Genomic organization of Borna disease virus

(central nervous system infection/behavioral disorders/negative-strand RNA viruses)

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ABSTRACT Borna disease virus is a neurotropic negative-strand RNA virus that infects a wide range of vertebrate hosts, causing disturbances in movement and behavior. We have cloned and sequenced the 8910-nucleotide viral genome by using RNA from Borna disease virus particles. The viral genome has complementary 3' and 5' termini and contains antisense information for five open reading frames. Homology to Filoviridae, Paramyxoviridae, and Rhabdoviridae is found in both cistronic and extracistronic regions. Northern analysis indicates that the virus transcribes mono- and polycistronic RNAs and uses termination/polyadenylation signals reminiscent of those observed in other negative-strand RNA viruses. Borna disease virus is likely to represent a previously unrecognized genus, bornaviruses, or family, Bornaviridae, within the order Mononegavirales.

Borna disease virus (BDV) is a neurotropic virus that causes an immune-mediated syndrome resulting in disturbances in movement and behavior. Originally described as a natural infection of horses in southern Germany (1), Borna disease has now been described in sheep (2), cats (3), and domestic fowl (4). Though natural infection has not been reported in primates, subhuman primates can be infected experimentally (5, 6). Antibodies to BDV proteins have been found in patients with neuropsychiatric disorders (7–9), suggesting that BDV or a related agent may be pathogenic in humans.

Because BDV grows only to low titer, it was difficult to purify for analysis. However, the identification of BDV cDNA clones by subtractive hybridization (10, 11) and, more recently, the advent of a method for isolation of virus particles (12) led to partial characterization of BDV as a negative-strand RNA virus which transcribes its RNA in the cell nucleus (12). We have now cloned and sequenced genomic RNA from virus particles.[¶] The genomic organization of BDV indicates that it is likely to represent a distinct virus genus or family within the order Mononegavirales.

MATERIALS AND METHODS

BDV cDNA Library Preparation and Screening. Genomic RNA template for library construction was obtained from an oligodendrocyte cell line (Oligo/TL) acutely infected with BDV strain V (12). For the first genomic library, RNA from one virus particle preparation was polyadenylated with poly(A) polymerase (GIBCO/BRL) to facilitate cloning from the 3' terminus by oligo(dT)-primed cDNA synthesis. Libraries were prepared in pSPORT with the SuperScript plasmid system (GIBCO/BRL). The first library was screened with pAB5 and pAF4 radiolabeled restriction fragments (10). Subsequent libraries were screened with radiolabeled restriction fragments from locations progressively 5' on the genomic

RNA. The 5'-terminal sequence from each library was used to design an oligonucleotide primer for construction of the next library.

DNA Sequencing and Sequence Analysis. Plasmid DNA was sequenced on both strands by the dideoxynucleotide chain-termination method (13) using a modified bacteriophage T7 DNA polymerase (Sequenase version 2.0; United States Biochemical). Five to 10 independent clones from each library were sequenced with overlap so that each region of the genomic RNA was covered by at least 2 clones. Four libraries were analyzed, yielding ≈ 8.9 kb of continuous sequence. Nucleic acid sequence was analyzed with a sequence-analysis software package (Genetics Computer Group, Madison, WI). Data base searches for related sequences and multiple sequence alignments were performed with FASTA and PILEUP.

Sequence Determination at the 3' and 5' Termini of BDV Genomic RNA. Genomic RNA from one virus particle preparation ($1\text{--}2 \times 10^8$ cells) was treated with tobacco acid pyrophosphatase (Epicentre Technologies, Madison, WI) and circularized with T4 RNA ligase (New England Biolabs) (14). The ligated RNA was reverse transcribed with SuperScript II (GIBCO/BRL) using a primer, 5'-GCCTCCCCTTAGCGACACCCTGTA-3', complementary to a region 465 nt from the 5' terminus of the BDV genome. A 2- μ l aliquot of the reverse transcription reaction was used to amplify the ligated region by the PCR using Stoffel fragment (Perkin-Elmer/Cetus). Primers used in the first round of PCR were 5'-GCCTCCCCTTAGCGACACCCTGTA-3' and 5'-GAAACATATCGCGCCGTGAC-3', located 241 nt from the 3' terminus of the BDV genome. Amplified products were subjected to a second round of PCR using a nested set of primers: 5'-TACGTTGGAGTTGTTAGGAAGC-3', 251 nt from the 5' terminus, and 5'-GAGCTTAGGGAGGC-TCGCTG-3', 120 nt from the 3' terminus. PCR products were cloned (15) and sequence across the 5'/3' junction was determined from five independent isolates.

Northern Hybridization. Poly(A)⁺ RNA extracted from acutely infected rat brain by using the FastTrack system (Invitrogen) was size-fractionated in 0.22 M formaldehyde/1% agarose gels (16), transferred to Zeta-Probe GT nylon membranes (Bio-Rad), and hybridized with random-primed ³²P-labeled restriction fragments (17) representing open reading frames (ORFs) across the BDV genome (see Fig. 4b). RNA transfer, hybridization, and washing were performed with the manufacturer's protocol (Bio-Rad).

RESULTS

Sequencing of Genomic BDV RNA. Beginning from the 3' terminus, a series of four overlapping cDNA libraries was

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Abbreviations: BDV, Borna disease virus; ORF, open reading frame.
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[§]The sequence reported in this paper has been deposited in the GenBank data base (accession no. U04608).

constructed by using BDV particle RNA (12) as template. Previous studies have shown that the genomic RNA is not polyadenylated (18). Thus, to construct the first library, genomic RNA was polyadenylated *in vitro* to facilitate oligo(dT)-primed cDNA synthesis. For the subsequent three libraries, genome-complementary oligonucleotide primers were designed based on 5'-terminal sequence determined in the previous round of cloning. Each region of the genome was sequenced by using a minimum of two independent clones. To determine the sequences at the termini, genomic RNA was circularized and sequenced across the junction by using five independent clones.

The 8910-nt BDV genome contained antisense information for five major ORFs flanked by 53 nt of noncoding sequence at the 3' terminus and 91 nt of noncoding sequence at the 5' terminus (Fig. 1). In 3'-5' order, the first three ORFs encoded two previously described viral proteins, p40 (19) and p23 (20). The third, fourth, and fifth ORFs had coding capacities of 16 kDa (gp18), 57 kDa (p57), and 190 kDa (p180), respectively (Fig. 1a). Predicted amino acid sequence for the 16-kDa ORF correlated with microsequence data for an 18-kDa BDV glycoprotein (S. Kliche, T.B., and W.I.L., unpublished data), originally described as the Borna disease-associated 14.5-kDa protein (21). The first three ORFs showed no overlap and were in frame with the fifth ORF (Fig. 1b). The 57-kDa ORF was in a +1/-2 frame relative to the other four ORFs and overlapped the adjacent ORF for gp18 by 28 codons and ORF p180 by 34 codons. All ORFs were located on the positive strand, complementary to the genomic RNA. ORF analysis of the genomic (negative) strand showed only

Fig. 1. (a) Organization of the BDV genome. Hatched boxes represent coding sequence complementary to ORFs for identified proteins (p40, p23, gp18) or putative proteins (p57, p180). Overlap is indicated by cross-hatched areas. Length of coding sequence corresponding to ORFs in nucleotides is indicated in brackets. Underlined italic numbers indicate length of sequence from stop codon complement to last templated uridine of termination/polyadenylation signal (black boxes). Italics with arrow indicate number of nucleotides in intervening sequence between p40 polyadenylation signal and p23 coding sequence and between p23 polyadenylation signal and gp18 coding sequence, respectively. Italics with dashed arrow indicate number of noncoding nucleotides at termini of the genome. (b) Coding potential of genome. Genomic sequence was translated in all six possible reading frames (3'-5' negative sense; 5'-3' positive sense) by using FRAMES (Genetics Computer Group). ORFs are indicated by bars and hatched boxes.

three small ORFs, each with a coding capacity of <16 kDa (Fig. 1b).

Homology Analysis of Coding Sequence. Predicted amino acid sequence for the identified ORFs was used to examine data bases for similarity to other proteins. Previous analysis of the ORF encoding p40 had revealed distant sequence similarity to L proteins of Paramyxoviridae and Rhabdoviridae (19). FASTA analysis of translated sequence from ORFs encoding p23, gp18, and p57 showed no apparent similarity to other viral sequences; however, the p180 ORF sequence consistently retrieved L polymerases of Paramyxo- and Rhabdoviridae. Alignment of the p180 ORF sequence with the sequences of RNA-dependent RNA polymerases of negative-strand RNA viruses showed conservation of both sequence and linear order of regions homologous among these proteins. Extensive conservation was found in the four characteristic motifs for L polymerases of negative-strand RNA viruses (A-D in Fig. 2) (22, 23). With the exception of the glycine residue in motif B (position 322 of the alignment), conservation was found for the individual amino acid residues postulated to participate in polymerase function (22). Conservation was also found for a motif (a in Fig. 2) proposed to participate in template recognition (23, 24). The alignment produced by the Genetics Computer Group's PILEUP program placed the p180 ORF sequence between polymerases of Paramyxo- and Rhabdoviridae. This intermediate position is reflected by the presence of conserved amino acids which are in agreement with either the rhabdo- or the paramyxovirus sequences (★ or ×, respectively; Fig. 2). The distance between conserved motifs a and A was found to be short in BDV as it is in rhabdoviruses, whereas this region is highly variable in length and sequence among paramyxoviruses (23). The PILEUP-generated dendrogram, obtained by using complete p180 ORF and L-protein sequences, indicated that the putative BDV polymerase was more closely related to L polymerases of Rhabdoviridae than to those of Paramyxoviridae (data not shown).

Analysis of Noncoding Sequence at the Genomic Termini. The 3'-terminal genomic sequence had a high A+U content, 60.5%, with an A/U ratio of ≈1:2, similar to 3' leader sequences of other negative-strand RNA viruses. At the extreme 3' end, filo-, paramyxo- and rhabdoviruses have a common G+U-rich region (Fig. 3a). In BDV, as in respiratory syncytial virus, rabies virus, and filoviruses, this region was not located at the 3' extremity. Comparison of the 3' and 5' termini of BDV genomic RNA revealed complementarity similar to that found in other negative-strand RNA viruses (25, 26) (Fig. 3b). Alignment of the genomic termini allowed formation of a terminal panhandle, with the first 3 nt unpaired. The subsequent complementary area of 6 nt (positions 4-9 and 8907-8902) could be extended by one gap insertion between positions 8901 and 8902 resulting in an additional 10-nt stretch of complementarity with a single mismatch (positions 18 and 8994; Fig. 3b).

Identification of Potential Termination/Polyadenylation Sites. Sequence preceding the poly(A) tracts of two cloned BDV mRNAs (UA₅) (19, 20) was used to analyze genomic sequence for homologous sites that could serve as potential termination/polyadenylation signals. Seven sites were found (Fig. 4c). Northern hybridization experiments supported use of four of these sites (T2, T3, T5, and T7) and allowed identification of a termination/polyadenylation signal consensus sequence CMNMYNNWA₆, where M is A or C, Y is C or U, and W is A or U. Only one of the three remaining sites (t6) matched the consensus sequence (Fig. 4c).

Northern Hybridization Analysis. Restriction fragments representing the five ORFs were used as probes for hybridization to poly(A)⁺ RNA isolated from acutely infected rat brain by FastTrack (Fig. 4a and b). Because this procedure does not entirely eliminate poly(A)⁻ RNAs, small levels of

Fig. 2. Alignment of the p180 ORF and negative-strand RNA virus L-polymerase amino acid sequences with FILEUP. Solid lines indicate conserved L-polymerase motifs (a, A, B, C, D). BDV sequence is indicated with double arrowheads. Rhabdoviridae: RaV, rabies virus; VSV, vesicular stomatitis virus; SYN, sonchus yellow net virus. Paramyxoviridae: MeV, measles virus; SeV, Sendai virus; NDV, Newcastle disease virus; RSV, respiratory syncytial virus. Filoviridae: MaV, Marburg virus. Numbers indicate amino acid range shown. Uppercase letters in viral sequence lines indicate residues conserved in more than six sequences. Uppercase letters in consensus line (Con) indicate presence of identical or conserved amino acids in BDV. Agreement of BDV sequence with either rabdoviruses or paramyxoviruses is indicated by * or x, respectively. +, Nonconserved glycine residue in BDV.

BDV genome-size RNA can usually be detected in these preparations. To allow determination of the relative abundance of RNAs detected by each probe, exposure times were normalized to the signal of the 8.9-kb RNA. Consistent with the 3'-to-5' transcriptional gradient found for other negative-strand RNA viruses, of the eight subgenomic RNAs identified, those detected by the 3'-most probes (genomic orientation), A and B, were more abundant than those detected by the more 5' probes (Fig. 4 a and b).

Mapping of the eight transcripts to the genome by Northern hybridization indicated use of only three sites for transcriptional initiation and four sites for termination. Probes C* and E* were used to distinguish between termination at T5 or t6

(Fig. 4b). The patterns of hybridization with probes C* and E* were identical to those obtained with probes C and E, respectively, indicating termination at T5 (data not shown).

Probes corresponding to p40 (A) and p23 (B) detected monocistronic RNAs of 1.2 kb and 0.75 kb, respectively (Fig. 4). Probes A and B also detected a 1.9-kb RNA consistent with failure of transcriptional termination at the p40 termination site (27). Transcriptional readthrough was also found for polycistronic transcripts of 3.5, 2.8, and 7.1 kb. The 3.5-kb RNA detected by probes B, C, D, and C* is likely to initiate at or near the beginning of the p23 ORF and terminate at T5. The 2.8-kb RNA detected by probes C, D, and C* is likely to initiate at or near the beginning of the gp18 ORF and

Fig. 3. Sequence analysis of BDV genomic termini. (a) Similarity of 3'-terminal BDV sequence to leader regions of Rhabdoviridae (RaV, VSV), Paramyxoviridae (MeV, SeV, NDV, RSV), and Filoviridae (MaV). Abbreviations are as in Fig. 2. EboV, Ebola virus. Sequences are shown in viral RNA (3'-5', negative sense) orientation. Sequences were prealigned with BESTFIT (Genetics Computer Group) and then manually aligned by using arbitrary gap insertion to optimize nucleotide matching. (b) Comparison of complementarity at 3' and 5' termini of BDV genomic RNA with that of four other nonsegmented, negative-strand RNA viruses. The 3'- and 5'-terminal sequences for each virus are shown in viral RNA (3'-5', negative sense) orientation. Underlined sequence refers to transcriptional start of first gene or end of the L gene, respectively (predicted for BDV). The end of the L gene of RaV is located outside the region shown.

FIG. 4. Map of BDV subgenomic RNAs relative to the viral antigenome. (a) Northern hybridization analysis of rat brain poly(A)⁺ RNA. Each lane was hybridized with a probe representing a major BDV ORF as indicated by the letters A–E (see b). Results of hybridization with probes C* and E* were identical to results of hybridization with probes C and E, respectively (data not shown). Numbers at left indicate size of RNA markers in kilobases. Numbers at right indicate estimated size of major transcripts. (b) Position of viral transcripts with respect to antigenome as determined by Northern hybridization and sequence analysis. Dashed lines indicate regions in the 1.5-kb RNA and the 6.1-kb RNA that contain a deletion. The boundaries of the deletions are not known. Relative positions of probes used for Northern hybridization are shown. On the ORF map, potential start codons are indicated with upward lines; ◇, start codons predicted to be functional; x, potential start codon present in strain V that is absent in strain He/80 (see text). Potential termination sites are indicated with downward lines. Use of T2 and T3 has been confirmed (19, 20); use of T5 and T7 is consistent with hybridization results. Termination at t1, t4, and t6 has not been observed (see a). (c) Alignment of the seven potential termination sites of BDV. Location of sites is indicated in the ORF map. Stop codons are underlined. Lowercase letters indicate termination/polyadenylation consensus sequence. No termination/polyadenylation site was found at or near the end of the gp18 ORF.

terminate at T5. The 7.1-kb detected by probes C, D, C*, E*, and E is likely to initiate at or near the beginning of the gp18 ORF and to continue through T5 until it terminates at T7. Probes C and C* hybridized to both a 1.5-kb RNA and a 6.1-kb RNA. Interestingly, neither the 1.5-kb RNA nor the 6.1-kb RNA was detected by probe D, located between C and C* on the viral genome. These findings are consistent with posttranscriptional modification resulting in a 1- to 1.3-kb deletion (Fig. 4).

DISCUSSION

The order Mononegavirales, which incorporates the families Filoviridae, Paramyxoviridae, and Rhabdoviridae, has distinct characteristics that include (i) a nonsegmented negative-sense RNA genome, (ii) linear genome organization in the order 3' untranslated region/core protein genes/envelope protein genes/polymerase gene/untranslated 5' region, (iii) a virion-associated RNA-dependent RNA polymerase, (iv) a helical nucleocapsid that serves as template for replication and transcription, (v) transcription of 5–10 discrete, unprocessed mRNAs by sequential interrupted synthesis from a single promoter, and (vi) replication by synthesis of a positive-sense antigenome (28).

The genomes of rhabdo-, paramyxo-, and filoviruses range in size from 11 to 20 kb. The BDV genome has been estimated to be between 8.5 kb (10, 18) and 10.5 kb (11, 29) in length. Our data confirm that the BDV genome, at only 8910 nt, is smaller than those of other negative-strand RNA viruses. Several features suggest that BDV is a member of the order Mononegavirales: organization of ORFs on the genome; extensive sequence similarities of the largest BDV ORF to L polymerases of rhabdo-, paramyxo-, and filoviruses; homology of 3' noncoding sequence to leader sequences of Mononegavirales; and complementarity of BDV genomic termini.

In 5'-to-3' antigenomic orientation, the first ORF contains 1110 nt. Due to a more favorable translation initiation context (30), it is likely that the second AUG codon, 39 nt inside the ORF, is used to express a 357-aa protein of 39.5 kDa (p40) (27). Twenty-six nucleotides downstream of the stop codon

is a polyadenylation signal (19) (T2, Fig. 4 b and c). The second ORF starts 79 nt from the p40 polyadenylation site. It has a length of 603 nt coding for a 201-aa protein of 22.5 kDa (p23). The stop codon of the p23 ORF is part of the polyadenylation signal (20) (T3, Fig. 4 b and c). Analysis of the intergenic region between the p40 and p23 ORFs has shown that this sequence is less conserved among different BDV isolates than coding sequences for p40 and p23 (15). Therefore, expression of a small ORF in this region (x, Fig. 1b) (11, 27) that overlaps with the p23 ORF seems unlikely (15). Ten nucleotides downstream of the p23 polyadenylation signal is the third ORF, 426 nt, coding for a 142-aa (16.2 kDa) protein. Due to glycosylation, the protein expressed from this ORF has an apparent size of ≈18 kDa (gp18) (S. Kliche, T.B., and W.I.L., unpublished data). No polyadenylation signal similar to those identified for p40 and p23 mRNAs (19, 20) was found near the end of the gp18 ORF (Fig. 4 b and c). Instead, the following ORF overlaps with the end of the gp18 ORF by 28 aa. It has a total size of 1509 nt that could code for a 503-aa protein of 56.7 kDa (p57). The ORF has two AUG codons in the overlap with gp18. A third AUG located outside the overlap is 451 nt from the beginning of the ORF. Which, if any, of these AUGs is used is unknown, as no protein has been identified. A potential polyadenylation site is located 28 nt downstream of the p57 ORF (t4). However, Northern hybridization results suggest that this site is a weak or nonfunctional signal, because no major transcript(s) was found to stop at this position (Fig. 4).

The fifth ORF encompasses more than half the genome. A potential polyadenylation site (T7) similar to that seen at the end of the p40 and p23 ORFs is found 33 nt from the stop codon of the p180 ORF (Fig. 4 b and c). The size of the protein expressed from this ORF cannot be determined from current data. In BDV strain V, the ORF overlaps 34 aa with the p57 ORF. In BDV strain He/80, there is no overlap, because the first AUG (x, Fig. 4b) is changed to GUG (data not shown). Therefore, it is more likely that the AUG 351 nt downstream (◇, Fig. 4b) is used to initiate translation of a 1608-aa protein of 180.3 kDa (p180). Though there are frequent point mutations in the 351 nt between these two AUG codons, the

predicted amino acid sequence is fully conserved between strain V and strain He/80. Such conservation is distinct from the level of divergence observed for the intervening sequence between the p40 and p23 ORFs (15) and suggests that this region is under selective pressure. Translation from the proposed start codon would result in a putative BDV polymerase ≈ 200 aa smaller than L polymerases of other members of the order Mononegavirales. While all domains described as critical to polymerase function are present in the p180 ORF, it is intriguing to consider the possibility that a larger protein is expressed from the 6.1-kb RNA. Deletions identified by Northern hybridization suggest that viral mRNAs may undergo posttranscriptional modification. Whether such a mechanism leads to extension of the p180 ORF coding sequence (6.1 kb RNA) or to expression of additional viral protein(s) (1.5 kb RNA) remains to be determined.

Although functional studies of BDV proteins have not been done, the organization of the viral genome, together with the limited biochemical data available, suggests possible roles for individual proteins in the virus life cycle. Four lines of evidence suggest that p40 is likely to be a structural protein: (i) like nucleocapsid (N) proteins of rhabdo- and paramyxoviruses (31) [except pneumoviruses (32)], p40 is found in the most 3' position on the genome; (ii) p40 is similar in size to N proteins; (iii) both p40 (27, 33) and N proteins (31) are abundant in infected cells and particles; and (iv) neither N proteins (31) nor p40 (34) is phosphorylated or glycosylated. p23, a phosphorylated protein (34), is in the next position on the genome. The p23 ORF corresponds in position to genes coding for phosphoproteins in Paramyxoviridae (P) and Rhabdoviridae (NS) (31). This suggests that p23 might serve a similar role in the BDV system. In support of this hypothesis, analysis with Genetics Computer Group software shows that the protein has a high Ser/Thr content (16%), is charged (pI 4.8), and contains an N-terminal cluster of acidic amino acids compatible with structural features of P/NS proteins (31). In previously described Mononegavirales, the next gene codes for matrix (M) protein (31). gp18 occupies this position on the BDV genome. Though small for a matrix protein, gp18 has a predicted pI, 10, that is close to the basic pI of M proteins, ≈ 9 , and its membrane-association would be compatible with a matrix protein function. For p57, computer analysis predicted similarities to glycoproteins of negative-strand RNA viruses: potential glycosylation sites as well as N-terminal and C-terminal hydrophobic "anchor" domains (data not shown). The largest ORF is located most 5' on the genome. Its coding potential (≈ 180 kDa), 5' position, and conservation of motifs considered critical to L-polymerase activity suggest that this ORF is likely to code for the BDV polymerase (Fig. 2) (22–24).

Analysis of Northern hybridization experiments in conjunction with genomic sequence data has allowed construction of a tentative transcription map (Fig. 4). While it has not been possible to identify signals for initiation of transcription by using consensus sequences of other negative-strand RNA viruses, we have identified a consensus sequence for termination/polyadenylation in BDV by using known ends of p40 and p23 mRNAs (19, 20) (Fig. 4c). These sequences appear to function as weak termination signals. Unlike other negative-strand RNA viruses, BDV shows a high frequency of readthrough transcripts.

Organization and sequence similarities to Filo-, Paramyxo-, and Rhabdoviridae suggest that BDV is a member of the order Mononegavirales. Dependent on the parameters and regions selected for homology analysis, BDV can be represented as being more closely related to filo-, paramyxo-, or rhabdoviruses. Overlap of coding sequence, high frequency of polycistronic readthrough transcripts, and post-transcriptional modification are properties of the BDV system not found in other members of the order Mononegavirales. These features could serve as independent mechanisms

for modulation of gene expression to achieve the persistent, noncytopathic infection that is a cardinal characteristic of this neurotropic virus.

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