JOURNAL OF VIROLOGY, Dec. 1994, p. 7669–7675
0022-538X/94/$04.00+0

Vol. 68, No. 12

MINIREVIEW

Molecular Biology of Borna Disease Virus: Prototype of a New Group of Animal Viruses

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Borna disease virus (BDV), the etiological agent of Borna disease (BD), causes a central nervous system disease that is manifested by profound behavioral abnormalities, accumulation of disease-specific antigens in limbic system neurons, and often, but not always, the presence of inflammatory cell infiltrates in the brain (35, 36, 47). BDV has been characterized recently as a nonsegmented, negative-stranded (NNS) RNA virus with the property, unique among NNS RNA animal viruses, of a nuclear site for replication and transcription of its genome, suggesting that BDV is the prototype of a new group of animal RNA viruses.

Originally described in the early 19th century in horses in Germany, BD has become an important model for the study of both the mechanisms and the biological consequences of persistent virus infection of the central nervous system (35). Naturally occurring infections with BDV have been confirmed as yet only in horses, sheep, cats, cattle, and ostriches (6, 31, 35–38). However, BD can be experimentally transmitted to a wide range of animal species from birds and rabbits to nonhuman primates, with a variable period of incubation, ranging from several weeks to many months, as well as diverse pathological manifestations, depending on the species, immune status, age of the host, route of infection, and the particular virus isolate used for the infection (35, 36, 41, 42, 47). BDV infection induces behavioral changes that resemble some types of affective neuropsychiatric disorders in humans. Furthermore, serological data suggest an association of BDV, or a related virus, with specific psychiatric diseases in humans (1, 7–9, 23, 49, 58), providing further impetus for the study of this neurotropic infectious agent.

Sensitivity to organic solvents, UV light, and detergents suggest that BDV is an enveloped virus, while results from filtration experiments estimate that BDV particles have a size of 80 to 125 nm (21, 35, 43). In addition, although different BDV isolates may display remarkable phenotypic differences in their interactions with the host, all BDV isolates are characterized by noncytolytic replication and by being highly neurotropic, with cells and tissues of nonneuronal origin exhibiting low susceptibility to BDV infection (12, 13, 30, 33, 35, 36, 40, 47).

Progress in understanding the pathogenesis of BDV has only recently been complemented by gains in knowledge about its molecular biology. The latter constitutes the focus of this review, as the pathogenesis and immunobiology of BDV have been the subject of several recent reviews (27, 35, 36, 47).

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BDV IS A SINGLE-STRANDED RNA VIRUS

The lack of cell-free virus associated with BDV infection has hampered its molecular characterization. However, the recent isolation of BDV-specific cDNAs corresponding to each of the two known major BDV-specific antigens, the 24-kDa p24 and 40-kDa p40 BDV polypeptides (39, 45, 55, 58), together with the availability of persistently infected cell lines that provide a homogeneously infected cell population with all cells expressing both BDV RNA and antigens (13, 20, 30) have furnished the tools to initiate the molecular characterization of BDV. Southern blot hybridization studies with genomic and episomal DNA from a cell line persistently infected with BDV (BDV-Pi) did not detect BDV-specific sequences under experimental conditions that allowed the detection of 0.5 copies of BDV-specific sequences per cell genome equivalent (20). Moreover, pretreatment of RNA obtained from BDV-Pi cells with low concentrations of RNase A at high ionic strength prevented hybridization to a BDV-specific probe (17), indicating that double-stranded RNA species are not associated with BDV infection. These results led to the conclusion that BDV was not a retrovirus or DNA virus but rather a single-stranded RNA virus (17, 20).

BDV REPLICATES AND TRANSCRIBES IN THE NUCLEUS

Studies addressing the subcellular distribution and polyadenylation of BDV RNAs detected by a BDV-specific probe corresponding to BDV p24 revealed that BDV poly(A)$^+$ RNAs of approximately 0.85, 2.1, and 3.5 kb were present in both the cytoplasmic and nuclear fractions but at much higher levels in the former (17). In contrast, an additional nonpolyadenylated BDV RNA of approximately 9 kb was found mostly associated with the nuclear fraction (17). The use of specific-stranded riboprobes showed that BDV poly(A)$^+$ RNAs have sense polarity, whereas the 9-kb nuclear poly(A)$^-$ BDV RNA has mostly antisense polarity (17, 20, 34). Furthermore, cytoplasmic poly(A)$^+$ RNA, but not nuclear poly(A)$^-$ RNA from BDV-Pi cells, directed the synthesis of 24- and 40-kDa BDV polypeptides in in vitro translation assays (17, 34, 58).

Analysis of RNA isolated from partially purified BDV infectious particles obtained by hypertonic treatment of BDV-Pi cells (10, 17, 44, 61), as well as by treatment with the lipid solvent Freon-113 (46), revealed the presence of one single BDV-specific RNA species of 9 kb (10, 17, 61) or 10 kb (46) characterized as poly(A)$^-$ and of negative polarity (10, 17, 46, 61). Although the difference in size reported by different groups remains to be explained, these results indicated that the 9-kb nuclear poly(A)$^-$ negative-polarity RNA represents a BDV genomic RNA species.

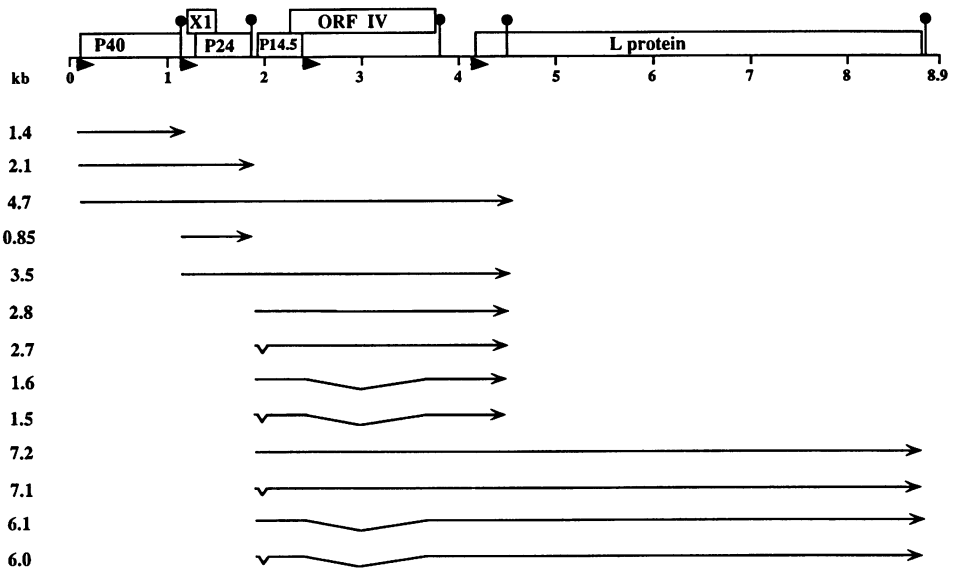

FIG. 1. Genomic organization and transcriptional map of the BDV genome RNA. Putative transcription initiation signals (▶) and transcription termination-polyadenylation signals (🎈) in the BDV genome are indicated (10, 17, 18, 52).

New synthesis of both BDV poly(A)$^+$ RNA and poly(A)$^-$ negative-polarity genomic RNA species was found to take place in the nucleus of infected cells (17), from which newly synthesized BDV poly(A)$^+$ RNAs are efficiently transported to the cytoplasmic compartment, whereas the 9-kb BDV genomic RNA species remains mostly associated with the nuclear fraction as determined by nucleocytoplasmic RNA transport assays (10). Thus, both transcription and replication of the BDV genome occur in the nuclei of infected cells.

BDV HAS AN NNS RNA GENOME

Negative-stranded RNA virus genomes are tightly associated with ribonucleoprotein (RNP) complexes, which constitute the minimal subviral structure able to carry out transcription and replication (26, 57). Nuclear RNPs isolated from BDV-Pi cells contained only one BDV-specific RNA species resistant to micrococcal nuclease digestion, as determined by Northern (RNA) blot hybridization using a BDV p24 probe (17). This encapsidated RNA had a size of 9 kb and was characterized as poly(A)$^-$, with more than 90% being of negative polarity as determined with strand-specific riboprobes (17). The significant amount of encapsidated 9-kb BDV RNA of positive polarity is likely to represent the full-length antigenomic template required for the replication of negative-stranded RNA virus genomes (26, 57).

As with other negative-stranded RNA viruses, BDV RNPs were also found to be infectious on the basis of an ability to direct the synthesis of BDV macromolecules, both protein and nucleic acids, as well as the production of BDV cell-associated infectivity upon transfection of BDV-susceptible cells (17). Infectious BDV RNPs were resistant to nonionic detergent treatment but were destroyed by protease treatment, whereas naked RNA extracted from infectious BDV RNPs lacks the ability to direct viral RNA and protein synthesis after transfection (17), a result anticipated for a negative-stranded RNA virus. Analysis of micrococcal nuclease-resistant RNA extracted from nuclear RNPs prepared from in vivo ^{32}P-radiolabeled BDV-Pi cells revealed that a single ^{32}P-RNA species of approximately 9 kb was associated with infectious BDV RNPs (17). The viral origin of this RNA was confirmed by its hybridization to a BDV-specific probe. These results indicated that BDV has an NNS RNA genome.

SEQUENCE AND GENOMIC ORGANIZATION OF BDV

Open reading frames (ORFs) predicted in the BDV genome sequence. The cloning and complete sequence of two BDV genomes have recently been reported (11, 18). One sequence was derived from RNA extracted from BDV RNPs prepared from C6 cells persistently infected with BDV Giessen strain (18), whereas the other was generated from RNA obtained from partially purified infectious BDV particles produced by an oligodendrocyte cell line infected with BDV strain V (11). The two sequences had 95% homology at the nucleotide level and predicted the same BDV genomic organization, similar to that of other members of the Mononegavirales order (11, 18, 57).

Five major ORFs are predicted in the BDV genome sequence (11, 18) (Fig. 1). ORFs I, II, and III correspond to the previously identified 40-kDa p40, 24-kDa p24, and 14.5-kDa p14.5 BDV proteins, respectively (34, 39, 45, 50, 55, 56). BDV p24 is an acidic polypeptide (pI, 4.8) that has a high Ser-Thr content (16%), with phosphorylation at serine residues (55), and an N-terminal cluster of acidic amino acids. p24 is thus a good candidate for the BDV counterpart of the phosphoprotein transcriptional activator found in other NNS RNA viruses (2, 3, 57). The polypeptides predicted by ORFs IV and V have not yet been identified in protein samples from BDV-infected material. ORF IV overlaps with the C terminus of p14.5 by 28 amino acids (aa) and is capable of encoding a 503-aa polypeptide with a predicted molecular mass of 56 kDa. This is likely to be a viral glycoprotein on the basis of sequence features that include several putative N-linked glycosylation sites as well as the presence of hydrophobic domains at both the N and C termini, similar to the glycoproteins of other NNS RNA viruses (11, 18). The first three AUGs within ORF IV, two of which lie within the overlap with p14.5, are located in a poor sequence context for translation initiation, whereas the fourth AUG, located 451 nucleotides (nt) downstream from the initiation of

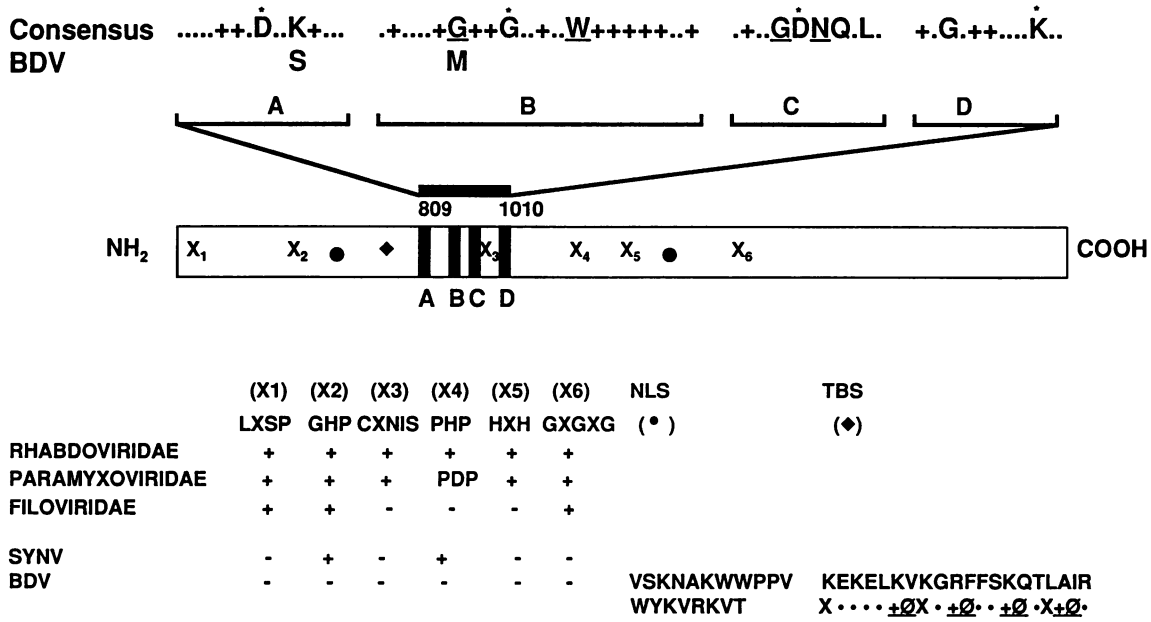

FIG. 2. Conserved amino acid sequence motifs in the BDV L protein and its comparison with other L proteins. NLS, putative nuclear localization signals; TBS, putative template binding site; +, positively charged amino acids; ø, hydrophobic amino acids.

ORF IV, has an optimal translational start (TS) signal (14). Which of these AUGs is used remains to be determined, as the polypeptide corresponding to ORF IV has not yet been detected. Interestingly, several genomic clones derived from BDV-Pi C6BV cells contained a single nucleotide deletion at nt 2366, generating a truncated ORF IV that lacks the 150 N-terminal aa and a small ORF ×2 capable of encoding a predicted protein of 12 kDa that overlaps with the C terminus of p14.5 (18). In addition to the putative viral glycoprotein predicted by ORF IV, binding studies using *Canavalia ensiformis* lectin have identified a glycoprotein of ~17 kDa associated with purified BDV infectious particles, which likely corresponds to ORF III (53). Finally, ORF V is capable of encoding a protein with a predicted molecular mass of 180 kDa, whose deduced amino acid sequence displays strong homology to other NNS RNA virus polymerases of the L protein family (11, 18). The highest level of homology corresponded to the putative catalytic domain, consisting of 5 invariant and 22 conservatively maintained residues clustered in four highly conserved motifs: A, B, C, and D (57) (Fig. 2). In addition, a putative template-binding site previously identified in the L proteins of NNS RNA viruses (57) is also found in BDV ORF V (18). Moreover, the sequences VSKNAKWPPV and WYKVRKVT, which correspond to aa 189 to 197 and 943 to 950, respectively, in BDV ORF V, are putative nuclear localization signals (25, 48) that could mediate transport of the BDV L protein to the nucleus where BDV replication and transcription take place.

The genomic polarity has a very limited coding capability, and none of the predicted ORFs has a favorable TS; further they are not flanked by putative transcription start and termination-polyadenylation signals (11, 18). The possibility that these ORFs direct synthesis of polypeptides in BDV-infected cells has not been formally eliminated; however, it presently seems unlikely that BDV has an ambisense coding strategy.

Putative transcription initiation (S) and termination-polyadenylation (E) signals. Like those of other NNS RNA viruses, BDV ORFs are flanked by nontranslated boundary sequences where putative regulatory signals involved in transcription initiation (S) and termination-polyadenylation (E) are located (2, 11, 18, 22, 24, 32a, 57). However, no evident S and E signals were found flanking p14.5 (11, 18). Both S and E putative signals showed minor variations among the different BDV ORFs. The deduced BDV S consensus sequence (3′UNC NNNUUNN5′) is identical to the one obtained when NNS RNA viruses of the three Mononegavirales families, *Filoviridae*, *Paramyxoviridae*, and *Rhabdoviridae*, are compared (18, 22). Similarly, the deduced BDV E consensus sequence [3′U(G/A)(G/A)(U/G)AUUUUU5′] also shows strong similarities to those found in other NNS RNA viruses (18, 22). Two additional putative E signals are found within ORF V around nt 4500 and 5228, respectively, in the BDV genome, whereas ORF V is not followed by an S signal (11, 18).

Analysis of noncoding sequences at the 3′ and 5′ ends of the BDV genome. Extracistronic sequences known as positive- and negative-stranded leader RNAs are found at the 3′ and 5′ ends, respectively, of the NNS RNA virus genomes (2, 22, 24, 32a). Putative positive- and negative-stranded leader RNAs are also predicted in the BDV genome sequence (11, 18). Similar to those of other NNS RNA viruses, BDV 3′-terminal genomic sequences have a high A+U content with a U/A ratio of ~2:1. The conserved motif found among 3′ leader sequences of members of the Mononegavirales, characterized by the presence of U residues at every second or third position (5, 22), is also found in BDV (18) as is the conserved sequence 3′UAUUU5′ located in the middle of the 3′-terminal genomic sequences of NNS RNA viruses (22). Moreover, comparison of the 3′ and 5′ termini of BDV genomic RNA revealed terminal sequence complementarity similar to that found in other negative-stranded RNA viruses (2, 11, 18, 24, 32a, 32b, 57). In rhabdoviruses, positive-stranded leader RNAs, the initial products of viral transcription, are essential elements in transcription and replication (2, 3), whereas positive-stranded leader RNAs may not always be essential operating elements during transcription and replication of paramyxoviruses (4). Neither positive- nor negative-stranded leader RNAs have yet

been detected in filovirus-infected cells (22) nor in BDV-infected cells.

TRANSCRIPTION OF BDV GENOME RNA

BDV RNA species detected in BDV-infected cells. Analysis of poly(A)$^+$ RNA obtained from BDV-infected cells and tissues by Northern blot hybridization with a set of overlapping probes spanning the entire BDV genome identified nine species of poly(A)$^+$ subgenomic RNAs with polarity complementary to the negative-stranded RNA genome (11, 18). Mapping of these transcripts to the genomic RNA indicated that only three different S signals and four different E signals appear to be used during BDV transcription (11, 18) (Fig. 1). Monocistronic transcripts were detected only for p40 and p24 (11, 18). In addition, bicistronic (p40 + p24), tricistronic (p24 + p14.5 + ORF IV), and tetracistronic (p40 + p24 + p14.5 + ORF IV) transcripts of 2.1, 3.5, and 4.7 kb, respectively, were also detected (11, 18). Four additional transcripts of 7.2, 6.1, 2.8, and 1.6 kb that were related in sequence were also detected (11, 18, 19, 52). Transcription of these four RNAs appears to initiate around the same position near the beginning of p14.5. The 7.2- and 6.1-kb transcripts use the E signal located after the stop codon of ORF V, whereas the 2.8- and 1.6-kb transcripts appear to use the E signal located within ORF V around position 4510 on the BDV genome (11, 18, 19, 51).

Sequential and polar transcription are key features of NNS RNA virus transcription (2, 3, 24, 32a, 57). The polarity in molar quantity of transcripts as a result of a progressive loss of efficiency of transcription from the 3′- to the 5′-encoded cistrons constitutes a major element of transcriptional regulation for many NNS RNA viruses (2, 3, 24, 32a, 57). BDV transcription appears to have rather unique features. The absence of monocistronic transcripts corresponding to several viral polypeptides (p14.5 as well as ORFs IV and V) represents a situation not previously described for other NNS RNA viruses. In addition, initiation of BDV 1.6-, 2.8-, 6.1-, and 7.2-kb transcripts appears to occur in an area of the BDV genome RNA devoid of recognizable S sequence motifs. Moreover, BDV polycistronic poly(A)$^+$ RNAs accounted for about 55% of the total synthesis of BDV poly(A)$^+$ transcripts, which is considerably higher than the levels of polycistronic poly(A)$^+$ RNA synthesis described during infection with rhabdoviruses and paramyxoviruses (24, 32a, 60). Furthermore, E signals for two abundant BDV transcripts, 2.8 and 1.6 kb, are located within ORF V, which is likely to influence the efficiency of transcription of BDV ORF V; this is similar to the situation described for respiratory syncytial virus (15).

RNA splicing contributes to the generation of mature BDV mRNAs. A more detailed mapping of BDV 7.2-, 6.1-, 2.8-, and 1.6-kb poly(A)$^+$ RNAs revealed that the 7.2- and 2.8-kb transcripts are colinear with the BDV genome, whereas the 6.1- and 1.6-kb RNAs lack sequences internal to the 7.2- and 2.8-kb transcripts, respectively (11, 19, 51). Reverse transcription-PCR experiments using both BDV genomic RNA and cytoplasmic poly(A)$^+$ RNA from BDV-infected cells confirmed an internal deletion of approximately 1.2 kb in both the 6.1- and 1.6-kb RNAs, as well as revealing the existence of BDV poly(A)$^+$ RNAs with a small internal deletion of approximately 100 nt (19, 51). Cloning and sequencing of the corresponding reverse transcription-PCR products determined the precise boundaries of these two internal deletions in the BDV genome. Inspection of the BDV antigenomic polarity sequence at the boundaries of the internal deletions revealed the presence of sequence motifs with similarities to consensus sequences of mammalian 5′ and 3′ splice sites (19, 28). Seven of nine nucleotide positions in the 5′ splice sites of both BDV introns I (93 nt) and II (1,293 nt) matched the mammalian consensus sequence for 5′ splice sites. In addition, sequences characteristic of 3′ splice sites (branch site region, polypyrimidine tract, and AG dinucleotide) preceded the 3′ ends of the breakpoints. Furthermore, purine-rich sequences with strong similarities to those previously described as splicing enhancer sequences (54, 59) were also found in the downstream exon close to the 3′ splice site of both BDV introns I and II (19). BDV introns I and II span nt 1932 to 2025 and 2410 to 3703, respectively, in the BDV antigenomic sequence (11, 18). Splicing of intron I would place the amino acid in position 13 of p14.5 next to a stop codon, whereas splicing of intron II would generate a truncated version of ORF IV containing its first 58 aa fused to a new C terminus of 20 aa. An additional ORF predicted in RNA species resulting from the splicing of intron II would encode a variant BDV L protein with 153 aa added to the N terminus (19, 51) (Fig. 3). Whether these new predicted BDV polypeptides are synthesized in infected cells and what their possible functions are remain to be determined. However, RNA splicing may play an important role in the regulation of BDV genome expression by increasing the versatility of its primary transcripts, as well as by providing the possibility for the controlled expression of specific BDV protein domains and synthesis of new BDV polypeptides (29, 52).

STRUCTURE OF BDV PARTICLES

Partial purification of BDV infectious particles released from BDV-infected oligo/TL cells by hypertonic treatment and further purification by cesium chloride gradient centrifugation lead to the recovery of most of the infectivity in a gradient fraction corresponding to a band with a density of 1.22 g/cm^3 (61). Treatment of this infectious gradient fraction with BDV-neutralizing antibodies prior to inoculation into rabbits and rats prevented the development of BD (61). Electron microscopy of negatively stained particles present in the gradient fraction at 1.22 g/cm^3 shows two distinct groups of spherical particles; one with a diameter of 90 nm ± 10 nm, the other approximately 50 to 60 nm in diameter. The larger particles are enveloped and contain an electron-dense core (50 to 60 nm). BDV-neutralizing antisera in combination with colloidal gold conjugates provided a distinct marking of BDV particles of both 90 nm ± 10 nm and 50 to 60 nm. Nonneutralizing antibodies did not label these BDV particles (61).

Thin sections of BDV-Pi cells revealed the presence of intracytoplasmic viruslike particles ranging from 50 to 100 nm in diameter (16). These particles were found in groups free in the cytoplasm, close to the plasma membrane. Most of the particles appeared roughly spherical, with a limiting outer membrane and an internal structure consisting of strandlike material which in some cases was condensed underneath the envelope. The particles showed no association with cisternae of the endoplasmic reticulum, the Golgi complex, nor other intracytoplasmic membranes, and the assembly process or site at which these particles are assembled has not been observed (16).

CONCLUDING REMARKS

It is now established that BDV has an NNS RNA genome, whose organization is similar to that displayed by other members of the Mononegavirales order, characterized by three conserved gene blocks (57). Block 1 codes for the nucleopro-

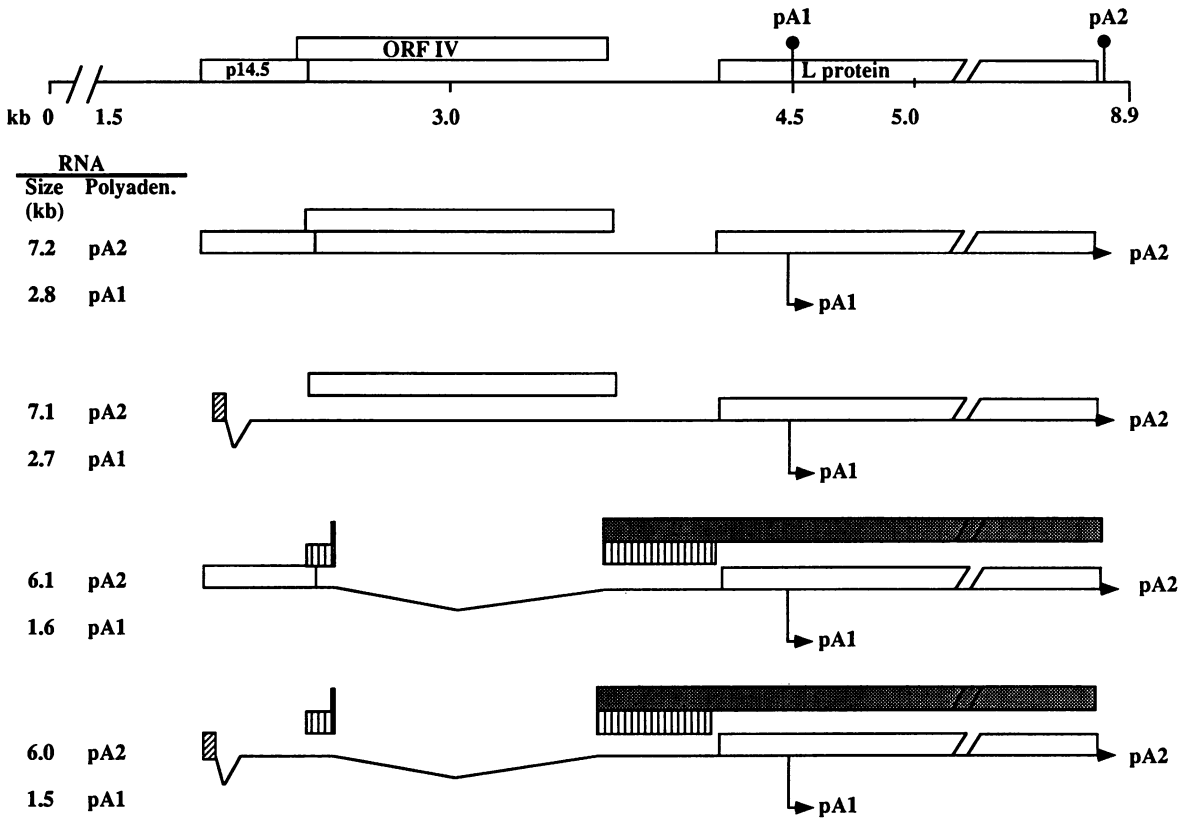

FIG. 3. Schematic of new predicted BDV polypeptides generated by RNA splicing.

teins and polymerase cofactors, which may correspond to p40 (ORF I) and p24 (ORF II) in the BDV genome; block 2 codes for the matrix and envelope proteins, whose likely counterparts in the BDV genome are p14.5 (ORF III) and ORF IV; and block 3 codes for the viral polymerase, which has been identified as ORF V in the BDV genome. However, BDV has the property, unique among NNS RNA animal viruses, of nuclear transcription and replication. Consequently, BDV may represent the prototype of a new group of animal RNA viruses. It is worth noting that multiplication of members of the plant genus *Nucleorhabdovirus*, among them sonchus yellow net virus (SYNV), also occurs in the nucleus, and interestingly the L proteins of BDV and SYNV are the most distantly related to the L proteins from animal rhabdoviruses (18), raising some interesting questions about the evolutionary origins of BDV.

The complete sequence of the BDV genome has been determined, and thus it is now possible to conduct a detailed molecular analysis of the genomic organization and gene products encoded by BDV as well as to investigate the regulation of BDV genome expression. The establishment of an in vitro system for the analysis of BDV transcription and replication, together with the ability to produce large amounts of individual and functional BDV polypeptides, will help to define the functions associated with each of the BDV polypeptides and to understand the molecular mechanisms involved in the control of BDV genome expression. Moreover, some recently developed experimental approaches for manipulation of negative-stranded RNA genomes (26) should be applicable to the BDV system, providing the tools to examine the role of *cis*- and *trans*-acting factors in BDV replication and transcription, as well as, eventually, assembly and pathogenesis. Studies aiming to define the biological activity and expression regulation of BDV polycistronic transcripts containing ORFs IV and V, the putative viral glycoprotein and polymerase, respectively, and the investigation of the role and regulation of RNA splicing in the generation of BDV mRNAs will provide important information for understanding BDV genome expression.

The cellular site and process of BDV morphogenesis, as well as the mechanisms of cell-to-cell transmission used by BDV, also constitute areas of great interest. Preliminary studies suggest that envelopes of BDV particles observed in infected cells are in direct contact with the cytoplasmic matrix, a topology which would not be expected to be compatible with the presence of a layer of external surface glycoproteins, a situation also described for rabies virus (16). The establishment of a cell culture system which produces higher yields of infectious virus particles would be of great value for the further investigation of the assembly process of BDV.

Information is now available that will allow the design of sensitive molecular probes and screening strategies for BDV. Such reagents should then permit molecular epidemiological studies to rigorously prove or disprove an association of BDV, or a related virus, with human psychiatric disorders. The possible role of BDV in human health together with the prospect of finding other BDV-like viruses, some of them possibly clinically and/or economically relevant, underscore the importance of developing effective antiviral therapies for the control and cure of persistent BDV infections, a task that will be facilitated by an increased knowledge of the molecular biology of BDV.

ACKNOWLEDGMENTS

I express my deep appreciation to I. Lipkin for introducing me to the BDV system, as well as for his support and encouragement of my laborarory's work on BDV. I thank P. Borrow, M. Eddleston, M. Oldstone, and J. Holland for helpful discussions. I also express my especial recognition to B. Cubitt and C. Oldstone, members of my laboratory.

This research was supported in part by USPHS grant NS-12428.

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