RNA Splicing in Borna Disease Virus, a Nonsegmented, Negative-Strand RNA Virus

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Borna disease virus (BDV) is a nonsegmented, negative-strand RNA virus related to rhabdoviruses and paramyxoviruses. Unlike animal viruses of these two families, BDV transcribes RNAs in the nuclei of infected cells and produces high levels of transcripts containing multiple open reading frames. Previous Northern blot analysis of RNA from BDV-infected rat brain tissue has shown that two viral transcripts, a 6.1-kb RNA and a 1.5-kb RNA, lack regions that are internal to two otherwise identical transcripts, the 7.1-kb RNA and the 2.8-kb RNA, respectively (T. Briesse, A. Schneemann, A. Lewis, Y. Park, S. Kim, H. Ludwig, and W. I. Lipkin, *Proc. Natl. Acad. Sci. USA* 91:4362–4366, 1994). To determine the precise location of this deletion, we performed reverse transcription PCR analysis using total RNA from BDV-infected rat brain tissue. This investigation resulted in the identification of two introns in the 7.1- and 2.8-kb RNAs, which can be alternatively spliced to yield additional RNA species, including the 6.1- and 1.5-kb RNAs. Transient transfection of COS-7 cells with a cDNA clone of the 2.8-kb RNA resulted in the production of both the 2.8-kb RNA and the 1.5-kb RNA, confirming the theory that the 2.8-kb RNA is a sufficient substrate for splicing in mammalian cells. Splicing has not previously been observed in nonsegmented, negative-strand RNA viruses and presumably serves as a mechanism by which expression of BDV proteins is regulated in infected cells.

Borna disease virus (BDV) is a neurotropic, negative-strand RNA virus that infects birds, rodents, primates, and possibly humans (2, 3, 5, 12, 19, 20, 27). It causes an immune system-mediated syndrome characterized by movement disorders and behavioral disturbances (23). In animal hosts and in cultured cells, BDV establishes a persistent infection that is characterized by low-level production of infectious virions (8, 14). Because of the difficulties in obtaining purified virus particles, only limited information concerning the morphology and the structural proteins of BDV is available. The virus has not yet been classified, but sequence analysis of the 8.9-kb viral genome revealed similarities to rhabdoviruses and paramyxoviruses (6, 10). The genome contains five open reading frames (ORFs) organized in a manner consistent with that of nonsegmented, negative-strand RNA viruses (*Mononegavirales*). Three Borna disease-associated proteins, p40, p23, and gp18, have been partially characterized and mapped to three of the five ORFs (Fig. 1A) (16, 25, 30). The two remaining ORFs have been predicted to encode a 57-kDa protein and a protein (pol) that is likely to serve as the viral RNA-dependent RNA polymerase (6).

Despite the similarities in sequence and genome organization to other members of the order *Mononegavirales*, BDV has features that clearly distinguish it from animal viruses of this order. One of the most striking characteristics of BDV is its nuclear localization for transcription (5). In contrast, animal viruses of the rhabdovirus, paramyxovirus, and filovirus families replicate and transcribe in the cell cytoplasm (1, 4, 11). Eight BDV transcripts have been identified in infected cells by Northern (RNA) blot analysis using probes complementary to each of the five ORFs (6). Four of these transcripts with apparent lengths of 7.1, 6.1, 2.8, and 1.5 kb initiate at the same position near the beginning of the gp18 ORF but end at two

different termination sites located approximately 2,600 bases (2.8- and 1.5-kb RNAs) and 7,000 bases (7.1- and 6.1-kb RNAs) downstream (6). Whereas the 7.1- and 2.8-kb RNAs are colinear with the BDV genome, the 6.1- and 1.5-kb RNAs lack regions internal to the 7.1- and 2.8-kb RNAs, respectively (6). To investigate this observation in more detail, we performed a series of reverse transcription PCR (RT-PCR) experiments using RNA isolated from BDV-infected rat brain tissue (28). Here we show that at least two primary BDV transcripts are posttranscriptionally modified by RNA splicing. This process yields several additional RNA species, including the 6.1- and 1.5-kb RNAs.

MATERIALS AND METHODS

Source of viral RNA. RNA was obtained from brains of rats acutely infected with BDV strain He80-1 (28) and from C6 cells persistently infected with the same strain. The procedure used for purification of total RNA from these sources has been described previously (19).

RT-PCR analysis. One microgram of total RNA from BDV-infected rat brain tissue was primed with primer A5 (5'-GAATTCAGGATCCGCGGCCG(T)₁₅-3') and reverse transcribed by standard procedures (28). The resulting cDNA was used as template for PCR amplification with the following primer pairs: S1 (5'-TCCTCGAGATGAATTCAAACATTCCTATG-3') and A1 (5'-GTCCTCTGGTGCTGAGTTGTT-3'); S1 and A2 (5'-GAGGGTTTGTTCACGACTT-3'); S1 and A3 (5'-CCATTGTAATCTACTGGAGG-3'); S1 and A4 (5'-ATACTTCAGGGGGCAATACA-3'). PCR amplification conditions have been described previously (28). A 10-μl aliquot of each reaction mixture was analyzed on a 1% agarose gel containing 100 ng of ethidium bromide per ml. PCR products were purified from the gel by using a USBioclean purification kit (U.S. Biochemicals, Cleveland, Ohio) and cloned into pBluescript SKII+ (Stratagene, La Jolla, Calif.) prepared with 3' T overhangs (21). Following transformation

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FIG. 1. RT-PCR and Northern blot analysis of total RNA from BDV-infected rat brain tissue indicating alternative splicing of the 7.1- and 2.8-kb RNA transcripts. (A) Schematic diagram of the BDV genome with the locations of ORFs. RNAs initiating near the start of the gp18 ORF and ending at the termination site at nt 4510 are shown as solid lines (2.8-kb RNA and splice products). Dashed lines indicate that some RNAs extend to the termination site at nt 8855 (7.1-kb RNA and splice products). The size of each transcript is shown to the left (those of longer RNAs are in parentheses). Primers (S1 and A1 through A4) used for PCR are represented as arrowheads. Locations of probes (I through IV) used in Northern blot analysis are shown below the transcripts. (B) RT-PCR analysis of total RNA from BDV-infected rat brain tissue. BDV-specific cDNA was amplified by using primers S1 and A1 (lane 1), S1 and A2 (lane 2), S1 and A3 (lane 3), or S1 and A4 (lane 4). In lane 4, bands representing RT-PCR products derived from the four larger templates (2.8, 2.7, 7.1, and 7.0 kb) are faint (indicated by the star), presumably because of preferential amplification of the two smaller products (labeled C and D). (C) Northern blot analysis of poly(A)⁺ RNA from BDV-infected rat brain tissue, using probes surrounding the boundaries of region 2.

of *Escherichia coli* DH5 α F', plasmids containing inserts were selected, and the sequences of the inserts were determined by the Sanger dideoxy chain termination method using Sequenase 2.0 (U.S. Biochemicals).

Northern blot analysis of poly(A)⁺ RNA. Poly(A)⁺ RNAs were purified from total RNA with oligo(dT)-coated magnetic beads (Dynabeads; Dynal) according to protocols provided by the manufacturer. Electrophoresis of RNA through a 1% agarose gel was performed as described previously (26) except that the gel and the buffer both contained 0.22 M formaldehyde (31). RNA was transferred to a nylon membrane (Zetaprobe; Bio-Rad, Hercules, Calif.) by capillary action and UV cross-linked. Membrane strips were hybridized overnight at 68°C in buffer containing 6 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5 $\times$ Denhardt's solution, 200 μ g of sheared, denatured salmon sperm DNA per ml, 0.5% sodium dodecyl sulfate, and 50% formamide. Probes I, II, III, and IV were digoxigenin-UTP-labeled antisense RNAs generated by in vitro transcription from linearized plasmids containing the

appropriate cDNAs (see below). After hybridization, blots were washed and developed with alkaline phosphatase-conjugated anti-digoxigenin antibodies and Lumiphos530 according to the manufacturer's protocols (Boehringer Mannheim, Indianapolis, Ind.).

Preparation of RNA probes. cDNA fragments representing the regions spanned by probes I through IV were generated by PCR from a plasmid that contained a cloned portion of the BDV genome (nucleotides [nt] 1376 to 4299 of BDV strain V, plasmid 6.31) (6). The following primers were used for PCR: 5'-AATTCAAAACATTCCTATG-3' and 5'-TAAGGCCCTG AAGATCGAAT-3' (probe I); 5'-TGCCTCAAGTACCACT GCAA-3' and 5'-GAGGGTTTTTGTTCACGACTT-3' (probe II); 5'-AGTCTCAACATGACCCCTCA-3' and 5'-TAGAAC CCCACCCAACCAGG-3' (probe III); and 5'-ATGTACG AGCACTAGGCCAGA-3' and 5'-ATTAGGAGATGGCAT CTGCTC-3' (probe IV). PCR conditions were as described previously (28) except that the cycle parameters were as follows: 5 min at 95°C, 5 min at 55°C, and 30 cycles of

FIG. 2. Comparison of BDV splice sequences with mammalian consensus sequences important for splice site recognition. Bases located in exons are in bold type. Y, pyrimidine; R, purine; N, any base; [^], branchpoint nucleotide.

amplification (2 min at 70°C, 1.5 min at 95°C, and 1 min at 55°C). PCR products were purified and cloned into pBluescript SKII+ as described above. Following transformation, plasmids containing inserts were selected, and the orientations of the inserts relative to the T7 and T3 promoters were determined. Depending on the orientation, plasmids were linearized with *Xba*I or *Xho*I and transcribed by using either T7 or T3 RNA polymerase. Transcription of digoxigenin-UTP-labeled antisense RNAs was performed according to the manufacturer's protocols (Boehringer Mannheim).

Cloning of the 2.8-kb RNA. RNAs containing intron 2-specific sequences were purified from total RNA of persistently infected C6 cells by using a biotinylated primer (5'-biotin-GAGGGGATTGAGGAAAGCAAATGAGGGGTAGGCCGATGTGCACC-3') complementary to intron 2 and streptavidin-coated magnetic beads (Dynabeads) according to protocols provided by the manufacturer. Primers S1 and A5 were used to amplify the 2.8-kb transcript from the purified RNA sample by RT-PCR; the PCR product was isolated and cloned into pBluescript SKII+ as described above.

Construction of p2.8Δ225. pBluescript SKII+, containing a full-length clone of the 2.8-kb RNA, was digested with *Not*I, which cuts within the 5' polylinker, and *Apa*I, which cuts within the viral sequence at nt 4288 upstream of the termination-polyadenylation signal. The fragment was subcloned into the *Not*I and *Apa*I sites of pCr/CMV (Invitrogen, La Jolla, Calif.) to create p2.8Δ225. After transformation of *E. coli* DH5αF', plasmid DNA was isolated by alkaline lysis (Wizard Minipreps; Promega, Madison, Wis.).

Transfection of COS-7 cells. COS-7 cells (5×10^7) were electroporated in the presence of 60 μg of p2.8Δ225 as described previously (7). Sixteen hours after electroporation, plasma membranes were disrupted with a nonionic detergent and poly(A)⁺ RNA was isolated from the cytoplasmic fraction by using oligo(dT)-coated magnetic beads (Dynabeads) according to the manufacturer's protocols. One-tenth of the purified RNA was used for RT-PCR, and the remainder was used for Northern blot analysis. PCR with primers S1 and A4 was performed after reverse transcription with oligo(dT) primer A5. Northern blot analysis of the RNA was performed with RNA probe IV as described above.

RESULTS

To determine the sizes and locations of the deletions in the 6.1- and 1.5-kb transcripts, total RNA from BDV-infected rat brain tissue was primed with oligo(dT), reverse transcribed, and then amplified by PCR with a sense primer (S1) that mapped to the extreme 5' end of the gp18 ORF and a series of downstream antisense primers (A1 through A4) (Fig. 1). Each PCR amplification resulted in the generation of two predominant DNA fragments that differed in length by approximately 100 bases; the sizes of the PCR products increased in direct proportion to the distance between the sense primer and the antisense primer on the genome. Use of primer pair S1 and A4 yielded two additional fragments (fragments C and D in Fig. 1B, lane 4) smaller than the distance spanned by the two primers on the genome. These two fragments, as well as fragments A and B generated with primers S1 and A1 (Fig. 1B, lane 1), were cloned and sequenced to determine the basis for the observed heterogeneity in length. Sequence analysis of at least two independent clones revealed that fragment A contained the entire sequence spanned by primers S1 and A1 on the BDV genome, whereas fragment B lacked the region from nt 1932 to 2025 (region 1, 94 nt; Fig. 1A). Fragment C contained region 1 but lacked the region from nt 2410 to 3703 (region 2, 1,293 nt). Fragment D lacked both region 1 and region 2. These results suggested that the 6.1-kb RNA and the 1.5-kb RNA were derived from the 7.1-kb RNA and the 2.8-kb RNA, respectively, by deletion of region 2 and that smaller versions of each of these four RNAs existed which lacked region 1. RNAs differing only in the presence or absence of region 1 (94 nt) would not be resolved as separate species under our electrophoresis conditions.

To confirm that the results obtained were not based on artifacts arising during PCR amplification, we performed Northern blot analysis of poly(A)⁺ RNA from BDV-infected rat brain tissue, using probes surrounding the boundaries of region 2 (probes I through IV in Fig. 1A). As predicted, probes complementary to sequences within region 2 (probes II and III) detected the 7.1- and 2.8-kb RNAs but not the 6.1- and 1.5-kb RNAs. Probes complementary to sequences outside region 2 (probes I and IV) detected all four RNAs (Fig. 1C).

FIG. 3. Evidence for splicing of BDV transcripts through the transient transfection of COS-7 cells with a plasmid (p2.8Δ225) containing a truncated BDV 2.8-kb RNA. **(A)** Schematic representation of p2.8Δ225 and the differential processing of its primary RNA product. A cDNA of the 2.8-kb BDV RNA was truncated by 225 nt at the 3' end and cloned into the eukaryotic expression vector pRc/CMV such that transcription was under the control of the cytomegalovirus promoter. Because of the presence of flanking vector sequences and a poly(A) tail, the primary transcript was predicted to be approximately 3 kb in length. The hatched box represents the BDV cDNA insert; shaded and open boxes in the primary RNA transcript and its processing products represent exons and introns, respectively. **(B)** RT-PCR analysis of poly(A)⁺ RNA extracted from COS-7 cells transfected with p2.8Δ225 (lane 1) or pRc/CMV (lane 2; negative control) using primers S1 and A4 (see panel A). **(C)** Northern blot analysis of poly(A)⁺ RNA isolated from COS-7 cells transfected with p2.8Δ225 (lane 1) or pRc/CMV (lane 2; negative control) using RNA probe IV (see panel A). Unspliced mRNA with an apparent length of 3.0 kb (product 1) and spliced mRNA species with apparent lengths of ≈1.7 kb (products 3 and 4) were detected. The identity of the other, smaller RNA, which was not seen in the negative control (COS-7 cells transfected with pRc/CMV; lane 2) or in RNA extracted from BDV-infected rat brain tissue (Fig. 1C, lane 4), is unknown.

Analysis of BDV antigenomic sequences contiguous to the two sites of deletion revealed similarities to consensus sequences of mammalian 5' and 3' splice sites (15), suggesting that regions 1 and 2 represented introns (hereafter referred to as intron 1 and intron 2) (Fig. 2). Seventy-eight percent of the positions in the 5' and 3' splice sites of both introns matched the mammalian consensus sequence, and branchpoint nucleotides were found 18 nt (intron 1) and 30 nt (intron 2) from the 3' splice site junctions (13).

To further test the hypothesis that BDV RNAs undergo splicing, a truncated cDNA copy of the 2.8-kb RNA was cloned into the eukaryotic expression vector pRc/CMV under the control of the cytomegalovirus promoter (Fig. 3). The poly(A) tail and 225 additional bases were removed from the 3' end of the 2.8-kb cDNA before cloning to prevent the possibility that the plasmid, called p2.8Δ225, could serve as a template during

oligo(dT)-primed RT-PCR. RNA transcribed from this plasmid was nevertheless expected to be polyadenylated because of the presence of a polyadenylation signal provided by the vector. Following transfection of COS-7 cells with p2.8Δ225, poly(A)⁺ RNA was isolated at 16 h and reverse transcribed with an oligo(dT) primer. BDV-specific cDNA was then amplified by using primers S1 and A4. Products identical to fragments C and D in Fig. 1B were detected (labeled 3' and 4' in Fig. 3B), indicating removal of either intron 2 or both introns from the primary p2.8Δ225 RNA transcript. Unspliced primary transcripts or transcripts that lacked only intron 1 were not detected under these conditions, presumably because amplification of the smaller products was more efficient. PCR analysis using primers S1 and A2 showed that unspliced transcripts were present in the poly(A)⁺ RNA sample (data not shown). Sequence analysis of all PCR products revealed

the presence of splice junctions identical to those found in *in vivo*-synthesized RNAs (data not shown). RNAs of lengths consistent with those of spliced (1.7-kb) and unspliced (3.0-kb) messages were also detected by Northern blot hybridization (Fig. 3C, lane 1). The identity of a third, smaller RNA, which was not seen in the negative control (COS-7 cells transfected with pRc/CMV only; lane 2) or in RNA extracted from BDV-infected rat brain tissue (Fig. 1C, lane 4), is unknown.

DISCUSSION

This is the first report of RNA splicing in a nonsegmented, negative-strand RNA virus. The results presented here are consistent with the observation that BDV transcription occurs in the nucleus and support the notion that this virus represents a previously unrecognized family or genus within the order *Mononegavirales*.

Northern blot analysis of RNA isolated from BDV-infected rat brain tissue showed that splicing was not 100% efficient, as both spliced and unspliced messages were present in infected cells. The splicing efficiency of the 7.1- and 2.8-kb RNAs may have been adversely affected by a slight deviation from the consensus sequence at the 3' splice site. Fifty-five percent (18) of all 3' exons in spliced RNAs start with a G residue; in BDV, however, these exons started with a U residue (exon 2) or a C residue (exon 3) (Fig. 2). The presence of these nucleotides may have decreased the recognition efficiency of the 3' splice sites. A decrease in the efficiency of splicing, presumably due to inaccessibility of the 3' splice site to components of the splicing machinery, has also been observed in influenza virus (24).

Transcription of the BDV genome results in the synthesis of at least eight subgenomic RNAs (6). Probes complementary to the gp18 ORF hybridize to the 7.1-, 6.1-, 2.8-, and 1.5-kb RNAs. Interestingly, probes complementary to the p57 and pol ORFs detect only a subset of the same RNAs but no additional RNA species (6). The lack of primary monocistronic RNAs for the p57 and pol ORFs and the synthesis of RNAs containing all three ORFs (gp18, p57, and pol) suggest that posttranscriptional modification may be necessary for the expression of p57 and pol. Though the possibility that the 2.8- and 7.1-kb RNAs serve as the messages for p57 or pol cannot be excluded, this would require that translation be initiated following internal entry of ribosomal preinitiation complexes. Such a mechanism may not need to be invoked in light of the data indicating that the 7.1- and 2.8-kb RNAs undergo RNA splicing.

Splicing of intron 1 effectively eliminates the gp18 ORF by removing 23% of its coding capacity and juxtaposing the 13th amino acid codon with a stop codon. It is conceivable that, after translation of this minicistron, ribosomes continue to scan along the RNA and reinitiate at a downstream p57 AUG codon (Fig. 4). A number of cellular and viral RNAs have been shown to use this strategy for internal initiation of translation (9, 17, 22, 29). Preliminary *in vitro* translation experiments indicate that transcripts lacking intron 1 (2.7-kb RNA) can indeed serve as the message for p57 as indicated by immunoprecipitation of a 57-kDa protein by sera from chronically infected rats (data not shown). Alternatively, it is possible that p57 constitutes part of a gp18-p57 fusion protein generated from a posttranscriptionally edited RNA or by ribosomal frameshift during gp18 translation.

In a manner similar to that of the events required for translation of p57, activation of the pol ORF might be accomplished by splicing of both intron 1 and intron 2 from the 7.1-kb RNA (Fig. 4). Removal of intron 2 extends the pol ORF by 459 nt, allowing the prediction of a protein of 190 kDa. Expression of the polymerase also appears to be regulated at the level of

FIG. 4. Model illustrating how differential splicing of the 7.1- and 2.8-kb transcripts yields RNAs that might serve as messages for the translation of p57 and pol. The organization of the gp18, p57, and pol ORFs (shaded boxes) on the BDV genome is shown at the top. Transcripts retaining intron 1 (i.e., the 7.1-, 6.1-, 2.8-, and 1.5-kb RNAs) can serve as messages for gp18. Splicing of intron 1 alone results in two RNAs that could serve as messages for the translation of p57 (7.0- and 2.7-kb RNAs). Splicing of both introns from the 7.1-kb RNA results in a 6.0-kb RNA that might serve as the message for pol. Removal of intron 2 extends the pol ORF by 459 nt (hatched box), allowing the prediction of a 190-kDa protein. It is not known whether a truncated polymerase protein is produced from the 1.4-kb RNA.

RNA transcription. Transcription starting at the gp18 ORF has to continue beyond a strong termination site at position 4510 on the BDV genome to generate the 7.1-kb RNA. This event appears to be infrequent, given the low levels of the 7.1- and 6.1-kb RNAs relative to those of the 2.8- and 1.5-kb RNAs (Fig. 1C). Alternatively, the lower levels might be due to a decrease in the overall stability of the 7.1- and 6.1-kb RNAs.

With a length of 8.9 kb, the BDV genome is smaller than those of rhabdoviruses and paramyxoviruses (>11 kb). RNA splicing has allowed the virus to achieve a comparable level of protein diversity through the use of overlapping reading frames. In addition, transcription and RNA splicing appear to be important components of the mechanism used to modulate expression of gp18, p57, and pol. They may also be critical for achieving key features of BDV biology such as low-level production of infectious virus and viral persistence.

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