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Implication of a cis-Acting Element in the Cytoplasmic Accumulation of Unspliced Borna Disease Virus RNAs

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Borna disease virus (BDV), the prototype of a new family within the order Mononegavirales, is unusual in its nuclear localization for replication and transcription and use of RNA splicing for gene expression. The BDV antigenome contains three transcription units and six major open reading frames. Multicistronic RNAs containing two introns are elaborated from the third transcription unit. Differential splicing of the two introns and cytoplasmic accumulation of the unspliced and partially spliced RNA are critical for the balanced expression of the putative matrix protein, glycoprotein, and polymerase. To investigate the mechanisms for cytoplasmic expression of unspliced and partially spliced BDV transcripts, the levels of these transcripts were measured in the cytoplasm of infected COS-7 cells and noninfected COS-7 cells transfected with plasmids containing 2.8-kb cDNA inserts representing either wild-type or mutant BDV RNA from the third transcription unit. Analysis of truncation mutations allowed the identification of a cis-acting element present within the 3′ end of the BDV 2.8-kb transcript that facilitated the cytoplasmic accumulation of unspliced BDV transcripts through nucleocytoplasmic transport. The nucleocytoplasmic transport activity was not dependent on the presence of BDV proteins. Gel-shift assays revealed that the cis-acting element binds specifically to host cytoplasmic and nuclear proteins.
fected with the plasmids pRc2.8, pRc2.8UUG, and pRc/CMV. Plasmid pRc2.8 was generated by releasing the 2.8-kb cDNA insert from a plasmid that contained cDNA that represented the entire 2.8-kb transcript (p2.8) by restriction digestion with NotI and ApaI. The digestion resulted in two fragments (2,250 bp, NotI/ApaI, and 456 bp, ApaI/ApaI) that were ligated into pRc/CMV prepared with NotI and ApaI. The resulting plasmids that contained both fragments in the forward orientation were designated pRc2.8. The plasmid pRc2.8UUG, in which the gp18 ORF initiation codon was converted to a UUG codon, was generated by releasing a cDNA fragment that contained the UUG mutation from the plasmid pT7UUG (34) by restriction digestion with XbaI and ligation of the fragment into plasmid pRc2.8 prepared by XbaI digestion. Plasmids pRc2.8, pRc2.8UUG, and pRc/CMV were transfected into COS-7 cells by electroporation (35). Total cellular RNA was isolated from the transfected and BDV-infected COS-7 cells with Tri-Reagent (Molecular Research Center) according to standard protocols. The relative ratios of spliced to unspliced intron 1 RNAs were determined by RPA with radiolabeled PBD transcript (36).

Identical results were obtained with the truncated pRc2.8 transcripts that contained 456 nt, which corresponded to the 3’ end of the 2.8-kb RNA (Fig. 3A). Reduced cytoplasmic expression of unspliced RNAs was also observed with constructs that contained the 456-nt region in an inverted orientation, suggesting that the cis-acting element functions in an orientation-dependent manner (data not shown).

The cis-acting element within the 456-nt region at the 3’ end of the 2.8-kb RNA responsible for cytoplasmic expression of unspliced transcripts was mapped by using a panel of deletion mutants (Fig. 3A and B). Plasmids representing 3’ truncations of the 2.8-kb cDNA were generated by PCR amplification of p2.8 with the primer pairs 631Sp6.4 and 841T7.6 (5’-AGCCTATGGTGGCAATAA, nt position 4175) for p-320 and primer pairs 631Sp6.4 and 841T7.6 (5’-GATTAGGAGAAGCCAGA, nt position 3830) and pr320.AS (5’-CTTGCTAGCCATGTTGGCGA, nt position 4248) for p-240. The PCR products were cloned into pBluescript SKII(+) to create plasmid pRPA-1 (33); a second portion was retained for use in RPA as a control for the capacity of unspliced transcripts to protect the radiolabeled probe transcribed from pRPA-1. To serve as a control for the quantity and integrity of RNA used in the RPA, porphobilinogen deaminase (PBD) RNA levels were examined using a radiolabeled PBD transcript (36).

The RPA revealed that the relative ratios of spliced to unspliced intron 1 RNAs were similar in infected and noninfected transfected cells (Fig. 2). This finding indicated that the BDV N, P, and Pol proteins were not required for the expression of unspliced transcripts. It did not, however, exclude a role for the gp18 or G protein, because both are encoded by the 2.8-kb cDNA and could be present in the transfected cells. To test for the presence of unspliced transcripts in the absence of M, transfection experiments were performed with the mutated 2.8-kb cDNA construct that lacked the M ORF initiation codon (pRc2.8UUG). Analysis of total RNA isolated from cells transfected with pRc2.8UUG indicated that the ratios of spliced to unspliced intron 1 RNAs were similar to those found in BDV-infected cells (Fig. 2). Because the G protein is not localized to the nucleus (17, 32), no additional studies were pursued to examine whether G could be implicated in regulating expression of unspliced or partially spliced transcripts.

Identification of a cis-acting element responsible for the cytoplasmic expression of unspliced and partially spliced RNA. The BDV genome is compact, and the presence of a large stretch (765 nucleotides [nt]) of untranslated sequence downstream of the G ORF in the 2.8-kb transcript was not anticipated. To determine whether this region contains sequences important for posttranscriptional RNA processing, the 2.8-kb cDNA was truncated at the 3’ end and the repertoire of RNAs expressed from the truncated construct (pRc2.8-456) was assessed in RPA. The pRc2.8-456 plasmid was generated by digestion of pRc2.8 with ApaI to release the last 456 nt representing the 3’ end of the 2.8-kb cDNA followed by recircularization of the vector. The cytoplasmic expression of unspliced RNA was markedly reduced in assays with truncated transcripts relative to wild-type transcripts, suggesting the presence of a cis-acting element within this 456-nt region at the 3’ end of the 2.8-kb RNA (Fig. 3A). Reduced cytoplasmic expression of unspliced RNAs was also observed with constructs that contained the 456-nt region in an inverted orientation, suggesting that the cis-acting element functions in an orientation-dependent manner (data not shown).

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megalovirus promoter. Whereas removal of 240 nt from the 3' end had no impact, removal of 320 nt abrogated cytoplasmic accumulation of unspliced RNAs. This localized the 3' end of the cis-acting element to a region between nt 4190 and 4269.

To map the 5' end of the minimal cis-acting element, linker-scanning deletion mutants were generated by PCR amplification of p2.8 with primers T7 and pΔ20cis (5'-CTTGGGCCCGCTAGCATTGCCTACCAGCGCATAG; for pRc2.8, nt position 4090, underlined text represents the ApaI restriction site) or pΔ50cis (5'-CTTGGGCCCGCTAGTCGTATAGCCGGGAAGCAG; for pRc2.8, nt position 4120, underlined text represents the ApaI restriction site). After diges-
TABLE 1. Ratio of unspliced to spliced intron 1 BDV transcripts in COS-7 cells transfected with constructs containing or lacking the PRCE element

<table>
<thead>
<tr>
<th>Construct</th>
<th>Unspliced to spliced intron 1 (± SEM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoplasm</td>
<td>Nucleus</td>
</tr>
<tr>
<td>pRc2.8a</td>
<td>0.70 (0.03)</td>
</tr>
<tr>
<td>pRc(−)456b</td>
<td>0.06 (0.01)</td>
</tr>
</tbody>
</table>

* Represents the average of at least three experiments.

Contains the PRCE element.

Lacks the PRCE element.

TABLE 2. Ratio of cytoplasmic to nuclear unspliced and spliced intron 1 transcripts in COS-7 cells transfected with constructs containing or lacking the PRCE element

<table>
<thead>
<tr>
<th>Construct</th>
<th>Cytoplasmic/ nuclear ratio</th>
<th>RNA ratio (± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unspliced</td>
<td>pRc2.8a</td>
<td>7.27 (0.58)</td>
</tr>
<tr>
<td>Intron 1</td>
<td>pRc(−)456b</td>
<td>0.69 (0.02)</td>
</tr>
<tr>
<td>pRc2.8a</td>
<td>13.7 (0.39)</td>
<td></td>
</tr>
<tr>
<td>pRc(−)456b</td>
<td>14.2 (0.43)</td>
<td></td>
</tr>
</tbody>
</table>

* Represents the average of at least three experiments.

Contains the PRCE element.

Lacks the PRCE element.
cytoplasmic transport. First, the ratios of unspliced to spliced transcripts in the nucleus were only slightly higher for transcripts that contained the PRCE element. In contrast to the situation observed with influenza virus, there was no increase in the nuclear levels of spliced transcripts which contain the PRCE element, suggesting that there was no interference with nucleocytoplasmic transport of spliced mRNAs. Second, whereas the cytoplasmic to nuclear ratio of spliced transcripts was independent of the presence of the PRCE element, the presence of the PRCE element was associated with an 11-fold increase in the cytoplasmic to nuclear ratio of unspliced transcripts (Table 2). The finding that the PRCE element only facilitated the expression of unspliced transcripts suggests a distinct nucleocytoplasmic transport pathway for unspliced transcripts and may account for the twofold difference in transport efficiency of spliced and unspliced intron 1 RNAs that contain the PRCE element (Table 2). Taken together, these observations are consistent with the model in which the PRCE element allows unspliced RNAs to bypass the spliceosome assembly or nuclear retention/degradation pathways and facilitates their nucleocytoplasmic transport (3).

cis-Acting elements involved in the cytoplasmic expression of unspliced transcripts have been identified in many viral systems (4, 7, 12, 18, 20, 25, 27, 30, 37). Although most of these cis-acting elements play a role in the cytoplasmic accumulation of unspliced transcripts and are functionally homologous, their mechanisms of action are variable and include enhanced stability of unspliced transcripts, interference with splicing, and facilitation of nucleocytoplasmic transport (4, 7, 12, 27, 29, 37). The BDV PRCE element appears to be most functionally similar to the PRE (hepatitis B virus) and PPE (herpes simplex virus) intron-independent nucleocytoplasmic transport se-

![Diagram](attachment:image.png)

**FIG. 4.** RNA that contains the PRCE element is retarded in an RNA gel mobility shift assay. Two specific complexes were identified when nuclear (Nuc) extracts from noninfected COS-7 cells were incubated with a radiolabeled RNA probe that contained the PRCE element (lane 1, complex I). Complex formation was inhibited by the addition of a 20-fold molar excess of unlabeled specific transcripts to nuclear (lanes 3 and 4) or cytoplasmic (lanes 6 and 7) extracts but not addition of a 20- or 40-fold molar excess of unlabeled nonspecific transcripts (lanes 8 to 11). Lane 1 represents radiolabeled transcripts that were incubated with buffer in the absence of cellular extracts.

shown). Whether the complex II observed in the RNA retardation assays is due to binding of the 90-kDa factor remains to be established.

Expression of the BDV gp18 and BDV G proteins is dependent on the presence of unspliced and partially spliced BDV transcripts in the cytoplasm (4, 6, 11, 22, 32, 35). Because incompletely spliced transcripts are typically retained in the nucleus, this study was initiated to determine the basis for the cytoplasmic accumulation of unspliced 2.8-kb and spliced 2.7-kb intron 1 BDV RNAs. The observation that the cytoplasmic expression of the unspliced 2.8-kb RNA was not dependent on the presence of replicating virus indicated that BDV proteins did not play essential roles in trans; however, studies performed with truncation mutations of the 2.8-kb RNA demonstrated dependence on the presence of a cis-acting element. Whereas the cis-acting element enhanced the cytoplasmic accumulation of unspliced RNA (2.8 kb), it did not affect the accumulation of the spliced intron 1 transcripts (2.7 kb). The pre-mRNA cytoplasmic expression (PRCE) element was mapped to a 200-nt region located at the 3′ end of the 2.8-kb transcript. UV cross-linking studies identified two host cell-specific factors that interacted with the PRCE sequences. We speculate that these factors may function in facilitating the cytoplasmic accumulation of pre-mRNAs.

Two observations support the hypothesis that the mechanism for the cytoplasmic accumulation of unspliced RNAs by the PRCE element is likely to be the enhancement of nucleo-

![Diagram](attachment:image.png)

**FIG. 5.** Identification of cellular factors that bind to RNAs which contain the PRCE element. A radiolabeled RNA probe containing the PRCE element was UV cross-linked to factors found in the nuclear and cytoplasmic extracts and then treated with RNase A and RNase T1 prior to analysis by SDS-PAGE and autoradiography. Two factors (90 and 16 kDa) were identified in nuclear extracts (lane 1). One factor (16 kDa) was identified in cytoplasmic extracts (lane 4). The binding of these factors to the RNA probe was disrupted in the presence of a 20-fold molar excess of unlabeled specific competitor (lanes 2, 3, 5, and 6). Lane 7 represents radiolabeled probe incubated with both nuclear and cytoplasmic extracts without UV cross-linking. Arrows indicate positions of the 90- and 16-kDa proteins, respectively.
quences, which facilitate export of viral RNAs from the nucleus in the absence of virus-specific factors (18, 20, 25, 30). However, the BDV PRCE element does not share sequence or secondary structure similarity with any of the known cis-acting elements that are involved in the cytoplasmic accumulation of unspliced transcripts.

RNA mobility retardation and UV cross-linking studies revealed that two host cell-specific factors bind the PRCE element. One factor migrates at an apparent molecular size of 16 kDa upon SDS-PAGE analysis. The 16-kDa factor is present in both the nucleus and cytoplasm and may be important in transport of unspliced RNAs from the nucleus. This factor may be functionally related to viral proteins found in other systems such as Rev and Rex (19, 26, 28). The 13-kDa HIV Rev protein facilitates the nuclear export of unspliced RNAs that contain the RRE by shuttling between the nucleus and cytoplasm (21, 26, 28). The 90-kDa nucleus-associated factor, which specifically interacted with the PRCE element, may also have functional homologs. Heterogeneous nuclear ribonucleoprotein (hnRNP) L is a 68-kDa protein that can bind chimeric RNAs that contain the herpes simplex virus thymidine kinase pre-mRNA processing element (PPE) and enhance intron-independent nucleocytoplasmic transport (25). Whether the 90-kDa factor, is a member of the hnRNP family remains to be determined.

Since intron 1 is located within the gp18 ORF, nuclear export of unspliced RNA is required for translation of the BDV gp18. Whereas splicing of intron 1 prevents translation of BDV G (34), it facilitates translation of BDV G (34). It is intriguing to speculate that the PRCE element and cell-associated transacting factors, which bind the PRCE element, play key roles in modulating the expression of BDV gp18 and BDV G by regulating cytoplasmic levels of unspliced and partially spliced BDV transcripts.

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3. Berthold, E., and F. Maldarelli. 1986. Nucleocytoplasmic RNA mobility retardation and UV cross-linking studies reveal that two host cell-specific factors bind the PRCE element and cell-associated transacting factors, which bind the PRCE element, play key roles in modulating the expression of BDV gp18 and BDV G by regulating cytoplasmic levels of unspliced and partially spliced BDV transcripts.

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