, formaldehyde and exposure to ultra violet radiation.

3. GENOME ORGANIZATION AND ENCODE PROTEINS

BDV has a non-segmented, negative-stranded (NNS) RNA genome (ca 8.9 Kb in size and Mr of ca $3x10^6$; refs. 12, 13). The RNA genome is not polyadenylated. Extracistronic sequences are found at the 3' (leader) and 5'(trailer) ends of the BDV genome. As with other negative strand RNA viruses, the ends of the BDV genome RNA exhibit partial inverted complementarity. Full-length plus-strand (antigenomic) RNA's are present in infected cells and viral ribonucleoproteins.

Six major open reading frames (ORFs) are found in the BDV genome sequence (refs. 12, 13; see figure 1). These ORF's code for polypeptides with predicted Mr of 40 kDa (p40), 24 kDa (p24), 10 kDa (p10), 16 kDa (p16), 56 kDa (p56) and 180 kDa (p180), respectively. Based on their positions in the viral genome and abundancy in infected cells and virion particles, together with their biochemical and sequence features, p40, p24 and p16 BDV polypeptides correspond to the viral nucleoprotein (N), the phosphoprotein (P) transcriptional activator, and matrix (M) proteins, respectively, found in other NNS RNA. Two isoforms of the BDV N (p40 and p38) are found in BDV-infected cells. These two forms of the viral N may be encoded by two different mRNA species (14), but differential usage of two in-frame initiation codons present in the BDV p40 gene could also contribute to the production of BDV p40/38. BDV p24 is an acidic polypeptide (predicted pI of 4.8), that has a high Ser-Thr content (16%), with phosphorylation at serine residues which is mediated by both protein kinase C and casein kinase II (15). In addition to p24, a second polypeptide (Mr 16 kDa), termed P' (p16), is also translated from the second in-frame AUG codon present within the P ORF . P' has been detected in BDV-infected cultured cells and brain cells of experimentally infected animal (16), but its role in the virus lifecycle is currently unknown. An additional ORF, p10 or X, encodes for a polypeptide of 10 kDa present in BDV-infected cells and tissues (17). BDV p10 starts within the same mRNA transcription unit, 49 nt upstream from p24 and overlaps, in a different frame, with the 71 N-terminal amino acids of p24. In contrast to other NNS RNA viruses, the putative BDV M protein appears to be glycosylated, and has a Mr of 18 kDa (gp18: ref. 17). M was initially characterized as a biantennary complex-type glycoprotein sensitive to endoglycosidase F (endo F), but not to endoglycosidase H or O-glycosidase (18). In addition, M was proposed to be present in the virus envelope and it to participate in viral attachment to the cell (18). Intriguingly, M does not contain any typical consensus sequence for N-glycosylation. In addition, recent evidence has shown that BDV M is a nonglycosylated protein which lines the inner side of a lipid-containing viral envelope (19). Therefore BDV M has similar features to those found in the matrix proteins of mononegaviruses. These new findings question the previously proposed location of BDV M on the virus surface and its role in virus entry. BDV ORF IV (p56), the putative virus surface glycoprotein (G), overlaps, in a different frame, with the C-terminus of ORF p16, and is capable of encoding a 503 amino acids polypeptide with a predicted molecular mass of 56 kDa. The p56 gene directs the synthesis of two glycosylated polypeptides of about 84-94 kDa (gp84/94) and 43 kDa (gp43), corresponding to the full length and C-terminus, respectively, of the p56 gene (10). The gp43 is produced via cleavage of the gp84/94 by the cellular protease furin. BDV ORF V (p180) is capable of encoding a polypeptide whose deduced amino acid sequence displays strong homology to other NNS-RNA virus polymerases, members of the L protein family (12, 13). Several additional viral polypeptides can be translated from the spliced forms of BDV mRNA's (see section 4.2), which increases the proteomic complexity of BDV.

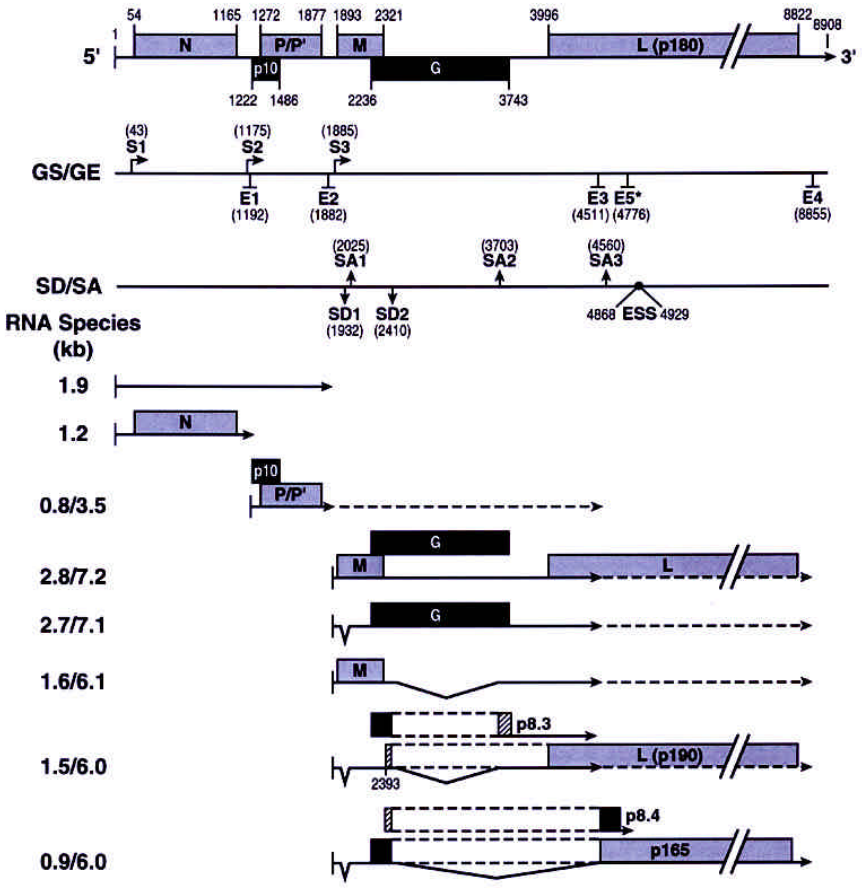

Figure 1. Genomic organization and transcriptional map of BDV. BDV open reading frames are represented by boxes at the top. Different shades correspond to usage of different reading frames within the antigenomic polarity of the BDV genomic RNA. The location of transcription initiation and transcription termination sites are indicated by S and E, respectively. Positions of BDV introns I, II and III are indicated.

The genomic polarity of the RNA has a very limited coding capability; which consists of only very small ORF's that are not flanked by recognizable putative transcription initiation and termination/polyadenylation signals. Thus, presently there is no evidence that BDV might use an ambisense coding strategy. The recent determination of the complete sequence of two additional strains of BDV have confirmed the previously proposed genome organization of BDV, showing also a high degree of conservation of the BDV genome coding potential (20).

4. CYCLE OF INFECTION

4.1. Virus adsorption and entry

There is evidence that a proteinaceous cellular receptor, of unknown identity, is required for BDV infection. Antibodies to both the virus G and M (gp18) proteins have neutralizing activity suggesting that both are implicated in BDV adsorption and, or, entry (10, 18). Nevertheless, monoclonal antibodies directed to gp84/94, but not to M, exhibited neutralizing activity against BDV (21). It is plausible that antibodies against M associated carbohydrates, which might be present also in gp84/94, could explain the reported neutralizing activity of anti-M antibody. Lysosomotropic drugs and chemical treatments that cause depletion of cellular energy block BDV infection, indicating that BDV entry occurs by receptor-mediated endocytosis, and requires an acidic intracellular compartment to allow the fusion between viral and cellular membranes (22). Reductions in the pH of the endocytic compartment triggers this fusion event, and releases the BDV ribonucleoprotein (RNP) which is then transported to the cell nucleus where viral transcription and replication occur.

Both gp84/94 and gp43 are associated with cell-free infectious BDV particles and appear to participate in virus entry. A plausible model for BDV cell entry is that the N-terminal part of the virion-associated gp84/94 is involved in BDV receptor binding and endocytosis, whereas gp43 would mediate the pH-dependent fusion event required for BDV infection. The role of gp43 in membrane fusion is supported by the observation that it is the only known BDV polypeptide that has been detected at the cell surface, and cells persistently infected with BDV form extensive syncytia upon exposure to low-pH medium (22). Interestingly, near the N-terminus of gp43 there is a highly hydrophobic sequence that is reminiscent of the

fusogenic domain described for the surface glycoproteins of other enveloped viruses. Nonetheless, we cannot rule out that other viral proteins not expressed at the cell surface could influence fusion, as has been described for the herpes simplex virus gK (23). Results from pseudotype studies have provided evidence that the N-terminal part (amino acids 1 to 244) of BDV p56 is sufficient for receptor recognition and virus entry (24). This pseudotype approach was based in a recently developed recombinant vesicular stomatitis virus (VSV) in which the gene for green fluorescent protein was substituted for the VSV G protein gene (VSV-G*). Complementation of VSV-G* with BDV p56 resulted in infectious VSV-G* pseudotypes that contained both BDV gp84 and gp43. A chimeric glycoprotein that contained the N-terminal 244 amino acids of BDV p56 fused to the transmembrane (TM) and cytoplasmic domain (CTD) of VSV G protein was efficiently incorporated into VSV-G* particles resulting in pseudotype infectious virions that were neutralized by BDV-specific antiserum (24). Consistent with these pseudotype findings, a recombinant VSV where the VSV-G was replaced by the BDV-G was infectious but its tropism paralleled that of BDV rather than that of VSV. Thus, in primary hippocampal cell cultures both astrocytes and neurons were readily and equally infected by VSV wild-type. In contrast, in the case of the recombinant VSV only neurons were infected at early times post-infection, whereas astrocytes became infected at later times.

4.2. Transcription and replication

BDV has the property, unique among known NNS RNA animal viruses, of a nuclear site for genome transcription and replication (12, 13). The nucleolus has been proposed as the site where BDV RNA synthesis occurs (25). The Genomic and antigenomic RNA molecules are neither capped nor polyadenylated. These RNA's exist as infectious RNP in the nucleus of infected cells (12).

Sequential and polar transcription results in decreasing molar quantity of BDV transcripts from the 3'- to the 5'-encoded cistrons. RNA species corresponding to the leader RNA have not been detected yet in BDV-infected cells. The viral mRNA's are polyadenylated and contain a 5' cap structure, but sequences at the 5' of the BDV mRNA's are homogeneous and genome encoded (26). Thus, it is unlikely that transcription initiation of BDV mRNA's involves a cap-snatching mechanism similar to the one used by influenza viruses. Monocistronic viral mRNA's in BDV-infected cells are detected only for the N gene (figure 1). The BDV genome contains three transcription initiation sites (S signals), and four transcription termination/polyadenylation sites (E signals: refs. 12, 13 and figure 1). The S signals contain a semiconserved uracil-rich motif that is partially copied into the respective transcripts. A similar motif is not found within the S signals of previously described NNS RNA viruses. BDV E signals consist of six or seven U residues preceded by a single adenine residue, resembling the E signal motif found in other NNS RNA viruses. The BDV genome lacks the characteristic configuration of E signal / intergenic (IG) region / S signal, found at the gene boundaries of other NNS RNA viruses. Instead, BDV transcription units and transcriptive signals frequently overlap. Thus, for example signal S2 of the P gene is located 18 nucleotudes upstream of signal E1 of the N gene. Likewise, BDV E2 signal for the P gene is completely contained within the S3 signal of the third transcription unit. Overlapping genes have been documented for several NNS RNA viruses, and such arrangement is thought to contribute to gene expression regulation (27). It is worth noting that the polymerase of the respiratory syncytial virus (RSV) is capable of efficiently transcribing from an L S signal placed downstream of the M2 E signal (27). This finding raises the possibility that the gene overlap exhibited by several mononegaviruses, including BDV, might not have a significant impact on the biology of these viruses.

Readthrough at transcriptional termination sites can be observed with several mononegaviruses. This phenomenon is usually considered to be aberrant and of low, or none, biological significance for the biology of the virus. However, for some viruses, transcriptional readthrough may be critical for their viability. Thus, readthrough of BDV E3 signal is strictly required for the synthesis of the virus L polymerase, which may provide an additional regulatory mechanism for the control of BDV genome expression.

Two of the BDV primary transcripts are post-transcriptionally processed by the cellular RNA splicing machinery (12, 13). Three introns (I, II and III) have been identified in the BDV genome. BDV introns I and II span nt 1932-2025 and 2410 to 3703, respectively, in the BDV antigenomic sequence. Splicing of intron I results in mRNA species where amino acid in position 13 of the M protein is followed by a stop codon, whereas splicing of intron II, and I+II, results in a mRNA containing an ORF that corresponds to the first 58 amino acids of G fused to a new C terminus of 20 amino acids, resulting in a polypeptide with a predicted Mr of 8.3 kDa (p8.3). RNA species resulting from splicing of intron II, and I+II, predict also an additional ORF that would consist of BDV ORF V (p180) extended by 153 amino acid residues at its N-terminus, resulting in a putative BDV L protein with a predicted molecular mass of 190 kDa (p190). Whether all these new predicted BDV polypeptides are synthesized in infected cells and tissues is unknown. However, recent evidence suggests that p190, rather than p180, is likely to be the BDV L polymerase preset in infected cells (28). Intron III is generated by alternative choice of the 3' splicing acceptor (SA) site (28-30). Intron-III spliced mRNA's were detected in cells from different types and species, and have coding capability for two new viral proteins with predicted molecular masses of 8.4 (p8.4) and 165 (p165) kDa. These findings underscore the proteomic complexity exhibited by BDV**.**

4.3. Assembly, release and cell-to-cell propagation

Thin sections of BDV-infected cells revealed the presence of intracytoplasmic virus-like particles with morphological characteristics similar to those described for partially purified cell-free BDV infectious particles (31).

These particles were observed below the plasma membrane of infected cells, and rarely seen in cytoplasmic vacuoles. They showed no association with cisternae of the endoplasmic reticulum (ER), the Golgi complex, or other intracytoplasmic membranes. The assembly process and site of BDV maturation have not been identified. BDV RNP's accumulate in the nuclei of infected cells. These RNP's are biologically active as determined by their ability to direct synthesis of BDV macromolecules and production of infectious virus upon transfection into susceptible cells (32). The nuclear envelope is continued by the ER, and BDV gp84 appears to accumulate at the ER and perinuclear space. This may facilitate the interaction of gp84 with viral RNP during their nuclear egress. Budding of BDV particles has only been observed at the cell surface of BDV-infected Madin-Darby canine kidney (MDCK) cells after treatment with n-butyrate (11). Whether this reflects a natural pathway for the exit of BDV remains to be determined.

An intriguing aspect of the biology of BDV is the mechanism by which BDV spreads within the CNS. BDV appears to propagate trans-synaptically but full virus particles have never been observed at the synaptic junctions (33, 34). A similar situation has been described for rabies virus (RV; ref. 33). These observations led to the attractive hypothesis that bare RNP could be the infectious unit being transported trans-synaptically within the CNS. However, recent findings have indicated that RV G is absolutely required for the propagation of RV in neuronal culture cells, as well as within the mouse CNS (35). Whether this finding applies also to the propagation of BDV is currently unknown. Interestingly, results from experiments where BDV-infected and non-infected cells were separated by a filter that did not allow cell-to-cell contact but permitted the pass of cell-free virus suggested that cell contact is required for efficient propagation of BDV at least in the case of rat astrocytes.

5. REGULATION OF GENE EXPRESSION

Mononegaviruses use a variety of mechanisms to direct and control expression of their genomes, including: overlap of transcription units and transcriptive signals, readthrough of transcription termination signals, differential use of translational initiation codons, and efficient translation of ORF's placed downstream within polycistronic mRNA's. Remarkably, BDV concurrently uses all these mechanisms, together with RNA splicing, for the control of the expression of its compact genome. This represents a unique situation among known mononegaviruses. Our current knowledge about the molecular mechanisms responsible for the control of BDV genome expression is very limited. Hence, we will review only a few selected processes to illustrate the complexity underlying the execution of the BDV gene expression program.

5.1. Regulation of BDV RNA splicing.

Alternative splicing of mRNA precursors is a versatile mechanism of gene expression regulation that accounts for a considerable proportion of proteomic complexity in higher eukaryotes. Its modulation is achieved through the combinatorial interplay of positive and negative regulatory signals present in the RNA, which are recognized by complexes composed of members of the hnRNP and SR protein families (36-38). Alternative splicing has been shown to play an important role in the lifecycle of several viruses, including influenza (39), adenovirus (40), human immunodeficiency virus (41), and papillomavirus type I (42). Moreover, in the case of BDV, RNA splicing can contribute to modulate the efficiency of termination-reinitiation of translation and leaky scanning mechanisms that are also involved in the regulation of the expression of BDV gene products.

Virus-derived BDV mRNA's are spliced with significant lower efficiency compared to the same plasmid-derived mRNA's (29, 30, 43). It has also been documented that splicing of plasmid-derived BDV 2.8 kb primary transcript is not influenced by co-expression of the BDV proteins N, P and p10, or in BDV-infected cells (43). These findings indicate that BDV-encoded factors, or virus-induced cellular factors, rather than *cis*-acting RNA elements determine the low efficiency of RNA splicing in virus-infected cells. In addition, a similar splicing pattern has been observed in different cell types from different species, and such pattern was not altered in response to osmotic shock-mediated stress (30). These findings suggest that BDV might have developed strategies to acquire certain degree of insulation from cellular influences that could have unwanted effects on the regulation of virus RNA processing. These mechanisms could also prevent BDV-induced disturbances in the regulation of the cellular RNA processing machinery. This, in turn, could facilitate virus persistence without compromising cell viability.

Introns II and III share the 5' splicing donor (SD) site (29, 30). Interestingly, alternative splicing of intron II and III is regulated by the use of an alternative transcription termination/polyadenylation signal (GE6), and a cis-acting exon splicing suppressor (ESS) element located within the L gene (29). This ESS resembles those found in other viral and cellular pre-mRNA's. The BDV mRNA's that terminate at GE6 will not contain the ESS, and can be spliced more efficiently than those pre-mRNA terminating at GE5. The regulation of the use of alternative GE signals by the BDV polymerase are presently unknown. Intron-III spliced mRNA's have coding capability for two new viral proteins with predicted molecular masses of 8.4 and 165 (p165) kDa. The p165 is a deleted form of the BDV L polymerase, containing three RGD motifs and a signal peptide signal that could target it into the secretory pathway. Consistent with this hypothesis, plasmid-derived p165 protein tagged with a *c-myc* epitope accumulated in the ER/Golgi apparatus. Expression of p8.3 and p165 proteins, and their possible secretion to the extracellular milieu, in virus-infected cells remain to be determined. However, it is worth noting the presence of three RGD motifs in p165, which could provided a secreted p165 protein with the ability to interact with integrin molecules present at the cell surface (44). This, in turn, could trigger cellular signal transduction pathways that might contribute to BDV-cell interactions. Thus, alternative splicing of BDV pre-mRNA may generate a "variant" L gene product with

functions other than those predicted for a RNA dependent RNA polymerase. A similar situation has been proposed for the cytomegalovirus DNA polymerase accessory protein ppM44 (45). These findings underscore the proteomic complexity exhibited by BDV.

5.2. Regulation of protein expression.

In eukaryotes, the majority of mRNA's are monocistronic. However, examples of downstream ORF's (dORF's) expression have been described for several cell and viral polycistronic transcripts. BDV polypeptides, with exception of N, are translated from polycistronic mRNA's, which represents a unique situation among known mononegaviruses. Expression of dORF can be achieved by a leaky ribosome scanning mechanism, resumption of scanning after termination of an upstream ORF (uORF), or cap-independent internal initiation. Experimental evidence indicates that a leaky ribosome scanning mechanism contributes to the expression of the dORFs P' and G (16, 35, 46), whereas at the present time there is no evidence that cap-independent internal initiation is used for translation of BDV mRNA's.

The expression and function of virus surface GPs of other mononegaviruses have been extensively studied. *Paramyxoviridae* possess two integral membrane proteins, one of which (HN) is involved in cell attachment and the other (F) in mediating pH-independent fusion of the viral envelope with cellular membranes (47). *Filoviridae* have a single GP making up the virion surface spike (48). Expression of this GP results from a complex regulatory mechanism involving both transcriptional editing and translational frameshifting (49). Finally, *Rhabdoviridae* also have a single GP present at the surface of the virus (50). In all cases, synthesis of these glycoproteins involves maturation by trafficking through the Golgi complex. Eventually, the GP's are expressed at the cell surface and assembly of infectious virions occurs through budding on plasma membranes. In contrast, expression of full length BDV G (gp84) appears to be mainly restricted to the ER and perhaps nuclear envelope. In contrast, the C-terminus (gp43), resulting from post-translational cleavage by the cellular protease furin, is detected at the cell surface (10, 22, 51). Both gp84 and gp43 products appear to be associated with infectious virus particles, and are involved in the initiation of infection by BDV. This situation resembles that of filoviruses. However, in the case of BDV the predicted N-terminal part of p56 (gp41), which is generated upon cleavage by furin, has not been detected yet in BDV infected cells, suggesting a rapid degradation of this product upon cleavage of gp84. In BDV-infected cells, both gp84 and gp43 remained sensitive to both Endo F and H (10), suggesting that these polypetides do not mature in the Golgi complex. Together, these features are indicative of a novel mechanism for the maturation of a NNS RNA virus surface glycoprotein and hence for the assembly of BDV particles. Moreover, G expression is usually detected only in a small percentage (1-10%) of BDV persistently infected cells. These features of BDV G expression might contribute to the paucity of production of infectious viral particles and exquisite ability of BDV to establish persistence

6. NUCLEOCYTOPLASMIC TRANSPORT DURING THE LIFECYCLE OF BDV

Completion of the BDV lifecycle requires a variety of nuclear transport events involving viral macromolecules, namely: (i) import of RNP containing the incoming viral genome; (ii) export of transcribed BDV mRNA for translation in the cytosol; (iii) import of viral proteins required for control of virus RNA synthesis and formation of RNP; (iv) export of assembled BDV RNP. Each of these processes is distinct, and the same viral components can be part of multiple transfer events in and out of the nucleus, each of which may utilize different mechanisms and different signals (52-54). The mechanisms involved in the control of trafficking of viral RNP across the nuclear envelope, and BDV mRNA nuclear export remain largely unknown. In contrast, several of the signals and interactions involved in nucleocytoplasmic transport of BDV proteins have been elucidated during the last few years, and will be the focus of this section.

The nucleprotein (N) is the most abundant BDV protein present in virus infected cells both in tissue culture systems and in BDV-infected animals. N is mainly found in the nucleus, although present also at lower levels in the cytoplasm of BDV-infected cells (53-57). Likewise, N predominantly localized in the nuclei of cells transfected with an N-expression plasmid (14, 58, 59). Based on computer predictions it was initially proposed that positively charged residues $K^{163}KRFK^{167}$ and $R^{338}YRRREISR^{346}$ within BDV N might serve as nuclear localization signals (NLS) because of their similarity to short basic amino acid previously found to be bona fide NLS that mediate nuclear localization of proteins in eukaryotes (60). However, BDV N mutants with amino acid substitutions within the $K^{163}KRFK^{167}$ and the $R^{338}YRRREISR^{346}$ motifs were found to accumulate in the nuclei of transiently transfected cells, indicating that these two motifs were dispensable for nuclear targeting activity of BDV N (14, 58). Cells transfected with plasmids independently encoding each of the two N isoforms, p40 and p38, revealed that p40 and p38 accumulated in the nucleus and cytoplasm, respectively (14, 58, 59). This finding suggested that the nuclear targeting activity of BDV N was associated with the 13 amino-terminal residues, containing the basic amino acid-rich sequence P^3KRR^6. The p40 mutants containing deletions or amino acid substitutions that disrupted the putative P^3KRR^6 NLS showed a cytoplasmic localization instead of the nuclear one associated with wild-type p40 isoform (58). In addition, beta-galactosidase, which normally resides in the cytoplasm, exhibited a nuclear localization after its fusion to the nonapeptide $P^3KRRLVDDA^{11}$ from the N-terminus of the p40 (58). These results demonstrated that $P^3KRRLVDDA^{11}$ represents a bona fide NLS of BDV N.

The isoform p38 of N lacks the NLS present in the p40 isoform, and as predicted p38 accumulates in the cytoplasm of cells transfected with the corresponding expression plasmid. Interestingly, immunoprecipitation of lysates prepared after subcellular fractionation of BDV-infected cells showed that p38 and p40 were present in both

nuclear and cytoplasmic fractions (14). Both p38-and p40 proteins were also found in the nucleus and cytoplasm of cells cotransfected with plasmids expressing p38 and p40, suggesting an interaction between the two N isoforms (58, 59). This intracellular interaction between p40 and p38 was further supported by results of coimmunoprecipation studies. These findings suggest that p38 can be imported into the nucleus through interaction with p40. Furthermore, the cytoplasmic retention of p40 when interacting with p38 raised the possibility that in addition to its nuclear targetting activity, BDV N also has a nuclear export activity. Cells were transfected with recombinant expression plasmids containing the DNA sequence encoding the green fluorescent protein (GFP) fused to the sequence encoding N. Analyses of the subcellular distribution of the recombinant GFP-N fusion proteins revealed that a segment of N corresponding to amino acids 128-145 was sufficient to mediate the cytoplasmic accumulation of the GFP chimeric protein (59). This N segment contained a leucine rich motif (L^{128}TELEISSIFSHCC^{141}) similar to those found in other well characterized nuclear export signals (NES). Mutations of the L or I positions within this putative NES, abrogated the nuclear export activity of N. Proteins containing a leucine-rich NES use CRM1/exportin 1 as the export receptor to travel through the nucleopore complex (NCP: (54). The cell permeable fungal metabolite leptomycin B (LMB), specifically inhibits the interaction beween the cargo and CRM1. LMB treatment abrogated the nuclear exclusion of GFP containing the NES of N, indicating that nuclear export of N is mediated by a CRM1-dependent pathway (54). Thus, BDV N exhibits the behaviour of a bona fide nucleocytoplasmic shuttling protein, and likely contributes to the nucleocytoplasmic transport of the BDV RNP.

The biological implications of the existence of two N isoforms with different subcellular targeting properties remains to be determined. The p38 co-localized also with P in the nuclei of cells co-transfected with the p38- and P-expression plasmids, suggesting a P/p38 interaction (14, 58, 59). *In vitro* binding studies have suggested that two regions of N (amino acids 56-100 and 131-155) are involved in binding to P (61). Hence, it seems likely that p38 lacking an NLS can be transported into the nucleus from the cytoplasm through hetero-dimerization with N, or P. Interestingly, p38 was retained in the nuclei of the co-transfected cells. This retention could be due to the overlap between the NES of p38 and one of its P binding sites, which might result in masking of the p38 NES. Therefore, it is possible that by interacting with p40 and P, p38 might play a role in the control of the bi-directional trafficking of BDV RNP.

The BDV P and P' can be detected in the cytoplasm and nucleus of BDV-infected cells (56, 62), and both P and P' are found in the nucleus of cells transfected with the corresponding expression plasmids (16, 56, 63). Inspection of the ORF II amino acid sequence identified a basic amino acid-rich sequence, R^{22}RKRSGSPRPRK^{33}, as a good candidate for being the NLS of P. Analysis of carboxy-and amino-terminal truncation mutants of P showed that amino acids 20-37 were sufficient to promote efficient nuclear accumulation of a P-GFP fusion protein (64). This NLS comprised of amino acids 20-37 of P, appeared to have a bipartite structure consisting of two basic amino acid-rich sequences: R^{22}RER^{25} and R^{30}PRKIPR^{36}, separated by four non-basic amino acids, S^{26}GSP^{29}. There is evidence that proline at position 29 may be also part of this NLS (63). The P' lacks the first 55 amino acid residues of P which include the N-terminal NLS. However, P' is capable of translocation into the nucleus, indicating the existence of an additional NLS within P/P', with the capability of operating independently of the N-terminal NLS. Results from the subcellular localization of a variety of P mutants, and of chimeric proteins consisting of various portions of P fused to beta-galactosidase provided evidence of the existence of an additional independent NLS (P^{181}PRIYPQLPSAPT^{193}) located in the in the carboxy-terminal region (63). Unlike other previously described NLS, frequently rich in basic amino acids, both P NLS are proline-rich instead. P was also found to bind to serpin and pendulin (karyopherin alpha/importin-60), proteins implicated in NLS binding during nuclear import, providing additional support of the nuclear targeting activity of P. The N-P complexes have been found in the nuclei of infected cells. Mutational analysis of P showed that its C-terminus (amino acid positions 197-201) contains a N-binding site. Therefore, nucleocytoplasmic transport of P could also be mediated by its interaction with N.

The BDV p10 is detected in brain cells of naturally and experimentally infected animals (17). Immunofluorescence analyses of BDV-infected cells using a rabbit polyclonal serum to p10 showed that p10 is present in the nucleus and cytoplasm of infected cells both in cultured cells and infected animals (17, 65). However, inspection of the amino acid sequence of p10 ORF failed to reveal the presence of a motif similar to previously identified NLS's of nuclear proteins. Moreover, in p10-transfected cells, p10 protein was detected predominantly in the cytoplasm. Results from the yeast two-hybrid system have failed to show and interaction of p10 with with serpin or pendulin, further suggesting that the nuclear localization of p10 is dependent on its interaction with other viral proteins. The p10 was found in the nuclei of cells co-transfected with p10 and P, and coimmunoprecipitation studies indicated that p10 forms a complex with P. Direct interaction between p10 and P, but not between p10 and N, has been also demonstrated in the yeast two-hybrid system, and in vitro binding assays using Ni-agarose-bound N (64, 66). In contrast to these findings, immunofluorescence studies documented a nuclear co-localization of p10 and N in cells co-transfected with p10 and N expression plasmids (65). Furthermore, in vitro protein-protein interaction studies on solid phase-bound N showed a direct interaction between p10 and N (65). Currently available evidence is conflicting and does not permit to reach a final conclusion on whether there is a direct interaction between p10 and N proteins. Subcellular localization studies showed co-localization of p10 with P' in BDV-infected cells. Whether this finding reflects a direct p10/P' interaction remains to be clarified. Results from coimmunoprecipitation studies

supported a direct interaction between p10 and P' (16). However, binding of P to p10 has been mapped to amino acid positions 33-115 of P (67). This domain is likely to be disrupted in P' because the 55 N-terminal amino acid residues of P are not present in P'.

The BDV p10 protein contains an amino-terminal short peptide motif that is similar to those previously characterized as bona fide NES in the Rev protein of HIV-1 and the NEP/NS2 proteins of influenza virus, as well as the cellular cAMP-dependent protein kinase inhibitor. This finding led to the proposal that p10 could play a role in nuclear export of BDV proteins and RNP (64). However, recent studies on p10-P interaction demonstrated that an amino-terminal motif rich in leucine, S^{2}SDLRLTLLELVRRL^{16}, of p10 mediates the p10-P interaction, and that S^{2} and L^{16} are important for this interaction (64, 67). Therefore, the putative NES of p10 would overlap with the P-binding site, S^{2}SDLRLTLLELVRRL^{16} which could interfere with the predicted nuclear export activity of p10. Moreover, a p10 mutant lacking this putative NES was also predominantly found in the cytoplasm (67), suggesting that this putative NES may not play an important role in localizing the p10 protein to the cytoplasm. Further studies will be needed to elucidate the nuclear export activity and the regulatory function(s) of p10.

Transcription and replication of the BDV genome take place in the nucleus. Therefore, BDV L protein, the predicted viral RNA-dependent RNA polymerase, should be present also in the nucleus. Within the BDV L amino acid sequence are motifs (V^{189}SKNAKWPPV^{197} and W^{943}YKVRKVT^{950}) that resemble those proposed to mediate nuclear import of the L protein of the plant nucleorhabdovirus sonchus yellow neat virus (SYNV). However, it is unknown whether these sequences actually mediate nuclear targeting of BDV L. Immunofluorescence analyses with antisera specific to synthetic L peptides demonstrated that, in the absence of other viral proteins, the L protein is predominantly present in the nuclei of cells transfected with the eukaryotic L expression plasmid (28). This suggests that the L protein may possess a functional NLS and is actively translocated to the nucleus after its synthesis in the cytoplasm.

As with other NNS RNA viruses, BDV P and L are expected to interact during the formation of the virus polymerase complex. Coimmunoprecipitation experiments have provided evidence for such interaction. In addition, P and L appear to co-localize in the nucleus of BDV-infected cells (28). Unexpectedly, when L and P were co-transfected in cells, L was found in the cytoplasm, whereas P was found in the cytoplasm and the nucleus. It was proposed that over-expression of L and P might have contributed to the observed phenomenon. However, this finding could also reflect our currently limited knowledge about the mechanisms involved in nucleocytoplasmic transport of BDV proteins.

A model for the nucleocytoplasmic trafficking of BDV RNP has been proposed based on the NLS and NES signals, as well as interacting domains, identified in BDV proteins (28). This model proposes that relative levels of p40, p38 and P may play a key role determining the directionality of BDV RNP movement. Increased levels of nuclear P, due to an overlap between the NES present in N and one of the N binding sites to P, could negatively affect nuclear export mediated by the NES of N. On the other hand, increased levels of p38 could result in a net increase in the density of NES in p40/p38 complexes. This, in turn, could enhance nuclear export over retention. Although very attractive, this model does not satisfactorily explain the observation that BDV RNP, containing the virus genome RNA, accumulate in the nucleus, and are barely detectable in the cytoplasm at any stage of infection. In addition, there is little evidence supporting significant changes in the ratio N/P during the first 96 hours following BDV infection of a variety of susceptible cell lines. However, during this time the virus is able to complete its lifecycle as determined by a steady state increase in the number of infected cells

7. PERSPECTIVES

BDV has unique genetic and biological features, representing the prototypic member of a new group of RNA animal viruses. Therefore, the investigation of its molecular and cellular biology may provide us with new insights about the biology of these infectious agents. As with other mononegaviruses, these investigations of the molecular biology of BDV will greatly benefit from the establishment of a reverse genetic system. Such system will facilitate a detailed analysis of the virus RNA *cis*-acting signals and viral trans-acting factors required for BDV transcription, RNA replication, assembly and budding of infectious particles. Furthermore, the possibility to generate predetermined specific mutations within the BDV genome, and analyze their phenotypic expression in animal models, will provide a new and powerful approach to elucidate the molecular mechanisms underlying BDV-host interactions, including BDV persistence and associated disease. Evidence indicates that BDV might infect humans. However, the prevalence of BDV in humans and its possible association with certain neuropsychiatric disorders remains to be solved. Increased knowledge about the molecular and cellular biology of BDV should help in the development of standardized sensitive and reliable serological and nucleic acid diagnostic tests to address this pressing question.

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